Understanding the role of the rumen microbiome for enhancing feed efficiency and reducing methane emissions in sheep

Steven McLoughlin MSc (Biomedical Genomics)

A thesis submitted to University of Galway in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

June 2023

[1]Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Athenry, Galway, Ireland

[2]Genetics and Biotechnology Laboratory, Plant and AgriBiosciences Research Centre (PABC), Ryan Institute, University of Galway, Galway, Ireland

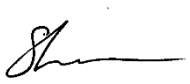
Research Supervisors:

Prof. Sinéad M. Waters[1,2] and Prof. Charles Spillane[2]

Table of Contents

Declaration

I certify that this thesis is my own work, and that I have not used this work in the course of another degree, either at University of Galway, or elsewhere.

Signed:  Steven McLoughlin

Acknowledgements

I would like to express my sincere gratitude to Prof. Sinead Waters and Prof. Charles Spillane for granting me the opportunity to embark on a research career in metagenomics. Their guidance, encouragement, and support throughout my PhD journey have been invaluable. I am deeply grateful for their mentorship and cannot thank them enough for everything they have done for me.

To Teagasc Walsh fellowship program for the funding throughout my PhD.

To Dr. Galina Brychkova for her help throughout the PhD.

To my GRC committee Dr. Sara Farrona, Dr. Peter McKeown and Dr. David Styles.

To my mother Mary and Eamonn Kelly, I want to extend my heartfelt gratitude for the immense help and unwavering support you have provided throughout my PhD journey. Your generosity and encouragement have been invaluable to me, and enabled me to get my PhD over the line. I am deeply grateful.

To my brother Jason, I would not have been able to embark on this journey it were not for your support, I honestly cannot thank you enough. You are an amzing person and I hope life will be as kind to you in return.

I extend my heartfelt appreciation to my partner Stephanie and our son Cuan Rí. Stephanie, your patience and support have been my rock throughout this challenging PhD journey. Cuan, your boundless energy and daily presence fill my life with immeasurable joy and motivation.

To Paul Smith and Stuart Kirwan for all your support and guidance throughout my PhD, and getting me away from the computer to do some rumen sampling. It was a pleasure collaborating with both of you, and I deeply appreciate your camaraderie and expertise.

To my father, David, and Jodie, I express my gratitude for the support and assistance your provided during my PhD journey.

To Stephen and Majella McConnellogue for your support and understanding, and off course taking Cuan on busy days. And I can’t forgot Andrea for taking Cuan at times, you are the best god mother.

To Mary Collins for my accommodation throughout my time in Galway.

To Andrew, Barry, Cathal, Tyler and Declan for providing all the fun and support in Galway during my studies.

To Jamie Rielly for his advice and guidance.

Communications and Publications

Presentations

Title: Breed and ruminal fraction effects on bacterial and archaeal community composition in sheep
Conference: *BSAS Annual conference* .2021. 'The Challenge of Change. The New Normal?'

Title: Trajectory of colon microbiota colonisation in the bovine neonate
Conference: INRAE 12th international Symposium on Gut microbiology 2021

Title: The Effect of time off feed on methane and the rumen microbiome
Conference: EAAP 73rd Annual Meeting

Courses attended

'Metagenomics , metatranscriptomics, and multi'omics for microbial community studies'. Online. 24th-28th May 2021 – Physalia & Huttenhower Lab

'R and Statistics Workshop'. Carlisle. 19th-20th Aug 2019

FACCE ERA-Gas Symposium and Summer School. Amsterdam. 12th -14th June 2019

Publications

McLoughlin, S., Spillane, C., Claffey, N., Smith, P.E., O'Rourke, T., Diskin, M.G. and Waters, S.M., 2020. Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Frontiers in microbiology*, 11, p.1981.

McLoughlin, S., Spillane, C., Campion, F.P., Claffey, N., Sosa, C.C., McNicholas, Y., Smith, P.E., Diskin, M.G. and Waters, S.M., 2023. Breed and ruminal fraction effects on bacterial and archaeal community composition in sheep. *Scientific Reports*, 13(1), p.3336.

McLoughlin, S., Spillane, C., Kirwan, S., Dunne, E., O'Connor, E., Roscam, E., Smith, PE., Admin, N., McGovern, F., and Waters, SM. (**In Prep**). Time off feed affects enteric methane and rumen microbiome in sheep. *Frontiers in microbiology.*

Abstract

Sheep play a vital role in global agricultural enterprises and support human livelihoods by converting human-inedible plant matter into human-edible products, such as meat and milk. However, the production of methane, a potent greenhouse gas, during the fermentation of feed by sheep's rumen microbiome contributes to anthropogenic greenhouse gas emissions. Therefore, the primary objective of this thesis was to investigate the role of the rumen microbiome in sheep to enhance feed utilisation, reduce methane emissions to promote the long-term sustainability of the sector. Chapter 2 of this thesis used 16S rRNA amplicon sequencing to investigate the bacterial and archaeal populations in both solid and liquid fractions of the rumen of sheep with different feed conversion rates. The study found that the rumen archaea diversity and composition differed between feed efficient cohorts, providing evidence for the link between methane production and dietary energy loss. Chapter 3 explored the impact of breed on the bacterial and archaeal populations in the solid, liquid, and epithelial rumen fractions of sheep using 16S rRNA amplicon sequencing. The study found that breed influenced feed efficiency and the rumen bacterial populations, with potential applications for breeding programs aimed at selecting microbiomes that can utilise feed efficiently and produce less methane. The study also found variations in the distribution of bacterial taxa between ruminal fractions, revealing a rumen fraction bias that has implications for sheep rumen sampling techniques. Chapter 4 used PACs to investigate the effect of time off feed (TOF) on methane emissions and employed meta-omics techniques to assess the influence of TOF on rumen bacterial and archaeal communities in pasture-grazed sheep. The study found that TOF can influence methane emissions and the composition of the rumen microbiome, which could have implications for methane-microbiome studies involving animals that spend variable amounts of time off feed. Overall, this thesis showed that the rumen microbiome is influenced by a range of factors such as feed efficiency, breed, ruminal fraction, and time off feed, with potential implications for improving feed conversion efficiency, reducing methane emissions, and optimising rumen sampling techniques.

Chapter 1
Literature Review

Prospects for adjusting rumen microbiome composition for improved productivity & sustainability of sheep production

McLoughlin S[1,2], Spillane C[2,*], Waters SM[1,*]

[1] Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Athenry, Co. Galway, Ireland

[2] Genetics and Biotechnology Laboratory, Agriculture, Food Systems and Bioeconomy Research Centre, Ryan Institute, University of Galway, Ireland

Keywords: Sheep, Rumen Microbiome, Feed efficiency, Methane. (Min.5-Max. 8)

Abstract

Sheep are an integral component of agricultural systems worldwide due to their ability to convert human-inedible plant biomass into valuable products like meat and milk. However, the contribution of livestock production to global anthropogenic greenhouse gas (GHG) emissions is a concern. The efficiency of feed utilisation and the extent of methane (CH_4) production are primarily associated with the metabolic activities of the diverse microbial community inhabiting the rumen, known as the rumen microbiome. Efforts directed towards unravelling the intricate interactions within the rumen microbiome can provide valuable insights into optimising feed efficiency, mitigating CH_4 emissions, and ensuring the long-term sustainability of the sheep sector.

Sheep evolution

The earliest ruminants originated approximately 50 million years ago during the late Eocene period as selenodont Artiodactyls. The evolutionary history within suborder Ruminantia is considered to have involved successive lineages from Hypertragulidae to Tragulidae to Leptomerycidae to Gelocidae to Moschidae, and eventually to horned ruminants (Webb and Taylor, 1980). Today, there are 6 extant ruminant families which include the Tragulidae (e.g chevrotains), Moschidae (e.g muskdeer), Giraffidae (e.g giraffe, okapi), Antilocapridae (e.g pronghorn) (Figure 1.1), Cervidae (e.g deer, moose) and Bovidae (e.g sheep, cattle) (Hackmann and Spain, 2010). Among the ruminant families Bovidae are the most species-rich ruminant subfamily, with 140 species that include common domestic livestock, such as sheep, goats, and cattle (Hackmann and Spain, 2010). The diversification of bovids and the divergence of sheep and goats corresponded with the proliferation of C4 grasses (grasses that utilise C4 photosynthesis) during the late Neogene period (Strömberg, 2011;Jiang et al., 2014). According to archaeological records of ungulate fossils, the sheep and

goat lineages diverged approximately 5-7 million years ago (Savage and Russell, 1983). The Caprinae lineage (including sheep and goat species) split from the Bovinae (Bos Taurus) branch around 15-20 million years ago. More recently, phylogenetic analysis based on single orthologous genes carried out by Delano et al. (2014) indicates that sheep and goats diverged from cattle approximately 19.9 million years ago (Ma) before present (BP), while sheep and goats diverged approximately 4.3 Ma BP (Jiang et al., 2014).

Figure 1.1 Pronghorn-Antelope. Image adapted from (Jones, 2018). Photographer Donald M. Jones

Domestication of sheep

The domestication of sheep is understood to have occurred in the Fertile Crescent region of the Middle East, which includes parts of modern-day Turkey, Iran, and Iraq (Zygoyiannis, 2006). Archaeological evidence suggests that sheep were first domesticated by humans in this region around 10,000-11,000 years ago, making them one of the oldest domesticated livestock (Zeder, 2008). Domesticated sheep are descended from wild Asian mouflon (*Ovis orientalis*) (Rezaei et al., 2010), which were hunted and later tamed by early humans for food and fiber (Fuks and Marom, 2021). Domestication reshaped the morphology, behavior, and genetics of the animals. Today, there are 1,143 different breeds of sheep, (FAO, 2023), each with their own unique characteristics and uses. They are well-suited to a variety of environments, from arid rangelands to lush pastures (Pearce et al., 2010;Arora et al., 2011), and are raised for their wool, meat, and milk in many countries around the world.

Global importance of sheep

Sheep production is a globally significant agricultural activity that plays a vital role in sustaining rural economies and supporting cultures in many regions globally (Ibrahim et al., 2019). Sheep provide highly valued products such as meat, milk, and wool, which are traded and consumed widely (Zygoyiannis,

2006). Between 1998 and 2018, the aggregate global economic output attributable to the sheep industry amounted to an estimated valuation of $34 billion, accounting for 2% of total output value of all farmed animals (Schrobback et al., 2023).

Sheep wool is a highly versatile and sustainable material and was among the earliest commodities to be traded internationally (Rajabinejad et al., 2019). Sheep wool has found applications in various industries such as textiles (Allafi et al., 2022) and construction (Mansour et al., 2016;Alyousef et al., 2022). Sheep are particularly significant for global food security, since as ruminants they are capable of transforming human inedible plant matter into high quality human edible protein products such as meat and milk. These products provide a source of protein, fat (Omega-6 and -3 fatty acids), vitamins (B, E) and minerals (Zn, Fe, and K) (Fowler et al., 2019), which are essential for maintaining human health and wellbeing, and as an energy source.

In recent years, there has been an increase in the volume of meat and milk produced by sheep production systems globally (FAOSTAT, 2022), a trend attributed to the growing global population and rising demand, particularly in emerging nations (Salter, 2017). Global production of sheep meat ranks fourth in the world, following chicken, pig, and beef, and is higher than that of goat meat, with approximately 9.9 million tonnes produced in 2020 (FAOSTAT, 2022). Data obtained from FAOSTAT (2022) indicates a consistent upward trend in the global sheep population over the course of the last two decades. In 2020, the worldwide sheep population reached 1.3 billion, representing a significant increase of 20% compared to the figures recorded in 2000. Furthermore, when compared to 2010, there was a 17% increase in the global sheep population (FAOSTAT, 2022). While sheep farming is a vital component of global agricultural systems, it is especially important in developing nations. According to data from FAOSTAT (2022), approximately 33% and 20% of the global sheep population are located in low-income food deficit and least developed countries (FAOSTAT, 2022). The largest sheep populations are found in Asia and Africa, with China having the largest sheep population globally, with over 173 million heads. The steady increase in the global sheep population underscores the critical importance of sheep production at a global scale, particularly in developing countries, where it plays a significant role in food security and economic stability.

Agriculture and sheep production in Ireland

Agriculture is Ireland’s oldest indigenous industry, and its national importance is evident from the fact that it covers a vast portion of the country’s land area. According to the Central Statistics Office (CSO), in 2018, agriculture occupied 58.4% of Ireland’s total land area (CSO, 2020), which was significantly higher than the EU average of 39.1% for the same year (Eurostat, 2022). The prominence of agriculture in Ireland is due to the favorable temperate climate and rainfall conditions that are suitable for grass growth, facilitating the establishment of animal farming systems that are primarily based on grazing and the production of grass-fed agri-products (O’Mara, 2012). These products have a high demand in the global market, as they are perceived to be healthier,

sustainable, and better for animal welfare when compared to non-grazing systems (Font-i-Furnols and Guerrero, 2014;Prache et al., 2020). As a result, the Irish agri-food sector has become a major contributor to the country's economic growth. The sector provides employment to around 174,000 individuals, which amounts to 7.1% of the country's labor force, according to the Department of Agriculture, Food, and the Marine (DAFM) (DAFM, 2022a). Additionally, the sector serves as a significant source of exports, with Irish agri-food products being exported to over 180 countries worldwide, generating €15.4 billion for the Irish economy in 2021 (DAFM, 2022a). Looking ahead, Ireland has set ambitious targets for the future development of its agri-food sector. As part of its Food Vision 2030 roadmap, Ireland aims to become a global leader in sustainable food production and increase agri-food exports to €21 billion by 2030 (DAFM, 2021). Achieving these goals will require continued investment in the sector, including in research and development, innovation, and sustainable farming practices.

Sheep production is an important component of Ireland's agricultural industry, contributing to the country's economy, generating employment opportunities, and sustaining rural communities (Ryan et al., 2016;O'Mara, 2022). Additionally, sheep farming plays a significant role in the conservation of Ireland's natural landscapes and biodiversity (O'Mara, 2022). Sheep are often used for grazing in areas with diverse and multifunctional ecosystems, such as upland and mountainous regions (O'Rourke et al., 2012). Grazing by sheep helps to maintain these ecosystems by controlling vegetation growth and providing habitats for wildlife. According to the latest report on Ireland's sheep and goat census, the national sheep population rose to 4.02 million in 2021, representing a 3.6% increase from the preceding year (DAFM, 2022b). This figure comprised 2.7 million breeding ewes over 1 year of age and 86,216 breeding rams, while lambs, wethers, and cull ewes accounted for 1.2 million of the national flock. The number of registered holdings also increased by 571 to 36,163 in 2021, compared to the previous year (DAFM, 2022b). The Irish sheep industry is known for producing high-quality, grass-fed lamb, which is sought after by consumers worldwide. In 2021 Ireland sheep meat exports were valued at €420 million, an increase of 15% on the previous year (Bia, 2022).

There are a range of sheep breeds that are used for production in Ireland, which are classified and distributed according to their adaptability to different environmental habitats and farming systems, such as mountain, upland and lowland breeds. For instance, some sheep are more suited to mountain or upland farming systems, while others are more suited to lowland farming systems. The Scottish Blackface breed are typically found along the western mountainous regions of Ireland (Teagasc, 2020). They are a dual-purpose breed that is known for high quality wool and meat, and popular for cross breeding. The Scottish Blackface have a thick heavy fleece that help them withstand harsh weather conditions. The Cheviot sheep breed is also a hill breed. However, while resilient they are not as hardy as the Scottish Blackface. Cheviots are used more in hill regions along the eastern side of the country (Teagasc, 2020) where the weather conditions are less harsh. They are slightly larger than the Scottish Blackface and produce fast maturing lambs (Kirton et al., 1995). The Belclare breed is a popular

composite breed that was developed in Ireland in the 1960s by successive crossbreeding trials with the Finnish landrace, Galway, Lleyn, and Texel breeds (Hanrahan, 1991;Hanrahan et al., 2004;Mullen et al., 2013). Belclares are known for being a docile, easy to manage, highly productive breed that requires minimal maintenance. Figure 1.2 shows three popular sheep breed in Ireland, Scottish Blackface, Cheviot and Belclare.

Figure 1.2 Popular sheep breeds in Ireland. Scottish Blackface (Left) adapted from (Zhou et al., 2019a), Wicklow Cheviot (Middle) adapted from (Geary, 2019), Belclare (Right) adapted from (Merrick, 2022).

Anthropogenic greenhouse gas emissions

In 1856, Eunice Newton Foote conducted a pioneering experiment that demonstrated the greenhouse effect of carbon dioxide, providing empirical evidence that sunlight could heat carbon dioxide to greater temperatures, and retain its heat for a longer period of time than other gases (Foote, 1856). This discovery led to further research and understanding of other gases that could also contribute to what is termed the "greenhouse effect", including CH_4 and nitrous oxide (N_2O) (Weart, 2010). Greenhouse gases (GHGs) are gases in the atmosphere that are able to absorb and emit thermal infrared radiation in the Earth's atmosphere (Cassia et al., 2018). The most important greenhouse gases contributing to planetary warming are carbon dioxide (CO_2), CH_4, N_2O, water vapour (H_2O), and fluorinated gases (such as hydrofluorocarbons and perfluorocarbons). It is important to note that they are essential for maintaining habitable temperatures on Earth (Kweku et al., 2018). Indeed, without atmospheric GHGs the temperature of the Earth would be approximately 33°C cooler (Kweku et al., 2018). GHGs can occur naturally, originating from natural causes such as volcanic eruptions and wildfires, or through human (anthropogenic) activities, such as agriculture, carbon combustion and landfills. For millennia the concentration of GHGs in the Earth's atmosphere have remained relatively stable (EPA, 2022). However, since the beginning of the industrial revolution in the 1700s the concentration of GHGs in the Earth's atmosphere has been steadily increasing as a result of human activity (Figure 1.3) (Letcher, 2021). Prior to the industrial revolution, the level of CO_2 in the atmosphere was circa 280ppm. Today, the concentration of CO_2 in the atmosphere is over 400ppm, which resulted in a 1.5 degree warming of the planet (Letcher, 2021;WMO, 2023). The United Nations (UN) Intergovernmental Panel on Climate Change (IPCC) projects that without major near-term reductions in GHG emission, global temperatures will rise by more than 1.5-2°C by 2100 in

the best case scenario, or >4°C in the worst case scenario (Masson-Delmotte et al., 2021; Lee et al., 2023). The increase in GHG concentration is the major factor in global climate change. Global warming changes weather patterns, while rising CO_2 levels also effects on biological and chemical processes. While climate change is leading to an increased frequency and intensity of adverse weather-related shocks, the impacts of climate change will be unevenly distributed across regions. Precipitation deficits and droughts, resource depletion, rising sea levels, and biodiversity loss are just a few of the impacts associated with climate change (Pörtner et al., 2022), which will have ramifications for the health and well-being, food and water security, and economic prosperity of individuals and nations globally (Masson-Delmotte et al., 2018). There is an urgent need to reduce GHG emissions through sustainable practices and the development of alternative, cleaner energy sources.

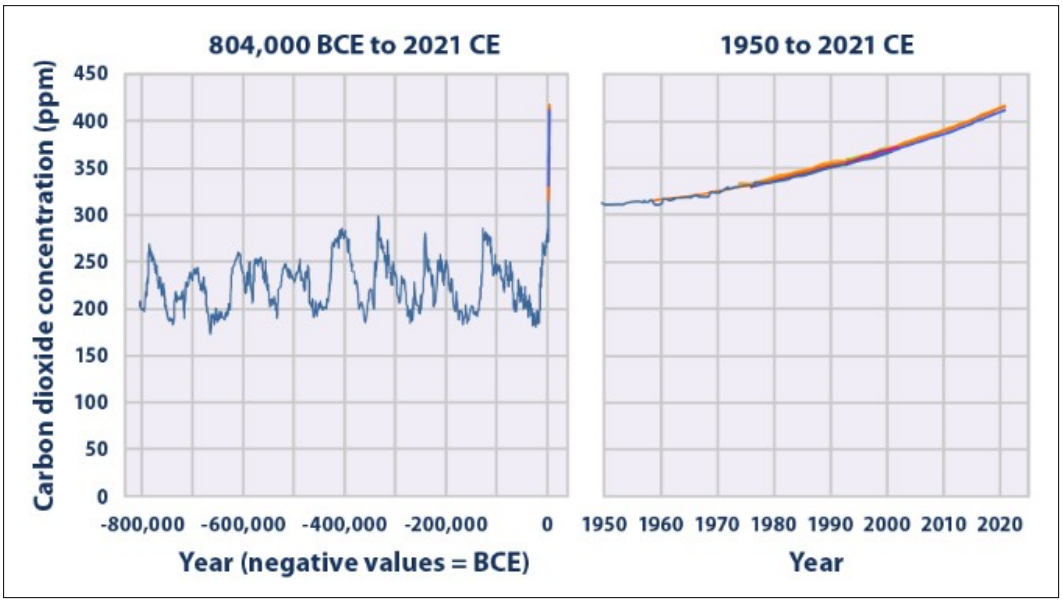


Figure 1.3: Global Atmospheric Concentrations of Carbon Dioxide Over Time (EPA, 2022).

Methane

$CH_4$is a potent greenhouse gas, with a global warming potential approximately 28 times higher than carbon dioxide over a period of 100 years (Liu et al., 2022), making it the second most significant greenhouse gas after carbon dioxide (Pachauri and Meyer, 2014). However, atmospheric CH_4 has a relatively short lifespan of approximately 9.1 ± 0.9 yrs (Prather et al., 2022), before it is eventually oxidised to carbon dioxide and water (Lashof and Ahuja, 1990). CH_4 is released to the atmosphere from sources such as wetlands, extraction and combustion of fossil fuels, the breakdown of organic waste in landfills, and ruminant livestock production. Over the past century, the concentration of CH_4 in the atmosphere has significantly increased, reaching approximately 1920 parts per billion (ppb) in 2022 (Lan, 2023). This represents a doubling of the levels recorded in 1918, which were at 966 ppb (Etheridge et al., 1998). The increase in

atmospheric CH_4 concentrations is largely attributed to anthropogenic activities, including livestock production, and the increasing CH_4 emissions trend is projected to continue (Kleinen et al., 2021). The ongoing increase in CH_4 concentrations in the atmosphere is a cause for concern, as it contributes to the warming of the planet and is a driver of climate change. Reducing CH_4 emissions is a key priority in the fight against climate change (Arias et al., 2021).

International and national greenhouse gas emissions

In 2019, global GHG emissions amounted to a total of 50 billion tonnes (Bt) of CO_2 equivalent. Among the countries with the highest emissions, China was the largest contributor producing 12.06Bt of CO_2 equivalent, followed by the United States (US) with 5.77Bt, India with 3.36Bt and the EU producing 3.15Bt CO_2eq (Figure 1.4). The majority of global GHG emissions are contributed by electricity and heat production (15.83Bt), transport (8.43Bt), manufacturing and construction (6.22Bt) and agriculture (5.80Bt) (Ritchie, 2020).

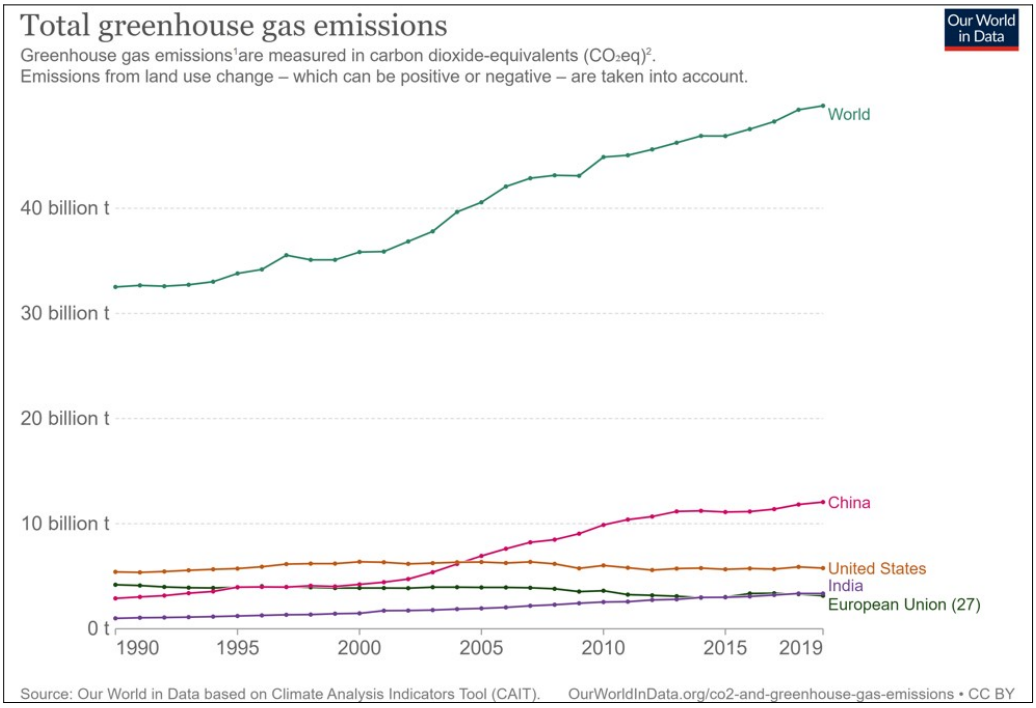


Figure 1.4: Total greenhouse gas emissions (CO_2eq) globally and in selected regions over past three decades (Our World in Data)

In 2019, Ireland generated 59.77 million tonnes (Mt) of CO_2eq of GHGs. Agricultural activities were the largest source of GHG emissions estimated at 21.48Mt of CO_2eq (**Figure 1.5**)., accounting for approximately 36% of national GHG emissions. This was followed by transport (12.237Mt) and energy industries (10.632Mt). Within agriculture, 17.04 and 5.92 Mt of CO_2eq were generated by the release of CH_4 and N_2O, respectively

Specifically, within the livestock sector, pre farm-gate emissions were mainly driven by enteric fermentation and manure management, which together contributed 14.1Mt of CO_2eq (**Figure 1.5**), constituting approximately 65% of total agricultural GHG emissions. Enteric fermentation, primarily occurring in the

digestive systems of ruminant livestock, was the most significant contributor to farm-gate emissions, accounting for 12.2Mt of CO_2eq, or approximately 57% of agricultural emissions (Duffy, 2021) (Figure 1.5).

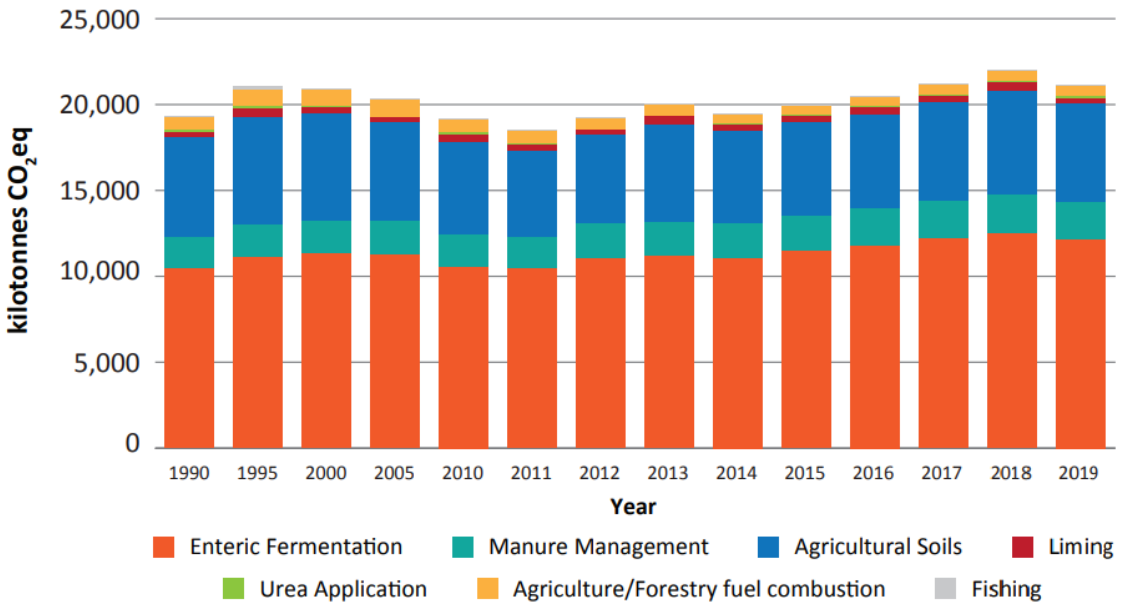


Figure 1.5: Ireland's agricultural GHG emissions from 1990 to 2019. Figure adapted from (EPA, 2021)

Global inter-governmental efforts to reduce greenhouse gas emissions

The Kyoto protocol, adopted in 1997 as part of the United Nations Framework on Climate Change UNFCC, was the first international agreement aimed at limiting GHG emissions to the atmosphere (UNFCC, 1997). Under the Kyoto protocol, participating countries agreed to reduce their GHG emissions by an average of 5% below 1990 levels, during the period from 2008 - 2012 (UNFCC, 1997). A second commitment period known as the Doha Amendment, was agreed in 2012, which committed 37 participating countries to further reduce their emissions by at least 18% below 1990 levels 2020 (period 2013-2020) (UNFCC, 2012;Erbach, 2015). In 2015, the Paris agreement (UNFCC, 2015) was adopted with the aim of strengthening the global response to climate change. The Paris agreement built on the foundations of the Kyoto protocol and laid forth the goal of limiting global temperatures to below 1.5 to 2 degrees Celsius over pre-industrial levels (UNFCC, 2015). It also includes provisions for financial support to reduce GHG emissions and adapt to the negative impacts of climate change (UNFCC, 2015;Horowitz, 2016). As part of the Paris agreement member countries of the UNFCC are required to submit Nationally Determined Contributions (NDCs) outlining their climate action plans, which include emissions reduction targets and strategies. More recently, at the 2021 UNFCCC's COP26, nearly 200 countries pledged to strengthen the fight against climate change, known as the Glasgow pact. The pledge includes a commitment to limit global warming to 1.5

°C, phase out the use of coal worldwide, and increase funding to help developing countries adapt to the effects of climate change (Cifuentes-Faura, 2022).

The European Union has been a leading player in the global fight against climate change and is an active participant in international climate change negotiations (Cifuentes-Faura, 2022). The EU played a key role in the ratification of the Kyoto Protocol in 2002 and the Paris Agreement in 2015. In line with the Paris Agreement targets, the EU has set internal targets to reduce the overall greenhouse gas emissions of its member states. This is supported by the EU's European Green Deal, which is a comprehensive plan launched by the European Commission in December 2019 (European-Commission, 2019). The EU Green Deal aims to reduce GHG emissions by at least 55% by 2030 compared to 1990 levels, and to achieve climate neutrality by 2050 (European-Commission, 2019). The plan includes a wide range of emissions mitigation measures across different sectors, such as energy, transport, agriculture, and buildings, with the ultimate goal of reducing greenhouse gas emissions and achieving a more sustainable and resource-efficient economy (European-Commission, 2019).

Greenhouse gas emissions in Ireland

The Irish government has developed a strategy to reduce GHG emissions and combat climate change, known as the Climate Action Plan and Low Carbon Development Act 2021 (Oireachtas, 2021) (Torney, 2021). The act is legally binding and targets a 51% reduction in GHG emissions by 2030 below 2018 levels, and in line the European green deal to achieve net-zero emissions by 2050 (Oireachtas, 2021). Ireland's Climate Action Plan 2023 (DECC, 2022) includes a wide range of measures across various sectors,ncludeing agriculture, transport, and energy as well as initiatives to increase the use of renewable energy, reduce waste, and promote sustainable practices (DECC, 2022). The plan is a key part of Ireland's commitment to the European Green deal and its efforts to mitigate the impacts of global warming (DECC, 2022). In the context of the agricultural sector, Climate Action Plan specifies that agri-emissions should not exceed 17.25 Mt CO_2eq by the end of 2030, compared to the 2018 baseline of 23 Mt CO_2eq (DECC, 2022). This will require a reduction in emissions of 5.75 Mt, or 25%, compared to 2018 levels. Ireland's Climate Action Plan is focused on implementing a range of measures to reduce GHG emissions from agriculture, such as sustainable land management, reducing the use of chemical nitrogen fertilisers, and improving the efficiency of livestock production (DECC, 2022). Achieving these objectives while sustainably expanding agri-food output by 2030, as outlined in FoodVision 2030, presents a significant challenge for Ireland's agricultural sector. Indeed, according to the Environmental Protection Agency (EPA), Ireland is projected to fall short of its emissions target reductions, with current forecasts suggesting a 29% reduction in greenhouse gas (GHG) emissions by 2030 (EPA, 2023).Nevertheless, reducing CH_4 emissions from livestock production is a key area for achieving both these objectives and the sectors long-term environmental, economic, and social sustainability.

Measuring and reporting of methane emissions

CH_4output is reported in in the literature in a variety of ways, e.g: daily CH_4 emissions (DME) which represents the daily CH_4 emissions in grams (g/day) or litres (l/day); CH_4yield (MY) accounts for the CH_4 emissions per unit of DMI (CH_4 (g)/DMI(kg)); CH_4intensity (MI) accounts for CH_4 per unit of animal product (CH_4 (g)/milk yield(kg) or (CH_4 (g)/carcass weight(kg))) and more recently residual CH_4 emissions (g/day) which is the difference in the animals actual and expected CH_4 output, based on its level of feed intake and body weight (Smith et al., 2022a).

Sheep digestive tract anatomy

The digestive tract of sheep (Figure 1.6) is similar to that of other ruminant animals such as cows, goats, and deer (Samir and Ghadbane, 2021). The evolution of this unique digestive anatomy and its organisation has enabled sheep to efficiently utilise plant matter as a source of energy (Van Soest, 1994). The sheep's digestive tract comprises broadly 4 regions; the mouth or oral cavity, the oesphagus, the stomach and the small and large intestines (Harfoot, 1981). Sheep use their mouths for the process of grazing and masticating their feed. In the mouth feed is mixed with saliva before moving through the digestive tract. The oesophagus is a muscular tube that connects the mouth to the stomach and serves to move the bolus of feed and saliva from the mouth to the stomach via peristaltic contractions (Harfoot, 1981). The stomach of sheep is organised into four compartments: the reticulum, the rumen, the omasum and the abomasum (Akester and Titchen, 1969). The reticulum receives the feed matter coming into the digestive system. It acts like a filter trapping undigested or large feed particles and compacting them into cuds which are later regurgitated and chewed again to aid digestion (Sejian et al., 2017). The reticulum is closely associated with the rumen, and the two compartments are often referred together as the reticulo-rumen (Sejian et al., 2017). The rumen is the largest compartment of the sheep's stomach and serves as the primary site for feed digestion (Samir and Ghadbane, 2021). The rumen contracts to move solid and liquid contents to subsequent chambers of the stomach and lower intestines. The omasum is lined with many thin folded plates that help strain the fluids and breakdown feed particles. The abomasum is similar to the stomach of monogastric animals (referred to as the 'true stomach'), it secretes gastric acids and enzymes that breakdown food further (Fenchel et al., 2012). The intestines function to absorb nutrients into the bloodstream, facilitated by the presence of villi and microvilli along the intestinal lining, which significantly increases the surface area available for nutrient absorption. The large intestine is responsible for the reabsorption of water and electrolytes and the formation of faeces.

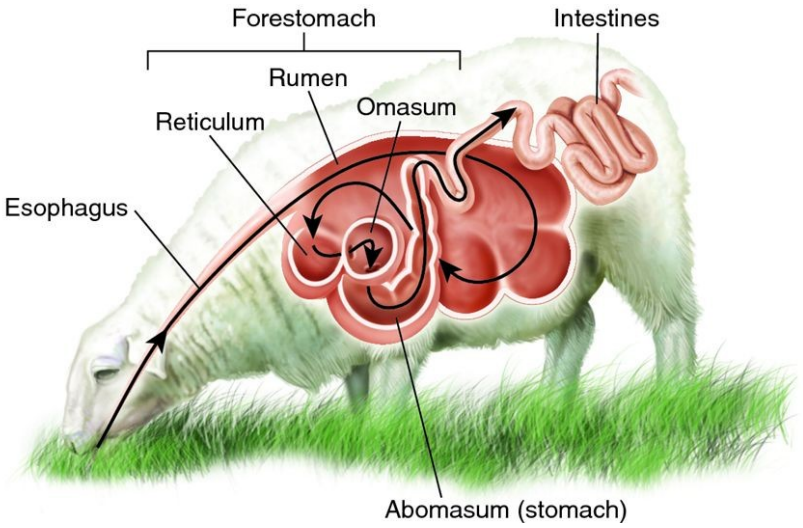


Figure 1.6: Illustration of the Digestive tract of sheep. Sourced From: https://australiansheepenterprise.weebly.com/digestive-system.html

The sheep rumen

The rumen is the largest compartment of the stomach and is a warm (38.4–39.8°C), anoxic and weakly acidic environment (Zhang et al., 2017a;Williams et al., 2020) conducive to the growth of anaerobic microorganisms, which the host depends for the digestion of cellulose-rich diets (Hungate, 1975). The anaerobic environment is crucial to the host because it limits the complete oxidation of carbohydrates to carbon dioxide and water (Ungerfeld, 2020). Instead, the carbohydrates are partially oxidised to volatile fatty acids (VFAs) and fermentative gases by ruminal microorganisms, which are important for the host's metabolic processes (Ungerfeld, 2020). The lining of the rumen is composed of stratified squamous epithelium through which VFAs can be readily absorbed. Papillae extend into the lumen and enhance nutrient absorption by increasing the overall surface area of the rumen epithelium (Harfoot, 1981). The rumen absorbs around 75% of VFA, with less than 10% entering the small intestine (Church, 1979;Harfoot, 1981).

The rumen harbours one of the most complex and diverse microbial ecosystems in the animal kingdom, comprising bacteria, protozoa, archaea, fungi, and viruses/bacteriophages (Sirohi et al., 2012;Newbold and Ramos-Morales, 2020). These microorganisms coexist in the rumen and engage in mutually beneficial interactions with the host and with each other (Singh et al., 2019). The microbial community, their genomes, and the rumen environment are collectively known as the rumen microbiome (Marchesi and Ravel, 2015). The rumen microbiome plays a vital role in the host's digestive system by encoding the necessary enzymes for the digestion and fermentation of complex structural polysaccharides, such as cellulose, hemicellulose, xylan, pectin, and starch (Stewart et al., 2019; Newbold and Ramos-Morales, 2020). The breakdown of these plant polymers into constituent monomers and oligomers facilitates

subsequent fermentation by resident microbes, generating volatile fatty acids (VFAs) such as acetate, butyrate, and propionate, as well as microbial protein (Hungate, 1975). VFAs serve as the primary energy source for the host (Bergman, 1990). Additionally, fermentative gases such as $CO_2$and CH_4 are produced, and are mainly eructated from the rumen (Hungate, 1975).

Rumen bacteria

Bacteria are the most abundant and diverse group of microorganisms present in the rumen. Their numbers are estimated to be approximately $10^{10\text{-}11}$ cells/ml of rumen fluid (Matthews et al., 2019). Bacteria present in the rumen are categorised according to the rumen fraction with which they are associated, i.e. solid-, liquid-, and epithelial- fractions (Li et al., 2020; Ren et al., 2020). Solid associated bacteria form attachments to particulate matter in the rumen and play an important role in fiber digestion (McAllister et al., 1994). Liquid associated community are typically free-floating planktonic bacteria involved in the metabolism of soluble nutrients present in the rumen fluid. In addition, the rumen fluid serves as a medium for solid adherent bacteria to travel to newly ingested feed (De Mulder et al., 2017). The epithelial associated bacteria attach to the epithelial lining of the rumen and are involved in oxygen scavenging, urea hydrolysis and epithelial cell turnover (Cheng et al., 1979; De Mulder et al., 2017). Bacteria in the rumen can be classified based on the substrates they utilise for growth or the products they produce during fermentation (Cammack et al., 2018). For example, bacteria can be classified as cellulolytic or non-cellulolytic based on their ability to break down cellulose. Other classifications include hemicellulolytic, amylolytic, proteolytic, and lipolytic, which indicate the ability of bacteria to digest hemicellulose, starch, protein, and lipids, respectively. In addition, bacteria can also be classified based on the fermentation products they produce. For instance, some bacteria produce acetate, propionate, and butyrate as primary fermentation end products, while others produce lactate, formate, and succinate.

The most prevalent bacterial phyla in the sheep rumen are Firmicutes and Bacteroidota (formerly Bacteroidetes) (Henderson et al., 2015). Firmicutes is a large phylum of Gram-positive bacteria, dominated by the families *Ruminococcaceae* and *Lachnospiraceae* within the rumen (Henderson et al., 2015;Martinez Boggio et al., 2023). These families include some of the most efficient cellulolytic and hemicellulolytic degraders such as *Ruminococcus albus*, *Ruminococcus flavefacians* (Koike and Kobayashi, 2001), *Eubacterium cellulosolvens* (Anderson and Blair, 1996), and *Butyrivibrio fibrisolvens* (Hernáez et al., 2018) (Figure 1.7). Bacteroidota is another large phylum composed mainly of Gram-negative bacteria and dominated by the *Prevotellaceae* family in the rumen (Henderson et al., 2015;Martinez Boggio et al., 2023), which is primarily driven by the abundance of the genus *Prevotella* (Henderson et al., 2015). Findings from the global rumen census (GRC) indicate that *Prevotella* accounted for approximately 21.5% of the microbial community in the rumen sheep, followed by unclassified Clostridiales (16.5%) and *Ruminococcaceae* (10.0%)

unclassified Bacteroidales (8.7%), unclassified *Lachnospiraceae* (6.0%), *Ruminococcus* (3.5%), *Butyrivibrio* (3.3%), unclassified *Veillonellaceae* (2.5%), and *Fibrobacter* (1.9%). Collectively, these bacterial groups represent the core microbiome in sheep (Henderson et al., 2015) (Figure 1.8).

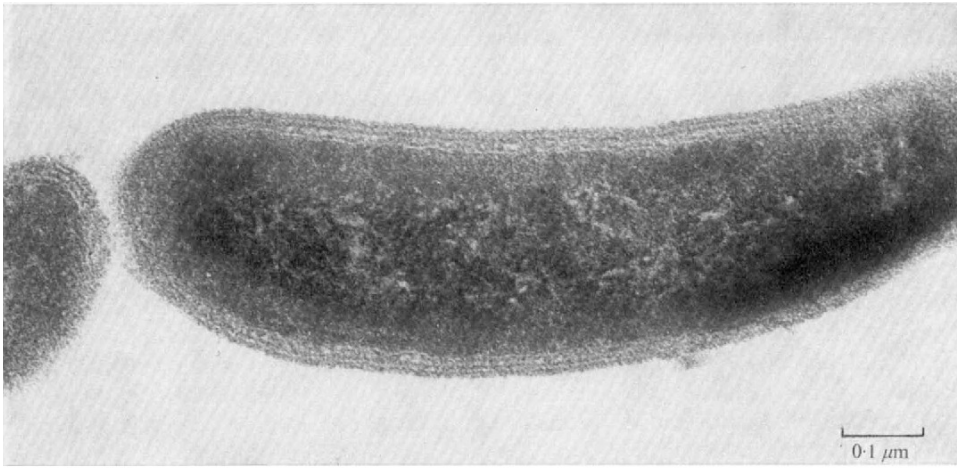


Figure 1.7: Electron micrograph image of sections of *Butyrivibrio fibrisolvens*. Image adapted from (Sharpe et al., 1975) and credited to Dr B. E. Brooker and Mr D. Hobbs.

Rumen fungi

Anaerobic fungi (AF) were first discovered in the rumen by Colin Orpin in the mid 1970s (Orpin, 1976). AF are classified under the phylum Neocallimastigomycota (Yücel and Ekİncİ, 2022) and are estimated to comprise approximately 10-20% of the microbial biomass in the rumen (Rezaeian et al., 2004; Chaucheyras-Durand and Ossa, 2014), with concentrations ranging from 10^2 to 10^4 mL^{-1} of rumen fluid (Singh et al., 2019). The phylum Neocallimastigomycota, which constitutes the class Neocallimastigomycetes, order Neocallimastigales and family Neocallimastigaceae, currently comprises 18 genera *Neocallimastix, Piromyces, Caecomyces, Agriosomyces, Aklioshbomyces, Buwchfawromyces, Capellomyces, Feramyces, Ghazallomyces, Joblinomyces., Liebetanzomyces, Khoyollomyces, Pecoramyces, Tahromyces, Aestipascuomyces, Orpinomyces, Anaeromyces,* and *Cyllamyces* (Bhagat et al. 2023).

AF residing in the rumen lack essential components such as mitochondria and cytochromes necessary for oxidative phosphorylation. Nonetheless, they possess distinctive organelles known as hydrogenosomes, which are vital for generating cellular energy under the anaerobic conditions of the rumen (da Silva et al., 2017; St-Pierre et al., 2018). AF propagate through the generation of zoospores, motile, flagellated spores capable of dispersing throughout the rumen environment. Although zoospores can remain mobile for hours, they typically attach to feed quickly and shed their flagella. Subsequently, these zoospores germinate, giving rise to a fungal thallus comprised of sporangium and a filamentous rhizomycelium or a bulbous holdfast, initiating fungal growth and proliferation (Hess et al. 2020).

AF play a key role in the ruminal degradation of plant matter in the rumen (Rabee et al., 2019). During growth, AF produce rhizoids, specialized thread-like structures capable of penetrating structural components of plant cell walls. This process is known to physically rupture lignocellulosic tissues, thereby increasing the surface area of plant matter. Consequently, this facilitates enhanced microbial colonization and promotes efficient forage utilization (Akin and Borneman, 1990). In addition, AF exhibiting a notable capacity to produce a diverse array of potent enzymes involved in the hydrolysis of cellulose and hemicellulose, including xylanases, endoglucanases, and cellobiohydrolases (Akin and Borneman, 1990; Wood et al., 1995; Gruninger et al., 2014). The primary end-products of AF fermentation process include H_2, CO_2, formate, lactate, succinate, ethanol, and acetate, derived from carbohydrate fermentation (da Silva, Pedezzi, and Souto, 2017). In addition, AF also play an important role in the process of methanogenesis. Indeed, methanogens have been observed via electron microscopy attached to the rhizoids of AF (Jin et al. 2011). Furthermore, co-cultures of methanogens and AF exhibited a notable decrease in H_2 levels and the presence of CH_4 compared to pure cultures of AF alone, suggesting the occurrence of interspecies hydrogen transfer.Rumen Protozoa

Protozoa are the largest of the microorganisms in the rumen, with cell sizes ranging from 10x20 – 10x200µm (Millen et al., 2016). As a result they can account for as much as 50% of total microbial biomass, despite being less numerous in terms of cell numbers than other microbial groups (Choudhury et al., 2015). Their cell numbers are estimated in the region of 10^{4-6} per mL of rumen fluid (Choudhury et al., 2015; Singh et al., 2019). Protozoa are classified as ciliates or flagellates, based on the presence of either flagella or cilia (Millen et al., 2016). Ciliate protozoa make up the majority of the protozoan community in the rumen and belong to two taxonomical groups: entodiniomorphs and holotrichs (Leschine, 1995;Millen et al., 2016).

Protozoa play important roles in the digestion and fermentation of feed in the rumen (Millen et al., 2016;Williams et al., 2020), and produce a variety of VFAs that provided energy to the host (Morgavi et al., 2010). Like fungi, protozoa also possess hydrogenosomes and can produce high amounts of hydrogen during metabolism (Williams and Coleman, 1997; Morgavi et al., 2010). They are known to engage in mutualistic interactions with archaea, whereby they are involved in interspecies hydrogen transfer that helps sustain a community of endobiont and ectobiont-associated archaea (Tan et al., 2020). In addition, protozoa are known to exhibit predatory behavior on bacteria, archaea and fungi in the rumen (Williams et al., 2020; Solomon and Jami, 2021). This behavior has been linked to horizontal gene transfer (HGT), which has been suggested to enhance the fibrolytic capabilities of rumen protozoa (Ricard et al., 2006).

Rumen archaea

Rumen archaea make up approximately ~2-4% (of cell number) of the microbial community present in the rumen (Millen et al., 2016). Similar to bacteria, rumen archaea have also known to associate with different fraction of the rumen (i.e solid, liquid and epithelium) (Morgavi et al., 2010). In addition, rumen archaea

also have a protozoal associated community, and are known to engage in endo- and ecto-symbiotic relationships with rumen protozoa (Williams and Coleman, 1997). While archaea appear morphologically similar to bacteria, they are metabolically distinct (Millen et al., 2016). The majority of rumen archaea are methanogenic, meaning they produce CH_4as a product of their metabolism (Morgavi et al., 2010). There are 3 broad groups of methanogens found in the rumen and are categorised based on the substrates they use during methanogenesis. Most methanogens are hydrogenotrophic, in that they reduce carbon dioxide with electrons from H_2 and formate for metabolism. Other methanogens are methylotrophic which utilise methyl compounds during methanogenesis. A few species are acetoclastic methanogens which utilise acetate for the methanogenesis. Methanogens play an important role in preventing the accumulation of H_2, which is produced during the fermentation process and maintain a balanced rumen ecosystem. Indeed, all three known pathways of methanogenesis rely on the availability of H_2 as a substrate (Morgavi et al., 2010). According to the global rumen census *Methanobrevibacter gottschalkii* are the most dominant methanogen accounting for on average 54% of the total archaeal community in sheep, followed by *Methanobrevibacter ruminantium* (25.7%) and *Methanosphaera sp. ISO3-F5* (7.3%) (Henderson et al., 2015) (Figure 1.8).

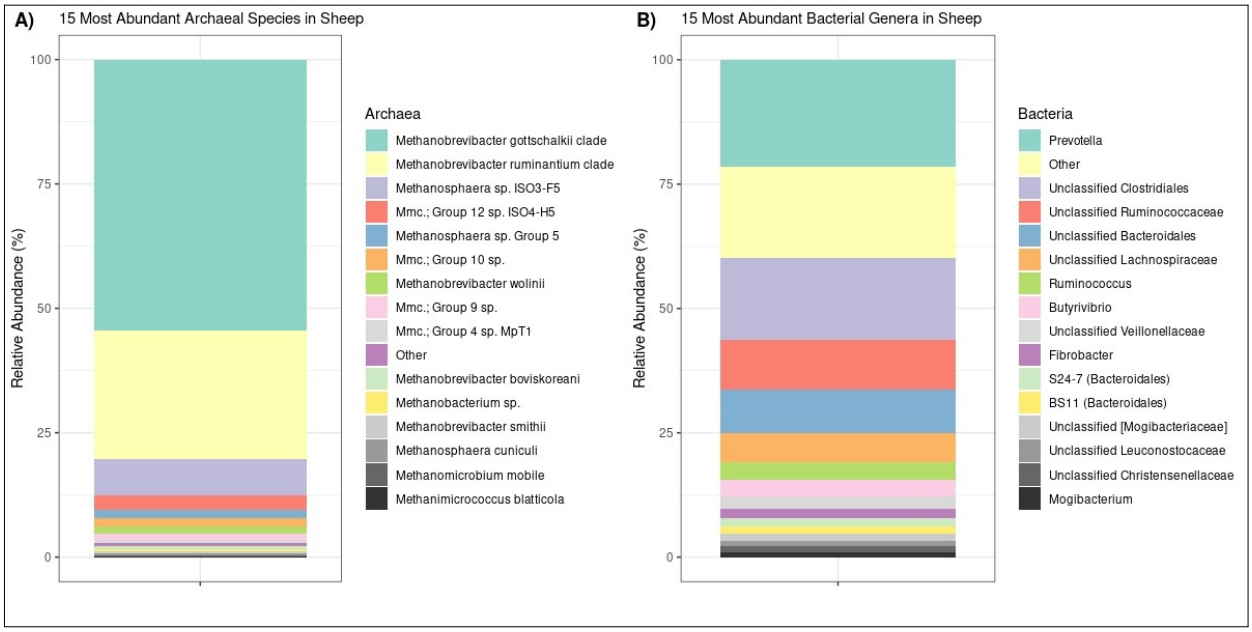


Figure 1.8: Stacked barcharts representing the relative abundance of the top 15 archaeal species (A) and bacterial genera (B) in sheep. Data used to generate this figure was obtained from the global rumen census (Henderson et al., 2015).

Microbial digestion and fermentation of plant matter

Plant cell walls are complex structures that provide structural support and protection to the plant. They are composed of various polysaccharides such as cellulose, hemicellulose, lignin, and pectin (Terry et al., 2019), with cellulose being the most abundant (Morrison, 1979). Lignin and hemicellulose form a matrix around the cellulose strands, which helps to protect it from degradation (Morrison, 1979). While rumen microorganisms such as bacteria, fungi, and protozoa are capable of breaking down hemicellulose, cellulose, and pectin, they are unable to degrade lignin due to its complex structure and resistance to enzymatic breakdown (Samir and Ghadbane, 2021). As the lignin content in plant material increases with maturity, the rate of degradation by rumen microorganisms decreases. Mastication mechanically damages plant fibers, exposing the inner structures and facilitating microbial colonisation when the feed enters the rumen (Terry et al., 2019).

Upon entering the rumen, the newly ingested plant matter undergoes a series of microbial colonisation events which lead to the gradual breakdown of organic matter over time, as indicated by *in sacco* studies (Huws et al., 2015). Primary phase colonisers rapidly attach to the surface of the ingested plant material and begin metabolizing the readily available nutrients. During the secondary phase of microbial colonisation, the microbial population undergoes a shift towards specialised microorganisms that are capable of breaking down the more complex structural components of plant cell walls (Huws et al., 2016). During the secondary phase, cellulolytic and hemicellulolytic microorganisms form attachments to feed particles (Huws et al., 2016), and produce a vast array of hydrolytic enzymes or enzyme complexes (e.g β-1,4-glucanase, cellobiohydrolase and β-glucosidase) that depolymerize the structural polysaccharides into constituent hexose and pentose sugars, such as glucose, xylose, and arabinose. These sugars represent essential substrates for microbial metabolism and fermentation within the rumen and the provision of energy to the host animal in the form of VFAs and microbial protein.

Glucose is the monomeric constituent of cellulose (and of starch) and is metabolised by rumen microbes via several different pathways (Hackmann et al., 2017). One of the most well-documented pathways is the Embden-Meyerhof-Parnas (EMP) pathway, which successively reduces glucose to pyruvate (Hackmann et al., 2017). Pyruvate is a crucial intermediate metabolite in the rumen, serving as a central branching point for the formation of various VFAs. Pyruvate undergoes oxidative decarboxylation to acetyl-CoA which can subsequently be converted to acetate. Alternatively, two molecules of acetyl-CoA can be used to form acetoacetyl-CoA which is the precursor to butyrate formation. In contrast, pyruvate can be metabolised to lactate or succinate, which are subsequently converted to propionate (Beauchemin et al., 2022a). The production of acetate and butyrate in the rumen is reported to result in a net

production of hydrogen, while the production of propionate results in a net use of hydrogen.

Hydrogen metabolism plays a central role in the energy metabolism of the rumen (Beauchemin et al., 2022a). Metabolic hydrogen is produced during the conversion of glucose to pyruvate through glycolysis and the subsequent oxidative decarboxylation of pyruvate to acetyl-CoA (Beauchemin et al., 2022a). This process results in the reduction of electron carriers NAD+ and ferredoxins, which must be re-oxidised to maintain the fermentation process. The re-oxidation of these electron carriers is facilitated by hydrogenase enzymes, which transfer electrons to H^+ or CO_2 to produce dihydrogen or formate, respectively (Ungerfeld, 2020). However, the accumulation of hydrogen in the rumen can limit the activity of hydrogenase enzymes and impede fermentation (Satyanagalakshmi et al., 2015). To dispose of hydrogen and maintain low rumen hydrogen levels, the rumen microbiome has evolved several hydrogen-incorporating pathways or "hydrogen sinks", including propionate production, nitrate or nitrite reduction to form ammonia, sulfate reduction to form hydrogen sulfide, and reductive acetogenesis that converts hydrogen and carbon dioxide to form acetate (Ungerfeld, 2020;Beauchemin et al., 2022a). However, the most significant hydrogen sink in the rumen is methanogenesis carried out by rumen methanogens (Figure 1.9).

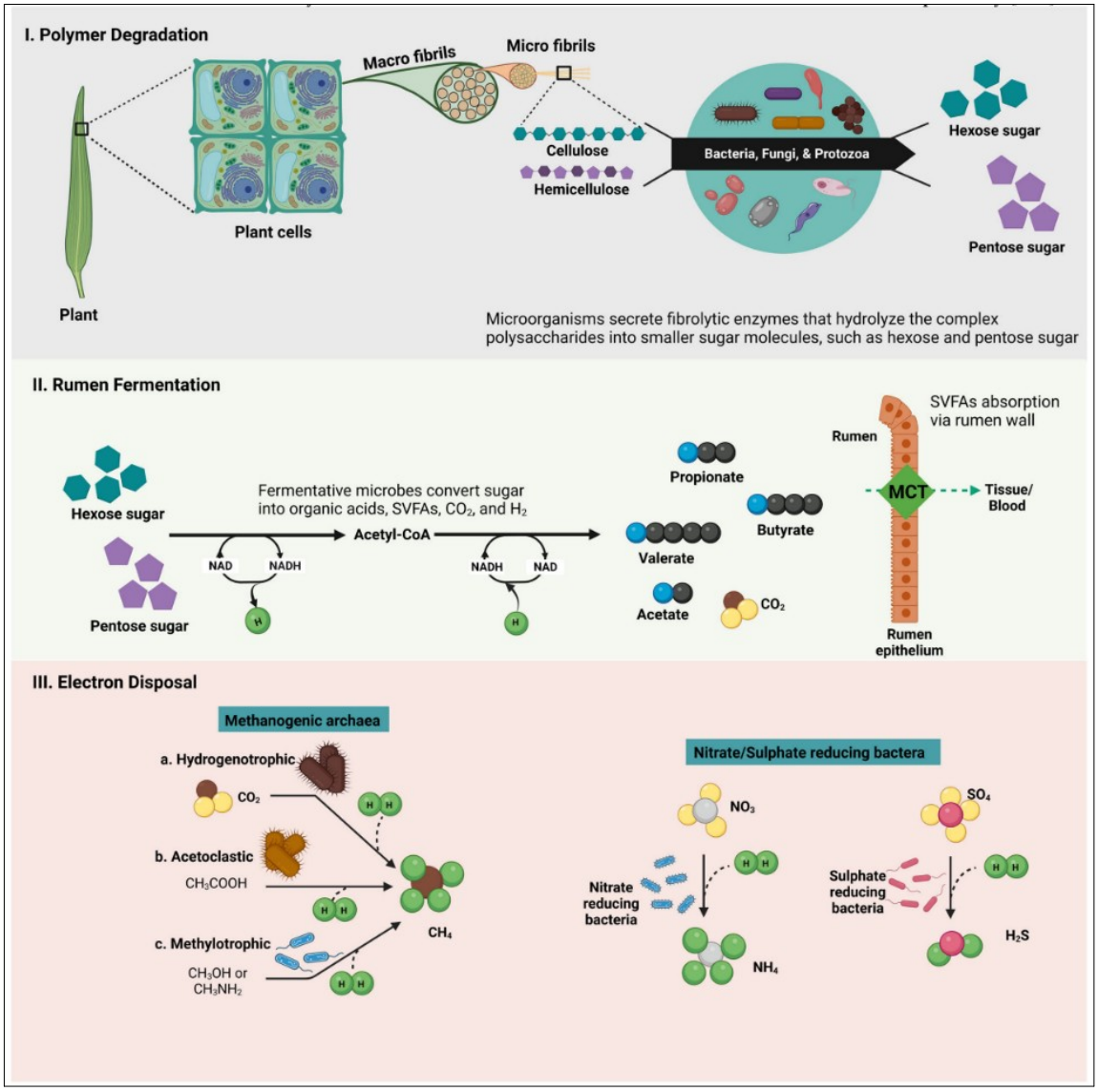


Figure 1.9: Microbial digestion, fermentation and hydrogen disposal in the rumen: polysaccharide degradation (I), rumen fermentation (II), and electron disposal (III). Coloured circles: dark gray is methyl groups, blue is carboxyl groups, brown is carbon, yellow is oxygen, green is hydrogen, light grey is nitrogen and pink is sulfur. SVFA: Short volatile fatty acids. MCT: monocarboxylate transporter. Figure and legend sourced from (Sanjorjo et al., 2023)

Methanogenesis pathways

CH_4 production in the rumen occurs primarily through three distinct methanogenic pathways (Figure 1.10), namely, hydrogenotrophic, methylotrophic, and acetoclastic pathways (Liu and Whitman, 2008). The hydrogenotrophic pathway, which involves the reduction of carbon dioxide using electrons derived from hydrogen or formate, is the central route for CH_4 synthesis within the rumen (Kurth et al., 2020).

The hydrogenotrophic pathway is a seven step metabolic cascade which begins with formate-dehydrogenase catalysing the reduction of CO_2 bound to methanofuran using electrons from reduced ferredoxin, forming formylmethanofuran (Wagner et al., 2018). Next, formyltransferase cleaves and transfers the formyl-group from formylmethanofuran to tetrahydromethanopterin forming formyl-tetrahydromethanopterin. Step 3 is catalysed by methenyltetrahydromethanopterin cyclohydrolase, which converts formyltetrahydromethanopterin to methenyltetrahydromethanopterin (Wagner et al., 2018). Step 4 is catalysed by 5,10-methylene-tetrahydromethanopterin dehydrogenase, which reduces the methenyl group to a methylene group forming methylene-tetrahydromethanopterin utilising electrons supplied by F_{420} (Goldman et al., 2009). Alternatively, the methenyl group can be reduced to a methylene group using electrons from H_2 which is catalyzed by H_2-forming methylenetetrahydromethanopterin dehydrogenase (Goldman et al., 2009). Step 5 is catalysed by methylene-tetrahydromethanopterin reductase, which reduces the methylene group to a methyl group using electrons from F_{420}, resulting in the formation of methyl-tetrahydromethanopterin. Step 6 is catalysed by a methyltransferase complex (MtrA-H), which transfers the methyl group from methyl-tetrahydromethanopterin to coenzyme M (CoM-SH), forming methyl-S-CoM. Finally, methyl-S-CoM is reduced with coenzyme B (CoB-SH) to CH_4 by methyl-coenzyme M reductase (MCR) (Wagner et al., 2018).

Methylotrophic methanogenesis from methanol is catalyzed by methanol coenzyme M methyltransferase, which transfers of the methyl group from methanol to coenzyme M, forming methyl-coenzyme M. Methyl-coenzyme M enters the central methanogenesis pathway and is subsequently reduced to CH_4 by the enzyme methyl-coenzyme M reductase (Thauer et al., 2008).

During acetoclastic methanogenesis acetate is converted to acetyl-CoA by acetyl-CoA synthetase. Acetyl-CoA is then dismuted to form a carboxyl group and a methyl group by acetyl-CoA decarbonylse/synthase complex. The carboxyl group is oxidised to form carbon dioxide and the methyl group is enters the central methanogenesis pathways and is reduced to CH_4 by methy-coenzyme M reductase (Kurth et al., 2020).

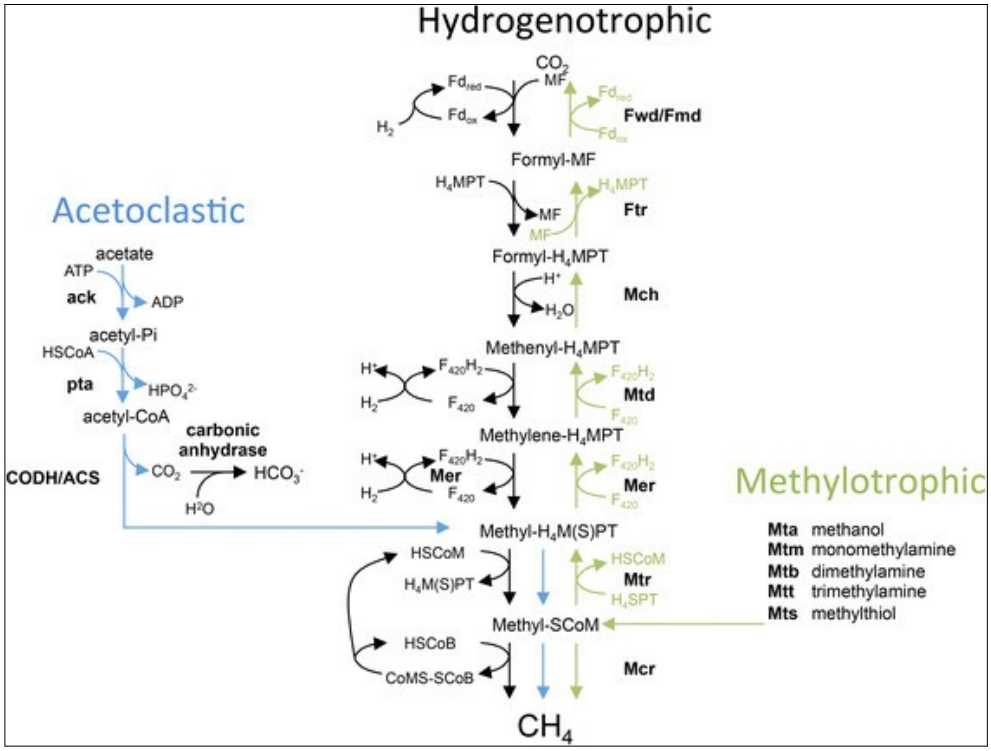


Figure 1.10: Depiction of the acetoclastic, hydrogenotrophic and methylotrophic methanogenesis pathways which were inferred from the genome of Methanosarcina barkeri CM1. Figure and legend sourced from (Lambie et al., 2015).

Methods for measuring methane emissions from sheep production

Accurate quantification of CH_4 emissions is critical for reducing CH_4 from sheep production systems and forming the basis for national inventories and mitigation strategies (Hill et al., 2016;Patra, 2016). Several technologies have been used to measure CH_4 emissions from sheep, including respiration chambers (RCs), portable accumulation chambers (PACs), sulphur hexafluoride (SF_6) tracers and GreenFeeds (GF) (Hammond et al., 2015). However, these methods differ in their scope and application, and each has its own advantages and disadvantages (Storm et al., 2012;Hammond et al., 2016). It is important to carefully evaluate which method is most appropriate for a particular study as no one method is suitable for every research problem (Hammond et al., 2016). A brief description of the above-mentioned techniques and their advantages and disadvantages is outlined below.

Respiration chambers

RCs have been used to measure CH_4 emissions from livestock for over 120 years (Hammond et al., 2016) and fall under two system categories: open-circuit and closed-circuit systems, with the former most frequently used in ruminant research (Storm et al., 2012). The usage of RCs involves holding animals for a period of day(s) in a large, airtight, and environmentally-controlled chamber

under slightly negative atmospheric pressure (Pickering et al., 2015; Hammond et al., 2016). The basic idea behind RCs is that outside air is drawn into the chamber via inlet air vent at the bottom of the chamber and mixes with the gases emitted from the animal inside the chamber. Air is drawn out of the chamber through an outlet air vent at the top of the chamber while sampling inlet and outlet air for gas analysis (Hammond et al., 2016). Flow rate is measured using a flow meter and gas concentrations are measured at specific and regular time intervals (e.g 12 mins) using gas sensors (Waghorn, 2014). CH_4 emissions are estimated multiplying the airflow through the system by the difference in the concentration inlet and outlet gases (Storm et al., 2012; Hammond et al., 2016; Zhao et al., 2020). Importantly, gas concentration and flow measurements are required to be corrected to a standard temperature and pressure and account for humidity which can affect emissions (Hammond et al., 2016). RCs quantify all gases emitted from animals, including those exhaled, eructated, and released through flatulence. When operated properly (Hristov et al., 2018), RCs are considered the gold standard for estimating CH_4 emission (Pickering et al., 2015; Hammond et al., 2016), due to the precision of measurements and the ability to account for feed intake (Waghorn, 2014). However, RCs have a number of drawbacks. They are limited in the use for large-scale research due to low throughput and high costs and do not measure the gases in animals under their natural conditions (Bhatta and Enishi, 2007; Garnsworthy et al., 2019). Furthermore, the use of RCs may result in a reduction of feed intake and alteration of feeding behavior in animals, which can lead to an underestimation of daily CH_4 emissions compared to what would be produced under normal conditions (Bickell et al., 2014).

Portable accumulation chambers

PACs were developed by Goopy et al. (2011) as a means to measure gaseous emissions from sheep (Goopy et al., 2011). The use of PACs involves enclosing an animal in a compact air-tight transportable chamber for a short period of time (~50mins-1hr) (Jonker et al., 2018;O’ Connor et al., 2021) (Figure 1.11). Gases, including CH_4 accumulate within the chamber and concentration readings are taken at regular intervals using a monitoring device attached to a one-way valve (Jonker et al., 2018). The CH_4 production is calculated by multiplying the concentration of CH_4 (corrected for background CH_4) by the net chamber volume (total chamber volume minus the live weight of the animal) (Goopy et al., 2011). Although measurements from PACs are short-term spot samples they have been shown to be repeatable and heritable (Goopy et al., 2015) and found to correlate well with RCs. Goopy et al. (2013) reported moderately-high correlations between CH_4 measurements taken in PACs for one hour and those taken in RCs over three days (r=0.71), one day (r=0.67) and one hour (r=0.69). In comparison to RCs, PACs have proven a cost-effective method of measuring CH_4 emissions, ranking animals and are suitable for on-farm use.Also PACs allow for the measurement of multiple animals simultaneously facilitating large-scale research (Goopy et al., 2011; Jonker et al., 2018; O’ Connor et al., 2021). While a single PAC can cost as much as €80,000 and a single RC €50,000-60,000 (Cummins et al. 2022), PACs have a much higher throughput, with animals only required to

spend 50mins in the chamber when compared to days with RCs. A major drawback of PACs is that they cannot capture fluctuations in daily CH_4 emissions, cannot measure CH_4 per unit of feed intake. Despite limitations PACs can still prove valuable for ranking animals for breeding studies aimed at reducing CH_4 emissions.

