Phenotypic characterisation of members of the *Lachnospiraceae* family isolated from ruminants

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Microbiology

at

Massey University, Manawatū, New Zealand

Krsana Rajasekaran

2023

Abstract

Microbial fermentation in the rumen employs the metabolic capacity of microorganisms to degrade lignocellulose from the diets consumed by ruminant animals. Advances in genomic, metagenomic and culture independent methods for studying microbiomes have caused a lag in the functional characterisation of isolated cultures. Moreover, understanding the interactions between microbes during rumen fermentation may help in producing strategies to improve animal productivity and address environmental impact issues such as enteric methane emissions. In this study it is demonstrated how cultured strains are required to accurately describe the functional traits of rumen bacteria. Members of the *Lachnospiraceae* family are one of the most abundant bacterial groups in the rumen, however, many of its isolated members are yet to be fully characterised or properly classified. In this study, the genomes of 45 *Lachnospiraceae* strains sequenced in the Hungate 1000 project were functionally annotated using the web-based annotation tool, Protologger. These predictions were then compared with phenotypic traits from the corresponding strains, uncovered using microscopy, carbon utilisation testing, and by analyses of short-chain fatty acid production, and headspace hydrogen. The results indicate how the genome can assist in the culturing and studying of rumen microorganisms but should not be solely relied on for the elucidation of functional traits.

Phenotypic characterisations of the 45 *Lachnospiraceae* strains revealed a preference for the resultant soluble components of cellulose degradation rather than hemicellulose. Starch and pectin were more readily fermented in comparison to cellulose and xylan. End product analysis revealed that the studied strains produce acetate, butyrate and propionate, products known to contribute to host health and nutrition. Ethanol, formate, lactate and less commonly succinate were produced as fermentation products demonstrating the potential of the strains to participate in interspecies metabolite transfers. A subset of the strains including members of the genera *Lachnospira*, *Eubacterium* and *Oribacterium* as well as unclassified *Lachnospiraceae* bacterium strains were shown to produce methanol from pectin degradation. End products of fibrolytic fermentation by the 45 *Lachnospiraceae* strains can potentially act as substrates for methanogenic archaea. The results of this study help to improve the knowledge surrounding the poorly studied *Lachnospiraceae* family and

increases the overall utility of the Hungate 1000 culture collection. Additionally, the comparison between genotypic predictions and the phenotyping, accentuates the importance of culture-based studies, providing an incentive to continue cultivating representative strains from the rumen environment to clarify how various microorganisms are contributing to rumen fermentation.

Acknowledgements

I would like to express my gratitude to my supervisors Dr Dragana Gagic, Dr Graeme Attwood, and Dr William (Bill) Kelly for their tremendous support and supervision in completing this project. I would like to thank Kerri Reilly, whose experience helped immensely in culturing the Hungate 1000 isolates and conducting the technical work. My thanks also go to Peter Reid, who helped me run the 1000+ VFA samples on the GCs so quickly.

Thank you to the funders of this project: the Master's Award was funded by the New Zealand Government to support the objectives of the Global Research Alliance on Agricultural Greenhouse Gases. Thanks also to the personnel at the New Zealand Agricultural Greenhouse Gas Centre for administering the funding support.

I would like to thank my lab mates, specifically: Dr Laureen Crouzet, Ryzzah Trinidad, Wen Lim, Dr Rachel Kaminsky, Priya Soni, Rebecca Tavendale, Rosemary Heathcott, Amy Wells, Catherine Andrews, and Kathy Wieliczko, who all helped me technically or with words of encouragement, without which, I could not have made it through all the long days in the lab. To all the people in the Rumen Microbiology team, thank you for your support and encouragement.

I would like to thank my family, especially Appa (who helped me look through my many drafts), Amma, Shrika, and Rayhan. And a very special thank you to my grandma, Saradha. Thank you all for continually being my greatest supporters.

Contents

List of Figures

List of Tables

Abbreviations

× g	times gravity
µg	microgram
µL	microlitre
µm	micrometre
Ack	acetate kinase
Adh	alcohol dehydrogenase
Aldh	aldehyde dehydrogenase
ANI	average nucleotide identity
Aor	aldehyde: ferredoxin oxidoreductase
Bcd	butyryl-CoA dehydrogenase
BdhAB	(butanol) dehydrogenase
CAZymes	carbohydrate active enzymes
CBH	cellobiohydrase
CBM	carbohydrate binding module
CE	carbohydrate esterase
CH_4	methane
CO_2	carbon dioxide
CoA	coenzyme A
CRISPR	clustered regularly interspaced short palindromic repeats
DNA	deoxyribose nucleic acid
EC	Enzyme Commission
EG	endoglucanase
FAs	fatty acids
Fd ox	oxidised ferredoxin
Fd red	reduced ferredoxin
g	gram
GC	gas chromatography
GenRFV	general sugar rumen fluid vitamin mix

GH	glycoside hydrolase
GIT	gastrointestinal tract
GLC	gas liquid chromatography
GT	glycosyl transferases
GTDB	Genome Taxonomy Database
H_2	dihydrogen
HGA	homogalacturonan
HTP	high throughput
IMG	Integrated Microbial Genomes and Microbiomes
ITS	internal transcribed spacer sequences
KEGG	Kyoto Encyclopedia of Genes and Genomes
M	molar
M2GSC	Modified Medium 2 of Hobson
MAG	metagenome assembled genomes
Mbp	megabase pairs
min	minutes
mL	millilitre
mm	millimetre
mM	millimolar
NoSubRFV	no substrate rumen fluid vitamin mix
OD	optical density
OD_{600}	optical density at 600nm
OTU	operational taxonomic units
PCR	polymerase chain reaction
Pdc	pyruvate decarboxylase
Pdh	phenylacetaldehyde dehydrogenase
PME	pectin methyl esterase
PL	polysaccharide lyase
POCP	percentage of conserved proteins

Por	pyruvate: ferredoxin oxidoreductase
Pta	phosphotransacetylase
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonan II
RDP	rumen degradable protein
RM02	rumen mineral medium 2
rRNA	ribosomal ribonucleic acid
SCFAs	short chain fatty acids
sec	seconds
TAE	tris base, acetic acid and ethylenediaminetetraacetic acid
TE	tris- ethylenediaminetetraacetic acid
V	volts
VFA	volatile fatty acid
w/v	weight/volume

Chapter 1. Introduction

Ruminant livestock rely on a diverse community of microorganisms in their forestomachs (the reticulo-rumen, referred to as the rumen) to digest lignocellulose in their diet (Wallace et al., 2019). The resident microorganisms of the rumen work synergistically to produce short-chain fatty acids (SCFAs) and protein, which contribute to approximately 70% of the nutrition for the host animals (Shen et al., 2019). Although many rumen microorganisms have been isolated and characterised through classic cultivation studies and more recently via genomic and metagenomic approaches, the cultivation and characterisation of rumen microorganisms remains an on-going task to gain a better understanding of the microbiology of the rumen.

Only ~4% of all the microbial operational taxonomic units (OTUs) identified via marker gene amplification and sequencing have representative strains in culture which clearly demonstrates the large gap which remains between gene-based and culture-dependent techniques (Creevey et al., 2014). The rumen microbial community consists of organisms that are specialised in the breakdown and bioconversion of plant material into usable end products for the host animal. To further understand the fermentative capabilities of these organisms, cultivation and characterisation in the laboratory is required. Furthering knowledge on rumen microbial strains and new isolates will help to understand the processes of rumen fermentation, potentially providing insights into how different groups of microorganisms work together interdependently.

The Hungate 1000 project was a collaborative initiative between several rumen microbiology laboratories worldwide which aimed to address this problem and produced a reference set of 501 rumen microbial strains. While all the Hungate cultures have draft or complete genome sequences, many of them have not been phenotypically characterised. Specifically, a large group of bacterial strains belonging to the family *Lachnospiraceae* are yet to be classified. *Lachnospiraceae* is a phenotypically heterogenous, fermentative group of bacteria (Cotta & Forster, 2006). There is a need to increase the knowledge surrounding species level diversity in terms of fibre degrading ability and metabolite production which will help to describe the role of this family in the rumen environment as well as other mammalian gastrointestinal tract (GIT) environments.

1.1. Rumen structure and function

The rumen accounts for approximately 12-25% of the live weight of ruminants and is the largest of the forestomach compartments of these animals (Waghorn et al., 1989). The rumen, reticulum, and the omasum are called pre-gastric compartments as they are located prior to the true gastric stomach, called the abomasum (Balch, 1959). Plant material is eaten, chewed and regurgitated by ruminants repeatedly so that the material is decreased in size. Endogenous enzymes in the saliva then assist to degrade protein and starch within the digesta (Sissons, 1981). The digesta remains in the reticulo-rumen for an extended period of time providing time for the complex rumen microbial community to degrade and digest this material. Here, most of the SCFAs and nutrients are absorbed. Circular muscle tissue in the rumen and reticulum contracts to churn and mix the digesta to push it up through the oesophagus for regurgitation (Pfannkuche et al., 2004). Finger like papillae protrude out in to the lumen of the rumen, increasing the surface area of the epithelium which improves the efficiency of nutrient uptake (Steele et al., 2016). The rumen has evolved as a highly specialised organ to contain and break down lignocellulose in coordination with the ruminal microbial community. Once the feed particle size is reduced sufficiently, the material passes out of the reticulo-rumen to the omasum where most of the fluid is recovered and the residual material moves through to the gastric abomasum where acid hydrolysis occurs (Balch, 1959).

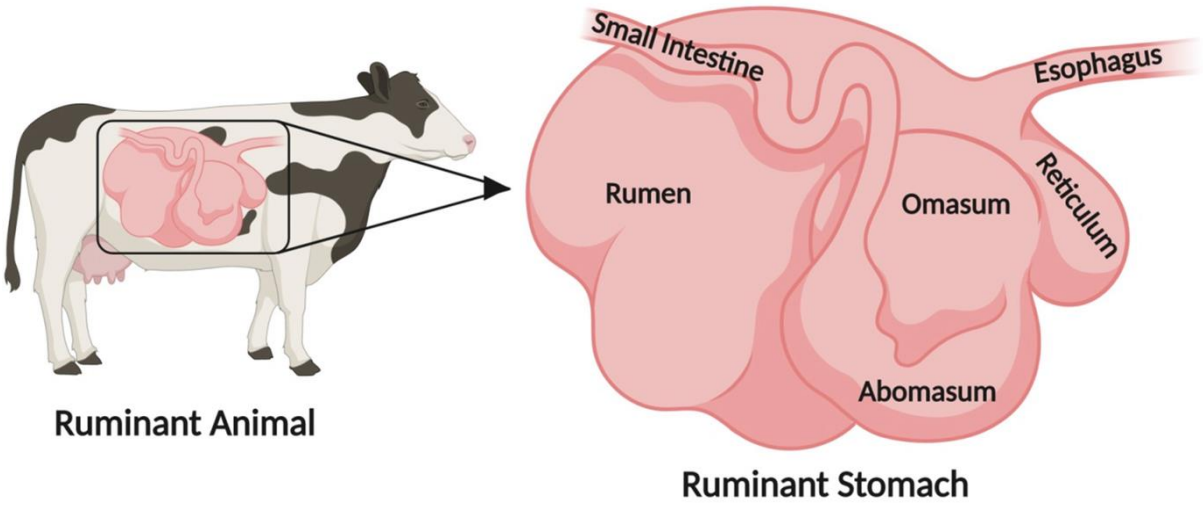


Figure 1.1 Schematic diagram of bovine rumen structure. Image obtained with permission from "Untangling the structure and function of rumen microbes in relation to ruminant health and exploring their biotechnological applications." By Gowtham, H.G., Priyanka, G., Hariprasad, P., 2022, *Animal Manure. Soil Biology, vol 64.* (https://doi.org/10.1007/978-3-030-97291-2_5).

1.2. Members of the rumen microbial community

1.2.1. Protozoa

Protozoa are single-celled eukaryotes that makeup almost 50% of the biomass of the rumen microbial community, however, they only account for ~1×10^6 cells/mL of rumen fluid, due to their relatively large size (Williams et al., 2020). Two groups of protozoa are known to inhabit the rumen; entodiniomorphs and holotrichs. Rumen protozoa are challenging to culture as the presence of extracellular and intra-cellular bacterial symbiotic partners makes it difficult to produce axenic protozoal cultures (Dehority, 2008). The role of protozoa in the rumen is still not fully understood as procuring functional data for these organisms remains difficult.

Defaunation (the removal of protozoa from the rumen using chemical or physical means) studies have provided insights into protozoal function in the rumen (Mosoni et al., 2011). A meta-analysis by Newbold et al. in 2015, showed that organic matter digestibility was significantly decreased in defaunated animals, suggesting that protozoa play an intermediate fibrolytic role to free substrates for other species. Defaunation could have detrimental effects on members of the rumen consortia which are reliant on protozoal communities to

produce an environmental niche for them (Solomon et al., 2022). Due to the limitations of studying protozoa in the rumen; there is still a lot to learn about what these organisms do and how they influence fermentation. Advancing culturing technologies of both bacteria and protozoa will help in providing reliable results.

1.2.2. Anaerobic rumen fungi

Six phyla of anaerobic rumen fungi have been described to date: *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces,* and *Piromyces* (Joshi et al., 2018). There is still some debate about how much microbial biomass in the rumen is contributed by fungi. Quantitative PCR data indicates that fungi represent 8-10% of the total biomass in the rumen (McSweeney & Mackie, 2012). Zoospores are detectable in the rumen fluid, but rhizoids that are associated with plant material are harder to detect. The close association of fungi with feed material suggest an important fibre-degrading role in the rumen (Hagen et al., 2021). Characterising the rumen fungal community has been challenging, again, due to the limited success in culturing these organisms. Advances in taxonomic analyses using internal transcribed spacer sequences (ITS I & II), means quantitative data on fungal abundances is now available (Blaalid et al., 2013; Kittelmann et al., 2013).

1.2.3. Archaea

The majority of the archaea in the rumen are strictly anaerobic methanogens (Patra et al., 2017). Archaea contribute 0.3-3% of the rumen microbiome as found using ribosomal RNA (rRNA) gene-targeted DNA probes (Janssen & Kirs, 2008). Archaea in the rumen fall into four different orders: *Methanobacteriales, Methanomicrobiales*, *Methanomassiliicoccales* and *Methanosarcinales*. The predominant genus is *Methanobrevibacter* from the order *Methanobacteriales* representing approximately 70% of the archaeal population (Janssen & Kirs, 2008). Ruminal methanogens are mainly hydrogenotrophic or methylotrophic and only a few isolated rumen methanogens are acetoclastic or able to use short chain alcohols as a source of reducing power (Hedderich & Whitman, 2013; Li et al., 2023). Methanogens occupy the lower trophic levels of rumen fermentation, and rely on interspecies transfer of H_2 (Hegarty & Klieve, 1999). Coculture experiments have demonstrated the close relationships between fermentative bacterial species and methanogens (Leahy et al., 2010; Sasaki et al., 2012).

Hydrogenotrophic methanogens are the most abundant; with 9 out of the 11 clades discovered predicted to be hydrogen utilisers (Henderson et al., 2015; Janssen & Kirs, 2008). Organisms belonging to the genera *Methanobrevibacter, Methanomicrobium, Methanobacterium, Methanosphaera*, and *Methanimicrococcus* are able to scavenge H_2 to reduce carbon dioxide (CO_2) into methane (CH_4) (Cord-Ruwisch et al., 1988).

Methylotrophic methanogens such as *Methanomassiliicoccales* and *Methanosphaera* possess methyltransferase systems that allow them to reduce methyl compounds such as methanol, methylamines, or methyl-sulphides into CH_4 (Söllinger et al., 2018). Additionally, some methanogens, such as members of *Methanobacteriales,* combine both the hydrogenotrophic and methylotrophic modes of methanogenesis, that allows cofactor bound methyl groups to be oxidised into CO_2 (Hedderich & Whitman, 2013).

Acetoclastic methanogens such as *Methanosarcina barkeri* are able to utilise acetate to produce CH_4 (He et al., 2019). In this instance, acetate is converted to acetyl-CoA and is attached to a methyl group to become methyl-H_4SPT, which is then shuttled in to the main methanogenesis pathway (Stams et al., 2019).

Alternative sources of reducing power for methanogenesis are formate and short chain alcohols which act as electron donors for methanogenesis. Only two rumen methanogen strains, *Mbb. boviskoreani* JH1 and the closely related AbM4 strain are known to use short chain alcohols as a source of reducing power to reduce CO_2 to CH_4, without the need of H_2 (Li et al., 2023).

1.2.4. Bacteria

Bacteria are the most numerous microorganisms in the rumen at a concentration between 1×10^{10} to 1×10^{11} cells/mL of rumen fluid (Russell & Hespell, 1981). The *Bacteroidetes* and *Firmicutes* phyla dominate the bacterial community (Matthews et al., 2019). The high fibre diets of ruminant animals require the presence of key fibre degraders to break down recalcitrant cellulose via enzymatic means (Emerson & Weimer, 2017). Fibre degrading bacteria are among the most studied microorganisms in the rumen and their colonisation of the rumen a few days after birth suggests they have an important role in rumen metabolism (Jami & Mizrahi, 2012).

16S rRNA gene amplicon sequencing and community analysis of 742 rumen samples from ruminants around the world revealed a conserved dominant group of bacterial families that were present at similar abundances, namely, *Prevotella, Butyrivibrio*, *Ruminococcus* and unclassified members from *Lachnospiraceae, Clostridiales*, *Ruminococcaceae* and *Bacteroidales* (Henderson et al., 2015). The full extent of bacterial diversity is yet to be determined and there is limited data available to describe their roles in rumen fermentation (Creevey et al., 2014).

1.3. The locations and functions of bacteria in the rumen

There are three overlapping microenvironments found in the foregut of ruminants creating different niches for rumen microbes; the liquid phase constitutes around 25% of the microbial mass, the solid phase which makes up around 70% of the microbial mass, and the final 5% includes microbial cells in close association with rumen epithelial cells (Pinnell et al., 2022). The primary role of these bacteria in these ruminal niches is to convert feed material into end products that can be used by the ruminant animal. SCFAs are the major end products of rumen fermentation and together with microbial protein provide 70-85% of the nutrition for ruminants (Loerch & Oke, 2018). Acetate, propionate, and butyrate are the primary SCFAs produced. Certain non-volatile fatty acids are also formed as intermediates, including lactate and succinate, which are further metabolised into the major SCFAs (Bergman, 1990). To better understand how microbial communities in these niches interact there needs to be more resolution of the functional capabilities of individual members of the rumen bacterial groups .