Figure 1.11: Portable accumulation chambers (PACs) used to measure methane emissions from sheep. Image sourced from (Teagasc, 2017)

SF_6 tracer technique

SF_6 tracers were developed by Patrick Zimmerman in 1993 to measure CH_4 emissions from cattle (Zimmerman, 1993) and was later adapted for use in sheep. The SF_6 system consists a small brass permeation tube that contains liquid SF_6 and a sampling capillary line/tubing fitted from the nostrils to a pre-evacuated canister harnessed to the sheep back (Berndt et al., 2014; Hammond et al., 2016). The permeation tube is administered orally into the animal's rumen and is designed to release the SF_6 gas at a relatively stable and constant rate. Breath samples are continuously collected via the sample line into the pre-evacuated canisters (Hammond et al., 2016). CH_4 output is calculated by multiplying the CH_4:SF_6 ratio by the SF_6 permeation rate (Berends et al., 2014). Calibration of permeation rate pre- and post-sampling and adjustment of the CH_4:SF_6 ratio for background concentrations of CH_4 and SF_6 are conducted and critical to ensure accurate estimations (Della Rosa et al., 2021). SF_6 is advantageous as animals are not confined to a chamber (Garnsworthy et al., 2019), which allows CH_4 emissions to be measured from grazing animals. Moreover, relative to RCs they are less expensive and are suitable for large scale

studies (Garnsworthy et al., 2019). Despite its usefulness in quantifying enteric CH_4 emissions using the SF_6 tracer technique has several limitations. Firstly, the method does not capture all of the CH_4 emitted from the animal (Bhatta and Enishi, 2007), which can result in the underestimation of total emissions. Additionally, the rate of SF_6release from the permeation tube can decline over time, potentially leading to errors in the data (Hammond et al., 2016). Furthermore, high variability of measurements within and between animals, interference with grazing behavior (Hammond et al., 2016), the fact that SF_6has a powerful global warming potential (GWP^{100}) of 23,500 are among other drawbacks of the technique (Bhatta and Enishi, 2007).

GreenFeed

The GreenFeed (GF) system (C-Lock Inc., Rapid City, South Dakota, USA) is a portable standalone unit that consists of a head chamber, a feeding dish, and an automated diet feeder linked to radio frequency identification (RFID) sensors to identify individual animals using RFID tags. The system dispenses feed pellets into the feeding tray, which is used as 'bait' to attract animals to the GF and encourage them to remain at the unit to allow exhaled gases to be sampled. An extractor fan at the top of the unit draws exhaled gases and air through the system and detected using sensors. Integration of air flow, gas concentrations, and muzzle position are used to measure CH_4 and CO_2 fluxes (Huhtanen et al., 2015). Breath samples are obtained from individual animals throughout the day, each time the animal visits the unit, and are generally measured over a 3-7 minute period (Hammond et al., 2016). GF systems are a reliable and useful way to collect methane emissions from either housed or grazing animals and are less expensive that RCs. However, a primary limitation of the GF systems is that it is dependent on animals voluntarily visiting the system, and animals may need training to use the system (Garnsworthy et al., 2019).

Research techniques for understanding the rumen microbiome

Traditionally, the rumen microbiome was studied using culture-based approaches pioneered by Robert Hungate (Krause et al., 2013), who developed procedures for isolating and cultivating anaerobic microorganisms present in the rumen. Over the years culture-based microbiology has provided a wealth of information and transformed our understanding of rumen ecology (Annison and Bryden, 1998), helping describe some of the most abundant and diverse bacterial members present in the rumen (Newbold and Ramos-Morales, 2020). Typically culture-based methodologies involve growing microorganisms in broths or on agar plates, isolating pure colonies after a period of incubation, and using biochemical tests for classification (Gupta et al., 2019). However, a major recognised limitation of culture-based microbiology is that a large proportion of rumen microorganisms that are not amenable to laboratory cultivation techniques (Krause et al., 2013). Indeed, Staley and Konopka (1985) observed that when complex microbial communities from natural environments were examined via microscopy, the number of viable cell colonies on agar were

substantially lower than the number visible under the microscope, which became known as the great plate count anomaly (Staley and Konopka, 1985). Given the rumen microbiome's immense diversity and complexity, culture-based techniques alone are insufficient for understanding the complexity of the rumen microbiome in terms of community composition, function, and interactions (Morgavi et al., 2013).

The advent of next-generation sequencing (NGS) technologies has revolutionised the study of the rumen microbiome by allowing high-throughput parallel sequencing of the genetic content contained within microbial communities and overcoming the limitations of culture-based techniques (Morgavi et al., 2013). NGS technologies have revealed a much higher level of microbial diversity, including previously uncultured microorganisms that constitute a significant portion of the rumen microbiome (Krause et al., 2013;McCabe et al., 2015). Moreover, NGS technologies provide a means to begin more functional characterization of microbial communities (Morgavi et al., 2013), enabling the study of the rumen microbiome's role in enhancing feed efficiency (Zhang et al., 2016) and reducing CH_4 production (Shi et al., 2014; Kamke et al., 2016). NGS technologies are central to current methodologies employed to study the rumen microbiome, including metataxonomics, metagenomics, metatranscriptomics, metabolomics, and culturomics (Zehavi et al., 2018; Xue et al., 2022). Among these methodologies, amplicon sequencing (metataxonomics) and whole shotgun metagenomic sequencing (metagenomics) are widely utilised and employ bioinformatics as means of analysis (Matthews et al., 2019; Liu et al., 2021). A brief description of both is provided below.

Metataxonomics

Metataxonomics is the study of microbial populations through targeted sequencing of phylogenetic marker genes (Wilkinson et al., 2018). This approach involves high-throughput sequencing of PCR-amplified taxonomic marker genes, which are termed amplicons (Denman et al., 2018; Weinroth et al., 2022). The most commonly used marker genes include the 16S ribosomal RNA gene for bacterial and archaeal communities, the 18S ribosomal RNA gene for eukaryotic organisms such as fungi and protozoa, and the internal transcribed spacer region (ITS) or the D1/D2 domain of the large ribosomal subunit for fungi (Dagar et al., 2011; Schoch et al., 2012;Denman et al., 2018). The 16S ribosomal RNA gene is highly conserved in both bacteria and archaea, is evolutionary stable, and contains enough variable information to delineate between different bacterial and archaeal taxonomic groups (Woese, 1987; Zeineldin et al., 2018). The gene encodes the RNA component of the small ribosomal subunit involved in protein synthesis (Kitahara and Miyazaki, 2013). Structurally, the 16S rRNA gene consists of around 1550 base pairs and contains nine hypervariable regions interspersed with highly conserved regions (Ramiro-Garcia et al., 2016) (Figure1.12). These conserved regions can be targeted with primers to sequence through variable regions of interest, allowing for the identification of different bacterial and archaeal taxa (Yang et al., 2016). In the past, long-read sequencing platforms such as Oxford Nanopore and PacBio have not been commonly used

for sequencing the 16S rRNA marker gene due to their high rate of sequencing errors (Pollock et al., 2018). However, recent studies have emerged demonstrating their potential usefulness for sequencing the 16S rRNA gene (Myer et al., 2016). Nevertheless, high quality, short-read sequencing technologies such as the Illumina MiSeq are more often preferred and are used to sequence targeted hypervariable region(s) within the gene (Kameoka et al., 2021). The selection of the hypervariable region is a crucial step in the analysis of 16S rRNA gene sequencing data as it can impact the results (Pollock et al., 2018; Kameoka et al., 2021). The V4 region of the 16S rRNA gene is a widely utilised target region for microbial analysis (Gruninger et al., 2019) owing to its relatively short length that allows for near-complete overlap of Illumina paired-end sequences and its ability to accurately identify taxa included in mock community standards (McGovern et al., 2018).

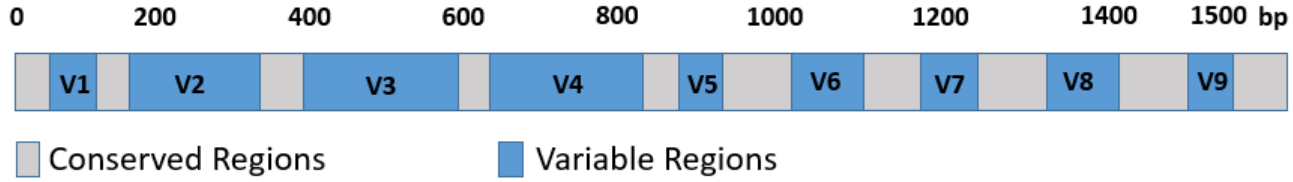


Figure 1.12: Depiction of the 16S rRNA gene. The numbers at the top indicate the estimated number of base pairs (bp) from the start to the end of the gene. Areas in gray depict conserved regions of the gene. Areas in blue depict the variable regions on the gene (V1-V9). Image adapted from (McAllister et al., 2018).

Metagenomics and Metatranscriptomics

Metagenomics is the study of the entire (meta) genetic material (genomes) obtained from a complex microbial community using untargeted (shotgun) high-throughput sequencing (Marchesi and Ravel, 2015). This approach generates large-scale sequencing data that can reveal insights into the genomes of bacteria, protozoa, fungi, archaea, and viruses simultaneously. Metagenomics sequencing has several applications, including taxonomic and functional profiling, draft genome assembly, and the identification of novel microorganisms (Quince et al., 2017; Latorre-Pérez et al., 2020). In addition, it is possible to study taxa at the species and strains level, and investigate their functional relationships with the host (Beghini et al., 2021). Similar to metagenomics, metatranscriptomics is the untargeted sequencing of all microbial mRNA (Moran, 2009) present in a biological sample using high throughput sequencing technologies. This method is used to profile the gene expression of the rumen microbiome to provide information on the functional activity of microbial communities.

The analysis of metagenomic and metatranscriptomic data requires greater computational resources and advanced bioinformatics tools when compared to amplicon-based metataxonomics (Niu et al., 2018), making it a more complex and challenging process. In addition, although sequencing cost has dropped over the decade, the cost of high-throughput metagenomics sequencing can still be

expensive (Teufel and Sobetzko, 2022) and may be a limiting factor, particularly in studies with a large number of samples. Despite these limitations, metagenomics and metatranscriptomics offers a powerful approach to study the rumen microbiome and has enabled researchers to gain valuable insights into the contribution of the rumen microbiome to the regulation of complex traits such as feed efficiency and CH_4 emissions (Shi et al., 2014; Kamke et al., 2016; Greening et al., 2019; Asselstine et al., 2021).

What associations between the rumen microbiome and methane emissions in sheep?

A number of studies have investigated associations between the rumen microbiome and CH_4 emissions in sheep (Kittelmann et al., 2014; Shi et al., 2014; Kamke et al., 2016; Ghanbari Maman et al., 2020). Although archaea are the exclusive producers of CH_4in the rumen, and are thus positively linked to its production (Wang et al., 2023), a clear association between community abundance and higher or lower CH_4 producing sheep has not been widely observed (Kittelmann et al., 2014;Shi et al., 2014). Instead it appears that the relationship between the archaeal community and CH_4 emissions is more tightly linked to differences in community composition rather than the overall size of the archaeal community (Tapio et al., 2017). For example, Kittelmann et al. (2014) and Shi et al. (2014) both reported no significant differences in total archaeal abundance in the rumen of high and low MY sheep. However, they did observe differences in the abundance of individual members of the archaeal community (Kittelmann et al., 2014;Shi et al., 2014). The abundance of *Methanosphaera* and *Methanosphaera stadtmanae* were found to be higher in the LMY cohort, while the abundance of *Methanobrevibacter smithii* was found to be increased in the rumen of the HMY cohort. Further analysis of the data produced by Shi et al (2014) showed increased abundance of *Methanobrevibacter smithii* and *Methanobrevibacter ruminantium* in higher CH_4 emitting sheep and *Methanosphaera stadtmanae* in lower CH_4 emitting sheep (Ghanbari Maman et al., 2020).

Differences in the abundance of archaeal taxa between high or low CH_4 emitting animals can be driven by hydrogen concentrations in the rumen. For example, hydrogenotrophic *Methanobrevibacter* species are phylogenetically classified into two distinct clades; SGMT (*M. smithii, M. millerae, M. thaueri and M. gottschalkii*) and RO (*M. ruminantium* and *M. olleyae*) clades. The SGMT clade are known to possess 2 isozymes of the methyl-coenzyme M reductase enzyme; *MCRI* and *MCRII*, while the RO clade only possesses *MCRI*. The catalytic properties of both enzymes are distinct with *MCRI* exhibiting higher substrate specificity than *MCRII*, while having a lower maximum turnover rate (Pitta et al., 2022). Indeed, the expression of *MCRI* and *MCRII* is dependent on the concentrations of dissolved hydrogen in the rumen, with *MCRII* only fully expressed when hydrogen concentrations are relatively high (Alvarado et al., 2014). Thus, the SGMT clade would have an energetic advantage over the RO

clade when the partial pressure of hydrogen in the rumen is elevated and contribute to higher CH_4 production. Moreover, methylotrophic *Methanosphaera* have a lower hydrogen threshold than hydrogenotrophic methanogens, which they can out-compete when the partial pressure of hydrogen in the rumen is low (Feldewert et al., 2020) and could explain its higher abundance in LMY sheep.

Methanogenesis is intricately linked to the production and transfer of hydrogen from microbial species such as bacteria, protozoa, and fungi to methanogens (Patra et al., 2017). Similarly, the relationship between these microbial groups is further complicated by the competition for methanogenic substrates, as evidenced by the presence of competing pathways in bacteria (Greening et al., 2019). As a result, the production of CH_4 in the rumen is dependent on the interplay between these microorganisms. Indeed, studies have found the bacterial community composition differing between high and low CH_4 yielding (MY) sheep (Kittelmann et al., 2014; Kamke et al., 2016). Kittelmann et al. (2014) investigated whether differences in rumen bacterial community were linked to high and low CH_4 emitting in sheep (Kittelmann et al., 2014). The study found that there were gradual transitions between three distinct bacterial ruminotypes of 236 sheep samples, which were linked to different CH_4 emissions. Two of these ruminotypes, Q and S, were associated with significantly lower CH_4 yields than the third ruminotype, H. The authors hypothesised that lower CH_4 yields were the result of bacterial communities that fermented ingested feed to relatively less hydrogen, which resulted in less CH_4 being formed (Kittelmann et al., 2014). Ruminotype Q was associated with a lower ruminal acetate to propionate ratio and high relative abundances of the propionate-producing *Quinella ovalis*, while ruminotype S was characterised by lactate- and succinate-producing *Fibrobacter spp., Kandleria vitulina*, *Olsenella spp.*, *Prevotella bryantii,* and *Sharpea azabuensis*. In contrast, high-CH4 ruminotype H had higher relative abundances of species known to form significant amounts of hydrogen, including *Ruminococcaceae* and *Lachnospiraceae* (Kittelmann et al., 2014). In a later study, Kamke et al. (2016) conducted a study to investigate the contribution of rumen bacteria to MY phenotypes and to identify specific microbial species and metabolic pathways associated with low MY in sheep (Kamke et al., 2016). The study found that HMY animals harbored a greater abundance of *Ruminococcace* and *Lachnospiraceae* families, while lactate-producing *Sharpea spp.* and lactate fermenting *Megasphaera elsdenii* were enriched in the rumen bacterial communities of low MY (LMY) sheep.

In addition, Kamke et al. (2016) observed differences in metabolic pathways, whereby the LMY sheep exhibited an upregulation of genes involved in the conversion of lactate to propionate, namely acyl-CoA-dehydrogenase, propionate-CoA-transferase, lactate-dehydrogenase, and lactyl-CoA-transferase. It is understood that propionate, which is produced from succinate and lactate precursors, functions as a hydrogen sink in the rumen (Kamke et al., 2016). Greening et al. (2019) found the expression profiles of H_2-evolving hydrogenases were comparable between the two divergent cohorts (Greening et al., 2019). On the other hand, differences in the expression of H_2 utilising pathways were observed between the cohorts. Specifically, the expression levels of

methanogenic hydrogenases and methyl-CoM reductase were found to be decreased in low MY sheep. In contrast, the gene expression levels of two prominent non-methanogenic H_2 sinks, namely acetogenesis and fumarate reduction, were found to be upregulated (Greening et al., 2019). The results of the studies suggest that disparities in MY between high and low MY sheep are not exclusively attributable to differences in hydrogen production. Instead, the upregulation of methanogenic and non-methanogenic H_2 utilising pathways, such as propionate production, acetogenesis and fumarate reduction, appears to be also contributing to the observed differences in MY between the two cohorts.

Ciliate protozoa play a central role in the supply of substrates for methanogenesis by producing significant amounts of hydrogen during fermentation. A meta-analysis conducted by Guyader et al. (2014) explored the correlation between rumen protozoa concentration and CH_4 production. The study found that a reduction in protozoa concentration by 0.12 log10 cells/ml led to a significant reduction in the amount of CH_4 produced (1 gram of CH_4 per kilogram of dry matter intake). However, it was noted that in 21% of the studies used in the meta-analysis, there was a reduction in CH_4 production even when the protozoa concentration were the same (Guyader et al., 2014). A further meta-analysis conducted by Newbold et al. (2015) found that the elimination of ciliate protozoa from the rumen reduced CH_4 production by as much as 11% (g/kg DMI) (Newbold et al., 2015). In a study carried out by Belanche et al. (2015) the effects of different types of protozoa (entodinium and holotrich protozoa) on the production of CH_4 in the rumen of sheep found that when defaunated sheep were refaunated with holotrich protozoa, there was an increase in the number of methanogens and CH_4 production. When the sheep were subsequently refaunated with a mixture of both holotrich and entodinium there was no differences in CH_4 production or the number of methanogens (Belanche et al., 2015).

Studies investigating the relationship between fungal communities and their impact on CH_4 emissions in sheep are lacking. However, one study conducted by Kittelmann et al. (2014) reported no differences in fungal or protozoal communities between HMY and LMY sheep (Kittelmann et al., 2014), however, this may be due to the limited representation of these microorganisms in genomic databases (Greening et al., 2019).

Methane mitigation strategies for more sustainable sheep production

The rumen microbiome is influenced by a range of factors, such as diet, feeding practices and host genetics, which alter fermentation in the rumen and consequently influence the amount of CH_4 produced. These factors can be targeted in mitigation strategies aimed at reducing CH_4 emissions from sheep production systems by improving dietary management, supplementing diet with feed additives, breeding for low CH_4 producing animals and/or enhancing feed efficiency.

Dietary management practices to reduce methane emissions

Dietary management practices can play an important role in reducing CH_4 emissions from sheep. One potential strategy for reducing CH_4 emissions is feeding diets with a high proportion of grains/or concentrates to forages. This is known to favourably alter the rumen fermentation, leading to increased ratio of propionate to acetate production, faster ruminal passage rate and lower CH_4 emissions per unit of feed consumed (Janssen, 2010). Gere et al. (2022) showed that supplementing low-quality forage (Rhodes grass hay) with dry distilled grains soluble (DDGS) (a DM ratio of 64:36) in sheep resulted in an increase in dry matter intake (DMI) by 22%, while reducing DME by 24% (g/d) and MY by 35% (g/kg DMI) (Gere et al., 2022). Indicating that supplementing low-quality forage with DDGS could improve productivity and reduce CH_4 emissions in livestock systems. However, increasing concentrate or grain feeding can lead to ruminal acidosis and negatively impact normal rumen physiology and host health (Snyder and Credille, 2017). A potentially safer alternative is to offer higher quality forages (Fraser et al., 2015; Wang et al., 2019;Thompson and Rowntree, 2020), such as young plants, which have lower levels of neutral detergent fiber (NDF) and higher amounts of easily fermentable carbohydrates compared to more mature and lower quality forages (Islam and Lee, 2019). Indeed, in a study conducted by Moss et al. (2019), it was found that supplementing sheep with 0.5 kg/d concentrates while on a high-quality perennial rye diet had no significant effect on DME or MY. Based on their research findings, the authors concluded that a diet consisting of high-quality grass can sustain high nutrient utilization efficiency as effectively as diets supplemented with concentrates in the context of ewe lamb production. In addition, Fraser et al. (2015) discovered that sheep offered fresh cut perennial ryegrass with a low NDF profile produced less CH_4 emissions per unit DMI and proportion of gross energy intake excreted as CH_4 compared to those fed fresh cut permanent pasture with a high NDF profile. This finding is consistent with the knowledge that higher quality forages are more readily digested and have a faster passage rate from the rumen, leading to a reduction in CH_4 production (Janssen, 2010). Some studies have also demonstrated that offering lotus and chicory to sheep can significantly reduce CH_4 emissions in sheep (Waghorn et al., 2002), while other studies have found no discernible effect of either forage type (Hammond et al., 2011; Sun et al., 2012). In summary, careful management of dietary practices can lead to reduced CH_4 emissions without negatively impacting animal health, welfare, or productivity, ultimately contributing sustainable production.

Plant extracts in sheep feeds to reduce methane emissions

Various plant extracts such as saponins, tannins, and essential oils are being studied as potential strategies to mitigate CH_4 emissions from sheep. These substances have antimicrobial properties that can alter fermentation in the rumen, leading to reduced CH_4 production (Patra et al., 2012). However, the use of these extracts can also have negative impacts on factors such as feed intake and nutrient utilization, which can affect animal performance (Vasta et al., 2009; Patra et al., 2012). Factors such as the type of extract, its source, molecular

weight dose administered and diet type have varying degrees of impact on both CH_4reduction and animal performance (Islam and Lee, 2019; Valenti et al., 2021), and optimizing the use of plant extracts to reduce CH_4while limiting effects on animal performance is a matter of ongoing research (Zhou et al., 2019b; Valenti et al., 2021). Studies in sheep have found that purified condensed tannins and tea saponins can significantly reduce CH_4 emissions without impacting animal performance (Yuan et al., 2007; Wang et al., 2009; Ngámbi et al., 2022). A meta-analysis comparing the effectiveness of various CH_4-reducing strategies found that tannins and lipids were the most effective additives in reducing CH_4 production, with the specific effectiveness varying based on the type and amount of additive, diet composition, and method of CH_4 production measurement (Torres et al., 2022; Santos Torres et al., 2023).

Dietary lipids in sheep feeds to reduce methane emissions

The inclusion of polyunsaturated fatty acids (PUFAs) has been found to effectively reduce CH_4 emissions in sheep (Machmüller, 2006). Different types of PUFAs, such as coconut oil, soya oil, rapeseed oil, and linseed oil, have been tested for their effectiveness in reducing CH_4 emissions (Machmüller et al., 2000; Mao et al., 2010). For instance, Mao et al. (2010) studied the effects of adding soybean oil on CH_4 production in growing lambs fed a diet composed of Chinese wild rye and concentrates. The finding showed that including 3% soybean oil reduced CH_4 emissions by 14% (from 26.2 to 22.6 L/kg DMI) (Mao et al., 2010). In another study, Machmüller et al. (2000) showed that including 6% coconut oil could reduce CH_4 emissions by 26% (662 to 489 ml kg^{-1} Live weight) in lambs fed maize silage, grass hay and concentrates (Machmüller et al., 2000). The reduction of CH_4through dietary lipid supplementation is mediated through combined effect of various mechanisms including the toxicity of lipids to protozoa and methanogens, the bio-hydrogenation of unsaturated fatty acids, and the promotion of propionate production through the shifting of rumen fermentation pathways (Newbold et al., 2015; Beauchemin et al., 2022b). While the use of PUFAs have been shown to effectively reduce CH_4 emissions in sheep, their use does have potential drawbacks. High concentration fats can also have a toxic effect on cellulolytic microorganisms in the rumen, which could have a negative impact on fiber digestibility, rumen fermentation and ultimately animal production (Behan et al., 2019). In addition, oils and fats can reduce feed intake by as much as 6% and digestibility of the diet by as much as 4% (Arndt et al., 2021), which can impact on production. Therefore, it is important to carefully balance the benefits of reducing CH_4 emissions with potential negative effects on productivity when incorporating PUFAs into sheep diets. Current recommendations is that dietary fat levels do not exceed 6-8% of the diets DM (FAO, 2022).

Ionophores in sheep feeds to reduce methane emissions

Ionophores, such as monensin, have been shown to be effective feed additives for reducing CH_4 production in sheep (Ushida et al., 1985). Ionophores are a type of antimicrobial agent that work by binding to the lipid bi-layer of microbial cells (Chow et al., 1994), allowing H^+ and metal ions to pass through the cell

membrane and ultimately leading to cell death (Russell and Strobel, 1989; Fellner et al., 1997). Monensin is known to selectively target and reduce the populations of gram-positive bacteria and protozoa in the rumen (Shen et al., 2017; Ogunade et al., 2018). This leads to changes in rumen fermentation patterns, shifting towards increased ratios of propionate to acetate, reduced ammonia and increased protein, reduced H+ and CH_4 production (Russell and Strobel, 1989; Russell and Houlihan, 2003). However, the use of antimicrobial agents in general is controversial due to the potential development of antimicrobial resistance. Therefore, the use of monensin has been prohibited in certain regions, including the EU. (Castillo et al., 2004).

3-NOP feed additive for methane reductions from ruminants

3-nitrooxypropanol (3-NOP), or Bovaer® as it is commercially known, is a synthetic compound that has been shown to reduce $CH_4$by inactivating the activity of methyl coenzyme M reductase, a key enzyme involved in the production of CH_4. A meta-analysis carried out by Jayanegara et al. (2018) examined the methane reducing effects of 3-nitrooxypropanol (3-NOP) on enteric CH_4 emissions from cattle and sheep. The study found that increasing levels of 3-NOP in ruminant diets decreased enteric MY by approximately 20% without negatively affecting animal performance or DMI (Jayanegara et al., 2018). Additionally, 3-NOP decreased A:P ratio and archaea population, and had little effect on nutrient digestibility of the diet (Jayanegara et al., 2018). Overall, the study concluded that 3-NOP is an effective feed additive for mitigating CH_4 emissions in ruminants without compromising animal productivity (Jayanegara et al., 2018). An analogous compound, ethyl-3-nitrooxypropionate (E-3NP), has also been shown to have CH_4-reducing potential in sheep (Martínez-Fernández et al., 2014). Martínez-Fernández showed that high doses of E-3NP (500mg/kg of DMI) reduce CH_4 production by as much as 29% in sheep (Martínez-Fernández et al., 2014). Lower doses of both E-3NP and 3-NOP have also been shown to reduce CH_4 emissions, albeit to a lesser extent (Martínez-Fernández et al., 2014). Overall, these findings provide a promising avenue for reducing methane emissions from sheep production systems.

Host genetics and breeding programs

Numerous studies have demonstrated that CH_4 emission in sheep is a heritable trait, indicating that host genetics can influence the amount of CH_4an animal produces (Ghavi Hossein-Zadeh, 2023; Pinares-Patiño et al., 2013). For example, heritability estimates for DME were found to range from 0.13 (Goopy et al., 2015) to 0.29 (Pinares-Patiño et al., 2013), while MY had a heritability of 0.13 (Pinares-Patiño et al., 2013). A recent meta-analysis showed that heritability estimates for MY was 0.137 (Ghavi Hossein-Zadeh, 2023), further supporting the idea that CH_4 emissions in sheep are genetically influenced. These findings highlight the potential for selective breeding as a strategy for reducing CH_4 emissions from sheep production systems. Rowe et al. (2019) established selection lines of sheep with high and low CH_4 emissions. After 10 years, the study reported a 12% reduction in CH_4 emissions between the high and low MY selection lines (Rowe et al., 2019), demonstrating the potential of selective breeding for cumulative and

permanent reductions in CH_4. Thus, selective breeding programs could be a sustainable and effective solution for mitigating CH_4 emissions from sheep production systems.

Feed efficiency in sheep production for methane emissions reduction

Feed efficiency is an important metric in livestock production as it measures the animal's ability to convert ingested feed into body weight or milk. Sheep that are feed efficient are able to maintain or increase their production while consuming less or comparable amounts of feed than their feed inefficient counterparts (Zhang et al., 2017b). Feed can account for a substantial proportion of total production costs which is estimated to be 60-70% in the sheep industry (Zhang et al., 2019), therefore, feed efficiency has significant implications for profitability within the sector. Indeed, feed efficient lambs have been demonstrated to save between 20% - 26% on feed costs in comparison to their feed inefficient counterparts (Ellison et al., 2022). In addition, research has shown that the quantity of feed consumed by sheep has a significant impact on CH_4 production, with dry matter intake (DMI) accounting for between 76% and 91% of the variation in CH_4 emissions from pasture-fed sheep. Studies have shown that more feed efficient sheep tend to produce less CH_4 per unit of feed consumed (Paganoni et al., 2017). This can be attributed to the fact that animals with higher feed efficiency produce less methane per unit of weight gain due to their reduced overall feed intake. Furthermore, ruminal methanogenesis is commonly associated with the loss of dietary energy, ranging from 2% to 12% according to various studies (Johnson and Johnson, 1995; Bhatt et al., 2019), thereby influencing feed efficiency of the host. Since CH_4 cannot be metabolised, it is eructated to the atmosphere, contributing to atmospheric GHG emissions (Naqvi and Sejian, 2011). Thus, enhancing feed efficiency in sheep has the potential to improve profitability and environmental sustainability of the sector.

Measures of feed efficiency in ruminant livestock

Feed efficiency can be measured in a number of different ways. Feed conversion ratio (FCR) and residual feed intake (RFI) are two of the most common indices measuring feed efficiency in sheep (Ellison et al., 2017; Claffey et al., 2018; Zhang et al., 2019). FCR is simply calculated as the kilogram ratio of the animal's average dry matter intake (DMI) to its average daily growth (ADG) (Berry and Crowley, 2013). In essence, FCR measures how many kilos of feed are consumed to produce one kilogram of animal product, thus a decrease in FCR is related with an increase in energy efficiency. However, one notable downside of using FCR as a measure of feed efficiency is that it is correlated to animal body weight and ADG, which can lead to the selection of larger, faster growing animals that would theoretically require more maintenance (Santana et al., 2012). Consequently, this can have negative economic and environmental ramifications. RFI is a more robust and reliable measure of feed efficiency (Zhang et al., 2017b). The concept of RFI was first introduced by (Koch et al., 1963) and is defined as the difference between observed feed intake and predicted feed

intake, which is calculated as the residuals from a multiple linear regression model of feed intake adjusted for production levels and maintenance requirements (Berry and Crowley, 2013). As a result RFI is independent on animal's growth rate and are better capable of selection animals that are truly more feed efficient.

Factors influencing feed efficiency of ruminant livestock

Feed efficiency is a complex trait that is influenced by various factors including host genetics (Tortereau et al., 2020) and the rumen microbiome (Ellison et al., 2017). In ruminants, including sheep, the rumen microbiome plays a crucial role in feed digestion and fermentation, which results in the production of volatile fatty acids (VFAs), the primary source of energy for the host (France and Siddons, 1993). As a result, the rumen microbiome is inextricably linked to the feed efficiency of the host. Indeed, a number of studies have established links between the rumen microbiome and feed efficiency in sheep (Ellison et al., 2017; Zhang et al., 2021b; Cheng et al., 2022). For instance, Zhang et al. (2021) conducted a study on concentrate-fed Hu lambs with divergent residual feed intake (RFI), using 16S rRNA amplicon sequencing to investigate the rumen microbiome. The study found that low-RFI (high feed efficiency) animals harbored a more diverse microbial community enriched with *Succinivibrio* and exhibited a higher Firmicutes-to-Bacteroidota ratio (F:B) than high-RFI (low feed efficiency) animals (Zhang et al., 2021b). Although, other studies have reported a higher F:B in feed inefficiencient animals (Lopes et al., 2021).Besides the rumen microbiome, feed efficiency has a genetic component, with RFI and FCR showing moderate heritability in sheep (Tortereau et al., 2020). According to a study conducted by Tortereau et al. (2020), both residual feed intake (RFI) and feed conversion ratio (FCR) were found to be heritable, with RFI exhibiting higher heritability (0.45) than FCR (0.30). The study further demonstrated that selecting sires with low RFI values led to the production of lambs that consumed 3% less feed, while maintaining comparable growth rates to those selected based on high RFI values (Tortereau et al., 2020). These findings suggest the potential for breeding strategies to improve feed efficiency and improve profitability within the sector. Further continued understanding the complex interactions among host genetics, diet, and the rumen microbiome can contribute to the development of effective strategies to enhance feed efficiency and reduce the environmental impact of sheep production systems.

Typical workflow for rumen microbiome studies

The rumen harbors a diverse microbial community that plays a crucial role in the feed efficiency, CH_4 production and host health. To understand the complex interactions between rumen microbes and their host, microbial studies are conducted which follow a typical workflow: the collection and storage of rumen samples, DNA extraction and library preparation, DNA sequencing, sequence analysis, and statistical analysis. The microbial community analysis workflow typically employs short-read Illumina sequencing technologies, and the following is an overview of this process.

Sample collection and storage

Conducting a rumen microbial study requires careful consideration of the sample collection process. The type and number of samples collected, as well as the method used to obtain them, should be determined based on the research hypothesis. A power analysis can be conducted to determine the necessary or minimum number of samples required to discern a biological effect (Ferdous et al., 2022). Methods for collecting rumen samples include trans-esophageal tubing, rumen fistulation, or collection at slaughter (Ramos-Morales et al., 2014; Fu et al., 2020). To ensure accurate biological inference, it's important to maintain the information present in the rumen sample at the time of collection. Rumen samples are typically snap frozen in liquid nitrogen immediately after collection and stored in laboratory freezers at -20 to -80°C (Martinez-Fernandez et al., 2019). Variations in storage methodologies, the timing of storage, and refreezing after thawing can all affect the microbial community composition and downstream analyses (Granja-Salcedo et al., 2017). Therefore, it is crucial to carefully consider the sample collection and preservation process in order to obtain reliable and meaningful results in rumen microbial studies.

DNA extraction and library preparation

DNA is isolated from microbial cells prior to sequencing using DNA extraction methodologies. This is achieved by lysing microbial cells by mechanical and/or chemical means (Gupta, 2019), which are incorporated into a variety of commercially available DNA extraction and purification kits, such as the Qiagen DNeasy PowerSoil® or FastDNA™ SPIN Kit for Soil. DNA extraction protocols aim to maximize yield, quality and purity of microbial DNA (Gupta, 2019). Mechanical lyses using bead beating technology has been shown to produce higher DNA yields, however, excessive bead beating can lead to DNA shearing (Zhang et al., 2021a), which can negatively impact downstream analysis (Cullen et al., 2022). The variability in microbial cell wall structures among different microorganisms complicates DNA extraction, which can bias sequencing and analysis (Zhang et al., 2021a; Lourenco and Welch, 2022). Therefore, it is essential to control potential biases during the DNA extraction process to ensure accuracy and reliability in downstream analysis (Lourenco and Welch, 2022). One approach to achieving this is by using the same extraction protocol and batch reagents for all samples (Lourenco and Welch, 2022) to minimise variations in DNA yield or quality that could skew the results. Additionally, the inclusion of proper negative and positive controls is crucial for mitigating potential biases (Lourenco and Welch, 2022). Negative controls enable researchers to monitor for potential contamination during the DNA extraction process, while positive controls, such as "mock communities", provide a known set of DNA sequences that can be used to validate the sequencing process and downstream processing and analysis (McGovern et al., 2018).

After DNA extraction, DNA libraries are prepared prior to sequencing. For amplicon sequencing, specific variable regions of interest (such as 16S rRNA V1-V9) are first amplified via PCR using suitable primers (Leray et al., 2016). Amplicons are then ligated with adapter sequences to prepare them for

sequencing (Leray et al., 2016). For metagenomics sequencing, DNA is fragmented, end-repaired and then ligated with adapter sequences (Bronner and Quail, 2019). The adapters contain a complementary sequence to oligonucleotides present on the surface of the sequencing flow cell, which anchors the DNA fragments for sequencing (Kozarewa et al., 2009). In addition, adapters also contain an index sequence that enables pooling and sample identification, as well as an annealing site for the sequencing primers to attach to the DNA template and initiate the sequencing reaction (Leray et al., 2016;Slatko et al., 2018). Size selection is performed either via gel electrophoresis or a bead-based size selection method to obtain the desired library sequencing sizes (Bronner and Quail, 2019). Finally, libraries are quantified and assessed prior to sequencing using a Agilent Bioanalyser (Bronner and Quail, 2019).

DNA sequencing

The choice of sequencing technology can depend on the type of study being carried out, as different sequencing technologies have their own strengths and weaknesses. Short-read sequencing technologies, such as the Illumina platforms (e.g MiSeq, NovaSeq HiSeq) (Caporaso et al., 2012), are widely used in rumen microbiome studies as they offer high sequencing depth at a comparatively low cost (Bharti and Grimm, 2021) Illumina's MiSeq sequencing platform has been widely adopted in amplicon sequencing studies, primarily owing to its high-throughput capabilities, fast turnaround times, extended sequence read lengths, and high accuracy (Wen et al., 2017). The MiSeq sequencing platform commonly generates 15GB of data with 25 million paired-end reads with a read length of 300bp (Illumina, nd), and facilitates parallel sequencing of roughly 400 samples with an average of 50,000 paired-end reads per sample (Dong et al., 2017). Illumina HiSeq and NovaSeq platforms are typically used for shotgun metagenomics sequencing, which allows for thousands of microbial genomes to be sequenced in parallel. For instance, the HiSeq 2500 model can generate up to 600 million high quality paired-end reads with a read length of 251bp reads, alternatively, the NovaSeq 6000 can produce up to 1.6 billion paired end reads with a read length of 251bp reads (Illumina, 2021). Illumina sequencing platforms use bridge amplification and sequencing by synthesis technology, which involves the iterative addition of individual nucleotides to DNA template strands in repetitive cycles (Buermans and den Dunnen, 2014). Each nucleotide is fluorescently labeled and detected after incorporation, allowing the sequence of the template strand to be determined (Buermans and den Dunnen, 2014).

Bioinformatic processing and analysis of amplicon data

After sequencing, reads are demultiplexed/sorted according to sample of origin, which is based on the adapter index/barcode sequences. This produces individual files (FastQ files) containing all the reads belonging to their respective samples. Reads then undergo a quality control step, to assess sequencing performance and inform pre-processing of the raw reads. FASTQC (Andrews, 2010) is an efficient and widely utilised tool for the quality assessment of sequencing data. Reads then pre-processed to trim off adapter sequences and

remove low-quality bases and reads. A variety of different tools have been developed for trimming sequence reads including Cutadapt (Martin, 2011), Trimmomatic (Bolger et al., 2014) and FASTP (Chen et al., 2018). Paired end reads can then be error corrected and merged using tools such as PANDAseq (Masella et al., 2012). QC and pre-processing are important components of the workflow which serves to improve the overall quality of the data and minimise overestimation of the microbial community prior to downstream analyses.

Following QC and processing steps, sequenced reads are clustered and classified. Traditionally, reads were clustered based on an arbitrary sequence similarity threshold of 97%, known as operational taxonomic units (OTUs) (Westcott and Schloss, 2015) using the UPARSE-OTU algorithm (Edgar, 2013). However, such criteria are incapable of distinguishing closely related species or genera (Pei et al., 2010). In addition, errors introduced by PCR and sequencing may compromise the ability to distinguish between distinct taxa (Galloway-Peña and Hanson, 2020).

Recently, there has been a shift away from arbitrary OTU clustering and towards amplicon sequence variants (ASVs), which provides greater resolution of the rumen microbiome (Callahan et al., 2017). ASV methods, such as DADA2 (https://benjjneb.github.io/dada2/index.html), infer biological sequencing by correcting errors introduced during PCR amplification and sequencing, and are capable of distinguishing sequence variants that differ by as little as one nucleotide (Callahan et al., 2016;Callahan et al., 2017). ASV are advantageous over OTUs as they can be compared across studies, they are not impacted by incomplete databases and are reproducible for future datasets (Callahan et al., 2017). ASVs or OTUs are then taxonomically classified by comparing representative DNA sequences to a reference database, such as the SILVA (Quast et al., 2012) or Greengenes (DeSantis et al., 2006) databases. A feature-count table is generated by quantifying the frequency of each ASV or OTU in each sample and serves as the basis for downstream analyses, such as alpha and beta diversity analysis or differential abundance analysis (Figure 1.13A).

One of the fundamental drawbacks of amplicon sequence analysis gene analysis is that it provides limited information on metabolic activity of microbial communities. However, software applications such as PICRUSt (Langille et al., 2013; Douglas et al., 2020) and CowPI (Wilkinson et al., 2018) have been to developed to help infer the functional potential from amplicon data. Functional prediction algorithms, however, are incapable of inferring the functional activity, which can only can only be measured via metatranscriptomic methodologies.

Metagenomic sequence analysis

Metagenomic data is quality assessed with bioinformatic tools, such as FASTQC (Andrews, 2010) and MultiQC (Ewels et al., 2016), which inform pre-processing of the raw data. Undesired sequences, low-quality bases, and adapter sequences can be removed using tools such as Trimmomatic (Bolger et al., 2014), Cutadapt (Martin, 2011) and FASTP (Chen et al., 2018). In addition, metagenomic datasets can contain contaminant reads from the host, and their presence in the dataset

can lead to erroneous downstream analysis and interpretation. Contaminant reads should therefore be removed, which can be achieved by aligning and filtering reads that map to the host reference genome using Bowtie2 (Langmead and Salzberg, 2012) or BWA (Burrows-Wheeler-Aligner) (Li and Durbin, 2009). Kneaddata (https://github.com/biobakery/kneaddata) is a recently developed bioinformatic pipeline that incorporates various bioinformatic tools such as FASTQC (Andrews, 2010), Trimmomatic (Bolger et al., 2014), and Bowtie2 (Langmead and Salzberg, 2012) for trimming and cleaning of metagenomic datasets. These quality control steps are crucial to ensure that the metagenomic data's quality is sufficient for downstream analysis and that any biases introduced by contamination or low-quality reads are minimised.

Cleaned metagenomics reads can follow one of two analytical paths, or both: assembly-based and/or reference-based analysis (Liu et al., 2021). In reference-based analysis, sequenced reads are classified by mapping them against curated databases. Kraken2 (Wood et al., 2019) and MetaPhaln3 (Beghini et al., 2021) are popular tools for taxonomic classification and quantification of metagenomics reads. Kraken2 is a fast and memory efficient tool that uses exact k-mer matching to the lowest common ancestor (LCA) to assign taxonomic labels to DNA sequences (Wood et al., 2019). MetaPhlAn3 performs taxonomy classification by aligning reads to a database of pre-defined clade specific markers genes (Beghini et al., 2021). A review by Ye et al. (2019) presents an evaluation of 20 taxonomic classification tools and provides benchmarks for their performance (Ye et al., 2019). In addition to taxonomy profiling, HUMAnN3 can be used to conduct functional profiling of metagenomics and/or metatranscriptomic data (Beghini et al., 2021). HUMAnN3 implements a tiered approach to functional classification, firstly it maps reads to a to clade specific marker genes to identify species within a sample, then it maps reads to functionally annotated pan-genomes of identified species, and finally aligns unclassified reads to a protein database (UniRef90 or UniRef50) using translated search (Franzosa et al., 2018). MEGAN is an another widely used bioinformatics tool which can be used for both taxonomic and functional analysis of metagenomic data (Huson et al., 2016).

Assembly-based metagenomics aims to reassemble short sequenced reads into longer contiguous sequences, known as contigs (Deng and Delwart, 2021). There are two different approaches to metagenome assembly: *de novo* assembly and reference guided assembly (Galloway-Peña and Hanson, 2020). *De novo* assembly methods aim to rebuild contigs using only the sequenced reads, independent of reference genomes, and can be done by either co-assembly or independent assembly. In co-assembly reads are assembled from multiple samples whereas independent assembly refers to the assembly of reads in each sample independently (Delgado and Andersson, 2022). *De novo* reconstruction using metagenomic reads is a computationally expensive, and complex procedure due to the size and heterogenous nature of metagenomic data (Sun et al., 2022; Vuong et al., 2022). Nevertheless, several heuristic-based algorithms for *de novo* assembly have been developed, including ‘greedy’, overlap-layout consensus (OLC), and De Bruijn graph (Ghurye et al., 2016). De Bruijn graph is

the most widely implemented algorithm in *de novo* assembly tools (Galloway-Peña and Hanson, 2020). In principle, the algorithm constructs a de Bruijn graph using k-mers of reads, where the nodes of the graph represent the prefixes and suffixes of the k-mers and edges represent the k-mers. Instead of explicitly aligning the reads, this method identifies their overlap by examining the shared k-mers. The de Bruijn graph finds the Eulerian path through the graph to assemble reads into contigs (Ghurye et al., 2016). Popular *de novo* assembling tools include Megahit (Li et al., 2015), MetaSPAdes (Nurk et al., 2017), MetaVelvet (Namiki et al., 2011) and SOAPdenovo2 (Luo et al., 2012). For reference-guided assembly, sequenced reads are aligned to reference genomes, and contigs are reassembled based on their relative alignment positions (Cepeda et al., 2017).

Once reads are assembled into contigs, gene predictions can be made using tools such as MetaGeneMark2 (Gemayel et al., 2022) and Prokka (Seemann, 2014), which identify protein coding regions within contigs. CD-HIT (Fu et al., 2012) can then be used to remove redundant genes and produce a reference database from the contig dataset (Fu et al., 2012). Gene abundance tables are then produced by aligning back the reads using alignment based tool such as Bowtie2 (Langmead and Salzberg, 2012) or BWA (Li and Durbin, 2009), or pseudo-aligners such as Salmon (Patro et al., 2015) and used for downstream exploratory and statistical analyses. In addition, genes can annotated functionally, for example with KEGG Orthology (KO) (Kanehisa et al., 2016), or pathways such as MetaCyc (Caspi et al., 2020) or KEGG pathways (Kanehisa et al., 2016;Liu et al., 2021). Alternatively, assembled contigs can be clustered based on similarity in composition, coverage, and tetranucleotide frequency to create genome bins (Alneberg et al., 2014). This process is referred to as binning, and the resulting genome bins can be used to assemble draft genomes known as metagenome-assembled genomes (MAGs). A variety of tools have been available for metagenomics binning such as MaxBin2 (Alneberg et al., 2014), CONCOCT (Alneberg et al., 2014) or MetaBAT2 (Kang et al., 2019).

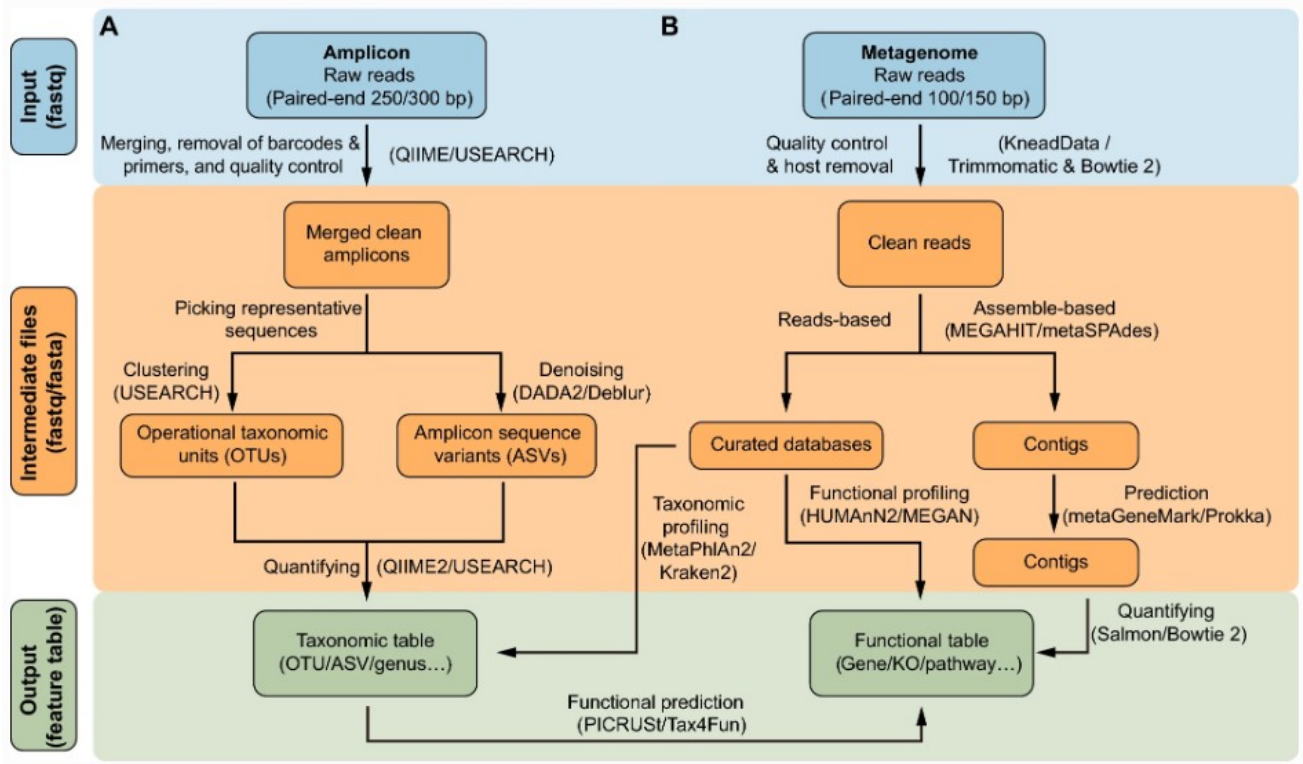


Figure 1.13: Workflow of commonly used methods for amplicon (A) and metagenomic (B) sequencing. Blue, orange, and green blocks represent input, intermediate, and output files, respectively. The text next to the arrow represents the method, with frequently used software shown in parentheses. Taxonomic and functional tables are collectively referred to as feature tables. Figure and legend sourced from (Liu et al., 2021).

Classification databases for rumen microbiome studies

Classification of microbial communities is an important step for the analysis of complex ecosystems such as those found in the rumen. Classification involves comparing DNA sequences obtained from rumen samples to reference databases to identify and annotate the closest matching sequences. The Ribosomal Database Project (RDP) (Cole et al., 2014), SILVA (Quast et al., 2012), GreenGenes (DeSantis et al., 2006), RefSeq (O'Leary et al., 2016) the Genome Taxonomy Database (GTDB) (Parks et al., 2018) are among the most commonly used publicly available reference databases for taxonomic classification. The choice of reference database can have an impact on sequence classification and subsequently on the interpretation of microbiota analysis (Pollock et al., 2018; Henderson et al., 2019). Many of the reference databases used in rumen microbiome studies are biased toward human-relevant microorganisms (Pollock et al., 2018), which can lead to misclassification and underestimation of rumen microbial diversity. Additionally, a large proportion of the rumen microbiome remains uncharacterised (McCabe et al., 2015), and current databases are not fully representative of the rumen microbiome (Smith et al., 2022b), which can result in an incomplete identification of the true microbial diversity present in the

rumen. To address this, efforts have been made to improve the representation of the rumen microbiome. The Hungate1000 project, launched in 2012, aims to isolate and sequence the genomes of 1,000 microorganisms cultured from the rumen (Seshadri et al., 2018). Currently more than 410 bacteria and archaea, from every known family, have been cultured and their genomes sequenced. Moreover, the genomes are estimated to represent up to 75% of all bacterial and archaeal genera present in the rumen (Seshadri et al., 2018). Further work is needed to improve the representation of the rumen microbiome in reference databases in order to accurately characterise rumen microbial diversity.

Statistical analysis of microbiome data

Feature count table

Amplicon and metagenomic sequencing technologies generate large amounts of sequencing data, which are subsequently processed through bioinformatics pipelines to produce feature-count tables that detail the abundance of microbial features (e.g. OTUs, genes, pathways) in each sample. Feature-count tables, along with sample metadata, are used for various exploratory and statistical analyses, including diversity calculations, differential abundance and correlation analysis, co-abundance network analysis, and data visualization (Liu et al., 2021).

Microbiome feature-count tables are often sparse, especially when it comes to taxonomy data (Pan, 2021). This means that many of the ASVs or OTUs have zero counts in most of the samples - up to 70%-90%, in some cases (Lin and Peddada, 2020; Yang and Chen, 2022). This sparsity is due to the fact that microbial communities are typically diverse, with many different taxa present but only a subset of these taxa are detected in any given sample due to limitations of sequencing depth or sampling variations, and are known as sampling zeros (Yang and Chen, 2022). However, some taxa may truly not be present, and are known as structural zeros (Zeng et al., 2022). In addition, microbiome data is highly variable with large variations in the abundance of specific taxa between samples (Pan, 2021). Furthermore, microbiome data is compositional in nature (Gloor et al., 2016; Gloor et al., 2017), which means that the data only provides information on the relative abundances of microbial taxa in a given sample and not their absolute abundances (Yang and Chen, 2022). Therefore, changes in the abundance of one taxon can affect the relative abundances of all others. Strong compositional effects can arise with presence of many low-abundance taxa alongside a few highly abundant taxa (Yang and Chen, 2022). Thus, microbiome feature count tables have complex characteristics that present challenges for normalization and statistical analyses (Yang and Chen, 2022).

To reduce the complexity of the data, it is often common practice to filter out features with low counts and low prevalence (Cao et al., 2021). Filtering is often implemented by setting arbitrary abundance and prevalence thresholds, informed by biological understanding and/or careful examination of the data. However, such criteria may result in a loss of informative data and influence the

results (Mokhtari and Ridenhour, 2022). To ensure reliability, it can be useful to apply and compare multiple filtering thresholds. Alternatively, various packages have been developed to perform unsupervised filtering, such as PERFect (Smirnova et al., 2019), which applies a statistical data-driven significance threshold to identify the most informative subset of taxa for further analysis (Smirnova et al., 2019). Another recently proposed method is the MI-based (mutual information based) method which uses information theoretic functionals and graph theory for filtering (Mokhtari and Ridenhour, 2022).

Data normalisation

Prior to downstream analysis, feature-counts are often normalised to account for differences in sequencing depth across samples, and ensure meaningful comparisons can be made between samples (Badri et al., 2020). There are various approaches to normalization, and the choice of normalization method may differ depending on the nature of data and analysis being conducted (Xia and Sun, 2017). Rarefying is common approach used to normalise feature-count data, and often performed prior to diversity analysis (Willis, 2019). Rarefying equalizes sequencing depth across samples by randomly subsampling counts without replacement to a specified depth, often determined by rarefaction curves, to ensure equal numbers of sequences are drawn from each sample (Lin and Peddada, 2020; Pan, 2021). However, rarefying the data can result in the loss of useful information and bias the results (Nearing et al., 2022). Total sum scaling (TSS) is a widely used approach, whereby the individual read counts within a sample are transformed into proportions, such as relative abundances, by dividing them by the total number of reads (Badri et al., 2020). Cumulative sum scaling (CSS) is an alternative method for normalizing counts in microbiome analysis. This approach involves scaling the counts of individual features based on their cumulative sum up to a percentile threshold, which is determined using a data-driven approach (Paulson et al., 2013). Others methods of normalization and scaling are also available and their strengths and limitations have been reviewed by Lin and Peddada (2020) (Lin and Peddada, 2020).

Diversity analysis

Alpha diversity analysis is a common first step in the analysis of microbiome data. Alpha diversity is a measure of the diversity within a sample or community (Sepkoski, 1988) and in essence describes the community structure. Alpha diversity can be calculated using different metrics, such as the number of species (richness) or the equitability of their distribution (evenness) in a sample (Thukral, 2017). The Shannon index (generally denoted as *H'*), developed by Claude Shannon in 1948, is one of the most widely used indices to measure alpha diversity in microbiome studies (Thukral, 2017;Xia et al., 2018). The Shannon index measures both the richness and evenness of the community (Gauthier and Derome, 2021). It calculates the natural logarithm of the proportion of individual species in a sample and multiplies it by the portion of individuals of each species (Xia et al., 2018). This results in a value that increases with increasing diversity. Another common metric for alpha diversity is the Simpson index, which is a measure of the diversity based on the probability

that two individuals randomly drawn from the community belong to the same species (Xia et al., 2018). This index is a measure of the inverse dominance, which means the higher the Simpson index, the lower the dominance of the species in the community and the greater the diversity (Xia et al., 2018). The Simpson diversity index ranges from 0 to 1, with a value of 0 indicating no diversity (i.e., all individuals belong to the same species) and a value of 1 indicating maximum diversity (i.e., all species are equally abundant). Alpha diversity measures can be computed using the R programming package Vegan (Oksanen et al., 2013). These measures are commonly compared between groups utilizing either classical parametric tests, provided certain assumptions are satisfied, or non-parametric tests in cases where parametric test assumptions are not satisfied.

Beta diversity refers to the measurement of diversity between different samples or communities (Koleff et al., 2003), describing variations in species composition (Xia et al., 2018). Beta diversity can be estimated by using a variety of methods such as the Jaccard or Sorensen indices which measure the similarity or dissimilarity based on the presence or absence of different species between different communities (Xia et al., 2018). Another commonly used method for measuring beta diversity is the Bray-Curtis dissimilarity metric, which calculates the compositional dissimilarity between two microbial communities based on the relative abundance of different species in each community (Xia et al., 2018). Another popular approach for calculating beta diversity is the UniFrac metric, which considers the evolutionary relationships between different species (Lozupone and Knight, 2005). To compare beta diversity across groups, a common method is to use permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2014), which can also be implemented using the R programming package Vegan (Oksanen et al., 2013).

Differential abundance analysis

Differential abundance analysis is a critical aspect of microbiome data analysis, which aims to identify taxa or features that are differentially abundant between two or more groups or conditions. However, this can be challenging due to the complex nature of the data, including high dimensionality, sparsity, and compositional structure. To perform differential abundance analysis, various statistical methods have been developed and applied, which can differ in their assumptions, power, and sensitivity. Classic parametric statistical tests, such as the t-test and analysis of variance (ANOVA) are considered inappropriate for differential abundance analysis due to the non-normal distribution of microbiome data which violates the test assumptions (Pan, 2021). Non-parametric test such as the Wilcoxon rank sum test and Kruskal-Wallace test, are often used as a result. However, non-parametric tests also have limitations. For example, ties can occur when multiple taxa have zero counts, which can limit the ability to detect differences between groups (Pan, 2021). In addition, nonparametric tests cannot adjust for covariates, and may have low statistical power, especially when working with sparse data and small sample sizes (Pan, 2021). A number of packages originally designed for RNA-Seq differential expression analysis, such

as EdgeR (Robinson et al., 2010) and DESeq2 (Love et al., 2014), have been used for differential abundance analysis in rumen microbiome studies (Glendinning et al., 2021; Neves et al., 2021). These packages use a generalised linear model with a negative binomial distribution but apply different normalization methods. A major limitation of these techniques is that the large number of zero counts in a microbiome dataset can exceed the assumptions of the negative binomial model, which can limit the statistical power (Pan, 2021). ANCOM-BC (Analysis of Compositions of Microbiomes with Bias Correction) (Lin and Peddada, 2020) is a recently developed method that extends upon the Analysis of Composition of Microbiomes (ANCOM) (Mandal et al., 2015). ANCOM-BC accounts for the compositional nature of microbiome data and offers improved handling of the high sparsity and zero-inflation commonly observed in these datasets. The method uses a bias correction technique to adjust for the effects of non-biological variation on microbiome data (Lin and Peddada, 2020). Various tools or packages are available for conducting differential abundance analysis. Therefore, the selection of a suitable method should take into account the specific characteristics of the dataset and the underlying assumptions of the chosen approach.

Conclusion

In summary, this review highlights the importance of sheep production, encompassing both its positive contributions and negative implications. Beyond its critical role in ensuring food security by providing a source of animal protein, sheep production also confronts environmental challenges, particularly with regards to CH_4 production through the enteric fermentation of feed within the rumen. Harnessing the power of next-generation sequencing (NGS) and metagenomics, this review underscores the potential of these technologies in unravelling the intricate dynamics of the rumen microbial community. Understanding the role of the rumen microbiome and factors influencing it offers promising avenues for dietary management practices and selective breeding programs aimed at optimizing feed efficiency while concurrently mitigating CH_4 emissions. The integration of NGS and metagenomics approaches in studying the rumen microbiome represents a pivotal step towards achieving sustainable and environmentally friendly sheep production systems.

Aims

The objective of this thesis is to employ metagenomic approaches to further investigate the role of the rumen microbiome in enhancing feed efficiency and reducing methane emissions in sheep. It aims to advance our current understanding of rumen microbiome and to facilitate the development of more effective strategies to improve livestock productivity and sustainability.

References

Akester, A., and Titchen, D. (1969). Radiographic studies of the reticulo-rumen in the sheep. *Journal of anatomy* 104**,** 137.

Akin, D.E., and Borneman, W.S. (1990). Role of rumen fungi in fiber degradation. *J Dairy Sci* 73**,** 3023-3032.

Allafi, F., Hossain, M.S., Lalung, J., Shaah, M., Salehabadi, A., Ahmad, M.I., and Shadi, A. (2022). Advancements in applications of natural wool fiber. *Journal of Natural Fibers* 19**,** 497-512.

Alneberg, J., Bjarnason, B.S., De Bruijn, I., Schirmer, M., Quick, J., Ijaz, U.Z., Lahti, L., Loman, N.J., Andersson, A.F., and Quince, C. (2014). Binning metagenomic contigs by coverage and composition. *Nature Methods* 11**,** 1144-1146.

Alvarado, A., Montañez-Hernández, L.E., Palacio-Molina, S.L., Oropeza-Navarro, R., Luévanos-Escareño, M.P., and Balagurusamy, N. (2014). Microbial trophic interactions and mcrA gene expression in monitoring of anaerobic digesters. *Frontiers in Microbiology* 5.

Alyousef, R., Mohammadhosseini, H., Ebid, A.a.K., and Alabduljabbar, H. (2022). An Integrated Approach to Using Sheep Wool as a Fibrous Material for Enhancing Strength and Transport Properties of Concrete Composites. *Materials (Basel)* 15.

Anderson, M.J. (2014). Permutational multivariate analysis of variance (PERMANOVA). *Wiley statsref: statistics reference online***,** 1-15.

Andrews, S. (2010). "FastQC: a quality control tool for high throughput sequence data". Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom).

Annison, E.F., and Bryden, W.L. (1998). Perspectives on ruminant nutrition and metabolism I. Metabolism in the Rumen. *Nutrition Research Reviews* 11**,** 173-198.

Arias, P., Bellouin, N., Coppola, E., Jones, R., Krinner, G., Marotzke, J., Naik, V., Palmer, M., Plattner, G.-K., and Rogelj, J. (2021). Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change; Technical Summary.

Arndt, C., Hristov, A.N., Price, W.J., Mcclelland, S.C., Pelaez, A.M., Cueva, S.F., Oh, J., Bannink, A., Bayat, A.R., and Crompton, L.A. (2021). Strategies to mitigate enteric methane emissions by ruminants-a way to approach the 2.0° C target. *AgriRxiv***,** 20210085288.

Arora, R., Bhatia, S., and Mishra, B.P. (2011). Genetic variation and relationship of six Indian sheep breeds adapted to the northwestern arid zone of Rajasthan. *Biochem Genet* 49**,** 449-457.

Asselstine, V., Lam, S., Miglior, F., Brito, L.F., Sweett, H., Guan, L., Waters, S.M., Plastow, G., and Cánovas, A. (2021). The potential for mitigation of methane emissions in ruminants through the application of metagenomics, metabolomics, and other -OMICS technologies. *Journal of Animal Science* 99.

Badri, M., Kurtz, Z.D., Bonneau, R., and Müller, C.L. (2020). Shrinkage improves estimation of microbial associations under different normalization methods. *NAR Genomics and Bioinformatics* 2**,** lqaa100.

Beauchemin, K.A., Ungerfeld, E., and Gruninger, R. (2022a). "Control of Methanogenesis in Dairy Animals," in *Encyclopedia of Dairy Sciences (Third Edition),* eds. P.L.H. Mcsweeney & J.P. Mcnamara. (Oxford: Academic Press), 65-78.

Beauchemin, K.A., Ungerfeld, E.M., Abdalla, A.L., Alvarez, C., Arndt, C., Becquet, P., Benchaar, C., Berndt, A., Mauricio, R.M., and Mcallister, T.A. (2022b). Invited review: Current enteric methane mitigation options. *Journal of Dairy Science*.

Beghini, F., Mciver, L.J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., Mailyan, A., Manghi, P., Scholz, M., and Thomas, A.M. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *elife* 10**,** e65088.

Behan, A.A., Loh, T.C., Fakurazi, S., Kaka, U., Kaka, A., and Samsudin, A.A. (2019). Effects of Supplementation of Rumen Protected Fats on Rumen Ecology and Digestibility of Nutrients in Sheep. *Animals (Basel)* 9.

Belanche, A., De La Fuente, G., and Newbold, C.J. (2015). Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiology Ecology* 91**,** fiu026.

Berends, H., Gerrits, W.J.J., France, J., Ellis, J.L., Van Zijderveld, S.M., and Dijkstra, J. (2014). Evaluation of the SF6 tracer technique for estimating methane emission rates with reference to dairy cows using a mechanistic model. *Journal of Theoretical Biology* 353**,** 1-8.

Bergman, E. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological reviews* 70**,** 567-590.

Berndt, A., Boland, T., Deighton, M., Gere, J., Grainger, C., Hegarty, R., Iwaasa, A., Koolaard, J., Lassey, K., and Luo, D. (2014). Guidelines for use of sulphur hexafluoride (SF6) tracer technique to measure enteric methane emissions from ruminants. *Ministry of Primary Industries: Wellington, New Zealand***,** 1-66.

Berry, D., and Crowley, J. (2013). Cell biology symposium: genetics of feed efficiency in dairy and beef cattle. *Journal of animal science* 91**,** 1594-1613.

Bhagat N.R., Kumar S., Kumari R., Bharti V.K. (2023). A Review on Rumen Anaerobic Fungi: Current Understanding on Carbohydrate Fermentation and Roughages Digestion in Ruminants. *Applied Biochemistry and Microbiology*. 2023/06/01;59:231-249.

Bhatt, R.S., Soni, L., and Sahoo, A. (2019). Methane production and microbial protein synthesis in adult sheep fed total mixed ration as mash and as complete feed block. *Carbon Management* 10**,** 241-253.

Bhatta, R., and Enishi, O. (2007). Measurement of methane production from ruminants. *Asian-australasian journal of animal sciences* 20**,** 1305-1318.

Bia, B. (2022). *Export* [Online]. Available: https://www.bordbia.ie/industry/irish-sector-profiles/meat-industry/ [Accessed 22/02/02].

Bickell, S., Revell, D., Toovey, A., and Vercoe, P. (2014). Feed intake of sheep when allowed ad libitum access to feed in methane respiration chambers. *Journal of Animal Science* 92**,** 2259-2264.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30**,** 2114-2120.

Bronner, I.F., and Quail, M.A. (2019). Best Practices for Illumina Library Preparation. *Current Protocols in Human Genetics* 102**,** e86.

Buermans, H.P.J., and Den Dunnen, J.T. (2014). Next generation sequencing technology: Advances and applications. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1842**,** 1932-1941.

Callahan, B.J., Mcmurdie, P.J., and Holmes, S.P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal* 11**,** 2639-2643.

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods* 13**,** 581-583.

Cammack, K.M., Austin, K.J., Lamberson, W.R., Conant, G.C., and Cunningham, H.C. (2018). Ruminnat nutrition symposium: Tiny but mighty: the role of the rumen microbes in livestock production1. *Journal of Animal Science* 96**,** 752-770.

Cao, Q., Sun, X., Rajesh, K., Chalasani, N., Gelow, K., Katz, B., Shah, V.H., Sanyal, A.J., and Smirnova, E. (2021). Effects of Rare Microbiome Taxa Filtering on Statistical Analysis. *Frontiers in Microbiology* 11.

DECC (2022). *CLIMATE ACTION PLAN 2023* [Online]. Available: https://www.gov.ie/pdf/?file=https://assets.gov.ie/243585/9942d689-2490-4ccf-9dc8-f50166bab0e7.pdf [Accessed 22/01/2023].

Caspi, R., Billington, R., Keseler, I.M., Kothari, A., Krummenacker, M., Midford, P.E., Ong, W.K., Paley, S., Subhraveti, P., and Karp, P.D. (2020). The MetaCyc database of metabolic pathways and enzymes-a 2019 update. *Nucleic acids research* 48**,** D445-D453.

Cassia, R., Nocioni, M., Correa-Aragunde, N., and Lamattina, L. (2018). Climate Change and the Impact of Greenhouse Gasses: CO(2) and NO, Friends and Foes of Plant Oxidative Stress. *Front Plant Sci* 9**,** 273.

Castillo, C., Benedito, J., Méndez, J., Pereira, V., Lopez-Alonso, M., Miranda, M., and Hernández, J. (2004). Organic acids as a substitute for monensin in diets for beef cattle. *Animal Feed Science and Technology* 115**,** 101-116.

Cepeda, V., Liu, B., Almeida, M., Hill, C.M., Koren, S., Treangen, T.J., and Pop, M. (2017). MetaCompass: reference-guided assembly of metagenomes. *BioRxiv***,** 212506.