1.4. Bacterial diversity in the rumen

The full extent of the bacterial diversity in the rumen is not yet known (Creevey et al., 2014). Molecular techniques have allowed researchers to estimate bacterial diversity and based on studies by Edwards et al. (2004), Sundset et al. (2007), and Henderson et al. (2015) it is apparent that as advances in this field are made, there is an ongoing re-evaluation of the diversity of bacteria in the rumen. Edwards et al. in 2004 reported 341 operational taxonomic units (OTUs) in cattle. However, 16S rRNA gene analysis in a study by Zhu et al. (2021) estimated there were around 6187 OTUs, their newer sequencing technologies revealed that the diversity in the rumen ecosystem was greater than previously thought (Zhu et al., 2021).

The study by Henderson et al. in 2015, where 742 rumen samples from 32 animal species, in 35 countries were analysed, showed that only 14% of OTUs were named species and 70% were not within identified genera. This study encompassed a wide range of samples providing a better indication of core microbial diversity in a range of ruminant species. It is apparent that there is an opportunity for further research to classify and characterise more of the ruminal bacterial community.

In 2018, Seshadri et al. produced a reference set of around 500 microbial isolates from the rumen and found the major taxonomic groups could be identified. However, uncultured bacteria make up large proportions of the fibre degrading potential in the rumen. Around 77% of bacterial fibre degraders are uncultured organisms (Seshadri et al., 2018). The presence of uncultivated and uncharacterised strains makes it difficult to draw conclusions about the function of the bacterial community.

1.5. *Lachnospiraceae*

The family *Lachnospiraceae* is one of the most abundant microbial groups in the rumen (Matthews et al., 2019), and makes up 32% of the bacteria within the Hungate 1000 project. Yet, many of the cultured *Lachnospiraceae* strains have not been phenotypically characterised. This family of microorganisms belongs to the order *Clostridiales* and is currently described as having 59 different genera (Vacca et al., 2020). There is a high level of heterogeneity amongst its members, so it is likely that the functional characterisation of these strains will lead to the delineation of novel genera and species which will help to better describe this family. As strict anaerobes, they are primarily found in oxygen-poor environments such as in mammalian digestive systems (Meehan & Beiko, 2014).

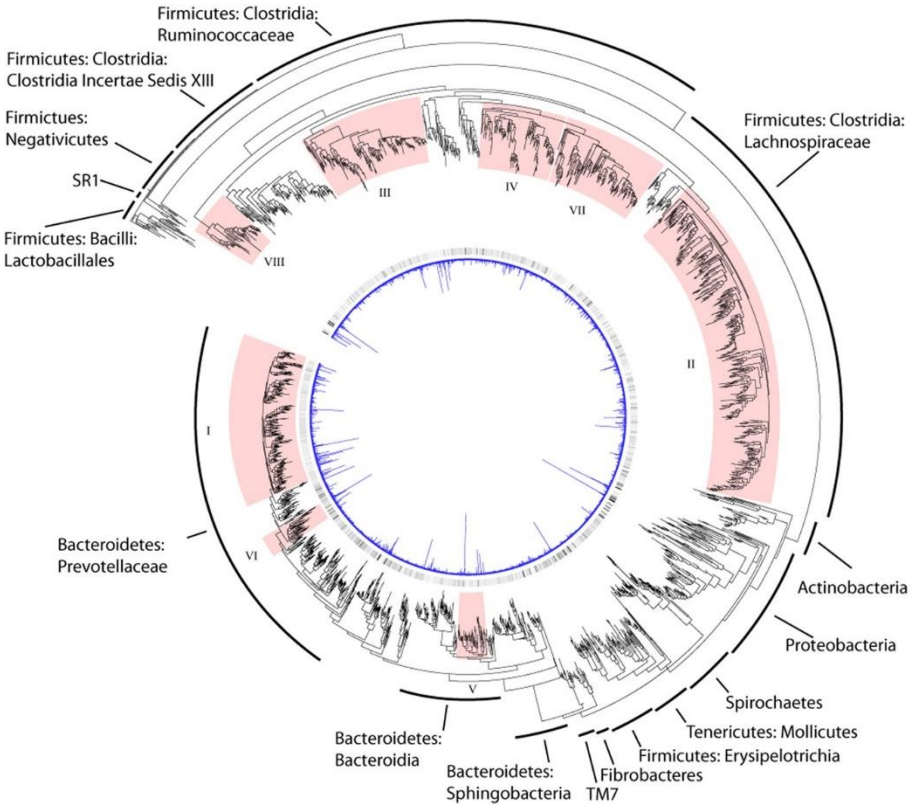


Figure 1.2 Phylogenetic tree representing 2405 rumen OTUs from the Ribosomal Database Project. Figure reprinted from 'Determining the culturability of the rumen bacterial microbiome' by C. J. Creevey, W. J. Kelly, G. Henderson and S. C. Leahy, 2014, *Applied Microbiology international, 7*(5), p. 467-479 (https://doi.org/10.1111/1751-7915.12141) .

1.5.1. Genomic diversity of *Lachnospiraceae*

There is a high level of morphological and phylogenetic heterogeneity in the *Lachnospiraceae* family. In a study carried out by Sorbara et al in 2020, it was found that only 397 protein coding genes were shared in a core genome of 273 human gut *Lachnospiraceae* strains . Additionally, the genes that were shared were mostly associated with fundamental metabolic processes such as peptidoglycan synthesis and glycolysis. Genes associated with more specific metabolic processes, such as sugar transport, were shared by organisms belonging to the same genus or species (Sorbara et al., 2020).

1.6. Fibre degradation in the rumen and the role of *Lachnospiraceae*

1.6.1. The fibre degrading potential of *Lachnospiraceae*

Metagenomic studies reveal a positive relationship between rumen *Lachnospiraceae* species and carbohydrate degrading metabolic pathways (Xie et al., 2022). This suggests a large capability to utilise polysaccharides derived from the diet of host animals. Additionally,

members of the *Lachnospiraceae* family possess large numbers of carbohydrate active enzymes (CAZymes) for example, *Lachnospiraceae* bacterium NLAE-zl-G231 has 296 glycoside hydrolases (GHs) and polysaccharide lysases (PLs) (Seshadri et al., 2018). The fermentation of carbohydrates in the rumen leads to the production of SCFAs. Although there are many physiologically diverse bacterial species in the rumen, the pathways involved in fermentation are conserved. Generally, rumen microbes use a range of CAZymes to breakdown carbohydrates. The CAZy database consists of experimentally characterised CAZymes associated with the assembly, deconstruction and modification of carbohydrates (Lombard et al., 2014). The main CAZymes involved in carbohydrate breakdown are GHs, carbohydrate esterases (CEs), PLs and carbohydrate binding modules (CBMs) (Cantarel et al., 2009). GHs are the most abundant CAZymes and include glycosidases and transglycosidases which are responsible for the hydrolysis and transglycosylation of glycosidic bonds. CEs act upon ester bonds attached to carbohydrate molecules which allows for the accelerated degradation of polymeric sugars and facilitates access to GHs for further degradation (Nakamura et al., 2017). PLs use elimination chemistry to cleave uronic acid containing polysaccharides such as pectin (Lombard et al., 2010). CBM are non-catalytic protein domains which can optimise catalytic hydrolases by binding them to substrates (Sidar et al., 2020). The presence of genes that code for GH, CE, PL enzymes and CBM proteins in microbial genomes are indicative of their carbohydrate fermentation capacity. Another type of CAZyme, the glycosyl transferases (GTs), are involved in the biosynthesis of glycosidic bonds in microbes; GTs are implicated in the formation of carbohydrates involved in adhesion to substrates and in cell wall synthesis rather than carbohydrate catabolism (Zhu et al., 2013). Rumen *Lachnospiraceae* have large repertoires of CAZymes that have been found via genomic sequencing, therefore these can be used to infer their strong fibre degrading ability (Seshadri et al., 2018).

Metagenomic analysis of *Lachnospiraceae* genomes by Biddle et al (2013) indicates that *Lachnospiraceae* members are equipped with GH families that are associated with degradation of hemicellulose and pectin sidechains (GH2, GH3, GH28,GH43 and GH51), and starch degradation (GH13, GH31). However, cellulolytic GH families (GH5, GH6, GH8, GH9, GH45 and GH48) were less common in the *Lachnospiraceae* genomes (Biddle et al., 2013). Additionally, many phosphorylases belonging to the GH 94 family were found in

Lachnospiraceae genomes, these enzymes are primarily associated with the cleavage of beta-glycosidic bonds of cellobiose, cellodextrin and chitobiose (Biddle et al., 2013).

1.6.1.1. Cellulose degradation

The lower number of cellulolytic GHs family genes found in *Lachnospiraceae* may indicate a reduced capability for cellulose degradation in comparison to other plant components. Plant cell walls are made up of cellulose fibrils encased in a matrix of hemicellulose, lignin, and pectin. Cellulose consists of chains of β1-4,-linked glucose units and extensive H_2 bonding occurring between glucose chains gives cellulose its crystalline structure which is recalcitrant to degradation (Roger et al., 1990). Cellulose is broken down by microbially derived cellulase enzymes which act on β1-4 linkages in the cellulose structure. Cellulases are classified into different types based on the nature of their hydrolysing functions. Endo -β-1,4-glucanases (EG, EC 3.2.1.4) act within cellulose chains to cleave β-1,4 linkages to produce glucose chains of smaller lengths . Exo- β-1,4-cellobiohydrases (CBH, EC 3.2.1.91) breakdown cellulose from the non-reducing end of the chains producing cellobiose units. Similarly, β-glucosidases act in the same way as CBHs but produce glucose units (Watanabe & Tokuda, 2001). These enzymes are grouped into GH families with GH5, GH6, GH8, GH9, GH45, and GH48 being the major GH families found in rumen bacteria (Liu et al., 2021).

Known cellulose degraders have various mechanisms for degrading cellulose, for example *Ruminococcus flavefaciens* uses an elaborate arrangement of its enzymatic machinery on its bacterial cell wall, known as a cellulosome, to degrade cellulose (Dassa et al., 2014). This complex has distinctive features: cohesin and dockerin subunits bind carbohydrate-degrading enzymes to structural subunits called scaffoldins in the bacterial cell wall (Lloyd-Price et al., 2019). This aggregate is bound to cellulose via the non-catalytic CBMs. In contrast*, Fibrobacter succinogenes* was found to have an alternative strategy of cellulose degradation . Burnet et al. (2015) demonstrated that *F. succinogenes* used cell surface pili to attach its cell walls to cellulose fibrils. Outer membrane vesicles of *F. succinogenes* were coupled with fibro-slime complexes, and secreted endoglucanases for degradation of cellulose (Burnet et al., 2015). These primary cellulose degraders carry the burden of producing high molecular weight specialised enzymes, so that they can free substrates for other secondary fibre degraders. Following genomic evidence, many *Lachnospiraceae* are likely to be poor cellulose degraders and engage in nutrient exchange with primary cellulose

degraders such as *F. succinogenes* and *R. flavefaciens* (Kelly et al., 2010; Moraïs & Mizrahi, 2019).

1.6.1.2. Hemicellulose degradation

Unlike cellulose, hemicellulose is made up of heterogeneous monosaccharide units and is highly branched. The polymer of hemicellulose is made up of various pentoses, hexoses and sugar acids (Mudgil, 2017). Hemicelluloses work in cohesion with pectic components to mechanically link the individual cellulose microfibrils together. New Zealand pastures are comprised of a mixture of perennial rye grass (*Lolium perenne)* and white clover (*Trifolium repens*) (Woodfield, 1999). White clover has Type I primary and secondary cell walls while perennial rye grass is made up of Type II primary and secondary cell walls. Type I cell walls contain xyloglucans (XyG) and pectin, whereas, Type II cell walls are made up of glucuronoarabinoxylan (GAX) and ferulic acid crosslinks (Hobson & Stewart, 1988; Levy et al., 1991).

Due to the heterogenous nature of hemicelluloses, a wide range of hemicellulases are required for its hydrolysis. Endoxylanases, such as endo-1,4-β-D xylanases (EC 3.2.8/156) cleave internal linkages of the xylan backbone resulting in the release of xylooligosaccharides. These are then further hydrolysed by β-D xylosidases (EC 3.2.137) (Shallom & Shoham, 2003). The varied side chains of hemicelluloses are cleaved by a collection of α- D-glucuronidases (EC 3.2.1.139/131), α-L arabinofuranosidases (EC 3.2.1.55) and acetyl-xylan esterases (EC 3.1.1.72/3) (Juturu & Wu, 2013). The CAZymes families that are most commonly associated with the hydrolysis and break down of hemicelluloses include GH families GH10, GH11, GH31, GH43, GH51, GH62 and the CE4 family (Østby & Várnai, 2023).

Based on genomic CAZyme analysis The *Lachnospiraceae* family are thought to be more effective hemicellulose degraders in comparison to cellulose (Xie et al., 2022). *Butyrivibrio* are known to contain repertoires of CAZymes associated with hemicellulose degradation, and *Butyrivibrio fibrisolvens* has shown strong xylanolytic activity in culture (Hespell & Whitehead, 1990). The genomic and phenotypic traits of *Lachnospiraceae* members suggest it is likely that there are other hemicellulolytic individuals within this bacterial group.

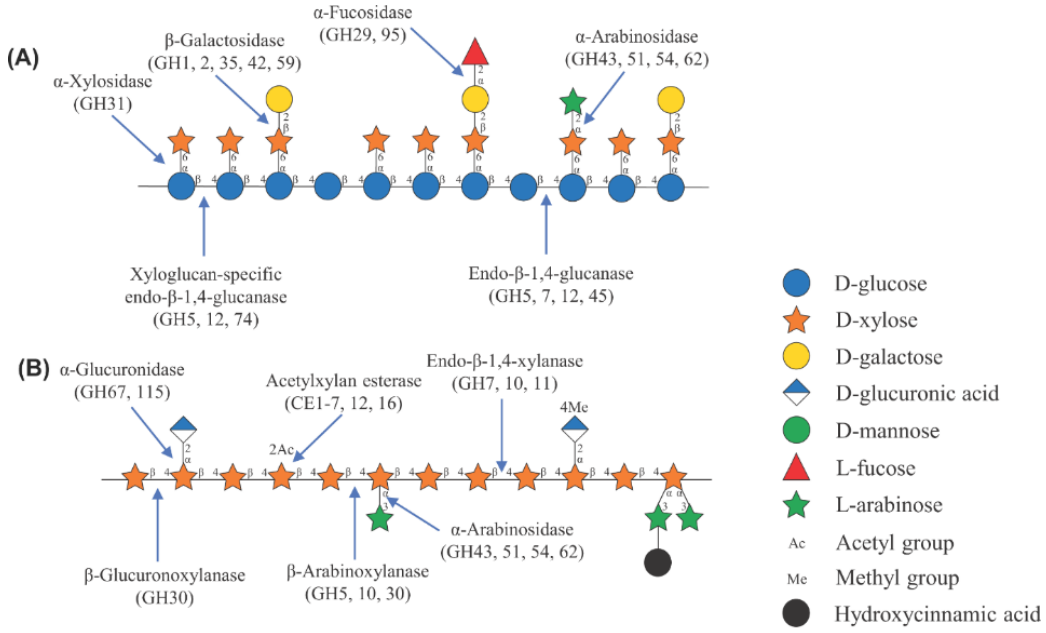


Figure 1.3 Graphical summary of the structure of hemicellulose and hemicellulase enzymes (A) xyloglucan (B) acetylated glucuronoarabinoxylan. From 'Hemicellulolytic enzymes in lignocellulose processing' by H. Østby, A. Várnai, 2023. *Essays in Biochemistry ,67*, 3, p.533-550 (https://doi.org/10.1042/EBC20220154).

1.6.1.3. Pectin degradation

Pectin is a structural polysaccharide that makes up the middle lamellae of plant cell walls (Goldberg et al., 1996). Pectin is associated with the primary cell walls of plants and facilitates the cell-to-cell interactions as well as control of cell wall structure. There are three main types of pectin: homogalacturonan (HGA), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII) (Thibault et al., 1993). HGA contains repeating α-1,4-D-galacturonic acid units whereas the rhamnogalacturonans have repeating units of both α-1,4-D-galacturonic acid and α-1,2-L-rhamnose (Keegstra et al., 1973). Xylogalacturonan,

arabinan, arabinogalactan I, are also considered pectins and are found in smaller amounts in the plant cell wall (Thibault et al., 1993).

Specialised PLs called pectate lyases (EC 4.2.2.2 and PL1, PL9, PL10) use β elimination to cleave the uronic acid residues in pectin backbones (Hugouvieux-Cotte-Pattat et al., 2014). Rhamnogalacturonan lyases (EC 4.2.2.23) belonging to the PL11 family cleave α-1,4 glycosidic bonds between L-rhamnose and D-galacturonic acid (McDonough et al., 2004). Additionally, GHs belonging to the GH28 family contain endo- and exo- polygalacturonases (EC 3.2.1.15) and rhamnogalacturonases (EC3.2.1.173) (Villarreal et al., 2022). CEs belonging to the CE 8 family (methylesterases, EC 3.1.1.11) and the CE12 family (acetylesterases, EC 3.1.1.11) work in concert to effectively cleave esterified pectin residues (Shevchik & Hugouvieux-Cotte-Pattat, 2003).

Of the studied members of the *Lachnospiraceae* family, many are known to be pectinolytic. Additionally, examination of the genomes of *Lachnospiraceae* reveal many pectin degrading CAZymes. For example, ruminal *Lachnospira multipara* strains have been shown to use the concerted action of pectin-specific CEs and PLs to degrade pectin (Kelly et al., 2019; Paggi et al., 2005).

1.6.2. Protein degradation by *Lachnospiraceae*

Rumen degradable protein (RDP) is sourced from the ruminant diet in the form of forage feeds and silage. Although the RDP is not directly utilised by the ruminant, microbial cells use this protein to generate microbial protein for growth. A portion of the microbial biomass in the rumen is washed into the abomasum of the ruminant, where microbial activity is halted by an acidic environment and post ruminal digestive enzymes (Harmon, 1993). Microbial protein makes up 60-90% of the protein required for growth, reproduction, and lactation in ruminants (Wallace et al., 1997). The rumen fermentable pool of proteins come from feed proteins but also, endogenous saliva, sloughed off epithelial cells and other lysed microbial cells (National Academies of Sciences & Medicine, 2021).

Rumen degradable proteins that are supplied in the diet are primarily degraded by bacterial proteases (Brock et al., 1982). Rumen bacterial genera within the family *Lachnospiraceae*, such as *Butyrivibrio*, *Eubacterium*, *Lachnobacterium* and *Lachnospira,* are known for their proteolytic abilities (Attwood & Reilly, 1995). For example, *Butyrivibrio proteoclasticus* B316

has been found to be strongly proteolytic employing mainly serine proteinases, but also cysteine and metallo- proteinases. This strain did not produce ammonia, indicating that it is only involved in the primary hydrolyses of peptides (Attwood et al., 1996). On the other hand, another species, *Butyrivibrio fibrisolvens*, another proteolytic *Lachnospiraceae* member , can ferment proteins through to ammonia (Wallace & McKain, 1991).

1.6.3. SCFA production by *Lachnospiraceae*

Much like other fibre degraders, *Lachnospiraceae* are known to produce SCFAs and H_2 as products of their metabolism. The role of *Lachnospiraceae* species has been studied more intensively in human GIT environments as they have been proven as important factors in human disease (Vacca et al., 2020). However, parallels can be drawn to the rumen gut ecosystem.

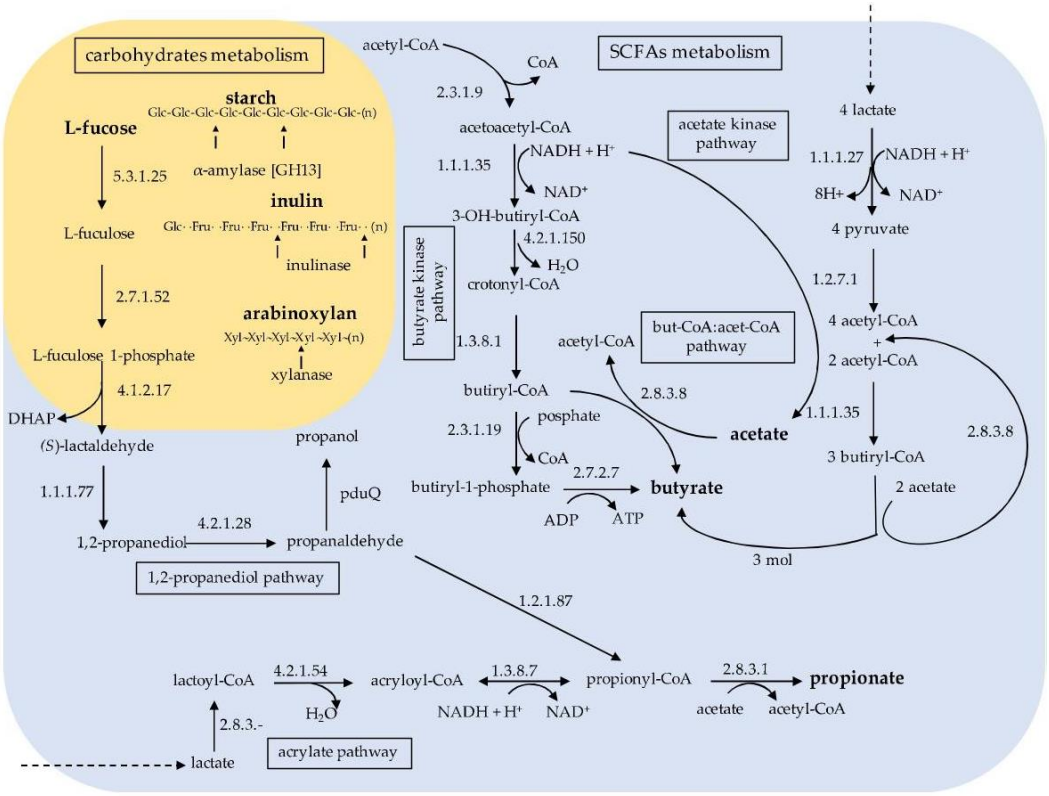


Figure 1.4 The main metabolic pathways for SCFA production in the family *Lachnospiraceae.* Reprinted from "The controversial role of human gut *Lachnospiraceae*" By M. Vacca, G. Celano, F. M. Calabrese, P. Portincasa, M. Gobbetti and M. De Angelis ,(2020). *Microorganisms*, *8*(4), p.573. (https://doi.org/10.3390/microorganisms8040573).

1.6.3.1 Acetate production

Acetate is the most abundant SCFA produced in the rumen, providing up to 50% of the energy provided from VFA metabolism to the host organism (Aiello et al., 1989). Some *Lachnospiraceae* are primary fibre degraders, and many genera are known to produce acetate (Seshadri et al., 2018). The breakdown of hexose and pentose sugars results in the production of pyruvate following glycolysis. Pyruvate is a key precursor for acetate formation via the acetate kinase pathway. This pathway is initiated by the formation of acetyl phosphate from acetyl- CoA facilitated by a phosphotransacetylase (EC 2.3.1.8). The resultant acyl phosphate is then metabolised into acetate; which is catalysed by acetate kinase (EC 2.7.2.1) (Fox et al., 1986).

1.6.3.2. Butyrate production

Butyrate production has been studied in detail, due to its beneficial effects on the intestinal barrier of mammals including ruminants (Yang et al., 2022). Specifically, butyrate encourages the redistribution of proteins that are associated with tight junctions in the rumen epithelium, namely ZO-1 and occludin (Peng et al., 2009).

The initial steps in butyrate formation are the conversion of carbohydrates into acetyl-CoA and acetoacetyl-CoA which are subsequently converted in to butyryl-CoA, the precursor for butyrate synthesis (Louis et al., 2004). The *Roseburia / Eubacterium rectale* group are the main producers of butyrate in human gut environments (Louis et al., 2004). There is variation in the ways certain *Lachnospiraceae* species can produce SCFAs which is also observed in the rumen ecosystem. An example of this is *Coprococcus eutactus* which produce butyrate via a butyrate kinase (EC 2.7.2.7) (Duncan et al., 2002). This enzyme works in concert with phosphate butyryltransferase (EC 2.3.1.19) which converts butyryl-CoA in to butanoyl phosphate, which is then acted upon by butyrate kinase to produce butyrate (Bachochin et al., 2020). On the other hand, certain *Roseburia* species and *Eubacterium rectale* use a butyryl CoA: acetate CoA transferase (EC. 2.8.3.8), this enzyme transfers the CoA moiety from butyryl CoA to a free acetate molecule, leaving butyrate as a product (Louis & Flint, 2009).

1.6.3.3. Propionate production

Propionate is another end-product which results from carbohydrate fermentation by anaerobes. Propionate is also important in the rumen owing to its role as a precursor required for gluconeogenesis (Yao et al., 2017). Propionate has been associated with increased weight gain in Holstein heifers as seen in a study by Ren et al in 2019. In this study, animals were fed increased amounts of steam flaked corn, resulting in an increased weight gain in contrast to control animals (Ren et al., 2019).

There are three main pathways by which propionate is produced in the rumen: the succinate, propanediol and acrylate pathways. The propanediol and acrylate pathways have been shown to be used by *Lachnospiraceae*. For example, in a study by Scott et al. in 2006, *Roseburia inulinivorans* when grown on fucose uses the propanediol pathway. Fucose is known as a propionigenic sugar and in this case is converted into propionaldehyde which

uses a CoA-dependent propionaldehyde dehydrogenase (EC 1.2.1.87) to be converted to propionyl- CoA which is then further reduced into propionate using a CoA-transferase (Prabhu et al., 2012).

The acrylate pathway uses lactate as a precursor to produce propionate. In a study by Reichhardt et al. in 2014, lactoyl CoA dehydratase (EC 4.2.1.54) was used a marker for the acrylate pathway. This marker was found in the *Lachnospiraceae* species, *Coproccocus catus.* Here, lactate is reduced to propionate (Reichardt et al., 2014). The conversion of acetyl- CoA from acetate occurs alongside this reaction. The simultaneous production of acetyl-CoA from acetate occurs because acetate is an electron sink for the NADH produced within the propionate formation pathway specifically where acrylyl-CoA is reduced to propionyl CoA.

1.6.3.4. Minor SCFAs

Minor SCFAs such as, isoacids (isobutyrate and isovalerate), valerate, and caproate are also microbial fermentation products produced by bacterial fermentation of lignocellulose. Isobutyrate, isovalerate and valerate are produced by the deamination of certain amino acids via Stickland metabolism (Nisman, 1954). The utilisation of valine, leucine, and isoleucine as H_2 acceptors by either strict proteolytic bacteria or in environments where usable carbohydrate substrates are lacking, results in the production of branched chain fatty acids (Nisman, 1954). Caproate is produced when there is a surplus of H_2 in the rumen environment as an additional H_2 sink via the reverse beta oxidation pathway or the fatty acid biosynthesis pathway (Ungerfeld, 2015). Production of some of these minor SCFAs may indicate protein fermentation by certain organisms and the state of the fermentation system.

1.6.4. Alcohol production by *Lachnospiraceae*

As a result of bacterial fermentation of lignocellulose, short chain alcohols are produced. *Lachnospiraceae* are known to produce ethanol, methanol, and butanol (Abdugheni et al., 2022).

1.6.4.1. Ethanol

Many *Lachnospiraceae* members produce ethanol as a product of carbohydrate fermentation (Cotta & Forster, 2006). There are four pathways that all use pyruvate as an initial intermediate product as described in Figure 1.5 (Hobson & Stewart, 1988).

Firstly, the Pdc-Adh pathway (in purple, Figure 1.5), which does not rely on the conversion of pyruvate in to acetyl-CoA and instead uses pyruvate decarboxylase (Pdc, EC 1.2.4.1) to catalyse the reduction of pyruvate into acetaldehyde and subsequently an alcohol dehydrogenase (Adh, EC 1.1.1.1) to further reduce acetaldehyde into ethanol (Eram & Ma, 2013). Secondly, the Pdh-Aldh-Adh pathway (in yellow, Figure 1.5), uses phenylacetaldehyde dehydrogenase (Pdh*,* EC 1.2.1.39) to reduce pyruvate into acetyl-CoA and then aldehyde dehydrogenase (Aldh*,* EC 1.2.1.3) to produce acetaldehyde and finally Adh to produce ethanol. The final two pathways both use a pyruvate: ferredoxin oxidoreductase (Por, EC 1.2.7.1) to convert pyruvate to acetyl- CoA with ferredoxin (Fd) as an electron acceptor. the Por-Aldh-Adh pathway (in green, Figure 1.5), continues through the aldh and Adh portion as seen in the Pdh-Aldh-Adh pathway. The fourth pathway, (Por-)Aor-Adh (in blue, Figure 1.5), diverges at acetyl-CoA where acetate is produced via phosphotransacetylase (Pta*,* EC 2.3.1.8) and acetate kinase (Ack*,* EC 2.7.2.1). Aldehyde ferredoxin oxidoreductase (Aor*,* EC 1.2.7.5) is then used to produce acetaldehyde and Adh mediates the production of ethanol (Nissen & Basen, 2019).

Ethanol is formed as an alternative electron sink when H_2 is allowed to accumulate in the system; this occurs primarily in pure culture. However, in the rumen environment, methanogenic archaea sequester H_2, through the more thermodynamically favourable methanogenesis route for electron disposal. Certain methanogens, specifically *Methanobrevibacter boviskoreani* JH1 can utilise as a source of reducing power ethanol in the absence of H_2 (Li et al., 2023).

1.6.4.2. Butanol

Bacterial genera within the family *Lachnospiraceae,* such as *Blautia,* can produce butanol from carbohydrate fermentation (Abdugheni et al., 2022). Strict anaerobes produce butanol via acetone- butanol-ethanol fermentation (in red, Figure 1.5) (Birgen et al., 2019). Here, pyruvate is reduced to acetyl-CoA using Por with the electron acceptor Fd. Butyryl-CoA

dehydrogenase (Bcd, EC 1.1.1.157) is then used to produce butryl-CoA which is finally converted to butanol via (butanol) dehydrogenase (BdhAB, EC 1.1.1-). This short chain alcohol can also be utilised by methanogens such as *Methanobrevibacter boviskoreani* JH1 (Li et al., 2023).

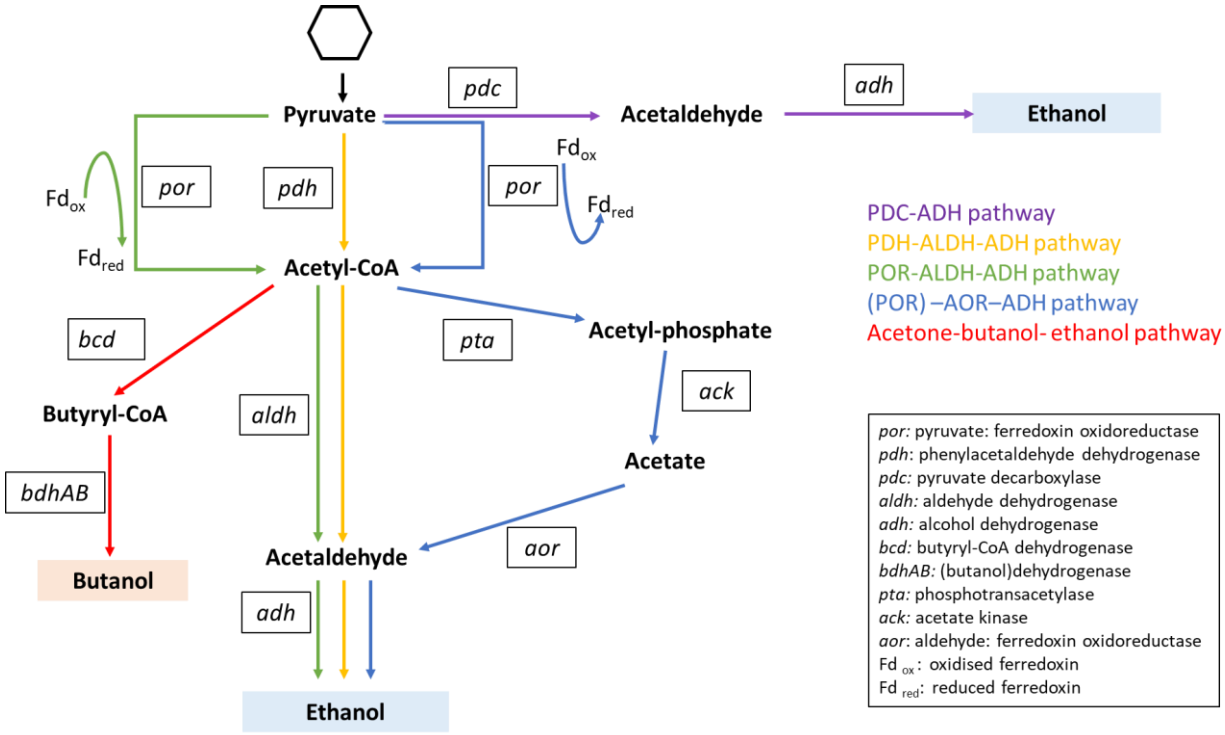


Figure 1.5 Metabolic pathways and enzymes responsible for ethanol and butanol production by *Lachnospiraceae.* Image adapted from 'The emerging role of aldehyde:ferredoxin oxidoreductases in microbially-catalyzed alcohol production' by L. S. Nissen and M. Basen, 2019, *Journal of Biotechnology, 306,* p. 105-117 (https://doi.org/10.1016/j.jbiotec.2019.09.005).

1.6.4.3. Methanol

Methanol production from carbohydrate fermentation in the rumen is primarily from pectin degradation (Vantcheva et al., 1970). HGA can be methylesterfied at the C-6 carboxyl. Pectin methyltransferases (PMEs, EC 3.1.1.11), specifically, a carbohydrate esterase belonging to

the CE 8 family is used to catalyse the demethylesterification of HGA in plant cell walls to produce methanol as an end product (Figure 1.6), (Pelloux et al., 2007).

Some *Lachnospiraceae* members are known to produce methanol as a result of pectin degradation. For example, *Lachnospira multipara* is one of the main pectinolytic species in the rumen (Dehority, 1973). In pure culture, *L. multipara* strains were shown to produce 3.7-9.6 mM of methanol in when grown on pectin (Dušková & Marounek, 2001). Methanol is an important substrate for methylotrophic methanogens therefore pectinolytic bacteria may form commensal relationships with these types of methanogens.

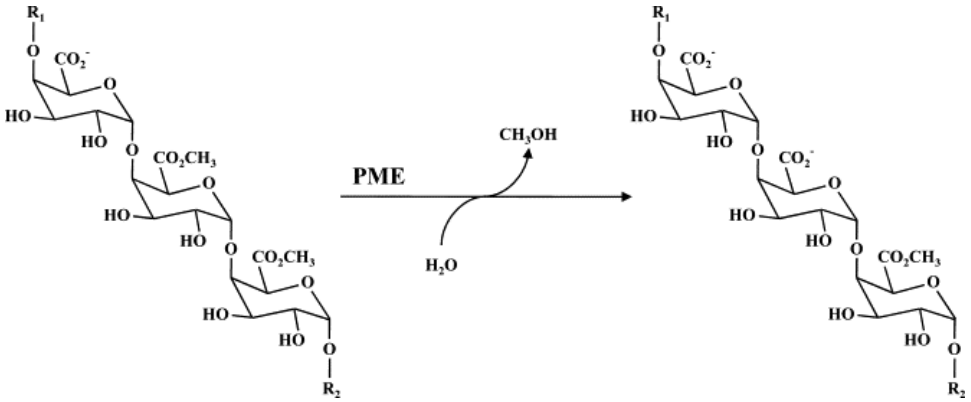


Figure 1.6 Action of pectin methyltransferase cleaving off methyl group from homogalacturonan. Reprinted with permission from 'Pectin methylesterase and its proteinaceous inhibitor: a review' by R. P. Jolie, T. Duvetter, A. M. Van Loey and M. E. Hendrickx, 2010, *Carbohydrate Research, 345* (18), p. 2583-2595. (https://doi.org/10.1016/j.carres.2010.10.002).

1.6.5. H_2 production by *Lachnospiraceae*

The production and use of H_2 is central to understanding rumen fermentation. Thermodynamically favourable flows of H_2 drive the fermentation; this is demonstrated in many of the carbohydrate breakdown pathways utilised by rumen bacteria (Kelly et al., 2022). For example, in the glycolytic pathway the oxidation of glyceraldehyde-3-phosphate to 1,3 bisphosphoglycerate is coupled to the reduction of NAD+ to NADH (van Lingen et al., 2016). H_2 is also produced during the oxidative decarboxylation of pyruvate to acetyl CoA which is the first step to producing acetate and butyrate, which are important for the health and productivity of the host animals (Schönfeld & Wojtczak, 2016). For carbohydrate metabolism to be maintained reduced co-factors such as NADH need to be re-oxidised, and

hydrogenases work to transfer electrons from reduced cofactors to H_2. H_2 is then shunted to various pathways, such as methanogenesis, so that it does not accumulate in the rumen. (Hackmann et al., 2017; Ungerfeld, 2020).