Chaucheyras-Durand, F., and Ossa, F. (2014). The rumen microbiome: Composition, abundance, diversity, and new investigative tools. *The Professional Animal Scientist* 30**,** 1-12.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34**,** i884-i890.

Cheng, J., Zhang, X., Xu, D., Zhang, D., Zhang, Y., Song, Q., Li, X., Zhao, Y., Zhao, L., Li, W., Wang, J., Zhou, B., Lin, C., Yang, X., Zhai, R., Cui, P., Zeng, X., Huang, Y., Ma, Z., Liu, J., and Wang, W. (2022). Relationship between rumen microbial differences and traits among Hu sheep, Tan sheep, and Dorper sheep. *Journal of Animal Science* 100.

Cheng, K., Mccowan, R., and Costerton, J. (1979). Adherent epithelial bacteria in ruminants and their roles in digestive tract function. *The American journal of clinical nutrition* 32**,** 139-148.

Choudhury, P.K., Salem, A.Z.M., Jena, R., Kumar, S., Singh, R., and Puniya, A.K. (2015). Rumen microbiology: An overview. *Rumen microbiology: from evolution to revolution***,** 3-16.

Chow, J.M., Van Kessel, J.A., and Russell, J.B. (1994). Binding of radiolabeled monensin and lasalocid to ruminal microorganisms and feed. *J Anim Sci* 72**,** 1630-1635.

Church, D.C. (1979). *Digestive physiology and nutrition of ruminants. Volume 2. Nutrition.* O & B Books, Inc.

Cifuentes-Faura, J. (2022). European Union policies and their role in combating climate change over the years. *Air Quality, Atmosphere & Health* 15**,** 1333-1340.

Claffey, N.A., Fahey, A.G., Gkarane, V., Moloney, A.P., Monahan, F.J., and Diskin, M.G. (2018). Effect of breed and castration on production and carcass traits of male lambs following an intensive finishing period. *Translational Animal Science* 2**,** 407-418.

Cso (2020). *Environmental Indicators Ireland 2020* [Online]. Available: https://www.cso.ie/en/releasesandpublications/ep/p-eii/environmentalindicatorsireland2020/landuse/ [Accessed 10/12/2022].

Cullen, J.T., Lawlor, P.G., Cormican, P., Crispie, F., and Gardiner, G.E. (2022). Optimisation of a bead-beating procedure for simultaneous extraction of

bacterial and fungal DNA from pig faeces and liquid feed for 16S and ITS2 rDNA amplicon sequencing. *Animal - Open Space* 1**,** 100012.

Cummins, S., Lanigan, G. J., Richards, K. G., Boland, T. M., Kirwan, S. F., Smith, P. E., & Waters, S. M. (2022). Solutions to enteric methane abatement in Ireland. *Irish Journal of Agricultural and Food Research* DOI: 10.15212/ijafr-2022-0014

Dafm (2021). *Food Vision 2030 - A World Leader in Sustainable Food Systems* [Online]. Available: https://www.gov.ie/pdf/?file=https://assets.gov.ie/179717/910771f6-05ed-43f1-b8ce-52ef7c602710.pdf#page=null [Accessed 22/11/2022].

Dafm (2022a). *Annual Review and Outlook for Agriculture, Food and the Marine 2022* [Online]. Available: https://www.gov.ie/en/publication/91e7e-annual-review-and-outlook-for-agriculture-food-and-the-marine-2020/ [Accessed 22/11/22].

da Silva R.R., Pedezzi R., Souto T.B. (2017). Exploring the bioprospecting and biotechnological potential of white-rot and anaerobic Neocallimastigomycota fungi: peptidases, esterases, and lignocellulolytic enzymes. *Applied Microbiology and Biotechnology*. 2017/04/01;101:3089-3101.

Dafm (2022b). *National Sheep and Goat Census 2021* [Online]. Available: https://assets.gov.ie/226265/1598c686-c575-414d-9719-33ff1ff3c72d.pdf [Accessed 22/02/23].

Dagar, S.S., Kumar, S., Mudgil, P., Singh, R., and Puniya, A.K. (2011). D1/D2 domain of large-subunit ribosomal DNA for differentiation of Orpinomyces spp. *Applied and environmental microbiology* 77**,** 6722-6725.

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., Ruttink, T., De Wiele, T.V., and De Campeneere, S. (2017). Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiology Ecology* 93**,** fiw251.

Delgado, L.F., and Andersson, A.F. (2022). Evaluating metagenomic assembly approaches for biome-specific gene catalogues. *Microbiome* 10**,** 72.

Della Rosa, M., Jonker, A., and Waghorn, G. (2021). A review of technical variations and protocols used to measure methane emissions from ruminants using respiration chambers, SF6 tracer technique and GreenFeed, to facilitate global integration of published data. *Animal Feed Science and Technology* 279**,** 115018.

Deng, Z., and Delwart, E. (2021). ContigExtender: a new approach to improving de novo sequence assembly for viral metagenomics data. *BMC Bioinformatics* 22**,** 119.

Denman, S.E., Morgavi, D.P., and Mcsweeney, C.S. (2018). Review: The application of omics to rumen microbiota function. *Animal* 12**,** s233-s245.

Desantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G.L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and environmental microbiology* 72**,** 5069-5072.

Dong, X., Kleiner, M., Sharp, C.E., Thorson, E., Li, C., Liu, D., and Strous, M. (2017). Fast and Simple Analysis of MiSeq Amplicon Sequencing Data with MetaAmp. *Frontiers in Microbiology* 8.

Douglas, G.M., Maffei, V.J., Zaneveld, J.R., Yurgel, S.N., Brown, J.R., Taylor, C.M., Huttenhower, C., and Langille, M.G. (2020). PICRUSt2 for prediction of metagenome functions. *Nature biotechnology* 38**,** 685-688.

Duffy, P., Black, K., Fahey, D., Hyde, B., Kehoe, A., Murphy, J., Quirke, B., Ryan A.M., and Ponzi, J. (2021). Greenhouse gas emissions 1990 - 2019 reported to the united nations framework convention on climate change. *Ireland - national inventory report 2021*.

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* 10**,** 996-998.

Ellison, M., Conant, G., Lamberson, W., Cockrum, R., Austin, K., Rule, D., and Cammack, K. (2017). Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Ruminant Research* 156**,** 12-19.

Ellison, M.J., Cockrum, R.R., Means, W.J., Meyer, A.M., Ritten, J., Austin, K.J., and Cammack, K.M. (2022). Effects of feed efficiency and diet on performance and carcass characteristics in growing wether lambs. *Small Ruminant Research* 207**,** 106611.

EPA(2021). *Ireland's Final Greenhouse Gas Emissions 1990-2019* [Online]. Available: https://www.epa.ie/publications/monitoring--assessment/climate-change/air-emissions/EPA-GHG-Inventory-Report-Final.pdf [Accessed 22/02/23].

EPA(2022). *Climate Change Indicators* [Online]. Available: https://www.epa.gov/climate-indicators/climate-change-indicators-atmospheric-concentrations-greenhouse-gases#ref5 [Accessed 22/02/2023].

EPA (2023). *Ireland projected to fall well short of climate targets, says EPA* [Online]. Available: https://www.epa.ie/news-releases/news-releases-2023/ireland-projected-to-fall-well-short-of-climate-targets-says-epa.php [Accessed 30/01/2024].

Erbach, G. (2015). Doha Amendment to the Kyoto Protocol.

Etheridge, D.M., Steele, L.P., Francey, R., and Langenfelds, R. (1998). Atmospheric methane between 1000 AD and present: Evidence of anthropogenic emissions and climatic variability. *Journal of Geophysical Research: Atmospheres* 103**,** 15979-15993.

European-Commission (2019). *Communication From The Commission - The European Green Deal* [Online]. Available: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1576150542719&uri=COM%3A2019%3A640%3AFIN [Accessed 19/01/2023].

Eurostat (2022). *Land use statistics* [Online]. Available: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Land_use_statistics [Accessed 10/12/2022].

Fao (2022). "Methane Emissions in Livestock and Rice Systems – Sources, quantification, mitigation and metrics (Draft for public review)", in: *Livestock Environmental Assessment and Performance (LEAP) Partnership.* (ed.) Fao. (Rome, Italy).

Fao (2023). *Status and trends of animal genetic resources - 2022* [Online]. Available: https://www.fao.org/3/cc3705en/cc3705en.pdf [Accessed 23/02/2023].

Faostat (2022). *Crops and livestock products* [Online]. Available: https://www.fao.org/faostat/en/#data/QCL [Accessed 10/10/2022].

Feldewert, C., Lang, K., and Brune, A. (2020). The hydrogen threshold of obligately methyl-reducing methanogens. *FEMS Microbiol Lett* 367.

Fellner, V., Sauer, F.D., and Kramer, J.K.G. (1997). Effect of Nigericin, Monensin, and Tetronasin on Biohydrogenation in Continuous Flow-Through Ruminal Fermenters1. *Journal of Dairy Science* 80**,** 921-928.

Fenchel, T., King, G.M., and Blackburn, T.H. (2012). "Chapter 9 - Symbiotic Systems," in *Bacterial Biogeochemistry (Third Edition),* eds. T. Fenchel, G.M. King & T.H. Blackburn. (Boston: Academic Press), 163-181.

Ferdous, T., Jiang, L., Dinu, I., Groizeleau, J., Kozyrskyj, A.L., Greenwood, C.M.T., and Arrieta, M.-C. (2022). The rise to power of the microbiome: power and sample size calculation for microbiome studies. *Mucosal Immunology* 15**,** 1060-1070.

Font-I-Furnols, M., and Guerrero, L. (2014). Consumer preference, behavior and perception about meat and meat products: An overview. *Meat Science* 98**,** 361-371.

Foote, E. (1856). Circumstances affecting the heat of the sun’s rays. *Am. J. Sci. Arts* 22**,** 382-383.

Fowler, S.M., Morris, S., and Hopkins, D.L. (2019). Nutritional composition of lamb retail cuts from the carcases of extensively finished lambs. *Meat Science* 154**,** 126-132.

France, J., and Siddons, R. (1993). Volatile fatty acid production. *Quantitative aspects of ruminant digestion and metabolism* 2**,** 157-175.

Franzosa, E.A., Mciver, L.J., Rahnavard, G., Thompson, L.R., Schirmer, M., Weingart, G., Lipson, K.S., Knight, R., Caporaso, J.G., Segata, N., and Huttenhower, C. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nature Methods* 15**,** 962-968.

Fraser, M.D., Fleming, H.R., Theobald, V.J., and Moorby, J.M. (2015). Effect of breed and pasture type on methane emissions from weaned lambs offered fresh forage. *J Agric Sci* 153**,** 1128-1134.

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28**,** 3150-3152.

Fu, Z., Xu, X., Zhang, J., and Zhang, L. (2020). Effect of different feeding methods on rumen microbes in growing Chinese Tan sheep. *Revista Brasileira de Zootecnia* 49.

Fuks, D., and Marom, N. (2021). Sheep and wheat domestication in southwest Asia: a meta-trajectory of intensification and loss. *Anim Front* 11**,** 20-29.

Galloway-Peña, J., and Hanson, B. (2020). Tools for analysis of the microbiome. *Digestive diseases and sciences* 65**,** 674-685.

Garnsworthy, P.C., Difford, G.F., Bell, M.J., Bayat, A.R., Huhtanen, P., Kuhla, B., Lassen, J., Peiren, N., Pszczola, M., Sorg, D., Visker, M., and Yan, T. (2019). Comparison of Methods to Measure Methane for Use in Genetic Evaluation of Dairy Cattle. *Animals (Basel)* 9.

Gauthier, J., and Derome, N. (2021). Evenness-Richness Scatter Plots: a Visual and Insightful Representation of Shannon Entropy Measurements for Ecological Community Analysis. *mSphere* 6**,** e01019-01020.

Geary, M. (2019). *Wicklow Cheviot Sheep Breeders getting ready for the upcoming show season* [Online]. Available: https://www.agriland.ie/farming-news/wicklow-cheviot-sheep-breeders-getting-ready-for-the-upcoming-show-season/ [Accessed 19/04/2023].

Gemayel, K., Lomsadze, A., and Borodovsky, M. (2022). MetaGeneMark-2: Improved Gene Prediction in Metagenomes. *bioRxiv***,** 2022.2007.2025.500264.

Gere, J.I., Feksa Frasson, M., Wawrzkiewicz, M., Fernández Pepi, M.G., Ramos, M.L., Bualó, R., Cerón-Cucchi, M.E., and Jaurena, G. (2022). Enteric Methane Emission from Sheep Fed with Rhodes Grass Hay (Chloris gayana) Alone or Supplemented with Dried Distillers’ Grains with Solubles. *Methane* 1**,** 210-217.

Ghanbari Maman, L., Palizban, F., Fallah Atanaki, F., Elmi Ghiasi, N., Ariaeenejad, S., Ghaffari, M.R., Hosseini Salekdeh, G., and Kavousi, K. (2020). Co-abundance analysis reveals hidden players associated with high methane yield phenotype in sheep rumen microbiome. *Sci Rep* 10**,** 4995.

Ghavi Hossein-Zadeh, N. (2023). A meta-analysis of the genetic contribution to greenhouse gas emission in sheep. *Journal of Animal Breeding and Genetics* 140**,** 49-59.

Ghurye, J.S., Cepeda-Espinoza, V., and Pop, M. (2016). Metagenomic Assembly: Overview, Challenges and Applications. *Yale J Biol Med* 89**,** 353-362.

Glendinning, L., Genç, B., Wallace, R.J., and Watson, M. (2021). Metagenomic analysis of the cow, sheep, reindeer and red deer rumen. *Scientific reports* 11**,** 1990.

Gloor, G.B., Macklaim, J.M., Pawlowsky-Glahn, V., and Egozcue, J.J. (2017). Microbiome Datasets Are Compositional: And This Is Not Optional. *Frontiers in Microbiology* 8.

Gloor, G.B., Wu, J.R., Pawlowsky-Glahn, V., and Egozcue, J.J. (2016). It's all relative: analyzing microbiome data as compositions. *Annals of Epidemiology* 26**,** 322-329.

Goldman, A.D., Leigh, J.A., and Samudrala, R. (2009). Comprehensive computational analysis of Hmd enzymes and paralogs in methanogenic Archaea. *BMC Evolutionary Biology* 9**,** 1-12.

Goopy, J.P., Robinson, D.L., Woodgate, R., Donaldson, A., Oddy, V., Vercoe, P., and Hegarty, R. (2015). Estimates of repeatability and heritability of methane production in sheep using portable accumulation chambers. *Animal Production Science* 56**,** 116-122.

Goopy, J.P., Woodgate, R., Donaldson, A., Robinson, D.L., and Hegarty, R. (2011). Validation of a short-term methane measurement using portable static chambers to estimate daily methane production in sheep. *Animal Feed Science and Technology* 166**,** 219-226.

Granja-Salcedo, Y.T., Ramirez-Uscategui, R.A., Machado, E.G., Duarte Messana, J., Takeshi Kishi, L., Lino Dias, A.V., and Berchielli, T.T. (2017). Studies on bacterial community composition are affected by the time and storage method of the rumen content. *PLOS ONE* 12**,** e0176701.

Greening, C., Geier, R., Wang, C., Woods, L.C., Morales, S.E., Mcdonald, M.J., Rushton-Green, R., Morgan, X.C., Koike, S., and Leahy, S.C. (2019). Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME journal* 13**,** 2617-2632.

Gruninger, R., Ribeiro, G., Cameron, A., and Mcallister, T. (2019). Invited review: Application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal* 13**,** 1843-1854.

Gruninger, R.J., Puniya, A.K., Callaghan, T.M., Edwards, J.E., Youssef, N., Dagar, S.S., Fliegerova, K., Griffith, G.W., Forster, R., Tsang, A., Mcallister, T., and Elshahed, M.S. (2014). Anaerobic fungi (phylum Neocallimastigomycota): advances in understanding their taxonomy, life cycle, ecology, role and biotechnological potential. *FEMS Microbiol Ecol* 90**,** 1-17.

Gupta, N. (2019). DNA Extraction and Polymerase Chain Reaction. *J Cytol* 36**,** 116-117.

Gupta, S., Mortensen, M.S., Schjørring, S., Trivedi, U., Vestergaard, G., Stokholm, J., Bisgaard, H., Krogfelt, K.A., and Sørensen, S.J. (2019). Amplicon

sequencing provides more accurate microbiome information in healthy children compared to culturing. *Communications Biology* 2**,** 291.

Guyader, J., Eugène, M., Noziere, P., Morgavi, D., Doreau, M., and Martin, C. (2014). Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach1. *Animal* 8**,** 1816-1825.

Hackmann, T.J., Ngugi, D.K., Firkins, J.L., and Tao, J. (2017). Genomes of rumen bacteria encode atypical pathways for fermenting hexoses to short-chain fatty acids. *Environmental Microbiology* 19**,** 4670-4683.

Hackmann, T.J., and Spain, J.N. (2010). Invited review: Ruminant ecology and evolution: Perspectives useful to ruminant livestock research and production. *Journal of Dairy Science* 93**,** 1320-1334.

Hammond, K., Hoskin, S., Burke, J., Waghorn, G., Koolaard, J., and Muetzel, S. (2011). Effects of feeding fresh white clover (Trifolium repens) or perennial ryegrass (Lolium perenne) on enteric methane emissions from sheep. *Animal Feed Science and Technology* 166**,** 398-404.

Hammond, K., Humphries, D., Crompton, L., Green, C., and Reynolds, C. (2015). Methane emissions from cattle: Estimates from short-term measurements using a GreenFeed system compared with measurements obtained using respiration chambers or sulphur hexafluoride tracer. *Animal Feed Science and Technology* 203**,** 41-52.

Hammond, K.J., Crompton, L.A., Bannink, A., Dijkstra, J., Yáñez-Ruiz, D.R., O'kiely, P., Kebreab, E., Eugène, M.A., Yu, Z., Shingfield, K.J., Schwarm, A., Hristov, A.N., and Reynolds, C.K. (2016). Review of current in vivo measurement techniques for quantifying enteric methane emission from ruminants. *Animal Feed Science and Technology* 219**,** 13-30.

Hanrahan, J. (1991). Evidence for single gene effects on ovulation rate in the Cambridge and Belclare breeds. *Colloques de l'INRA (France)*.

Hanrahan, J.P., Gregan, S.M., Mulsant, P., Mullen, M., Davis, G.H., Powell, R., and Galloway, S.M. (2004). Mutations in the Genes for Oocyte-Derived Growth Factors GDF9 and BMP15 Are Associated with Both Increased Ovulation Rate and Sterility in Cambridge and Belclare Sheep (Ovis aries)1. *Biology of Reproduction* 70**,** 900-909.

Harfoot, C. (1981). Anatomy, physiology and microbiology of the ruminant digestive tract. *Lipid metabolism in ruminant animals***,** 1-19.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Abecia, L., Angarita, E., Aravena, P., Nora Arenas, G., Ariza, C., Attwood, G.T., Mauricio Avila, J., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M.F., Brown-Kav, A., Carvajal, A.M., Cersosimo, L., Vieira Chaves, A., Church, J., Clipson, N., Cobos-Peralta, M.A., Cookson, A.L., Cravero, S., Cristobal Carballo, O., Crosley, K., Cruz, G., Cerón Cucchi, M., De La Barra, R., De Menezes, A.B., Detmann, E., Dieho, K., Dijkstra, J., Dos Reis, W.L.S., Dugan, M.E.R., Hadi Ebrahimi, S., Eythórsdóttir, E., Nde Fon, F., Fraga, M., Franco, F., Friedeman, C., Fukuma, N., Gagić, D., Gangnat, I., Javier Grilli, D., Guan,

L.L., Heidarian Miri, V., Hernandez-Sanabria, E., Gomez, A.X.I., Isah, O.A., Ishaq, S., Jami, E., Jelincic, J., Kantanen, J., Kelly, W.J., Kim, S.-H., Klieve, A., Kobayashi, Y., Koike, S., Kopecny, J., Nygaard Kristensen, T., Julie Krizsan, S., Lachance, H., Lachman, M., Lamberson, W.R., Lambie, S., Lassen, J., Leahy, S.C., Lee, S.-S., Leiber, F., Lewis, E., Lin, B., Lira, R., Lund, P., Macipe, E., Mamuad, L.L., Cuquetto Mantovani, H., Marcoppido, G.A., Márquez, C., Martin, C., Martinez, G., Eugenia Martinez, M., Lucía Mayorga, O., Mcallister, T.A., Mcsweeney, C., Mestre, L., Minnee, E., Mitsumori, M., Mizrahi, I., Molina, I., Muenger, A., Muñoz, C., Murovec, B., Newbold, J., Nsereko, V., O'donovan, M., Okunade, S., O'neill, B., et al. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* 5**,** 14567.

Hess M, Paul SS, Puniya AK, Van der Giezen M, Shaw C, Edwards JE, Fliegerová K. (2020). Anaerobic fungi: past, present, and future. *Frontiers in Microbiology*.11:584893.

Hill, J., Mcsweeney, C., Wright, A.-D.G., Bishop-Hurley, G., and Kalantar-Zadeh, K. (2016). Measuring Methane Production from Ruminants. *Trends in Biotechnology* 34**,** 26-35.

Horowitz, C.A. (2016). Paris Agreement. *International Legal Materials* 55**,** 740-755.

Hristov, A.N., Kebreab, E., Niu, M., Oh, J., Bannink, A., Bayat, A.R., Boland, T.M., Brito, A.F., Casper, D.P., Crompton, L.A., Dijkstra, J., Eugène, M., Garnsworthy, P.C., Haque, N., Hellwing, A.L.F., Huhtanen, P., Kreuzer, M., Kuhla, B., Lund, P., Madsen, J., Martin, C., Moate, P.J., Muetzel, S., Muñoz, C., Peiren, N., Powell, J.M., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Storlien, T.M., Weisbjerg, M.R., Yáñez-Ruiz, D.R., and Yu, Z. (2018). Symposium review: Uncertainties in enteric methane inventories, measurement techniques, and prediction models. *J Dairy Sci* 101**,** 6655-6674.

Huhtanen P., Cabezas-Garcia E.H., Utsumi S., Zimmerman S. (2015). Comparison of methods to determine methane emissions from dairy cows in farm conditions. *Journal of Dairy Science*. 2015/05/01/;98:3394-3409.

Hungate, R. (1975). The rumen microbial ecosystem. *Annual Review of Ecology and Systematics* 6**,** 39-66.

Huson, D.H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.-J., and Tappu, R. (2016). MEGAN community edition-interactive exploration and analysis of large-scale microbiome sequencing data. *PLoS computational biology* 12**,** e1004957.

Huws, S.A., Edwards, J.E., Creevey, C.J., Rees Stevens, P., Lin, W., Girdwood, S.E., Pachebat, J.A., and Kingston-Smith, A.H. (2015). Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiology Ecology* 92.

Huws, S.A., Edwards, J.E., Creevey, C.J., Rees Stevens, P., Lin, W., Girdwood, S.E., Pachebat, J.A., and Kingston-Smith, A.H. (2016). Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS Microbiology Ecology* 92**,** fiv137.

Ibrahim, A., Budisatria, I.G.S., Widayanti, R., and Artama, W.T. (2019). The impact of religious festival on roadside livestock traders in urban and peri-urban areas of Yogyakarta, Indonesia. *Veterinary World* 12**,** 1408.

Illumina (2021). *Long read lengths for shotgun metagenomics* [Online]. Available: https://www.illumina.com/content/dam/illumina/gcs/assembled-assets/marketing-literature/novaseq-shotgun-metagenomics-app-note-m-na-00010/novaseq-shotgun-metagenomics-app-note-m-na-00010.pdf [Accessed 01/02/2023].

Illumina (nd). *Focused power on the MiSeq System* [Online]. Available: https://www.illumina.com/systems/sequencing-platforms/miseq.html [Accessed 02/02/2023].

Islam, M., and Lee, S.S. (2019). Advanced estimation and mitigation strategies: a cumulative approach to enteric methane abatement from ruminants. *J Anim Sci Technol* 61**,** 122-137.

Janssen, P.H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160**,** 1-22.

Jayanegara, A., Sarwono, K.A., Kondo, M., Matsui, H., Ridla, M., Laconi, E.B., and Nahrowi (2018). Use of 3-nitrooxypropanol as feed additive for mitigating enteric methane emissions from ruminants: a meta-analysis. *Italian Journal of Animal Science* 17**,** 650-656.

Jiang, Y., Xie, M., Chen, W., Talbot, R., Maddox, J.F., Faraut, T., Wu, C., Muzny, D.M., Li, Y., and Zhang, W. (2014). The sheep genome illuminates biology of the rumen and lipid metabolism. *Science* 344**,** 1168-1173.

Jin W., Cheng Y.F., Mao S.Y., Zhu W.Y. (2011). Isolation of natural cultures of anaerobic fungi and indigenously associated methanogens from herbivores and their bioconversion of lignocellulosic materials to methane. *Bioresource Technology*. 2011/09/01/;102:7925-7931.

Johnson, K.A., and Johnson, D.E. (1995). Methane emissions from cattle. *Journal of animal science* 73**,** 2483-2492.

Jones, D.M. (2018). *Pronghorn-Antelope* [Online]. Donald M. Jones Photography: Donald M. Jones. Available: https://donaldmjones.com/portfolios/pronghorn-antelope [Accessed 10/04/23].

Jonker, A., Hickey, S.M., Rowe, S.J., Janssen, P.H., Shackell, G.H., Elmes, S., Bain, W.E., Wing, J., Greer, G.J., Bryson, B., Maclean, S., Dodds, K.G., Pinares-Patiño, C.S., Young, E.A., Knowler, K., Pickering, N.K., and Mcewan, J.C. (2018). Genetic parameters of methane emissions determined using

portable accumulation chambers in lambs and ewes grazing pasture and genetic correlations with emissions determined in respiration chambers1. *Journal of Animal Science* 96**,** 3031-3042.

Kameoka, S., Motooka, D., Watanabe, S., Kubo, R., Jung, N., Midorikawa, Y., Shinozaki, N.O., Sawai, Y., Takeda, A.K., and Nakamura, S. (2021). Benchmark of 16S rRNA gene amplicon sequencing using Japanese gut microbiome data from the V1–V2 and V3–V4 primer sets. *BMC Genomics* 22**,** 527.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P.H., Shi, W., Froula, J., Rubin, E.M., and Attwood, G.T. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4**,** 56.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic acids research* 44**,** D457-D462.

Kang, D.D., Li, F., Kirton, E., Thomas, A., Egan, R., An, H., and Wang, Z. (2019). MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *PeerJ* 7**,** e7359.

Kirton, A.H., Carter, A.H., Clarke, J.N., Sinclair, D.P., Mercer, G.J.K., and Duganzich, D.M. (1995). A comparison between 15 ram breeds for export lamb production 1. Liveweights, body components, carcass measurements, and composition. *New Zealand Journal of Agricultural Research* 38**,** 347-360.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., Mcewan, J.C., and Janssen, P.H. (2014). Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. *PLOS ONE* 9**,** e103171.

Kleinen, T., Gromov, S., Steil, B., and Brovkin, V. (2021). Atmospheric methane underestimated in future climate projections. *Environmental Research Letters* 16**,** 094006.

Koch, R.M., Swiger, L.A., Chambers, D., and Gregory, K.E. (1963). Efficiency of feed use in beef cattle. *Journal of animal science* 22**,** 486-494.

Koike, S., and Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus flavefaciens. *FEMS microbiology letters* 204**,** 361-366.

Koleff, P., Gaston, K.J., and Lennon, J.J. (2003). Measuring beta diversity for presence–absence data. *Journal of Animal Ecology* 72**,** 367-382.

Kozarewa, I., Ning, Z., Quail, M.A., Sanders, M.J., Berriman, M., and Turner, D.J. (2009). Amplification-free Illumina sequencing-library preparation

facilitates improved mapping and assembly of (G+C)-biased genomes. *Nat Methods* 6**,** 291-295.

Krause, D., Nagaraja, T., Wright, A., and Callaway, T. (2013). Board-invited review: rumen microbiology: leading the way in microbial ecology. *Journal of animal science* 91**,** 331-341.

Kurth, J.M., Op Den Camp, H.J.M., and Welte, C.U. (2020). Several ways one goal—methanogenesis from unconventional substrates. *Applied Microbiology and Biotechnology* 104**,** 6839-6854.

Kweku, D.W., Bismark, O., Maxwell, A., Desmond, K.A., Danso, K.B., Oti-Mensah, E.A., Quachie, A.T., and Adormaa, B.B. (2018). Greenhouse effect: greenhouse gases and their impact on global warming. *Journal of Scientific research and reports* 17**,** 1-9.

Lambie, S.C., Kelly, W.J., Leahy, S.C., Li, D., Reilly, K., Mcallister, T.A., Valle, E.R., Attwood, G.T., and Altermann, E. (2015). The complete genome sequence of the rumen methanogen Methanosarcina barkeri CM1. *Standards in Genomic Sciences* 10**,** 57.

Lan, X., K.W. Thoning, and E.J. Dlugokencky (2023). *Trends in globally-averaged CH4, N2O, and SF6 determined from NOAA Global Monitoring Laboratory measurements* [Online]. Available: https://doi.org/10.15138/P8XG-AA10 [Accessed 19/02/2023].

Langille, M.G., Zaneveld, J., Caporaso, J.G., Mcdonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., and Knight, R. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology* 31**,** 814-821.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods* 9**,** 357-359.

Lashof, D.A., and Ahuja, D.R. (1990). Relative contributions of greenhouse gas emissions to global warming. *Nature* 344**,** 529-531.

Latorre-Pérez, A., Pascual, J., Porcar, M., and Vilanova, C. (2020). A lab in the field: applications of real-time, in situ metagenomic sequencing. *Biology Methods and Protocols* 5**,** bpaa016.

Lee, H., Calvin, K., Dasgupta, D., Krinner, G., Mukherji, A., Thorne, P., Trisos, C., Romero, J., Aldunce, P., Barret, K. and Blanco, G., (2023). IPCC, 2023: Climate Change 2023: Synthesis Report, Summary for Policymakers. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. *IPCC*, Geneva, Switzerland.

Leray, M., Haenel, Q., and Bourlat, S. (2016). "Preparation of Amplicon Libraries for Metabarcoding of Marine Eukaryotes Using Illumina MiSeq: The Adapter Ligation Method."), 209-218.

Leschine, S.B. (1995). Cellulose degradation in anaerobic environments. *Annual review of microbiology* 49**,** 399-426.

Letcher, T.M. (2021). "Global warming—a complex situation," in *Climate Change*. Elsevier), 3-17.

Li, D., Liu, C.-M., Luo, R., Sadakane, K., and Lam, T.-W. (2015). MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. *Bioinformatics* 31**,** 1674-1676.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler transform. *bioinformatics* 25**,** 1754-1760.

Li, Z., Mu, C., Xu, Y., Shen, J., and Zhu, W. (2020). Changes in the solid-, liquid-, and epithelium-associated bacterial communities in the rumen of hu lambs in response to dietary urea supplementation. *Frontiers in microbiology* 11**,** 244.

Lin, H., and Peddada, S.D. (2020). Analysis of microbial compositions: a review of normalization and differential abundance analysis. *npj Biofilms and Microbiomes* 6**,** 60.

Liu, L.-Y., Xie, G.-J., Ding, J., Liu, B.-F., Xing, D.-F., Ren, N.-Q., and Wang, Q. (2022). Microbial methane emissions from the non-methanogenesis processes: A critical review. *Science of The Total Environment* 806**,** 151362.

Liu, Y.-X., Qin, Y., Chen, T., Lu, M., Qian, X., Guo, X., and Bai, Y. (2021). A practical guide to amplicon and metagenomic analysis of microbiome data. *Protein & Cell* 12**,** 315-330.

Liu, Y., and Whitman, W.B. (2008). Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Annals of the New York Academy of Sciences* 1125**,** 171-189.

Lopes, D. R. G., de Souza Duarte, M., La Reau, A. J., Chaves, I. Z., de Oliveira Mendes, T. A., Detmann, E., and Mantovani, H. C. (2021). Assessing the relationship between the rumen microbiota and feed efficiency in Nellore steers. *Journal of Animal Science and Biotechnology*, *12*, 1-17.

Lourenco, J.M., and Welch, C.B. (2022). Using microbiome information to understand and improve animal performance. *Italian Journal of Animal Science* 21**,** 899-913.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15**,** 1-21.

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and environmental microbiology* 71**,** 8228-8235.

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., He, G., Chen, Y., Pan, Q., and Liu, Y. (2012). SOAPdenovo2: an empirically improved memory-efficient short-read de novo assembler. *Gigascience* 1**,** 2047-2217X-2041-2018.

Machmüller, A. (2006). Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. *Agriculture, Ecosystems & Environment* 112**,** 107-114.

Machmüller, A., Ossowski, D., and Kreuzer, M. (2000). Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. *Animal Feed Science and Technology* 85**,** 41-60.

Mandal, S., Van Treuren, W., White, R.A., Eggesbø, M., Knight, R., and Peddada, S.D. (2015). Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease* 26**,** 27663.

Mansour, E., Curling, S., Stéphan, A., and Ormondroyd, G. (2016). Absorption of volatile organic compounds by different wool types. *Green Materials* 4**,** 1-7.

Mao, H.-L., Wang, J.-K., Zhou, Y.-Y., and Liu, J.-X. (2010). Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livestock Science* 129**,** 56-62.

Marchesi, J.R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome* 3**,** 31.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* 17**,** 10-12.

Martínez-Fernández, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martín-García, A.I., Molina-Alcaide, E., Kindermann, M., Duval, S., and Yáñez-Ruiz, D.R. (2014). Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. *Journal of Dairy Science* 97**,** 3790-3799.

Martinez-Fernandez, G., Denman, S.E., and Mcsweeney, C.S. (2019). Sample Processing Methods Impacts on Rumen Microbiome. *Front Microbiol* 10**,** 861.

Martinez Boggio, G., Marie-Etancelin, C., Menras, J.-M., Tomas, R., Chemit, M.-L., Gabinaud, B., Pascal, G., and Meynadier, A. (2023). A large database linking the rumen bacterial composition and milk traits in Lacaune sheep. *Scientific Data* 10**,** 17.

Masella, A.P., Bartram, A.K., Truszkowski, J.M., Brown, D.G., and Neufeld, J.D. (2012). PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13**,** 31.

Masson-Delmotte, V., Zhai, P., Pirani, A., Connors, S.L., Péan, C., Berger, S., Caud, N., Chen, Y., Goldfarb, L., and Gomis, M. (2021). Climate change 2021: the physical science basis. *Contribution of working group I to the sixth assessment report of the intergovernmental panel on climate change* 2.

Masson-Delmotte, V., Zhai, P., Pörtner, H.-O., Roberts, D., Skea, J., Shukla, P.R., Pirani, A., Moufouma-Okia, W., Péan, C., and Pidcock, R. (2018). Global warming of 1.5 C. *An IPCC Special Report on the impacts of global warming of* 1**,** 43-50.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'toole, P.W., and Cotter, P.D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10**,** 115-132.

Mcallister, T., Bae, H., Jones, G., and Cheng, K.-J. (1994). Microbial attachment and feed digestion in the rumen. *Journal of animal science* 72**,** 3004-3018.

Mcallister, T.A., Dunière, L., Drouin, P., Xu, S., Wang, Y., Munns, K., and Zaheer, R. (2018). Silage review: Using molecular approaches to define the microbial ecology of silage. *Journal of Dairy Science* 101**,** 4060-4074.

Mccabe, M.S., Cormican, P., Keogh, K., O'connor, A., O'hara, E., Palladino, R.A., Kenny, D.A., and Waters, S.M. (2015). Illumina MiSeq Phylogenetic Amplicon Sequencing Shows a Large Reduction of an Uncharacterised Succinivibrionaceae and an Increase of the Methanobrevibacter gottschalkii Clade in Feed Restricted Cattle. *PLOS ONE* 10**,** e0133234.

Mcgovern, E., Waters, S.M., Blackshields, G., and Mccabe, M.S. (2018). Evaluating Established Methods for Rumen 16S rRNA Amplicon Sequencing With Mock Microbial Populations. *Front Microbiol* 9**,** 1365.

Merrick, M. (2022). *Belclare sheep: bred for Irish systems to increase flock profitability* [Online]. Farmers Journal. Available: https://www.farmersjournal.ie/belclare-sheep-bred-for-irish-systems-to-increase-flock-profitability-720860 [Accessed 19/04/2023].

Millen, D.D., Arrigoni, M.D.B., and Pacheco, R.D.L. (2016). *Rumenology.* Springer.

Mokhtari, E.B., and Ridenhour, B.J. (2022). Filtering ASVs/OTUs via mutual information-based microbiome network analysis. *BMC bioinformatics* 23**,** 380.

Moran, M.A. (2009). Metatranscriptomics: eavesdropping on complex microbial communities. *Microbe* 4**,** 7.

Morgavi, D.P., Forano, E., Martin, C., and Newbold, C.J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal* 4**,** 1024-1036.

Morgavi, D.P., Kelly, W., Janssen, P., and Attwood, G. (2013). Rumen microbial (meta) genomics and its application to ruminant production. *animal* 7**,** 184-201.

Morrison, I. (1979). Carbohydrate chemistry and rumen digestion. *Proceedings of the Nutrition Society* 38**,** 269-274.

Mullen, M.P., Hanrahan, J.P., Howard, D.J., and Powell, R. (2013). Investigation of Prolific Sheep from UK and Ireland for Evidence on Origin of the Mutations in BMP15 (FecXG, FecXB) and GDF9 (FecGH) in Belclare and Cambridge Sheep. *PLOS ONE* 8**,** e53172.

Myer, P.R., Kim, M., Freetly, H.C., and Smith, T.P.L. (2016). Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. *Journal of Microbiological Methods* 127**,** 132-140.

Namiki, T., Hachiya, T., Tanaka, H., and Sakakibara, Y. (Year). "MetaVelvet: an extension of Velvet assembler to de novo metagenome assembly from short sequence reads", in: *Proceedings of the 2nd ACM conference on bioinformatics, computational biology and biomedicine*), 116-124.

Naqvi, S., and Sejian, V. (2011). Global climate change: role of livestock. *Asian Journal of Agricultural Sciences* 3**,** 19-25.

Nearing, J.T., Douglas, G.M., Hayes, M.G., Macdonald, J., Desai, D.K., Allward, N., Jones, C.M.A., Wright, R.J., Dhanani, A.S., Comeau, A.M., and Langille, M.G.I. (2022). Microbiome differential abundance methods produce different results across 38 datasets. *Nature Communications* 13**,** 342.

Neves, A.L., Yu, J., Suzuki, Y., Baez-Magana, M., Arutyunova, E., O'hara, E., Mcallister, T., Ominski, K.H., Lemieux, M.J., and Guan, L.L. (2021). Accelerated discovery of novel glycoside hydrolases using targeted functional profiling and selective pressure on the rumen microbiome. *Microbiome* 9**,** 1-16.

Newbold, C., and Ramos-Morales, E. (2020). Ruminal microbiome and microbial metabolome: effects of diet and ruminant host. *Animal* 14**,** s78-s86.

Newbold, C.J., De La Fuente, G., Belanche, A., Ramos-Morales, E., and Mcewan, N.R. (2015). The role of ciliate protozoa in the rumen. *Frontiers in microbiology* 6**,** 1313.

Ngámbi, J.W., Selapa, M.J., Brown, D., and Manyelo, T.G. (2022). The effect of varying levels of purified condensed tannins on performance, blood profile, meat quality and methane emission in male Bapedi sheep fed grass hay and pellet-based diet. *Trop Anim Health Prod* 54**,** 263.

Nguyen S.H., Nguyen H.D.T., Hegarty RS. (2020). Defaunation and its impacts on ruminal fermentation, enteric methane production and animal productivity. *Livestock Research for Rural Development*.32:1-9

Niu, S.-Y., Yang, J., Mcdermaid, A., Zhao, J., Kang, Y., and Ma, Q. (2018). Bioinformatics tools for quantitative and functional metagenome and metatranscriptome data analysis in microbes. *Briefings in Bioinformatics* 19**,** 1415-1429.

Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P.A. (2017). metaSPAdes: a new versatile metagenomic assembler. *Genome research* 27**,** 824-834.

O'mara, F. (2022). *Teagasc Hill Sheep Conference 2022 (Foreword)* [Online]. Available: https://www.teagasc.ie/media/website/publications/2022/Teagasc-National-Hill-Sheep-Conference-2022-full-web.pdf [Accessed 22/02/2023].

O'mara, F.P. (2012). The role of grasslands in food security and climate change. *Annals of Botany* 110**,** 1263-1270.

O' Connor, E., Mchugh, N., Boland, T.M., Dunne, E., and Mcgovern, F.M. (2021). Investigation of intra-day variability of gaseous measurements in sheep using portable accumulation chambers. *Journal of Animal Science* 99.

O'rourke, E., Kramm, N., and Chisholm, N. (2012). The influence of farming styles on the management of the Iveragh uplands, southwest Ireland. *Land Use Policy* 29**,** 805-816.

Ogunade, I., Schweickart, H., Andries, K., Lay, J., and Adeyemi, J. (2018). Monensin Alters the Functional and Metabolomic Profile of Rumen Microbiota in Beef Cattle. *Animals (Basel)* 8.

Oireachtas (2021). *Climate Action and Low Carbon Development (Amendment) Act 2021* [Online]. Available: https://data.oireachtas.ie/ie/oireachtas/act/2021/32/eng/enacted/a3221.pdf [Accessed 19/01/2023].

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., and Wagner, H. (2013). Package 'vegan'. *Community ecology package, version* 2**,** 1-295.

Pachauri, R., and Meyer, L. (2014). Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change.

Paganoni, B., Rose, G., Macleay, C., Jones, C., Brown, D., Kearney, G., Ferguson, M., and Thompson, A. (2017). More feed efficient sheep produce less methane and carbon dioxide when eating high-quality pellets. *Journal of Animal Science* 95**,** 3839-3850.

Pan, A.Y. (2021). Statistical analysis of microbiome data: The challenge of sparsity. *Current Opinion in Endocrine and Metabolic Research* 19**,** 35-40.

Patra, A., Min, B., and Saxena, J. (2012). "Dietary Tannins on Microbial Ecology of the Gastrointestinal Tract in Ruminants."), 237-262.

Patra, A., Park, T., Kim, M., and Yu, Z. (2017). Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *Journal of Animal Science and Biotechnology* 8**,** 13.

Patra, A.K. (2016). Recent Advances in Measurement and Dietary Mitigation of Enteric Methane Emissions in Ruminants. *Frontiers in Veterinary Science* 3.

Patro, R., Duggal, G., and Kingsford, C. (2015). Salmon: accurate, versatile and ultrafast quantification from RNA-seq data using lightweight-alignment.

Paulson, J.N., Stine, O.C., Bravo, H.C., and Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nat Methods* 10**,** 1200-1202.

Pearce, K., Norman, H., and Hopkins, D. (2010). The role of saltbush-based pasture systems for the production of high quality sheep and goat meat. *Small Ruminant Research* 91**,** 29-38.

Pei, A.Y., Oberdorf, W.E., Nossa, C.W., Agarwal, A., Chokshi, P., Gerz, E.A., Jin, Z., Lee, P., Yang, L., Poles, M., Brown, S.M., Sotero, S., Desantis, T., Brodie, E., Nelson, K., and Pei, Z. (2010). Diversity of 16S rRNA Genes within Individual Prokaryotic Genomes. *Applied and Environmental Microbiology* 76**,** 3886-3897.

Pickering, N., Oddy, V., Basarab, J., Cammack, K., Hayes, B., Hegarty, R., Lassen, J., Mcewan, J., Miller, S., and Pinares-Patiño, C. (2015). Animal board invited review: genetic possibilities to reduce enteric methane emissions from ruminants. *animal* 9**,** 1431-1440.

Pinares-Patiño, C., Hickey, S., Young, E., Dodds, K., Maclean, S., Molano, G., Sandoval, E., Kjestrup, H., Harland, R., and Hunt, C. (2013). Heritability estimates of methane emissions from sheep. *animal* 7**,** 316-321.

Pitta, D., Indugu, N., Narayan, K., and Hennessy, M. (2022). Symposium review: Understanding the role of the rumen microbiome in enteric methane mitigation and productivity in dairy cows. *Journal of Dairy Science* 105**,** 8569-8585.

Pollock, J., Glendinning, L., Wisedchanwet, T., and Watson, M. (2018). The Madness of Microbiome: Attempting To Find Consensus "Best Practice" for 16S Microbiome Studies. *Appl Environ Microbiol* 84.

Pörtner, H.-O., Roberts, D.C., Adams, H., Adler, C., Aldunce, P., Ali, E., Begum, R.A., Betts, R., Kerr, R.B., and Biesbroek, R. (2022). Climate change 2022: Impacts, adaptation and vulnerability. *IPCC Sixth Assessment Report*.

Prache, S., Martin, B., and Coppa, M. (2020). Review: Authentication of grass-fed meat and dairy products from cattle and sheep. *Animal* 14**,** 854-863.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F.O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* 41**,** D590-D596.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature biotechnology* 35**,** 833-844.

Rabee, A.E., Forster, R.J., Elekwachi, C.O., Kewan, K.Z., Sabra, E.A., Shawket, S.M., Mahrous, H.A., and Khamiss, O.A. (2019). Community structure and fibrolytic activities of anaerobic rumen fungi in dromedary camels. *Journal of Basic Microbiology* 59**,** 101-110.

Rajabinejad, H., Bucişcanu, I.-I., and Maier, S.S. (2019). CURRENT APPROACHES FOR RAW WOOL WASTE MANAGEMENT AND UNCONVENTIONAL VALORIZATION: A REVIEW. *Environmental Engineering & Management Journal (EEMJ)* 18.

Ramos-Morales, E., Arco-Pérez, A., Martín-García, A.I., Yáñez-Ruiz, D.R., Frutos, P., and Hervás, G. (2014). Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Animal Feed Science and Technology* 198**,** 57-66.

Ren, Q., Si, H., Yan, X., Liu, C., Ding, L., Long, R., Li, Z., and Qiu, Q. (2020). Bacterial communities in the solid, liquid, dorsal, and ventral epithelium fractions of yak (Bos grunniens) rumen. *Microbiologyopen* 9**,** e963.

Rezaei, H.R., Naderi, S., Chintauan-Marquier, I.C., Taberlet, P., Virk, A.T., Naghash, H.R., Rioux, D., Kaboli, M., and Pompanon, F. (2010). Evolution and taxonomy of the wild species of the genus Ovis (Mammalia, Artiodactyla, Bovidae). *Molecular Phylogenetics and Evolution* 54**,** 315-326.

Rezaeian, M., Beakes, G.W., and Parker, D.S. (2004). Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. *Mycological Research* 108**,** 1227-1233.

Ritchie, H., Roser, M., and Rosado, P. (2020). OurWorldInData.org. Available: https://ourworldindata.org/co2-and-greenhouse-gas-emissions [Accessed 23/02/2023].

Robinson, M.D., Mccarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *bioinformatics* 26**,** 139-140.

Rowe, S., Hickey, S., Jonker, A., Hess, M., Janssen, P., Johnson, T., Bryson, B., Knowler, K., Pinares-Patino, C., and Bain, W. (Year). "Selection for divergent methane yield in New Zealand sheep–a ten-year perspective", in: *Proc. Assoc. Adv. Anim. Breed. Genet*), 306-309.

Russell, J.B., and Houlihan, A.J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health1. *FEMS Microbiology Reviews* 27**,** 65-74.

Russell, J.B., and Strobel, H. (1989). Effect of ionophores on ruminal fermentation. *Applied and environmental microbiology* 55**,** 1-6.

Ryan, M., Hennessy, T., Buckley, C., Dillon, E.J., Donnellan, T., Hanrahan, K., and Moran, B. (2016). Developing farm-level sustainability indicators for Ireland using the Teagasc National Farm Survey. *Irish Journal of Agricultural and Food Research* 55**,** 112-125.

Salter, A.M. (2017). Improving the sustainability of global meat and milk production. *Proceedings of the Nutrition Society* 76**,** 22-27.

Samir, M., and Ghadbane, M. (2021). "Sheep Digestive Physiology and Constituents of Feeds.").

Sanjorjo, R.A., Tseten, T., Kang, M.-K., Kwon, M., and Kim, S.-W. 2023. In Pursuit of Understanding the Rumen Microbiome. *Fermentation* [Online], 9.

Santana, M., Rossi Jr, P., Almeida, R., and Cucco, D. (2012). Feed efficiency and its correlations with carcass traits measured by ultrasound in Nellore bulls. *Livestock Science* 145**,** 252-257.

Santos Torres, R.D.N., Coelho, L.D.M., Ghedini, C.P., Neto, O.R.M., Chardulo, L.a.L., Torrecilhas, J.A., De Lima Valença, R., Baldassini, W.A., and Almeida, M.T.C. (2023). Potential of nutritional strategies to reduce enteric methane emission in feedlot sheep: A meta-analysis and multivariate analysis. *Small Ruminant Research* 220**,** 106919.

Satyanagalakshmi, K., Sridhar, G., and Sirohi, S. (2015). An overview of the role of rumen methanogens in methane emission and its reduction strategies. *African Journal of Biotechnology* 14**,** 1427-1438.

Savage, D.E., and Russell, D.E. (1983). Mammalian paleofaunas of the world. *BENJAMIN/CUMMINGS, MENLO PARK, CA(USA). 1983.*

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., Consortium, F.B., List, F.B.C.A., and Bolchacova, E. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the national academy of Sciences* 109**,** 6241-6246.

Schrobback, P., Dennis, G., Li, Y., Mayberry, D., Shaw, A., Knight-Jones, T., Herrero, M., Marsh TL, Pendell DL, Torgerson PR, Gilbert W, and Huntington B (2023). Approximating the global economic (market) value of farmed animals. *Global food security*, *39*, 100722.

Seemann, T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 30**,** 2068-2069.

Sejian, V., Bhatta, R., Gaughan, J., Malik, P.K., Naqvi, S., and Lal, R. (2017). *Sheep production adapting to climate change.* Springer.

Sepkoski, J.J. (1988). Alpha, beta, or gamma: where does all the diversity go? *Paleobiology* 14**,** 221-234.

Seshadri, R., Leahy, S.C., Attwood, G.T., Teh, K.H., Lambie, S.C., Cookson, A.L., Eloe-Fadrosh, E.A., Pavlopoulos, G.A., Hadjithomas, M., Varghese, N.J., Paez-Espino, D., Perry, R., Henderson, G., Creevey, C.J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N.C., Henrissat, B., Woyke, T., Ivanova, N.N., and Kelly, W.J. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat Biotechnol* 36**,** 359-367.

Sharpe, M.E., Brock, J.H., and Phillips, B.A. (1975). Glycerol teichoic acid as an antigenic determinant in a Gram-negative bacterium Butyrivibrio fibrisolvens. *Journal of general microbiology* 88 2**,** 355-363.

Shen, J., Liu, Z., Yu, Z., and Zhu, W. (2017). Monensin and Nisin Affect Rumen Fermentation and Microbiota Differently In Vitro. *Frontiers in Microbiology* 8.

Shi, W., Moon, C.D., Leahy, S.C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., and Seedorf, H. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome research* 24**,** 1517-1525.

Singh, B., Mal, G., Gautam, S.K., and Mukesh, M. (2019). "Gut/Rumen Microbiome —A Livestock and Industrial Perspective," in *Advances in animal biotechnology*. Springer), 17-29.

Sirohi, S.K., Singh, N., Dagar, S.S., and Puniya, A.K. (2012). Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. *Applied Microbiology and Biotechnology* 95**,** 1135-1154.

Slatko, B.E., Gardner, A.F., and Ausubel, F.M. (2018). Overview of Next-Generation Sequencing Technologies. *Curr Protoc Mol Biol* 122**,** e59.

Smirnova, E., Huzurbazar, S., and Jafari, F. (2019). PERFect: PERmutation Filtering test for microbiome data. *Biostatistics* 20**,** 615-631.

Smith, P.E., Kelly, A.K., Kenny, D.A., and Waters, S.M. (2022a). Enteric methane research and mitigation strategies for pastoral-based beef cattle production systems. *Frontiers in Veterinary Science* 9.

Smith, R.H., Glendinning, L., Walker, A.W., and Watson, M. (2022b). Investigating the impact of database choice on the accuracy of metagenomic read classification for the rumen microbiome. *Animal Microbiome* 4**,** 57.

Snyder, E., and Credille, B. (2017). Diagnosis and treatment of clinical rumen acidosis. *Veterinary Clinics: Food Animal Practice* 33**,** 451-461.

Solomon, R., and Jami, E. (2021). Rumen protozoa: from background actors to featured role in microbiome research. *Environmental Microbiology Reports* 13**,** 45-49.

Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual review of microbiology* 39**,** 321-346.

Stewart, R.D., Auffret, M.D., Warr, A., Walker, A.W., Roehe, R., and Watson, M. (2019). Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nature biotechnology* 37**,** 953-961.

Storm, I.M.L.D., Hellwing, A.L.F., Nielsen, N.I., and Madsen, J. (2012). Methods for Measuring and Estimating Methane Emission from Ruminants. *Animals* 2**,** 160-183.

Strömberg, C.A. (2011). Evolution of grasses and grassland ecosystems. *Annual review of Earth and planetary sciences* 39**,** 517-544.

Sun, J., Qiu, Z., Egan, R., Ho, H., Li, Y., and Wang, Z. (2022). Persistent memory as an effective alternative to random access memory in metagenome assembly. *BMC Bioinformatics* 23**,** 513.

Sun, X.Z., Hoskin, S.O., Zhang, G.G., Molano, G., Muetzel, S., Pinares-Patiño, C.S., Clark, H., and Pacheco, D. (2012). Sheep fed forage chicory (Cichorium intybus) or perennial ryegrass (Lolium perenne) have similar methane emissions. *Animal Feed Science and Technology* 172**,** 217-225.

Tan, C., Ramírez-Restrepo, C.A., Shah, A.M., Hu, R., Bell, M., Wang, Z., and Mcsweeney, C. (2020). The community structure and microbial linkage of rumen protozoa and methanogens in response to the addition of tea seed saponins in the diet of beef cattle. *Journal of Animal Science and Biotechnology* 11**,** 80.

Tapio, I., Snelling, T.J., Strozzi, F., and Wallace, R.J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of animal science and biotechnology* 8**,** 1-11.

Teagasc (2017). *Methane* [Online]. Teagasc. Available: https://www.teagasc.ie/environment/climate-change--air-quality/methane/ [Accessed 19/04/2023].

Teagasc (2020). *The Hill Sheep Sector in Ireland* [Online]. Available: https://www.teagasc.ie/news--events/daily/sheep/the-hill-sheep-sector-in-ireland.php [Accessed 20/01/23].

Terry, S.A., Badhan, A., Wang, Y., Chaves, A.V., and Mcallister, T.A. (2019). Fibre digestion by rumen microbiota — a review of recent metagenomic and metatranscriptomic studies. *Canadian Journal of Animal Science* 99**,** 678-692.

Teufel, M., and Sobetzko, P. (2022). Reducing costs for DNA and RNA sequencing by sample pooling using a metagenomic approach. *BMC Genomics* 23**,** 613.

Thauer, R.K., Kaster, A.-K., Seedorf, H., Buckel, W., and Hedderich, R. (2008). Methanogenic archaea: ecologically relevant differences in energy conservation. *Nature Reviews Microbiology* 6**,** 579-591.

Thompson, L.R., and Rowntree, J.E. (2020). Invited Review: Methane sources, quantification, and mitigation in grazing beef systems. *Applied Animal Science* 36**,** 556-573.

Thukral, A.K. (2017). A review on measurement of Alpha diversity in biology. *Agric. Res. J* 54**,** 1-10.

Torney, D. (2021). Comparative Assessment of the Climate Action and Low Carbon Development (Amendment) Act 2021.

Torres, R.N.S., Ghedini, C.P., Paschoaloto, J.R., Da Silva, D.a.V., Coelho, L.M., Almeida Junior, G.A., Ezequiel, J.M.B., Machado Neto, O.R., and Almeida, M.T.C. (2022). Effects of tannins supplementation to sheep diets on their performance, carcass parameters and meat fatty acid profile: A meta-analysis study. *Small Ruminant Research* 206**,** 106585.

Tortereau, F., Marie-Etancelin, C., Weisbecker, J.L., Marcon, D., Bouvier, F., Moreno-Romieux, C., and François, D. (2020). Genetic parameters for feed

efficiency in Romane rams and responses to single-generation selection. *animal* 14**,** 681-687.

Unfcc (1997). *Kyoto protocol to the United Nations Framework Convention on Climate change* [Online]. Available: https://unfccc.int/sites/default/files/resource/docs/cop3/l07a01.pdf [Accessed 28/11/2022].

Unfcc (2012). *DOHA amendment to the Kyoto protocol* [Online]. Available: https://treaties.un.org/doc/Publication/CN/2012/CN.718.2012-Eng.pdf [Accessed 28/11/2022].

Unfcc (2015). *Paris agreement* [Online]. Available: https://unfccc.int/sites/default/files/english_paris_agreement.pdf [Accessed 28/11/2022].

Ungerfeld, E.M. (2020). Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology* 11.

Ushida, K., Miyazaki, A., and Kawashima, R. (1985). Effect of monensin on ruminal VFA and gas production of sheep fed high concentrate diet.

Valenti, B., Campidonico, L., Natalello, A., Lanza, M., Salami, S.A., Priolo, A., Serra, A., Pauselli, M., and Luciano, G. (2021). Fatty acid metabolism in lambs supplemented with different condensed and hydrolysable tannin extracts. *PLoS One* 16**,** e0258265.

Van Soest, P.J. (1994). *Nutritional ecology of the ruminant.* Cornell university press.

Vasta, V., Mele, M., Serra, A., Scerra, M., Luciano, G., Lanza, M., and Priolo, A. (2009). Metabolic fate of fatty acids involved in ruminal biohydrogenation in sheep fed concentrate or herbage with or without tannins1. *Journal of Animal Science* 87**,** 2674-2684.

Vuong, P., Wise, M.J., Whiteley, A.S., and Kaur, P. (2022). Ten simple rules for investigating (meta)genomic data from environmental ecosystems. *PLOS Computational Biology* 18**,** e1010675.

Waghorn, G. (2014). Technical Manual on Respiration Chamber Designs.

Waghorn, G., Tavendale, M., and Woodfield, D. (Year). "Methanogenesis from forages fed to sheep", in: *Proceedings of the New Zealand Grassland Association*), 167-171.

Wagner, T., Watanabe, T., and Shima, S. (2018). "Hydrogenotrophic Methanogenesis," in *Biogenesis of Hydrocarbons,* eds. A.J.M. Stams & D. Sousa. (Cham: Springer International Publishing), 1-29.

Wang, C., Zhao, Y., Aubry, A., Arnott, G., Hou, F., and Yan, T. (2019). Effects of concentrate input on nutrient utilization and methane emissions of two breeds of ewe lambs fed fresh ryegrass. *Translational Animal Science* 3**,** 485-492.

Wang, C.J., Wang, S.P., and Zhou, H. (2009). Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. *Animal Feed Science and Technology* 148**,** 157-166.

Wang, K., Xiong, B., and Zhao, X. (2023). Could propionate formation be used to reduce enteric methane emission in ruminants? *Science of The Total Environment* 855**,** 158867.

Wang L., Abu-Doleh A., Plank J., Catalyurek UV., Firkins J.L., Yu Z. (2019). The transcriptome of the rumen ciliate Entodinium caudatum reveals some of its metabolic features. *Bmc Genomics*.20:1-18

Weart, S.R. (2010). The idea of anthropogenic global climate change in the 20th century. *WIREs Climate Change* 1**,** 67-81.

Webb, S.D., and Taylor, B.E. (1980). The phylogeny of hornless ruminants and a description of the cranium of Archaeomeryx. Bulletin of the AMNH; v. 167, article 3.

Weinroth, M.D., Belk, A.D., Dean, C., Noyes, N., Dittoe, D.K., Rothrock, M.J., Jr., Ricke, S.C., Myer, P.R., Henniger, M.T., Ramírez, G.A., Oakley, B.B., Summers, K.L., Miles, A.M., Ault-Seay, T.B., Yu, Z., Metcalf, J.L., and Wells, J.E. (2022). Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies. *Journal of Animal Science* 100**,** skab346.

Wen, C., Wu, L., Qin, Y., Van Nostrand, J.D., Ning, D., Sun, B., Xue, K., Liu, F., Deng, Y., Liang, Y., and Zhou, J. (2017). Evaluation of the reproducibility of amplicon sequencing with Illumina MiSeq platform. *PLOS ONE* 12**,** e0176716.

Westcott, S.L., and Schloss, P.D. (2015). De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ* 3**,** e1487.

Wilkinson, T.J., Huws, S.A., Edwards, J.E., Kingston-Smith, A.H., Siu-Ting, K., Hughes, M., Rubino, F., Friedersdorff, M., and Creevey, C.J. (2018). CowPI: a rumen microbiome focussed version of the PICRUSt functional inference software. *Frontiers in microbiology* 9**,** 1095.

Williams, A.G., and Coleman, G.S. (1997). *The rumen protozoa.* Springer.

Williams, C.L., Thomas, B.J., Mcewan, N.R., Rees Stevens, P., Creevey, C.J., and Huws, S.A. (2020). Rumen Protozoa Play a Significant Role in Fungal Predation and Plant Carbohydrate Breakdown. *Frontiers in Microbiology* 11.

Willis, A.D. (2019). Rarefaction, Alpha Diversity, and Statistics. *Frontiers in Microbiology* 10.

Wmo (2023). Early Warning For All - Bulletin. *World Meteorological Organisation*.

Woese, C.R. (1987). Bacterial evolution. *Microbiological reviews* 51**,** 221-271.

Wood, D.E., Lu, J., and Langmead, B. (2019). Improved metagenomic analysis with Kraken 2. *Genome Biol* 20**,** 257.

Wood, T.M., Wilson, C.A., and Mccrae, S.I. (1995). The cellulose system of the anaerobic rumen fungus Neocallimastix frontalis: studies on the properties of fractions rich in endo-(1→4)-β-d-glucanase activity. *Applied Microbiology and Biotechnology* 44**,** 177-184.

Xia, Y., and Sun, J. (2017). Hypothesis testing and statistical analysis of microbiome. *Genes & Diseases* 4**,** 138-148.

Xia, Y., Sun, J., and Chen, D.-G. (2018). *Statistical analysis of microbiome data with R.* Springer.

Xue, M.Y., Xie, Y.Y., Zhong, Y., Ma, X.J., Sun, H.Z., and Liu, J.X. (2022). Integrated meta-omics reveals new ruminal microbial features associated with feed efficiency in dairy cattle. *Microbiome* 10**,** 32.

Yang, L., and Chen, J. (2022). A comprehensive evaluation of microbial differential abundance analysis methods: current status and potential solutions. *Microbiome* 10**,** 130.

Ye, S.H., Siddle, K.J., Park, D.J., and Sabeti, P.C. (2019). Benchmarking Metagenomics Tools for Taxonomic Classification. *Cell* 178**,** 779-794.

Yuan, Z.P., Zhang, C.M., Zhou, L., Zou, C.X., Guo, Y.Q., Li, W.T., Liu, J.X., and Wu, Y.M. (2007). Inhibition of methanogenesis by tea saponin and tea saponin plus disodium fumarate in sheep. *Journal of Animal and Feed Sciences* 16**,** 560-565.

Zeder, M.A. (2008). Domestication and early agriculture in the Mediterranean Basin: Origins, diffusion, and impact. *Proceedings of the national Academy of Sciences* 105**,** 11597-11604.

Zehavi, T., Probst, M., and Mizrahi, I. (2018). Insights Into Culturomics of the Rumen Microbiome. *Frontiers in Microbiology* 9.

Zeineldin, M., Barakat, R., Elolimy, A., Salem, A.Z.M., Elghandour, M.M.Y., and Monroy, J.C. (2018). Synergetic action between the rumen microbiota and bovine health. *Microbial Pathogenesis* 124**,** 106-115.

Zeng, Y., Li, J., Wei, C., Zhao, H., and Tao, W. (2022). mbDenoise: microbiome data denoising using zero-inflated probabilistic principal components analysis. *Genome Biology* 23**,** 94.

Zhang, B., Brock, M., Arana, C., Dende, C., Van Oers, N.S., Hooper, L.V., and Raj, P. (2021a). Impact of Bead-Beating Intensity on the Genus- and Species-Level Characterization of the Gut Microbiome Using Amplicon and Complete 16S rRNA Gene Sequencing. *Frontiers in Cellular and Infection Microbiology* 11.

Zhang, D., Zhang, X., Li, F., Li, C., La, Y., Mo, F., Li, G., Zhang, Y., Li, X., and Song, Q. (2019). Transcriptome analysis identifies candidate genes and pathways associated with feed efficiency in Hu Sheep. *Frontiers in Genetics* 10**,** 1183.

Zhang, L., Chung, J., Jiang, Q., Sun, R., Zhang, J., Zhong, Y., and Ren, N. (2017a). Characteristics of rumen microorganisms involved in anaerobic degradation of cellulose at various pH values. *Rsc Advances* 7**,** 40303-40310.

Zhang, X., Wang, W., Mo, F., La, Y., Li, C., and Li, F. (2017b). Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. *Sci Rep* 7**,** 12681.

Zhang, Y.K., Zhang, X.X., Li, F.D., Li, C., Li, G.Z., Zhang, D.Y., Song, Q.Z., Li, X.L., Zhao, Y., and Wang, W.M. (2021b). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15**,** 100161.

Zhang, Z., Xu, D., Wang, L., Hao, J., Wang, J., Zhou, X., Wang, W., Qiu, Q., Huang, X., and Zhou, J. (2016). Convergent evolution of rumen microbiomes in high-altitude mammals. *Current Biology* 26**,** 1873-1879.

Zhao, Y., Nan, X., Yang, L., Zheng, S., Jiang, L., and Xiong, B. (2020). A Review of Enteric Methane Emission Measurement Techniques in Ruminants. *Animals* 10**,** 1004.

Zhou, P., Mcevoy, T.G., Gill, A.C., Lambe, N.R., Morgan-Davies, C.R., Hurst, E., Sargison, N.D., and Mellanby, R.J. (2019a). Investigation of relationship between vitamin D status and reproductive fitness in Scottish hill sheep. *Scientific Reports* 9**,** 1162.

Zhou, R., Wu, J., Zhang, L., Liu, L., Casper, D.P., Jiao, T., Liu, T., Wang, J., Lang, X., and Song, S. (2019b). Effects of oregano essential oil on the ruminal pH and microbial population of sheep. *PloS one* 14**,** e0217054.

Zimmerman, P.R. (1993). "System for measuring metabolic gas emissions from animals". Google Patents).

Zygoyiannis, D. (2006). Sheep production in the world and in Greece. *Small ruminant research* 62**,** 143-147.

`

Chapter 2

Rumen microbiome composition is altered in sheep divergent in feed efficiency

Steven McLoughlin[1,2], Charles Spillane[2*], Noel Claffey[1], Paul E. Smith[1], Tommy O'Rourke[1], Michael G. Diskin[1], Sinéad M. Waters[1,2,*]

[1]Teagasc, Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre Athenry, Co. Galway, Ireland.

[2]Genetics and Biotechnology Laboratory, Plant and AgriBioscience Research Centre (PABC), Ryan Institute, National University of Ireland Galway, University Road, Galway H91 REW4, Ireland.

Keywords: Greenhouse Gas, Feed Efficiency, Sheep, Ruminant, Microbiome, Metagenomics, 16S RNA

Abstract

Rumen microbiome composition and functioning is linked to animal feed efficiency, particularly for bovine ruminants. To investigate this in sheep, we compared rumen bacterial and archaeal populations (and predicted metabolic processes) of sheep divergent for the feed efficiency trait feed conversion ratio (FCR). In our study 50 Texel cross Scottish Blackface (TXSB) ram lambs were selected from an original cohort of 200 lambs. From these, 26 were further selected for experimentation based on their extreme feed conversion ratio (High Feed Efficiency, HFE=13; Low Feed Efficiency, LFE=13). Animals were fed a 95% concentrate diet *ad libitum* over 36 days. 16S rRNA amplicon sequencing was used to investigate the rumen bacterial and archaeal communities in the liquid and solid rumen fractions of sheep divergent for FCR. Weighted UniFrac distances separated HFE and LFE archaea communities from the liquid rumen fraction (Permanova, $P<0.05$), with greater variation observed for the LFE cohort (Permdisp, $P<0.05$). LFE animals exhibited greater Shannon and Simpson diversity indices, which was significant for the liquid rumen fraction ($P<0.05$). *Methanobrevibacter olleyae* (in liquid and solid fractions) and *Methanobrevibacter millerae* (liquid fraction) were differentially abundant, and increased in the LFE cohort ($P.adj<0.05$), while *Methanobrevibacter wolinii* (liquid fraction) was increased in the HFE cohort ($P.adj<0.05$). This suggests that methanogenic archaea may be responsible for a potential loss of energy for the LFE cohort. Bacterial community composition (Permanova, $P>0.1$) and diversity ($P>0.1$) was not affected by the FCR phenotype. Only the genus *Prevotella 1* was differentially abundant between HFE and LFE cohorts. Although no major compositional shifts of bacterial populations were identified amongst the feed

efficient cohorts (*FDR*>0.05), correlation analysis identified putative drivers of feed efficiency with *Ruminococcaceae UCG-014* (liquid, *rho*=-0.53; solid, *rho*=-0.56) and *Olsenella* (solid, *rho*=-0.40) exhibiting significant negative association with FCR (*P*<0.05). *Bifidobacterium* and *Megasphera* showed significant positive correlations with ADG. Major cellulolytic bacteria *Fibrobacter* (liquid, *rho*=0.43) and *Ruminococcus 1* (liquid, *rho*=0.41; solid, *rho*=41) correlated positively with FCR (*P*<0.05). Our study provides evidence that feed efficiency in sheep is likely influenced by compositional changes in archaea community, and abundance changes of specific bacteria, rather than major overall shifts within the rumen microbiome.

Introduction

The world's population is expected to increase by 2 billion persons in the next 30 years, from 7.7 billion currently to 9.7 billion in 2050 (UN, 2019). In addition, rising gross domestic product (GDP) in developing countries and urbanization is driving dietary shifts towards animal-based protein products (Thornton, 2010; Henchion et al., 2017). There is increasing demand on livestock production systems to support the dietary requirements and demand of a rapidly growing population (Hunter et al., 2017). Feed is the largest economic factor influencing profitability in livestock enterprises, accounting for up to 70% of total direct costs (Kenny et al., 2018). Due to the cost of feed as an external input, improving profitability of livestock systems has significantly focused on the identification of animals capable of maximizing the utilization of feed (McGovern et al., 2018). Research to date provides evidence that highly feed efficient animals consume less feed, while at the same time maintaining the same level of production as less efficient animals (Carberry et al., 2012; Shabat et al., 2016; Claffey et al., 2018). Additionally, highly efficient animals produce less methane and less manure due to reduced consumption of feed (Kenny et al., 2018). Therefore, improving feed efficiency has the potential to simultaneously increase profitability within the livestock sector while reducing the environmental impact of livestock production.

Feed Conversion Ratio (FCR) and Residual Feed Intake (RFI) are two widely used measures of feed efficiency (Bhatt et al., 2013; Zhang et al., 2017; Claffey et al., 2018; McGovern et al., 2018). FCR is calculated as the kilogram ratio of dry matter intake (DMI) to average daily gain (ADG), while RFI measures the residual difference between observed and predicted feed intake against bodyweight maintenance and animal performance (Berry and Crowley, 2013). FCR and RFI have an inverse relationship with feed efficiency, with superior FCR and RFI measures corresponding to poorer animal production, and vice versa (Cannas et al., 2019). Both measures are related as they both require feed intake as a variable. However, a major limitation of FCR is that it is dependent on ADG, which can result in the selection of larger and faster growing animals that require more maintenance. In contrast, RFI is independent of growth rate and considered a more robust measurement of feed efficiency (Santana et al., 2012).

Ruminants depend on the microbes (composed mainly of bacteria, archaea, fungi and protozoa) residing in the rumen to ferment and transform their feed into volatile fatty acids (VFAs), proteins and vitamins. The primary VFAs produced (butyrate, propionate and acetate) contribute approximately 80% of the hosts metabolised energy requirements (Keogh et al., 2017; Li and Guan, 2017; Abecia et al., 2018; Zeineldin et al., 2018). Furthermore, the concentrations of different VFAs within the rumen have been associated with feed efficiency of the host (Li and Guan, 2017). The underlying biological mechanisms regulating production efficiency are dependent on a number of internal and external factors including age, sex, genotype and diet, all of which are known to influence rumen microbial structure and function (Henderson et al., 2015; Shabat et al., 2016; Claffey et al., 2018; Thomas et al., 2019). Hence, there is a potential association between feed efficiency and the rumen microbiome. Indeed, previous research performed by our group and others has identified links between the rumen microbiome and animal variation in feed efficiency phenotypes (Carberry et al., 2012; Jewell et al., 2015; Shabat et al., 2016; Ellison et al., 2017; McGovern et al., 2018).

Understanding of microbiome composition and functioning has advanced in recent years through the application of high-throughput next-generation sequencing (NGS) technologies for metagenomic analyses (Quince et al., 2017). Popular NGS platforms such as the MISeq (Illumina) and MINion (Oxford Nanopore) coupled with metagenomic approaches that either target specific genes (16S rRNA) or the whole bacterial genome, are providing insights into complex microbial populations in the rumen, which are otherwise difficult to identify using culture-dependent approaches (Zhou et al., 2015; Kachiprath et al., 2018; Gu et al., 2019). Additionally, the development of user-friendly computational software is enabling researchers to extrapolate more information from biological data. For instance, CowPI, a functional prediction tool, can infer the functional potential of different rumen microbiome profiles using 16S rRNA data (Wilkinson et al., 2018).
There are approximately 1.2 billion sheep in the world that are primarily reared for commodities such as meat, milk and wool (Pulina et al., 2018). Sheep production remains an important agricultural enterprise internationally, which is exemplified by continual annual growth of the sheep dairy sector (Pulina et al., 2018). To date, most research investigating the relationship between feed efficiency and the rumen microbiome has been conducted in cattle. However, sheep are less expensive, require less feed, reach maturity quicker and are more manageable than cattle, making sheep a practical and economical model for ruminant research (Delano et al., 2002).

In a previous study by our group (Claffey et al., 2018), FCR was measured for a cohort of Texel cross Scottish Blackface (TXSB) lamb rams over 36 days and was found to vary across the group. While the rumen microbiome has been shown to be associated with feed efficiency in cattle (Carberry et al., 2012; Jewell et al., 2015; McGovern et al., 2018) such a relationship has not been extensively examined in sheep. Therefore, the objective of the current study was to investigate the bacterial and archaeal populations present in both solid and

liquid fractions of the rumen of sheep that are divergent for the FCR phenotype, using amplicon sequencing targeting the 16S rRNA gene. In addition, the archaeal and bacterial populations identified were correlated with FCR to further identify possible microbial drivers of feed efficiency. To determine the potential functionality of the microbiome taxa that are differentially abundant due to FCR, CowPI (Wilkinson et al., 2018) was used to predict functional genes of metabolic pathways associated with feed efficiency in sheep.

Material and Methods

Animal model

All animal procedures used in this study were conducted under experimental license from Ireland's Health Product Regulatory Authority (HPRA) in accordance with the European Union (EU) protection of animals used for scientific purposes regulations 2012 (S.I. No 543 of 2012). This study was conducted as part of a larger study designed to examine the production efficiency of purebred Scottish Blackface and Texel cross Scottish Blackface (TXSB) wether and ram lambs (n=200) (Claffey et al., 2018). The current study focused on the rams of the TXSB breeds of sheep used in that study. Briefly, twenty-six lambs of the TXSB were separated into two highest and lowest feed conversion efficiency quartile cohorts (high and low feed efficiency animals with 13 animals in each group) according to their extreme feed conversion ratio (FCR) values, from an original group of 50 individuals (HFE = 3.83 ± 0.40, LFE=6.05 ± 0.92, ($p < 0.05$)). The experiment was performed over a period of 36 days of intensive indoor feeding. Lambs were individually penned on expanded metal-floored feeding pens (182 cm L × 122 cm W) and allowed tactile, olfactory, and visual contact with each other through the pen partitions. Lambs were allowed a 12-d pre-experimental acclimatization period to adapt to a 95% concentrate diet. Relative to commencement of *ad libitum*, concentrate feeding (day 0), lambs were offered 150-g/d fresh weight of concentrate feed on days −12, −11, and −10 increasing by 100-g/d fresh weight concentrate on each day from days −9 to d −1 to minimise the risk of any digestive upsets. For the duration of the finishing period, lambs were offered 100-g/d DM of silage and had *ad libitum* access to concentrates; *ad libitum* concentrate was described as access to concentrate feed at all times over the 36-d experimental period. Concentrate and silage samples were collected weekly and dried overnight at 55 °C and pooled for determination of CP, ADF, NDF, and ash. Concentrate and silage were offered daily with individual lamb refusals recorded twice weekly (Claffey et al., 2018). Lambs were transported to the slaughter facility on the morning of slaughter. Animals were slaughtered at a mean age of 292 days old. Production variables (average daily feed intake (ADI), total weight gain (TWG), feed conversion ratio

(FCR), and average daily gain (ADG)) were calculated post slaughter. All production data used in the study has been previously described (Claffey et al., 2018).

Rumen sampling, DNA extraction and 16s rDNA library preparation

Liquid and solid fractions from rumen content were collected immediately after slaughter. Fractions were separated by squeezing rumen digesta through four layers of sterile cheesecloth, which were collected in 250ml centrifuge bottles. Both fractions were frozen immediately in liquid nitrogen after separation and then stored at -80ºC. Under liquid nitrogen, each sample was homogenized to a fine frozen powder using a pestle and mortar. Extraction of microbial DNA from the samples was performed using the method described by Yu and Morrison, (2004) (Yu and Morrison, 2004). DNA purity was assessed using Nanodrop 1000 spectrophotometer. The 260/280 ratio averaged 1.83. To generate the PCR amplicons of the V4 hyper-variable region (of the 16SrDNA), 515F-806R primers were used on a template of 25ng of rumen microbial DNA (Caporaso et al., 2011). 515F-806R primers target both bacterial and archaeal populations (Willis et al., 2019). The 515F-806R primers were designed with Nextera overhang adapters. The PCR amplification was conducted using 2X KAPA HiFi HotStart ReadyMix DNA polymerase (Roche Diagnostics, West Sussex, United Kingdom). The PCR conditions were as described in McGovern et al. (2018). Finally, the amplicons were sequenced on an Illumina MiSeq platform using the 500-cycle version 2 MiSeq reagent kit (Illumina, San Diego, CA, United States).

Bioinformatic analysis

Raw paired-end sequenced reads were quality checked with FASTQC (version 0.11.5) (Andrews, 2010). Primers and ambiguous basecalls were removed using Cutadapt (version 1.18) (Martin, 2011). Processing and analysis of amplicon reads was performed using Divisive Amplicon Denoising Algorithm 2 (DADA2), as described in (Callahan et al., 2017). Read filtering, dereplication, sample inference, chimera removal, merging of paired end reads and taxonomic classification were all performed following the DADA2 tutorial from https://benjjneb.github.io/dada2/tutorial.html (version 1.12) with minor alterations. Taxonomic classification was performed to the genus level using the SILVA classification database (sourced from https://zenodo.org/record/1172783#.XWLkpd-YW6A) (Callahan, 2018). The final output from DADA2 was an Amplicon Sequence Variant (ASV) table and a corresponding taxonomy table. A phylogenetic tree was constructed using the phangorn package (Schliep, 2011). A phyloseq object containing the ASV table, taxonomy table, phylogenetic tree and experimental metadata was built using the R/Bioconductor package Phyloseq (version 1.26) (Mcmurdie and Holmes, 2013) prior to downstream analysis. Finally, CowPI was used to predict the functional processes of the microbial community within each sample using the ASVs generated from the DADA2 pipeline (Wilkinson et al., 2018). Basic local alignment search tool (BLAST) against the rRNA/ITS database was used to further

classify methanogens representative ASV sequences of interest (Johnson et al., 2008).

Compositional and Statistical Analysis

Compositional and statistical analyses were carried out using various libraries/packages in R studio (running R version 3.6.1). Samples were separated according to rumen phase (liquid and solid) for independent analysis and compared between the feed efficient cohorts (low feed efficient (LFE) v high feed efficient (HFE)). Taxa unassigned at the phylum level, with less than 5 counts and prevalent in 3 or less samples were filtered from the data. For the analysis of alpha and beta diversity counts were normalised by subsampling to the minimum sampling depth; bacteria reads (liquid=63,924, solid=75,873) and archaea reads (liquid=896, solid=1182). Principle coordinate analysis (PCoA) based on weighted and unweighted UniFrac distances was performed for ordination analysis to visualize compositional differences between the two cohorts for both rumen fractions. PERMANOVA analysis with 9999 permutations was conducted using the Adonis function from the R/Bioconductor package Vegan (version 2.5-5) (Oksanen et al., 2019). Vegans betadisper and permutest functions were used to test for homogeneity of variance. Alpha diversity indices Shannon, Simpson and observed ASVs were obtained for each of the rumen samples and compared between cohorts using the non-parametric Wilcoxon rank sum test. Alpha and beta diversity analysis was conducted at the ASV level for both bacterial and archaeal populations.

To profile the bacterial community populations, taxa were agglomerated to higher taxonomic ranks (i.e. phylum to genus) due to poor classification at the species level and counts were transformed to relative abundances. Archaeal populations were assessed at the genus and ASV level. Differential relative abundance analysis was conducted from phylum to genus level for bacteria populations and conducted at the genus to ASV level for archaea populations. For lower taxonomic ranks (i.e. genus and ASV) analysis was only conducted on taxa had a relative abundance greater than 0.1% and were prevalent at least 30% of samples. The Wilcoxon rank sum test was implemented to test for differences in relative abundance of taxa between the cohorts, and Benjamini-Hochberg (B-H) was used to correct for multiple testing. Spearman’s correlation analysis was also performed to test for associations between relative abundance of taxa and production traits of feed efficiency (FCR and ADG).

STAMP (v.2.1.3) (Parks et al., 2014) was used to conduct principal component and statistical analysis following functional prediction using CowPI (Wilkinson et al., 2018). The relative abundance of reads mapped to each functional process was compared between cohorts using Whites non-parametric t-test with B-H correction for multiple testing.

Results

Animal production traits differed across the divergent feed efficiency cohorts

This study focused on twenty-six TXSB ram lambs divergent for feed efficiency (HFE *n*=13; LFE *n*=13). A Wilcoxon rank sum test was performed to test the null hypothesis that production traits; feed conversion ratio (FCR), average daily gain (ADG), average daily intake (ADI), total weight gain (TWG), did not differ between the two cohorts. For the four production traits significant differences were found in their medians ($p < 0.05$), confirming that production traits were statistically different between feed efficiency cohorts (Table 2.1) (Claffey et al., 2018).

Over 1600 unique ASVs identified in both rumen fractions

Following data processing, quality filtering and chimera removal, and a total of 6,326,753 amplicon reads remained for analysis (solid phase=3,061,130, liquid phase=3,265,623). The average number of reads per sample in the liquid rumen phase was 125,600, and 117,735 in the solid rumen phase. 1691 uniquely identified ASVs were obtained from the reads in both rumen fractions. After prevalence filtering and removal of unclassified ASVs at the phylum level 560 and 513 ASV's mapped to kingdom bacteria, while 12 and 11 ASV's mapped to kingdom archaea for liquid and solid rumen fractions, respectively. Initial exploratory analysis using PCoA ordination based on weighted UniFrac distances detected two samples from the LFE cohort as outliers (Animal ID: 10707 and 10835) (supplementary Figure 1). Further investigation revealed that in both fractions the genus *Prevotella 1* had a relative abundance of approximately 70%. The samples from both animals were considered highly biased and removed prior to downstream analysis.

Effect of rumen fraction and FCR on microbial community composition and diversity

Ruminal fraction (i.e liquid and solid) had no effect on microbial profiles ($P<0.05$). Similar microbial composition, diversity and relative abundances were observed between the two fractions (Supplementary Figure 2.2). PCoA analysis on bacteria community composition showed considerable overlap between HFE and LFE samples, based on weighted (liquid, $P=0.28$, $R2=0.05$, $PermDisp=0.91$; solid, $P=0.48$, $R2=0.03$, $PermDisp=0.71$) (Figure 2.1) and unweighted (liquid, $P=0.10$, $R2=0.06$, $PermDisp=0.37$; solid, $P=0.15$, $R2=0.06$, $PermDisp=0.69$) UniFrac distances (Supplementary Figure 2.3). Alpha diversity indicators; Shannon, Simpson and observed ASVs were not significant between HFE and LFE cohorts for either rumen fraction ($P > 0.05$), although LFE cohort exhibited greater diversity (Figure 2.2). For archaea populations greater variation in community composition was observed in the LFE cohort and found to be significantly divergent from the HFE cohort in the liquid rumen fraction based on weighted UniFrac distances (liquid, $P=0.01$, $R2=0.18$, $PermDisp=0.01$; solid, $P=0.12$, $R2=0.08$, $PermDisp=0.10$) (Figure 2.1). Shannon and Simpson indices were increased in the LFE liquid fraction ($P > 0.05$) and observed ASV was

increased in the LFE solid fraction ($P > 0.05$) when compared to the HFE cohort (Figure 2.2).

Significant effects of FCR on microbial abundance

After filtering a total of 13 bacterial phyla were identified in both rumen fractions, and their relative abundances were not affected by the FCR phenotype. *Firmicutes,* and *Bacteroidetes* constituted the most abundant phyla, respectively. Together they represented 77% and 83% mean relative abundance in the HFE cohort, and 82% and 83% mean relative abundance in the LFE cohort, for liquid and solid rumen fractions respectively (Table 2.2). The *Firmicutes* and *Bacteroidetes* ratio (F:B) was not affected by FCR phenotype ($P > 0.1$) in either the liquid or solid rumen fractions. *Proteobacteria and Actinobacteria* were the next most abundant phyla respectively. The mean relative abundance of *Fibrobacter* was increased in both HFE and LFE solid rumen fractions compared with liquid fractions, while also exhibiting a greater mean relative abundance in the LFE cohort compared the HFE cohort ($P>0.05$) (Table 2.2).

A total of 104 and 99 bacterial genera were identified in the liquid and solid rumen fraction, respectively. The most dominant genera in both fractions were *Prevotella 7*, *Succinivibrionaceae UCG-001* and *Lachnospiraceae NK3A20 group* (Figure 2.4) and their abundance did not differ between feed efficient cohorts ($P>0.05$). The genus *Prevotella 1* was the only bacterial taxa differentially abundant, increased in the LFE liquid fraction ($FDR=0.02$) (Figure 2.3). Genera were predominantly enriched to families *Lachnospiraceae* (liquid=24.0%; solid=24.0%), *Ruminococcaceae* (liquid=16.3%; solid=17.0%), *Veillonellaceae* (liquid=8.7%; solid=9.0%), *Eryspielotrichaceae* (liquid=8.7%; solid=9.0%) and *Prevotellaceae* (liquid=8.7%; solid=9.0%).

After profiling of the archaeal population three genera were identified. *Methanobrevibacter* was the most dominant, followed by *Methanosphera* and *Candidatus Methanomethylophilus,* respectively, and their abundances were not affected by the FCR phenotype ($P>0.05$). At the ASV level, 11 taxa were identified from the liquid rumen fraction and 10 from the solid rumen fraction. Three archaeal ASVs showed difference in relative abundance between the divergent FCR cohorts. In the liquid rumen fraction ASV58 and ASV118, identified to closely match *Methanobrevibacter olleyae* (98.8%) and *Methanobrevibacter millerae* (99.2%), respectively, were increased in the LFE cohort. Whereas ASV18, identified as *Methanobrevibacter wolinii* (100%) was increased in the HFE cohort. In the solid rumen fraction *Methanobrevibater olleyae* was increased in the LFE cohort (Figure 2.3).

Significant associations of different bacterial taxa with FCR and ADG

Spearman's correlation analysis was performed to identify putative bacterial drivers of feed efficiency. At the genus level, relationships were only explored for genera that were prevalent in greater than 30% of samples and had minimum

relative abundance of 0.1%. At the genus level, in the rumen liquid phase the relative abundance of *Ruminococcaceae UCG-014* (*rho*=-0.51) exhibited the strongest negative correlation with FCR (*P* <0.05), while *Prevotella 1* (*rho*=0.56), *Coprococcus 1* (*rho*=0.44), *Ruminococcus 1* (*rho*=0.41) and *Fibrobacter* (*rho*=0.43) exhibited the strongest positive correlation with FCR (*P* <0.05). Only *Bifidobacterium (rho*=0.41) exhibited a significant positive association with ADG (*P*<0.05), while *Prevotella 1* (*rho*=-0.72), *Pseudoramibacter* (*rho*=-0.56), *Coprococcus 1* (*rho*=-0.51)*, Ruminococcus 1* (*rho*=-0.50)*, Ruminococcus 2* (*rho*=-0.43)*, Acetitomaculum* (*rho*=-0.45), *Rikenellaceae RC9 gut group* (*rho*=-0.42), *Fibrobacter* (*rho*=-0.61), and *Treponema 2* (*rho*=-0.57) exhibited significant negative associations with ADG (*P* <0.05) (Table 2.3).

In the solid rumen phase, *Ruminococcaceae UCG-014* (*rho*=-0.55) and *Olsenella* (*rho*=-0.40) exhibited a significant negative association with FCR (*P* <0.05), while *Pyramidobacter* (*rho*=0.53), *Pseudoramidobacter* (*rho*=0.42), *Ruminococcus 1* (rho=0.43), *Acetitomaculum* (*rho*=-0.42), *Prevotella 1* (*rho*=0.39) and *Coprococcus 1* (*rho*=0.42) exhibited significant positive associations with FCR (*P* <0.05). *Bifidobacterium* (*rho*=0.44) and *Megasphera* (*rho*=0.43) were significantly positively associated with ADG (*P* <0.05), while *Prevotella 1* (*rho*=-0.56), *Coprococcus 1* (*rho*=-0.41)*, Ruminococcus 1* (*rho*=-0.53)*,* and *Acetitomaculum* (*rho*=-0.41), *Roseburia* (*rho*=-0.45), *Pseudoramibacter* (*rho*= -0.45), *Fibrobacter* (*rho*=-0.51), *Pyramidobacter* (*rho*=-0.52) and *Treponema 2* (*rho*=-0.48) were among those showing significant negative associations with ADG (*P* <0.05) (Table 2.3).

At the phylum level, *Fibrobacter* (liquid, *rho*=0.44), *Synergistetes* (liquid, *rho*=0.038; solid, *rho*=0.51) and *Elusimicrobia* (solid, *rho*=0.43) exhibited a significant positive relationship with FCR (*P* <0.05), whereas *Tenericutes* (solid, *rho*=-0.44) showed a significant negative relationship with FCR (*P* <0.05) (Table 2.2).

Functional potential and microbial processes similar between low and high feed efficient cohorts

CowPI was used for functional prediction analysis of the microbial community for each of the rumen samples. Principal component analysis, which captured over 75% variation with the first 2 principal components, indicated no separation in functional potential between feed efficient cohorts (Supplementary Figures 2.4 and 2.5). Metabolic processes identified by CowPI were compared between the feed efficient cohorts using White's non-parametric t-test and adjusted for multiple testing using B-H method. No significant differences in metabolic processes were observed between cohorts for both liquid and solid rumen fractions (*P.adj*>0.1).

Discussion

The current study examined the effect of the FCR phenotype on ruminal bacteria and archaeal populations obtained from the liquid and solid rumen fractions of

TxSB ram lambs offered a high concentrate diet *ad libitum.* Rumen liquid and solid fractions are widely used for microbiome research (De Mulder et al., 2016; Ji et al., 2017) and for animal feed efficiency analyses (McGovern et al., 2018). Both are differentiated ecological niches, which can contribute to feed efficiency in different ways. The solid rumen fraction is largely composed of plant and grain biomass and selects for various adherent cellulolytic and saccharolytic microbial species that play a significant role in the breakdown of feed within the rumen (De Mulder et al., 2016). While the liquid rumen fraction is considered to contribute less to the metabolic activity of rumen, it does provide readily available nutrients for free living microbes and facilitates their movement to newly consumed feed (De Mulder et al., 2017). Our analysis reveals a similar microbial community composition, diversity and relative abundance profiles between the liquid and solid rumen fractions. A range of studies have also reported comparable findings in microbial diversity and community composition between liquid and solid rumen fractions in both cattle and sheep (Schären et al., 2017; McGovern et al., 2018; Li et al., 2020). The high degree of similarity observed between the fractions may be attributed to the method used to separate the fractions (McGovern et al., 2018). Alternatively, it may also reflect the frequent interchange of microbes between the liquid and solid fractions (De Mulder et al., 2016; Schären et al., 2017). Ultimately, due to the large degree of homogeneity observed between the two rumen fractions it is difficult to determine whether either plays a distinctive role towards feed efficiency in the current study.

In the rumen, methane production is considered beneficial to the host as it regulates the partial pressure of hydrogen facilitating microbial growth and digestion within the rumen (Wallace et al., 2015; Lan and Yang, 2019). However, production of methane is known to result in a loss of dietary energy to the host, of circa 2-12% depending on the diet (Johnson and Johnson, 1995), thereby impacting on the production performance of the animal. Several studies have linked higher methane emission to feed inefficiency in ruminants (Nkrumah et al., 2006; Zhou et al., 2010; Fitzsimons et al., 2013). The abundance of methanogenic archaea has also been correlated with higher levels of methane emissions (Wallace et al., 2015) and poorer feed efficiency (Zhou et al., 2010). As a result, numerous approaches have been developed to target rumen archaea to reduce methane emissions and improve animal production, including vaccines and small molecule enzyme inhibitors (Matthews et al., 2019). Although numerous studies in ruminants have reported no correlation between the overall abundance of methanogens and methane emissions, they have shown positive correlations between methane production and compositional changes within the archaea community (Danielsson et al., 2012; Shi et al., 2014; Tapio et al., 2017). In particular, increased abundance of taxa assigned to the *Methanobrevibacter* SGMT clade (i.e *M. gottschalkii, M. millerae* and *M. smithii)* is strongly correlated with methane emissions compared to those within the RO clade (i.e *M. ruminantium, M. olleyae, M. wolinii*) (Tapio et al., 2017). Members of the SGMT clade harbour 2 methyl coenzyme M reductase isozymes McrI and McrII, enabling them to utilise hydrogen more efficiently than those within the RO clade, which

solely express McrI (Tapio et al., 2017). In line with such studies, our results show no major shifts in the relative abundance of archaea taxa at the genus level or higher taxonomic ranks between the feed efficient cohorts. However, compositional changes were observed at the ASV level. LFE animals exhibited greater variation in community composition (based on weighted UniFrac distances) and increased diversity (as calculated by Shannon and Simpson and observed ASVs) compared to their HFE counterparts. The relative abundance of *Methanobrevibacter millerae* (SGMT clade) and *Methanobrevibacter olleyae* (RO clade) was increased in the LFE liquid fraction, while *Methanobrevibacter wolinii* (RO clade) was increased in the HFE liquid fraction. *Methanobrevibacter olleyae* was also increased in the LFE solid fraction. Compositional changes within the *Methanobrevibacter* genus between divergent cohorts may partially explain the observed differences in feed conversion and animal production in our study.

Bacteria are the most diverse microbial domain found within the rumen and are capable of extracting energy from a wide variety of dietary substrates, including fiber, starch, sugars and protein (Tapio et al., 2017). Due to the dependence of the host on bacterial fermentation it can be considered that the rumen bacterial population plays a critical role in the feed efficiency of the animal. Indeed, previous studies in both cattle and sheep have reported significant association between the feed efficiency of the host and rumen bacterial populations (Jewell et al., 2015; Shabat et al., 2016; Ellison et al., 2017). In our study, no significant differences in bacterial alpha diversity between HFE and LFE lambs were observed. This is consistent with a number of studies in cattle (Myer et al., 2015; McGovern et al., 2018). Although, differences in alpha diversity were not significant in the present study, the HFE cohort exhibited a less diverse bacterial community than their LFE counterparts. This finding is in agreement with a larger study in cattle that reported lower bacterial diversity associated with higher feed efficiency (Shabat et al., 2016). Furthermore, we found no major shifts in community composition and relative abundance of taxa between feed efficient cohorts in either liquid or solid rumen fractions. Weighted UniFrac distances was unable differentiate bacterial community composition between HFE and LFE cohorts with only a small percentage of the variation explained by the FCR phenotype (Figure 1). This finding was supported by our differential relative abundance analysis, which identified the genus *Prevotella 1* as the only bacterial taxa differentially abundant between the two feed efficient cohorts, increased in the LFE liquid fraction (Figure 3). While we detected no major differences in the relative abundance of taxa, we have identified several taxa exhibiting significant correlations of relative abundance with FCR and/or ADG (Table 3).

Prevotella 1, *Fibrobacter*, *Ruminococcus 1, Corprococcus*, *Pseudoramibacter* and *Pyramidobacter* all exhibited significant positive associations with FCR and negative associations with ADG. *Prevotella* species are known to ferment a wide variety of substrates including starches, peptides, proteins and hemicellulose (Matsui et al., 1998; Xie et al., 2019), which contribute to the feed efficiency of the host. Indeed, different *Prevotella* species have been associated with both higher and lower feed efficiency in cattle and sheep (Ellison et al., 2017; Brooke

et al., 2019; Delgado et al., 2019). For instance, Ellison et al. (2017) reported that the abundance of *Prevotella ruminicola* increased significantly for L-RFI lambs when fed a concentrate based diet and decreased in L-RFI lambs when fed a forage-based diet. The opposite was reported for *Prevotella bryantii* (Ellison et al., 2017). This indicates a dietary effect on the abundance of *Prevotella* species, which is likely attributed to the metabolic divergence observed within the genus *Prevotella* (Matsui et al., 2000). Matsui et al. (2000) reported differential production of polysaccharide degrading enzymes and growth rates among *Prevotella* species when grown on various growth substrates *in vitro*. A recent study identified *Prevotella 1* as the most dominant genus in both the liquid and solid rumen fractions of lambs (Li et al., 2020). However, Li et al. (2020) offered lambs a higher ratios of forage to concentrate (45:55), which contrasts with the 95% concentrate diet provided to lambs in the current study. This suggests that *Prevotella 1* may require fibrous tissue or substrates released following fiber degradation for optimal growth, where the association with poorer feed efficiency in the current study may be driven by differences in the quantity of dietary intake observed between the two feed efficient cohorts.

Members of the genera *Fibrobacter* and *Ruminococcus* are predominant fiber-digesting bacteria in the rumen, specifically the species *Fibrobacter succinogens*, *Ruminococcus flavefacians* and *Ruminococcus albus* (Koike and Kobayashi, 2001). All of these three species largely depend on cellulose for growth and energy, although *Ruminococcus albus* can utilise more efficiently a variety of other substrates produced following breakdown of plant fibers (La Reau and Suen, 2018). In contrast to our findings (Table 3), McGovern et al (2018) found negative associations between RFI and the relative abundance of *Fibrobacter* and *Ruminococcus* OTUs. This may have resulted from variations in the ratio of dietary concentrates to forage fed to the animals during the two studies, in addition to differences in how feed efficiency was measured (FCR compared to RFI). Indeed, the abundance of these cellulolytic bacteria in the rumen has previously been shown to diminish with reductions in the ratio of dietary forages (Carberry et al., 2012; Henderson et al., 2015; Zhang et al., 2017). This suggests that the cellulolytic activities of *Fibrobacter* and *Ruminococcus* may become redundant in high concentrate-based diets, and that their increased abundance in the LFE cohort may confer inefficiency in energy extraction from feed.

Species within the genus *Coprococcus* metabolise carbohydrates for growth and energy, producing predominantly butyrate and acetate as fermentation end products (Whitman, 2015). In contrast to our findings, previous studies in cattle have shown positive associations between *Coprococcus* and host feed efficiency (Jewell et al., 2015; McGovern et al., 2018). This may be explained by differences in animal models or diets used in the studies. Indeed, Kim et al. (2014) profiled the fecal microbiota from steers fed three different diets (high grain, moderate grain, and silage/forage) and found three distinct OTU's belonging to *Coprococcus* that differed significantly between the treatment groups (Kim et al., 2014).

Associations between *Pseudoramibacter* and ruminant feed efficiency are not well reported in the literature. *Pseudoramibacter* is a member of the *Eubacteriaceae* family and can utilise carbohydrates for energy (Deusch et al., 2017), while producing fermentation end products butyrate, acetate, formate and hydrogen (Deusch et al., 2017; Palakawong Na Ayudthaya et al., 2018).

The phylum *Synergistetes* was negatively correlated with feed efficiency in the solid rumen fraction (Table 2). This association was primarily driven by the genus *Pyramidobacter*. Members of the *Pyramidobacter* genus are asaccharolytic, nonmotile and produce acetic acid and isovaleric acid (Downes et al., 2009). The abundance of *Pyramidobacter* has previously been associated with low RFI in Simmental bulls (McGovern et al., 2018) and isolated from higher methane emitting steers (Wallace et al., 2015). McGovern et al. (2018) found the relative abundance of *Pyramidobacter* and *Fibrobacter* to be positively correlated. This may indicate that *Pyramidobacter* relies on co-dependence with *Fibrobacter* for nutrient utilization following fiber degradation in the rumen.

The genera *Roseburia*, *Treponema 2*, *Mogibacterium, Rikenellaceae RC9 gut group*, *Acetitomaculum* and *Ruminococcus 2* all exhibited significant negative associations with ADG in either or both rumen fractions in the current study, but showed no significant associations with FCR. *Roseburia* utilises carbohydrates for growth and its abundance is known to increase with greater ratios of dietary concentrates (Mccann et al., 2014; Zhang et al., 2018). Butyrate is the primary VFA produced by *Roseburia* and its production is largely dependent on the availability of acetate (Duncan et al., 2002). Supporting the findings of our study, Li et al. (2019) identified a greater abundance of *Roseburia* in feed inefficient Kinsella composite hybrid steers fed a high-energy diet (Li et al., 2019). In contrast, Ellison et al. (2017) reported a greater abundance of *Roseburia* in feed efficient lambs fed a concentrate diet. Other studies in this area have not reported any association between *Roseburia* and feed efficiency (Jewell et al., 2015; McGovern et al., 2018; Carberry et al., 2012). Given the *Roseburia* saccharolytic activity it is unclear why the genus correlated negatively with feed efficiency in lambs fed a high concentrate diet. One suggestion is that a greater availability of acetate may be present in the rumen of lower feed efficient lambs. *Roseburia* has been reported to be a net utiliser of acetate during growth (Duncan et al., 2002). The correlation between the abundance of *Acetitomaculum* and lower feed efficiency may support this possibility. The genus *Acetitomaculum* is capable of utilizing hydrogen to reduce carbon dioxide for the formation of acetate in a process known as acetogenesis (Greening and Leedle, 1989; Le Van et al., 1998).

Mogibacterium has previously been identified in the rumen of both sheep (Mi et al., 2018) and cattle (Myer et al., 2015; Freetly et al., 2020). *Mogibacterium* belongs to the order *Clostridiales* from the phylum *Firmicutes* and is described as incapable of breaking down carbohydrates for energy (Whitman, 2015). Similar to the finding presented in the current study, a recent study found *Mogibacterium* to be enriched in the jejunum of lower ADG steers fed a high-

energy diet (Freetly et al., 2020). In addition, *Mogibacterium* has also been associated with higher methane-emitting steers (Wallace et al., 2015).

Members of the genus *Treponema* have been associated with pathological conditions including digital dermatitis (Wilson-Welder et al., 2015), yaws disease and syphilis (Newbrook et al., 2017), while others are part of the normal microflora in the GI tract of animals (Newbrook et al., 2017). *Treponema* species such as *T. bryantii* and *T. succinifaciens* ferment carbohydrates (Stanton, 1984; Cwyk and Canale-Parola, 1979), but are also known to be involved in the breakdown of fiber (Xie et al., 2018). In a recent study (Ellison et al., 2019) identified a greater abundance of the species *Treponema maltophilum* in the rumen of feed efficient lambs fed a forage-based diet. McGovern et al. (2018) also identified two *Treponema* OTU's positively correlating with feed efficiency in Simmental bulls. This contrasts with the findings of our study, which found *Treponema* associating significantly with poorer ADG and tending towards feed inefficiency in lambs fed a high concentrate diet.

Rikenellaceae RC9 gut group has previously been identified in the rumen of domesticated livestock (Petri et al., 2013; Ren et al., 2019) (Ishaq et al., 2019). Petri et al. (2013) observed a reduction in the abundance of *Rikenellaceae* in heifers treated with a diet comprising mixed forage and concentrate to those fed forage alone (Petri et al., 2013). Additionally, the abundance of unclassified *Rikenellaceae* was found to decrease in goats fed with high grain diets compared to hay based diets (Liu et al., 2015). The finding from these studies may indicate a preference for forage-based diets for the *Rikenellaceae RC9 gut group* and could explain its correlation with reduced ADG in our study where animals were fed a concentrate diet.

Ruminococcaceae UCG-014 and *Olsenella* also exhibited significant negative associations with FCR and were not found to be significantly associated with ADG. The uncultivable genus *Ruminococcaceae UCG-014* (family *Ruminococcaceae*) showed the strongest negative associations with FCR. *Ruminococcaceae* is considered a dominant family within the rumen of livestock (Creevey et al., 2014; Henderson et al., 2015) and generally more abundant in animals fed forage-based diets (Henderson et al., 2015). Within the *Ruminococcaceae* family certain members are known cellulolytic fermenters, such as *Ruminococcus albus* and *Ruminococcus flavefaciens* (Perea et al., 2017). However, other members are non-cellulolytic and actively ferment various forms of polysaccharides (Hook et al., 2011; Petri et al., 2012; La Reau and Suen, 2018). Indeed, Ellison et al. (2017) found particular *Ruminococcus* species to be more enriched in sheep fed a concentrate diet, compared to those fed a forage-based diet and vice versa. Additionally, in a study carried on dairy cows the abundance of *Ruminococcaceae NK4A214* was increased in a high grain diet (Pan et al., 2017). It is unclear why *Ruminococcaceae UCG-014* shows significant associations with FCR in our study. One possibility is that it may indicate that *Ruminococcaceae UCG-014* is associated with improved carbohydrate metabolism in the rumen.

Olsenella ferment starch and glycogen substrates and produce lactic, acetic and formic acid (Göker et al., 2010). Members of the genus *Olsenella* have been identified in oral cavities and GIT of humans and animals (Kraatz et al., 2011; Ellison et al., 2017; Kubasova et al., 2018; Elolimy et al., 2020). In line with our findings, Elolimy et al. (2020) reported a greater abundance of *Olsenella* in hindguts of feed efficient Holstein heifer calves (Elolimy et al., 2020). However, other studies have reported a greater abundance of *Olsenella* in the rumen microbiota of low feed efficient lambs when fed a concentrate diet (Ellison et al., 2017) and in the fecal microbiota of low feed efficient piglets (Kubasova et al., 2018).

The genera *Megasphera* and *Bifidobacteria* exhibited significant positive associations with ADG but no significant associations with FCR (Table 3). *Megasphera* has previously been associated with high feed efficiency in Holstein dairy cattle (Shabat et al., 2016), and also exhibited greater abundance in lower methane emitting sheep (Kamke et al., 2016). *Megasphera* is known to metabolise lactate within the rumen, which it utilises for production of important VFAs for animal growth (e.g acetate, propionate, and butyrate) (Chen et al., 2019), indicating its association with ADG in ruminants. Removal of lactate is important mechanism in regulating pH levels within the rumen, preventing rumen lactic acidosis and maintaining rumen health and function (Hernández et al., 2014; Chen et al., 2019).

Bifidobacterium species are known to produce a broad spectrum of carbohydrate modifying enzymes, which facilitate the metabolism of a wide variety of dietary carbohydrates. This enables members of the *Bifidobacterium* genus to efficiently adapt, extract energy and contribute to the feed efficiency of the host when offered a high-energy diet (Pokusaeva et al., 2011). Indeed, Ellison et al. (2017) found *Bifidobacterium* to be significantly more abundant in the rumen of feed efficient lambs when fed a concentrate diet. Furthermore, a study conducted by Abe et al. (1995) showed that oral administration of *Bifidobacterium* improved daily weight gain and FCR of young calves (Abe et al., 1995). Our findings are consistent with both of these studies, suggesting that *Bifidobacterium* may contribute significantly in extracting energy from carbohydrate based diets.

The relationship of feed efficiency with the rumen microbiota composition and abundance has not been extensively researched in sheep. In one study, Ellison et al. (2017) examined the effect of feed efficiency, diet and breed on the rumen microbial populations from the rumen of sheep. That study differed from our study in several key aspects. Firstly, in the study by Ellison et al. (2017) RFI (Residual Feed Intake) was used to distinguish feed efficient cohorts, which contrasts with the FCR measurement used in our study. Secondly, Ellison et al. (2017) used wether lambs spanning three different breeds of sheep - Rambouillet, Hampshire, and Suffolk, whereas TXSB ram lambs were used in our study. Thirdly, Ellison et al. (2017) fed animals with both concentrate and forage based diets. Although animals were not treated with a forage-based diet in this

study, the composition of concentrates in the diets used in both studies varied. A further study carried out by Perea et al. (2017) also examined the effect of RFI on the microbial populations in the rumen, as well as multiple other sites from the digestive tract, (including the rumen) of wether lambs fed a forage-based diet. Differences in diet, breeds, sex, measures of feed efficiency and analytical methodologies used between the studies may have contributed to the differences in the findings between the studies.

The dominance of *Firmicutes* and *Bacteroidetes* in the rumen of ruminants is widely reported throughout the literature (Paz et al., 2018; McGovern et al., 2018; Liu et al., 2019; Bo Trabi et al., 2019). Consistent with those studies *Firmicutes* and *Bacteroidetes* were identified as the most abundant phyla in the rumen of ram lambs fed a high concentrate diet. *Prevotella 7*, *Succinivibrionaceae UCG-001* and *Lachnospiraceae NK3A20* group were found to be the three most abundant genera in our study (Figure 3). This finding is also in line with a large global study set out to characterise the core rumen microbiota in small and large ruminants (Henderson et al., 2015). Henderson et al. (2015) identified *Prevotella* and unclassified *Lachnospiraceae* among the most abundant bacterial groups in the rumen. In addition, *Prevotella* and unclassified *Succinivibrionaceae* were found to be the most abundant bacterial groups in ruminants when fed a concentrate diet (Henderson et al., 2015). The degree of similarity between the taxa of sheep and cattle indicate that sheep models may serve as a useful and robust model for rumen microbiome research, as they are less expensive and more manageable than cattle (Delano et al., 2002).

To limit global warming to below 1.5°C above pre-industrial levels by 2050, in line with the Paris Agreement (UNFCCC, 2015), as well as feeding a growing population, there is an urgent requirement to increase production while reducing methane emissions intensity from livestock (Islam and Lee, 2019). In 2018, the IPCC's Special Report on Global Warming of 1.5°C detailed reduction targets for global biogenic methane to between 24% and 47% of 2010 levels by 2050 (IPCC, 2018). Reducing methane emissions from livestock production represents a promising mitigation strategy that can be achieved by sustainable intensification livestock production and/or reduced livestock product consumption (Herrero et al., 2016). In the context of sustainable intensification of ruminant livestock production, our findings indicate that genetically selecting for feed efficient animals can be a potential route for improving production and reducing feeding costs, while achieving methane emissions reductions in ruminant production systems.

Conclusion

In summary, our study investigated the rumen bacterial and archaeal populations in the rumen of ram lambs divergent for the FCR phenotype, which were fed a concentrate diet. Although lambs were found to be significantly divergent for feed efficiency, no major shifts in the rumen bacterial composition

were observed. Correlation analysis suggests that the differences in feed efficiency may be attributed to a number of specific bacterial taxa within the rumen rather than the community as a whole. On the other hand, archaea community composition, diversity and the relative abundance of a *Methanobrevibacter* species differed between HFE and LFE cohorts, which may partially explain a loss of energy in the LFE cohort. Whilst no significant difference in predicted metabolic processes was detected using CowPI, a limitation of this technique is that it infers microbial metabolic pathways based of 16S rDNA data and does not measure the microbial transcriptome or proteome (Wilkinson et al., 2018). Our study was also somewhat limited by poor classification at the species level. The aforementioned limitations can potentially be overcome by the use of both shotgun metagenomics for greater resolution of taxonomic classification (Quince et al., 2017; Brumfield et al., 2020) and metatranscriptomics uncovering the functional potential of the rumen microbiome (Shakya et al., 2019). Our current study focused exclusively on interrogating bacterial and archaeal populations from the rumen. A more comprehensive understanding of the contribution of the sheep rumen microbiota to animal feed efficiency would ideally investigate all major microbial populations within the rumen, including protozoa and fungi (Newbold et al., 2015; Tapio et al., 2017).

Figures

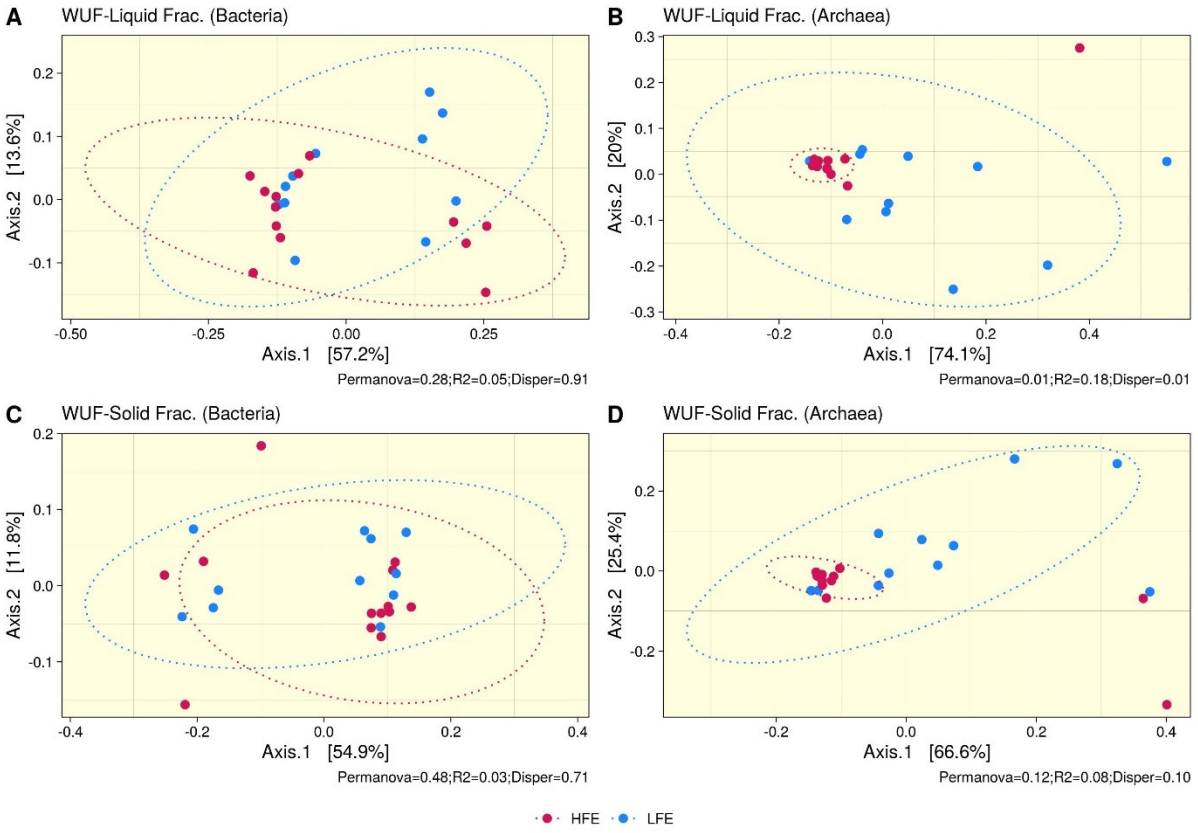


Figure 2.1: Beta diversity analysis. PCoA ordination plots based on weighted UniFrac distances for bacteria (A and C) and archaea (B and D) populations, for liquid (A and B) and solid (C and D) rumen fractions. Permanova P-value

(Permanova), R2 and homogeneity of dispersion analysis (Disper) is provided for each analysis. Dots represent the different microbial samples and colours represent different feed efficient cohorts, HFE (Dark Pink) and LFE (Blue). HFE *n*=13, LFE *n*=11.

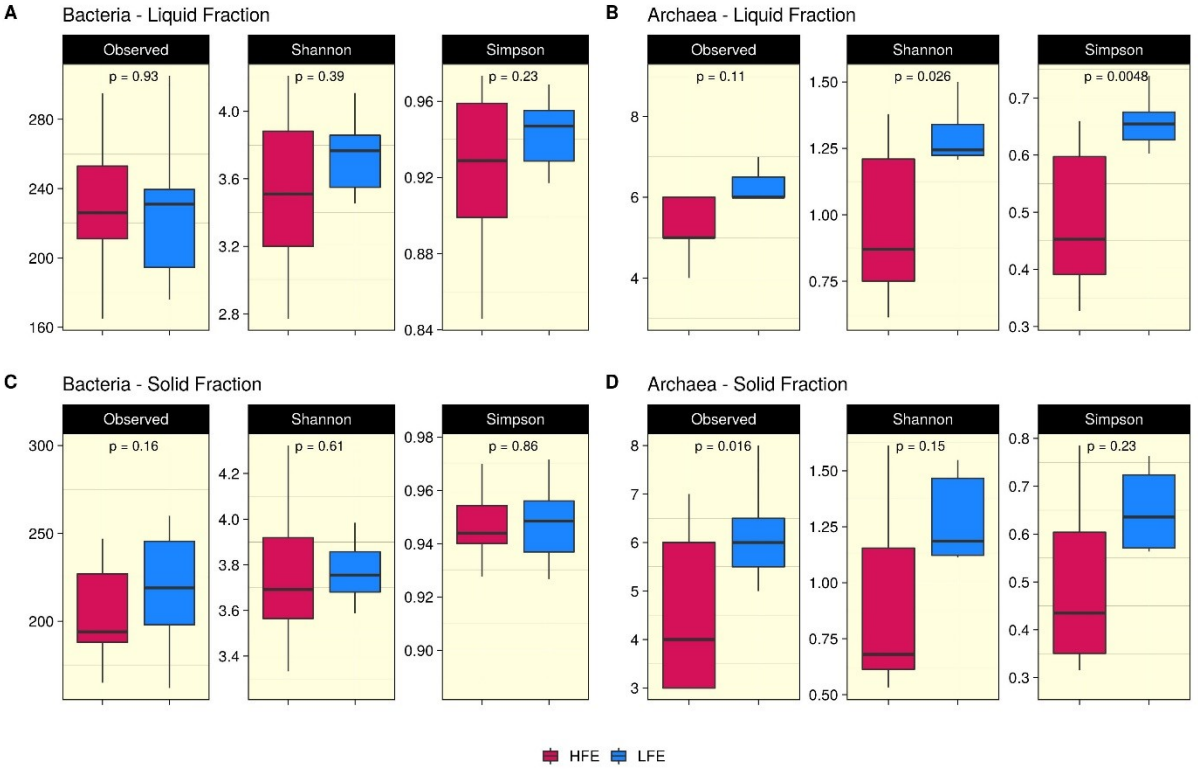


Figure 2.2: Alpha diversity analysis. Boxplots representing variations in alpha diversity in the rumen liquid (A and B) and solid (C and D) rumen fractions between high (Dark Pink) and low (Blue) feed efficiency cohorts. Alpha diversity metrics include Shannon, Simpson, and observed ASVs for both bacteria (A and C) and archaea (B and D) populations. HFE *n*=13, LFE *n*=11.

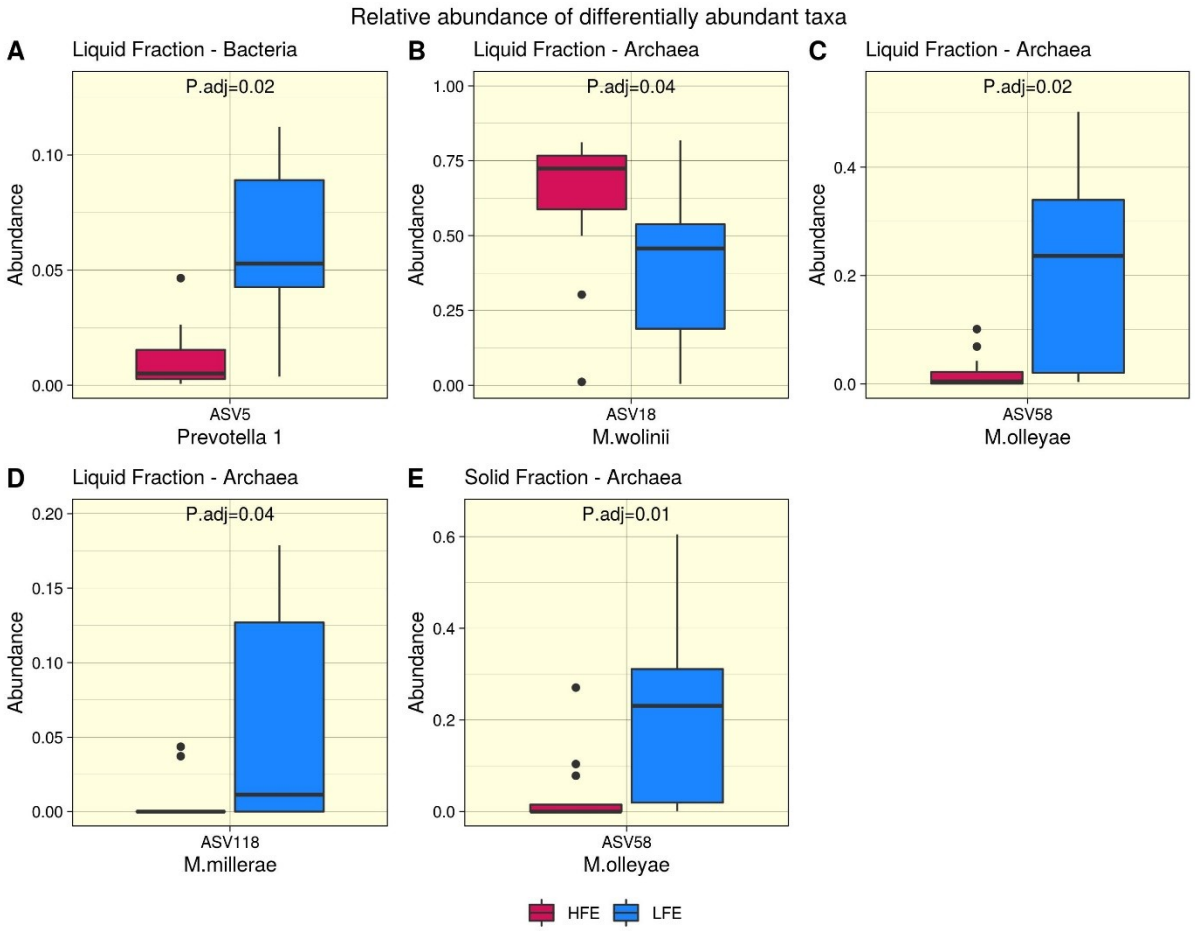


Figure 2.3: Differential abundance analysis. Bacteria (A) and archaea (B, C, D, E) taxa found to be differentially abundant between high (Dark Pink) and low (Blue) feed efficiency cohorts for liquid (A, B, C and D) and solid (E) rumen fractions. HFE *n*=13, LFE *n*=11.

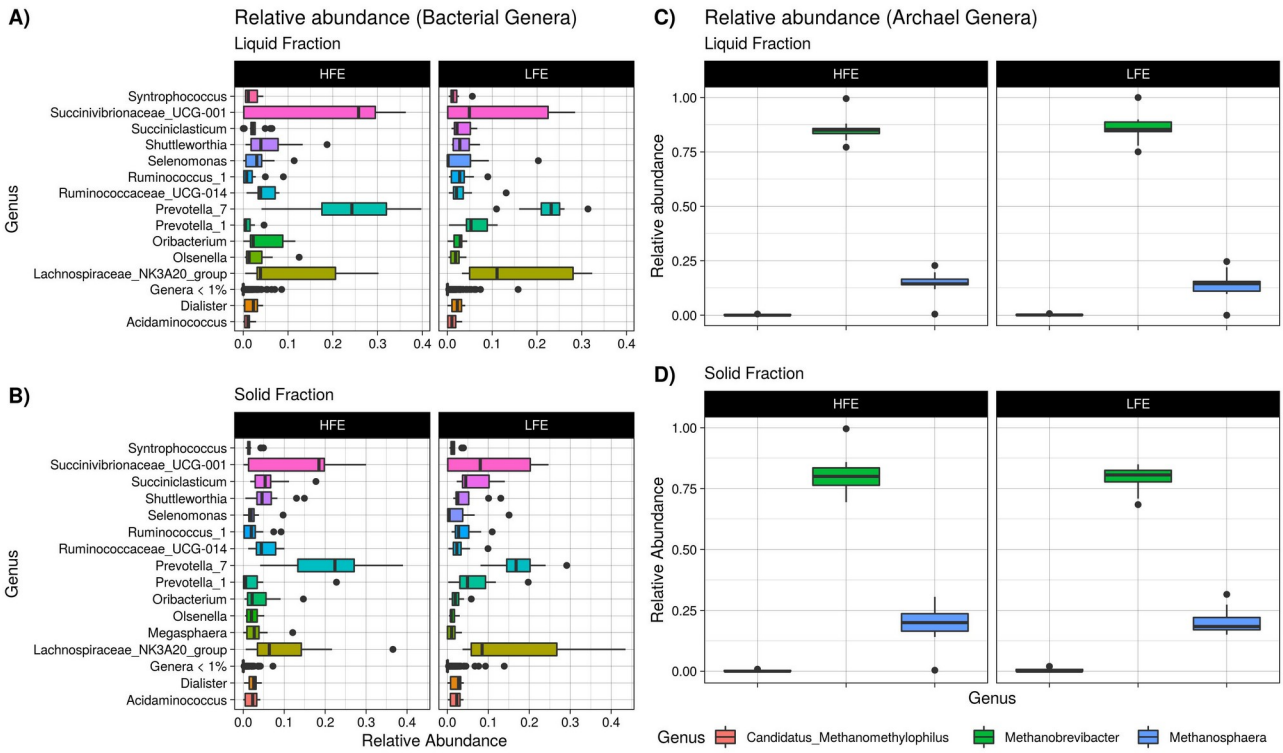


Figure 2.4: Relative abundance boxplots**.** Boxplots representing the variations in relative abundance of dominant bacterial genera (>1% relative abundance) (A and B) and archaea genera (C and D) in the rumen of HFE and LFE cohorts for liquid (A and C) and solid (B and D) rumen fractions. HFE *n*=13, LFE *n*=11.

Tables

Table 2.1: Production traits related to feed efficiency (FCR, ADG, ADI, TWG) analyzed per feed efficiency cohort. A Wilcoxon rank sum test was used to test for significance. HFE *n*=13, LFE *n*=13.

Production traits

Production Traits	HFE (mean±sd)	LFE (mean±sd)	Wilcox. Pvalue
FCR	3.83±0.40	6.05±0.92	6.41E-10
ADG	0.47±0.08	0.27±0.04	9.43E-09
TWG	17.03±2.79	9.70±1.58	9.43E-09
ADI	1.79±0.25	1.63±0.38	4.50E-02

Table 2.2: Mean relative abundance and standard deviation of bacterial phyla in rumen liquid phase for both HFE and LFE cohorts. P-values are derived from Wilcoxon rank sum test and adjusted for false discovery rate using B-H method. Correlation coefficient derived using Spearman correlation to find associations between relative abundance and FCR. F:B denotes *Firmicicutes to Bacteroidetes ratio.*

Rumen Fraction	***Phylum***	***HFE (mean)***	***HFE (sd)***	***LFE (mean)***	***LFE (sd)***	***Wilcoxon Pvalue***	***BHFDR***	***Spearman Rho (FCR)***	***Spearman Pvalue***
Liquid									
	F:B	0.67	0.25	0.69	0.20	1.00	NS	0.01	0.98
	Proteobacteria	0.18	0.13	0.12	0.11	0.07	0.30	-0.26	0.22
	Bacteroidetes	0.29	0.06	0.33	0.07	0.23	0.49	0.18	0.41
	Firmicutes	0.48	0.14	0.50	0.10	0.57	0.77	0.13	0.55
	Actinobacteria	0.04	0.04	0.02	0.01	0.57	0.77	-0.21	0.33
	Cyanobacteria	0.00	0.00	0.01	0.01	0.73	0.79	-0.07	0.73
	Fibrobacteres	0.00	0.00	0.01	0.02	0.09	0.30	0.44	0.03
	Spirochaetes	0.00	0.00	0.01	0.01	0.03	0.30	0.34	0.10
	Patescibacteria	0.00	0.00	0.00	0.01	0.65	0.77	-0.00	0.99
	Tenericutes	0.00	0.01	0.00	0.00	1.00	1.00	-0.33	0.12
	Synergistetes	0.00	0.00	0.00	0.00	0.27	0.50	0.38	0.06
	Kiritimatiellaeota	0.00	0.00	0.00	0.00	0.13	0.34	0.39	0.06
	Epsilonbacteraeota	0.00	0.00	0.00	0.00	0.60	0.77	-0.02	0.93
	Elusimicrobia	0.00	0.00	0.00	0.00	0.06	0.30	0.28	0.18
Solid									
	F:B	0.57	0.21	0.58	0.19	0.91	NS	0.18	0.39
	Proteobacteria	0.13	0.09	0.11	0.10	0.42	0.75	-0.06	0.79
	Bacteroidetes	0.28	0.07	0.29	0.04	0.91	0.91	0.19	0.38
	Firmicutes	0.54	0.12	0.54	0.13	0.91	0.91	-0.14	0.52
	Actinobacteria	0.03	0.02	0.01	0.01	0.12	0.39	-0.39	0.06
	Cyanobacteria	0.01	0.00	0.01	0.01	0.69	0.91	0.11	0.61
	Fibrobacteres	0.01	0.01	0.02	0.03	0.30	0.75	0.28	0.18
	Spirochaetes	0.01	0.01	0.01	0.02	0.11	0.39	0.28	0.19
	Patescibacteria	0.00	0.00	0.00	0.00	0.91	0.91	-0.10	0.65
	Tenericutes	0.01	0.01	0.00	0.00	0.36	0.75	-0.44	0.03

Synergistetes	0.00	0.00	0.00	0.00	0.05	0.31	0.51	0.01
Epsilonbacteraeota	0.00	0.00	0.00	0.00	0.72	0.91	-0.06	0.77
Elusimicrobia	0.00	0.00	0.00	0.00	0.01	0.12	0.43	0.04
Kiritimatiellaeota	0.00	0.00	0.00	0.00	0.46	0.75	0.13	0.54

Table 2.**3:** Spearman's rank correlation showing dominant bacterial genera (>0.1%) that had a significant relationship with either FCR and/or ADG in the liquid rumen phase. For analysis, only genera prevalent in more than 40% of samples were explored. HFE *n*=13, LFE *n*=11.

	Genus	***HFE mean***	***HFE (sd)***	***LFE (mean)***	***LFE (sd)***	***Rho (FCR)***	***Pvalue (FCR)***	***Rho (ADG)***	***Pvalue (ADG)***
Liquid									
	Prevotella_1	0.01	0.01	0.06	0.04	0.56	0.01	-0.71	0.00
	Fibrobacter	0.00	0.01	0.02	0.02	0.43	0.03	-0.61	0.00
	Treponema_2	0.00	0.01	0.01	0.01	0.34	0.10	-0.57	0.00
	Pseudoramibacter	0.00	0.00	0.00	0.00	0.34	0.10	-0.56	0.01
	Coprococcus_1	0.00	0.00	0.00	0.01	0.43	0.03	-0.51	0.01
	Ruminococcus_1	0.02	0.03	0.03	0.03	0.41	0.05	-0.50	0.01
	Acetitomaculum	0.01	0.01	0.01	0.01	0.41	0.05	-0.45	0.03
	Ruminococcus_2	0.00	0.00	0.01	0.02	0.31	0.15	-0.43	0.04
	Rikenellaceae_RC9_gut_group	0.00	0.00	0.01	0.02	0.16	0.47	-0.42	0.04
	Bifidobacterium	0.01	0.01	0.00	0.00	-0.32	0.12	0.41	0.05
	Ruminococcaceae_UCG-014	0.05	0.03	0.03	0.04	-0.51	0.01	0.19	0.38
Solid									
	Prevotella_1	0.03	0.07	0.06	0.06	0.39	0.06	-0.56	0.01
	Ruminococcus_1	0.03	0.04	0.03	0.03	0.43	0.04	-0.53	0.01
	Pyramidobacter	0.00	0.00	0.00	0.00	0.53	0.01	-0.52	0.01
	Fibrobacter	0.01	0.02	0.01	0.03	0.28	0.18	-0.51	0.01
	Treponema_2	0.01	0.01	0.01	0.02	0.28	0.19	-0.48	0.02
	Roseburia	0.01	0.01	0.01	0.01	0.37	0.08	-0.45	0.03
	Pseudoramibacter	0.00	0.00	0.00	0.00	0.42	0.04	-0.45	0.03
	Bifidobacterium	0.00	0.00	0.01	0.00	-0.34	0.10	0.44	0.03
	Megasphaera	0.03	0.01	0.03	0.01	-0.2	0.36	0.43	0.04

					0			
Acetitomaculum	0.01	0.02	0.01	0.01	0.42	0.04	-0.41	0.05
Coprococcus_1	0.00	0.00	0.00	0.00	0.42	0.04	-0.41	0.05
Olsenella	0.02	0.01	0.02	0.01	-0.40	0.05	0.33	0.12
Ruminococcaceae_UCG-014	0.06	0.03	0.03	0.03	-0.55	0.01	0.29	0.17

References

Abe, F., Ishibashi, N., and Shimamura, S. (1995). Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. *Journal of dairy science* 78**,** 2838-2846.

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A.I., Pinloche, E., Creevey, C.J., Denman, S.E., Newbold, C.J., and Yáñez-Ruiz, D.R. (2018). Analysis of the Rumen Microbiome and Metabolome to Study the Effect of an Antimethanogenic Treatment Applied in Early Life of Kid Goats. *Frontiers in microbiology* 9**,** 2227-2227.

Andrews, S. (2010). "FastQC: a quality control tool for high throughput sequence data". Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom).

Berry, D.P., and Crowley, J.J. (2013). Cell Biology Symposium: genetics of feed efficiency in dairy and beef cattle. *J Anim Sci* 91**,** 1594-1613.

Bhatt, R.S., Karim, S.A., Sahoo, A., and Shinde, A.K. (2013). Growth performance of lambs fed diet supplemented with rice bran oil as such or as calcium soap. *Asian-Australasian journal of animal sciences* 26**,** 812-819.

Brooke, C.G., Najafi, N., Dykier, K.C., and Hess, M. (2019). Prevotella copri, a potential indicator for high feed efficiency in western steers. *Animal Science Journal* 90**,** 696-701.

Brumfield, K.D., Huq, A., Colwell, R.R., Olds, J.L., and Leddy, M.B. (2020). Microbial resolution of whole genome shotgun and 16S amplicon metagenomic sequencing using publicly available NEON data. *PLOS ONE* 15**,** e0228899.

Callahan, B. (2018). Silva taxonomic training data formatted for DADA2. *Silva version* 132.

Callahan, B.J., Mcmurdie, P.J., and Holmes, S.P. (2017). Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal* 11**,** 2639-2643.

Cannas, A., Tedeschi, L.O., Atzori, A.S., and Lunesu, M.F. (2019). How can nutrition models increase the production efficiency of sheep and goat operations? *Animal frontiers : the review magazine of animal agriculture* 9**,** 33-44.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences* 108**,** 4516.

Carberry, C.A., Kenny, D.A., Han, S., Mccabe, M.S., and Waters, S.M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Applied and environmental microbiology* 78**,** 4949-4958.

Chen, L., Shen, Y., Wang, C., Ding, L., Zhao, F., Wang, M., Fu, J., and Wang, H. (2019). Megasphaera elsdenii Lactate Degradation Pattern Shifts in Rumen Acidosis Models. *Frontiers in Microbiology* 10**,** 162.

Claffey, N.A., Fahey, A.G., Gkarane, V., Moloney, A.P., Monahan, F.J., and Diskin, M.G. (2018). Effect of breed and castration on production and carcass traits of male lambs following an intensive finishing period. *Translational Animal Science* 2**,** 407-418.

Creevey, C.J., Kelly, W.J., Henderson, G., and Leahy, S.C. (2014). Determining the culturability of the rumen bacterial microbiome. *Microbial biotechnology* 7**,** 467-479.

Danielsson, R., Schnürer, A., Arthurson, V., and Bertilsson, J. (2012). Methanogenic Population and CH₄ Production in Swedish Dairy Cows Fed Different Levels of Forage. *Applied and Environmental Microbiology* 78**,** 6172.

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., Ruttink, T., De Wiele, T.V., and De Campeneere, S. (2016). Exploring the methanogen and bacterial communities of rumen environments: solid adherent, fluid and epimural. *FEMS Microbiology Ecology* 93.

Delano, M.L., Mischler, S.A., and Underwood, W.J. (2002). "Chapter 14 - Biology and Diseases of Ruminants: Sheep, Goats, and Cattle," in *Laboratory Animal Medicine (Second Edition),* eds. J.G. Fox, L.C. Anderson, F.M. Loew & F.W. Quimby. (Burlington: Academic Press), 519-614.

Delgado, B., Bach, A., Guasch, I., González, C., Elcoso, G., Pryce, J.E., and Gonzalez-Recio, O. (2019). Whole rumen metagenome sequencing allows classifying and predicting feed efficiency and intake levels in cattle. *Scientific Reports* 9.

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. (2017). A Structural and Functional Elucidation of the Rumen Microbiome Influenced by Various Diets and Microenvironments. *Frontiers in microbiology* 8**,** 1605-1605.

Downes, J., Vartoukian, S.R., Dewhirst, F.E., Izard, J., Chen, T., Yu, W.-H., Sutcliffe, I.C., and Wade, W.G. (2009). Pyramidobacter piscolens gen. nov., sp. nov., a member of the phylum 'Synergistetes' isolated from the human oral cavity. *International journal of systematic and evolutionary microbiology* 59**,** 972-980.

Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E., and Flint, H.J. (2002). Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine. *Applied and environmental microbiology* 68**,** 5186-5190.

Ellison, M.J., Conant, G.C., Lamberson, W.R., Austin, K.J., Van Kirk, E., Cunningham, H.C., Rule, D.C., and Cammack, K.M. (2019). Predicting residual feed intake status using rumen microbial profiles in ewe lambs1. *J Anim Sci* 97**,** 2878-2888.