1.6.5.1 Hydrogenases

Hydrogenases are a diverse range of enzymes found in bacteria, archaea, and eukaryotes in the rumen. Hydrogen metabolism is an important feature of rumen fermentation and is facilitated by hydrogenases. Three main types of hydrogenases have been identified in the genomes of the Hungate 1000 culture collection (Greening et al., 2019; Seshadri et al., 2018), including [FeFe], [NiFe] and [Fe] hydrogenases which can be subclassified into 11 smaller subgroups based on their function and distribution in rumen microbes (Greening et al., 2016). A3 [FeFe] hydrogenases have been identified as the primary catalyst for H_2 production in ruminants (Greening et al., 2019). A3 [FeFe] hydrogenases were encoded in the genomes of the major carbohydrate fermenters in the rumen, including many members of the *Lachnospiraceae* family (Greening et al., 2019). These enzymes are responsible for H_2 sensing, consumption, and production. With many of these hydrogenases being found in genomes of uncharacterised *Lachnospiraceae* species there is a need for increased phenotypic characterisation of these fermentative bacteria.

1.7. Bacterial characterisation techniques

1.7.1. Culture-independent techniques

DNA-based methods for identification and analysis of microorganisms have revolutionised the study of the rumen ecological community (Leahy et al., 2013). Next-generation sequencing provides information on microbial diversity and relative abundance of organisms, allowing the identification of significant microbial populations within the rumen microbiome (Morgavi et al., 2013). Bioinformatic analysis of metagenomic and meta-transcriptomic data can also be used to understand the genes present and which genes are expressed (Creevey et al., 2014). In addition, deep sequencing of metagenomic DNA allows for identification of novel taxa through metagenome assembled genomes (MAGs) providing genomic information on difficult-to- culture or uncultured organisms. The 16S rRNA gene catalogues assembled from such studies always far outweigh the number of cultured representatives, and this mismatch illustrates the large knowledge gap between the identified diversity and the cultured and characterised minority of strains in the lab. This lack

of representative strains for experimental validation of functional characteristics, makes species delineation very challenging (Hitch et al., 2021). The culture-independent techniques still rely on the availability of cultured representatives to provide reference information and classify these microorganisms to species level.

1.7.2. Culture-dependent techniques

The reasons for lab-cultured bacteria representing only 20% of the microbial community known to be present in the rumen are related to the strict anaerobic requirements of rumen microorganisms which make it difficult to grow them in culture media in a laboratory setting (Creevey et al., 2014). Rumen microorganisms also occupy specific niches within the rumen ecosystem which means mimicking these conditions is required to grow them as pure cultures. This was recognised by Robert Hungate, a pioneer in the anaerobic rumen microbiology field, who developed habitat-simulating media prepared under strictly anaerobic conditions in broth tubes (the “Hungate technique”) and agar roll tubes (Hungate, 1950). Many different media targeting the cultivation of different groups of rumen microorganisms have since been developed (Joblin, 2005; Kenters et al., 2011; Leedle & Hespell, 1980) .

Having representative strains of rumen bacteria aids in-depth metabolic analysis to understand their metabolism and functional roles in rumen fermentation processes. For example, it was observed that goats in Hawaii could graze the tree forage plant *Leucaena leucocephala* despite it containing the toxic, non-protein, amino acid, mimosine, which in other locations caused poisoning of ruminant species. Subsequently, Allison et al. in 1992, isolated four strains of rumen bacteria from using the roll tube method (Hungate, 1950). Using 16S rRNA sequencing it was apparent that these strains belonged to a novel taxon. Phenotypic characterisation techniques including gas liquid chromatography (GLC), transmission electron microscopy, Gram staining, and substrate utilisation testing were used to propose a novel genus and species: *Synergistes jonesii* (Allison et al., 1992*)*. In addition to species delineation, the functional characterisation led to the discovery that this species can degrade toxic pyridinediols found in *Leucaena leucocephala*. The strains in this study were isolated from a Hawaiian goat species that were fed *L. leucocephala* as a forage crop, however this forage is considered toxic to ruminants grown in the USA and Australia due to the compound 3-hydroxy-4(IH)-pyridone released from the degradation of mimosine

(Hagarty et al., 1976). Here, researchers were able to isolate and culture a bacterial species that aided in the discovery of previously unseen metabolic degradation of a toxic amino acid, further highlighting the importance of functionally characterising culturable strains.

In the last 15 years, the development of high throughput (HTP) sequencing technologies has resulted in a shift to culture-independent approaches. However, in more recent years, there has been a revival of culturing with or led by various "-omics" techniques and has been referred to as "culturomics".

1.8. Hungate 1000 project

Culture collections are the repository for culturable microorganisms and they support the long-term conservation of the biological diversity of rumen microorganisms. These collections provide ease of access to reference strains and other microbial resources. This is key for researchers to find biotechnological solutions for issues facing the agricultural industry, such as food security and CH_4 production. It is important to make culture collections accessible but also to provide data including functional and ecological niche characterisations (Díaz-Rodríguez et al., 2021). The Global Research Alliance on Agricultural Greenhouse Gases aims to use international flagship projects to isolate and phenotype novel rumen bacteria (Huws, 2022). These projects will help to address the opportunities for further research highlighted in this review. The Hungate 1000 was a global study combining culturing and genome sequencing to create a reference set of rumen microbial cultures and genomes (Seshadri et al., 2018). The outcome was the Hungate 1000 culture collection, a community resource that encompasses nearly all the available cultured bacterial and archaeal taxa that have been isolated from a range of ruminants worldwide.

Prior to HTP sequencing, research in rumen microbiology was mainly focused on the isolation and cultivation of strains; however, many genera were lacking reference genomic data (Creevey et al., 2014). First, culture-independent techniques including metagenomics and metatranscriptomics were used to derive functional data. Finally, this data was combined with approximately 500 isolates to form a culture collection. The reference genomes represent approximately 75% of the genus level taxa that have been reported in the rumen (Seshadri et al., 2018). However, detailed functional characterisation has only been carried out on a limited number of the Hungate 1000 strains.

1.9. Aims and objectives of thesis

As the global population grows, meeting nutritional demands remains a priority and is often met with increased animal numbers and productivity (van Dijk et al., 2021). However, the downside to this is increased enteric methane production, one of the major end products of rumen fermentation. Rumen fermentation currently contributes to 16% of global methane emissions (Tseten et al., 2022). The key to creating workable solutions to these issues; lies in understanding the underpinnings of rumen metabolism. Substantial research efforts go into manipulating the rumen microbiota to address greenhouse gas emissions and animal productivity; however, the unknowns of microbial community structure and function stand in the way. Inherent challenges in culturing rumen microorganisms have left gaps in classification and characterisation of strains. Of the 20 bacterial phyla that have been detected in the rumen, only nine phyla have cultured representatives (Creevey et al., 2014). As only 20% of the rumen microbial community has been classified, it is evident that further advances are required. As mentioned in the literature review there is strong interdependency between bacteria and other members of the rumen consortia, therefore experimental validation of predicted genomic traits of bacterial strains could be instrumental in understanding their contribution to rumen fermentation.

The *Lachnospiraceae* family, are one of the most abundant but poorly characterised bacterial groups in the rumen and should be prioritised as a group of interest. Increasing this resolution and applying it to the rest of the fibre-degrading network in the rumen could uncover new modes of metabolism and help to better understand what their roles are in the rumen microbial community.

The aims of this thesis are:

1. To phenotypically characterise 45 *Lachnospiraceae* strains from the Hungate 1000 culture collection to evaluate cell morphology, substrate utilisation capabilities, VFA production, alcohol production, and headspace H_2 production.
2. To compare the genomic data produced in the Hungate 1000 study using Protologger to the experimental findings from the phenotypic characterisation.

1.10. Hypothesis

It is hypothesised that the functional characterisation of the 45 *Lachnospiraceae* strains will provide a more in-depth view of the metabolic capabilities of the *Lachnospiraceae* family. Furthermore, it is hypothesised that there will be mismatches in the genomic prediction provided by the genome annotation tool Protologger and the phenotypic characterisation results showing that culture independent characterisation alone is not enough to describe the physiological capabilities of the 45 *Lachnospiraceae* strains.

Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Media and components

2.1.1.1. Rumen fluid

Rumen fluid was collected from ruminally fistulated cows which had been exclusively hay fed for three days. On the third day, feed was withheld from 4 pm until the following day. At 9-10 am on the fourth day, animals were placed into a crush and their rumen contents were collected and squeezed through 1x layer of cheese cloth (Cheese Cloth, 3.5kg Fine Roll 1742865 from Packaging House). The liquid portion was kept for use in the laboratory while the solid portion was placed back into the rumen. Up to 10 L of liquid rumen fluid was taken from each animal. The collected rumen fluid was frozen at -20°C until further use.

2.1.1.2. Processed rumen fluid

Frozen rumen fluid was thawed and centrifuged at 10,000 × g at 4 °C to remove fine particulate material. Subsequently, the rumen fluid was bubbled under 100% nitrogen (N_2) gas for 20 min, before being added to a high-pressure Schott bottle and autoclaved at 121 °C for 20 min. The autoclaved rumen fluid material was used for media preparation.

2.1.1.3. Clarified rumen fluid

Processed rumen fluid was added to a beaker alongside 0.08M $MgCl_2.6H_2O$ (1.63 g per 100 mL) and 0.08M $CaCl_2.2\ H_2O$ (1.18 g per 100 mL) and stirred to form a heavy precipitate. This rumen fluid mixture was centrifuged at 10,000 ×*g* at 4 °C for 20 min and the clarified supernatant (Clarified Rumen Fluid) was decanted and frozen at -20 °C until use.

2.1.2. Media solutions

2.1.2.1. Salt solution A

Table 2.1. Salt Solution A Components

Components	**g L^{-1}**
NaCl	6 g
$(NH_4)_2SO_4$	3 g
KH_4PO_4	1.5 g
$CaCl_2$	0.79 g
$MgSO_4.7H_2O$	1.2 g
Distilled Water	Up to 1 L

2.1.2.2. Salt solution 2B

Table 2.2. Salt Solution 2B Components

Components	**g L^{-1}**
K_2HPO_4	6 g
Distilled Water	1 L

The salt solutions above were prepared by adding the components to 1 L of distilled water and stirred on a magnetic stirrer with a stirring bar until dissolved.

2.1.2.3. Selenite/ tungstate solution

Table 2.3 Selenite/ tungstate solution components

Components	**mg L^{-1}**
NaOH	500 mg
$Na_2SeO_3.5H_2O$	3 mg
$Na_2WO_4.\ 2H_2O$	4 mg
Distilled Water	Up to 1 L

The components listed above were dissolved in distilled water and made up to 1 L. This mixture was then stirred on a magnetic stirrer with a stirring bar until dissolved.

2.1.2.4. Trace element solution SL10

Table 2.4. Trace element solution SL10 components

Components	**Mg, g, or mL L^{-1}**
25% HCl	10 mL
$FeCl_2{\cdot}4H_2O$	1.5 g
$CoCl_2{\cdot}6H_2O$	190 mL
$MnCl_2{\cdot}4H_2O$	100 mg
$ZnCl_2$	70 mg
H_3BO_3	6 mg
$Na_2MoO_4.2H_2O$	36 mg
$NiCl_2.6H_2O$	24 mg
$CuCl_2.2H_2O$	2 mg
Distilled Water	Up to 1 L

The components listed above were dissolved in distilled water and made up to 1 L and stirred on a magnetic stirrer with a stirring bar until dissolved.

2.1.2.5. Vitamin 10 concentrate

Table 2.5 Vitamin 10 concentrate components

Components	**mg L^{-1}**
4-aminobenzoate	40 mg
D- (+)- biotin	10 mg
Nicotinic Acid	100 mg
Hemicalcium D-(+)- pantothenate	50 mg
Pryidoxamine hydrochloride	150 mg
Thiamine chloride Hydrochloride	100 mg
Cyanocobalamin	50 mg
D,L-6,8- thioctic acid	30 mg
Riboflavin	30 mg
Folic Acid	10 mg
Distilled Water	Up to1 L

For the preparation of the Vitamin 10 concentrate, the components listed in Table 2.5 above were combined in a beaker and bubbled under 100% N_2 gas for 20 min, then 100 mL aliquots were anaerobically sterilised by filtration through a 0.22 µm polyethersulfone ((PES) Merck, Darmstadt, Germany) filter into autoclaved N_2-filled serum vials via syringe and needle.

2.1.2.6. No substrate rumen fluid mix (NoSubRFV)

Yeast extract (2 g, 2% wt/vol) was added and dissolved in 100 mL of clarified rumen fluid (Section 2.1.1.3). This mixture was bubbled under 100% N_2 gas for 15 min and transferred anaerobically to an N_2 -flushed, sterile serum vial through a 0.22 µm PES filter. Vitamin 10 concentrate (2 mL) was added anaerobically to this mix through a 0.22 µm filter using a syringe and needle.

2.1.2.7. General Sugar Rumen Fluid Vitamin Mix (GenRFV)

Table 2.6 General sugar rumen fluid vitamin mix components

Components	Weight/ Volume
D-Glucose	0.36 g
D-Cellobiose	0.34 g
D-Xylose	0.30 g
L-Arabinose	0.30 g
Sodium Lactate Syrup	2 g
Casamino acids	2 g
Peptone	2 g
Yeast Extract	2 g
Clarified Rumen Fluid (see Section 2.1.1.3)	100 mL
Vitamin 10 Concentrate solution*	2 mL

* Added after preparation of anaerobic mixture above.

The GenRFV solution was made up using the components in Table 2.6 to a final volume of 100 mL. The mixture was made anaerobic by bubbling under 100% N_2 gas for 15 min, then anaerobically filter sterilised through 0.22 µm PES filter, into a sterile, N_2-flushed, serum vial sealed with a butyl rubber stopper and an aluminium crimp.

Using a syringe and needle, this mixture was anaerobically added to modified RM02 medium (Section 2.1.3.1), giving a final concentration of 5% rumen fluid (wt/vol), 0.5 mM

cellobiose, 1 mM each arabinose, glucose, and xylose, 5 mM of lactate, 4 mM each of Ca^{2+} and Mg^{2+}, and 0.1% (wt/vol) peptone and yeast extract.

2.1.3. Media

2.1.3.1. Modified rumen mineral medium 2 (RM02)

Table 2.7 Modified rumen mineral medium 2 components

Components	**g or mL L^{-1}**
KH_2PO_4	1.4 g
$(NH_4)_2SO_4$	0.6 g
KCl	1.5 g
Trace Element solution SL10	1.0 mL
Selenite/Tungstate solution	1.0 mL
0.1% (w/v) Resazurin solution	0.8 mL
$NaHCO_3$*	4.2 g
L-cysteine·HCl·H_2O*	0.5 g
Distilled H_2O	950 mL

*These components were added after the medium had cooled.

RM02 is a growth medium that simulates the mineral conditions within the rumen (Kenters et al., 2011).

The components listed above were mixed in a 2 L Erlenmeyer flask and boiled. The contents of the flask were then bubbled under 100% CO_2. Once the medium had cooled to room temperature, the reducing agents ($NaHCO_3$ and L-cysteine·HCl·H_2O) and were added and dissolved. The medium was then dispensed into 16 mL Hungate or 8 mL Hungate tubes flushed with 100% CO_2 and sealed with butyl rubber stopper and screw cap. The tubes were autoclaved at 121°C for 20 min.

2.1.3.2. Modified medium 2 of Hobson (M2GSC)

Table 2.8 M2GSC medium components (Miyazaki et al., 1997)

Components	**g or mL L^{-1}**
Casitone (Bacto™)	10 g
Yeast Extract	2.5 g
Soluble Starch	2.0 g
D- Glucose	2.0 g
D- Cellobiose	2.0 g
Processed Rumen Fluid	300 mL
Mineral I Solution	150 mL
Mineral II solution	150 mL
Resazurin (0.1%)	1.0 mL
L-cysteine·HCl·H2O*	1.0g
$NaHCO_3$*	4.2 g
Distilled H_2O	Up to 1 L

*These components were added after the medium had been boiled and cooled.

The components listed above were mixed in a 2 L Erlenmeyer flask and boiled. The contents of the flask were then bubbled under 100% CO_2, and once the medium had cooled to room temperature, the $NaHCO_3$ and L-cysteine·HCl·H_2O were added and dissolved. The medium was dispensed in 9.5 mL aliquots into 16 mL Hungate tubes flushed with 100% CO_2 and sealed with butyl rubber stopper and screw cap. The tubes were autoclaved at 121°C for 20 min.

2.1.3.3. K broth

Table 2.9 K broth components (Makkar & McSweeney, 2005)

Components	**g or mL L^{-1}**
Casitone (Bacto™ [1])	10 g
Yeast Extract	2.0 g
Peptone	2.0 g
Salt Solution 1	150 mL
Salt solution 2B	150 mL
Processed Rumen Fluid	300 mL
D- Glucose	1.0 g
D- Cellobiose	1.0 g
L- Arabinose	1.0 g
Lactate Syrup	1.0 g
Resazurin	1.0 mL
$NaHCO_3$*	5 g
L-cysteine·HCl·H_2O*	0.5 g

*These components were added after medium had been boiled and cooled.

The components listed above were mixed in a 2 L Erlenmeyer flask and boiled. The contents of the flask were then bubbled under 100% CO_2, once the medium had cooled to room temperature the $NaHCO_3$ and L-cysteine·HCl·H_2O were added and dissolved. The medium was dispensed in 9.5 mL aliquots into 16 mL Hungate tubes flushed with 100% CO_2 and sealed with butyl rubber stopper and screw cap. The tubes were autoclaved at 121°C for 20 min.

2.1.4. Substrate solutions

2.1.4.1. Soluble substrate solutions

NoSubRFV (Section 2.1.2.6) was used as the base for the substrate solutions. Each of the 15 soluble sugar substrates were added to NoSubRFV at a concentration of 10 mM. A 0.25 mL volume of these solutions were added to 4.75 mL of Modified RM02 medium (Section 2.1.3.1.) to give a final concentration of 0.5 mM of the substrate in each tube.