Ellison, M.J., Conant, G.C., Lamberson, W.R., Cockrum, R.R., Austin, K.J., Rule, D.C., and Cammack, K.M. (2017). Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Ruminant Research* 156**,** 12-19.

Elolimy, A., Alharthi, A., Zeineldin, M., Parys, C., and Loor, J.J. (2020). Residual feed intake divergence during the preweaning period is associated with unique hindgut microbiome and metabolome profiles in neonatal Holstein heifer calves. *Journal of Animal Science and Biotechnology* 11**,** 13.

Fitzsimons, C., Kenny, D.A., Deighton, M.H., Fahey, A.G., and Mcgee, M. (2013). Methane emissions, body composition, and rumen fermentation traits of beef heifers differing in residual feed intake1. *Journal of Animal Science* 91**,** 5789-5800.

Freetly, H.C., Dickey, A., Lindholm-Perry, A.K., Thallman, R.M., Keele, J.W., Foote, A.P., and Wells, J.E. (2020). Digestive tract microbiota of beef cattle that differed in feed efficiency. *Journal of Animal Science*.

Göker, M., Held, B., Lucas, S., Nolan, M., Yasawong, M., Glavina Del Rio, T., Tice, H., Cheng, J.-F., Bruce, D., Detter, J.C., Tapia, R., Han, C., Goodwin, L., Pitluck, S., Liolios, K., Ivanova, N., Mavromatis, K., Mikhailova, N., Pati, A., Chen, A., Palaniappan, K., Land, M., Hauser, L., Chang, Y.-J., Jeffries, C.D., Rohde, M., Sikorski, J., Pukall, R., Woyke, T., Bristow, J., Eisen, J.A., Markowitz, V., Hugenholtz, P., Kyrpides, N.C., Klenk, H.-P., and Lapidus, A. (2010). Complete genome sequence of Olsenella uli type strain (VPI D76D-27C). *Standards in genomic sciences* 3**,** 76-84.

Greening, R.C., and Leedle, J.A. (1989). Enrichment and isolation of Acetitomaculum ruminis, gen. nov., sp. nov.: acetogenic bacteria from the bovine rumen. *Arch Microbiol* 151**,** 399-406.

Gu, W., Miller, S., and Chiu, C.Y. (2019). Clinical metagenomic next-generation sequencing for pathogen detection. *Annual Review of Pathology: Mechanisms of Disease* 14**,** 319-338.

Henchion, M., Hayes, M., Mullen, A.M., Fenelon, M., and Tiwari, B. (2017). Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods (Basel, Switzerland)* 6**,** 53.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Global Rumen Census, C., and Janssen, P.H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific reports* 5**,** 14567-14567.

Hernández, J., Benedito, J.L., Abuelo, A., and Castillo, C. (2014). Ruminal acidosis in feedlot: from aetiology to prevention. *The Scientific World Journal* 2014.

Herrero, M., Henderson, B., Havlík, P., Thornton, P.K., Conant, R.T., Smith, P., Wirsenius, S., Hristov, A.N., Gerber, P., Gill, M., Butterbach-Bahl, K., Valin, H., Garnett, T., and Stehfest, E. (2016). Greenhouse gas mitigation potentials in the livestock sector. *Nature Climate Change* 6**,** 452-461.

Hook, S.E., Steele, M.A., Northwood, K.S., Dijkstra, J., France, J., Wright, A.-D.G., and Mcbride, B.W. (2011). Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. *FEMS Microbiology Ecology* 78**,** 275-284.

Hunter, M.C., Smith, R.G., Schipanski, M.E., Atwood, L.W., and Mortensen, D.A. (2017). Agriculture in 2050: recalibrating targets for sustainable intensification. *Bioscience* 67**,** 386-391.

Ishaq, S.L., Lachman, M.M., Wenner, B.A., Baeza, A., Butler, M., Gates, E., Olivo, S., Geddes, J.B., Hatfield, P., and Yeoman, C.J. (2019). Pelleted-hay alfalfa feed increases sheep wether weight gain and rumen bacterial richness over loose-hay alfalfa feed. *PLOS ONE* 14**,** e0215797.

Islam, M., and Lee, S.-S. (2019). Advanced estimation and mitigation strategies: a cumulative approach to enteric methane abatement from ruminants. *Journal of animal science and technology* 61**,** 122-137.

Jewell, K.A., Mccormick, C.A., Odt, C.L., Weimer, P.J., and Suen, G. (2015). Ruminal Bacterial Community Composition in Dairy Cows Is Dynamic over the Course of Two Lactations and Correlates with Feed Efficiency. *Applied and environmental microbiology* 81**,** 4697-4710.

Ji, S., Zhang, H., Yan, H., Azarfar, A., Shi, H., Alugongo, G., Li, S., Cao, Z., and Wang, Y. (2017). Comparison of rumen bacteria distribution in original rumen digesta, rumen liquid and solid fractions in lactating Holstein cows. *Journal of animal science and biotechnology* 8**,** 16-16.

Johnson, K.A., and Johnson, D.E. (1995). Methane emissions from cattle. *Journal of Animal Science* 73**,** 2483-2492.

Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., Mcginnis, S., and Madden, T.L. (2008). NCBI BLAST: a better web interface. *Nucleic Acids Research* 36**,** W5-W9.

Kachiprath, B., Puthumana, J., Gopi, J., Solomon, S., Krishnan, K.P., and Philip, R. (2018). Amplicon sequencing based profiling of bacterial diversity from Krossfjorden, Arctic. *Data in brief* 21**,** 2522-2525.

Kenny, D.A., Fitzsimons, C., Waters, S.M., and Mcgee, M. (2018). Invited review: Improving feed efficiency of beef cattle–the current state of the art and future challenges. *animal* 12**,** 1815-1826.

Keogh, K., Waters, S.M., Cormican, P., Kelly, A.K., O'shea, E., and Kenny, D.A. (2017). Effect of dietary restriction and subsequent re-alimentation on the transcriptional profile of bovine ruminal epithelium. *PloS one* 12.

Kim, M., Kim, J., Kuehn, L.A., Bono, J.L., Berry, E.D., Kalchayanand, N., Freetly, H.C., Benson, A.K., and Wells, J.E. (2014). Investigation of bacterial diversity in the feces of cattle fed different diets1. *Journal of Animal Science* 92**,** 683-694.

Koike, S., and Kobayashi, Y. (2001). Development and use of competitive PCR assays for the rumen cellulolytic bacteria: Fibrobacter succinogenes, Ruminococcus albus and Ruminococcus flavefaciens. *FEMS Microbiology Letters* 204**,** 361-366.

Kraatz, M., Wallace, R.J., and Svensson, L. (2011). Olsenella umbonata sp. nov., a microaerotolerant anaerobic lactic acid bacterium from the sheep rumen and pig jejunum, and emended descriptions of Olsenella, Olsenella uli and Olsenella profusa. *International Journal of Systematic and Evolutionary Microbiology* 61**,** 795-803.

Kubasova, T., Davidova-Gerzova, L., Babak, V., Cejkova, D., Montagne, L., Le-Floc'h, N., and Rychlik, I. (2018). Effects of host genetics and environmental conditions on fecal microbiota composition of pigs. *PloS one* 13**,** e0201901-e0201901.

La Reau, A.J., and Suen, G. (2018). The Ruminococci: key symbionts of the gut ecosystem. *Journal of Microbiology* 56**,** 199-208.

Lan, W., and Yang, C. (2019). Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation. *Science of The Total Environment* 654**,** 1270-1283.

Le Van, T.D., Robinson, J.A., Ralph, J., Greening, R.C., Smolenski, W.J., Leedle, J.A., and Schaefer, D.M. (1998). Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and Acetitomaculum ruminis. *Appl Environ Microbiol* 64**,** 3429-3436.

Li, F., and Guan, L.L. (2017). Metatranscriptomic Profiling Reveals Linkages between the Active Rumen Microbiome and Feed Efficiency in Beef Cattle. *Applied and environmental microbiology* 83**,** e00061-00017.

Li, F., Hitch, T.C.A., Chen, Y., Creevey, C.J., and Guan, L.L. (2019). Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle. *Microbiome* 7**,** 6.

Li, Z., Mu, C., Xu, Y., Shen, J., and Zhu, W. (2020). Changes in the Solid-, Liquid-, and Epithelium-Associated Bacterial Communities in the Rumen of Hu Lambs in Response to Dietary Urea Supplementation. *Frontiers in Microbiology* 11.

Liu, J.-H., Bian, G.-R., Zhu, W.-Y., and Mao, S.-Y. (2015). High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of Toll-like receptor genes in goats. *Frontiers in microbiology* 6**,** 167-167.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* 17**,** 10-12.

Matsui, H., Ogata, K., Tajima, K., Nakamura, M., Nagamine, T., Aminov, R.I., and Benno, Y. (2000). Phenotypic Characterization of Polysaccharidases Produced by Four Prevotella Type Strains. *Current Microbiology* 41**,** 45-49.

Matsui, H., Ushida, K., Miyazaki, K., and Kojima, Y. (1998). Use of ratio of digested xylan to digested cellulose (X/C) as an index of fiber digestion in plant cell-wall material by ruminal microorganisms. *Animal Feed Science and Technology* 71**,** 207-215.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'toole, P.W., and Cotter, P.D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* 10**,** 115-132.

Mccann, J.C., Wickersham, T.A., and Loor, J.J. (2014). High-throughput Methods Redefine the Rumen Microbiome and Its Relationship with Nutrition and Metabolism. *Bioinformatics and biology insights* 8**,** 109-125.

Mcgovern, E., Kenny, D.A., Mccabe, M.S., Fitzsimons, C., Mcgee, M., Kelly, A.K., and Waters, S.M. (2018). 16S rRNA Sequencing Reveals Relationship Between Potent Cellulolytic Genera and Feed Efficiency in the Rumen of Bulls. *Frontiers in microbiology* 9**,** 1842-1842.

Mcmurdie, P.J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* 8.

Mi, L., Yang, B., Hu, X., Luo, Y., Liu, J., Yu, Z., and Wang, J. (2018). Comparative Analysis of the Microbiota Between Sheep Rumen and Rabbit Cecum Provides New Insight Into Their Differential Methane Production. *Frontiers in microbiology* 9**,** 575-575.

Myer, P.R., Wells, J.E., Smith, T.P.L., Kuehn, L.A., and Freetly, H.C. (2015a). Microbial community profiles of the colon from steers differing in feed efficiency. *SpringerPlus* 4**,** 454-454.

Newbold, C.J., De La Fuente, G., Belanche, A., Ramos-Morales, E., and Mcewan, N.R. (2015). The Role of Ciliate Protozoa in the Rumen. *Frontiers in microbiology* 6**,** 1313-1313.

Newbrook, K., Staton, G.J., Clegg, S.R., Birtles, R.J., Carter, S.D., and Evans, N.J. (2017). Treponema ruminis sp. nov., a spirochaete isolated from the bovine rumen. *International Journal of Systematic and Evolutionary Microbiology* 67**,** 1349-1354.

Nkrumah, J.D., Okine, E.K., Mathison, G.W., Schmid, K., Li, C., Basarab, J.A., Price, M.A., Wang, Z., and Moore, S.S. (2006). Relationships of feedlot feed efficiency, performance, and feeding behavior with metabolic rate, methane production, and energy partitioning in beef cattle1. *Journal of Animal Science* 84**,** 145-153.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., and Mcglinn, D. (2019). "vegan: Community Ecology Package. R package version 2.5-3. 2018".).

Palakawong Na Ayudthaya, S., Van De Weijer, A.H.P., Van Gelder, A.H., Stams, A.J.M., De Vos, W.M., and Plugge, C.M. (2018). Organic acid production from potato starch waste fermentation by rumen microbial communities from Dutch and Thai dairy cows. *Biotechnology for Biofuels* 11**,** 13.

Pan, X., Xue, F., Nan, X., Tang, Z., Wang, K., Beckers, Y., Jiang, L., and Xiong, B. (2017). Illumina Sequencing Approach to Characterize Thiamine Metabolism Related Bacteria and the Impacts of Thiamine Supplementation on Ruminal Microbiota in Dairy Cows Fed High-Grain Diets. *Frontiers in Microbiology* 8**,** 1818.

Parks, D.H., Tyson, G.W., Hugenholtz, P., and Beiko, R.G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics (Oxford, England)* 30**,** 3123-3124.

Perea, K., Perz, K., Olivo, S.K., Williams, A., Lachman, M., Ishaq, S.L., Thomson, J., and Yeoman, C.J. (2017). Feed efficiency phenotypes in lambs involve changes in ruminal, colonic, and small-intestine-located microbiota1. *Journal of Animal Science* 95**,** 2585-2592.

Petri, R.M., Forster, R.J., Yang, W., Mckinnon, J.J., and Mcallister, T.A. (2012). Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage. *Journal of Applied Microbiology* 112**,** 1152-1162.

Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., Mckinnon, J.J., and Mcallister, T.A. (2013). Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge. *PloS one* 8**,** e83424-e83424.

Pokusaeva, K., Fitzgerald, G.F., and Van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. *Genes & nutrition* 6**,** 285-306.

Pulina, G., Milán, M.J., Lavín, M.P., Theodoridis, A., Morin, E., Capote, J., Thomas, D.L., Francesconi, A.H.D., and Caja, G. (2018). Invited review: Current

production trends, farm structures, and economics of the dairy sheep and goat sectors. *Journal of Dairy Science* 101**,** 6715-6729.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., and Segata, N. (2017). Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology* 35**,** 833-844.

Ren, H., Su, X., Bai, H., Yang, Y., Wang, H., Dan, Z., Lu, J., Wu, S., Cai, C., Cao, Y., Lei, X., and Yao, J. (2019). Specific enrichment of microbes and increased ruminal propionate production: the potential mechanism underlying the high energy efficiency of Holstein heifers fed steam-flaked corn. *AMB Express* 9**,** 209.

Santana, M.H.A., Rossi, P., Almeida, R., and Cucco, D.C. (2012). Feed efficiency and its correlations with carcass traits measured by ultrasound in Nellore bulls. *Livestock Science* 145**,** 252-257.

Schären, M., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., Urich, T., Breves, G., and Dänicke, S. (2017). Alterations in the Rumen Liquid-, Particle- and Epithelium-Associated Microbiota of Dairy Cows during the Transition from a Silage- and Concentrate-Based Ration to Pasture in Spring. *Frontiers in microbiology* 8**,** 744-744.

Schliep, K.P. (2011). phangorn: phylogenetic analysis in R. *Bioinformatics (Oxford, England)* 27**,** 592-593.

Shabat, S.K.B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M.E., White, B.A., Shterzer, N., and Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME journal* 10**,** 2958-2972.

Shakya, M., Lo, C.-C., and Chain, P.S.G. (2019). Advances and Challenges in Metatranscriptomic Analysis. *Frontiers in Genetics* 10.

Shi, W., Moon, C.D., Leahy, S.C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., and Seedorf, H. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome research* 24**,** 1517-1525.

Tapio, I., Snelling, T.J., Strozzi, F., and Wallace, R.J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of Animal Science and Biotechnology* 8**,** 7.

Thomas, N.A., Olvera-Ramírez, A.M., Abecia, L., Adam, C.L., Edwards, J.E., Cox, G.F., Findlay, P.A., Destables, E., Wood, T.A., and Mcewan, N.R. (2019). Characterisation of the effect of day length, and associated differences in dietary intake, on the gut microbiota of Soay sheep. *Archives of microbiology* 201**,** 889-896.

Thornton, P.K. (2010). Livestock production: recent trends, future prospects. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 365**,** 2853-2867.

Un (2019). World population prospects 2019: Highlights (st/esa/ser. A/423).

Wallace, R.J., Rooke, J.A., Mckain, N., Duthie, C.-A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., and Roehe, R. (2015). The rumen microbial metagenome associated with high methane production in cattle. *BMC Genomics* 16**,** 839.

Whitman, W.B. (2015). *Bergey's manual of systematics of Archaea and Bacteria.* Wiley Online Library.

Wilkinson, T.J., Huws, S.A., Edwards, J.E., Kingston-Smith, A.H., Siu-Ting, K., Hughes, M., Rubino, F., Friedersdorff, M., and Creevey, C.J. (2018). CowPI: a rumen microbiome focussed version of the PICRUSt functional inference software. *Frontiers in microbiology* 9**,** 1095.

Willis, C., Desai, D., and Laroche, J. (2019). Influence of 16S rRNA variable region on perceived diversity of marine microbial communities of the Northern North Atlantic. *FEMS microbiology letters* 366**,** fnz152.

Wilson-Welder, J.H., Alt, D.P., and Nally, J.E. (2015). Digital Dermatitis in Cattle: Current Bacterial and Immunological Findings. *Animals : an open access journal from MDPI* 5**,** 1114-1135.

Xie, F., Zhang, L., Jin, W., Meng, Z., Cheng, Y., Wang, J., and Zhu, W. (2019). Methane Emission, Rumen Fermentation, and Microbial Community Response to a Nitrooxy Compound in Low-Quality Forage Fed Hu Sheep. *Current Microbiology* 76**,** 435-441.

Xie, X., Yang, C., Guan, L.L., Wang, J., Xue, M., and Liu, J.X. (2018). Persistence of Cellulolytic Bacteria Fibrobacter and Treponema After Short-Term Corn Stover-Based Dietary Intervention Reveals the Potential to Improve Rumen Fibrolytic Function. *Frontiers in microbiology* 9**,** 1363-1363.

Yu, Z., and Morrison, M. (2004). Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 70**,** 4800-4806.

Zeineldin, M., Barakat, R., Elolimy, A., Salem, A.Z.M., Elghandour, M.M.Y., and Monroy, J.C. (2018). Synergetic action between the rumen microbiota and bovine health. *Microbial pathogenesis* 124**,** 106-115.

Zhang, J., Shi, H., Wang, Y., Cao, Z., Yang, H., and Li, S. (2018). Effect of Limit-Fed Diets With Different Forage to Concentrate Ratios on Fecal Bacterial and Archaeal Community Composition in Holstein Heifers. *Frontiers in microbiology* 9**,** 976-976.

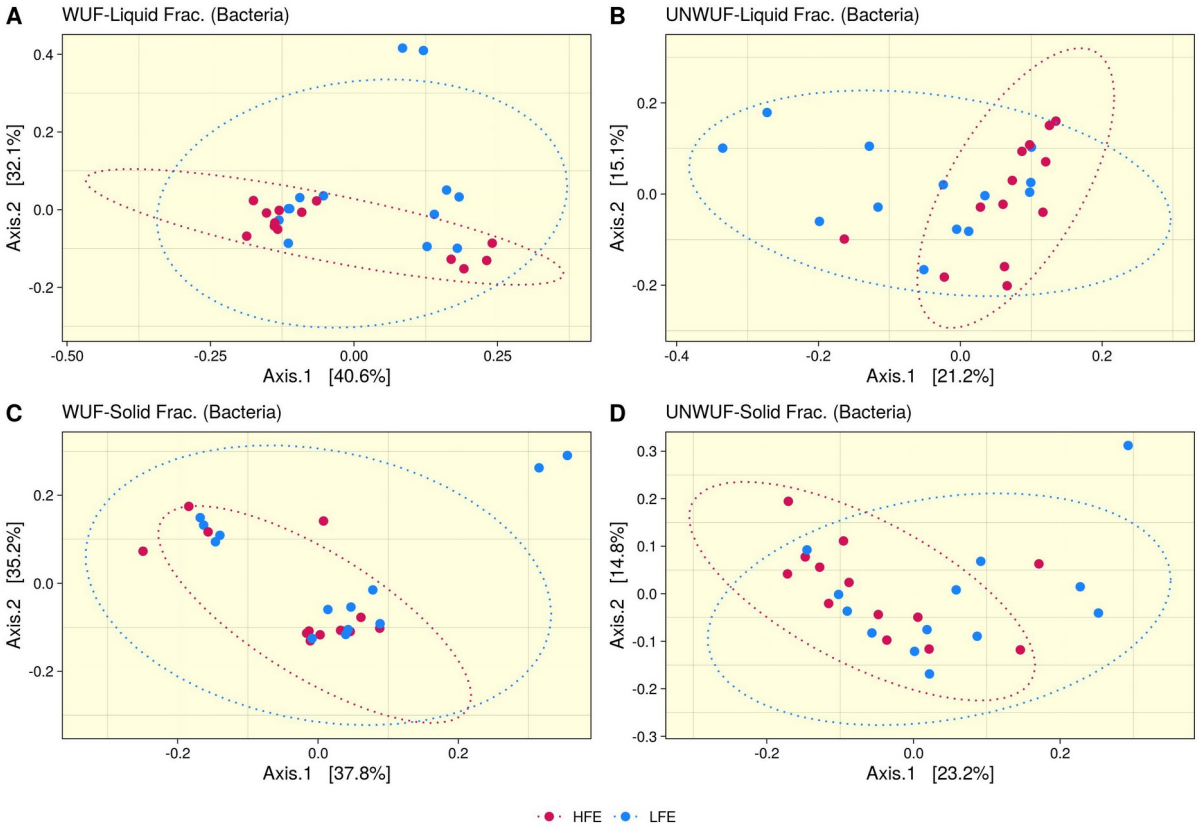
Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., He, Y., and Zhang, H. (2017). Effect of Dietary Forage to Concentrate Ratios on Dynamic Profile Changes and Interactions of Ruminal Microbiota and Metabolites in Holstein Heifers. *Frontiers in microbiology* 8**,** 2206-2206.

Zhou, J., He, Z., Yang, Y., Deng, Y., Tringe, S.G., and Alvarez-Cohen, L. (2015). High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. *MBio* 6**,** e02288-02214.

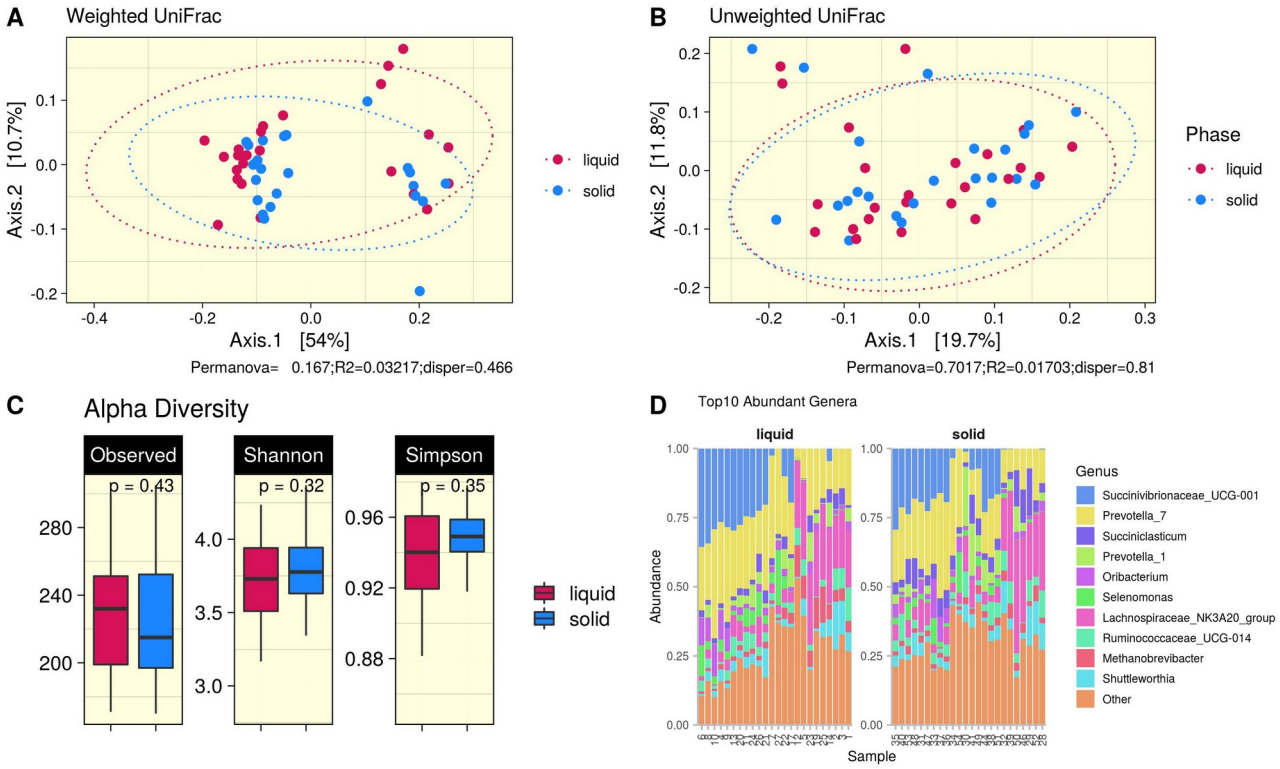
Zhou, M., Hernandez-Sanabria, E., and Guan, L.L. (2010). Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Applied and environmental microbiology* 76**,** 3776-3786.

Chapter 2 Supplementary Figures

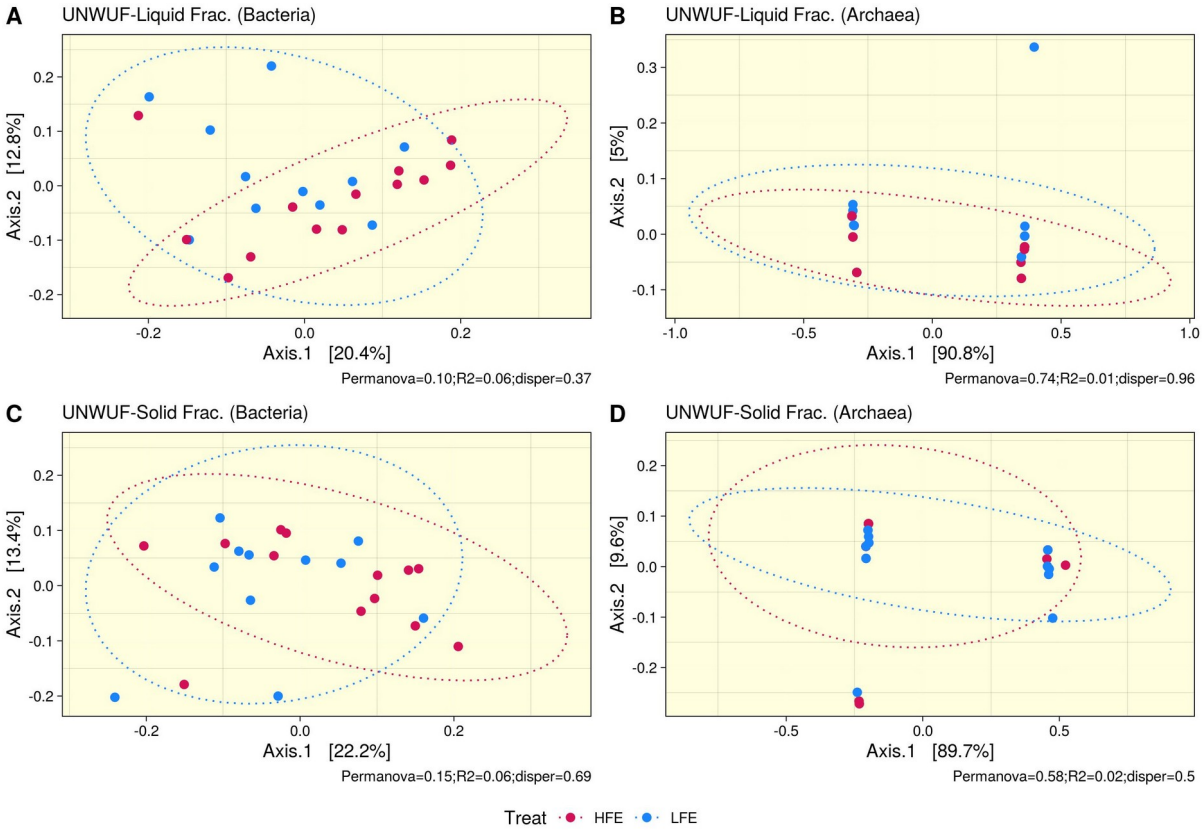
Figures



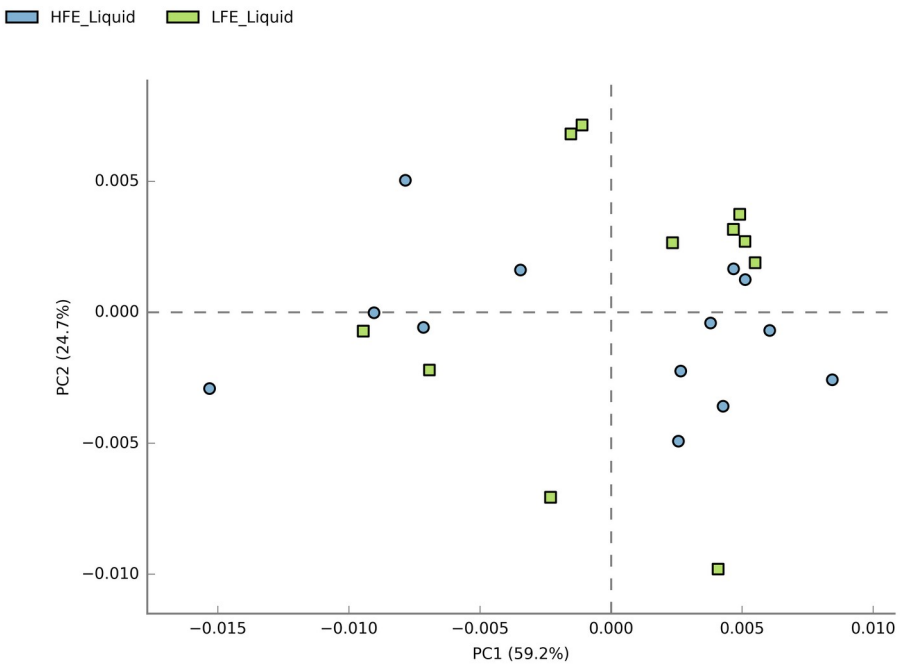
Supplementary Figure 2.1: Beta diversity analysis. NMDS ordination plot based weighted and unweighted UniFrac distances. Dots represent the different samples. Colours represent different feed efficient cohorts, HFE (pink) and LFE (Blue). The plots show outlying samples (10707, 10835), which were removed prior to downstream analysis. HFE n=13, LFE n=13.



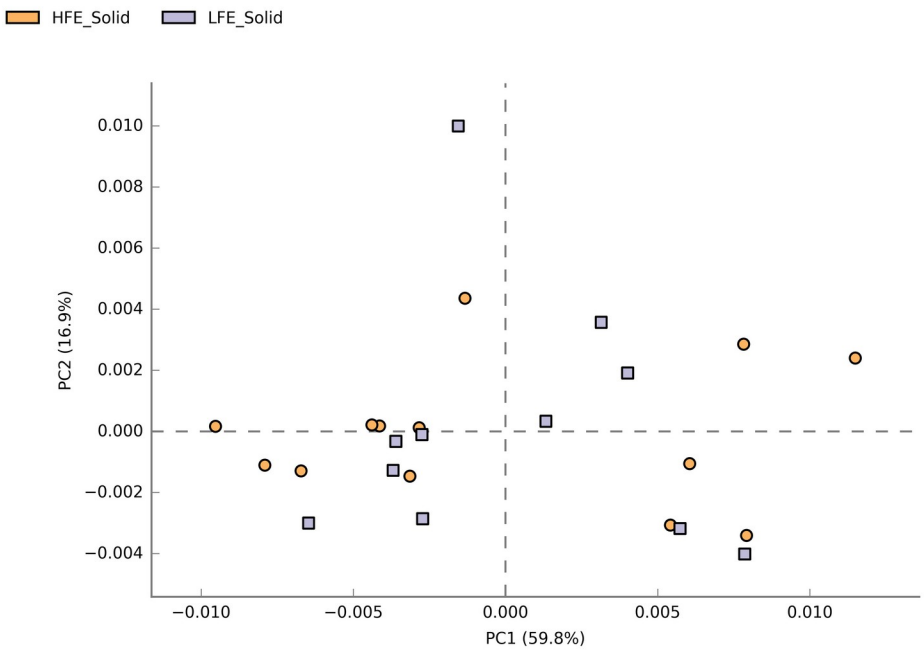
Supplementary Figure 2.2: Analysis of ruminal fractions. PCoA ordination plots with weighted (A) and unweighted (B) UniFrac distances. Alpha diversity analysis; Shannon, Simpson and observed ASV for solid and liquid rumen fractions (C). Colours represent different feed efficient cohorts, HFE (Dark Pink) and LFE (Blue). Stacked barchart representing the relative abundance of 10 most abundant genera (D). HFE n=13, LFE n=11.



Supplementary Figure 2.3: Beta diversity analysis**.** PCoA ordination plots based on weighted UniFrac distances for bacteria (A and C) and archaea (B and D) populations, for liquid (A and B) and solid (C and D) rumen fractions. Permanova P-value (Permanova), R2 and homogeneity of dispersion analysis (Disper) is provided for each analysis. Dots represent the different microbial samples and colours represent different feed efficient cohorts, HFE (Dark Pink) and LFE (Blue). HFE *n*=13, LFE *n*=11.



Supplementary Figure 2.4: PCA plot comparing microbiome functional profiles, from COWPi, for each sample from the rumen liquid phase. HFE *n*=13, LFE *n*=11.



Supplementary Figure 2.5: PCA plot comparing microbiome functional profiles, from COWPi, for each sample from the rumen solid phase. HFE *n*=13, LFE *n*=11.

Chapter 3

Breed and ruminal fraction effects on bacterial and archaeal community composition in sheep

Steven McLoughlin[1,2], Charles Spillane*[2], Francis P. Campion[1], Noel Claffey[1], Chrystian C. Sosa[1], Yvonne McNicholas[1], Paul E. Smith[1], Michael G. Diskin[1], and Sinéad M. Waters*[1,2]

[1]Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Athenry, Co. Galway, H65 R718, Ireland

[2]Genetics and Biotechnology Laboratory, Plant and AgriBiosciences Research Centre (PABC), Ryan Institute, National University of Ireland Galway, University Road, Galway H91 REW4, Ireland

Abstract

While the breed of cattle can impact on the composition and structure of microbial communities in the rumen, breed-specific effects on rumen microbial communities have rarely been examined in sheep. In addition, rumen microbial composition can differ between ruminal fractions, and be associated with ruminant feed efficiency and methane emissions. In this study, 16S rRNA amplicon sequencing was used to investigate the effects of breed and ruminal fraction on bacterial and archaeal communities in sheep. Solid, liquid and epithelial rumen samples were obtained from a total of 36 lambs, across 4 different sheep breeds (Cheviot (n=10), Connemara (n=6), Lanark (n=10) and Perth (n=10)), undergoing detailed measurements of feed efficiency – who were offered a nut based cereal *ad-libitum* supplemented with grass silage. Our results demonstrate that the feed conversion ratio (FCR) was lowest for the Cheviot (most efficient), and highest for the Connemara breed (least efficient). In the solid fraction, bacterial community richness was lowest in the Cheviot breed, while *Sharpea azabuensis* was most abundant in the Perth breed. Lanark, Cheviot and Perth breeds exhibited a significantly higher abundance of epithelial associated *Succiniclasticum* compared to the Connemara breed. When comparing ruminal fractions, *Campylobacter*, Family XIII, *Mogibacterium*, and Lachnospiraceae *UCG-008* were most abundant in the epithelial fraction. Our findings indicate that breed can impact the abundance of specific bacterial taxa in sheep while having little effect on the overall composition of the microbial community. This finding has implications for genetic selection breeding programs

aimed at improving feed conversion efficiency of sheep. Furthermore, the variations in the distribution of bacterial species identified between ruminal fractions, notably between solid and epithelial fractions, reveals a rumen fraction bias, which has implications for sheep rumen sampling techniques.

Introduction

Ruminant livestock contribute significantly to food security by converting human indigestible plant matter, into high quality sources of dairy and meat proteins, for human consumption (Oltjen and Beckett, 1996). Sustainable supply of animal derived protein over the next decades will be key to meeting the nutritional requirements of an estimated nine billion people by 2050 (Henchion et al., 2017). However, livestock production systems are also a major source of anthropogenic greenhouse gas emissions with enteric fermentation estimated to contribute to 35-40% of global methane emissions (Dopelt et al., 2019). As a result, there is an urgent need to increase animal protein production to fulfil nutritional demand while simultaneously improving the livestock industry's environmental sustainability metrics. Increasing the feed conversion efficiency of livestock is proposed as a mitigation solution for the livestock industry, as more feed efficient ruminants emit less methane than their less efficient counterparts (Hegarty et al., 2007;Fitzsimons et al., 2013;Beauchemin et al., 2020). In addition, improvements to feed efficiency are likely to benefit farm profitability (Kenny et al., 2018) while reducing the quantity of global land dedicated to producing feed for the livestock industry (van Zanten et al., 2016).

Mountain or hill sheep production is a significant agricultural enterprise that provides social and economic health in rural areas across the globe, while also protecting natural habitats and promoting biodiversity (Byrne et al., 2017). In Ireland and the UK, popular hill sheep breeds include the Scottish Blackface (SB) and the Cheviot. SB are mountain breeds which display adaptive tolerance to harsh environmental conditions and challenging terrains with low-energy vegetation (Carlyle, 1979). The wide distribution of SB breeds across the UK and Ireland has led to evolutionary changes within the breed, influenced largely by environmental pressures between different habitats (Carlyle, 1979). As a result, a range of different strains of SB breed exist today including the Lanark, Perth and Connemara breed types which all vary in body and wool composition. The Cheviot breed is also well-suited to highland farming, and while not as resilient as the SB (Blaxter et al., 1966), they are slightly larger and produce lambs that mature quickly (Kirton et al., 1995).

Sheep, like all ruminants, rely on a complex and dynamic microbial ecosystem (anaerobic bacteria, archaea, fungi and protozoa) within their rumen to derive energy from feed (Huws et al., 2018). The rumen is composed of three environmental niches, namely the solid-, liquid-, and epithelial-fractions (Ji et al., 2017;Li et al., 2020;Ren et al., 2020). The solid fraction, comprised of ingested feed, is primarily colonised by feed adherent microbes that breakdown fibrous matter (Li et al., 2020). The liquid fraction consists of the fluid within the rumen and provides an environment for free living microbes involved in the metabolism of soluble nutrients (McGovern et al., 2018). Finally, the epithelial fraction refers

to the epithelial lining of the rumen, which harbours microbes active in tissue recycling (Dinsdale et al., 1980), oxygen scavenging (Cheng et al., 1979), and urea hydrolysis (Cheng and Wallace, 1979) and is critically important for bioconversions and nutrient uptake as the cellular interface with the host animal. Previous research in both bovine and ovine models have reported differences in the microbial taxonomic profiles between ruminal fractions (Li et al., 2020;Ren et al., 2020) with the epithelial being mostly distinct from the solid and liquid fractions, whereas the solid and liquid fractions tend to more similar (McGovern et al., 2018;McLoughlin et al., 2020). To date, most studies investigating breed effects have been conducted using the rumen digesta samples and have not been explored using the epithelial fraction.

Previous research has revealed links between the rumen microbiota, feed efficiency and methane emissions in both cattle and sheep, with differences in microbial diversity and abundances between divergent animal cohorts (Myer et al., 2015;Shabat et al., 2016;Ellison et al., 2017;McLoughlin et al., 2020). Understanding factors that influence the composition and diversity of the rumen microbiome is critical for improving strategies to enhance feed efficiency and reduce ruminant methane emissions. Recently, studies in cattle have shown that microbial taxonomic profiles differ between breeds (Li et al., 2019a;Noel et al., 2019), suggesting that host genetics may regulate the composition of the rumen microbiome. However, such effects have not been explored in sheep.

It is unclear whether breed specific findings in cattle can be translated to sheep. Taxa-specific research is imperative given the importance of the global sheep industry from environmental, economic and social perspectives. In addition, while cattle studies have provided some indication that breed plays an important role in shaping the rumen microbiome, to date, such effects have not been investigated across all three ruminal fractions. Hence, the objectives of the current study are twofold. Firstly, to investigate the effect of breed on bacterial and archaeal populations in the solid, liquid and epithelial rumen fractions of sheep, and secondly to investigate the effect of the ruminal fraction on the microbial populations in each of the breeds, using 16S rRNA amplicon sequencing.

Methods

Animal model

Teagasc Animal Ethics Committee authorised all treatments involving animals in this investigation, which was conducted under experimental licence (No:P19132/P028) from Ireland’s Health Product Regulatory Authority (HPRA) in compliance with ARRIVE guidelines and the European Union protection of animals used for scientific purposes regulations 2012 (S.I. No 543 of 2012).

Over a 3-month period, data was collected on 36 ram lambs enrolled in a feed efficiency measurement test. Lambs included in this study originated from four different breeds: Cheviot (n=10), Connemara (n=6), Lanark (n=10) and Perth (n=10). After weaning, lambs were individually penned on plastic slat-floored feeding pens (182cm L x 122cm W). Lambs were allowed tactile, olfactory, and

visual contact with each other through the pen partitions. The mean body weight of animals at the beginning of the measurement period was 29.6 kg (SD=3.7). Throughout the trial period, all lambs were offered a cereal-based nut *ad libitum*, with fresh concentrates supplied daily and refusals removed (i.e., troughs emptied and cleaned) weekly. Concentrates were weighed daily in the morning, and daily intake was estimated by subtracting the weekly total intake from the number of refusals and dividing by seven. Concentrates were supplemented with unrestricted access to perennial rye-grass silage (*Lolium perenne*) to maintain rumen health (100-g/d DM). Silage was offered fresh daily and refusals removed twice weekly during morning feeding. At no point were animals without access to concentrates or silage during the *ad libitum* feeding period. Silage intake was not measured as consumption was low. Table 3.1 contains the ingredients and chemical composition of concentrate and silage used in the study. At all times throughout the measurement period lambs had access to fresh drinking water. The feed intake measurement period ceased when lambs reached a target slaughter weight of >40kg. Lambs were slaughtered at the Kepak Ltd abattoir in Athleague in Co. Roscommon on three separate dates when lamb maturity was reached; 29th November 2017, 13th December 2017 and 17th January 2018. The abattoir was approximately 56km (55min) from the Teagasc research farm in Athenry, Co. Galway, Ireland. Prior to slaughter, feed and water (at the farm) were withheld for 2 hours for all sheep in the study, since differences in time off feed may have affected rumen microbial community composition.

Phenotypic data collected throughout the trial period included the animals weight at the beginning of the trial period (Start weight); dry matter intake (DMI), described as the amount of feed (kg) the lambs consumed; average daily gain (ADG) was calculated by dividing the total weight gain over the trial period divided by the number of days animals were on trial before slaughter; feed conversion ratio (FCR) was calculated by dividing DMI by ADG. Live weight (LW) was the weight of lambs before slaughter. LW gain was the difference in weight at the beginning and end of the trial period. Carcass weight refers to the weight of the carcass after the offal has been removed following slaughter. KO% refers to the weight of the carcass as a percentage of the animal's live weight prior to slaughter.

Sample collection

Samples of ruminal fractions were collected immediately after slaughter. Rumen fluid and solid fractions were separated into 25ml tubes, by filtering rumen digesta through four layers of sterile cheesecloth. To collect rumen epithelial samples, papillae were cut from dorsal, ventral, cariad and caudal regions of the rumen wall using sterilised scissors, approx. 1cm^2, and subsequently rinsed with cold sterile saline solution (0.9% w/v NaCl). Samples from all three ruminal fractions were frozen immediately in liquid nitrogen after separation and then stored at −80°C. A total of 90 samples were available for the current study, 28 epithelial samples (Cheviot n=9, Connemara n=3, Lanark n=7, Perth n=9), 30

liquid (Cheviot n=9, Connemara n=5, Lanark n=9, Perth n=7), and 32 solid ruminal samples (Cheviot n=8, Connemara n=6, Lanark n=9, Perth n=9).

Rumen microbial DNA extraction and library preparation

Under liquid nitrogen, each sample was homogenised to a fine frozen powder using a pestle and mortar. Extraction of microbial DNA from the samples was performed using the method described by Yu and Morrison (Yu and Morrison, 2004). Amplicon libraries were created from 25ng of rumen microbial DNA using two rounds of PCR amplification as described in the Illumina Miseq 16S Sample Preparation Guide, with minor alterations to cycle length as described by McGovern et al. (2018) (McGovern et al., 2018). 515F/806R primers (Caporaso et al., 2011), built with Nextera over hang adapters, and 2x KAPA Hifi HotStart ReadyMix DNA polymerase were used for the first round of PCR amplification, targeting the V4 hyper-variable region of the 16S rDNA (Roche Diagnositics, West Sussex, UK). The first round of PCR was performed at 95°C for 3 minutes, then 20 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes. To enable the attachment of dual indices and Illumina sequencing adapters using the Nextera XT indexing kit, a second round of PCR was conducted at 95°C for 3 minutes, followed by 8 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes (Illumina, San Diego, CA, USA). Following PCR rounds 1 and 2, the amplicons were purified using the Qiaquick PCR Purification Kit (Qiagen, Manchester, UK). To remove adaptor primers, amplicons were pooled together in identical concentrations and gel purified using the Qiagen Gel Extraction Kit (Qiagen, Manchester, UK). Using the QIAquick PCR purification kit, the amplicons were again purified to eliminate any agarose residues (Qiagen, Manchester, UK). Amplicon purity was measured using the Nanodrop 1000, followed by confirmation using the Qubit fluorometer and the KAPA SYBR FAST universal kit with Illumina Primer Premix (Roche Diagnositics, West Sussex, UK). Amplicon libraries were diluted and denatured according to the Illumina Miseq 16S Sample Preparation Guide, and sequencing was performed on the Illumina MiSeq using the 500 cycle version 2 MiSeq reagent kit (Illumina, San Diego, CA, USA).

Bioinformatics

Amplicon reads were quality assessed using FASTQC (version 0.11.5) (Andrews, 2017). Adapters and ambiguous basecalls were subsequently removed using Cutadapt (version 1.18)(Martin, 2011). The amplicon reads were processed and analyzed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (DADA2) (version 1.18.0), as described in Callahan et al. (2016) (Callahan et al., 2016). The DADA2 tutorial available at https://benjjneb.github.io/dada2/tutorial.html (version 1.12) was followed for read filtering, dereplication, sample inference, chimera elimination, paired end read merging, and taxonomy categorization. Taxonomic classification was performed to the species level using the SILVA classification databases (version 132) (Callahan, 2018). The final output from DADA2 was an Amplicon Sequence Variant (ASV) table and a corresponding taxonomy table. A phylogenetic tree was created using the R package Phangorn (Schliep, 2011). Prior to downstream analysis, a Phyloseq object including the

ASV table, taxonomy table, phylogenetic tree, and experimental metadata was created using the R/Bioconductor package Phyloseq (version 1.26) (McMURDIE and Holmes, 2012). Six samples (2 solid, 2 liquid and 2 epithelial) were removed prior to downstream analysis as a result of having a substantially reduced number of reads (<100 reads).

Compositional and statistical analysis

Animal production trait data were first verified for normality and homogeneity using the Shapiro Wilks and Levenes tests, respectively, and then compared among breeds using a two-way ANOVA followed by a *post-hoc* Tukey HSD. DMI records were missing from one animal in each of the Connemara, Perth and Lanark breeds, as a result those animals were omitted from DMI and FCR comparisons.

To profile dominant bacterial and archaea taxa, raw counts were converted to relative abundances and the mean and standard deviation relative abundance of dominant phyla and genera were reported. To examine the effect of breed on bacterial and archaeal populations the data was first stratified according to ruminal fraction (i.e. solid, liquid and epithelial) and then compared between breeds (i.e Cheviot, Connemara, Lanark and Perth). Similarly, to examine the effect of ruminal fraction on microbial profiles data was stratified according to breed and compared between fractions. For fraction analysis only animals where all three ruminal fraction were available were considered. Due to low numbers of biological replicates the Connemara breed was excluded from ruminal fractions analysis. Prior to diversity analysis raw counts were normalised to even sampling depth using the scaling with ranked subsampling (SRS) method (Beule and Karlovsky, 2020) and rarefaction curves were generated to assess sequencing effort. Following assessment of rarefaction curves, sample 2083-Solid, which had a sequencing depth of 13,922, was removed due to a loss in community diversity when rarefying. Shannon, inverse Simpson, Faiths phylogenetic diversity (PD), and observed ASVs (richness) diversity indices were used to generate within-sample (alpha) diversity metrics. Alpha diversity data were checked for normality and homogeneity using Shapiro Wilks test and Levenes test prior to statistical analysis. A two-way ANOVA was used to test the null hypothesis that no difference on mean alpha diversity measures existed between groups. For beta diversity analysis, dissimilarities in community composition were measured using both weighted and unweighted UniFrac distances and visualised using principle coordinate analysis (PcoA). Differences in community composition was tested with PERMANOVA and conducted with 9999 permutations using the Adonis function from the R/Bioconductor package Vegan (version 2.5-5) (Dixon, 2003). Differential abundance (DA) analysis was conducted using both the likelihood ratio test (LRT) and the Wald's test from the DESeq2 package (Love et al., 2014). Only taxa with a relative abundance larger than 0.01 percent and a prevalence greater than 50 percent were considered for DA analysis. An *a priori* q-value threshold was set at 0.05. The date of sample collection was included as a covariate to adjust for variations in abundance associated with different slaughter dates. Lastly, Spearman correlation coefficients ($P < 0.05$) were done to

assess the correlation between bacterial and archaeal genera and animal production traits; FCR and ADG, in order to identify potential feed efficiency drivers. Only genera with a relative abundance larger than 0.01 percent and a prevalence greater than 50 percent were considered for correlation analysis.

Results

Breed differences in animal feed conversion and economic trait performance

Throughout the feed intake measurement period, summary statistics shows animals on test had an average DMI of 1.113Kg/d (SD=0.18), ADG of 0.27Kg/d (SD=0.1), FCR of 4.04 Kg of DMI/ Kg of ADG (SD=0.1), start weight of 29.6Kg (SD=3.7), final live weight of 46.0 Kg (SD=2.9), carcass weight of 20.2 Kg (SD=1.6), and a KO% of 44.1% (SD=3.3). ADG (P=0.005), FCR (P=0.035), CW (P<0.04) and start weight (P<0.036) were all significantly affected by breed. Summary statistics, along with comparisons amongst breeds for animal performance, feed intake and feed efficiency are displayed in Table 3.2. In summary, the Cheviot breed had the lowest FCR and the highest ADG, carcass weight, and start weight among all breeds, with differences in ADG and FCR being significant when compared to the Connemara breed and differences in carcass and start weight being significant when compared to the Lanark breed. In addition, the Cheviot breed had the fastest maturing lambs with 80% of lambs reaching maturity within the first 42 days (data not shown) and a mean LW of 47.1kg (Table 3.2).

Overall microbial community structure

After data processing, filtering, and removal of chimeras and lowly sequenced samples a total of 5,411,353 reads remained, with an average of 91.3% of reads surviving. The average number of reads per sample was 64,420, which mapped to 2547 ASVs. After removal of taxa unassigned at the phylum level 2434 ASV's remained. Analysis of the ASVs across all samples revealed that bacteria and archaea represented 95.4 and 4.6% of the microbial population, respectively. A total of 19 bacterial taxa were classified at the phylum level, with Firmicutes being the most abundant (45.8%), followed by Bacteroidetes (33.4%) and Proteobacteria (8.0%). There were 192 taxa classified at the genus level, with *Prevotella_1* (13.1%) and *Prevotella_7* (13.1%) being the most dominant followed by *Succinivibrio* (6.3%). Methanobrevibacter was shown to be the most abundant archaeal genus. (78.1%). In this study, no non-methanogenic archaeal taxa were identified.

Breed effects on bacterial and archaeal populations in the solid ruminal fraction

In the solid ruminal fraction, a total of 1706 bacterial ASVs agglomerated to 227 genera, 89 families, 51 orders, 27 classes and 16 phyla. Firmicutes (48.2%) Bacteroidetes (30.1%), Fibrobacterota (6.1%) were the three most abundant bacteria phyla (Figure 1). *Prevotella_7* (9.7%), *Prevotella_1* (9.2%), unclassified Lachnospiraceae (7.6%), *Fibrobacter* (6.1%) and *Ruminococcus_1* (5.6%) were

the 5 most dominant bacteria genera (Figure 2). A total of 27 archaeal ASVs were identified and agglomerated to 4 genera (*Methanobrevibacter*, *Methanosphera*, *Methanimicrococcus* and *Candidatus methanomethylophilus*), 3 families, 3 orders 3 classes and 1 phylum. *Methanobrevibacter* was the most dominant archaeal genus (72.9%).

Alpha diversity analysis revealed that breed had an effect on solid associated bacterial and archaeal community richness and bacteria community PD (ANOVA, $P<0.05$) (Table 3.3). Such differences were observed between the Cheviot and Lanark breeds, with the Cheviot exhibiting the least and the Lanark exhibiting the most rumen microbial diversity among the breeds. Based on weighted and unweighted UniFrac distances, beta diversity analysis showed no differences in overall community composition across the breeds (PERMANOVA, $P>0.05$) for either bacterial or archaeal communities (Table 3.4).

The abundance of *Sharpea* at the genus level, *Sharpea azabuensis* at the species level and an unclassified ASV (ASV37) belonging to the family Lachnospiraceae were affected by breed (LRT, *P.adj*<0.05) (Table 3.5). *Sharpea* (Wald, *P.*adj<0.001; Log2FC=4.37) and *Sharpea azabuensis* (Wald, *P.adj*<0.001; Log2FC=4.58) were higher in Perth compared to Cheviot. ASV37 (family Lachnospiraceae) was more abundant in Cheviot (Wald, *P.adj*<0.01; Log2FC=3.22) and Perth (Wald, *P.adj*<0.001; Log2FC=3.4) compared to Lanark (Table 3.5). Pairwise analysis between each of the breeds revealed a further 2 bacterial ASVs as differentially abundant. ASV48 classified to the genus *Prevotella_9* was higher in Lanark compared to Perth (Wald, *P.adj*<0.0001; Log2FC=7.88) and Cheviot (Wald, *P.adj*<0.01; Log2FC=9.11), and ASV329 classified to the genus *Pyramidobacter* was higher in Lanark compared to Cheviot (Wald, *P.adj*<0.05; Log2FC=5.52). At the genus level *P-2534-18B5_gut group* (ASV17), belonging to phylum Bacteroidetes, was higher in the Perth (Wald, *P.adj*<0.05; Log2FC=5.78) and Lanark (Wald, *P.adj*<0.05; Log2FC=6.82) breeds compared to Cheviot, and *Candidatus Saccharimonas* was higher in the Lanark compared to the Cheviot (Wald, *P.adj*<0.05; Log2FC=5.66). Similarly, at the family level P-2534-18B5_gut group (ASV17) was higher in the Perth (Wald, *P.adj*<0.01; Log2FC=5.88) and Lanark (Wald, *P.adj*<0.01; Log2FC=6.80) breeds compared to Cheviot, and Saccharimonadaceae (ASV317) was higher in the Lanark (Wald, *P.adj*<0.01; Log2FC=5.48) and Connemara (Wald, *P.adj*<0.05; Log2FC=6.89) breeds compared to the Cheviot. At the order level Coriobacteriales was higher in the Lanark compared to the Perth (Wald, *P.adj*<0.05; Log2FC=1.17) (Table 3.6). One archaea ASV belonging to the genus *Candidatus Methanomethylophilus* (ASV337) was higher in the Perth (Wald, *P.adj*<0.01; Log2FC=3.12) and Lanark (Wald, *P.adj*<0.05; Log2FC=3.21) compared to Cheviot (Table 3.6).

Breed effects on bacterial and archaeal populations in the liquid ruminal fraction

For the liquid ruminal fraction, a total of 1790 bacteria ASVs agglomerated to 236 genera, 95 families, 57 orders, 29 classes and 17 phyla. Firmicutes (43.1%), Bacteroidetes (37.1%), and Proteobacteria (8.9%) were the most dominant phyla

(Figure 1). *Prevotella 7* (12.2%), *Prevotella 1* (11.7%), unclassified Lachnospiraceae (6.2%), *Succinivibrio* (5.8%) and *Succiniclasticum* (4.4%) were the 5 most dominant genera (Figure 3.2). 26 archaea ASVs were available for analysis, which agglomerated to 4 genera (*Methanobrevibacter*, *Methanosphera,* unclassified Methanomethylophilaceae and *Candidatus Methanomethylophilus*), 2 families, 2 orders 2 classes and 1 phylum. Methanobrevibacter was the most dominant genus (78.9%).

Although there was no effect of breed on alpha diversity indices for bacteria communities (ANOVA, $P>0.05$), breed did have an impact on the richness of archaeal communities. (ANOVA, $P<0.05$) (Table 3.2). The Lanark breed had the highest level of archaeal community richness, whereas the Cheviot breed had the lowest level (Table 3.6). Based on weighted and unweighted UniFrac distances, the analysis of beta diversity showed no differences in overall community composition among breeds (PERMANOVA, $P>0.05$) for either bacterial or archaeal communities (Table 3.4).

The likelihood ratio test detected no breed effect (LRT, $P.adj>0.05$) on the abundance of bacterial or archaeal taxa across all taxonomic ranks. Pairwise analysis between each of the breeds revealed 5 taxa at the ASV level as differentially abundant. Two ASVs, ASV23 (Wald, $P.adj<0.01$; Log2FC=5.94) and ASV43 (Wald, $P.adj<0.05$; Log2FC=2.51) classified to the family Muribaculaceae were higher in Cheviot compared to Perth, ASV44 classified to the genus *Acetitomaculum* was higher in Cheviot (Wald, $P.adj<0.05$; Log2FC=10.26) compared to Connemara, ASV55 classified as *Sharpea azabuensis* was higher in Perth (Wald, $P.adj<0.01$; Log2FC=4.81) compared to Cheviot, and ASV20 classified to Lachnospiraceae *NK3A20 group* was higher in the Lanark (Wald, $P.adj<0.05$; Log2FC=3.06) compared to Connemara. At the genus level *Sharpea* (Wald, $P.adj<0.001$; Log2FC=5.05) was higher in the Perth compared to the Cheviot, ASV223 classified to order Rhodospirillales (Wald, $P.adj<0.01$; Log2FC=7.03) was higher in the Cheviot compared to the Perth, and ASV461 classified to Clostridiales_vadinBB60 group was higher in the Lanark (Wald, $P.adj<0.05$; Log2FC=5.86) compared to the Connemara. At the family level Muribaculaceae (Wald, $P.adj<0.05$; Log2FC=2.23) was higher in Cheviot compared to Perth, ASV223 classified to order Rhodospirillales was higher in the Cheviot compared to the Connemara (Wald, $P.adj<0.05$; Log2FC=7.11) and Perth (Wald, $P.adj<0.01$; Log2FC=6.81), ASV461 classified to Clostridiales_vadinBB60 group was higher in the Cheviot (Wald, $P.adj<0.05$; Log2FC=5.82) and Lanark (Wald, $P.adj<0.01$; Log2FC=6.09) compared to the Connemara, and ASV17 classified to P-2534-18B5_gut group was higher in the Perth (Wald, $P.adj<0.05$; Log2FC=4.75) and Lanark (Wald, $P.adj<0.05$; Log2FC=5.87) compared to the Cheviot. At the order level Rhodospirillales was higher in the Cheviot compared to the Connemara (Wald, $P.adj<0.05$; Log2FC=7.29) and Perth (Wald, $P.adj<0.01$; Log2FC=6.93), and Betaproteobacteriales was higher in the Cheviot compared to the Connemara (Wald, $P.adj<0.05$; Log2FC=3.03). At the class level the abundance of Alphaproteobacteria was higher in the Cheviot compared to the Perth (Wald, $P.adj<0.01$; Log2FC=7.11) and Connemara (Wald, $P.adj<0.05$; Log2FC=7.52). Finally at the Phylum level the abundance of Proteobacteria was

higher in the Perth compared to the Connemara (Wald, *P.adj*<0.05; Log2FC=2.62) (Table 3.6).

Breed effects on bacterial and archaeal populations in the epithelial ruminal fraction

In the epithelial ruminal fraction, a total of 1891 bacteria ASVs agglomerated to 231 genera, 89 families, 52 orders, 29 classes and 17 phyla. Firmicutes (46.3%), Bacteroidetes (33.4%), and Proteobacteria (10.1%) were the most dominant phyla (Figure 1). *Prevotella 1* (10.0%), *Prevotella 7* (8.9%), *Succinvibrio* (5.9%), unclassified Lachnospiraceae (5.7%), and *Ruminococcus 2* (5.0%) were the 5 most dominant genera (Figure 2). 28 archaeal ASVs were available for analysis, which agglomerated to 5 genera (*Methanobrevibacter*, *Methanosphera*, *Methanimicrococcus,* unclassified Methanomethylophilaceae and *Candidatus Methanomethylophilus*), 3 families, 3 orders 3 classes and 1 phylum. *Methanobrevibacter* was the most dominant genus (82.0%).

Alpha diversity analysis revealed that while breed had no effect on epithelial associated archaeal community indices, it had a significant effect on bacteria community richness and inverse Simpson diversity (ANOVA, *P*<0.05) (Table 3.3). Beta diversity analysis based on weighted and unweighted UniFrac distances, found no differences in community composition among the breeds (PERMANOVA, *P*>0.05), for either bacterial or archaeal communities (Table 3.4).

The abundance of Family XIII at the family level and an unclassified ASV (ASV379) belonging to Family XIII at the ASV level were affected by breed (LRT, *P.adj*<0.01) (Table 3.5). Family XIII was higher in Lanark compared to Cheviot (Wald, *P.adj*<0.05; Log2FC=1.41) and Perth (Wald, *P.adj*<0.05; Log2FC=1.13), and ASV379, belonging to Family XIII, was higher in the Lanark breed (Wald, *P.adj*<0.05; Log2FC=2.88) when compared to Perth breed (Table 3.6). Pairwise analysis revealed a further 7 bacterial ASVs as differentially abundant. ASV37 classified to the Lachnospiraceae family was higher in the Perth (Wald, *P.adj*<0.05; Log2FC=2.72) compared to the Lanark, ASV123 classified to the genus *Prevotella_1* was higher in the Connemara (Wald, *P.adj*<0.01; Log2FC=7.85) compared to the Lanark, ASV633 classified to the genus Ruminococcacese *UCG-010* was higher in the Perth (Wald, *P.adj*<0.05; Log2FC=4.0) compared to the Lanark, ASV24 classified to the genus *Succiniclasticum* was lower in the Connemara compared to Cheviot (Wald, *P.adj*<0.0001; Log2FC=24.14), Lanark (Wald, P.adj<0.0001; Log2FC=23.67) and Perth (Wald, P.adj<0.0001; Log2FC=25.98) breeds, ASV74 classified to the genus *Syntrophococcus* was higher in the Lanark breed compared to Connemara (Wald, *P.adj*<0.05; Log2FC=5.42), ASV33 classified to the genus *Ruminococcus_1* was higher in Perth (Wald, *P.adj*<0.05; Log2FC=4.81) and Connemara (Wald, *P.adj*<0.01; Log2FC=7.80) compared to Cheviot, and ASV118 also classified to the genus *Ruminococcus_1* was higher in Perth (Wald, *P.adj*<0.05; Log2FC=5.52) compared to Lanark. At the genus level, *Sharpea* was higher in the Perth compared to the Cheviot (Wald, *P.adj*<0.05; Log2FC=3.13), ASV361 classified to Family XIII *AD3011 group* was higher in the Lanark (Wald, *P.adj*<0.01; Log2FC=2.28) when compared to the Cheviot, while ASV69 classified to

Ruminococcaceae *UCG-014* (Wald, *P.adj*<0.05; Log2FC=2.77) and ASV406 classified to Family XIII *UCG-001* (Wald, *P.adj*<0.05; Log2FC=3.10) were both higher in the Cheviot when compared to the Lanark. At the family level Atopobiaceae was higher in the Lanark (Wald, *P.adj*<0.05; Log2FC=1.96) compared to Perth, and Synergistaceae was higher in the Lanark compared to the Cheviot (Wald, *P.adj*<0.05; Log2FC=2.19) (Table 3.6).

Effect of ruminal fraction on bacterial and archaeal populations across breeds

Bacterial and archaeal populations across ruminal fractions were investigated for Cheviot, Lanark and Perth breeds, and only included animals where all three ruminal fractions were available. Firmicutes was the most abundant phylum in the Cheviot (mean, solid=51%, liquid=41%, epithelial=45%), Lanark (mean, solid=44%, liquid=39%, epithelial=43%) and Perth (mean, solid=49%, liquid=46%, epithelial=49%) breeds (Figure 3). In the epithelial fraction *Prevotella*_7 was the most abundant genus in the Cheviot (10.6%) and Lanark (8.3%) breeds, while *Prevotella_1* (10.0%) was the most dominant genus for the Perth breed. In the liquid fraction *Prevotella_1* was the most dominant in the Cheviot (12.1%) breed, while *Prevotella_7* was most dominant in Lanark (11.1%) and Perth (13.1%) breeds. In the solid ruminal fraction unclassified Lachnospiracheae, *Prevotella_1* and *Prevotella_7* were most abundant in the Cheviot (9.9%), Lanark (10.5%) and Perth (11.1%) breeds, respectively (Figure 3.4).

For the Cheviot breed, bacterial community alpha diversity measures were not affected by ruminal fraction (ANOVA, *P*>0.1). For the Lanark breed, bacterial community Shannon diversity was affected by ruminal fraction (ANOVA, *P*<0.05). For the Perth breed, bacterial community richness (observed ASV) and phylogenetic diversity (PD) were affected by ruminal fraction (ANOVA, *P*<0.05), with the rumen epithelial fraction exhibiting greater diversity than solid and liquid ruminal fractions (Table 3.7). For all three breeds, archaeal community alpha diversity measures were not affected by ruminal fraction (ANOVA, *P*>0.1). Beta diversity analysis showed that bacterial and archaeal community composition were also unaffected by ruminal fraction for all breeds analysed (PERMANOVA, *P*>0.1) (Table 3.8).

Overall, ruminal fraction influenced 36 taxonomic groups across all ranks, representing 19 distinct ASVs, in the three breeds studied (LRT, *P*<0.05). Ruminal fraction influenced the abundance of 18 taxa (11 distinct ASVs) in the Lanark breed, the most of any of breeds studied. ASV141, classified to the phylum Epsilonbacteraeota and the genus *Campylobacter,* was affected by ruminal fraction (LRT, *P*<0.05) at all taxonomic ranks (i.e phylum to ASV) and found to be significantly more abundant in the epithelial fraction when compared to the solid fraction. The abundance of ASV449, classified to the genus *Desulfobulbus,* was affected by ruminal fraction at the order, family and genus taxonomic ranks (LRT, *P.adj*<0.05), found to be significantly more abundan in the epithelial ruminal fraction. At the genus level the abundance of *Butyrivibrio 2*, *Fretibacterium*, *Howardella,* and an unclassified ASV (ASV219) belonging to

family Neisseriaceae were all affected by ruminal fraction (LRT, *P*<0.05) and significantly higher in the epithelial fraction. Conversely, the abundance of *Shutterella* and two unclassified ASVs belonging to families Family XII *UCG-001* and Eggerthellaceae were highest in the solid ruminal fraction (Wald, *P.adj*<0.05). At the ASV level, the abundance of 2 unclassified ASVs; ASV210 and ASV239, belonging to genus *Mogibacterium* and family Family XIII were affected by ruminal fraction (LRT, *P*<0.05) and highest in the epithelial ruminal fraction (Wald, *P*<0.05). In the Cheviot breed, the abundance of 15 taxa (8 unique ASVs) were affected by ruminal fraction. ASV141 (*Campylobacter*) from taxonomic ranks phylum to genus and ASV449 (*Desulfobulbus*) from order to genus were differentially abundant and significantly more abundant in the epithelial ruminal fraction (Wald, *P*<0.05). At the family level, Neisseriaceae and an unclassified ASV, ASV198, belonging to the order Coriobacteriales, were affected by ruminal fraction, with the epithelial and solid ruminal fractions, respectively, containing a higher proportion of these bacteria. At the genus level, the abundance of *Mogibacterium*, *Butyrivibro 2* and two unclassified ASVs, ASV142 (F_Erysipelotrichaceae_UCG-004) and ASV263 (F_Burkholderiaceae), were affected by ruminal fraction (LRT, *P*<0.05), with highest abundances observed in the epithelial ruminal fraction. In the Perth breed, the abundance of the bacterial phylum Tenericutes and an unclassified archaeal genus, ASV475, belonging to the family Methanomethylophilaceae were impacted by ruminal fraction (LRT, P0.05) (Table 3.9) Taken together, the majority of differences in microbial abundance were observed between the solid and epithelial ruminal fractions, as shown in Table 10, which summarises all the results of pairwise analysis between fractions.