Table 2.10 Soluble substrate amounts in grams per 100mL

Substrate	**g (100 mL)$^{-1}$**
D- Raffinose	5.044 g
D- Cellobiose	3.423 g
D- Melibiose	3.430 g
D- Sucrose	3.423 g
D- Lactose	3.423 g
D- Maltose	3.423 g
D- Glucose	1.802 g
D- Galactose	1.802 g
L-Arabinose	1.501 g
L-Rhamnose	1.642 g
D- Xylose	1.501 g
D- Mannose	1.802 g
Dextrin	5.044 g
D-Galacturonic acid	1.944 g
D-Glucuronic acid	1.944 g

2.1.4.2. Insoluble substrate mixtures

Table 2.11 Insoluble substrate mixtures components

Substrate	**g (100 mL)$^{-1}$**
Microcrystalline Cellulose (Sigma Aldrich[1])	2 g
Pectin (from apple, Sigma Aldrich[1])	2 g
Polygalacturonic Acid (synthetic, Sigma Aldrich[1])	2 g
Starch (from Potato, BDH[2])	2 g
Xylan (from oat spelt, Sigma Aldrich[1])	2 g

[1]Sigma Aldrich, St Louis, MO, USA

[2]BDH, Dubai, UAE

Each insoluble substrate was made up as a 2% (wt/vol) mixture in water, the mixture was bubbled under CO_2 for 20 minutes in a 125 mL serum bottle and sealed using a butyl rubber stopper and an aluminium crimp seal. The serum bottles were autoclaved at 121°C for 20 min. A 0.25 mL volume of these solutions were added to 4.25 mL of RM02 medium for the insoluble substrate screen.

2.1.5. Storage solutions

2.1.5.1. Anaerobic Glycerol solution

Table 2.12 Anaerobic Glycerol solution components

Components	**mL L^{-1}**
Salt Solution A	85 mL
Salt Solution 2B	85 mL
Glycerol	200 mL
Resazurin (0.1% solution)	1.0 mL
L-cysteine·HCl·H_2O*	250 mg
$NaHCO_3$*	2.5 g
Distilled water	130 mL

*These components were added after media had been boiled and cooled.

The components listed above were mixed in a 2 L Erlenmeyer flask and were brought to a boil in a microwave and then immediately bubbled under CO_2. The mixture was then allowed to cool on ice till it was at room temperature, at which point the reducing agents were added. The anaerobic glycerol was then dispensed using a sterile strippette flushed with CO_2 into 125 mL serum bottles also filled with CO_2. The serum bottles were sealed with a butyl rubber stopper, an aluminium cap and sealed with a crimper. The glycerol mixture was autoclaved at 121^{o} C for 20 min.

2.1.6. PCR materials

2.1.6.1. Invitrogen Platinum II Taq Hot Start PCR Master Mix (2×)

The Taq Polymerase Master Mix was supplied by Thermo Fisher (Catalogue number: 14000012, Petone, Wellington).

2.1.6.2. Milli Q water

Water used in the PCR protocol was purified using a Millipore Milli Q Lab Water System and then aliquoted in to sterile 50 mL Falcon tubes.

2.1.6.3. Primers

Primers for amplification of bacterial 16S ribosomal RNA genes, (Weisburg et al., 1991), were supplied by Thermo Fisher NZ (Petone, Wellington) via their Custom DNA Oligonucleotide Synthesis service. The primer sequences were as follows:

Table 2.13 Primer sequences

Primer Name	Sequence (5' – 3')
1492r	GGYTACCTTGTTACGACTT
27f	GAGTTTGATCMTGGCTCAG

2.1.6.4. 1× TAE buffer

Tris- Acetate- EDTA buffer was made up as a 50× concentrated solution compromised of 2M Tris, 1M Acetic acid and 50 mM Ethylenediamine tetra-acetic acid (EDTA). To make up the working solution, a 50-fold dilution in sterile water was prepared and stored at room temperature.

Table 2.14 50× TAE buffer components

Components	g L^{-1}
Tris	242 g
Glacial Acetic Acid	57.1 mL
0.5M EDTA	100 mL
Distilled water	Up to 1L

2.1.7. Gram stain solutions

2.1.7.1. Crystal violet stock solution

Solution A:

20 g of Crystal Violet (85% dye) was dissolved in 100 mL of 95% ethanol.

Solution B:

1g of ammonium oxalate ($(NH_4)_2C_2O_4$) was dissolved in 100 mL distilled water.

Crystal violet working solution:

Solution A was diluted at a 1:10 ratio in distilled water. This was mixed with four volumes of Solution B. The working solution was then stored at room temperature for up to six months.

2.1.7.2. Gram's iodine solution

Sodium bicarbonate solution (5%)

5 g of sodium bicarbonate was dissolved in 100mL of water.

Iodine working solution

To produce the working solution, 1 g of iodine crystals and 2 g of potassium iodide were added to 5 mL of water. To this mixture 60 mL of the sodium bicarbonate solution was added. Finally, 240 mL of water was added, and this mixture was stored in an amber Schott bottle at room temperature for up to 6 months.

2.1.7.3. Decolourising solution

Equal volumes of 95% ethanol and acetone were mixed and stored in a 500 mL Schott bottle at room temperature for up to one year.

2.1.7.4. Safranin counterstain

2.5 g of Safranin O was mixed with 100 mL of 95% ethanol in a 250 mL Schott bottle and stored at room temperature for up to one year.

2.1.8. Gas liquid chromatography (GLC)

2.1.8.1. Internal standard

The ethyl butyrate (20% vol/vol) internal standard was made by adding 20 mL of ethyl butyrate to distilled water and made up to 100 mL. Aliquots of this solution were kept refrigerated at 4 ^{o}C sealed in 50 mL Falcon tubes.

2.2. Methods

2.2.1. Anaerobic culturing techniques

To culture anaerobic bacteria, equipment such as needles, syringes, and media, need to be free of oxygen. All syringes and needles are flushed with CO_2 or N_2 before they were used to subculture anaerobic strains. Media and additives were all bubbled with CO_2 and dispensed into CO_2 filled vessels that were sealed with a butyl rubber stopper and a screw cap.

2.2.2. Strain revival

Previously isolated Hungate strains are stored as glycerol stocks in -80°C freezers at the AgResearch, Grasslands Facility. The glycerol stocks were placed on ice and as the top of the tubes thawed, a sterile syringe and needle flushed with CO_2 was used and 0.3 mL of culture was removed 0.1 mL was injected in to three different types of media in Hungate tubes (RM02 and GenRFV, M2GSC and K Broth). The cultures were then incubated at 39°C.

2.2.3. PCR protocol

The components listed in the table below were mixed to produce a master mix which was dispensed in to 0.5 mL PCR tubes.

The bacterial strains were diluted by a 1:10 ratio with MilliQ water, and 2 µL was added to the master mix producing a final volume of 50 µL.

Table 2.15. PCR reaction components

Components	Volume
Invitrogen Platinum II Taq Hot Start PCR Master Mix (2x)	25 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Milli Q Water	22 µL

Table 2.15A Test reaction

Components	Volume
Taq Mix	48 µL
Diluted culture	2 µL

Table 2.15B Control reaction

Components	Volume
Taq mix	48 µL
Water / Extracted DNA	2 µL

2.2.3.1. PCR programme

Table 2.16 PCR programme steps

	Step	Temperature	Time
	Initial Denaturation	94 °C	2 min
30 PCR cycles	Denature	94 °C	15 sec
	Anneal	60 °C	15 sec
	Extend	68 °C	22.5 sec
	Hold	4 °C	Hold

The samples were placed in to Biometra Tadvanced Thermocycler (Analytik Jena, Jena, Germany) and the programme above was run.

2.2.3.2. Agarose gel electrophoresis

Agarose gel electrophoresis was used as the quantification method for the PCR products. Agarose gels (0.8% wt/vol) were made by adding 0.8 g of molecular grade agarose to 100 ml 1× TAE buffer (Section 2.1.6.4.) and boiled in a microwave until the agarose had melted and dissolved. The mixture was allowed to cool under running water, once cooled, 2 µL of SYBR safe (Thermo Fisher, Wellington, NZ) was added. The molten agarose was then transferred into a gel cast with a comb inserted, and once set, 1× TAE buffer was added into a Horizon 58 horizontal electrophoresis apparatus (Apogee, Baltimore, USA). The 16S rRNA gene amplified PCR products were separated by electrophoresis at 100 V for 20 min.

2.2.3.3. Gel image capture

To visualise the PCR products, the gels were observed under ultraviolet light using a gel documentation system (Biolab Scientific, Ontario, Canada). Images were taken using a Digital Single-Lens Reflex Nikon D-700 camera (Nikon, Tokyo, Japan) via digiCamControl, a remote camera control software.

2.2.3.4. PCR purification

The purification of PCR products was undertaken using the Qiagen MinElute PCR Purification Kit, which uses a column purification method (the full protocol can be found on the Qiagen website:

(https://www.qiagen.com/us/Resources/ResourceDetail?id=521feb84-9cfa-45ea-9a8f-54c2999aa9a9&lang=en).

2.2.4. Carbon source utilisation assay

2.2.4.1. Soluble substrate assay

The medium used for this assay was modified RM02 (Section 2.1.2.1); 4.5 mL was aliquoted into 8 mL Hungate tubes. The 0.1M substrate solution (Section 2.1.4.1) (0.25 mL) and test inoculum at 0.1 OD_{600} (0.25 mL) were added to the medium giving a final concentration of 0.5mM substrate in a 5 mL final volume.

The dilution of the test culture was achieved by incrementally increasing the amount of overnight culture added to a dilution tube (RM02) until an OD_{600} of 0.1 was reached. An equal amount of sterile growth media was added to the blank tube to account for the background OD_{600} of the growth media when the culture was added to the dilution tube.

To measure the growth of the test culture on the various substrates, the optical density of each of the tubes was measured at time 0 (immediately after inoculation) and then again after a 48-hr growth period at 39 °C. To ensure that the tubes were measured at the same position, and orientation a mark was made at the point of measurement.

The controls for this experiment were as follows:

- The **negative control** contained 4.5 mL modified RM02 medium and 0.25 mL and NoSubRFV (Section 2.1.2.6.). This tube was not inoculated with culture.
- The **positive control** contained 4.5 mL modified RM02 medium and 0.25mL GenRFV (Section 2.1.2.7.) to which 0.25 mL of the test inoculum was added.
- The **media only control** contained 4.5 mL modified RM02 medium and 0.25 mL of NoSubRFV, to which 0.25 mL of the test inoculum was added.

All components were added to the tubes anaerobically.

2.2.4.2. Insoluble substrate assay

OD_{600} measurements were not possible for the insoluble substrates assay as the OD_{600} of the substrates would mask any bacterial growth. Therefore, total SCFAs in the medium were measured via GLC and used as an indicator of growth.

Firstly, a 2% anaerobic solution of each of the five insoluble substrates (Section 2.1.4.2) was added at a volume of 0.25 mL to 4.25 mL of modified RM02 medium in an 8 mL Hungate tube. Secondly, 0.25 mL of NoSubRFV (Section 2.1.2.6.) was added. Finally, 0.25 mL of the standardised 0.1 OD_{600} test inoculum was added. The final volume in the tube was 5 mL and the final concentration of substrate was 0.1%.

The substrates were tested in triplicate with a 0-hour inoculated tube as a control and an inoculated media only tube as an indicator of background growth on media components (Section 2.2.4.1, media only control). The inoculum control tube was not incubated and 1 mL from this tube was taken for GC analysis immediately. The other tubes were incubated at 39 °C for 48 hrs. After the incubation period, H_2 measurements were taken from each of the tubes using Gas Chromatography (GC) and 1mL of the culture was taken for SCFA measurement via GLC.

2.2.5. Fermentation product analysis methods

2.2.5.1. VFA sample processing

For the insoluble substrate assay, the VFA concentration was measured to determine the level of growth whilst also providing insight into the fermentation profile of the organisms tested. For the soluble substrate assay the VFA profile of the bacterial growth on glucose or cellobiose was measured.

For VFA measurement via GLC, 1 mL of each treatment tube was sampled and added to a 1.5 mL Eppendorf tube and centrifuged at 10,000 × *g* for 20 min at 4 °C. A 900 µL aliquot of the supernatant was removed and added to 100 µL 20% (vol/vol) orthophosphoric acid containing the internal VFA standard, 2-ethylbutyric acid in an Eppendorf tube, which was frozen for a minimum of 24 hr.

2.2.5.2. Aqueous VFA processing and GLC analysis

A 750 µL aliquot of the VFA sample with the internal standard added was transferred to a 1.5 mL glass crimp top GC vial for analysis using a Shimadzu GC-2010 Plus via a Flame Ionisation Detection (FID) with helium as a carrier gas (BOC, NZ). The sample was injected into the column (Phenomenex Zebron ZB-FFAP Capilliary GC Column; 30 M length x 0.53 mm I.D. × 1.00 µm film thickness (Phenomenex, Torrence, CA, USA)) using an AOC 20i auto injector. The GC programme was set as follows; 1 µL of the sample was directly injected on to the column with the injection temperature set at 90 °C and the FID detector set to 240 °C. The column temperature programme is described in the table below.

Table 2.17. Column temperature programme aqueous VFA GLC analysis

Column Temperature	Time (min)	Rate of Increase (°C / min)
60 °C	3	-
120 °C	4	30 °C / min
185 °C	18.5	10 °C / min
200 °C	13.3	15 °C / min
200 °C	3	Hold

The protocol above was used to measure the following VFAs: acetic, butyric, propionic, caproic, valeric, *iso*-butyric and *iso*-valeric acids.

2.2.5.3 SCFA derivatisation and GLC analysis

A 1.5 mL Eppendorf tube was set up with 200 µL of the sample and internal standard to which 0.1 mL of 33% hydrochloric acid (Sigma Aldrich, St. Louis, MO, USA), 5 µL of 1 % resazurin dye and 800 µL of diethyl ether (Thermo Fisher NZ, Petone, Wellington), was added and vortexed for 5 sec. After mixing, the sample settled into two layers; the esterified SCFAs were in the top layer of ether. The uppermost phase was removed into a 2 mL Eppendorf. A further 800 µL diethyl ether was added to the original Eppendorf tube to ensure all the required SCFAs were extracted. The vortex step was repeated, and the top layer was again removed and added to the previously collected supernatant. Next, 100 µL of *N*-methyl-*N*-t-butyldemetzylsilytriflouroacetamide (Sigma Aldrich) was added to a glass GC vial along with 800 µL of the SCFA sample. The vial was then sealed with a crimp top and heated to 80 °C for 20 min. The sample was allowed to incubate for 48 hr at room temperature before being analysed on a Shimadzu GC-2010 Plus equipped with a Dielectric-Barrier Discharge Ionization Detector (BID), Phenomenex Zebron ZB-5MS Capilliary GC Column; 30 M length ×0.25 mm I.D × 0.25 µm film thickness (Phenomenex, Torrence, CA, USA), AOC 6000 auto-sampler and a VICI heated helium purifier (Valco Instruments Co. Inc. Houston, TX, USA).

The GC programme was as follows; 1 µL of the derivatised SCFA sample was injected at a split ratio of 20:1 with the injection temperature set to 240 °C and the BID temperature set to 250 °C. The column conditions are described in the table below.

Table 2.18. Column temperature programme derivatised FA GLC analysis

Column Temperature	Time (min)	Rate of Increase (°C / min)
50 °C	2	-
130 °C	26	5 °C / min
240°C	16	15 °C / min
240°C	4.67	Hold

The protocol above was used to measure formate, lactate, and succinate.

2.2.5.4. GLC analysis of alcohols

Alcohols produced during the fermentation of various substrates were measured using GLC. The alcohols measured were methanol, ethanol, 1-propanol, 2-propanol, and butanol.

The same vial used for the analysis of Aqueous VFAs was used for the measurement of alcohols. A Shimadzu GC-2010 Plus, AOC-6000 autosampler (Shimadzu Corporation, Kyoto, Japan), Restek Rtx-Volatiles GC column; 60 M length × 0.32 mm I.D × 1.5 µm film thickness (Restek, Bellefonte, PA, USA) and helium carrier gas (BOC, Palmerston North, NZ) were used.

The GC settings were as follows; 1 µL of the sample was injected at a temperature of 200 °C, with the FID detector set to 230 °C.

The column conditions are described in the table below.

Table 2.19. Column temperature programme alcohol GLC analysis

Column Temperature	Time (min)	Rate of Increase (°C / min)
50 °C	4	-
80 °C	10	8 °C / min
200 °C	8	40 °C / min
230 °C	3	Hold

2.2.5.5. GLC analyte quantification

Millimolar (mM) amounts of analytes from the samples are quantified using the Lab Solutions software (Shimadzu). This analysis is carried out by comparing the analyte peaks detected to the ratio of response between the analyte and the internal standard (20 mM ethylbutyrate in 20% (w/v) H_3PO_4) added to each sample.

2.2.5.6. Head space gas measurement using GC

For all the treatment tubes that exhibited growth for the soluble substrate assay, and all the insoluble sugar treatment tubes, H_2 production was measured in the culture headspace by GC. A 0.5 mL sample of the headspace gases was injected into an Aerograph 660 (Varian Associates, Palo Alto, USA) containing aPORAPAK Q 80/100 2MX1/8/TOGA column at room temperature with N_2 as the carrier gas at 12 cm^3/min. The detector was a thermal conductivity detector which was set to 100 °C. A set of 10 standards (30% hydrogen: 5% methane) were run before each measurement on the GC.

Equation 1, was used to find the total gas volume produced by 5 mL liquid culture using the average peak height produced by each strain grown on various substrates.

Equation 1.

$$\frac{\left(\frac{Sample\ peak\ height}{Volume\ loaded\ in\ mL}\right) \times \left(\frac{Sample\ attenuation}{Standard\ attenuation}\right) \times (Vessel\ volume\ mL)}{\left(\frac{(Standard\ mean\ peak\ height\ cm \times 100)}{(Volume\ loaded\ mL\ \times Standard\ Concentration\ \%)}\right)}$$

2.2.6. Microscopy methods

2.2.6.1. Gram staining method

To carry out the Gram staining procedure, the cells were heat fixed onto a standard microscope slide (25.4mm X 76.2 mm) by placing a drop of culture onto the slide and passing it through a Bunsen flame until completely dry. The heat fixed slide was then flooded with the Crystal Violet working solution and left for one min. After rinsing with tap water, the slide was flooded with the Iodine solution and left for one min. To rinse off excess Crystal Violet, a decolorising solution was applied to the slide for approximately 5 sec

and then rinsed away with tap water. Finally, the slide was flooded with the counter stain Safranin O and allowed to sit for 30 sec before rinsing with tap water. The slides were then allowed to dry completely before being observed under a light microscope using bright field microscopy at 100× magnification.

2.2.6.2. Agarose gel slides

Agarose-coated microscope slides were prepared by pipetting 1.5% molten agarose over a standard microscope slide. Once completely dry, a drop of culture was placed in the middle of the slide and covered with a coverslip. Images were taken under phase contrast using a light microscope at 100×magnification.

2.2.7. Genome annotation using Protologger

To summarise the potential phenotypic traits encoded in the genomes of the 45 *Lachnospiraceae* strains, an online web tool called Protologger was used (http://www.protologger.de/). Protologger aims to provide taxonomic, functional, and ecological features of a species which includes all the information required to produce a protologue (Hitch et al., 2021). The 16S rRNA and genome sequence files were downloaded from the National Center for Biotechnology Information (NCBI) database in FASTA format and fed in to Protologger. The pipeline described in Figure 2.1 was followed and the result was a protologue that summarised the potential substrate utilisation, SCFA production and taxonomic placement of each of the strains.

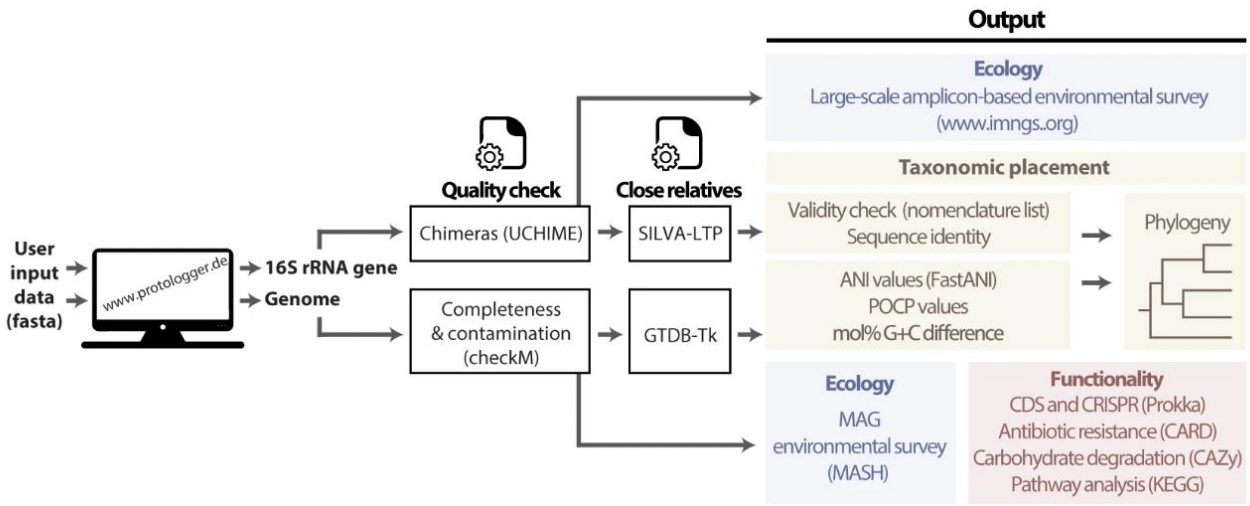


Figure 2.1 Pipeline of processes for producing Protologger output. Reprinted from 'Automated analysis of genomic sequences facilitates high-throughput and comprehensive description of bacteria' by T. C. A. Hitch, T. Riedel, A. Oren, J. Overmann, T. D. Lawley, and T. Clavel, 2020, *ISME Communications , 1*, p. 16. (https://doi.org/10.1038/s43705-021-00017-z)

Chapter 3. Phenotypic characterisation of ruminal members of the *Lachnospiraceae* family

3.1. Strain selection

The Hungate 1000 collection contains a total of 162 *Lachnospiraceae* strains, so to maximise the benefit of the phenotyping effort, strains that had not been characterised previously were chosen for study. *Butyrivibrio* and *Pseudobutyrivibrio* were excluded, as previous research has covered characterisation of many of the strains within this genus (Palevich, Kelly, Ganesh, et al., 2019; Palevich et al., 2017; Palevich, Kelly, Leahy, et al., 2019; Sengupta et al., 2022). From the remaining 89 strains, 45 were chosen based on their phylogenetic relationships to ensure representation from the large inter- and intra-species diversity evident within the family. To begin, a set of five type strains, within the *Lachnospiraceae* family, (highlighted in green in Table 3.1.), were selected as control cultures to ensure that the assays and phenotypic tests were consistent with previously published information for these strains (Bryant & Small, 1956; Chamkha et al., 2001; Cornick et al., 1994; Greening & Leedle, 1989; Haas & Blanchard, 2020).

3.2. Strains overview

Table 3.1 Overview of strain information taken from Genome Taxonomy Database (GTDB) (Parks et al., 2021; Parks et al., 2018).

Species	IMG #	GTDB Taxonomy	Genome Size	Genes	Scaffolds	G+C%
***Acetitomaculum ruminis* DSM 5522 (139B)**	2651870361	*Acetitomaculum ruminis*	3,082,721	2,710	54	34.2
***Blautia schinkii* DSM 10518 (B)**	2561511230	*Blautia_A schinkii_A*	6,679,327	5,907	65	45.6
***Blautia wexlerae* AGR2146**	2524614712	*Blautia_A wexlerae*	3,583,102	3,372	189	41.58
***[Clostridium] clostridioforme* AGR2157**	2524614699	*Enterocloster clostridioformis_A*	4,943,165	5,003	133	49.05
***Dorea longicatena* AGR2136**	2524614701	*Dorea_A longicatena*	3,595,079	3,624	137	40.99
***Dorea sp.* AGR2135**	2524614702	*Dorea formicigenerans*	3,509,498	3,548	73	40.56
***Enterocloster clostridioformis* ATCC 25537 (T90)**	2593339147	*Enterocloster clostridioformis*	5,465,751	5,305	148	48.95
***Eubacterium ruminantium* FB3002**	2623620486	*Eubacterium_Q ruminantium_A*	3,504,514	2,890	40	40.07
***Eubacterium ruminatium* HUN269**	2593339210	*Eubacterium_Q ruminantium*	2,878,992	2,603	36	37.27
***Lachnobacterium bovis* AE2004**	2558860969	*Lachnobacterium bovis_A*	2,844,447	2,688	22	31.27
***Lachnobacterium bovis* NK4B19**	2524614717	*Lachnobacterium bovis_A*	2,514,800	2,358	130	30.76
***Lachnoclostridium aminophilum* F**	2593339256	*Clostridium_R aminophilum*	3,113,820	2,662	31	50.76
***Lachnoclostridium aminophilum* KH1P1**	2606217764	*Clostridium_R aminophilum_A*	3,198,475	2,830	108	50.67
***Lachnospira multipara* ATCC 19207 (D32)**	2524614802	*Lachnospira multipara*	2,871,860	2,612	31	35.27
***Lachnospira multipara* D15d**	2651870315	*Lachnospira multipara*	2,841,494	2,589	35	35.21
***Lachnospira multipara* LB2003**	2562617004	*Lachnospira multipara_A*	2,579,700	2,322	21	33.51
***Lachnospira multipara* MC2003**	2558860134	*Lachnospira multipara_A*	2,606,430	2,368	26	33.32
***Lachnospira pectinoschiza* M83**	2657245269	*Lachnospira pectinoschiza*	2,704,308	2,365	12	35.95
Lachnospiraceae* bacterium A*C2012	2561511219	*FD2005 sp000702525*	2,555,436	2,379	2	41.29
***Lachnospiraceae* bacterium AC2014**	2562617014	*Agathobacter ruminis*	2,760,118	2,550	14	43.49
***Lachnospiraceae* bacterium AC2028**	2561511225	*AC2028 sp000702685*	2,772,364	2,557	14	46.23
Lachnospiraceae* bacterium AC*2029	2561511233	*Eubacterium_Q sp000703065*	3,859,732	3,177	1	49.86
***Lachnospiraceae* bacterium AC2031**	2558309034	*Eubacterium_Q sp000687975*	3,191,482	2,827	1	39.47
***Lachnospiraceae* bacterium C10**	2593339216	*Weimeria sp900100095*	2,946,042	2,740	70	44.6
***Lachnospiraceae* bacterium C6A11**	2561511098	*Eubacterium_Q sp000702245*	2,895,073	2,395	17	44
***Lachnospiraceae* bacterium C7**	2608642107	*Lachnobacterium sp900113385*	2,844,513	2,581	91	32.11
***Lachnospiraceae* bacterium FE2018**	2562617195	*Bilifractor sp000702845*	3,213,641	2,907	119	48.71
***Lachnospiraceae* bacterium KH1P17**	2606217738	*NK4A144 sp900168235*	3,790,302	3,359	28	43.53
***Lachnospiraceae* bacterium KH1T2**	2654588176	*NK4A144 sp900114405*	3,871,245	3,493	63	39.43
***Lachnospiraceae* bacterium MA2020**	2571042015	*NK4A144 sp000746015*	3,724,716	3,384	49	39.93
***Lachnospiraceae* bacterium MC2017**	2561511145	*NK4A144 sp000701625*	4,255,528	3,815	56	41.42
***Lachnospiraceae* bacterium NC2004**	2558860167	*NC2004 sp000621905*	3,373,099	2,899	56	39.37
***Lachnospiraceae* bacterium NC2008**	2558860166	*NK4A136 sp000687675*	2,718,916	2,506	26	36.84
***Lachnospiraceae* bacterium NK4A136**	2524614706	*NK4A136 sp000687675*	2,730,030	2,553	78	36.91
***Lachnospiraceae* bacterium NK4A144**	2524614703	*NK4A144 sp000424365*	4,092,878	3,796	68	41.62
***Lachnospiraceae* bacterium NK4A179**	2524614704	*NK4A136 sp000421045*	3,222,863	2,981	73	43.28
***Lachnospiraceae* bacterium P6A3**	2562617029	*AC2028 sp000702085*	2,978,537	2,721	9	44.2
***Lachnospiraceae* bacterium XPB1003**	2593339154	*NK4A136 sp900102065*	3,145,544	2,896	42	43.34
***Lachnospiraceae* bacterium YSB2008**	2558860136	*Eubacterium_Q sp000687655*	2,686,267	2,331	29	40.74
***Lachnospiraceae* bacterium YSD2013**	2596583542	*UBA1258 sp900100895*	3,242,758	2,956	54	45.07
***Lacrimispora aerotolerans* DSM 5434 (XBA62)**	2558860131	*Lacrimispora aerotolerans*	4,732,373	4,369	50	42.37
***Oribacterium* sp. FC2011**	2561511144	*Oribacterium sp900107835*	4,166,765	3,611	67	42.18
***Oribacterium* sp. P6A1**	2561511133	*Oribacterium sp000702885*	3,697,107	3,277	95	42.46
Oribacterium sp. WCC10	2651870359	*Oribacterium sp900113415*	3,642,652	3,164	53	41.47
Ruminococcus gnavus AGR2154	2545555826	*Ruminococcus_ gnavus*	3,724,077	3,784	5	42.52

GTDB annotations: “Q”= Query (taxonomic assignment for a genome is uncertain) , “A”- Awaiting (taxonomic assignment for genome is pending) , “R”= Reclassified (genome has been reclassified based on new data)

3.3. Phylogenetic tree of 45 *Lachnospiraceae* strains

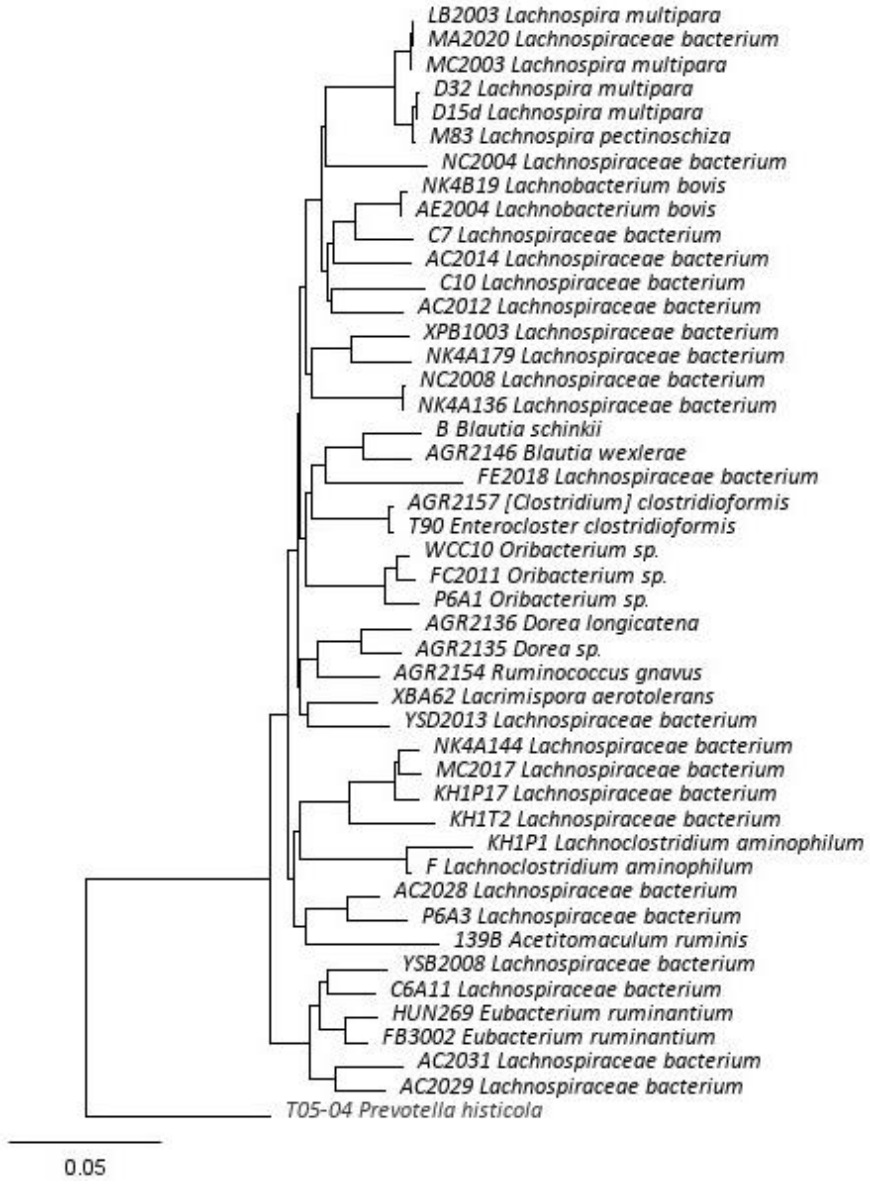


Figure 3.1 A phylogenetic tree based on 16S rRNA gene sequences of the 45 *Lachnospiraceae* strains used in this study. A global alignment was produced using a 93% similarity cost matrix, Jukes-Cantor genetic distance model, and a Neighbour Joining tree build method. *Prevotella histicola* T05-04, was used as an outgroup.

3.4. Gram stain results of 45 *Lachnospiraceae* strains

Gram staining was used to differentiate the strains as either Gram positive or Gram negative. The strains that retained the crystal violet dye were considered Gram positive, indicating they have a thick peptidoglycan cell wall (50- 90% of the cellular envelope). Gram negative cells are unable to retain the crystal violet dye and instead stained pink with the safranin counter stain. Gram negative cells have a thinner, double layered cellular envelope (Bartholomew & Mittwer, 1952). The slight majority of strains (25/45) were Gram positive (Table 3.2).

Table 3.2 Gram staining results

Species	Gram Stain Result
Acetitomaculum ruminis 139B	+
Blautia schinkii B	+
Blautia wexlerae AGR2146	+
Dorea longicatena AGR2136	+
Dorea sp. AGR2135	-
[Clostridium] clostridioforme AGR2157	-
Enterocloster clostridioformis T90	-
Eubacterium ruminantium FB3002	+
Eubacterium ruminantium HUN269	+
Lachnobacterium bovis AE2004	+
Lachnobacterium bovis NK4B19	+
Lachnoclostridium aminophilum F	+
Lachnoclostridium aminophilum KH1P1	+
Lachnospira multipara D15d	+
Lachnospira multipara D32	+
Lachnospira multipara LB2003	-
Lachnospira multipara MC2003	-
Lachnospira pectinoschiza M83	+
Lachnospiraceae bacterium AC2012	+
Lachnospiraceae bacterium AC2014	-
Lachnospiraceae bacterium AC2028	+
Lachnospiraceae bacterium AC2029	+
Lachnospiraceae bacterium AC2031	+
Lachnospiraceae bacterium C10	-
Lachnospiraceae bacterium C6A11	-
Lachnospiraceae bacterium C7	-
Lachnospiraceae bacterium FE2018	-
Lachnospiraceae bacterium KH1P17	+
Lachnospiraceae bacterium KH1T2	+
Lachnospiraceae bacterium MA2020	-
Lachnospiraceae bacterium MC2017	-
Lachnospiraceae bacterium NC2004	-
Lachnospiraceae bacterium NC2008	-
Lachnospiraceae bacterium NK4A136	-
Lachnospiraceae bacterium NK4A144	+
Lachnospiraceae bacterium NK4A179	-
Lachnospiraceae bacterium P6A3	-
Lachnospiraceae bacterium XPB1003	-
Lachnospiraceae bacterium YSB2008	+
Lachnospiraceae bacterium YSD2013	-
Lacrimispora aerotolerans X8A62	+
Oribacterium sp. FC2011	+
Oribacterium sp. P6A1	+
Oribacterium sp. WCC10	-
[Ruminococcus] gnavus AGR2154	+

3.5. Carbon source utilisation assays

A growth-based, carbon source utilisation assay was used to provide insight into the substrate utilisation patterns of the 45 *Lachnospiraceae* strains. The carbon sources were selected to cover the range of hexoses and pentoses likely encountered in the rumen and to identify phenotypic differences between *Lachnospiraceae* strains.

3.5.1. Soluble substrate utilisation assay

The presence of genes in the genomes of the 45 *Lachnospiraceae* strains encoding enzymes that are involved in the breakdown of these polysaccharides and metabolism of the released sugars could predict their fibre degradation capabilities (Seshadri et al., 2018). Here, a growth-based, carbon source utilisation assay was used to provide insight into their phenotypic substrate utilisation.