Bacterial and archaeal genera associated with FCR and ADG

We performed a Spearman correlation analysis between the relative abundance of genera and animal production traits; FCR and ADG to find potential drivers of feed efficiency in the solid, liquid and epithelial fractions. After adjusting for repeated hypotheses testing, no genera were determined to be statistically significant. Therefore, putative drivers of FCR and ADG were considered to have a (P<0.05). In the solid fraction, 4 bacterial genera showed significant negative correlations with FCR: *Succinivibrionaceae* (ρ=-4.1), *Lachnospira* (ρ=-3.9), *Syntrophococcus* (ρ=-3.8) and an unclassified genus (ASV9) belonging to the order Gastranaerophilales (ρ=-4.1). Ruminococcaceae *UCG-013* (ρ=-4.1) positively associated with ADG, while Lachnospiraceae *NK3A20 group* (ρ=-3.8) was negatively correlated with ADG. In the liquid ruminal fraction the genus *Acetitomaculum* (ρ=-3.8) an unclassified ASV belonging to the order Gastranaerophilales (ρ=-4.4) and the archaeal genus *Candidatus Methanomethylophilus* (ρ=-3.8) negatively correlated with FCR. Prevotella 9 (ρ=3.8), Roseburia (ρ=4.9), and 5 unclassified genera belonging to the families Ruminococcaceae -*UCG-013* (ρ=4.5), -*UCG-002* (ρ=4.4), - *UCG-014* (ρ=4.1), -*UCG-010* (ρ=4.1), and Lachnospiraceae (ρ=3.9), and an unclassified genus belonging to order Mollicutes (ρ=4.9) positively associated with ADG. In the epithelial fraction we observed no significant associations with FCR. *Prevotella 9* (ρ=4.8), 4 unclassified genera belonging to the families Ruminococcaceae -*UCG-*

013 (ρ=4.9), -*UCG-009* (ρ=4.7), - *UCG-014* (ρ=4.1) and Lachnospiraceae (ρ=4.5), an unclassified genus belonging to order Mollicutes_RF39 (ρ=4.6) and the archaeal genus *Methanosphera* (ρ=4.6) positively associated with ADG. *Mogibacterium* (ρ=-4.1) and 2 unclassified genera belonging to the families Prevotellaceae (ρ=-4.3) and Christensenellaceae (ρ=4.3) negatively associated with ADG (Table 3.11).

Discussion

The effects of breed genotype on shaping the composition and diversity of the rumen microbiota in hill sheep are unknown. However, recent studies in cattle have demonstrated that microbial taxonomic profiles vary between breeds, where the abundance of particular microbial species are regulated by host genetics (Li et al., 2019b). Given that the rumen comprises of 3 interconnecting microbial ecosystems; solid-, liquid- and epithelial ruminal fractions, we investigated 1) the effect of sheep breed on bacterial and archaeal populations in all three ruminal fractions, and 2) the effect of ruminal fraction on those populations in three breeds of sheep (i.e Cheviot, Lanark and Perth). Our results provide the first report that diversity and abundance of bacterial and archaeal taxa in the solid, liquid and epithelial rumen fractions of sheep are influenced by breed. Our results expand and reinforce previous research in cattle showing differences in bacteria populations between breeds and ruminal fractions.

In the current study, breed was found to influence important production traits related to host feed efficiency, including FCR and ADG. Cheviot lambs were found to have the lowest mean FCR, indicating that it was the most feed efficient breed. However, the difference was only significant when compared to the Connemara breed, which had the highest mean FCR. Additionally, the Cheviot breed also had the fastest maturing lambs in the study, with 80% of lambs reaching maturity (>40kg) within the first 42 days of the study, with a mean LW of 47.1kg. Among the SB strains the Perth had the lowest FCR. No differences in FCR and ADG between the Cheviot, Lanark and Perth were found. Although this is the first study to compare FCR and ADG between these mountain/hill sheep breeds, the findings are in line with a previous study that found that metabolic differences between six British sheep breeds (i.e. SB, Welsh Mountain, Cheviot, Suffolk Down, Kent, and Hampshire Down) were mostly similar (Blaxter et al., 1966). However, when subjected to environmental stresses such as wind and rain, differences were apparent, with the SB found to more stress-tolerant than the Cheviot (Blaxter et al., 1966).

Metagenomic studies investigating host genetic effects on the rumen microbiota have to date been performed using original rumen digesta (Paz et al., 2016;Li et al., 2019a), which comprises both the liquid and solid ruminal fractions. The purpose of this study was to investigate the influence of sheep breed on bacteria associated with each of the three fractions independently. Our findings demonstrate that breed contributed to significant variations in alpha diversity (i.e. observed ASV’s and PD) in the solid, but not in the liquid, ruminal fraction. In the solid fraction the Cheviot breed harbored a bacterial community that was less rich and more phylogenetically related than those of the Scottish Blackface

strains, which was significant when compared to the Lanark breed. Previously, lower rumen microbial alpha diversity and richness were linked to higher feed efficiency in cattle (Shabat et al., 2016). An efficient rumen microbiome is considered to be less diverse and more specialised in metabolizing feed and delivering energy to the host (Shabat et al., 2016), which could be a factor influencing the greater FCR observed for the Cheviot breed in the current study. Our beta diversity analysis showed that bacterial communities associated with the liquid and solid fractions were not affected by breed, suggesting a large overlap of community representatives among breeds. Taken together our findings on bacteria diversity and composition contrast with an analogous study conducted in cattle (Li et al., 2019a). Li et al. (2019) reported no significant differences in alpha diversity among three breeds of cattle; while PcoA based on Bray Curtis distances revealed that the Kinsella Hybrid breed exhibited a distinct bacteria community composition to that of the Angus and Charlaois breeds used in the study (Li et al., 2019a). Conversely, an earlier study in cattle found both alpha and beta diversities differing between Holstein and Jersey cows (Paz et al., 2016). Variations in community composition and diversity may be attributed to differences of animal model, management practice, diet, environment, age or analytical approaches used. We consider that a combination of these factors might explain differences between the current study and those studies mentioned. Although no major differences in bacteria community composition were observed, the abundance of several taxonomic groups were affected by breed in the solid ruminal fraction: *Sharpea* at the genus level, and *Sharpea azabuensis* and an unclassified ASV belonging to the family *Lachnospiraceae* at the species level. The Perth breed exhibited highest abundance of *Sharpea azabuensis* which was significant in comparison to the Cheviot breed. *Sharpea azabuensis* is a strictly anaerobic gram-positive bacterium that can metabolise a variety of sugars including D-glucose, D-fructose, D-galactose and sucrose producing lactate as the primary end product (Kumar et al., 2018). Previous studies investigating the rumen microbiota of sheep divergent for methane emissions have reported an enrichment of *Sharpea azabuensis* in lower methane emitting cohorts (Kittelmann et al., 2014;Kamke et al., 2016). As a result, we suspect that the greater abundance of *Sharpea azabuensis* in the Perth breed may be suggestive of lower methane production, however, due to the lack of methane emissions data recorded in this study, this should be considered with caution. *Acetitomaculum* was identified as a dominant bacterial genus in the liquid ruminal fraction, with a mean relative abundance of 3.5%. Its abundance was found to be negatively correlated with FCR, indicating a potential role in enhancing host feed efficiency. Indeed, the abundance of an unclassified ASV within the genus (ASV44) was found to be higher in feed efficient Cheviots, which was significant when compared to the Connemara breed. *Acetitomaculum* has one known species *A. ruminis*, an acetogenic bacterium capable of heterotrophic and autotrophic growth (Le Van et al., 1998). It possible that its higher abundance may be contributing to Cheviot feed efficiency by shifting H_2 away from methanogenesis and towards acetogenesis, reducing dietary energy loss (Karekar et al., 2022). Alternatively, *Acetitomaculum* may be contributing to Cheviots improved FCR through metabolic pathways other than reductive

acetogenesis due to the organism's ability to metabolise a wide range of substrates and its inability to compete with methanogens for H_2, especially at low H_2 concentrations (Le Van et al., 1998).

Bacteria associated with the rumen epithelium maintain close interactions with the host and have been shown to correlate with ruminal epithelial tissue gene expression (Chen et al., 2012;Liu et al., 2021), suggesting that host genetics may influence this bacterial population more than those in the solid and liquid fractions. In the current study, sheep breed was found to significantly contribute to differences in the alpha diversity (i.e. observed ASVs and inverse Simpson), but not beta diversity of the epithelial-associated bacterial community. In the context of alpha diversity, the Cheviot breed harbored the fewest number of observable ASVs, while the Connemara harbored the most. Moreover, when compared to Connemara and Perth, the Cheviot and Lanark breeds had a significantly higher mean inverse Simpson index. This finding suggests that, while epithelial community richness was lowest for the Cheviot breed, the community was more uniformly distributed with respect to species abundance than those of the Connemara and Perth breeds. Firmicutes, Bacteroidetes, and Proteobacteria were the most predominant bacterial phyla, which is consistent with previous studies exploring epithelial bacterial communities in cattle (Wetzels et al., 2017;Anderson et al., 2021). ASV24 classified to the genus *Succiniclasticum* was shown to be more abundant in the epithelia of the Cheviot, Lanark, and Perth breeds when compared to the Connemara breed. *Succiniclasticum* is a gram-negative rod-shaped anaerobe that ferments succinate and converts it to propionate (Van Gylswyk, 1995), an important precursor of glucose in the rumen (Elliot, 1980). The higher abundance of *Succiniclasticum* in those breeds may have contributed to their enhanced FCR compared to the Connemara breed by supplying enough extra propionate to boost gluconeogenesis, which is important for animal growth and production (Young, 1977). The abundance of *Ruminococcus 1* was significantly higher in the Perth and Connemara breeds relative to the Cheviot breed. *Ruminococcus* spp. Are core members of the rumen microbiome (Wirth et al., 2018), and its association with the rumen epithelial could indicate that its abundance is under host genetic regulation. Indeed, previous research carried out by Li et al (2019) showed *Ruminococcus* was heritable in cattle ($h2 = 0.16 \pm 0.08$; mean ± SE), and variations in its abundance were associated with a single nucleotide polymorphism (SNP) in the RAPH1 gene(Li et al., 2019b). The genus comprises some of the most proficient and best described cellulolytic degraders, including *R. albus* and *R. flavefacians* (Yeoman et al., 2021). Consequently, it is probable that the Connemara and Perth breed are genetically selecting for a higher abundance of *Ruminococcus*, which may have allowed these SB breeds to evolve into successful mountain sheep able to thrive in poor grazing areas with low-energy vegetation.

Bacterial community profiles can differ across ruminal fractions in both bovine and ovine ruminants (Li et al., 2020;Ren et al., 2020). Therefore, we investigated the influence of ruminal fraction on bacterial populations in the Cheviot, Connemara, and Perth breeds individually. Our findings show that ruminal

fraction had no effect of alpha diversity measures in the Cheviot breed. However, in the Lanark breed the liquid fraction exhibited a significantly lower Shannon diversity, while in the Perth breed the epithelial fraction exhibited a significantly higher community richness and PD when compared to the other fractions, respectively. It is widely reported in the literature that the bacterial community composition of the epithelial fraction is distinct from those communities associated with rumen content (Cammack et al., 2018;Li et al., 2020;Ren et al., 2020). In contrast to prior studies[16,18], our beta diversity analysis revealed no significant differences in community composition across ruminal fractions for any of the breeds studied. The reason for this finding is unclear, though it could be related to variations in dietary management between studies (Petri et al., 2013;Zhang et al., 2017a;Zhang et al., 2017b). While no major compositional differences were seen in this study, ruminal fraction had a significant impact on the abundance of taxa commonly associated with the rumen epithelium, with the majority of differences occurring between the epithelial and solid fractions in all breeds. When considering the Lanark and Cheviot breeds, several taxa within the order Clostridiales (Family XII*, Butyrivibrio 2, Mogibacterium* and Lachnospiraceae *UCG-008*) were significantly more abundant in the epithelial ruminal fraction. Clostridiales are obligate anaerobes that have previously been discovered to interact with the rumen epithelium (Chen et al., 2011;Tan et al., 2021) and reported to be key components of its core microbiota (Petri et al., 2013). In addition to Clostridiales, *Campylobacter* was also found to be significantly abundant in the epithelial fraction of the Cheviot and Lanark breeds. *Campylobacter* is an asaccharolytic microaerophilic bacterium that has frequently been identified as associating with the rumen epithelium (Schären et al., 2017;Ren et al., 2020;Pacífico et al., 2021), and its capacity to consume oxygen demonstrates its functional significance in maintaining the rumen's anaerobic environment (Mann et al., 2018).

Archaea are the sole producers of methane within the rumen, which is an important homeostatic process that regulates the partial pressure of hydrogen (Moss et al., 2000). However, methanogenesis is estimated to result in a 2-12% loss in feed efficiency to the host (Johnson and Johnson, 1995), and is further supported by research that has revealed associations between ruminant methane emissions and host feed efficiency (Alemu et al., 2017). Moreover, the abundance of methanogenic archaea in the rumen has been linked to both methane emissions (Wallace et al., 2015) and feed efficiency (Zhou and Hernandez-Sanabria, 2010). To date, research in cattle has shown that archaea taxonomic abundances vary between breeds of cattle (Li et al., 2019a) and some archaeal species have been found to be heritable (Goodrich et al., 2016;Li et al., 2019b), signalling a potential host genetic effect on the community. Therefore, in the present study we explored the effect of sheep breed on archaeal populations associated with the solid, liquid and epithelial fractions. Alpha diversity analysis showed that breed had a significant effect on the richness of the solid and liquid associated communities, whereby the Cheviot breed exhibited the lowest community richness. Beta diversity analysis revealed no significant effect of breed on community composition, suggesting the presence of a shared core

archaea community among breeds. Similarly, differential abundance analysis revealed no overall influence of breed on taxonomic abundances; however, pairwise comparison between breeds shows that the genus *Candidatus Methanomethylophilus* was significantly more abundant in Perth and Lanark breeds compared to the Cheviot breed in the solid ruminal fraction. Furthermore, results from our correlation analysis showed *Methanosphaera* and *Candidatus Methanomethylophilus* associated with improved ADG and feed efficiency in the epithelial and liquid ruminal fractions, respectively. *Candidatus Methanomethylophilus* is a H_2-dependent methylotrophic methanogen, which derives its energy from the metabolism of methanol and methylaimines (Borrel et al., 2012;Noel et al., 2016). In our previous study with sheep, *Candidatus Methanomethylophilus* was identified in both the solid and liquid ruminal fractions, but significant correlation was not observed between its abundance and FCR (McLoughlin et al., 2020). In contrast, Li et al. (2019) found the abundance of *Candidatus Methanomethylophilus* significantly higher in H-RFI Charlois steers when compared to L-RFI counterparts, however, similar findings were not observed in the Angus or Hybrid Kinsella breeds used in that same study(Li et al., 2019a). Furthermore, *Methanosphaera* and *Candidatus Methanomethylophilus* have previously been linked to lower methane emitting in sheep and cattle (Kittelmann et al., 2014;Martínez-Álvaro et al., 2020), respectively. However, given the complexity of methane synthesis in the rumen, associating higher or lower methane production to individual taxonomic groups may be unrealistic (Greening et al., 2019;Ghanbari Maman et al., 2020).

The results in the current study further support findings in the literature that breed/host genetics can influence the microbial community structure within the rumen. This could have applications for breeding programs, where microbiomes that are better at utilizing feed and producing less methane could potentially be selected for (Difford et al., 2018;Gonzalez-Recio et al., 2018). However, the heritability of rumen microbiome composition across generations needs further investigation in livestock ruminants, including with microbiome transplant experiments. Because of the functional redundancy of the rumen microbiome (Weimer, 2015), where phylogenetically distant microbes may have identical metabolic capabilities, taxonomic differences observed between breeds in the current study may not necessarily reflect functional divergence. Future research would benefit from coupling microbial community composition with rumen chemistry, in addition to multi-omics approaches (i.e meta-genomics/transcriptomics), which would give a better indication of the rumen microbiota's varied metabolic capacity between sheep breeds.

Conclusions

In summary, we demonstrate that the breed of sheep has an effect on the bacterial and archaea taxonomic abundance within the rumen, which can have significant implications for improving feed efficiency and reducing methane emissions. However, further research is required to determine if the taxonomic differences observed signifies functional variation between the breeds. Furthermore, we observed differences in the distribution of bacterial taxa

between ruminal fractions, which supports previous studies and highlights a rumen fraction bias which may have application for rumen sampling strategies. Finally, due to the limited power of the study we urge reader to not to over interpret the results.

Figures

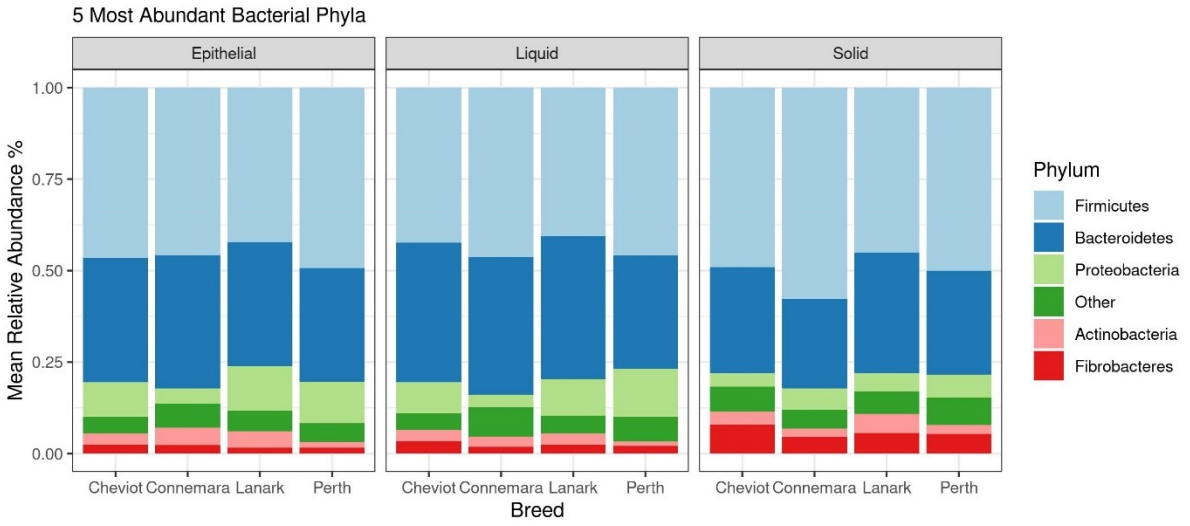


Figure 3.1: Stack barchart representing the mean relative abundance of the 5 most dominant phyla across breeds (i.e Cheviot, Connemara, Lanark, Perth) for solid liquid and epithelial ruminal fractions. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

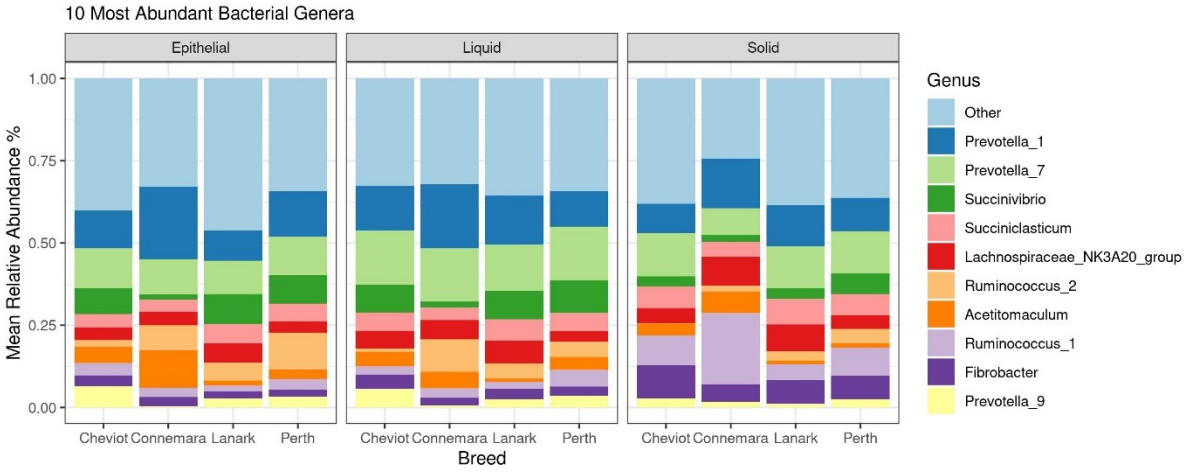


Figure 3.2: Stack barchart representing the mean relative abundance of the 10 most dominant genera across breeds (i.e Cheviot, Connemara, Lanark, Perth) for solid liquid and epithelial ruminal fractions. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

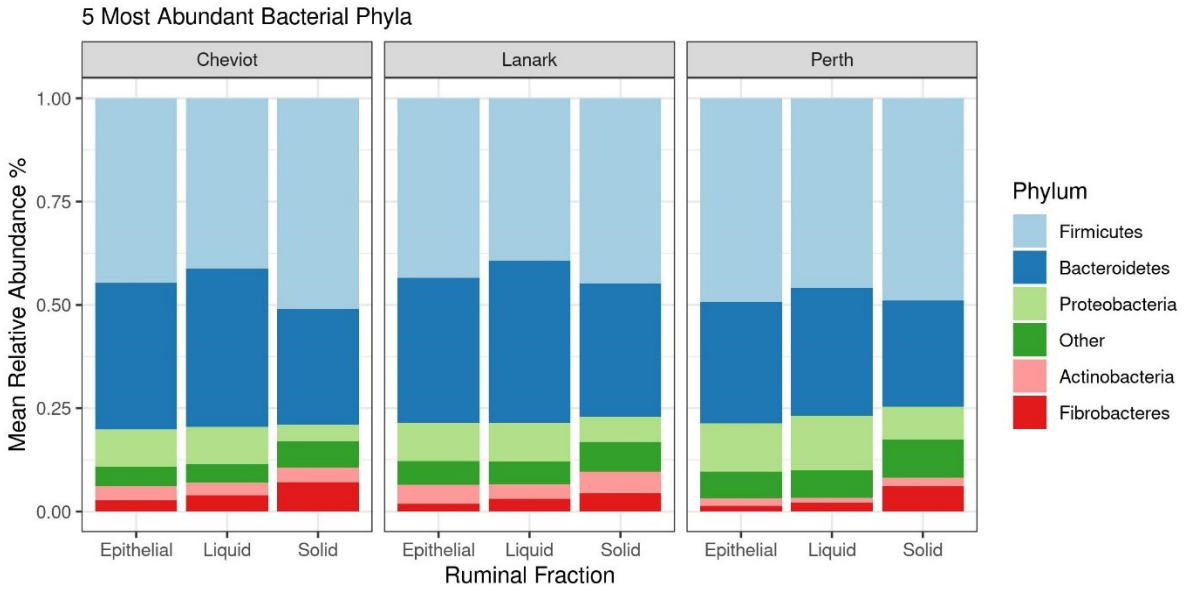


Figure 3.3: Stack barchart representing the mean relative abundance of the 5 most dominant phyla across ruminal fractions (i.e solid, liquid and epithelial) for Cheviot, Lanark and Perth breeds. Cheviot (solid *n*=7, liquid *n*=5, epithelial *n*=5), Lanark (solid *n*=7, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=7, liquid *n*=5, epithelial *n*=5)

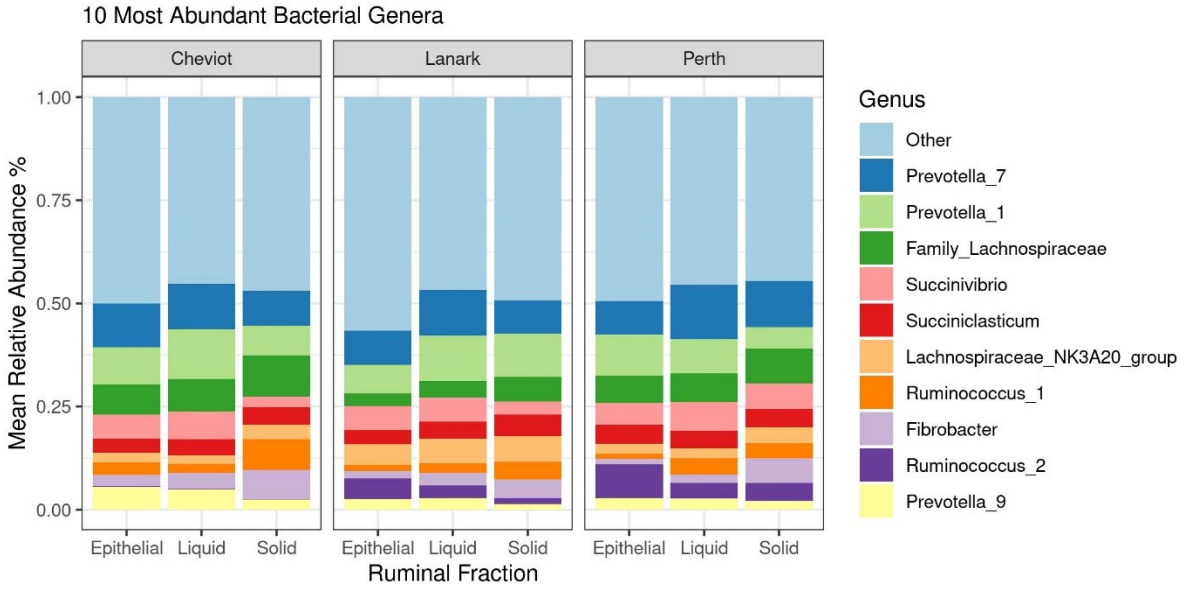


Figure 3.4: Stack barchart representing the mean relative abundance of the 10 most dominant bacterial genera across ruminal fractions (i.e solid, liquid and epithelial) for Cheviot, Lanark and Perth breeds. Cheviot (solid *n*=7, liquid *n*=5, epithelial *n*=5), Lanark (solid *n*=7, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=7, liquid *n*=5, epithelial *n*=5)

Tables

Table 3.1: Ingredient and chemical composition of concentrate and silage offered to lambs

Concentrate		Silage
Ingredient (kg/tonne)		
Maize	300	-
Barley	300	-
Soya hulls	165	-
Soya bean meal	155	-
Molasses	50	-
Minerals	30	-
Chemical Composition		
DM, g/kg	850	255
DMD	-	740
Composition of DM, g/kg		
CP	172	133
NDF	278	642
ADF	145	364
Ash	62	100

DMD=Dry matter digestibility

Table 3.2: Animal production traits for Cheviot, Connemara, Lanark, and Perth. Mean±Sd, ANOVA P value and Tukey HSD pairwise comparisons (superscripts) presented in table. ADG (Average Daily Gain), DMI (Dry Matter Intake) FCR (Feed Conversion Ratio) LW (Live Weight) CW (Carcass Weight) LW Gain (Live Weight Gain) and kill out percentage (KO%).

	Cheviot mean±sd	**Connemara** mean±sd	**Lanark** mean±sd	**Perth** mean±sd	**Anova Pvalue**	**Overall** mean±sd
ADG (Kg/d)	0.3±0.06^{a}	0.2±0.06^{b}	0.3±0.08^{ab}	0.3±0.05^{a}	0.005	0.27±0.1
DMI (Kg/d)	1.151±0.21	1.125±0.16	1.090±0.17	1.086±0.17	0.843	1.113±0.18
FCR (DMI/ADG)	3.7±0.54^{b}	5.1±1.46^{a}	4.1±0.95^{ab}	3.8±0.75^{ab}	0.035	4.04±0.1
LW (Kg)	47.1±3.37	45.6±2.84	44.5±1.47	46.8±3.17	0.161	46.0±2.9
CW (Kg)	21.3±1.73^{a}	20.2±0.65^{ab}	19.4±0.88^{b}	19.9±1.86^{ab}	0.040	20.2±1.6
LW Gain (Kg)	15.1±4.19	14.3±6.35	14.5±5.16	16.4±4.47	0.717	15.2±4.8
Start weight (Kg)	31.6±3.88^{a}	29.2±3.19^{ab}	27.7±2.28^{b}	29.3±4.10^{ab}	0.036	29.6±3.7
KO%	45.4±2.19	44.5±2.82	43.7±1.88	42.7±1.79	0.072	44.1±2.3

Table 3.3: Alpha diversity analysis. Measures of alpha diversity (Shannon, Simpson, Phylogenetic diversity and Observed ASV) for bacterial and archaeal communities, Mean±Sd. Effect of breed on alpha diversity measures tested using two way ANOVA. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

Bacteria community alpha diversity					
	Cheviot Mean±Sd	**Connemara** Mean±Sd	**Lanark** Mean±Sd	**Perth** Mean±Sd	**Anova, Pvalue**
Solid Fraction					
Shannon	4.1±0.27	3.9±0.24	4.2±0.20	4.2±0.27	0.202
InvSimpson	31.0±8.75	20.0±6.06	31.9±7.02	32.8±9.27	0.054
PD	48.3±5.15[b]	54.3±4.45[ab]	57.9±6.97[a]	52.5±7.10[ab]	0.036
Observed ASV	255.0±41.5[b]	300.6±33.73[ab]	334.9±55.69[a]	291.6±55.98[ab]	0.028
Liquid fraction					
Shannon	3.9±0.27	3.9±0.26	4.1±0.21	3.9±0.39	0.579
InvSimpson	23.2±8.12	21.2±7.71	28.1±6.21	22.7±13.05	0.464
PD	53.4±7.68	52.6±6.85	59.9±6.81	52.4±7.42	0.164
Observed ASV	284.4±57.44	297.0±49.15	338.7±57.30	287.4±51.21	0.157
Epithelial fraction					
Shannon	4.3±0.19	4.1±0.23	4.3±0.28	4.1±0.20	0.083
InvSimpson	33.5±7.70[a]	17.7±2.18[b]	34.9±12.68[a]	19.6±5.52[b]	0.001
PD	56.3±3.53	65.8±10.37	60.2±6.19	63.0±4.83	0.055
Observed ASV	313.7±33.08[b]	417.7±92.50[a]	340.0±51.15[ab]	396.1±48.58[ab]	0.032
Archaea community alpha diversity					
	Cheviot Mean±Sd	**Connemara** Mean±Sd	**Lanark** Mean±Sd	**Perth** Mean±Sd	**Anova, Pvalue**
Solid fraction					
Shannon	1.1±0.25	1.1±0.41	1.3±0.38	1.3±0.25	0.389
InvSimpson	2.4±0.52	2.5±1.05	3.1±0.98	3.0±0.70	0.392
PD	1.6±0.05	1.6±0.10	1.7±0.13	1.7±0.15	0.129
Observed ASV	5.9±1.13[b]	7.2±1.10[ab]	9.3±2.50[a]	8.9±2.27[a]	0.001
Liquid fraction					
Shannon	1.1±0.30	1.0±0.38	1.2±0.46	1.2±0.36	0.636
InvSimpson	2.5±0.74	2.1±0.82	2.9±1.19	3.1±1.04	0.377
PD	1.6±0.07	1.3±0.55	1.7±0.12	1.6±0.07	0.085
Observed ASV	6.2±1.39[b]	7.2±2.49[ab]	9.4±2.30[a]	7.0±1.22[ab]	0.016
Epithelial fraction					
Shannon	1.2±0.35	1.2±0.29	1.3±0.31	1.4±0.30	0.785

InvSimpson	2.9±1.27	2.3±0.48	2.7±0.99	3.2±1.04	0.627
PD	1.6±0.06	1.7±0.14	1.7±0.15	1.7±0.18	0.157
Observed ASV	7.1±2.47	9.3±3.51	9.2±1.83	9.4±1.92	0.170

Table 3.4: Beta diversity analysis. Effect of breed on bacterial and archaeal community composition in solid, liquid and epithelial ruminal fractions. Community dissimilarities calculated using weighted and unweighted UniFrac distances and compared among breeds using PERMANOVA, with P values and R2 values reported. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

	Weighted UniFrac		**Unweighted UniFrac**	
Fraction	**PERMANOVA *P* value**	**R2**	**PERMANOVA *P* value**	**R2**
Bacteria				
Solid	0.57	0.10	0.24	0.11
Liquid	0.59	0.10	0.27	0.12
Epithelial	0.47	0.12	0.09	0.14
Archaea				
Solid	0.72	0.08	0.45	0.11
Liquid	0.75	0.07	0.15	0.17
Epithelial	0.57	0.10	0.19	0.16

Table 3.5: Differential abundance analysis investigating the effect of breed on the abundance bacterial and archaeal taxa in solid, liquid and epithelial ruminal fractions. Analysis was conducted across all taxonomic ranks for bacterial populations (Phylum, Class, Order, Family, Genus and ASV), and lower taxonomic ranks for archaeal populations (Genus and ASV) using the LRT and Wald's test from DESeq2. Table reports significant findings along with the log10 of normalised counts (Mean±Sd), BH adjusted P values, pairwise comparisons (superscripts), taxonomic rank and classification. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

	Rank	**Classification**	**Cheviot** Mean±Sd	**Connemara** Mean±Sd	**Lanark** Mean±Sd	**Perth** Mean±Sd	**P.adj**
Solid fraction							
ASV55	Genus	*Sharpea*	1.37 ± 0.35^{b}	1.90 ± 0.71^{ab}	1.55 ± 0.84^{ab}	2.28 ± 0.51^{a}	0.009
ASV55	ASV	*Sharpea azabuensis*	1.34 ± 0.34^{b}	1.93 ± 0.73^{ab}	1.58 ± 0.87^{ab}	2.24 ± 0.51^{a}	0.023
ASV37	ASV	F_Lachnospiraceae	2.22 ± 0.46^{a}	2.40 ± 0.49^{ab}	1.92 ± 0.50^{b}	2.60 ± 0.53^{a}	0.024
Epithelial fraction							

ASV247	Family	Family XIII	2.84±0.22[b]	3.03±0.17[ab]	3.33±0.28[a]	2.95±0.18[b]	<0.001
ASV379	ASV	F_Family XIII	1.15±0.39[ab]	1.44±0.19[ab]	2.02±0.47[a]	1.05±0.43[b]	0.005

Table 3.6: Differential abundance analysis investigating the pairwise differences in bacterial and archaeal abundances between each of the breeds (i.e Cheviot, Connemara, Lanark and Perth) in solid, liquid and epithelial ruminal fractions. Analysis was conducted across all taxonomic ranks for bacterial populations (Phylum, Class, Order, Family, Genus and ASV), and lower taxonomic ranks for archaeal populations (Genus and ASV) using the Wald's pairwise test from DESeq2. Table reports the log10 of normalised counts (Mean±Sd), BH adjusted P values, Log2 fold change, taxonomic rank and classification, breeds compared and the breed the abundance was increased in for significant findings. Solid (Cheviot *n*=8, Connemara *n*=5, Lanark *n*=9, Perth *n*=7), liquid (Cheviot *n*=9, Connemara *n*=5, Lanark *n*=9, Perth *n*=5), epithelial (Cheviot *n*=9, Connemara *n*=3, Lanark *n*=6, Perth *n*=8)

	Kingdom	Classification	Rank	Log2FC	P.adj	Comparison	Higher In
Solid							
ASV27	Bacteria	Coriobacteriales	Order	1.17	0.041	Perth v Lanark	Lanark
ASV17	Bacteria	p-2534-18B5_gut_group (Bacteroidetes)	Family	5.88	0.007	Perth v Cheviot	Perth
ASV17	Bacteria	p-2534-18B5_gut_group (Bacteroidetes)	Family	6.80	0.007	Lanark v Cheviot	Lanark
ASV317	Bacteria	Saccharimonadaceae	Family	5.48	0.007	Lanark v Cheviot	Lanark
ASV317	Bacteria	Saccharimonadaceae	Family	6.89	0.023	Conn. v Cheviot	Connemara
ASV17	Bacteria	F_p-2534-18B5_gut_group (Bacteroidetes)	Genus	5.78	0.012	Perth v Cheviot	Perth
ASV17	Bacteria	F_p-2534-18B5_gut_group (Bacteroidetes)	Genus	6.82	0.014	Lanark v Cheviot	Lanark
ASV55	Bacteria	*Sharpea*	Genus	4.37	0.000	Perth v Cheviot	Perth
ASV317	Bacteria	Candidatus_Saccharimonas	Genus	5.66	0.014	Lanark v Cheviot	Lanark
ASV37	Bacteria	F_Lachnospiraceae	ASV	3.22	0.010	Lanark v Cheviot	Cheviot
ASV48	Bacteria	G_*Prevotella 9*	ASV	9.11	0.010	Lanark v Cheviot	Lanark
ASV48	Bacteria	G_*Prevotella 9*	ASV	7.88	0.009	Perth v Lanark	Lanark
ASV37	Bacteria	F_Lachnospiraceae	ASV	3.38	0.000	Perth v Lanark	Perth
ASV55	Bacteria	*Sharpea azabuensis*	ASV	4.58	0.000	Perth v Cheviot	Perth
ASV329	Bacteria	G_*Pyramidobacter*	ASV	5.52	0.018	Lanark v Cheviot	Lanark
ASV337	Archaea	G_*Candidatus Methanomethylophilus*	ASV	3.21	0.023	Lanark v Cheviot	Lanark
ASV337	Archaea	G_*Candidatus Methanomethylophilus*	ASV	3.12	0.006	Perth v Cheviot	Perth
Liquid							
ASV2	Bacteria	Proteobacteria	Phylum	2.62	0.017	Perth v Conn.	Perth
ASV22	Bacteri	Alphaproteobacteria	Class	7.11	0.00	Perth v	Cheviot

3	a				2	Cheviot	
ASV223	Bacteria	Alphaproteobacteria	Class	7.52	0.012	Conn. v Cheviot	Cheviot
ASV223	Bacteria	Rhodospirillales	Order	6.93	0.005	Perth v Cheviot	Cheviot
ASV223	Bacteria	Rhodospirillales	Order	7.29	0.024	Conn. v Cheviot	Cheviot
ASV219	Bacteria	Betaproteobacteriales	Order	3.03	0.045	Conn. v Cheviot	Cheviot
ASV223	Bacteria	O_Rhodospirillales	Family	7.11	0.021	Conn. v Cheviot	Cheviot
ASV223	Bacteria	O_Rhodospirillales	Family	6.81	0.003	Perth v Cheviot	Cheviot
ASV17	Bacteria	p-2534-18B5_gut_group (Bacteroidetes)	Family	4.75	0.047	Perth v Cheviot	Perth
ASV17	Bacteria	p-2534-18B5_gut_group (Bacteroidetes)	Family	5.87	0.019	Lanark v Cheviot	Lanark
ASV23	Bacteria	Muribaculaceae	Family	2.23	0.015	Perth v Cheviot	Cheviot
ASV461	Bacteria	O_Clostridiales *vadinBB60_group*	Family	5.82	0.049	Conn. v Cheviot	Cheviot
ASV461	Bacteria	O_Clostridiales *vadinBB60_group*	Family	6.09	0.004	Lanark v Conn.	Lanark
ASV55	Bacteria	*Sharpea*	Genus	5.05	0.003	Perth v Cheviot	Perth
ASV223	Bacteria	O_Rhodospirillales	Genus	7.03	0.005	Perth v Cheviot	Cheviot
ASV461	Bacteria	O_Clostridiales *vadinBB60_group*	Genus	5.86	0.013	Lanark v Conn.	Lanark
ASV20	Bacteria	F_Lachnospiraceae *NK3A20 group*	ASV	3.06	0.049	Lanark v Conn.	Lanark
ASV23	Bacteria	F_Muribaculaceae	ASV	5.94	0.002	Perth v Cheviot	Cheviot
ASV43	Bacteria	F_Muribaculaceae	ASV	2.51	0.023	Perth v Cheviot	Cheviot
ASV44	Bacteria	G_*Acetitomaculum*	ASV	10.26	0.034	Conn. v Cheviot	Cheviot
ASV55	Bacteria	*Sharpea azabuensis*	ASV	4.81	0.006	Perth v Cheviot	Perth
Epithelial							
ASV38	Bacteria	Atopobiaceae	Family	1.96	0.014	Perth v Lanark	Lanark
ASV162	Bacteria	Synergistaceae	Family	2.19	0.018	Lanark v Cheviot	Lanark
ASV247	Bacteria	Family XIII	Family	1.41	0.000	Lanark v Cheviot	Lanark
ASV247	Bacteria	Family XIII	Family	1.13	0.008	Perth v Lanark	Lanark
ASV55	Bacteria	*Sharpea*	Genus	3.13	0.028	Perth v Cheviot	Perth
ASV69	Bacteria	F_Ruminococcaceae *UCG-014*	Genus	2.77	0.049	Lanark v Cheviot	Cheviot
ASV361	Bacteria	F_Family_XIII *AD3011_group*	Genus	2.28	0.006	Lanark v Cheviot	Lanark
ASV406	Bacteria	F_Family XIII *UCG-001*	Genus	3.10	0.049	Lanark v Cheviot	Cheviot
ASV24	Bacteria	G_*Succiniclasticum*	ASV	24.14	0.000	Conn. v Cheviot	Cheviot
ASV24	Bacteria	G_*Succiniclasticum*	ASV	23.67	0.000	Lanark v Conn.	Lanark
ASV24	Bacteria	G_*Succiniclasticum*	ASV	25.98	0.000	Perth v Conn.	Perth
ASV33	Bacteria	G_*Ruminococcus 1*	ASV	7.80	0.005	Conn. v Cheviot	Connemara

ASV33	Bacteria	G_*Ruminococcus 1*	ASV	4.81	0.023	Perth v Cheviot	Perth
ASV37	Bacteria	F_Lachnospiraceae	ASV	2.72	0.046	Perth v Lanark	Perth
ASV74	Bacteria	G_*Syntrophococcus*	ASV	5.42	0.049	Lanark v Conn.	Lanark
ASV118	Bacteria	G_*Ruminococcus 1*	ASV	5.52	0.046	Perth v Lanark	Perth
ASV123	Bacteria	G_*Prevotella 1*	ASV	7.85	0.006	Lanark v Conn.	Connemara
ASV379	Bacteria	F_Family_XIII	ASV	2.88	0.019	Perth v Lanark	Lanark
ASV633	Bacteria	F_Ruminococcaceae *UCG-010*	ASV	4.00	0.046	Perth v Lanark	Perth

Table 3.7: Alpha diversity analysis. Measures of alpha diversity (Shannon, Inverse Simpson, Phylogenetic diversity and Observed ASV) for bacterial and archaeal communities, reported as Mean±Sd. Effect of breed on alpha diversity tested using 2 way ANOVA. Cheviot (solid *n*=7, liquid *n*=7, epithelial *n*=7), Lanark (solid *n*=5, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=5, liquid *n*=5, epithelial *n*=5)

Bacteria community alpha diversity				
	Epithelial Mean±Sd	**Liquid** Mean±Sd	**Solid** Mean±Sd	**Anova, Pvalue**
Cheviot				
Shannon	4.2±0.20	4.0±0.28	4.1±0.18	0.118
InvSimpson	31.7±6.86	25.0±8.41	33.1±6.78	0.121
PD	55.7±3.01	53.8±8.53	48.7±5.43	0.112
Observed ASV	305.4±32.99	288.1±65.09	257.4±44.20	0.21
Lanark				
Shannon	4.4±0.16[a]	4.2±0.16[b]	4.4±0.13[ab]	0.037
InvSimpson	38.2±10.95	29.5±5.97	35.7±4.04	0.174
PD	61.1±6.54	59.6±4.82	59.6±7.59	0.908
Observed ASV	344.8±55.66	341.0±34.40	354.0±54.83	0.904
Perth				
Shannon	4.1±0.14	3.9±0.39	4.2±0.31	0.476
InvSimpson	21.2±5.38	22.7±13.05	32.0±10.69	0.264
PD	62.1±5.63[a]	52.4±7.42[ab]	51.5±8.35[b]	0.03
Observed ASV	362.8±61.58[a]	287.4±51.21[b]	285.8±65.14[b]	0.027
Archaea community alpha diversity				
	Epithelial Mean±Sd	**Liquid** Mean±Sd	**Solid** Mean±Sd	**Anova, Pval**

				ue
Cheviot				
Shannon	1.2±0.24	1.1±0.30	1.1±0.25	0.752
InvSimpson	2.6±0.76	2.5±0.75	2.4±0.53	0.800
PD	1.6±0.06	1.6±0.07	1.5±0.04	0.122
Observed ASV	6.4±1.27	6.6±1.27	5.9±1.07	0.517
Lanark				
Shannon	1.3±0.31	1.2±0.46	1.3±0.38	0.900
InvSimpson	2.7±0.99	2.9±1.19	3.1±0.98	0.847
PD	1.7±0.15	1.7±0.12	1.7±0.13	0.737
Observed ASV	9.2±1.83	9.9±2.37	9.3±50	0.818
Perth				
Shannon	1.5±0.05	1.2±0.36	1.3±0.30	0.320
InvSimpson	3.6±0.50	3.1±1.04	3.1±0.84	0.565
PD	1.8±0.20	1.6±0.07	1.7±0.16	0.534
Observed ASV	8.8±2.17	7.0±1.22	8.2±2.17	0.293

Table 3.8: Beta diversity analysis. Effect of Fraction on bacterial and archaeal community composition for Cheviot, Lanark and Perth breeds. Community dissimilarities calculated using weighted and unweighted UniFrac distances and compared among breeds using PERMANOVA. *P* values and R2 values reported. Cheviot (solid *n*=7, liquid *n*=7, epithelial *n*=7), Lanark (solid *n*=5, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=5, liquid *n*=5, epithelial *n*=5)

	Weighted UniFrac		**Unweighted UniFrac**	
Breed	**PERMANOVA *P* value**	**R2**	**PERMANOVA *P* value**	**R2**
Bacteria				
Cheviot	0.49	0.09	0.89	0.06
Perth	0.68	0.09	0.64	0.11
Lanark	0.8	0.1	0.85	0.08
Archaea				
Cheviot	0.95	0.02	0.89	0.03
Perth	0.3	0.17	0.82	0.06
Lanark	0.6	0.9	0.83	0.06

Table 3.9: Differential abundance analysis investigating the effect of ruminal fraction on the abundance bacterial and archaeal taxa in Cheviot, Lanark and

Perth breeds. Analysis was conducted across all taxonomic ranks for bacterial populations (Phylum, Class, Order, Family, Genus and ASV), and lower taxonomic ranks for archaeal populations (Genus and ASV) using the LRT from DESeq2. Table reports significant findings along with the log10 of normalised counts (Mean±Sd), BH adjusted P values, pairwise comparisons (superscripts). taxonomic rank and classification. Cheviot (solid *n*=7, liquid *n*=7, epithelial *n*=7), Lanark (solid *n*=5, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=5, liquid *n*=5, epithelial *n*=5)

	Kingdom	Classification	Rank	P.adj	Epithelial	Liquid	Solid
Cheviot							
ASV141	Bacteria	Epsilonbacteraeota	Phylum	0.003	1.72±0.89^{a}	1.14±0.71^{ab}	0.68±0.48^{b}
ASV141	Bacteria	Campylobacteria	Class	0.002	1.72±0.89^{a}	1.14±0.71^{ab}	0.65±0.48^{b}
ASV141	Bacteria	Campylobacterales	Order	0.005	1.71±0.92^{a}	1.11±0.70^{ab}	0.67±0.50^{b}
ASV219	Bacteria	Betaproteobacteriales	Order	0.028	2.32±0.52^{a}	1.98±0.44^{ab}	1.80±0.24^{b}
ASV449	Bacteria	Desulfobacterales	Order	0.028	1.79±0.45^{a}	1.19±0.71^{ab}	0.84±0.45^{b}
ASV141	Bacteria	Campylobacteraceae	Family	0.002	1.72±0.89^{a}	1.12±0.71^{ab}	0.64±0.48^{b}
ASV198	Bacteria	O_Coriobacteriales	Family	0.000	1.50±0.26^{b}	1.56±0.17^{b}	2.03±0.13^{a}
ASV219	Bacteria	Neisseriaceae	Family	0.000	1.84±0.60^{a}	1.42±0.58^{b}	1.00±0.53^{b}
ASV449	Bacteria	Desulfobulbaceae	Family	0.014	1.81±0.49^{a}	1.19±0.73^{ab}	0.81±0.44^{b}
ASV141	Bacteria	*Campylobacter*	Genus	0.011	1.74±0.97^{a}	1.15±0.73^{ab}	0.64±0.50^{b}
ASV142	Bacteria	F_Erysipelotrichaceae *UCG-004*	Genus	0.030	1.65±0.75^{a}	1.88±0.86^{b}	1.84±0.47^{ab}
ASV199	Bacteria	*Mogibacterium*	Genus	0.030	1.69±0.55^{a}	1.38±0.52^{ab}	0.94±0.92^{b}
ASV254	Bacteria	*Butyrivibrio 2*	Genus	0.022	2.22±0.62^{a}	1.43±0.78^{ab}	0.96±0.63^{b}
ASV263	Bacteria	F_Burkholderiaceae	Genus	0.022	1.52±0.58^{a}	1.00±0.64^{ab}	0.55±0.42^{b}
ASV449	Bacteria	*Desulfobulbus*	Genus	0.030	1.82±0.40^{a}	1.22±0.75^{ab}	0.80±0.43^{b}
Lanark							
ASV141	Bacteria	Epsilonbacteraeota	Phylum	0.002	2.94±0.54^{a}	1.03±0.72^{b}	0.57±0.61^{b}
ASV141	Bacteria	Campylobacteria	Class	0.009	2.36±0.54^{a}	1.03±0.72^{ab}	0.57±0.61^{b}
ASV141	Bacteria	Campylobacterales	Order	0.003	2.34±0.58^{a}	1.01±0.66^{b}	0.58±0.62^{b}
ASV421	Bacteria	Desulfobacterales	Order	0.000	2.26±0.45^{a}	0.84±0.61^{b}	0.64±0.42^{b}
ASV141	Bacteria	Campylobacteraceae	Family	0.003	2.33±0.58^{a}	1.04±0.69^{b}	0.59±0.63^{b}
ASV421	Bacteria	Desulfobulbaceae	Family	0.000	2.25±0.42^{a}	0.85±0.61^{b}	0.62±0.39^{b}
ASV141	Bacteria	*Campylobacter*	Genus	0.001	2.41±0.62^{a}	1.02±0.67^{b}	0.53±0.56^{b}
ASV219	Bacteria	F_Neisseriaceae	Genus	0.013	1.70±0.64^{a}	0.87±0.35^{ab}	0.37±0.51^{b}
ASV23	Bacteri	*Butyrivibrio 2*	Genu	0.00	2.05±1.	0.79±0.7	0.48±0.5

9	a		s	8	30[a]	4[b]	0[b]
ASV391	Bacteria	*Fretibacterium*	Genus	0.000	2.02±1.16[a]	1.00±0.56[b]	0.40±0.39[b]
ASV406	Bacteria	Family XIII *UCG-001*	Genus	0.000	0.55±0.54[b]	0.90±0.59[ab]	1.43±0.41[a]
ASV421	Bacteria	*Desulfobulbus*	Genus	0.000	2.33±0.51[a]	0.85±0.62[b]	0.59±0.38[b]
ASV523	Bacteria	F_Eggerthellaceae	Genus	0.012	1.33±0.20[b]	1.39±0.15[ab]	1.75±0.11[a]
ASV587	Bacteria	*Shuttleworthia*	Genus	0.017	0.69±0.46[b]	1.03±0.43[ab]	1.47±0.14[a]
ASV846	Bacteria	*Howardella*	Genus	0.018	1.68±0.13[a]	0.74±0.70[ab]	0.87±0.24[b]
ASV141	Bacteria	*Campylobacter*	ASV	0.002	2.43±0.63[a]	1.01±0.65[b]	0.50±0.54[b]
ASV210	Bacteria	*Mogibacterium*	ASV	0.024	2.28±0.37[a]	1.59±0.40[ab]	0.97±0.63[b]
ASV379	Bacteria	F_Family XIII	ASV	0.000	2.06±0.44[a]	0.82±0.53[b]	0.20±0.29[b]
Perth							
ASV126	Bacteria	Tenericutes	Phylum	0.015	2.33±0.25[b]	2.6±0.26[a]	2.55±0.19[ab]
ASV475	Archaea	F_Methanomethylophilaceae	Genus	0.019	1.43±1.03[a]	0.52±0.48[b]	0.42±0.45[b]

Table 3.10: Differential abundance analysis investigating the pairwise differences in bacterial and archaeal abundances between each of the fractions (i.e solid, liquid and epithelial) in the Cheviot, Lanark and Perth breeds. Analysis was conducted across all taxonomic ranks for bacterial populations (Phylum, Class, Order, Family, Genus and ASV), and lower taxonomic ranks for archaeal populations (Genus and ASV) using the Wald's pairwise test from DESeq2. Table reports significant findings along with the log10 of normalised counts (Mean±Sd), BH adjusted P values, Log2 fold change, taxonomic rank and classification, fractions compared, and the fraction the abundance was increased in. Cheviot (solid *n*=7, liquid *n*=7, epithelial *n*=7), Lanark (solid *n*=5, liquid *n*=5, epithelial *n*=5), Perth (solid *n*=5, liquid *n*=5, epithelial *n*=5)

	Kingdom	**Classification**	**Rank**	**Log2FC**	**P.adj**	**Comparison**	**Higher in**
Cheviot							
ASV141	Bacteria	Epsilonbacteraeota	Phylum	5.42	0.000	Solid v Epithelial	Epithelial
ASV3	Bacteria	Firmicutes	Phylum	0.63	0.023	Solid v Epithelial	Solid
ASV14	Bacteria	Fibrobacter	Phylum	1.95	0.023	Solid v Epithelial	Solid
ASV81	Bacteria	Spirochaetes	Phylum	1.97	0.050	Solid v Epithelial	Solid
ASV141	Bacteria	Campylobacteria	Class	5.51	0.000	Solid v Epithelial	Epi
ASV141	Bacteria	Campylobacterales	Order	5.26	0.000	Solid v Epithelial	Epi
ASV449	Bacteria	Desulfobacterales	Order	3.48	0.002	Solid v Epithelial	Epi
ASV219	Bacteria	Betaproteobacteriales	Order	2.53	0.005	Solid v Epithelial	Epi
ASV219	Bacteri	Neisseriaceae	Famil	3.31	0.00	Liquid v	Epi

	a		y		2	Epithelial	
ASV141	Bacteria	Campylobacteraceae	Family	5.15	0.000	Solid v Epithelial	Epi
ASV449	Bacteria	Desulfobulbaceae	Family	3.74	0.001	Solid v Epithelial	Epi
ASV219	Bacteria	Neisseriaceae	Family	3.51	0.000	Solid v Epithelial	Epi
ASV3	Bacteria	Acidaminococcaceae	Family	0.98	0.042	Solid v Epithelial	Solid
ASV198	Bacteria	O_Coriobacteriales	Family	1.59	0.001	Solid v Epithelial	Solid
ASV81	Bacteria	Spirochaetaceae	Family	2.12	0.042	Solid v Epithelial	Solid
ASV198	Bacteria	O_Coriobacteriales	Family	1.57	0.005	Solid v Liquid	Solid
ASV142	Bacteria	F_Erysipelotrichaceae *UCG-004*	Genus	3.50	0.025	Liquid v Epithelial	Liq
ASV141	Bacteria	Campylobacter	Genus	5.70	0.000	Solid v Epithelial	Epi
ASV254	Bacteria	Butyrivibrio_2	Genus	4.16	0.001	Solid v Epithelial	Epi
ASV263	Bacteria	F_Burkholderiaceae	Genus	4.02	0.001	Solid v Epithelial	Epi
ASV128	Bacteria	F_Lachnospiraceae *UCG-008*	Genus	3.73	0.018	Solid v Epithelial	Epi
ASV199	Bacteria	Mogibacterium	Genus	3.70	0.002	Solid v Epithelial	Epi
ASV449	Bacteria	Desulfobulbus	Genus	3.63	0.002	Solid v Epithelial	Epi
ASV347	Bacteria	Family XIII *AD3011 group*	Genus	2.56	0.032	Solid v Epithelial	Epi
ASV141	Bacteria	G_Campylobacter	ASV	6.15	0.001	Solid v Epithelial	Epi
ASV263	Bacteria	F_Burkholderiaceae	ASV	4.32	0.006	Solid v Epithelial	Epi
Lanark							
ASV141	Bacteria	Epsilonbacteraeota	Phylum	4.09	0.023	Liquid v Epithelial	Epi
ASV141	Bacteria	Epsilonbacteraeota	Phylum	5.88	0.000	Solid v Epithelial	Epi
ASV41	Bacteria	Fibrobacter	Phylum	1.34	0.035	Solid v Epithelial	Solid
ASV141	Bacteria	Campylobacteria	Class	5.68	0.001	Solid v Epithelial	Epi
ASV421	Bacteria	Desulfobacterales	Order	4.57	0.001	Liquid v Epithelial	Epi
ASV141	Bacteria	Campylobacterales	Order	4.39	0.011	Liquid v Epithelial	Epi
ASV1	Bacteria	Bacteroidales	Order	0.63	0.021	Liquid v Epithelial	Liq
ASV141	Bacteria	Campylobacterales	Order	5.76	0.000	Solid v Epithelial	Epi
ASV421	Bacteria	Desulfobacterales	Order	5.63	0.000	Solid v Epithelial	Epi
ASV41	Bacteria	Fibrobacterales	Order	1.55	0.011	Solid v Epithelial	Solid
ASV421	Bacteria	Desulfobulbaceae	Family	4.45	0.001	Liquid v Epithelial	Epi
ASV141	Bacteria	Campylobacteraceae	Family	4.20	0.024	Liquid v Epithelial	Epi
ASV141	Bacteria	Campylobacteraceae	Family	5.77	0.000	Solid v Epithelial	Epi
ASV421	Bacteria	Desulfobulbaceae	Family	5.77	0.000	Solid v Epithelial	Epi

ASV219	Bacteria	Neisseriaceae	Family	4.38	0.005	Solid v Epithelial	Epi
ASV41	Bacteria	Fibrobacteraceae	Family	1.49	0.019	Solid v Epithelial	Solid
ASV239	Bacteria	Butyrivibrio 2	Genus	5.41	0.031	Liquid v Epithelial	Epi
ASV421	Bacteria	Desulfobulbus	Genus	4.95	0.002	Liquid v Epithelial	Epi
ASV391	Bacteria	Fretibacterium	Genus	4.78	0.014	Liquid v Epithelial	Epi
ASV141	Bacteria	Campylobacter	Genus	4.73	0.014	Liquid v Epithelial	Epi
ASV391	Bacteria	Fretibacterium	Genus	7.48	0.000	Solid v Epithelial	Epi
ASV239	Bacteria	Butyrivibrio 2	Genus	7.07	0.001	Solid v Epithelial	Epi
ASV141	Bacteria	Campylobacter	Genus	6.56	0.000	Solid v Epithelial	Epi
ASV421	Bacteria	Desulfobulbus	Genus	6.37	0.000	Solid v Epithelial	Epi
ASV219	Bacteria	F_Neisseriaceae	Genus	5.21	0.001	Solid v Epithelial	Epi
ASV708	Bacteria	Bacteroides	Genus	3.70	0.020	Solid v Epithelial	Epi
ASV568	Bacteria	Alistipes	Genus	3.39	0.016	Solid v Epithelial	Epi
ASV846	Bacteria	Howardella	Genus	2.78	0.011	Solid v Epithelial	Epi
ASV523	Bacteria	F_Eggerthellaceae	Genus	1.39	0.011	Solid v Epithelial	Solid
ASV587	Bacteria	Shuttleworthia	Genus	2.49	0.005	Solid v Epithelial	Solid
ASV406	Bacteria	Family XIII *UCG-001*	Genus	3.19	0.000	Solid v Epithelial	Solid
ASV141	Bacteria	G_Campylobacter	ASV	5.02	0.017	Liquid v Epithelial	Epi
ASV379	Bacteria	F_Family XIII	ASV	4.40	0.014	Liquid v Epithelial	Epi
ASV379	Bacteria	F_Family XIII	ASV	7.53	0.000	Solid v Epithelial	Epi
ASV141	Bacteria	G_Campylobacter	ASV	6.93	0.000	Solid v Epithelial	Epi
ASV247	Bacteria	F_Family XIII	ASV	5.87	0.005	Solid v Epithelial	Epi
ASV361	Bacteria	F_Family XIII *AD3011 group*	ASV	5.72	0.004	Solid v Epithelial	Epi
ASV210	Bacteria	G_Mogibacterium	ASV	4.09	0.002	Solid v Epithelial	Epi
Perth							
ASV126	Bacteria	Tenericutes	Phylum	0.97	0.003	Liquid v Epithelial	Liq
ASV421	Bacteria	Desulfobacterales	Order	3.73	0.007	Solid v Epithelial	Epi
ASV102	Bacteria	Family XIII	Family	1.27	0.050	Solid v Liquid	Solid
ASV219	Bacteria	Neisseriaceae	Family	4.24	0.027	Solid v Epithelial	Epi
ASV391	Bacteria	Fretibacterium	Genus	6.89	0.006	Solid v Epithelial	Epi
ASV421	Bacteria	Desulfobulbus	Genus	4.25	0.006	Solid v Epithelial	Epi
ASV276	Bacteria	O_Clostridiales	ASV	6.27	0.050	Solid v Epithelial	Epi
ASV128	Bacteri	F_Lachnospiraceae *UCG-008*	ASV	4.63	0.03	Solid v	Epi

	a				6	Epithelial	

Table 3.11 Spearman's rank correlation of bacterial and archaeal genera in the solid, liquid, and epithelial ruminal fractions that had a significant association with animal production traits FCR and/or ADG.

	Kingdom	**Classification**	**ρ**	**P value**	**P.adj**	**Trait**
Solid						
ASV21	Bacteria	Succinivibrionaceae	-0.41	0.025	0.707	FCR
ASV71	Bacteria	*Syntrophococcus*	-0.38	0.037	0.707	FCR
ASV149	Bacteria	*Lachnospira*	-0.39	0.033	0.707	FCR
ASV9	Bacteria	O_Gastranaerophilales	-0.41	0.026	0.707	FCR
ASV16	Bacteria	F_Lachnospiraceae *NK3A20 group*	-0.38	0.038	0.913	ADG
ASV366	Bacteria	F_Ruminococcaceae *UCG-013*	0.38	0.040	0.913	ADG
Liquid						
ASV9	Bacteria	O_Gastranaerophilales	-0.44	0.018	0.904	FCR
ASV44	Bacteria	*Acetitomaculum*	-0.38	0.043	0.904	FCR
ASV337	Archaea	Candidatus_Methanomethylophilus	-0.38	0.044	0.177	FCR
ASV10	Bacteria	F_Lachnospiraceae	0.39	0.038	0.434	ADG
ASV32	Bacteria	*Prevotella 9*	0.38	0.044	0.439	ADG
ASV69	Bacteria	F_Ruminococcaceae *UCG-014*	0.41	0.028	0.397	ADG
ASV82	Bacteria	Roseburia	0.49	0.009	0.340	ADG
ASV126	Bacteria	O_Mollicutes	0.49	0.008	0.340	ADG
ASV207	Bacteria	F_Ruminococcaceae *UCG-002*	0.44	0.020	0.397	ADG
ASV366	Bacteria	F_Ruminococcaceae *UCG-013*	0.45	0.018	0.397	ADG
ASV633	Bacteria	F_Ruminococcaceae *UCG-010*	0.41	0.030	0.397	ADG
Epithelial						
ASV10	Bacteria	F_Lachnospiraceae	0.45	0.021	0.356	ADG
ASV25	Archaea	Methanosphaera	0.44	0.024	0.096	ADG
ASV28	Bacteria	F_Prevotellaceae	-0.43	0.028	0.356	ADG
ASV32	Bacteria	Prevotella_9	0.48	0.013	0.356	ADG
ASV69	Bacteria	Ruminococcaceae *UCG-014*	0.41	0.036	0.391	ADG
ASV181	Bacteria	O_Mollicutes *RF39*	0.46	0.017	0.356	ADG
ASV210	Bacteria	Mogibacterium	-0.41	0.039	0.391	ADG
ASV366	Bacteria	F_Ruminococcaceae *UCG-013*	0.49	0.012	0.356	ADG
ASV426	Bacteria	F_Ruminococcaceae *UCG-009*	0.47	0.016	0.356	ADG
ASV912	Bacteria	F_Christensenellaceae	-0.43	0.027	0.356	ADG

References

Alemu, A.W., Vyas, D., Manafiazar, G., Basarab, J.A., and Beauchemin, K.A. (2017). Enteric methane emissions from low– and high–residual feed intake beef heifers measured using GreenFeed and respiration chamber techniques1,2. *Journal of Animal Science* 95**,** 3727-3737.

Anderson, C.J., Koester, L.R., and Schmitz-Esser, S. (2021). Rumen epithelial communities share a core bacterial microbiota: A meta-analysis of 16S rRNA gene Illumina MiSeq sequencing datasets. *Frontiers in Microbiology* 12.

Andrews, S. (2017). "FastQC: a quality control tool for high throughput sequence data. 2010".).

Beauchemin, K.A., Ungerfeld, E.M., Eckard, R.J., and Wang, M. (2020). Review: Fifty years of research on rumen methanogenesis: lessons learned and future challenges for mitigation. *Animal* 14**,** 2-16.

Beule, L., and Karlovsky, P. (2020). Improved normalization of species count data in ecology by scaling with ranked subsampling (SRS): application to microbial communities. *PeerJ* 8**,** e9593.

Blaxter, K.L., Clapperton, J.L., and Wainman, F.W. (1966). The extent of differences between six British breeds of sheep in their metabolism, feed intake and utilization, and resistance to climatic stress. *British Journal of Nutrition* 20**,** 283-294.

Borrel, G., Harris, H.M., Tottey, W., Mihajlovski, A., Parisot, N., Peyretaillade, E., Peyret, P., Gribaldo, S., O'toole, P.W., and Brugère, J.-F. (2012). "Genome sequence of “Candidatus Methanomethylophilus alvus” Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens". Am Soc Microbiol).

Byrne, D., Keena, C., Maguire, F., Sheridan, H., and Gorman, M. (2017). Farming the uplands-where to from here? *Teagasc Hill Sheep Conference*.

Callahan, B. (2018). Silva taxonomic training data formatted for DADA2. *Silva version* 132.

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: high-resolution sample inference from Illumina amplicon data. *Nature methods* 13**,** 581-583.

Cammack, K.M., Austin, K.J., Lamberson, W.R., Conant, G.C., and Cunningham, H.C. (2018). Ruminant nutrition symposium: Tiny but mighty: the role of the rumen microbes in livestock production. *Journal of Animal Science* 96**,** 752-770.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences* 108**,** 4516-4522.

Carlyle, W.J. (1979). The changing distribution of breeds of sheep in Scotland, 1795-1965. *The Agricultural History Review* 27**,** 19-29.

Chen, Y., Oba, M., and Guan, L.L. (2012). Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. *Veterinary Microbiology* 159**,** 451-459.

Chen, Y., Penner, G.B., Li, M., Oba, M., and Guan, L.L. (2011). Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. *Applied and environmental microbiology* 77**,** 5770-5781.

Cheng, K.J., Mccowan, R.P., and Costerton, J.W. (1979). Adherent epithelial bacteria in ruminants and their roles in digestive tract function. *The American Journal of Clinical Nutrition* 32**,** 139-148.

Cheng, K.J., and Wallace, R.J. (1979). The mechanism of passage of endogenous urea through the rumen wall and the role of ureolytic epithelial bacteria in the urea flux. *British Journal of Nutrition* 42**,** 553-557.

Difford, G.F., Plichta, D.R., Løvendahl, P., Lassen, J., Noel, S.J., Højberg, O., Wright, A.-D.G., Zhu, Z., Kristensen, L., Nielsen, H.B., Guldbrandtsen, B., and Sahana, G. (2018). Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. *PLOS Genetics* 14**,** e1007580.

Dinsdale, D., Cheng, K.J., Wallace, R.J., and Goodlad, R.A. (1980). Digestion of epithelial tissue of the rumen wall by adherent bacteria in infused and conventionally fed sheep. *Applied and environmental microbiology* 39**,** 1059-1066.

Dixon, P. (2003). VEGAN, A Package of R Functions for Community Ecology. *Journal of Vegetation Science* 14**,** 927-930.

Dopelt, K., Radon, P., and Davidovitch, N. (2019). Environmental effects of the livestock industry: The relationship between knowledge, attitudes, and behavior among students in Israel. *International journal of environmental research and public health* 16**,** 1359.

Elliot, J.M. (1980). "Propionate metabolism and vitamin B12," in *Digestive Physiology and Metabolism in Ruminants: Proceedings of the 5th International Symposium on Ruminant Physiology, held at Clermont — Ferrand, on 3rd–7th September, 1979,* eds. Y. Ruckebusch & P. Thivend. (Dordrecht: Springer Netherlands), 485-503.

Ellison, M.J., Conant, G.C., Lamberson, W.R., Cockrum, R.R., Austin, K.J., Rule, D.C., and Cammack, K.M. (2017). Diet and feed efficiency status affect rumen microbial profiles of sheep. *Small Ruminant Research* 156**,** 12-19.

Fitzsimons, C., Kenny, D.A., Deighton, M.H., Fahey, A.G., and Mcgee, M. (2013). Methane emissions, body composition, and rumen fermentation traits of beef heifers differing in residual feed intake. *Journal of Animal Science* 91**,** 5789-5800.

Ghanbari Maman, L., Palizban, F., Fallah Atanaki, F., Elmi Ghiasi, N., Ariaeenejad, S., Ghaffari, M.R., Hosseini Salekdeh, G., and Kavousi, K. (2020). Co-abundance analysis reveals hidden players associated with high methane yield phenotype in sheep rumen microbiome. *Scientific Reports* 10**,** 4995.

Gonzalez-Recio, O., Zubiria, I., García-Rodríguez, A., Hurtado, A., and Atxaerandio, R. (2018). Short communication: Signs of host genetic regulation in the microbiome composition in 2 dairy breeds: Holstein and Brown Swiss. *Journal of Dairy Science* 101**,** 2285-2292.

Goodrich, J.K., Davenport, E.R., Beaumont, M., Jackson, M.A., Knight, R., Ober, C., Spector, T.D., Bell, J.T., Clark, A.G., and Ley, R.E. (2016). Genetic determinants of the gut microbiome in UK twins. *Cell host & microbe* 19**,** 731-743.

Greening, C., Geier, R., Wang, C., Woods, L.C., Morales, S.E., Mcdonald, M.J., Rushton-Green, R., Morgan, X.C., Koike, S., Leahy, S.C., Kelly, W.J., Cann, I., Attwood, G.T., Cook, G.M., and Mackie, R.I. (2019). Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME Journal* 13**,** 2617-2632.

Hegarty, R.S., Goopy, J.P., Herd, R.M., and Mccorkell, B. (2007). Cattle selected for lower residual feed intake have reduced daily methane production1,2. *Journal of Animal Science* 85**,** 1479-1486.

Henchion, M., Hayes, M., Mullen, A.M., Fenelon, M., and Tiwari, B. (2017). Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods (Basel, Switzerland)* 6**,** 53.

Huws, S.A., Creevey, C.J., Oyama, L.B., Mizrahi, I., Denman, S.E., Popova, M., Muñoz-Tamayo, R., Forano, E., Waters, S.M., Hess, M., Tapio, I., Smidt, H., Krizsan, S.J., Yáñez-Ruiz, D.R., Belanche, A., Guan, L., Gruninger, R.J., Mcallister, T.A., Newbold, C.J., Roehe, R., Dewhurst, R.J., Snelling, T.J., Watson, M., Suen, G., Hart, E.H., Kingston-Smith, A.H., Scollan, N.D., Do Prado, R.M., Pilau, E.J., Mantovani, H.C., Attwood, G.T., Edwards, J.E., Mcewan, N.R., Morrisson, S., Mayorga, O.L., Elliott, C., and Morgavi, D.P. (2018). Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology* 9.

Ji, S., Zhang, H., Yan, H., Azarfar, A., Shi, H., Alugongo, G., Li, S., Cao, Z., and Wang, Y. (2017). Comparison of rumen bacteria distribution in original

rumen digesta, rumen liquid and solid fractions in lactating Holstein cows. *Journal of Animal Science and Biotechnology* 8**,** 16.

Johnson, K.A., and Johnson, D.E. (1995). Methane emissions from cattle. *Journal of Animal Science* 73**,** 2483-2492.

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P.H., Shi, W., Froula, J., Rubin, E.M., and Attwood, G.T. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome* 4**,** 56.

Karekar, S., Stefanini, R., and Ahring, B. (2022). Homo-Acetogens: Their Metabolism and Competitive Relationship with Hydrogenotrophic Methanogens. *Microorganisms* 10**,** 397.

Kenny, D.A., Fitzsimons, C., Waters, S.M., and Mcgee, M. (2018). Invited review: Improving feed efficiency of beef cattle – the current state of the art and future challenges. *Animal* 12**,** 1815-1826.

Kirton, A., Carter, A., Clarke, J., Sinclair, D., Mercer, G., and Duganzich, D. (1995). A comparison between 15 ram breeds for export lamb production 1. Liveweights, body components, carcass measurements, and composition. *New Zealand Journal of Agricultural Research* 38**,** 347-360.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., Mcewan, J.C., and Janssen, P.H. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLOS ONE* 9**,** e103171.

Kumar, S., Treloar, B.P., Teh, K.H., Mckenzie, C.M., Henderson, G., Attwood, G.T., Waters, S.M., Patchett, M.L., and Janssen, P.H. (2018). Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. *Anaerobe* 54**,** 31-38.

Le Van, T.D., Robinson, J.A., Ralph, J., Greening, R.C., Smolenski, W.J., Leedle, J.A., and Schaefer, D.M. (1998). Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and Acetitomaculum ruminis. *Appl Environ Microbiol* 64**,** 3429-3436.

Li, F., Hitch, T.C.A., Chen, Y., Creevey, C.J., and Guan, L.L. (2019a). Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle. *Microbiome* 7**,** 6-6.

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G., and Guan, L.L. (2019b). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7**,** 92.

Li, Z., Mu, C., Xu, Y., Shen, J., and Zhu, W. (2020). Changes in the solid-, liquid-, and epithelium-associated bacterial communities in the rumen of Hu

lambs in response to dietary urea supplementation. *Frontiers in Microbiology* 11**,** 244.

Liu, K., Zhang, Y., Yu, Z., Xu, Q., Zheng, N., Zhao, S., Huang, G., and Wang, J. (2021). Ruminal microbiota–host interaction and its effect on nutrient metabolism. *Animal Nutrition* 7**,** 49-55.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* 15**,** 550-550.

Mann, E., Wetzels, S.U., Wagner, M., Zebeli, Q., and Schmitz-Esser, S. (2018). Metatranscriptome sequencing reveals insights into the gene expression and functional potential of rumen wall bacteria. *Frontiers in Microbiology* 9.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal* 17**,** 10-12.

Martínez-Álvaro, M., Auffret, M.D., Stewart, R.D., Dewhurst, R.J., Duthie, C.-A., Rooke, J.A., Wallace, R.J., Shih, B., Freeman, T.C., Watson, M., and Roehe, R. (2020). Identification of complex rumen microbiome interaction within diverse functional niches as mechanisms affecting the variation of methane emissions in bovine. *Frontiers in Microbiology* 11.

Mcgovern, E., Kenny, D.A., Mccabe, M.S., Fitzsimons, C., Mcgee, M., Kelly, A.K., and Waters, S.M. (2018). 16S rRNA sequencing reveals relationship between potent cellulolytic genera and feed efficiency in the rumen of bulls. *Frontiers in microbiology* 9**,** 1842-1842.

Mcloughlin, S., Spillane, C., Claffey, N., Smith, P.E., O'rourke, T., Diskin, M.G., and Waters, S.M. (2020). Rumen microbiome composition is altered in sheep divergent in feed efficiency. *Frontiers in Microbiology* 11.

Mcmurdie, P.J., and Holmes, S. (2012). "Phyloseq: a bioconductor package for handling and analysis of high-throughput phylogenetic sequence data," in *Biocomputing 2012*. World Scientific), 235-246.

Moss, A.R., Jouany, J.-P., and Newbold, J. (2000). Methane production by ruminants: its contribution to global warming. *Ann. Zootech.* 49**,** 231-253.

Myer, P.R., Smith, T.P.L., Wells, J.E., Kuehn, L.A., and Freetly, H.C. (2015). Rumen microbiome from steers differing in feed efficiency. *PloS one* 10**,** e0129174-e0129174.

Noel, S.J., Højberg, O., Urich, T., and Poulsen, M. (2016). Draft genome sequence of "Candidatus Methanomethylophilus" sp. 1R26, enriched from bovine rumen, a methanogenic archaeon belonging to the Methanomassiliicoccales order. *Genome announcements* 4**,** e01734-01715.

Noel, S.J., Olijhoek, D.W., Mclean, F., Løvendahl, P., Lund, P., and Højberg, O. (2019). Rumen and fecal microbial community structure of Holstein and

Jersey dairy cows as affected by breed, diet, and residual feed intake. *Animals* 9**,** 498.

Oltjen, J.W., and Beckett, J.L. (1996). Role of ruminant livestock in sustainable agricultural systems. *Journal of Animal Science* 74**,** 1406-1409.

Pacífico, C., Petri, R.M., Ricci, S., Mickdam, E., Wetzels, S.U., Neubauer, V., and Zebeli, Q. (2021). Unveiling the bovine epimural microbiota composition and putative function. *Microorganisms* 9**,** 342.

Paz, H.A., Anderson, C.L., Muller, M.J., Kononoff, P.J., and Fernando, S.C. (2016). Rumen bacterial community composition in Holstein and Jersey cows is different under same dietary condition and is not affected by sampling method. *Frontiers in microbiology* 7**,** 1206-1206.

Petri, R.M., Schwaiger, T., Penner, G.B., Beauchemin, K.A., Forster, R.J., Mckinnon, J.J., and Mcallister, T.A. (2013). Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis. *Applied and Environmental Microbiology* 79**,** 3744-3755.

Ren, Q., Si, H., Yan, X., Liu, C., Ding, L., Long, R., Li, Z., and Qiu, Q. (2020). Bacterial communities in the solid, liquid, dorsal, and ventral epithelium fractions of yak (Bos grunniens) rumen. *MicrobiologyOpen* 9**,** e963.

Schären, M., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., Urich, T., Breves, G., and Dänicke, S. (2017). Alterations in the rumen liquid-, particle- and epithelium-associated microbiota of dairy cows during the transition from a silage- and concentrate-based ration to pasture in spring. *Frontiers in microbiology* 8**,** 744-744.

Schliep, K.P. (2011). phangorn: phylogenetic analysis in R. *Bioinformatics* 27**,** 592-593.

Shabat, S.K.B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M.E., White, B.A., Shterzer, N., and Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *The ISME Journal* 10**,** 2958-2972.

Tan, R.S.G., Zhou, M., Li, F., and Guan, L.L. (2021). Identifying active rumen epithelial associated bacteria and archaea in beef cattle divergent in feed efficiency using total RNA-seq. *Current Research in Microbial Sciences* 2**,** 100064.

Van Gylswyk, N.O. (1995). Succiniclasticum ruminis gen. nov., sp. nov., a Ruminal Bacterium Converting Succinate to Propionate as the Sole Energy-Yielding Mechanism. *International Journal of Systematic and Evolutionary Microbiology* 45**,** 297-300.

Van Zanten, H.H.E., Mollenhorst, H., Klootwijk, C.W., Van Middelaar, C.E., and De Boer, I.J.M. (2016). Global food supply: land use efficiency of livestock systems. *The International Journal of Life Cycle Assessment* 21**,** 747-758.

Wallace, R.J., Rooke, J.A., Mckain, N., Duthie, C.-A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., and Roehe, R. (2015). The rumen microbial

metagenome associated with high methane production in cattle. *BMC genomics* 16**,** 1-14.

Weimer, P.J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: implications for engineering improved ruminal fermentations. *Frontiers in microbiology* 6**,** 296.

Wetzels, S.U., Mann, E., Pourazad, P., Qumar, M., Pinior, B., Metzler-Zebeli, B.U., Wagner, M., Schmitz-Esser, S., and Zebeli, Q. (2017). Epimural bacterial community structure in the rumen of Holstein cows with different responses to a long-term subacute ruminal acidosis diet challenge. *Journal of Dairy Science* 100**,** 1829-1844.

Wirth, R., Kádár, G., Kakuk, B., Maróti, G., Bagi, Z., Szilágyi, Á., Rákhely, G., Horváth, J., and Kovács, K.L. (2018). The planktonic core microbiome and core functions in the cattle rumen by next generation sequencing. *Frontiers in Microbiology* 9.

Yeoman, C.J., Fields, C.J., Lepercq, P., Ruiz, P., Forano, E., White, B.A., and Mosoni, P. (2021). In vivo competitions between Fibrobacter succinogenes, Ruminococcus flavefaciens, and Ruminoccus albus in a gnotobiotic sheep model revealed by multi-omic analyses. *mBio* 12**,** e03533-03520.

Young, J.W. (1977). Gluconeogenesis in cattle: significance and methodology1. *Journal of Dairy Science* 60**,** 1-15.

Yu, Z., and Morrison, M. (2004). Improved extraction of PCR-quality community DNA from digesta and fecal samples. *BioTechniques* 36**,** 808-812.

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., He, Y., and Zhang, H. (2017a). Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. *Frontiers in Microbiology* 8.

Zhang, R., Ye, H., Liu, J., and Mao, S. (2017b). High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Applied Microbiology and Biotechnology* 101**,** 6981-6992.

Zhou, M., and Hernandez-Sanabria, E. (2010). Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. *Applied and environmental microbiology* 76**,** 3776-3786.

Chapter 4

Time off feed affects enteric methane and rumen microbiome in sheep

Steven McLoughlin[1,2], Charles Spillane[2], Stuart Kirwin[1], Eoin Dunne[1], Edel O'Connor[1], Emily Roscam[1], Paul Smith[1], Nida admin[1], Fiona McGovern[1], Sinead M. Waters[1,2]

[1] Animal and Bioscience Research Department, Animal and Grassland Research and Innovation Centre, Teagasc, Athenry, Ireland

[2] Genetics and Biotechnology Laboratory, Agriculture and Bioeconomy Research Centre, Ryan Institute, University of Galway, University Road, Galway H91 REW4, Ireland

Keywords: Methane, Microbiome, Sheep, Portable Accumulation Chambers, Pasture, metagenomics, Amplicon, Time Off Feed (Min.5-Max. 8)

Abstract

Methane emissions from ruminant pasture-based production systems account for a significant proportion of agricultural greenhouse gas (GHG) emissions. Studies utilizing portable accumulation chambers (PACs) have shown that time off feed (TOF) has an influence on methane emissions, where TOF is defined in this study as the duration of time animals were taken from pasture to entering the PACs. In this study, we utilised PACs to determine the effect of TOF on daily methane output from sheep, and a meta-omics approach to investigate associated bacterial and archaeal populations in the sheep rumen. A total of 94 Belclare ewes grazed on permanent pasture were used for the purpose of this study. Methane emissions and rumen samples were collected over 2 days from animals spending varying TOF (TOF, 1hr n=24, 2hr n=24, 3hr n=22, 5hr n=12, 6hr n=12). 16S rRNA amplicon sequencing was used to taxonomically profile the rumen bacterial and archaeal communities. Whole shotgun metagenomic and metranscriptomic sequencing data from a subset of 17 samples were functionally profiled using Humann3. We demonstrate that daily methane emissions (g/d) (DME) decreased with increasing TOF (P<0.0001). Bacterial and archaeal community alpha diversity measures were unaffected by TOF ($P.adj$>0.1). PERMANOVA analysis revealed that bacterial community composition was affected by TOF (P<0.05). Maaslin2 (Microbiome Multivariable Associations with Linear Models) was used to test relationships between TOF and the abundance of microbial taxa (bacteria and archaea). Our findings demonstrate that core fibrolytic members of the rumen microbiome, including *Butyrivibrio*, *Pseudobutyrivibrio* and *Eubacterium cellulosolevens* at the genus level, and *Ruminococcus albus, Selenomonas ruminantium* and *Prevotella spp.*at the ASV (amplicon sequence variant) level, are negatively associated with TOF (P<0.05).

In contrast, non-fibrolytic bacteria, including *Eubacterium coprostanegenes, Succiniclasticum* and *Christensenellaceae R7 group*, were positively associated with TOF ($P<0.05$). The relative abundance of archaeal taxa at the genus and ASV levels were not influenced by TOF ($P>0.05$), although the relative abundance of overall archaeal and bacterial communities was found to increase and decrease with increasing TOF, respectively ($P<0.05$). TOF was found to have no effect on either metacyc pathways or gene families. However, when accounting for gene copy numbers the transcriptional activity of the hydrogenotrophic methanogenesis pathway tended to decrease with increasing TOF ($P=0.09$). Our study shows that TOF can influence methane emissions and the composition of the rumen microbiome, which has implications for methane-microbiome studies involving animals that spend variable amounts of time off feed prior to measurement.

Introduction

The ability of ruminant livestock to convert low quality plant matter into high-quality protein products fit for human consumption is critical to supporting food security (Oltjen and Beckett, 1996). However, ruminant production systems are an important source of anthropogenic greenhouse gas (GHG) emissions, particularly methane (Czerkawski, 1969; Johnson and Johnson, 1995), which is reported to have a global warming effect 28 times greater than that of CO_2 over a 100 year period and is a significant contributor to global warming and climate change (IPCC, 2014). The IPCC indicates that a reduction in global methane emissions is required to mitigate increases in global temperature (Arias et al., 2021). Enteric fermentation of feed is the primary source of methane emissions from agriculture, which accounts for approximately a third of total anthropogenic methane emissions and ~90% of all livestock derived methane emissions (Saunois et al., 2020). The demand for animal products is anticipated to rise in the future decades due to a growing global population and rising affluence, where dietary transitions are anticipated to lead to an increase in atmospheric methane emissions (Hayek and Miller, 2021). As a result, ruminant production systems confront challenging obstacles to reduce emissions while meeting the growing demand for animal protein products. Therefore, research on methods to reduce methane emissions from livestock production systems have emerged as a pressing necessity for countries to fulfil their emissions reduction pledges under the Paris agreement (Wollenberg et al., 2016).

Degradation of plant matter in the rumen is time dependent driven by successive microbial colonisation events (Huws et al., 2016) and the gradual breakdown of plant polysaccharides. Plant cell walls are organised in such a way that cellulose, the most abundant polysaccharide component in plants, is caged in an outer matrix of hemicellulose, lignin and pectin (Morrison, 1979). The rate and the extent to which cellulose is utilised in the rumen is known to be dependent on the interaction and digestibility of the ligno-hemicellulose complex (Weimer, 1992;Hatfield, 1993). Enzymatic hydrolysis of cellulose would therefore increase with time as the ligno-hemicellulolytic matrix is depolymerised and cellulose

becomes more exposed to cellulase activity. (Waters et al., 2020). In the rumen, the degradation of hemicellulose is carried out by both highly specialised cellulolytic bacteria (e.g *F. succinogenes, R. flavefacians, R. albus*) and specific non-cellulolytic bacteria (e.g *B. fibrosolvens, P. ruminis, P. ruminantium)* (Weimer, 2022). These bacteria produce an array of cellulase and/or hemicellulases, which hydrolyze glycosidic linkages in plant polysaccharides producing shorter oligosaccharide fragments, which are subsequently fermented by ruminal microbes to produce VFAs. In addition, fermentation end-products such as hydrogen, formate, carbon dioxide, acetate and various methyl-compounds are utilised by methanogenic archaea for the production of methane.

Methane production in the rumen is inextricably linked and dependent on the coordinated action of both bacteria and archaea. Methane, which cannot be utilised by the host for energy, is expelled to the atmosphere and is reported to result in a loss of dietary energy to the host (Giger-Reverdin and Sauvant, 2000). Diet is an important factor influencing methane emissions from ruminant livestock. The quantity, quality and type of feed are all known to exert an effect on methane emissions. For instance, increasing the ratio of forage to concentrates has been shown to increase methane emissions in domestic livestock (Aguerre et al., 2011; Li et al., 2019). Furthermore, previous work by our group found that methane emissions fluctuate in tandem with diurnal feeding patterns in cattle (Smith et al., 2021). Similar findings were also reported in sheep (Lockyer, 1997). In addition, diet also has a substantial impact on the composition of the rumen microbiota (Carberry et al., 2012; Henderson et al., 2015). Findings from the global rumen census show that animals offered forage and concentrate diets harbor distinct bacterial communities (Henderson et al., 2015). In many temperate regions of the world, including Ireland and New Zealand, ruminant livestock are predominantly forage-fed on pasture-based systems (Knaus, 2016). Consequently, methane emissions from pasture-based systems can account for a significant proportion of national GHG emissions. However, there are a dearth of methane-microbiome related studies from pasture grazed animals, particularly in sheep. This may partly be due to the difficulties in accounting for dietary intake (Beauchemin et al., 2022). Indeed, previous research in sheep has demonstrated that dry matter intake (DMI) is the principal factor driving methane production from the rumen (Muetzel and Clark, 2015). Nevertheless, methane-microbiome studies from pasture-fed animals are required to better understand the factors influencing methane emissions from current agricultural production systems and guide the development of future methane mitigation strategies.

The ability to accurately quantify methane is required for methane mitigation solutions. A range of approaches have been developed to quantify methane emissions from ruminant livestock (Zhao et al., 2020). Most methane-microbiome studies have used respiration chambers (RCs) due to the precision and repeatability of measurements and the ability to control DMI (Kittelmann et al., 2014; Shi et al., 2014; Muetzel and Clark, 2015). However, because animals are not measured under on farm circumstances, RCs may underestimate daily methane output relative to what would occur on a farm, due to altered feeding

behaviour and feed intake (Bickell et al., 2014; Jonker et al., 2014). Alternative technologies have been developed for estimating methane emissions on-site, including SF_6 tracers (Johnson et al., 1994), GreenFeed systems (Rapid City, South Dakota; C-Lock Inc. (Hammond et al., 2015)) and portable accumulation chambers (PACs) (Jonker et al., 2018). In the present study, PACs were utilised due to their portability, suitability for small ruminants, high throughput, and correlation with RCs (Jonker et al., 2018). One notable finding from PAC studies is that the length of time sheep are kept off feed has a significant impact on methane output (Robinson et al., 2014). However, the effect of time off feed (TOF) on the associated rumen microbial populations have not yet been explored. To address this knowledge gap, the objectives of this study were to utilise PACs to investigate the effect of TOF on methane emissions and to use meta-omics techniques to assess the influence of TOF on rumen bacterial and archaeal communities in pasture-grazed sheep.

Methods

Animal model, methane measurements and rumen sampling

All animal procedures used in this study were conducted under experimental license from Ireland's Health Products Regulatory Authority (HPRA, Licence No: AE19132/P116) in accordance with the European Union (EU) protection of animals used for scientific purposes regulations 2012 (S.I. No. 543 of 2012).

A total of 94 Belclare non-lactating ewes were used for the purpose of this study. All animals were grazed on permanent pasture (perennial ryegrass) with a cover of approximately 5cm at Teagasc Athenry, Co. Galway. Ewe age ranged from 2-7 years with the mean age per ewe of 3.5 years. Methane was measured over 2 consecutive days using PACs (x12). On each day ewes were taken from pasture at 8:00am and held/enclosed in a concrete yard before entering the PAC. Prior to entering PACs all ewes were weighed to determine animals body weight (BW).

The PAC used in this study were air tight chambers made of aluminum sheets and with an internal volume of 853L (1.1 m width × 1.07 m average height × 0.77 m length). Each chamber was fitted with a manometer to monitor pressure and leaks, and a sampling valve to allow methane concentrations to be recorded. Methane concentrations within the chamber were measured using an RKI Eagle2 monitor (Weatherall Equipment and Instruments Ltd, UK), whereby the probe of the detector was inserted into the sampling valve and a stable reading was recorded. After completion of the gaseous measurements the sampling valve was closed immediately. To ensure the accuracy of gas measurements, daily gas checks (at the start and end of the day) of the Eagle 2 monitor were conducted using standard calibration gases of 100 ppm, 1,000 ppm, and 5,000 ppm methane.

For PAC methane measurements, 5 lots (or groups) of 12 ewes were randomly selected from a cohort of 60, and 3 lots of ewes from a cohort of 34 ewes on day 1, and day 2, respectively, and randomly assigned to 1 of the 12 individual PACs.

PAC measurements did not start until 1 hour after animals were taken off-pasture (approx. 9:00 a.m.). Methane output in the chamber was measured at 0, 25 and 50 mins (O' Connor et al., 2021). Final methane readings from each lot on day 1 were obtained at approximately 9:50 a.m., 10:50 a.m., 11:50 a.m., 12:50 p.m., and 13:50 p.m., corresponding to 1,2,3,5 and 6 hours off feed. Similar operations were carried out on day 2, with final methane emissions recorded at 9:50 a.m., 10:50 a.m., and 11:50 a.m., corresponding to 1,2 and 3 hours off feed. As soon as the animals were removed from the PACs, rumen samples were obtained using a transoesophageal intubation apparatus and transferred to 2x25ml tubes. 500ul aliquots of rumen fluid were aliquoted from 25ml samples and placed in 1ml tubes for DNA extraction. All samples were snap frozen in liquid nitrogen, then stored at -20 °C during transit before being stored at -80 °C until DNA was extracted.

Gaseous measurements of CH_4 obtained for each animal were converted to liter/hour (l/hour) using the equation:

$$CH_4(l/hour) = \left(\frac{(Methane\ x - Methane\ y)}{(Time\ x - Time\ y)} \times 60 ¿ \times (853 - (Bodywight)) ¿¿ 1{,}000{,}000\right)$$

'CH_4 (l/hour)' is the CH_4 emissions quantified in liters per hour, '$Methane_x$' is methane output in ppm at time point x, '$Methane_y$' is CH_4 output in ppm at time point y, '$Time_x$' is the time at time point x, '$Time_y$' is the time at time point y, and 'live-weight' is the live-weight of the animal in kg

Gas volume obtained in liters per hour were extrapolated to grams per day values using an equation analogous to the ideal gas law, as described by Jonker et al. (2018).

$$CH_4\,(g/day) = CH_4(l/hour) \times (Press \times 0.1)/(8.3145 \times (Temp + 273.15)) \times 16 \times 1440$$

'CH_4 (l/hour)' is CH_4 emissions quantified in liters per hour, 'press' is the pressure expressed in hectopascals and 'temp' is the temperature expressed in degrees Celsius, '16' is the molecular weight of CH_4, and '1,440' is the number of minutes in the day.

Microbial DNA extraction, 16S rRNA amplicon library preparation and sequencing

Microbial DNA was extracted from 500 µl of frozen rumen fluid sample (n=90) using a Qiagen DNAeasy Powersoil kit (Qiagen, Manchester, UK). DNA quality was assessed using agarose gels (0.8%) and a 1-kb DNA ladder (Bioline GmbH, Luckenwalde, Germany). The concentration of extracted DNA was quantified on the Nanodrop 1000 spectrophotometer and diluted to 100ng/µl before running agarose gels. A blank extraction control, subject to the same procedures as rumen fluid samples, was performed for each extraction kit (n=2). The absence of reagents contamination was visually confirmed on agarose gels and with the use of the Nanodrop.

Amplicon libraries were generated targeting the V4 hypervariable region of the 16S rRNA gene using the 515F/806R primers (Caporaso et al., 2011). Two rounds of PCR amplification were performed, as outlined in the *Illumina Miseq 16S Sample Preparation Guide,* using Herculase II Fusion DNA Polymerase Nextera XT Index V2 Kit (Illumina, San Diego, CA, United States). Following this libraries were pooled and sequenced Illumina MiSeq using the 500 cycle version 2 kit (Illumina, San Diego, CA, United States). 16SrRNA amplicon libraries were generated and sequenced by Macrogen (Seoul,Rep. Korea).

Note: A total of 90 rumen content samples were available for amplicon processing and analysis (TOF, 1h *n*=23, 2h *n*=24, 3h *n*=21, 5h *n*=12, 6h *n*=11).

Metagenomics and metatranscriptomic library preparation and sequencing

To enhance our understanding of the effects of TOF on the activity of the rumen microbial community, 17 samples (TOF-1h *n*=3; -2h *n*=4; -3h *n*=4; -5h *n*=3; -6h *n*=3) collected on day 1 of sampling were subjected to metagenomics and metatranscriptomic sequencing.

Metagenomic libraries (rumen n=17; controls n=4) were prepared using the Illumina TruSeq DNA PCR-Free kit and sequenced on an Illumina Novaseq (150bp PE) sequencing instrument by Macrogen (Seoul,Rep. Korea), at a targeted sequencing depth of 100M PE reads per sample. All negative controls were pooled and a single library was prepared for sequencing. Three positive internal sequencing standards were included with the run and consisted of the ZymoBIOMICS™ Microbial Community Standard (Zymo Research Corp., Irvine, CA, United States) and isolated DNA from *Methanobrevibacter olleyae* and *M. millerae*. The number of paired end reads generate per sample ranged from 22.3 and 50Gbps, excluding the negative control.

Under liquid nitrogen, rumen samples were ground to a fine powder using a pestle and mortar. Microbial RNA was extracted from 200ng of homogenised frozen rumen digesta using the Qiagen RNeasy plus kit (Qiagen, Manchester, UK). Following this, extracted RNA was DNase treated using a TURBO DNA-free™ Kit (Thermo Fisher Scientific, MA, USA) to remove genomic DNA. DNase treated RNA was then purified with Zymo RNA clean and concentrate kit (Zymo Research Corp, Irvine, CA, USA). RNA integrity (RIN) was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) with the concentration and purity of extracted RNA assessed using the Qubit™ RNA BR Assay Kit (BioSciences, Dublin, Ireland) and Nanodrop 1000 spectrophotometer, respectively. The absence of genomic DNA was confirmed by qPCR targeting the rumen methanogen population using the Mlas forward/ mcrA reverse primers described by Poulsen et al. (2013). The high Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA) was used for cDNA synthesis with qPCR performed using the Fast SYBR® Green Master Mix (Thermo Fisher Scientific, MA, USA). A blank extraction control, subject to the same procedures as rumen fluid samples, was performed for each extraction kit. The absence of reagents contamination was visually confirmed with the Agilent 2100

Bioanalyzer and with the use of the Nanodrop and Qubit. All negative controls were pooled and a single library was prepared for sequencing. Extracted RNA, with a RIN value of >7 were forwarded to Macrogen (Seoul, Rep. Korea) for library preparation and sequencing. Metatranscriptomic libraries were prepared with the use of the Illumina Truseq Stranded Total RNA library preparation with rRNA depletion conducted with the use of the Ribozero GOLD rRNA removal protocol and NEB bacterial rRNA removal kit (New England Biolabs, Ipswich, UK). Following this sequencing was completed on an Illumina NovaSeq (100bp PE) at a targeted sequencing depth of 50M PE reads per sample.

Bioinformatic analysis

Amplicon processing

16S rRNA gene amplicons were processed in R (version 3.4.2) using DADA2 (version 1.9.0) (Callahan et al., 2016) and submitted to the pipeline as described with minor alterations (https://benjjneb.github.io/dada2/tutorial.html). The average Q score for each sample was examined, and quality checks were then performed to maintain Q scores of > 30 for both forward and reverse reads. Forward reads were trimmed to a length of 240bp and reverse reads trimmed to 200bp. The removal of primer sequences was conducted using the trimLeft function. Identical sequences were combined using the dereplication function followed by the merging of forward and reverse reads. Following this an amplicon sequence variant (ASV) table was constructed after which chimeric sequences were removed and taxonomy assigned to sequences variants using the SILVA database (version 132) (Quast et al., 2012; Glöckner, 2019). Sample metadata, sequence taxonomy, and ASVs were combined into a phyloseq object using phyloseq (version 1.22.3) (McMurdie and Holmes, 2013) for downstream analysis.

Metagenomic and metatranscriptomic analysis

Raw sequenced Illumina paired-end reads were quality assessed using FASTQC (version 0.11.8) (Andrews, 2010). Illumina Truseq Adapters, low quality bases and poly-g tails were removed using the FASTP algorithm (Version X) (Chen et al., 2018). Processed reads were mapped to the sheep reference genome (Oar v4.0) using bowtie2 (Langmead and Salzberg, 2012) and mapped reads (~0.5%) were subsequently removed to deplete host contamination. Metaphlan 3 was used to assess the performance of the metagenomic pipeline by comparing the microbiological relative abundance of the ZymoBIOMICS™ standards to the theoretical relative abundance, which showed high correspondence (**Supplementary Figure 1**). For metatranscriptome data, SortMeRNA (v2.1b) (Kopylova et al., 2012) was used to separate rRNA and tRNA from mRNA reads. Trimmed and filtered metagenomic and metatranscriptomic data were functionally profiled using Humann3 (Franzosa et al., 2018), which was run with default parameters with translated search against the UniRef50 database. Taxonomy profiles derived from metagenome data were utilised when running Humann3 on metatranscriptome data.

Community and Statistical analysis

Compositional and statistical analysis were conducted using various libraries/packages in RStudio (running R version 4.2.0). The effect of TOF, ewe age and bodyweight on DME was tested using a linear model. Prior to taxonomic analysis, ASVs unassigned at the kingdom and phylum level, and ASVs with a relative abundance <0.001% and not prevalent in >5% of samples (n=6) were filtered from the data. Bacterial and archaeal ASV data were separated for the independent analysis of each microbial community. Alpha and beta diversity analysis was conducted on bacterial (genus and ASV level) and archaeal (ASV level) communities with counts normalised using the scaling with ranked subsampling (SRS) method (Beule and Karlovsky, 2020). For alpha diversity, Shannon and inverse Simpson diversity indices for each sample were obtained using diversity function from the R/Bioconductor package Vegan (Oksanen et al., 2013). Microbiome Multivariable Associations with Linear Models (Maaslin2) (Mallick et al., 2021) was used test associations between TOF and alpha diversity measures. For beta diversity analysis, Bray Curtis distances were used to calculate the dissimilarity in community composition between samples. The effect of TOF on bacterial and archaeal community composition was tested using the PERMANOVA test with 9999 permutations using the adonis function in Vegan (Oksanen et al., 2013). To profile dominant taxa at the phylum and genus levels feature counts were converted to relative abundances. Maaslin2 was used to test for associations between bacterial (phylum, genus, and ASV taxonomic ranks) and archaeal (genus, and ASV taxonomic ranks) taxa and TOF. This analysis was limited to taxa with relative abundances >0.01% and prevalence >50%. Basic local alignment search tool (BLAST) against the rRNA/ITS database was used to further classify taxa at the ASV level that exhibited a significant association with TOF. Similarly, relationship between gene families and metacyc pathways with TOF were tested Maaslin2. The BH method was used to adjust p-values for all multiple hypothesis testing, and significant relationships were defined as having a $P.adj < 0.05$. For all microbial statistical analysis ewe age was included as a fixed effect to control for variations attributable to animals age.

Results

Methane output from sheep is affected by time off feed

Animals body weight (BW) ranged from 63.0kg to 106.5kg, with a mean BW of 78.98kg, a median of 78.0kg and a standard deviation of 8.38kg. Ewe BW was found to have a significant effect on DME (lm, $P=0.04$). DME ranged from 3.88g/d to 13.88g/d, with a mean of 7.82g/d, a median of 7.40 and a standard deviation of 2.20g/d. Average DME was 9.43, 8.13, 7.31, 6.55 and 6.14 (g/d) for 1, 2, 3, 5 and 6 hours off feed, respectively, and was found to be significantly affected by TOF (lm, $P<0.0001$) (Figure 4.1).

Over 10,000 Unique Amplicon Sequence Variants (ASVs) identified in the rumen of sheep

Following data processing, quality filtering and chimera removal, and a total of 8,630,693 amplicon reads remained, representing 10,663 uniquely identified

ASVs. The min number of reads per sample was 68,824 and the max number of reads was 135,982, with an average read count per sample of 95,896. After removal of unassigned taxa at the kingdom and phylum level a total of 8,537,203 amplicon reads remained for analysis, representing 9,457 uniquely identified ASVs remained, 65 archaea ASVs and 9,392 bacteria ASVs. The min number of reads was 68,005 and the max number of reads was 134,632, with an average number of reads per sample of 94,857. Lastly, after filtering ASVs with a relative abundance <0.001% and not present in >5% of samples a total of 8,252,081 reads remained, representing 3468 uniquely identified ASVs; 33 archaea ASVs and 3435 bacteria ASVs.

Bacterial community composition in sheep is affected by time off feed

Bacterial and archaeal community Shannon and inverse Simpson diversity measures were not affected by TOF, when assessed at the genus and ASV levels (Table 4.1). According to our beta diversity analysis, TOF had no impact on the bacterial and archaeal community compositions at the ASV level. (Permanova: Bacteria P=0.07, Archaea P=0.9). However, bacterial community composition at the genus level was found to be significantly affected by TOF (Permanova, P=0.008) (Table 4.2). Pairwise permanova revealed that differences in bacterial community composition was primarily driven by differences between 1 and 6 hours off feed (Pairwise Permanova, P=0.03) (Supplementary Table 4.1).

Firmicutes and Prevotella dominate the rumen microbial communities at phylum and genus levels

In the rumen, bacteria made up on average of 92.8% of the microbial population, whereas archaea made up 7.2% of the microbial population. 3,435 bacterial ASVs agglomerated to 20 phyla, 37 classes, 75 orders, 121 families and 236 genera. Our findings showed that Firmicutes (mean, 54.2%), Bacteroidota (mean, 38.3%), Verrucomicrobiota (mean, 2.0%), Actinobacteriota (mean, 1.3%) and Fibrobacterota (mean, 0.6%) were the 5 most abundant bacterial phyla. *Prevotella* (mean, 23.2%), *Christensenellaceae R7 gut group* (mean, 8.1%), an unclassified *Lachnospiraceae* (mean, 5.8%), NK4214 group (mean, 3.8%) and *Rikenellaceae RC9 gut group* (mean, 3.7%) were identified as the most abundant bacterial genera (Figure 4.2). Additionally, 33 archaeal ASVs agglomerated to 2 phyla, 2 classes, 2 orders, 2 families and 3 genera. Euryarchaeota (99.3%) and Thermoplasmatota (0.7%) were the three archaeal phyla identified, while *Methanobrevibacter* (88.3%), *Methanosphaera* (11.1%) and an unclassified genus belonging to family Methanomethylophilaceae (0.6%) were the 3 genera identified.

Microbial associations with Time off Feed (TOF)

The relative abundance of the overall bacterial community was found to negatively associate with TOF (Maaslin2, *P.adj* <0.0001), while the relative abundance of archaeal community was found to positively associate with TOF (*P.adj*<0.0001) (Table 4.3). At the phylum level, the relative abundance of Verrucomicrobiota (*P.adj* = 0.004), Actinobacteriota (*P.adj* = 0.02) and

Planctomyceota (*P.adj*, 0.02) were found to positively associate with TOF (Table 4.3). At the genus level the abundance of FD2005 (Lachnospiraceae) (*P.adj* = 0.04), *Eubacterium cellulosolvens group* (*P.adj* = 0.02), *Anaeroplasma* (*P.adj* = 0.02), *Shuttleworthia* (*P.adj* = 0.01), *Butyrivibrio* (*P.adj* = 0.02), *Pseudobutyrivibrio* (*P.adj* = 0.01), *Selenomonas* (*P.adj* = 0.04) and unclassified Ruminococcaceae (*P.adj* = 0.02) were found to negatively associate with TOF. The relative abundance of *Succiniclasticum* (*P.adj* = 0.04), Christensenellaceae R-7 group (*P.adj* = 0.02), *Ruminococcus gauvreauii* group (*P.adj* = 0.004), *Eubacterium nodatum* group (*P.adj* = 0.04), *Eubacterium coprostanoligenes* group (*P.adj* = 0.02), WCHB1.41 (class Kiritimatiellae) (*P.adj* = 0.004), *P-1088.a5 gut group* (family *Pirellulaceae*) (*P.adj* = 0.04) and an unclassified Oscillospirales (UCG-010) (*P.adj* = 0.006) were found to positively associate with TOF (Table 4.3). At the ASV level the abundance of 10 bacterial ASV associated negatively with TOF, while 7 associated positively with TOF. Results are summarised in Table 4.4.

Activity of methanogenesis pathway decreased with increasing TOF

We performed an exploratory analysis on metabolic pathways and gene families utilising meta-genomic and -transcriptomic data from an ongoing larger study (collected on day 1 of sampling) (n=17: TOF-1h *n*=3; -2h *n*=4; -3h *n*=4; -5h *n*=3; -6h *n*=3). While a significant association between methane and TOF was found (lm, *P*=0.04) (Supplementary Figure 4.2), no significant effects were observed between between TOF and metabolic pathways, or TOF and gene families (*P*>0.05) were observed in the current study (Supplementary Figure 4.3 and 4.4). Although not significant, the abundance of the methanogenesis from hydrogen and carbon dioxide pathway (hydrogenotrophic methanogenesis) (Maaslin2, *P*=0.12) and the abundance of the *MCRA* gene (Maaslin2, *P*=0.11) tended to increase with increasing TOF. However, when accounting for gene copy number (RNA/DNA) the transcript abundance of hydrogenotrophic methanogenesis pathway (Maaslin2, *P*=0.09) and *MCRA* gene (Maaslin2, *P*=0.14) tended to decrease with increasing TOF (Supplementary Figure 4.5 and 4.6).

Discussion

This study aimed to examine the influence of TOF on DME and the composition of rumen bacterial and archaeal populations in pasture-fed sheep. The results demonstrated significant effects of TOF on both DME and the rumen microbiome. Robinson et al. (2014) analysed PAC methane data collected from pasture fed sheep and found methane emission to linearly decrease with increasing TOF (Robinson et al., 2014). Similarly, Sollinger et al. (2018) found methane emissions to continually decrease from 1 hour post feeding in lactating dairy cows (Söllinger et al., 2018). The findings from the current study corroborate with previous studies in both sheep and cattle where methane output linearly decreased with increasing TOF. Our results showed that there was a ~35% decrease in average DME between sheep held 1 and 6 hours off feed. Although, TOF has been shown to influence methane emissions (Robinson et al.,

2014;Söllinger et al., 2018), no research has yet investigated accompanying changes in the associated rumen microbiome in sheep.

Bacterial and archaeal population were taxonomically profiled in this study using 16S rRNA sequencing. Consistent with previous studies on ruminant livestock, our analysis identified Firmicutes and Bacteroidota as the two most prevalent bacterial phyla, which together accounted for ~92% of the rumen microbial population. Interestingly, Proteobacteria, which is commonly reported as the third most dominant bacterial phylum in the rumen, was not identified among the top 10 most abundant phyla in the present study, accounting for only ~0.3% of the bacterial population. Verrucamicrobiota was found to be the third most abundant bacterial phyla. Verrucomicrobiota has been identified in the rumen and its abundance shown to positively associate with cellulose content in the diet (Gharechahi et al., 2021). Thus, the prominence of Verrucomicrobiota in the current study could be dietary related. At the genus level, *Prevotella* was found to be the most dominant bacterial genus accounting for ~23% of the bacterial community followed by *Christensenellaceae R7 group* (8.1%) and unclassified *Lachnospiraceae* (5.8%). Our findings are in line with findings from the global rumen census (Henderson et al., 2015), whereby *Prevotella* was found to account for on average 21.5% of the rumen bacterial population and unclassified *Lachnospiraceae* represented 6.0%. While *Christensenellaceae* are core representatives of the rumen microbiome in sheep (Henderson et al., 2015), the prominence of the *Christensenellaceae R7 group* in the current study could also be dietary.

The effect of TOF on the rumen microbiome has previously been analysed in cattle. Kim et al. (2019) examined the effects of 24hr fasting on the rumen microbiota in Holstein steers fed a rice straw and concentrate diet (Kim et al., 2019). Kim et al. (2019) showed that fasting had an effect on the abundance of bacteria but not archaea, fungi or protozoa when examined using Denaturing Gradient Gel Electrophoresis (DGGE). In another study, Welch et al. (2021) examined the effects of pre-slaughter fasting on the rumen bacterial population in cattle fed a finishing diet using 16S rRNA gene sequencing. The researchers observed significant increase in bacterial community diversity following a 24 hour fast in HRFI and LRFI cohorts and changes in bacterial and archaeal abundances. Moreover, no significant impact on VFA concentrations were observed. However, Welch et al. (2021) did note a marked reduction in butyrate concentrations, although not statistically significant (Welch et al., 2021). Taken together, the results from both studies indicate that TOF can influence the rumen microbiome and fermentation profiles following a 24h fast. Over a shorter timeframe, Söllinger et al. (2018) found that rumen bacterial community remained relatively stable following 6 hours post feeding in 4 lactating dairy cows (Söllinger et al., 2018). However, the impact of time off feed (TOF) on the rumen microbiome in pasture-grazed sheep remains largely unexplored. To address this gap, we employed 16S rRNA sequencing to investigate the bacterial and archaeal populations in pasture-fed sheep during the period of 1-6 hours off feed. Our analysis revealed that TOF did not significantly affect the alpha diversity measures for both bacterial and archaeal communities. However, beta

diversity analysis revealed that the composition of the bacterial community was shifting with increasing TOF, mostly driven by compositional dissimilarities between 1 and 6 hours off feed. Thus, the findings from our study aligns with previous work by Kim et al. (2019) and Welch et al. (2021) in that fasting can impact on bacterial community composition in the rumen. The effects of TOF on the sheep rumen microbiota composition became apparent after approximately 6-7 hours without feed. The observed compositional changes in the bacterial community composition during increasing TOF could potentially be attributed to changes in substrate availability. As sheep remain off feed, there is a decline in the availability of nutrients and substrates in the rumen, which may trigger shifts in the growth and metabolism of specific bacterial taxa, subsequently influencing the overall community composition.

In the current study, at both the genus and ASV level, the abundance of core ruminal bacteria primarily involved in the degradation and utilsation of hemicellulose were found to negatively associate with TOF, including species of *Prevotella* and *Pseudobutyrivibrio*, *Butyrivibrio fibrisolvens, Ruminococcus albus* and *Eubacterium cellulosolvens*. *Prevotella spp.* belong to the most dominant genus in the rumen and are capable of utilising a variety of substrates for energy, including starch, protein, peptides, hemicellulose, and pectin (Flint et al., 2000;Emerson and Weimer, 2017). In *in vitro* studies, *Prevotella* species have been shown to breakdown plant cell wall hemicellulose components xylan, beta-glucan, glucomannan, and xyloglucan (Emerson and Weimer, 2017). *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio spp*. are recognised highly efficient hemicellulolytic degraders in the rumen (Grilli et al., 2015; Emerson and Weimer, 2017). They possess a large repertoire of polysaccharide degrading enzymes principally involved in the breakdown xylans and pectin (Palevich et al., 2019). *Ruminococcus albus* comprise strains that are both cellulolytic and hemicellulolytic, however, microbiological studies have reported that *R. albus* may rely more on hemicellulose rather than cellulose for growth and survival in the rumen (van der Toorn and van Gylswyk, 1985). *Eubacterium cellulosolvens* also possesses hemicellulolytic and cellulotyic activities through the production of a diverse set of glycosidic hydrolases (Luís et al., 2011). However, while known to be celluloltyic Anderson and Blair (1996) found that cellulose metabolism by *E. cellulosolvens* was inhibited in the presence of a glucose analogue, leading the authors to suggest that glucose could be the preferred substrate (Anderson and Blair, 1996). *Selenomonas ruminantium* was among the non-fibrolytic bacteria negatively associating with TOF. Although *S. ruminantium* is involved in the metabolism of non-structural carbohydrates, multiple investigations have revealed that *S. ruminantium* interacts with fibrolytic bacteria to enhance fibre digestion and utilsation (Sawanon and kobayashl, 2006; Sawanon et al., 2011). For instance, when grown on a variety of fiber sources the degradative abilities of *Ruminococcus flavefacians* were found to significantly increase when co-cultured with *S. ruminantium,* as compared to *R. flavefacians* mono-cultures (Sawanon and kobayashl, 2006). Thus, *S. ruminantium's* negative correlation with TOF may be due to its close interaction with fibrolytic bacteria. When considered collectively, these results could be a

natural reflection of, and happening as a result of, the gradual breakdown of hemicellulose and exposure of cellulose overtime.

A number of bacteria negatively associating with TOF have previously been linked with increased methane emissions in sheep and cattle. *In vivo* findings from our group and others have provided associations between *Butyrivibrio*, *Pseudobutyrivibrio* and *Ruminococcus* with higher methane emitting animals (Kittelmann et al., 2014; Auffret et al., 2018; Smith et al., 2022). Moreover, *in vitro* studies have verified symbiotic interaction between hydrogen and formate producing *Butyrivibria* and *Ruminococcus* species and hydrogen and formate utilising *Methanobrevibacter* species. For instance, Leahy et al. (2010) observed a >2 fold increase in methanogneseis genes when *M. ruminantium* was co-cultured with *Butyrivibrio proteoclasticus* grown in xylan compared to *M. ruminantium* grown in monocultures with hydrogen and carbon dioxide. (Leahy et al., 2010). Furthermore, Ng et al. (2017) demonstrated that adhesion proteins encoded by *M. ruminantium* are capable of attaching to *Butrivibrio* (Ng et al., 2016), indicating a potential evolutionary mechanism facilitating hydrogen transfer between these hydrogen generating and hydrogen using microbes. In addition, hydrogen transfer from *Ruminococcus albus* to methanogens and subsequent methane production have also been observed *in vitro* (Pavlostathis et al., 1990; Hino et al., 1995). Therefore, it is probable that the reduction in methane emissions with increasing TOF may be related to a decrease in hydrogen production and transfer from fibrolytic bacteria to methanogens.

Plant biomass in the rumen is broken down into monomeric and oligomeric components, which are subsequently fermented by a variety of rumen microorganisms (Stevens and Hume, 1998). In the current study, bacterial taxa at the genus and ASV level showing positive associations with TOF are primarily non-fibrolytic and specialised in the fermentation of oligosaccharides, amino acids, and lipids. At the genus level, TOF was found to promote the abundance of *Eubacterium coprostanoligenes* group, *Eubacterium nodatum* group, *Ruminococcus gauvreauii* group, *Succiniclasticum*, WCHB1-41 (phylum Virrucomicrobiota), Family Oscillospiraceae_UCG-010, Christensenellaceae R7 group, and p-1088-a5 gut group (family Pirelluaceae). Culture-based analysis suggests that *E. coprostanoligenes* plays a role in converting cholesterol to coprostanol, and shown to weakly ferment cellobiose, fructose, and glucose, producing acetic, fomic, and succinic acids (Freier et al., 1994). *E. nodatum* is a known amino acid-metabolizing, acetate- and butyrate-producing, non-saccharolytic bacteria (Uematsu and Hoshino, 1996). *Ruminococcus gauvreauii* group are principally involved in the metabolism of simple sugars and produce acetate as a primary end product (Domingo et al., 2008). Unclassified *WCHB1-41* genus is a member of the Kiritimatiellae class within the phylum Verrucomicrobiota. While, the metabolic activities of *WCHB1-41* are not presently understood a recent in vitro study found *WCHB1-41* abundance inversely correlated with butyrate production and positively correlated with acetate and propionate production (Zhou et al., 2022). Additionally, the abundance of Kiritimatiellae was found negatively correlated with DMI in sheep (Zhang et al., 2021), which is in line with the findings from the current study.

Christensenellaceae are known to metabolise a variety of sugars producing acetate and butyrate as fermentation end-products (Morotomi et al., 2012). Some members of the *Christensenellaceae* family have been shown to possess the fibrolytic digesting enzymes beta-arabinosidase, beta-galactosidase, and beta-glucosidase (Morotomi et al., 2012), suggesting that a *Christensenellaceae R7 group* also have fibre degradation abilities in the rumen. *Succiniclasticum* is a specialised bacteria that plays a key role in the rumen by fermenting succinate and converting it to propionate (Van Gylswyk, 1995). At the ASV level, *Mediterraneibacter lactaris*, *Faecalimonas umbilicata, Synthrococcus sucromutans Kiritimatiella glycovorans* and *Olsenella intestinalis* showed positive associations with TOF*. Syntropococcus sucromutans* is an acetogenic bacterium that ferments carbohydrates or pyruvate in the presence of formate (Doré and Bryant, 1990). *Olsenella intestinalis* is a recently identified bacterium isolated from cow faeces; it is catalase-negative and possesses alkaline phosphatase, beta-glucosidase, and arginine dihydeolase (Guan et al., 2022)*. Faecalimonas umbilicata* is a saccharolytic bacterium associated with acetate production and vitamin B12 biosynthesis (Sakamoto et al., 2018). *Mediterraneibacter lactaris* is a carbohydrate-metabolizing bacterium that has been known to quickly ferment lactose (Togo et al., 2018). *Kiritimatiella glycovorans* is a member of the phylum Viruccomicrobiota and ferments simple carbohydrates and sulfated polysaccharides. (Spring et al., 2016; van Vliet et al., 2020).

Although, archaea represent ~1-4% of the microbial population in the rumen (Wallace et al., 2017), they are the sole producers of methane (Leahy et al., 2013). Therefore, it is possible to reason, at least on a simplistic level, that the relative abundance of archaea in the present study would decrease in conjunction with the observed decrease in methane emissions. However, the relative abundance of the archaeal community was found to increase with increasing TOF, contrasting with the decrease in CH_4 emissions. A possible explanation for the increasing relative abundance of the archaeal community may be due to a decreasing relative abundance of the bacterial community. Alternatively, studies in cattle have found that the abundance of methanogens increased in the rumen of cattle under restricted dietary intake (McCabe et al., 2015), which was suggested to be related to a slower passage rate of feed through the rumen (Janssen, 2010; McCabe et al., 2015). Moreover, previous research in sheep have found no clear associations between the overall archaeal abundance and methane emissions (Kittelmann et al., 2014; Shi et al., 2014). It has been considered that the composition of the archaeal community at the species level is more closely related to methane emissions (Tapio et al., 2017). However, in the current study no significant relationships were found between TOF and the abundance of archaeal taxa at either the genus or ASV taxonomic ranks. Methane is produced via three pathways in the rumen; the hydrogenotrophic, aectoclastic and methylotrophic pathways (Lambie et al., 2015). The hydrogenotrophic pathway is the dominant methanogenesis pathway (Wirth et al., 2018) that converts carbon dioxide to methane using electrons from hydrogen or, to a lesser degree, formate (Friedman et al., 2017). Although not found to be significant, our preliminary research revealed that, like archaeal

abundance, the abundance of MCRA genes and the hydrogenogenotrophic methanogenesis pathway tended to increase with rising TOF. However, when transcript abundances were adjusted for gene copy number, the rate of methanogenesis tended to decrease with rising TOF. This finding could help to explain why methane emissions were decreasing with TOF in the current study. However, more research with a larger sample size is needed. Indeed, earlier research by Shi et al. (2014), found differences in the transcriptional abundance of the hydrogenotrophic methanogenesis pathway between high and low methane emitting sheep, despite finding no differences in overall archaeal abundance or gene abundance of enzymes involved in methanogenesis (Shi et al., 2014).

Conclusion

In summary, our study demonstrates that TOF has an impact on both methane emissions and the composition of rumen bacterial population. This study supports previous research that showed a decrease in methane emissions the longer animals are kept off feed. In addition, the abundance of key fibrolytic bacteria were found to decrease with increasing TOF in the current study. Our findings have implications for methane-microbiome studies in which animals are kept off feed for varying lengths of time, such as those using PACs, and future studies should seek to control for temporal effects.

Figures

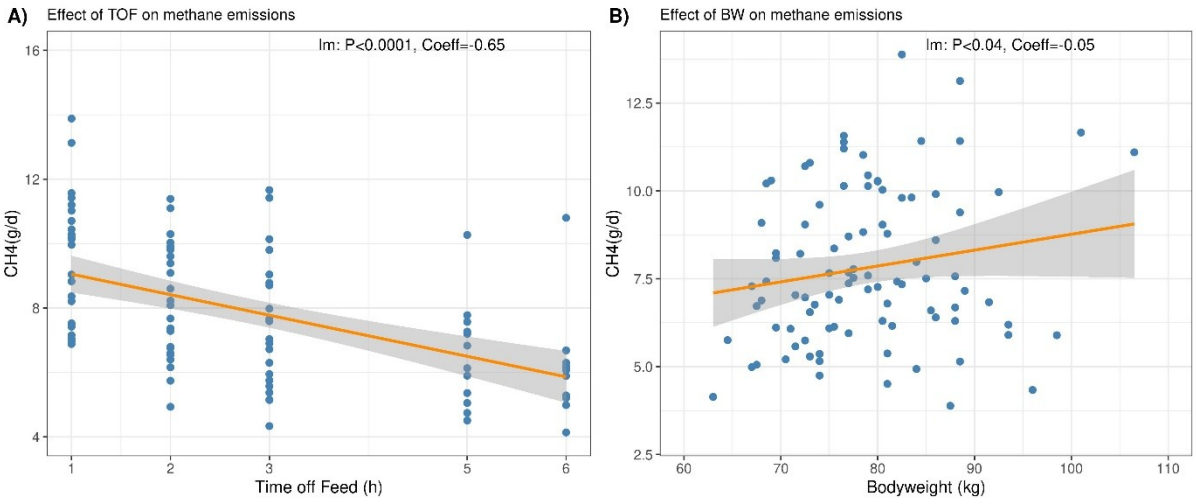


Figure 4.1: Figure presents the results of the linear model analysis conducted to examine the impact of TOF and BW on daily methane emissions. The x-axis represents the TOF (1-6h) (**A**), and animal's bodyweight (kg) (**B**), while the y-axis represents methane emissions (g/d).

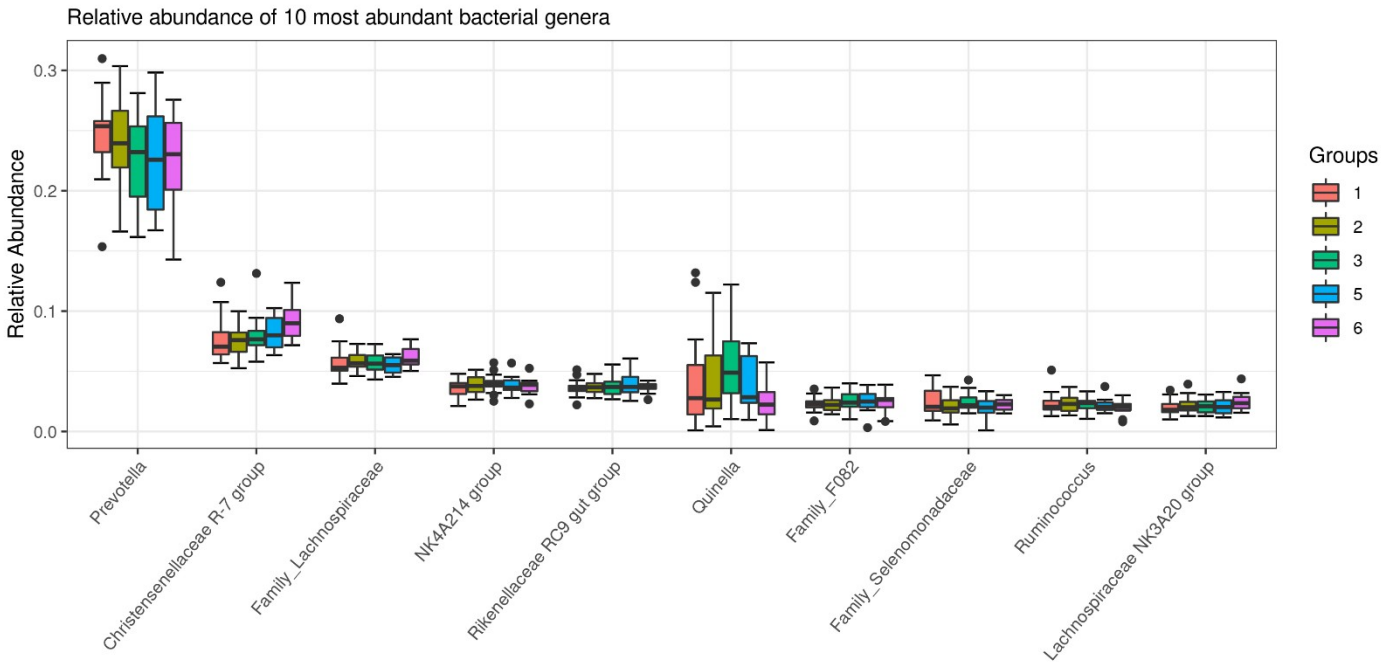


Figure 4.2: Boxplots representing the relative abundance of dominant bacterial genera in pasture grazed sheep.

Tables

Table 4.1: Table presents the associations between alpha diversity measures, specifically Shannon and inverse Simpson diversity, and time of feeding (TOF) using Maaslin2.

Diversity metric	Taxonomy & Rank	Coef	P.value	Q.value
Inverse Simpson	Bacteria Genus	0.355	0.028	0.112
Shannon	Bacteria Genus	0.012	0.066	0.124
Inverse Simpson	Bacteria ASV	2.848	0.558	0.744
Shannon	Bacteria ASV	-0.002	0.894	0.894
Inverse Simpson	Archaea ASV	-0.034	0.620	0.827
Shannon	Archaea ASV	0.000	0.969	0.969

Table 4.2: Table summarises the results of the beta diversity analysis, investigating the influence of Time of Feeding (TOF) on the composition of bacterial and archaeal communities. Community dissimilarities were calculated using Bray Curtis distances, and the statistical significance of the differences was tested using Permanova. Homogeneity of variance was assessed using Permdisp.

Permanova	R2	P.value	Permdisp P.value
Bacterial Genus	0.072	0.008	0.627
Bacterial ASV	0.050	0.073	0.731
Archaeal ASV	0.031	0.714	0.993

Table 4.3: Table presents the results of the Maaslin2 analysis, highlighting the significant associations between bacterial ASVs with Time of Feeding (TOF). ASVs were classified using BLAST.

feature	*Blast Classification*	*%ID*	*coef*	*stderr*	*N*	*N.not.0*	*P.value*	*Q.value*
ASV64	*Pseudobutyrivibrio ruminis; xylanivorans*	97.24 %; 97.22 %	-0.261	0.071	90	89	0.000	0.008
ASV87	*Prevotella brevis*	92.10 %	-0.256	0.063	90	87	0.000	0.003
ASV68	*Prevotella ruminocola*	96.44 %	-0.238	0.083	90	85	0.005	0.042
ASV88	*Pseudobutyrivibrio ruminis*	98.81 %	-0.210	0.042	90	90	0.000	0.000
ASV107	*Ruminococcus albus*	97.23 %	-0.193	0.044	90	90	0.000	0.002

ASV44	*Butyrivibrio fibrisolvens*	99.60%	-0.176	0.054	90	90	0.002	0.023
ASV113	*Gallalistipes aquisgranensis*	84.40%	-0.152	0.051	90	89	0.004	0.036
ASV98	*Shuttleworthia satelles*	91.70%	-0.135	0.045	90	90	0.003	0.033
ASV9	*Selenomonas ruminantium*	100.00%	-0.096	0.034	90	90	0.005	0.042
ASV99	*Marseillibacter massiliensis*	92.50%	-0.094	0.031	90	90	0.003	0.033
ASV40	*Faecalimonas umbilicata*	96.10%	0.107	0.036	90	89	0.004	0.036
ASV57	*Ruminococcus Lactaris*	93.20%	0.119	0.038	90	90	0.002	0.027
ASV106	*Syntrophococcus sucromutans*	96.40%	0.127	0.044	90	80	0.005	0.042
ASV147	*Ruminococcus Lactaris*	94.50%	0.138	0.047	90	90	0.004	0.036
ASV58	*Kiritimatiella glycovorans*	80.70%	0.172	0.039	90	90	0.000	0.002
ASV137	*Ruminococcus Lactaris*	93.70%	0.189	0.057	90	81	0.001	0.020
ASV39	*Olsenella intestinalis*	98.02%	0.307	0.091	90	87	0.001	0.016

Table 4.4: Table presents the results of the Maaslin2 analysis, highlighting significant associations between kingdoms bacteria and archaea, and bacterial phyla and genera with Time of Feeding (TOF).

feature	*Coef*	*stderr*	*N*	*N.not.0*	*P.value*	*Q.value*
Kingdom						
Bacteria	0.006	0.001	90	90	<0.001	<0.001
Archaea	0.088	0.020	90	90	<0.001	<0.001
Phylum						
Verrucomicrobiota	0.155	0.039	90	90	<0.001	0.004
Actinobacteriota	0.114	0.036	90	90	0.002	0.020
Planctomycetota	0.166	0.053	90	90	0.002	0.020
Genus						
FD2005 (Lachnospiraceae)	-0.208	0.072	90	89	0.005	0.039
Eubacterium..cellulosolvens.group	-0.206	0.061	90	90	0.001	0.015
Anaeroplasma	-0.156	0.042	90	90	<0.001	0.007
Shuttleworthia	-0.144	0.041	90	90	0.001	0.010
Butyrivibrio	-0.117	0.038	90	90	0.002	0.021
Pseudobutyrivibrio	-	0.03	9	90	0.001	0.014

	0.107	1	0			
Family_Ruminococcaceae	-0.087	0.028	90		0.003	0.022
Selenomonas	-0.076	0.028	90		0.007	0.040
Succiniclasticum	0.051	0.019	90		0.008	0.043
Christensenellaceae.R.7.group	0.053	0.016	90		0.001	0.015
Family_.Eubacterium..coprostanoligenes.group	0.069	0.021	90		0.002	0.017
Family_UCG.010	0.089	0.023	90		0.000	0.006
Ruminococcus..gauvreauii.group	0.090	0.022	90		0.000	0.004
Eubacterium..nodatum.group	0.101	0.036	90		0.006	0.040
Order_WCHB1.41	0.162	0.039	90		0.000	0.004
p.1088.a5.gut.group	0.167	0.040	90		0.000	0.004

References

Aguerre, M.J., Wattiaux, M.A., Powell, J.M., Broderick, G.A., and Arndt, C. (2011). Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. *Journal of Dairy Science* 94**,** 3081-3093.

Andrews, S. (2010). "FastQC: a quality control tool for high throughput sequence data". Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom).

Arias, P., Bellouin, N., Coppola, E., Jones, R., Krinner, G., Marotzke, J., Naik, V., Palmer, M., Plattner, G.-K., and Rogelj, J. (2021). Climate Change 2021: The Physical Science Basis. Contribution of Working Group14 I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change; Technical Summary.

Auffret, M.D., Stewart, R., Dewhurst, R.J., Duthie, C.-A., Rooke, J.A., Wallace, R.J., Freeman, T.C., Snelling, T.J., Watson, M., and Roehe, R. (2018). Identification, comparison, and validation of robust rumen microbial biomarkers for methane emissions using diverse Bos Taurus breeds and basal diets. *Frontiers in Microbiology* 8**,** 2642.

Beauchemin, K.A., Ungerfeld, E.M., Abdalla, A.L., Alvarez, C., Arndt, C., Becquet, P., Benchaar, C., Berndt, A., Mauricio, R.M., and Mcallister, T.A. (2022). Invited review: Current enteric methane mitigation options. *Journal of Dairy Science*.

Beule, L., and Karlovsky, P. (2020). Improved normalization of species count data in ecology by scaling with ranked subsampling (SRS): application to microbial communities. *PeerJ* 8**,** e9593.

Bickell, S., Revell, D., Toovey, A., and Vercoe, P. (2014). Feed intake of sheep when allowed ad libitum access to feed in methane respiration chambers. *Journal of Animal Science* 92**,** 2259-2264.

Callahan, B.J., Mcmurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods* 13**,** 581-583.

Carberry, C.A., Kenny, D.A., Han, S., Mccabe, M.S., and Waters, S.M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Applied and environmental microbiology* 78**,** 4949-4958.

Chen, S., Zhou, Y., Chen, Y., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34**,** i884-i890.

Czerkawski, J.W. (1969). Methane production in ruminants and its significance. *World Rev Nutr Diet* 11**,** 240-282.

Domingo, M.-C., Huletsky, A., Boissinot, M., Bernard, K., Picard, F., and Bergeron, M. (2008). Ruminococcus gauvreauii sp. nov., a glycopeptide-resistant species isolated from a human faecal specimen. *International journal of systematic and evolutionary microbiology* 58**,** 1393-1397.

Doré, J., and Bryant, M.P. (1990). Metabolism of one-carbon compounds by the ruminal acetogen syntrophococcus sucromutans. *Appl Environ Microbiol* 56**,** 984-989.

Emerson, E.L., and Weimer, P.J. (2017). Fermentation of model hemicelluloses by Prevotella strains and Butyrivibrio fibrisolvens in pure culture and in ruminal enrichment cultures. *Applied Microbiology and Biotechnology* 101**,** 4269-4278.

Flint, H.J., Martin, J.C., and Thomson, A.M. (2000). "Prevotella bryantii, P. ruminicola and bacteroides strains," in *Electrotransformation of Bacteria*. Springer), 140-149.

Franzosa, E.A., Mciver, L.J., Rahnavard, G., Thompson, L.R., Schirmer, M., Weingart, G., Lipson, K.S., Knight, R., Caporaso, J.G., and Segata, N. (2018). Species-level functional profiling of metagenomes and metatranscriptomes. *Nature methods* 15**,** 962-968.

Freier, T.A., Beitz, D.C., Li, L., and Hartman, P.A. (1994). Characterization of eubacterium coprostanoligenes sp. Nov., a cholesterol-reducing anaerobe. *International Journal of Systematic and Evolutionary Microbiology* 44**,** 137-142.

Friedman, N., Jami, E., and Mizrahi, I. (2017). Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. *Environmental microbiology* 19**,** 3365-3373.

Gharechahi, J., Vahidi, M.F., Bahram, M., Han, J.-L., Ding, X.-Z., and Salekdeh, G.H. (2021). Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilise high lignocellulosic forages in the cattle rumen. *The ISME Journal* 15**,** 1108-1120.

Giger-Reverdin, S., and Sauvant, D. (2000). Methane production in sheep in relation to concentrate feed composition from bibliographic data. *Eighth Seminar of the Sub-network on Nutrition of the FAO-CIHEAM Inter-regional Cooperative Research and Development Network on Sheep and Goats*.

Glöckner, F.O. (2019). The SILVA database project: an ELIXIR core data resource for high-quality ribosomal RNA sequences. *Biodiversity Information Science and Standards* 3**,** e36125.

Grilli, D., Egea, V., Paez, L., Carcano, D., Allegretti, L., Escudero, M., and Arenas, G. (2015). Degradation and utilization of hemicellulose from species forage by Pseudobutyrivibrio ruminis and Pseudobutyrivibrio xylanivorans. *Revista de la Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo* 47**,** 231-243.

Guan, Y., Li, Z., Kim, M.-J., Lee, J.-Y., Choe, H., Park, S.-H., Kang, S.W., Lee, J., Lee, J.H., Kim, H.B., Lee, J.-H., Kim, E., and Lee, M.-K. (2022). Olsenella intestinalis sp. nov., isolated from cow feces. *Archives of Microbiology* 204**,** 384.

Hammond, K., Humphries, D., Crompton, L., Green, C., and Reynolds, C. (2015). Methane emissions from cattle: Estimates from short-term measurements using a GreenFeed system compared with measurements obtained using respiration chambers or sulphur hexafluoride tracer. *Animal Feed Science and Technology* 203**,** 41-52.

Hatfield, R.D. (1993). "Cell wall polysaccharide interactions and degradability," in *Forage Cell Wall Structure and Digestibility*.), 285-313.

Hayek, M.N., and Miller, S.M. (2021). Underestimates of methane from intensively raised animals could undermine goals of sustainable development. *Environmental Research Letters* 16**,** 063006.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P.H. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific reports* 5**,** 1-15.