Although there was no single substrate that was commonly used amongst all strains, cellobiose, glucose and sucrose were utilised most widely (Figure 3.2). The substrates that were utilised by the least number of strains were D-glucuronic acid, D-galacturonic acid and rhamnose. Additionally, there appeared to be a preference for hexoses over the three pentose substrates tested (xylose, arabinose and rhamnose).

For those organisms with a classification, their substrate utilisation profiles mostly align with their assigned taxonomic groups. For example, the two *Dorea* species (AGR2136 and AGR2135), *Lachnoclostridium aminophilum* strains (F and KH1P1), *Lachnospira multipara* strains (LB2003, MC2003, D32 and D15d) and two *Blautia* strains (B and AGR2146) shared similar substrate utilisation profiles within their genera. In contrast, each of the two *Eubacterium ruminantium* strains (FB2003 and HUN269) and the *Entercloster clostridioformis* strains and [*Clostridium*] *clostridioforme* strains (T90 and AGR2157), appeared to have different carbohydrate fermentation capabilities.

Some strains grew poorly on all the substrates, for example, *Lachnoclostridium aminophilum* strains F and KH1P1. Both strains did not reach an OD_{600} of above 0.1 on any of the substrates, apart from sucrose which produced a OD_{600} of 0.177 on sucrose. *Lachnospiraceae* bacterium FE2018 also showed minimal growth on all the substrates tested.

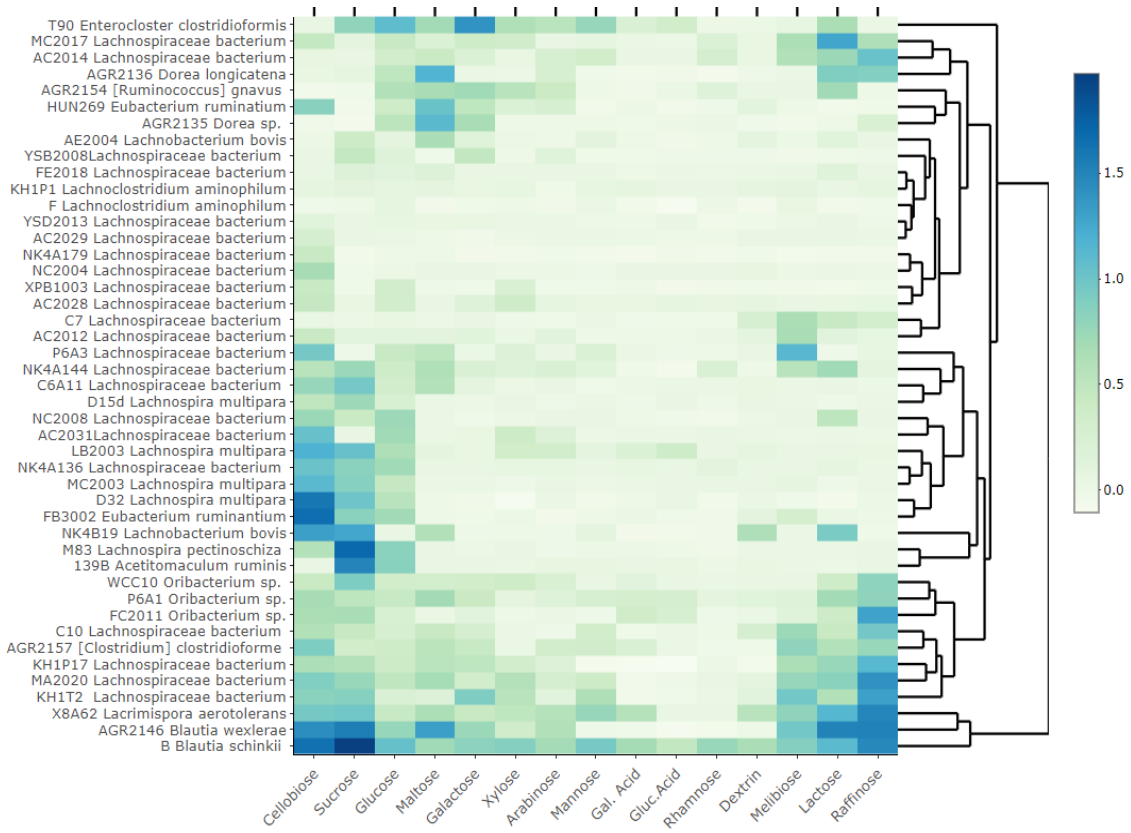


Figure 3.2. Heatmap representing growth of *Lachnospiraceae* strains on various soluble substrates. The OD_{600} of each triplicate culture after 48 hr growth at 39°C was averaged and the average growth on the media-substrate control was deducted to provide each data point. The dendrogram on the right-hand side of the heat map was produced using Euclidean Distances and Single Linkage as a hierarchical clustering method. (Gal. Acid = D-Galacturonic acid, Gluc. Acid = D-Glucuronic Acid). The heatmap was produced in R Studio using the Heatmaply package.

3.5.2. Insoluble substrate utilisation assay

The growth of the *Lachnospiraceae* strains on insoluble substrates (microcrystalline cellulose, pectin from apple, synthetic polygalacturonic acid, starch from potato, and xylan from oat spelt) was assessed by total SCFA production as measured by GLC after a 48-hr growth period on each of the substrates.

Pectin was the most utilised substrate followed by polygalacturonic acid, starch, and cellulose, while the least utilised substrate was xylan (Figure 3.3). The two clusters at the top of the dendrogram produced the most SCFAs on the widest range of substrates whereas the clusters at the bottom of the dendrogram represent organisms that grew better on pectin and polygalacturonic acid. Some strains did not grow on any of the five insoluble substrates tested, for example: *Lachnobacterium bovis* AE2004, *Lachnospiraceae* bacterium MA2020, and *Lachnospiraceae* bacterium NC2008. The organisms that produced the greatest amount of SCFAs was *Oribacterium* sp. WCC10 which produced a total of 9.82 mM of total SCFAs when grown on polygalacturonic acid. *Enterocloster clostridioformis* T90 produced more total SCFAs on xylan (8.53 mM), cellulose (7.1 mM) and starch (8.48 mM) than any other isolate, (Figure 3.3). *Lachnospira pectinoschiza* M83 produced the highest amount of total SCFAs (8.77 mM) on pectin compared to any of the other strains (Figure 3.3).

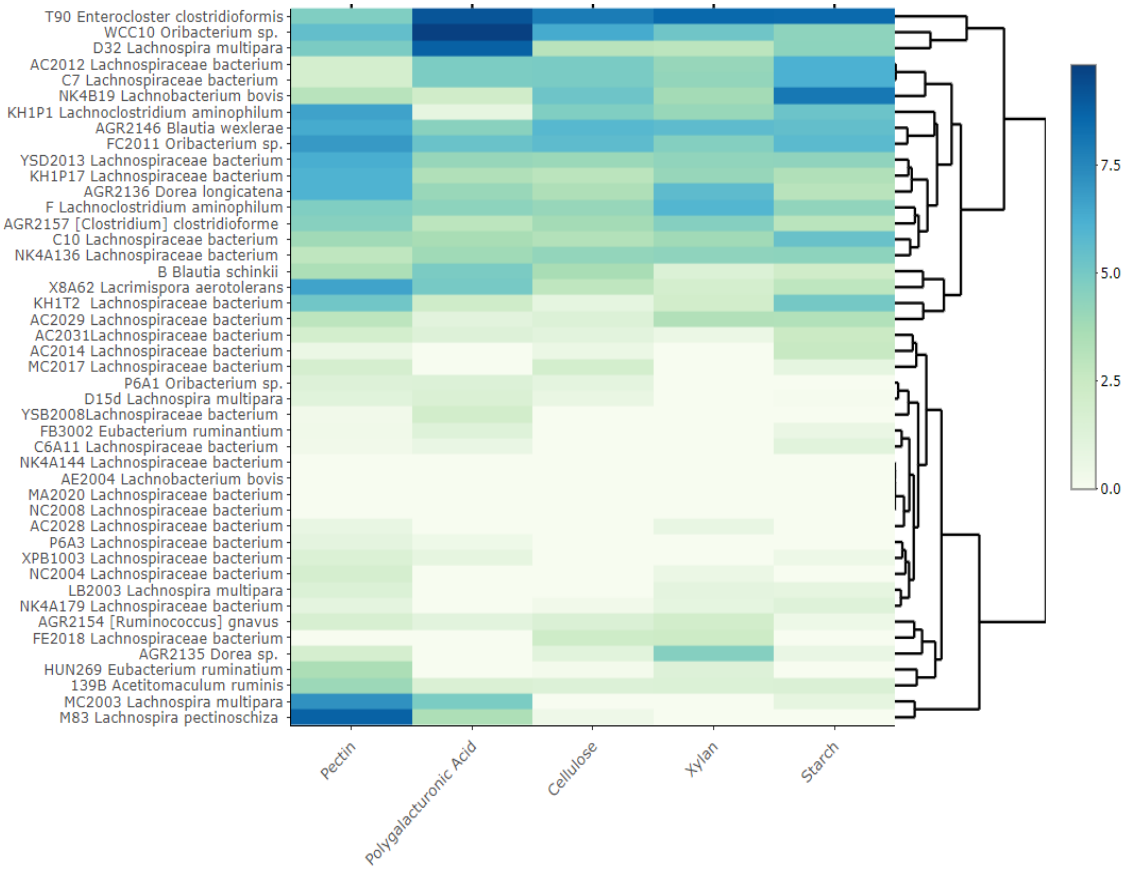


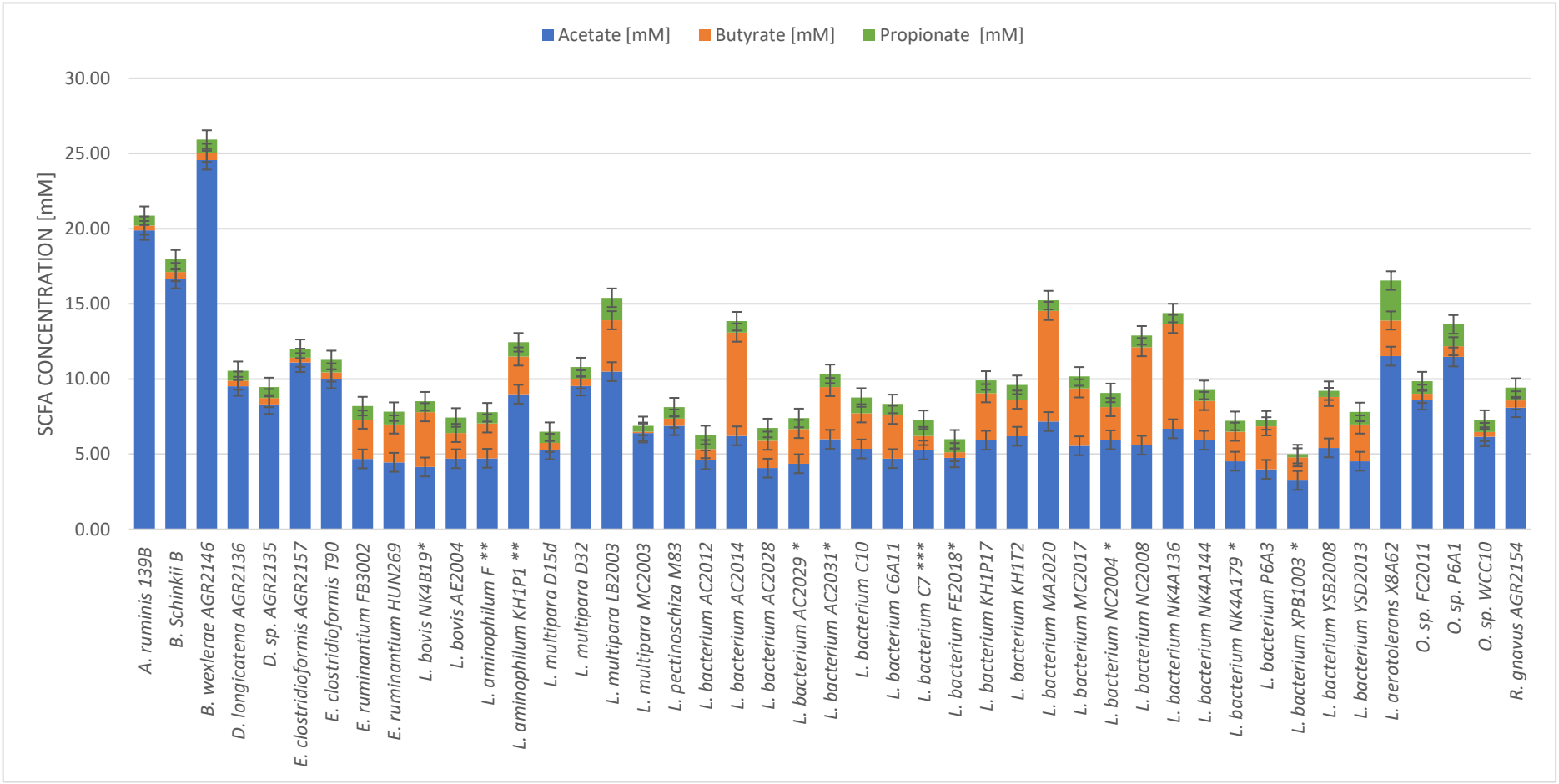
Figure 3.3 Heatmap representing SCFA production as an indicator of growth of *Lachnospiraceae* strains tested on five different insoluble substrates; pectin, starch, cellulose, xylan and polygalacturonic acid. The SCFA production (mM total SCFAs) of each of the triplicate cultures after 48 hr growth at 39°C was averaged and the average SCFA production on the media-substrate control was deducted to provide each data point. The dendrogram on the right of the heat map depicts the similarity between the SCFA production profiles of each of the 45 strains. Euclidean distance was used as a distance metric and Single Linkage was used as the hierarchical clustering method. The heatmap was produced in R Studio using the Heatmaply package.

3.5.3. Volatile SCFA profiles on soluble substrates

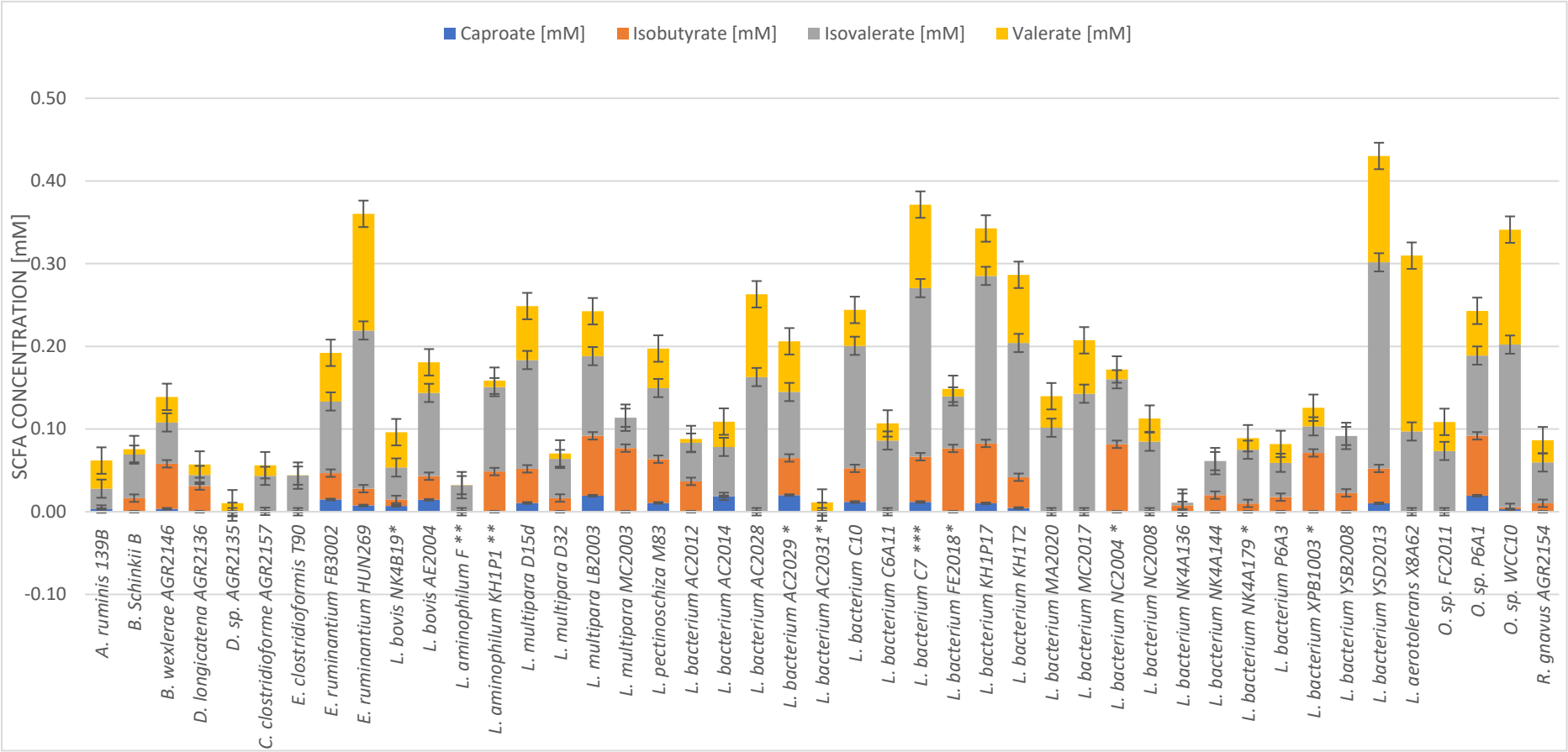
SCFAs were measured using GLC in samples of culture supernatants of each of the strains when grown on glucose or in some cases where growth on glucose was poor, on cellobiose and in the case of *Lachnospiraceae* bacterium C7, melibiose.

For 41 out of the 45 strains, acetate made up the majority of the total SCFA produced (>50%). The greatest acetate producers were *Blautia wexlerae* AGR2146 (24.55 mM), *Acetitomaculum ruminis* 139B (19.88 mM), and *Blautia schinkii* B (16.66 mM), (Figure 3.4A). In the remaining four strains, *Lachnobacterium bovis* NK4B19, *Lachnospiraceae* bacterium NK4A136, *Lachnospiraceae* bacterium AC2014, and *Lachnospiraceae* bacterium MA2020, butyrate was the main product. Butyrate and propionate were produced in lesser quantities by all the strains, the greatest propionate producer was *Lacrimispora aerotolerans* X8A62 at 2.65 mM, (Figure 3.4A). Butyrate was generally produced in greater concentrations in comparison to propionate, with the greatest producer (*Lachnospiraceae* bacterium MA2020) generating 7.32 mM of butyrate, (Figure 3.4A).