Hino, T., Miwa, T., Saitoh, H., Esaki, H., Takagi, S., Asanuma, N., and Umemori, J. (1995). Effect of methane production on plant fiber digestion by rumen microorganisms. *Bulletin of the Faculty of Agriculture-Meiji University (Japan)*.

Huws, S.A., Edwards, J.E., Creevey, C.J., Rees Stevens, P., Lin, W., Girdwood, S.E., Pachebat, J.A., and Kingston-Smith, A.H. (2016). Temporal dynamics of the metabolically active rumen bacteria colonizing fresh perennial ryegrass. *FEMS microbiology ecology* 92**,** fiv137.

Ipcc, C.C. (2014). Mitigation of climate change. *Contribution of working group III to the fifth assessment report of the intergovernmental panel on climate change***,** 1454.

Janssen, P.H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160**,** 1-22.

Johnson, K., Huyler, M., Westberg, H., Lamb, B., and Zimmerman, P. (1994). Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. *Environmental science & technology* 28**,** 359-362.

Johnson, K.A., and Johnson, D.E. (1995). Methane emissions from cattle. *J Anim Sci* 73**,** 2483-2492.

Jonker, A., Hickey, S.M., Rowe, S.J., Janssen, P.H., Shackell, G.H., Elmes, S., Bain, W.E., Wing, J., Greer, G.J., Bryson, B., Maclean, S., Dodds, K.G., Pinares-

Patiño, C.S., Young, E.A., Knowler, K., Pickering, N.K., and Mcewan, J.C. (2018). Genetic parameters of methane emissions determined using portable accumulation chambers in lambs and ewes grazing pasture and genetic correlations with emissions determined in respiration chambers1. *Journal of Animal Science* 96**,** 3031-3042.

Jonker, A., Molano, G., Antwi, C., and Waghorn, G. (2014). Feeding lucerne silage to beef cattle at three allowances and four feeding frequencies affects circadian patterns of methane emissions, but not emissions per unit of intake. *Animal Production Science* 54**,** 1350-1353.

Kim, J.N., Song, J., Kim, E.J., Chang, J., Kim, C.H., Seo, S., Chang, M.B., and Bae, G.S. (2019). Effects of short-term fasting on in vivo rumen microbiota and in vitro rumen fermentation characteristics. *Asian-Australas J Anim Sci* 32**,** 776-782.

Kittelmann, S., Pinares-Patiño, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., Mcewan, J.C., and Janssen, P.H. (2014). Two different bacterial community types are linked with the low-methane emission trait in sheep. *PLOS ONE* 9**,** e103171.

Knaus, W. (2016). Perspectives on pasture versus indoor feeding of dairy cows. *Journal of the Science of Food and Agriculture* 96**,** 9-17.

Kopylova, E., Noé, L., and Touzet, H. (2012). SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. *Bioinformatics* 28**,** 3211-3217.

Lambie, S.C., Kelly, W.J., Leahy, S.C., Li, D., Reilly, K., Mcallister, T.A., Valle, E.R., Attwood, G.T., and Altermann, E. (2015). The complete genome sequence of the rumen methanogen Methanosarcina barkeri CM1. *Standards in genomic sciences* 10**,** 1-8.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods* 9**,** 357-359.

Leahy, S., Kelly, W., Ronimus, R., Wedlock, N., Altermann, E., and Attwood, G. (2013). Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal* 7**,** 235-243.

Leahy, S.C., Kelly, W.J., Altermann, E., Ronimus, R.S., Yeoman, C.J., Pacheco, D.M., Li, D., Kong, Z., Mctavish, S., and Sang, C. (2010). The genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions. *PloS one* 5**,** e8926.

Li, R., Teng, Z., Lang, C., Zhou, H., Zhong, W., Ban, Z., Yan, X., Yang, H., Farouk, M.H., and Lou, Y. (2019). Effect of different forage-to-concentrate ratios on ruminal bacterial structure and real-time methane production in sheep. *PLoS One* 14**,** e0214777.

Lockyer, D. (1997). Methane emissions from grazing sheep and calves. *Agriculture, ecosystems & environment* 66**,** 11-18.

Luís, A.S., Alves, V.D., Romao, M.J., Prates, J.A., Fontes, C.M., and Najmudin, S. (2011). Overproduction, purification, crystallization and preliminary X-ray characterization of a novel carbohydrate-binding module of endoglucanase Cel5A from Eubacterium cellulosolvens. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* 67**,** 491-493.

Mallick, H., Rahnavard, A., Mciver, L.J., Ma, S., Zhang, Y., Nguyen, L.H., Tickle, T.L., Weingart, G., Ren, B., Schwager, E.H., Chatterjee, S., Thompson, K.N., Wilkinson, J.E., Subramanian, A., Lu, Y., Waldron, L., Paulson, J.N., Franzosa, E.A., Bravo, H.C., and Huttenhower, C. (2021). Multivariable association discovery in population-scale meta-omics studies. *PLOS Computational Biology* 17**,** e1009442.

Mccabe, M.S., Cormican, P., Keogh, K., O'connor, A., O'hara, E., Palladino, R.A., Kenny, D.A., and Waters, S.M. (2015). Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised Succinivibrionaceae and an increase of the Methanobrevibacter gottschalkii clade in feed restricted cattle. *PloS one* 10**,** e0133234.

Mcmurdie, P.J., and Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* 8**,** e61217.

Morotomi, M., Nagai, F., and Watanabe, Y. (2012). Description of Christensenella minuta gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order Clostridiales, and proposal of Christensenellaceae fam. nov. *International journal of systematic and evolutionary microbiology* 62**,** 144-149.

Morrison, I.M. (1979). Carbohydrate chemistry and rumen digestion. *Proceedings of the Nutrition Society* 38**,** 269-274.

Muetzel, S., and Clark, H. (2015). Methane emissions from sheep fed fresh pasture. *New Zealand Journal of Agricultural Research* 58**,** 472-489.

Ng, F., Kittelmann, S., Patchett, M.L., Attwood, G.T., Janssen, P.H., Rakonjac, J., and Gagic, D. (2016). An adhesin from hydrogen-utilizing rumen methanogen Methanobrevibacter ruminantium M1 binds a broad range of hydrogen-producing microorganisms. *Environmental Microbiology* 18**,** 3010-3021.

O' Connor, E., Mchugh, N., Boland, T.M., Dunne, E., and Mcgovern, F.M. (2021). Investigation of intra-day variability of gaseous measurements in sheep using portable accumulation chambers. *Journal of animal science* 99**,** skab132.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H., and Wagner, H. (2013). Package 'vegan'. *Community ecology package, version* 2**,** 1-295.

Oltjen, J.W., and Beckett, J.L. (1996). Role of ruminant livestock in sustainable agricultural systems. *Journal of Animal Science* 74**,** 1406-1409.

Palevich, N., Kelly, W.J., Leahy, S.C., Denman, S., Altermann, E., Rakonjac, J., and Attwood, G.T. (2019). Comparative Genomics of Rumen Butyrivibrio spp. Uncovers a Continuum of Polysaccharide-Degrading Capabilities. *Applied and Environmental Microbiology* 86**,** e01993-01919.

Pavlostathis, S.G., Miller, T.L., and Wolin, M.J. (1990). Cellulose fermentation by continuous cultures of Ruminococcus albus and Methanobrevibacter smithii. *Applied microbiology and biotechnology* 33**,** 109-116.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F.O. (2012). The SILVA ribosomal RNA gene database

project: improved data processing and web-based tools. *Nucleic acids research* 41**,** D590-D596.

Robinson, D.L., Goopy, J.P., Hegarty, R., Oddy, V., Thompson, A., Toovey, A., Macleay, C., Briegal, J., Woodgate, R., and Donaldson, A. (2014). Genetic and environmental variation in methane emissions of sheep at pasture. *Journal of Animal Science* 92**,** 4349-4363.

Sakamoto, M., Ikeyama, N., Yuki, M., and Ohkuma, M. (2018). Draft genome sequence of faecalimonas umbilicata jcm 30896t, an acetate-producing bacterium isolated from human feces. *Microbiology Resource Announcements* 7**,** e01091-01018.

Saunois, M., Stavert, A.R., Poulter, B., Bousquet, P., Canadell, J.G., Jackson, R.B., Raymond, P.A., Dlugokencky, E.J., Houweling, S., and Patra, P.K. (2020). The global methane budget 2000–2017. *Earth system science data* 12**,** 1561-1623.

Sawanon, S., and Kobayashl, Y. (2006). Synergistic fibrolysis in the rumen by cellulolytic Ruminococcus flavefaciens and non-cellulolytic Selenomonas ruminantium: Evidence in defined cultures. *Animal Science Journal* 77**,** 208-214.

Sawanon, S., Koike, S., and Kobayashi, Y. (2011). Evidence for the possible involvement of Selenomonas ruminantium in rumen fiber digestion. *FEMS microbiology letters* 325**,** 170-179.

Shi, W., Moon, C.D., Leahy, S.C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W.J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C.S., Mcewan, J.C., Janssen, P.H., Chen, F., Visel, A., Wang, Z., Attwood, G.T., and Rubin, E.M. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome research* 24**,** 1517-1525.

Smith, P.E., Kelly, A.K., Kenny, D.A., and Waters, S.M. (2022). Differences in the composition of the rumen microbiota of finishing beef cattle divergently ranked for residual methane emissions. *Frontiers in microbiology* 13.

Smith, P.E., Waters, S.M., Kenny, D.A., Kirwan, S.F., Conroy, S., and Kelly, A.K. (2021). Effect of divergence in residual methane emissions on feed intake and efficiency, growth and carcass performance, and indices of rumen fermentation and methane emissions in finishing beef cattle. *Journal of Animal Science* 99.

Söllinger, A., Tveit, A.T., Poulsen, M., Noel, S.J., Bengtsson, M., Bernhardt, J., Hellwing, A.L.F., Lund, P., Riedel, K., Schleper, C., Højberg, O., and Urich, T. (2018). Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems* 3**,** e00038-00018.

Spring, S., Bunk, B., Spröer, C., Schumann, P., Rohde, M., Tindall, B.J., and Klenk, H.-P. (2016). Characterization of the first cultured representative of Verrucomicrobia subdivision 5 indicates the proposal of a novel phylum. *The ISME Journal* 10**,** 2801-2816.

Stevens, C.E., and Hume, I.D. (1998). Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. *Physiological reviews* 78**,** 393-427.

Tapio, I., Snelling, T.J., Strozzi, F., and Wallace, R.J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. *Journal of Animal Science and Biotechnology* 8**,** 7.

Togo, A.H., Diop, A., Bittar, F., Maraninchi, M., Valero, R., Armstrong, N., Dubourg, G., Labas, N., Richez, M., and Delerce, J. (2018). Description of Mediterraneibacter massiliensis, gen. nov., sp. nov., a new genus isolated from the gut microbiota of an obese patient and reclassification of Ruminococcus faecis, Ruminococcus lactaris, Ruminococcus torques, Ruminococcus gnavus and Clostridium glycyrrhizinilyticum as Mediterraneibacter faecis comb. nov., Mediterraneibacter lactaris comb. nov., Mediterraneibacter torques comb. nov., Mediterraneibacter gnavus comb. nov. and Mediterraneibacter glycyrrhizinilyticus comb. nov. *Antonie Van Leeuwenhoek* 111**,** 2107-2128.

Uematsu, H., and Hoshino, E. (1996). Degradation of arginine and other amino acids by eubacterium nodatum atcc 33099. *Microbial Ecology in Health and Disease* 9**,** 305-311.

Van Der Toorn, J.J.T.K., and Van Gylswyk, N.O. (1985). Xylan-digesting bacteria from the rumen of sheep fed maize straw diets. *Microbiology* 131**,** 2601-2607.

Van Gylswyk, N.O. (1995). Succiniclasticum ruminis gen. Nov., sp. Nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *International Journal of Systematic and Evolutionary Microbiology* 45**,** 297-300.

Van Vliet, D.M., Lin, Y., Bale, N.J., Koenen, M., Villanueva, L., Stams, A.J., and Sánchez-Andrea, I. (2020). Pontiella desulfatans gen. nov., sp. nov., and Pontiella sulfatireligans sp. nov., two marine anaerobes of the Pontiellaceae fam. nov. producing sulfated glycosaminoglycan-like exopolymers. *Microorganisms* 8**,** 920.

Wallace, R.J., Snelling, T.J., Mccartney, C.A., Tapio, I., and Strozzi, F. (2017). Application of meta-omics techniques to understand greenhouse gas emissions originating from ruminal metabolism. *Genetics Selection Evolution* 49**,** 9.

Waters, S.M., Kenny, D.A., and Smith, P.E. (2020). "Role of the rumen microbiome in pasture-fed ruminant production systems," in *Improving rumen function*. Burleigh Dodds Science Publishing), 591-650.

Weimer, P.J. (1992). Cellulose degradation by ruminal microorganisms. *Critical Reviews in Biotechnology* 12**,** 189-223.

Weimer, P.J. (2022). Degradation of Cellulose and Hemicellulose by Ruminal Microorganisms. *Microorganisms* 10**,** 2345.

Welch, C.B., Lourenco, J.M., Seidel, D.S., Krause, T.R., Rothrock, M.J., Pringle, T.D., and Callaway, T.R. (2021). The impact of pre-slaughter fasting on the ruminal microbial population of commercial angus steers. *Microorganisms* 9**,** 2625.

Wirth, R., Kádár, G., Kakuk, B., Maróti, G., Bagi, Z., Szilágyi, Á., Rákhely, G., Horváth, J., and Kovács, K.L. (2018). The planktonic core microbiome and core functions in the cattle rumen by next generation sequencing. *Frontiers in Microbiology* 9**,** 2285.

Wollenberg, E., Richards, M., Smith, P., Havlík, P., Obersteiner, M., Tubiello, F.N., Herold, M., Gerber, P., Carter, S., and Reisinger, A. (2016). Reducing emissions from agriculture to meet the 2 C target. *Global change biology* 22**,** 3859-3864.

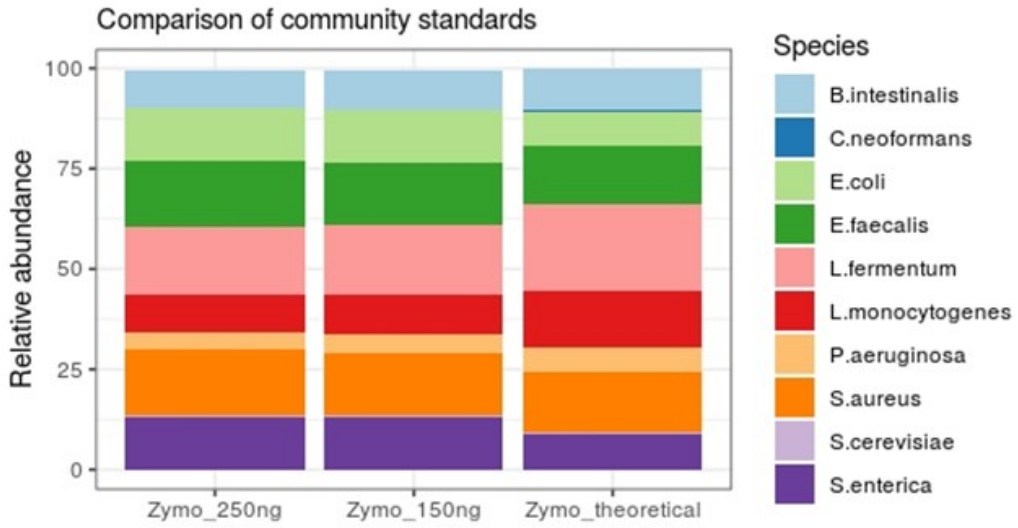
Zhang, Y.K., Zhang, X.X., Li, F.D., Li, C., Li, G.Z., Zhang, D.Y., Song, Q.Z., Li, X.L., Zhao, Y., and Wang, W.M. (2021). Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal* 15**,** 100161.

Zhao, Y., Nan, X., Yang, L., Zheng, S., Jiang, L., and Xiong, B. (2020). A review of enteric methane emission measurement techniques in ruminants. *Animals* 10**,** 1004.

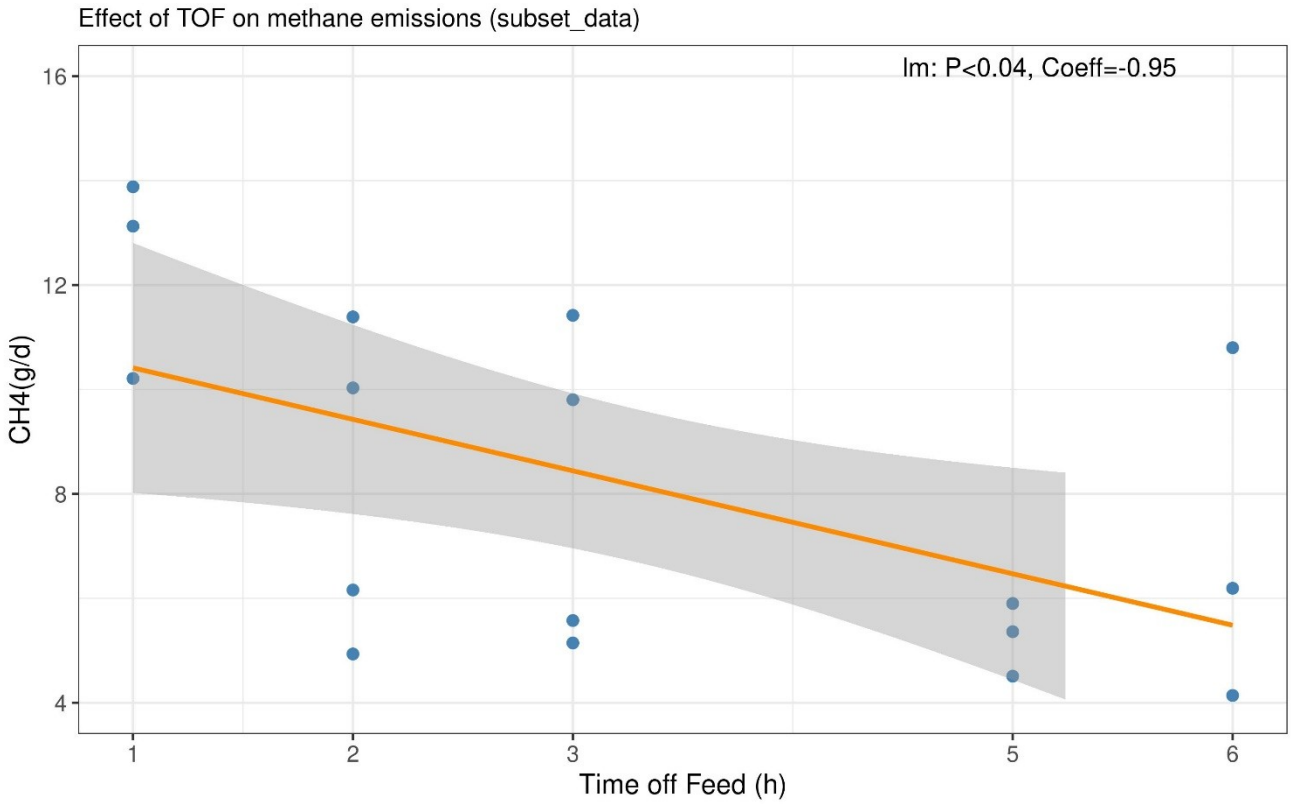
Zhou, J., Ding, Z., Pu, Q., Xue, B., Yue, S., Guan, S., Wang, Z., Wang, L., Peng, Q., and Xue, B. (2022). Rumen Fermentation and Microbiome Responses to Enzymatic Hydrolysate of Cottonseed Protein Supplementation in Continuous In Vitro Culture. *Animals* 12**,** 2113.

Chapter 4 Supplementary Figures and Tables

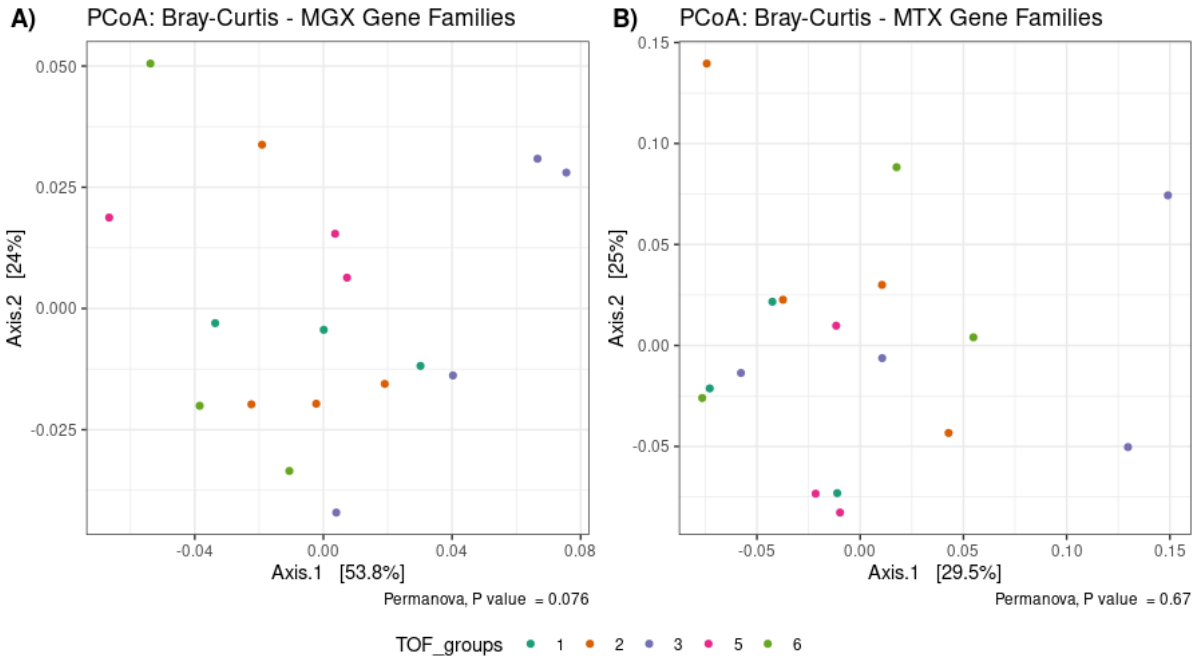
Supplementary Figures



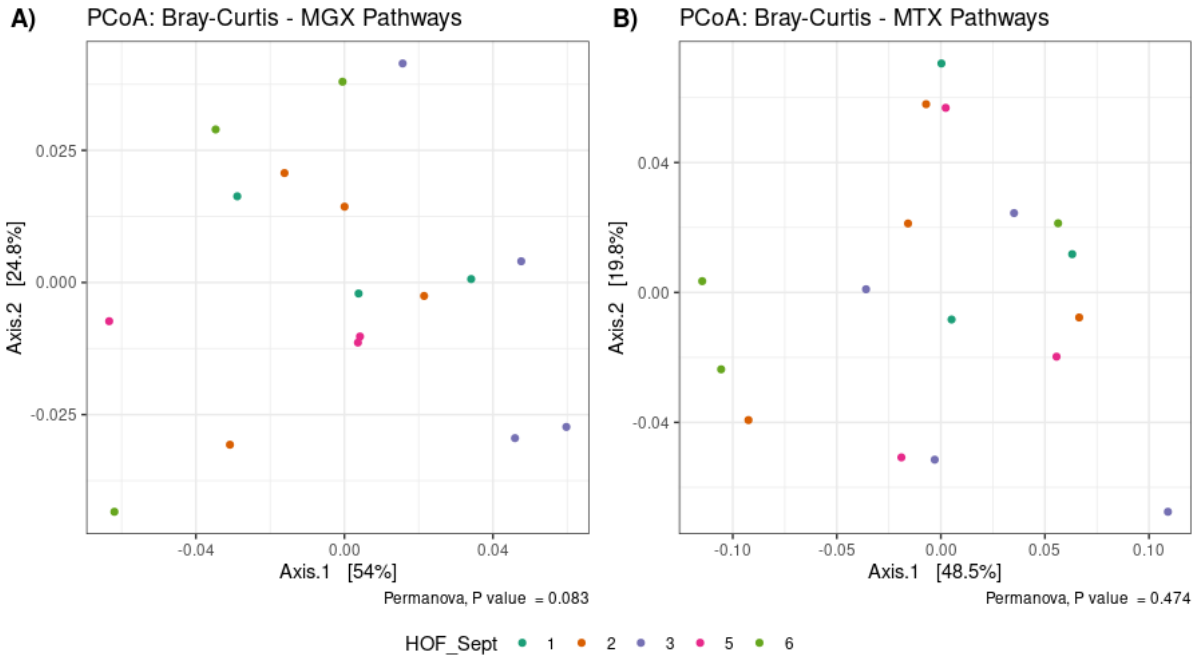
Supplementary Figure 4.1: Comparison of relative the abundance of ZymoBIOTICS™ standards run through our metagenomics pipeline with ZymoBIOTICS™ theoretical relative abundances. Species classified using Metaphlan 3.



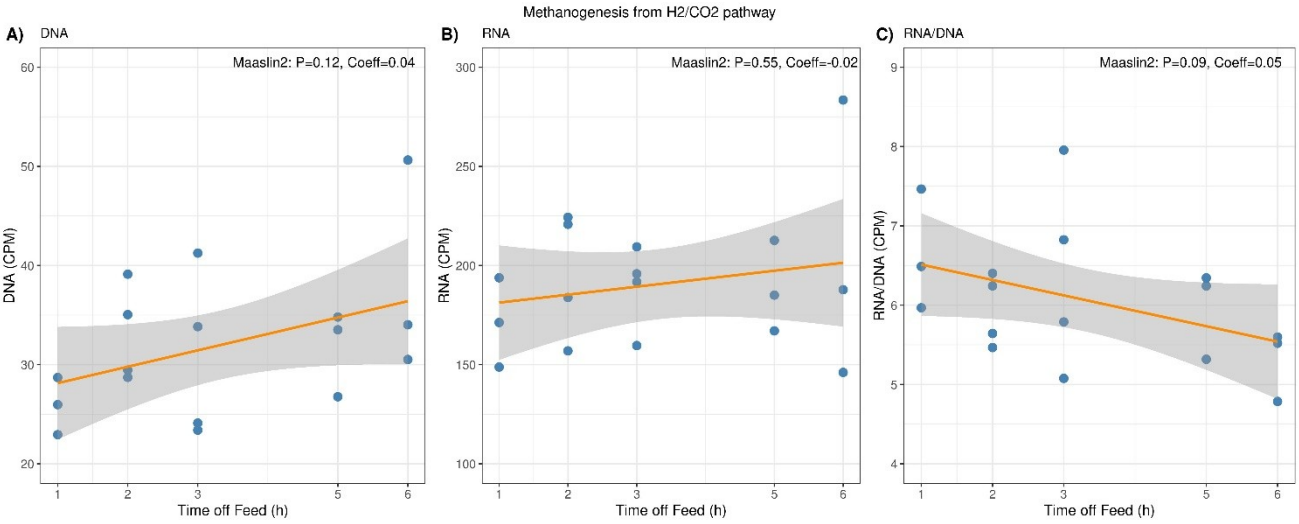
Supplementary Figure 4.2: Figure presents the results of the linear model analysis conducted to examine the impact of TOF on daily methane emissions. The x-axis represents the TOF (1-6h) and the y-axis represents methane emissions (g/d).



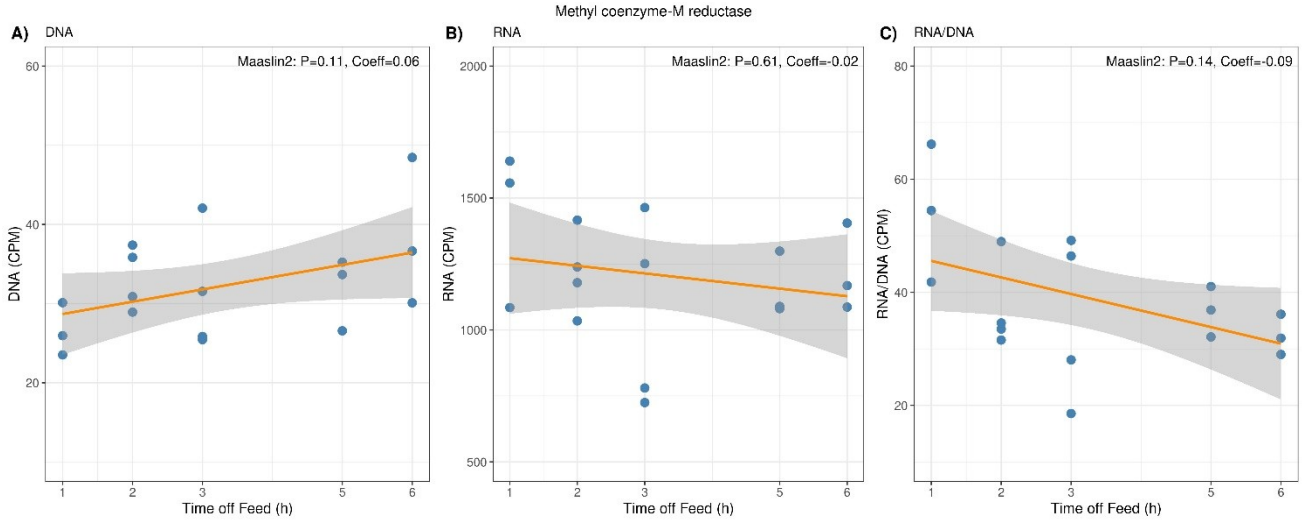
Supplementary Figure 4.3: PCoA on Bray Curtis distances for gene families identified by Humann3 on the metagenomic data set (A) and metranscriptomic dataset (B). Differences between groups were tested using Permanova.



Supplementary Figure 4.4: PCoA on Bray Curtis distances for meta-cyc pathways identified by Humann3 on the metagenomic data set (A) and metranscriptomic dataset (B). Differences between groups were tested using Permanova.



Supplementary Figure 4.5: Figure illustrating the results from Maaslin2 showing association between TOF and the hydrogenotrophic methanogenesis pathway at the DNA level (A), RNA level (B) and RNA transcripts adjusted for gene copy numbers (C).



Supplementary Figure 4.6: Figure illustrating the results from Maaslin2 showing association between TOF and Methyl-coenzyme-M reductase at the DNA level (A), RNA level (B) and RNA transcripts adjusted for gene copy numbers (C).

Supplementary Tables

Supplementary Table 4.1: Table present results from pairwise PERMANOVA. Comparisons made between TOF groups, testing the differences in bacterial community composition at the genus level

Pairwise Permanova: Bacterial Genera					
Pairs	Sums OfSqs	F.Model	R2	P.value	P.adjusted
3 vs 1	0.037	2.207	0.051	0.024	0.079
3 vs 2	0.021	1.392	0.032	0.171	0.284
3 vs 6	0.032	1.948	0.063	0.042	0.104
3 vs 5	0.010	0.581	0.019	0.849	0.849
1 vs 2	0.012	0.736	0.016	0.716	0.795
1 vs 6	0.059	3.502	0.099	0.000	0.003
1 vs 5	0.030	1.716	0.049	0.075	0.151
2 vs 6	0.038	2.498	0.070	0.008	0.042
2 vs 5	0.014	0.866	0.025	0.537	0.671
6 vs 5	0.017	0.934	0.043	0.474	0.671

Supplementary Table 4.2: Table present results from pairwise PERMANOVA. Comparisons made between TOF groups, testing the differences in bacterial community composition at the ASV level.

Pairwise Permanova: Bacterial ASVs					
pairs	Sums OfSqs	F.Model	R2	p.value	p.adjusted
3 vs 1	0.121	1.113	0.026	0.226	0.453
3 vs 2	0.115	1.134	0.026	0.203	0.453
3 vs 6	0.130	1.162	0.039	0.170	0.453
3 vs 5	0.102	0.919	0.030	0.584	0.730
1 vs 2	0.083	0.798	0.017	0.899	0.899
1 vs 6	0.196	1.719	0.051	0.008	0.078
1 vs 5	0.118	1.042	0.031	0.341	0.568
2 vs 6	0.167	1.598	0.046	0.022	0.112
2 vs 5	0.098	0.948	0.027	0.521	0.730
6 vs 5	0.101	0.850	0.039	0.707	0.786

Supplementary Table 4.3: Table present results from pairwise PERMANOVA. Comparisons made between TOF groups, testing the differences in archaeal community composition at the ASV level.

Pairwise Permanova: Archaeal ASVs					
pairs	Sums OfSqs	F.Model	R2	p.value	p.adjusted
3 vs 1	0.031	0.668	0.016	0.614	0.883
3 vs 2	0.020	0.423	0.010	0.794	0.883
3 vs 6	0.027	0.570	0.019	0.669	0.883
3 vs 5	0.038	0.790	0.026	0.517	0.883
1 vs 2	0.052	1.085	0.024	0.306	0.883
1 vs 6	0.014	0.283	0.009	0.927	0.927
1 vs 5	0.025	0.493	0.015	0.795	0.883
2 vs 6	0.024	0.480	0.014	0.746	0.883
2 vs 5	0.061	1.216	0.035	0.269	0.883
6 vs 5	0.028	0.537	0.025	0.739	0.883

Chapter 5

Discussion

Overall summary

This PhD thesis research aimed to understand the role of the rumen microbiome for enhancing feed efficiency and reducing methane emissions in sheep. In Chapter 1, an extensive review of the literature was conducted on the current state-of-the-art. The chapter provides insights into the evolution of sheep, their global and national economic importance, and their contribution to anthropogenic GHG and methane emissions. Additionally, the chapter overviews feed digestion in the sheep rumen, including details on the digestive anatomy, providing a comprehensive overview of the rumen microbiome, fermentation and methanogenesis. Furthermore, Chapter 1 indicates the methods that are employed to measure methane emissions, the factors that impact methane production from ruminants, and the strategies to reduce methane emissions in sheep and other ruminants. Finally, it provides an overview of how the rumen microbiome can be studied and the intricacies of typical research workflows associated with rumen studies, from sample collection to data analysis.

Chapter 2 investigated the bacterial and archaeal populations in sheep breeds that are divergent for feed efficiency (as a measured phenotype), connecting the feed conversion ratio (FCR) to the microbiome composition of the rumen using 16S rRNA gene sequencing. The research findings from Chapter 2 revealed no major changes in the overall composition of the bacterial community between high and low feed efficient sheep, but did discover significant relationships between the abundance of specific bacterial taxa and the feed efficiency traits FCR and ADG. For example, the abundances of *Fibrobacter* and *Ruminococcus* were negatively associated with the FCR, indicating that these prominent fibrolytic bacteria may be conferring inefficiency in terms of energy utilisation when sheep are fed a high concentrate diet. On the other hand *Bifidobacterium* and *Megasphaera* were associated with increased ADG, while *Ruminococcaceae UCG-014* and *Olsenella* were associated with improved FCR. Notably, this PhD research demonstrates that the composition of the archaeal community differed between the high and low feed efficiency cohorts. The composition of the archaeal community had previously been shown to be associated with methane production in ruminant livestock (Shi et al., 2014), and methanogenesis is known to result in a loss of dietary energy to the host (Giger-Reverdin and Sauvant, 2000). Therefore, differences in the composition of the archaeal community may have been a contributing factor driving the observed differences in FCR between the divergent cohorts. The overall findings from this PhD research supports interventions for manipulating the rumen microbial community to improve feed

efficiency and reduce methane emissions, such as the use of feed additives (Eger et al., 2018) or through selective breeding programs (Li et al., 2019b).

Understanding the factors that shape the composition and diversity of the rumen microbiome in sheep is critical for informing the rational development of more effective strategies to enhance feed efficiency and mitigate ruminant methane emissions. Previous studies in cattle have shown that breed/host genetic variation (i.e. genotypes) can contribute to variation in the composition of the rumen microbiome (Li et al., 2019a; Noel et al., 2019). However, breed effects on the rumen microbiome of sheep have not been widely investigated. Chapter 3, therefore investigated whether sheep breed `(as a genetic factor) could have an effect on the rumen bacterial and archaeal populations in the associated various fractions of the rumen (i.e solid, liquid and epithelial) from four popular hill sheep breeds in Ireland; the Cheviot, Connemara, Lanark and Perth (the latter three being strains of Scottish Blackface). This PhD study indicates that the Cheviot breed produceds the fastest maturing lambs, and had the lowest FCR (most feed efficient). However, the difference in FCR was only significant when compared to the Connemara sheep breed. The production traits between the Cheviot, Lanark and Perth breeds were largely similar, suggesting that the choice of breed utilised by producers may not have a major impact on production outcomes. However, factors such as environmental conditions are important to consider for hill producers, and could have potential implications for productivity and profitability. For example, a study carried out by Blaxter et al. (1966) showed that while metabolic differences between six different sheep breeds were small, the Scottish Blackface breed was found to be more tolerant to environmental stresses when compared to the Cheviot breed (Blaxter et al., 1966), which could have an influence on production outcomes.

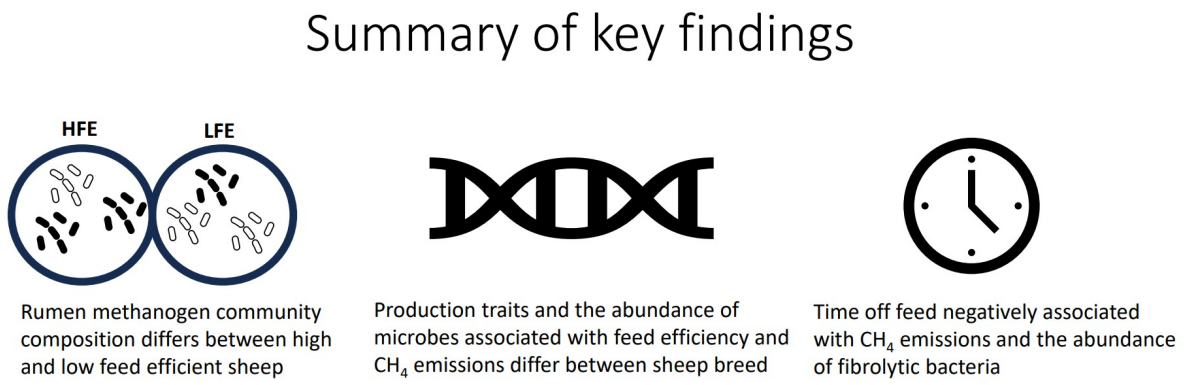
In Chapter 3, the impact of breed on the bacterial and archaeal community composition associated with the solid, liquid, and epithelial ruminal fractions was found not to be statistically significant, with only a minor proportion of variation in community composition being attributed to breed, i.e. ~10%. However, it was observed that breed had a marginally higher influence on the composition of the epithelial-associated communities, which could be of importance for future studies aimed at modulating the rumen microbiome for enhancing feed efficiency through selective breeding programs. Indeed, bacteria associated with the rumen epithelium maintain close interactions with the host and have been shown to correlate with ruminal epithelial tissue gene expression (Chen and Oba, 2012; Liu et al., 2021). Despite having no effect on the overall community composition, breed was found to have an effect on the abundance of specific ruminal taxa that could be related to feed efficiency. For instance, the abundance of the acetogenic bacterium *Acetitomaculum* was significantly higher in the Cheviot breed compared to the Connemara in the liquid ruminal fraction, which could be contributing to the enhanced FCR in the Cheviot breed by directing H_2 away from methanogenesis and towards acetate production. In addition, the study observed that the abundance of *Succiniclasticum* in the epithelial ruminal fraction was greater in the Cheviot, Perth, and Lanark breeds in comparison to the Connemara breed. *Succiniclasticum* is a specialised

bacterium that facilitates the conversion of succinate to propionate (Van Gylswyk, 1995). The higher abundance of this bacterium and its proximity to the rumen epithelium in these breeds could have contributed to their improved FCR by providing additional propionate, an essential glucogenic precursor required for growth and production (Young, 1977). The findings from this PhD research demonstrate that breed/host genetics can influence the rumen microbial community structure and has potential implications for breeding programs to select for microbiomes that can better utilise feed and produce less methane.

Chapter 2 and Chapter 3 also investigated the effect of the ruminal fraction on the composition of the rumen bacterial and archaeal populations. The results from both studies showed a high degree of similarity between solid and liquid ruminal fractions. Several studies have shown that these two fractions also tend to be more similar in composition when compared to the epithelial ruminal fraction (Schaeren et al., 2017; Li et al., 2020), which could be a consequence of the separation method used separate the two ruminal fractions or could be reflective of the frequent interchange of microbes between those ruminal fractions. Based on the beta diversity analysis, Chapter 3 found no compositional differences between the solid, liquid and epithelial ruminal fractions, however, the abundance of numerous bacterial taxa were found to be differentially abundant, particularly between the solid and epithelial fractions, including specific ruminal taxa known to be associated with the ruminal epithelial fraction such as *Campylobacter*, *Neisseriaceae*, *Desulfobulbus* and *Butyrivibrio* (Anderson et al., 2021). The bias of bacterial species distribution among ruminal fractions, particularly between solid and epithelial fractions, has implications for sheep rumen sampling techniques.

In Ireland, and many temperate regions of the world, livestock production systems are pasture-based (Knaus, 2016; O'Brien et al., 2018; O´Mara et al., 2021). Therefore, to improve our understanding of the factors driving methane emissions in current agricultural production systems, and inform the development of effective methane mitigation strategies, it is imperative to conduct methane-microbiome related studies on pasture-fed animals. Studies utilising portable accumulation chambers (PACs) have shown that the duration animals are retained off feed results in a decrease in methane output (Robinson et al., 2014). However, the impact of time off feed (TOF) on the rumen microbiome has not been investigated in sheep. Hence, Chapter 4 of this PhD thesis investigated the impact of time off feed (TOF) on methane emissions, as well as the taxonomic composition and functional activity of the rumen microbiome in pasture-grazed sheep. PACs were used to quantify methane output, and a multi-omics approach was adopted to characterise the composition and function of the rumen microbiome. Consistent with earlier studies (Robinson et al., 2014), our findings reveal a linear decline in methane emissions with increasing TOF. The abundance of major ruminal fibrolytic bacteria, including species of *Prevotella*, *Butyrivibrio*, *Pseudobutyribirio*, *Ruminococcus albus*, and *Eubacterium cellulosolvens,* showed a negative correlation with TOF, which aligned with the observed decrease in methane emissions during the study. These bacteria play a significant role in hemicellulose degradation (Emerson and

Weimer, 2017), relying on fermentable fiber as a nutrient source for their growth and proliferation. The study suggests that the decline in the abundance of these bacteria with increasing TOF could be attributed to the insufficient intake of fiber, leading to nutrient scarcity over time and potentially limiting their growth. This decline in abundance of key fibrolytic bacteria may be linked to the observed reduction in methane emissions as a number of the bacteria are known to produce hydrogen and/or formate during fermentation, including *Butyrivibrio*, *Pseudobutyrivibrio*, *Eubacterium cellulosolvens* and *Ruminococcus albus* (Prins et al., 1972; Van Gylswyk et al., 1996; Sengupta et al., 2022), which are utilised by methanogens for methanogenesis. Indeed, the decrease in the abundance of these fibrolytic bacteria during TOF could be a contributing factor to the observed decrease in methane emissions. Metagenomic and metatranscriptomic analysis revealed no significant differences in gene abundance, transcript abundance or the abundance or activity of metabolic pathways. Interestingly, when controlling for gene copy number the transcript abundance of the methyl-coenzyme-M reductase gene and the hydrogenotrophic methanogenesis pathway tended to decrease with increasing TOF. A graphical summary of the key finding from each of the chapters can be seen below in Figure 5.1.



Figue 5.1: Graphic summarising key findings from each of the chapters.

Limitations and recommendations for future studies

All research carried out as part of this PhD thesis centred on partial sequencing of the 16S rRNA gene to conduct metataxonomic analysis of the rumen bacterial and archaeal populations in sheep. This approach was chosen due to its culture independence, cost-effectiveness, and the ability to generate large amounts of microbial genomic data. However, while this technique was able provide informative data relating to taxonomy, it was unable to provide sufficient insights into the functional activity of the rumen microbiome. Given that feed efficiency and methane emissions are complex and dynamic traits regulated by the presence and activity of various biochemical pathways (Greening et al., 2019), the use of 16S rRNA gene sequencing could therefore be considered a limitation in Chapters 2 and 3. A more comprehensive and detailed understanding of the rumen microbiome and feed efficiency could have been provided with the use of metagenomics and/or metatranscriptomics sequencing. However, due to high costs associated with these approaches, 16S rRNA gene sequencing was

considered a more practical choice for providing an initial assessment of the rumen microbiome and its relationship with feed efficiency. In addition, although metagenomic and metatranscriptomic approaches were utilised for analysis in Chapter 4 to examine the functional activity of the rumen microbiome in relation to methane emissions and TOF, the number of samples available for analysis was considerably low, which impacted on the statistical power to detect biological effects, despite revealing taxonomic associations with TOF using 16S rRNA sequencing.

The research conducted in Chapters 2, 3 and 4 did not include examination of protozoa and fungi, which are also integral members of the rumen microbiota and hold crucial functions in feed digestion and the generation of substrates essential for methanogenesis (Bauchop, 1981; Guyader et al., 2014). However, due to the limited representation of rumen protozoa and fungi in publicly available databases and the predominant role of both bacterial and archaea play in feed utilisation and methane production, respectively, only bacterial and archaeal groups were targeted for study as part of this PhD thesis. Future studies should involve targeting protozoa and fungi to provide a more comprehensive view of the rumen microbiome.

Targeted sequencing of the V4 region of the 16S rRNA gene allowed for sufficient taxonomic classification of the bacterial and archaeal communities at higher taxonomic ranks (i.e phylum to genus) in Chapters 2, 3 and 4. However, there was poor taxonomic resolution at the species level in each of the studies. Greater taxonomic resolution could have been provided through metagenomic sequencing, although, as revealed when conducting analysis in Chapter 4, the mapping rates of rumen metagenome sequences were notably low, underscoring the inherent constraints associated with reference databases for classifying the rumen microbiome. Other approaches utilizing core genes have been proposed to increase discriminatory power at the species level. For instance, the *rpoB* gene, which encodes for the subunit of the bacterial RNA polymerase enzyme, has been suggested as a suitable candidate for phylogenetic analysis (Case et al., 2007; Ogier et al., 2019; Hassler et al., 2022). Studies have shown the *rpoB* gene to have superior discriminatory ability at the species level when compared to the 16S gene (Ogier et al., 2019; Hassler et al., 2022). However, this approach has not been widely utilised in the context of the rumen and work would be required to determine the suitability of this method for phylogenetic analysis of the rumen microbiota.

FCR was used in Chapters 2 and 3 to measure the feed efficiency of sheep, and to investigate the relationship between the rumen microbiota and FCR. FCR, calculated as the ratio of dry matter intake (DMI) to average daily gain (ADG), is a widely employed metric used to assess an animal's ability to convert feed into body mass (Fahmy et al., 1992). Studies have found FCR to be a heritable trait in sheep (Tortereau et al., 2020), suggesting that selective breeding for animals with superior FCR values could lead to improvements in the trait in future generations. Moreover, Li et al. (2020) found that specific microbial features that were heritable in cattle were also correlated with FCR, ADG and DMI, indicating

that breeding could be used to manipulate and select for efficient microbiomes in ruminants (Li et al., 2019b). While the research findings from Chapters 2 and 3 indicate relationships between the rumen microbiome and FCR, there are potential limitations to using FCR as a selection trait for breeding programs, as it is correlated with an animal's body weight and ADG (Tortereau et al., 2020). Therefore, selective breeding for enhanced FCR values may inadvertently result in selecting larger and faster-growing animals with increased nutritional requirements for maintenance (Arthur et al., 2004; Santana et al., 2012;Zhang et al., 2017), which could have negative implications for the sustainability of the industry in the long term. To address these limitations, residual feed intake (RFI) could be used as an alternative measure of feed efficiency. RFI quantifies the difference between the expected feed intake for maintenance and growth and the actual feed intake, and is independent of an animal's body weight and ADG (Zhang et al., 2017). Furthermore, RFI has been shown to have a higher heritability estimate than FCR (Tortereau et al., 2020; Zhao et al., 2022). These properties make RFI a more reliable indicator of feed efficiency and more suitable for selective breeding programs. Therefore, future FE-microbiome related studies would benefit from utilizing RFI as a measure of feed efficiency, as it offers a more robust and a sustainable alternative to FCR for breeding programs.

Chapter 3 of this PhD thesis represents the first investigation into the impact of breed on bacterial and archaeal populations across all three ruminal fractions. The research findings demonstrate that breed can have an effect on the abundance of specific bacterial taxa rather than on the overall community composition in the solid, liquid and epithelial ruminal fractions. A limitation of the study was the relatively small number of samples available for analysis, which was further reduced by the exclusion of samples due to poor sequencing quality. As a result, the statistical power of the study may have been diminished (Andrade, 2020), and the findings need to be underpinned by a larger-scale investigation. Despite these limitations, the study provides valuable insights into the impact of breed on rumen microbial populations and highlights the need for further research in this area.

Chapter 4 demonstrated that TOF influenced methane emissions and the rumen microbiota in pasture grazed sheep, held from 1 to 6 hours off feed. One limitation was that information on when individual animals were last fed prior to removal from pasture or the quantity of feed intake for each animal could not be accurately obtained. It is well established that dietary intake can have a significant influence on both the rumen microbiome and methane emissions (Janssen, 2010). This would have introduced variation into the data which could not be accounted for. Various approaches have been developed to estimate feed intake including mathematical models or the use of internal and external marker such as the n-alkane approach (Andriarimalala et al., 2020; Wright et al., 2020). However, it currently remains difficult to accurately determine intake from animals grazed on pasture. One alternative approach would be to conduct a feeding trial similar to that of Fraser et al. (2015), where animals spent a period of time grazing on pasture prior to being housed and offered freshly cut forage,

from the same pasture, allowing feed intake to be measured (Fraser et al., 2015). In addition, Chapter 4 was limited by the number of metagenome and metatranscriptome samples for functional analysis of the rumen microbiome, which would have impacted on the statistical power on that aspect of the study.

Recommendations and considerations for follow-up studies

Chapter 2 indicates that the composition of the rumen microbiome in sheep is altered between high and low feed efficient sheep. However, the functional basis underpinning rumen microbiome differences between the high and low feed-efficient cohorts was not investigated. Therefore, a potential future study could employ a multi-omics approach, including metagenomics, metatranscriptomics, and metabolomics, to shed light on possible functional possibilities underlying the phenotypic differences between the divergent cohorts. In addition, given that the rumen microbiome is a dynamic ecosystem that is constantly changing in response to the prevailing conditions in the rumen (Janssen, 2010), future research aimed at understanding the relationship between the rumen microbiome and feed efficiency could examine the stability of feed-efficient microbiomes over time through longitudinal-based studies. While such studies may be expensive, they would provide valuable insights into the dynamic relationship between the rumen microbiome and feed efficiency.

The findings of Chapters 2 and 3 of this PhD thesis indicate that there is a potential for breeding programs to select for more feed efficient microbiomes to improve feed efficiency and sustainability within the sheep industry. Indeed, Chapter 3 reveals the impact of sheep breed on specific microbial taxa within the rumen, while Chapters 2 and 3 identified associations between the rumen microbiome and FCR. However, there is a need for further investigation to determine the heritability of rumen microbial phenotypes in sheep and to identify the genotypes associated with these phenotypes to facilitate the development of genomic selection programs. It is important to note that while FCR was used as a measure of feed efficiency in both studies, its limitations for genomic selection suggest that residual feed intake (RFI) may be a more suitable trait for future studies.

Chapter 4 of the PhD thesis demonstrated that TOF could influence methane emissions and the composition of the rumen microbiome in pasture-fed sheep, and shed light on the temporal dynamics of the rumen microbiome and identified potential bacterial taxa that may play a role in methane production. However, this study found no associations between the TOF and the functionality of the rumen microbiome, which may have been attributable to the small number of samples available for metagenomic and metatranscriptomic analysis. Increasing the sample size for functional profiling of the rumen microbiome and investigating its relationship with TOF could be investigated further, and could be enhanced with the integration of metabolomics. Such a study would be useful for understanding the dynamic role of the rumen microbiome in relation to feed degradation, fermentation and methane generation. Information on feed intake would be necessary to account for the influence of diet.

Future perspectives

The use of next-generation sequencing (NGS) sequencing technologies will continue to be central methodologies for understanding the complex interactions between host and microbiome, and ultimately for understanding the role of the rumen microbiome for enhancing feed efficiency and reducing methane emissions in sheep. However, there is still considerable variation in pre- and post-sequencing approaches used to study the rumen microbiome (Szóstak et al., 2022). Technical variations in sample collection, DNA extraction, sequencing, and bioinformatic pipelines introduce bias (Siegwald et al., 2019) and make it difficult to compare and replicate studies in order to draw concrete and informative conclusions. Thus, there is a need for continued efforts to promote standardisation in protocols (McGovern et al., 2020; Szóstak et al., 2022) to reduce technical variation and improve the comparability and reproducibility of rumen microbiome studies. The use of mock community standards as positive controls therefore should be of utmost importance for future microbiome studies for the identification of errors and biases and the validation of microbiome workflows.

Metagenomics and metatranscriptomics are powerful tools for examining the composition and gene expression profiles of the rumen microbiome. Given the continued reduction in the cost DNA sequencing and the limitations associated with 16S rRNA profiling (Hassler et al., 2022), metagenomic and metatranscriptomic techniques can be expected to become more common place. However, it is important to acknowledge that these methods have limitations and that a more comprehensive understanding of the functional activity and metabolic pathways of the rumen microbiome requires the integration of other -omics approaches. For instance, gene expression does not necessarily guarantee successful translation of mRNA into a functional proteins, therefore, investigations of functionality at the protein level would be necessary (Hart et al., 2018). Similarly, metabolomics can be used to identify different metabolites produced by the rumen microbiome (Touitou et al., 2022), which can be linked to specific microbial processes or pathways. Combining information about the microbiome's composition, gene expression, protein production, and metabolite production, can provide a more holistic understanding of the functional workings of the rumen microbiome. In recent years, there has been a growing trend toward the integration of multiple -omics approaches to better understand complex microbial communities (Xue et al., 2020; Liu et al., 2022;Sasson et al., 2022) and this trend is likely to be of benefit for improving understanding of the rumen microbiome and its relationship with feed efficiency and methane production (Andersen et al., 2021).

Despite significant advancement in microbiome research, the rumen microbiome still remains incompletely characterised, meaning that reference databases still have important short-comings for rumen microbial research (Smith et al., 2022). Therefore, continued efforts to further characterise the rumen microbiome, such as the Hungate1000 project (Seshadri et al., 2018), are imperative for future studies. To date such advancements have been limited due to difficulties in

culturing members of the rumen microbiome. In addition, the heterogeneous nature of short read metagenome data makes it difficult to accurately assemble whole microbial genomes. Microbial single-cell (SC) sequencing (Woyke et al., 2017) and culturomics (Zehavi et al., 2018) are promising approaches that could be used to characterise individual members of the rumen microbiome. The information obtained from these approaches can be used to improve representation of the rumen microbiome in reference databases, which is essential for accurate analysis of metagenomics data, as well as the validation of findings from NGS approaches.

Sheep enteric methane data is currently reported in Ireland's national GHG inventories using Tier 1 methodologies (EPA, 2022). In contrast, enteric methane from cattle has been reported using Tier 2 methodologies since 2006. Tier 1 methods are less precise than Tier 2 and Tier 3 methods (Lokupitiya and Paustian, 2006) because they do not take into account detailed characteristics on dietary management and animal performance (Wilkes and Dijk, 2018). Thus, there is a need to transition to Tier 2 methodologies for reporting of enteric methane data from sheep. This transition is possible as there are available data on various aspects of Irish sheep that can be used to move towards Tier 2 methodologies, including sheep populations, finishing ages, concentrate usage, housing periods and manure storage systems (EPA, 2016). Because Tier 2 methods are more detailed, this would allow for the tracking of progress towards mitigation goals and the identification of areas requiring additional action. Therefore, transitioning from Tier 1 to Tier 2 methods for estimating sheep methane emissions in national inventories is essential for reducing greenhouse gas emissions and achieving mitigation targets.

Overall, this PhD thesis research has generated novel findings that advance our understanding of the role and relationship of the sheep microbiome to greenhouse gas emissions from ruminant animals, which is a pre-requisite for rational development of strategies and methods for reducing greenhouse gas emissions from ruminant livestock.

References

Andersen, T.O., Kunath, B.J., Hagen, L.H., Arntzen, M.Ø., and Pope, P.B. (2021). Rumen metaproteomics: Closer to linking rumen microbial function to animal productivity traits. *Methods* 186**,** 42-51.

Anderson, C.J., Koester, L.R., and Schmitz-Esser, S. (2021). Rumen Epithelial Communities Share a Core Bacterial Microbiota: A Meta-Analysis of 16S rRNA Gene Illumina MiSeq Sequencing Datasets. *Front Microbiol* 12**,** 625400.

Andrade, C. (2020). Sample size and its importance in research. *Indian J Psychol Med* 42**,** 102-103.

Andriarimalala, J.H., Dubeux Jr, J.C.B., Dilorenzo, N., Jaramillo, D.M., Rakotozandriny, J.D.N., and Salgado, P. (2020). Use of n-alkanes to

estimate feed intake in ruminants: A meta-analysis. *Journal of Animal Science* 98**,** skaa304.

Arthur, P., Archer, J., and Herd, R. (2004). Feed intake and efficiency in beef cattle: overview of recent Australian research and challenges for the future. *Australian Journal of Experimental Agriculture* 44**,** 361-369.

Bauchop, T. (1981). The anaerobic fungi in rumen fibre digestion. *Agriculture and Environment* 6**,** 339-348.

Blaxter, K., Clapperton, J., and Wainman, F. (1966). The extent of differences between six British breeds of sheep in their metabolism, feed intake and utilization, and resistance to climatic stress. *British journal of Nutrition* 20**,** 283-294.

Case, R.J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W.F., and Kjelleberg, S. (2007). Use of 16S rRNA and rpoB Genes as Molecular Markers for Microbial Ecology Studies. *Applied and Environmental Microbiology* 73**,** 278-288.

Chen, Y., and Oba, M. (2012). Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. *Veterinary microbiology* 159**,** 451-459.

Emerson, E.L., and Weimer, P.J. (2017). Fermentation of model hemicelluloses by Prevotella strains and Butyrivibrio fibrisolvens in pure culture and in ruminal enrichment cultures. *Appl Microbiol Biotechnol* 101**,** 4269-4278.

Epa (2016). *A Review of Livestock Methane Emission Factors* [Online]. Available: https://www.epa.ie/publications/research/climate-change/Research_Report_288.pdf [Accessed 06/04/23].

Epa (2022). *Ireland's National Inventory Report 2022* [Online]. Available: https://www.epa.ie/publications/monitoring--assessment/climate-change/air-emissions/Ireland-NIR-2022_Merge_v2..pdf [Accessed 04/04/23].

Fahmy, M.H., Boucher, J.M., Poste, L.M., Grégoire, R., Butler, G., and Comeau, J.E. (1992). Feed efficiency, carcass characteristics, and sensory quality of lambs, with or without prolific ancestry, fed diets with different protein supplements1. *Journal of Animal Science* 70**,** 1365-1374.

Fraser, M.D., Fleming, H.R., Theobald, V.J., and Moorby, J.M. (2015). Effect of breed and pasture type on methane emissions from weaned lambs offered fresh forage. *J Agric Sci* 153**,** 1128-1134.

Giger-Reverdin, S., and Sauvant, D. (2000). Methane production in sheep in relation to concentrate feed composition from bibliographic data. *Sheep and goat nutrition: Intake, digestion, quality of products and rangelands Zaragoza. Zaragoza: Ciheam-Iamz***,** 43-46.

Greening, C., Geier, R., Wang, C., Woods, L.C., Morales, S.E., Mcdonald, M.J., Rushton-Green, R., Morgan, X.C., Koike, S., and Leahy, S.C. (2019). Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME journal* 13**,** 2617-2632.

Guyader, J., Eugène, M., Nozière, P., Morgavi, D.P., Doreau, M., and Martin, C. (2014). Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. *animal* 8**,** 1816-1825.

Hart, E.H., Creevey, C.J., Hitch, T., and Kingston-Smith, A.H. (2018). Meta-proteomics of rumen microbiota indicates niche compartmentalisation and

functional dominance in a limited number of metabolic pathways between abundant bacteria. *Scientific Reports* 8**,** 10504.

Hassler, H.B., Probert, B., Moore, C., Lawson, E., Jackson, R.W., Russell, B.T., and Richards, V.P. (2022). Phylogenies of the 16S rRNA gene and its hypervariable regions lack concordance with core genome phylogenies. *Microbiome* 10**,** 104.

Janssen, P.H. (2010). Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160**,** 1-22.

Knaus, W. (2016). Perspectives on pasture versus indoor feeding of dairy cows. *Journal of the Science of Food and Agriculture* 96**,** 9-17.

Li, F., Hitch, T.C., Chen, Y., Creevey, C.J., and Guan, L.L. (2019a). Comparative metagenomic and metatranscriptomic analyses reveal the breed effect on the rumen microbiome and its associations with feed efficiency in beef cattle. *Microbiome* 7**,** 1-21.

Li, F., Li, C., Chen, Y., Liu, J., Zhang, C., Irving, B., Fitzsimmons, C., Plastow, G., and Guan, L.L. (2019b). Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle. *Microbiome* 7**,** 92.

Li, Z., Mu, C., Xu, Y., Shen, J., and Zhu, W. (2020). Changes in the solid-, liquid-, and epithelium-associated bacterial communities in the rumen of hu lambs in response to dietary urea supplementation. *Frontiers in microbiology* 11**,** 244.

Liu, K., Zhang, Y., Yu, Z., Xu, Q., Zheng, N., Zhao, S., Huang, G., and Wang, J. (2021). Ruminal microbiota–host interaction and its effect on nutrient metabolism. *Animal Nutrition* 7**,** 49-55.

Liu, Y., Wu, H., Chen, W., Liu, C., Meng, Q., and Zhou, Z. (2022). Rumen Microbiome and Metabolome of High and Low Residual Feed Intake Angus Heifers. *Frontiers in Veterinary Science* 9.

Lokupitiya, E., and Paustian, K. (2006). Agricultural Soil Greenhouse Gas Emissions. *Journal of Environmental Quality* 35**,** 1413-1427.

Mcgovern, E., Mcgee, M., Byrne, C.J., Kenny, D.A., Kelly, A.K., and Waters, S.M. (2020). Investigation into the effect of divergent feed efficiency phenotype on the bovine rumen microbiota across diet and breed. *Scientific Reports* 10**,** 1-11.

Noel, S.J., Olijhoek, D.W., Mclean, F., Løvendahl, P., Lund, P., and Højberg, O. (2019). Rumen and fecal microbial community structure of Holstein and Jersey dairy cows as affected by breed, diet, and residual feed intake. *Animals* 9**,** 498.

O'brien, D., Moran, B., and Shalloo, L. (2018). A national methodology to quantify the diet of grazing dairy cows. *Journal of Dairy Science* 101**,** 8595-8604.

O′Mara, F., Richards, K.G., Shalloo, L., Donnellan, T., Finn, J.A., and Lanigan, G. (2021). Sustainability of ruminant livestock production in Ireland. *Animal Frontiers* 11**,** 32-43.

Ogier, J.-C., Pagès, S., Galan, M., Barret, M., and Gaudriault, S. (2019). rpoB, a promising marker for analyzing the diversity of bacterial communities by amplicon sequencing. *BMC Microbiology* 19**,** 171.

Prins, R.A., Van Vugt, F., Hungate, R.E., and Van Vorstenbosch, C.J.a.H.V. (1972). A comparison of strains ofEubacterium cellulosolvens from the rumen. *Antonie van Leeuwenhoek* 38**,** 153-161.

Robinson, D.L., Goopy, J.P., Hegarty, R., Oddy, V., Thompson, A., Toovey, A., Macleay, C., Briegal, J., Woodgate, R., and Donaldson, A. (2014). Genetic and environmental variation in methane emissions of sheep at pasture. *Journal of Animal Science* 92**,** 4349-4363.

Santana, M., Rossi Jr, P., Almeida, R., and Cucco, D. (2012). Feed efficiency and its correlations with carcass traits measured by ultrasound in Nellore bulls. *Livestock Science* 145**,** 252-257.

Sasson, G., Moraïs, S., Kokou, F., Plate, K., Trautwein-Schult, A., Jami, E., Bayer, E.A., Becher, D., and Mizrahi, I. (2022). Metaproteome plasticity sheds light on the ecology of the rumen microbiome and its connection to host traits. *The ISME Journal* 16**,** 2610-2621.

Schaeren, M., Kiri, K., Riede, S., Gardener, M., Meyer, U., Hummel, J., Urich, T., Breves, G., and Daenicke, S. (2017). Alterations in the rumen liquid-, particle-and epithelium-associated microbiota of dairy cows during the transition from a silage-and concentrate-based ration to pasture in spring. *Frontiers in microbiology* 8**,** 744.

Sengupta, K., Hivarkar, S.S., Palevich, N., Chaudhary, P.P., Dhakephalkar, P.K., and Dagar, S.S. (2022). Genomic architecture of three newly isolated unclassified Butyrivibrio species elucidate their potential role in the rumen ecosystem. *Genomics* 114**,** 110281.

Seshadri, R., Leahy, S.C., Attwood, G.T., Teh, K.H., Lambie, S.C., Cookson, A.L., Eloe-Fadrosh, E.A., Pavlopoulos, G.A., Hadjithomas, M., Varghese, N.J., Paez-Espino, D., Perry, R., Henderson, G., Creevey, C.J., Terrapon, N., Lapebie, P., Drula, E., Lombard, V., Rubin, E., Kyrpides, N.C., Henrissat, B., Woyke, T., Ivanova, N.N., and Kelly, W.J. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nat Biotechnol* 36**,** 359-367.

Shi, W., Moon, C.D., Leahy, S.C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., and Seedorf, H. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. *Genome research* 24**,** 1517-1525.

Siegwald, L., Caboche, S., Even, G., Viscogliosi, E., Audebert, C., and Chabé, M. (2019). The Impact of Bioinformatics Pipelines on Microbiota Studies: Does the Analytical "Microscope" Affect the Biological Interpretation? *Microorganisms* 7.

Smith, R.H., Glendinning, L., Walker, A.W., and Watson, M. (2022). Investigating the impact of database choice on the accuracy of metagenomic read classification for the rumen microbiome. *Animal Microbiome* 4**,** 57.

Szóstak, N., Szymanek, A., Havránek, J., Tomela, K., Rakoczy, M., Samelak-Czajka, A., Schmidt, M., Figlerowicz, M., Majta, J., Milanowska-Zabel, K., Handschuh, L., and Philips, A. (2022). The standardisation of the approach to metagenomic human gut analysis: from sample collection to microbiome profiling. *Scientific Reports* 12**,** 8470.

Tortereau, F., Marie-Etancelin, C., Weisbecker, J.-L., Marcon, D., Bouvier, F., Moreno-Romieux, C., and François, D. (2020). Genetic parameters for feed

efficiency in Romane rams and responses to single-generation selection. *Animal* 14**,** 681-687.

Touitou, F., Tortereau, F., Bret, L., Marty-Gasset, N., Marcon, D., and Meynadier, A. (2022). Evaluation of the Links between Lamb Feed Efficiency and Rumen and Plasma Metabolomic Data. *Metabolites* 12.

Van Gylswyk, N. (1995). Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *International Journal of Systematic and Evolutionary Microbiology* 45**,** 297-300.

Van Gylswyk, N.O., Hippe, H., and Rainey, F.A. (1996). Pseudobutyrivibrio ruminis gen. nov., sp. nov., a Butyrate-Producing Bacterium from the Rumen That Closely Resembles Butyrivibrio fibrisolvens in Phenotype. *International Journal of Systematic and Evolutionary Microbiology* 46**,** 559-563.

Wilkes, A., and Dijk, S.V. (2018). Tier 2 inventory approaches in the livestock sector: a collection of agricultural greenhouse gas inventory practices.

Woyke, T., Doud, D.F.R., and Schulz, F. (2017). The trajectory of microbial single-cell sequencing. *Nature Methods* 14**,** 1045-1054.

Wright, M., Auldist, M., Kennedy, E., Dunshea, F., Galvin, N., Hannah, M., and Wales, W. (2020). Evaluation of the n-alkane technique for estimating the individual intake of dairy cows consuming diets containing herbage and a partial mixed ration. *Animal Feed Science and Technology* 265**,** 114524.

Xue, M.-Y., Sun, H.-Z., Wu, X.-H., Liu, J.-X., and Guan, L.L. (2020). Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8**,** 64.

Young, J. (1977). Gluconeogenesis in cattle: significance and methodology. *Journal of dairy science* 60**,** 1-15.

Zehavi, T., Probst, M., and Mizrahi, I. (2018). Insights Into Culturomics of the Rumen Microbiome. *Frontiers in Microbiology* 9.

Zhang, X., Wang, W., Mo, F., La, Y., Li, C., and Li, F. (2017). Association of residual feed intake with growth and slaughtering performance, blood metabolism, and body composition in growing lambs. *Scientific Reports* 7**,** 12681.

Zhao, Y., Zhang, X., Li, F., Zhang, D., Zhang, Y., Li, X., Song, Q., Li, C., Zhao, L., Wang, J., Xu, D., Cheng, J., Li, W., Lin, C., Zhou, B., and Wang, W. (2022). Estimation of genetic correlations of two key feed efficiency traits with production traits in male Hu sheep. *Anim Biotechnol***,** 1-12.