The results show that minor SCFAs were produced in much smaller quantities than the major SCFAs, with *iso*-valerate the most produced minor SCFA, whereas caproate was the least produced. *Lachnospiraceae* bacterium YSD2013 produced the highest quantity of total minor SCFA (0.43 mM) with *iso*-valerate making the majority of this at 0.25mM (Figure 3.4B). *Dorea* sp. AGR2135 and *Lachnospiraceae* bacterium NK4A136 produced the least amount of minor SCFAs.


Acetate [mM]
Butyrate [mM]
Propionate [mM]
SCFA CONCENTRATION [mM]
30.00
25.00
20.00
15.00
10.00
5.00
0.00
A. ruminis 139B
B. Schinkii B
B. wexlerae AGR2146
D. longicatena AGR2136
D. sp. AGR2135
E. clostridioformis AGR2157
E. clostridioformis T90
E. ruminantium FB3002
E. ruminantium HUN269
L. bovis NK4B19*
L. bovis AE2004
L. aminophilum F **
L. aminophilum KH1P1 **
L. multipara D15d
L. multipara D32
L. multipara LB2003
L. multipara MC2003
L. pectinoschiza M83
L. bacterium AC2012
L. bacterium AC2014
L. bacterium AC2028
L. bacterium AC2029 *
L. bacterium AC2031*
L. bacterium C10
L. bacterium C6A11
L. bacterium C7 ***
L. bacterium FE2018*
L. bacterium KH1P17
L. bacterium KH1T2
L. bacterium MA2020
L. bacterium MC2017
L. bacterium NC2004 *
L. bacterium NC2008
L. bacterium NK4A136
L. bacterium NK4A144
L. bacterium NK4A179 *
L. bacterium P6A3
L. bacterium XPB1003 *
L. bacterium YSB2008
L. bacterium YSD2013
L. aerotolerans X8A62
O. sp. FC2011
O. sp. P6A1
O. sp. WCC10
R. gnavus AGR2154

A



B

Figure 3.4. Bar graph depicting the average major **(A)** and minor **(B)** volatile SCFA concentration (mM), produced by 45 *Lachnospiraceae* strains after a 48-hour growth period at 39 °C. The strains were grown on glucose; or cellobiose (*) or grew poorly (<0.1 OD_{600})on glucose (**) or grew only on melibiose (***). Error bars show the ± standard error of the mean of each of the data points

3.5.4. Formate and non-volatile FA profiles on soluble substrates

The non-volatile FAs (fatty acids) and formate were measured after an ether extraction using barrier ion detection via GLC. Non-volatile FAs are succinate, and lactate. Formate and the non-volatile FAs are often formed as intermediary metabolites in the rumen that incorporate H_2 into their formation, making them intercellular electron carriers (Ungerfeld, 2015). This could be implicated in the disposal of electrons into metabolic H_2 sinks other than methanogenesis.

Four out of the 45 strains did not produce any of the non-volatile fatty acids, namely, *Lachnospiraceae* bacterium AC2029, *Lachnospiraceae* bacterium AC2012 and *Acetitomaculum ruminis* 139B. Of the strains that produced non-volatile-SCFA, formate was produced by most of them apart from *Lachnospiraceae* bacterium C7, *Lachnospiraceae* bacterium C10, *Lachnoclostridium aminophilum* F, *Lachnospiraceae* bacterium FE2018; these organisms only produced lactate, (Figure 3.5).

Lachnobacterium bovis NKB19 produced a large amount of both lactate (16.6 mM) and formate (16.54mM), (Figure 3.5). This strain stood out as the highest non-volatile SCFA producer. There were only a few strains that produced succinic acid in small amounts, with the greatest amount produced by *Blautia wexlerae* AGR2156 (2.14mM), (Figure 3.5).

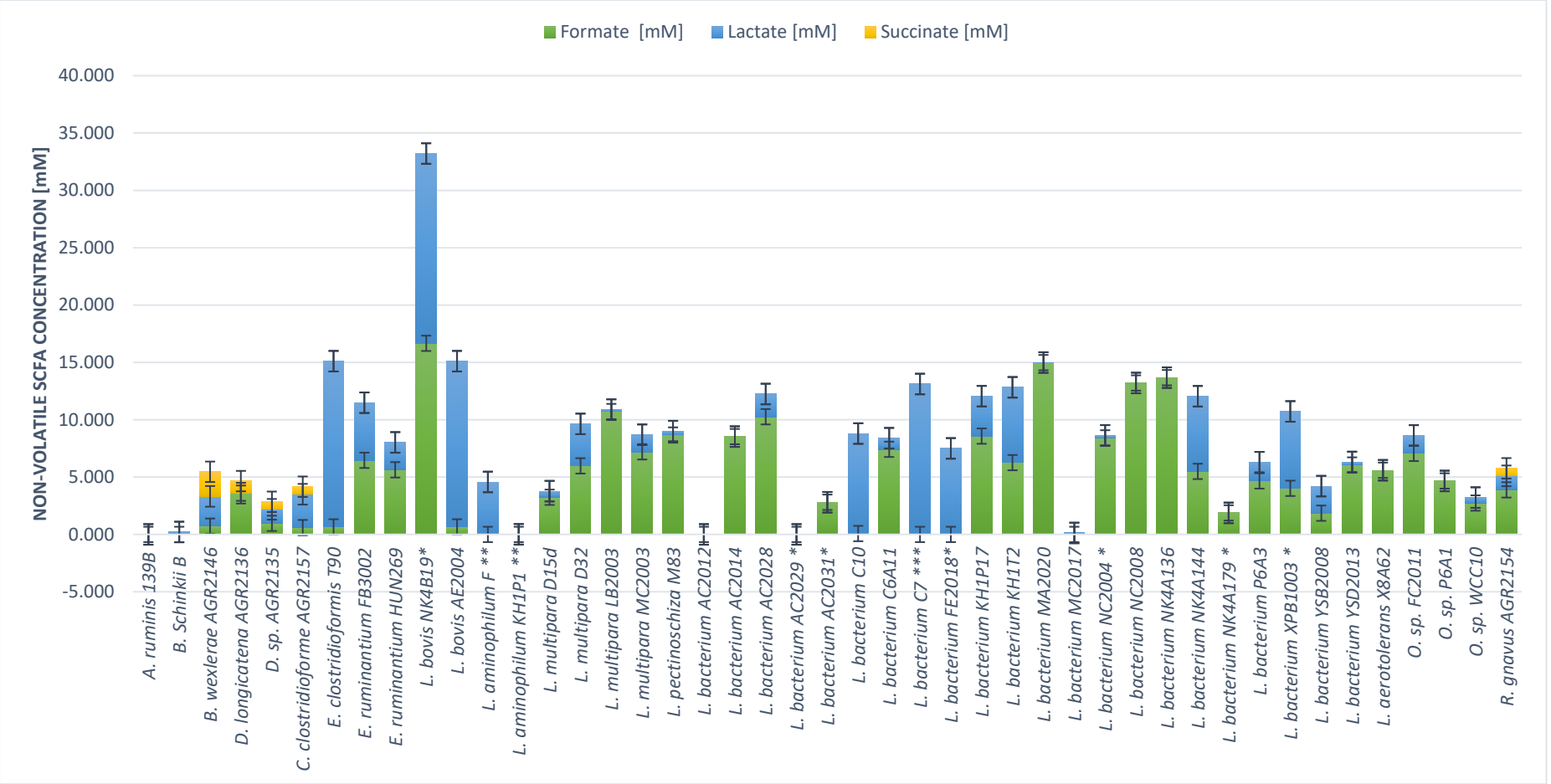


Figure 3.5 Bar graph depicting the average formate and non-volatile FAs (mM) produced by the 45 *Lachnospiraceae* strains after 48 hr growth at 39 °C. The strains were grown on glucose; or cellobiose (*), grew poorly (<0.1 OD_{600}) on glucose (**)or grew only on melibiose (***). Error bars show ± standard error of the mean of each of the data points

3.5.5. Short chain alcohol profiles on soluble substrates

Alcohols were measured via GLC in the same manner as the VFAs and the non-VFAs. Understanding which alcohols are produced by these organisms is important and can help to understand interspecies electron transfer and shows how these organisms can make use of alternative H_2 sinks.

None of the strains produced methanol when grown on glucose (cellobiose or melibiose). Ethanol was the most produced alcohol and the two *Oribacterium* species were the greatest ethanol producers with P6A1 producing 31.49 mM and WCC10 producing 27.86 mM. Only one strain produced a small amount of butanol which was AE2004 *Lachnobacterium bovis* (0.5mM).

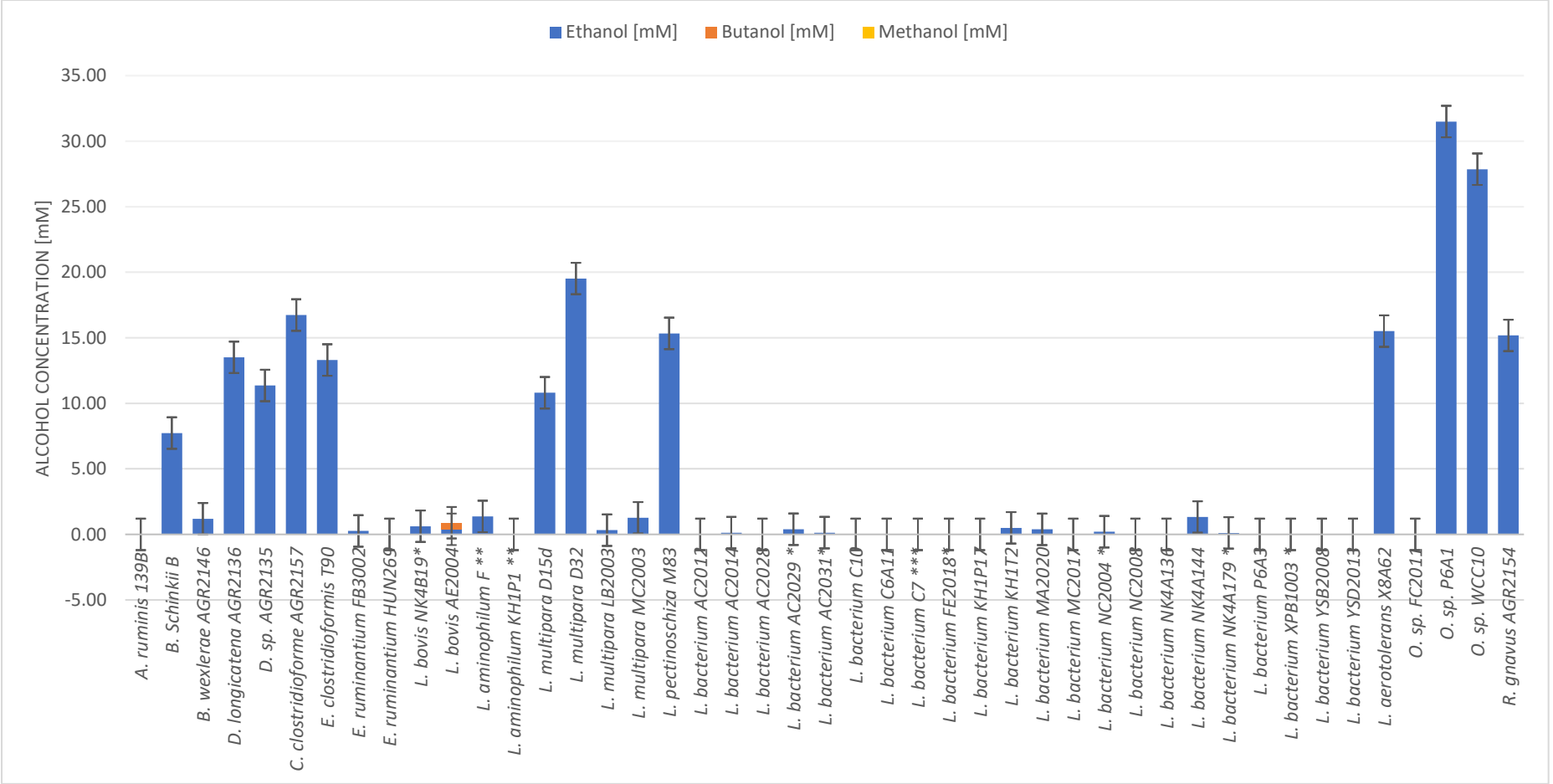


Figure 3.6. Bar graph depicting the average short chain alcohol concentration (mM) produced by the 45 *Lachnospiraceae* strains after 48 hr growth at 39 °C. The strains were grown on glucose, cellobiose (*), grew poorly (<0.1 OD_{600}) on glucose (**) or grew only on melibiose (***). Error bars show ± standard error of the mean of each of the data points.

3.5.6. Analysis of SCFAs produced by growth on insoluble substrates

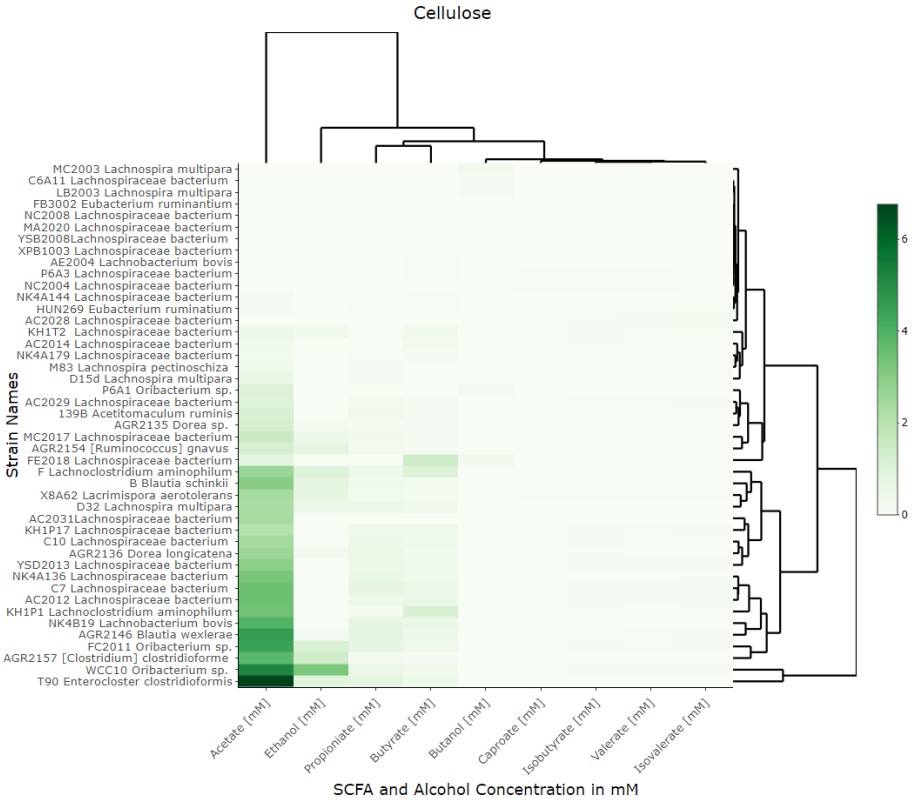
The production of SCFAs and alcohols after 48 hr of growth on five different insoluble substrates (microcrystalline cellulose, pectin from apple, polygalacturonic acid, starch from potato, and xylan from oat spelt) are summarised in Figure 3.7.

Apart from growth on pectin, *Oribacterium* sp. WCC10 and *Enterocloster clostridioformis* T90, cluster together as the greatest producers of acetate. *Oribacterium* sp. WCC10 also produced more ethanol than any other strain on all the substrates apart from pectin. On pectin, *Lachnospira pectinoschiza* M83 was the greatest producer of acetate (8.77 mM, Figure 3.7C).

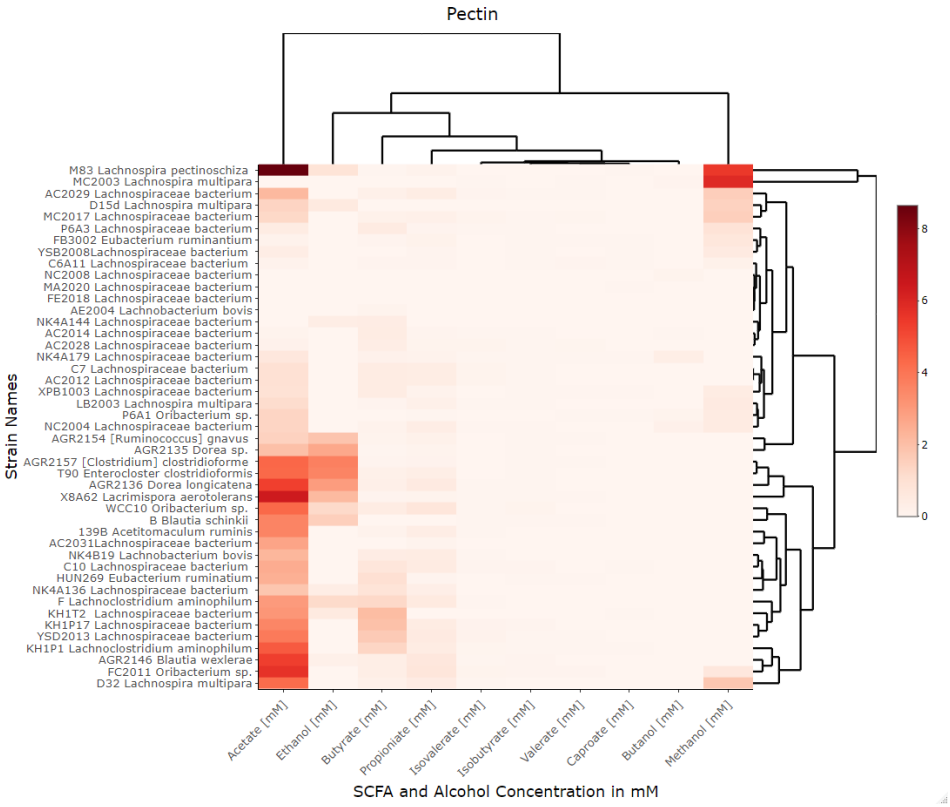
For cellulose, polygalacturonic acid, and xylan the end products produced in the highest concentration were acetate and ethanol. However, on starch, acetate and butyrate were the main fermentation products.

Growth on pectin produced mostly acetate and ethanol and was the only substrate that had methanol as an end-product; specifically, in *Lachnospira pectinoschiza* M83 (5.50 mM) and *Lachnospira multipara* MC2003 (5.90 mM, Figure 3.7C).

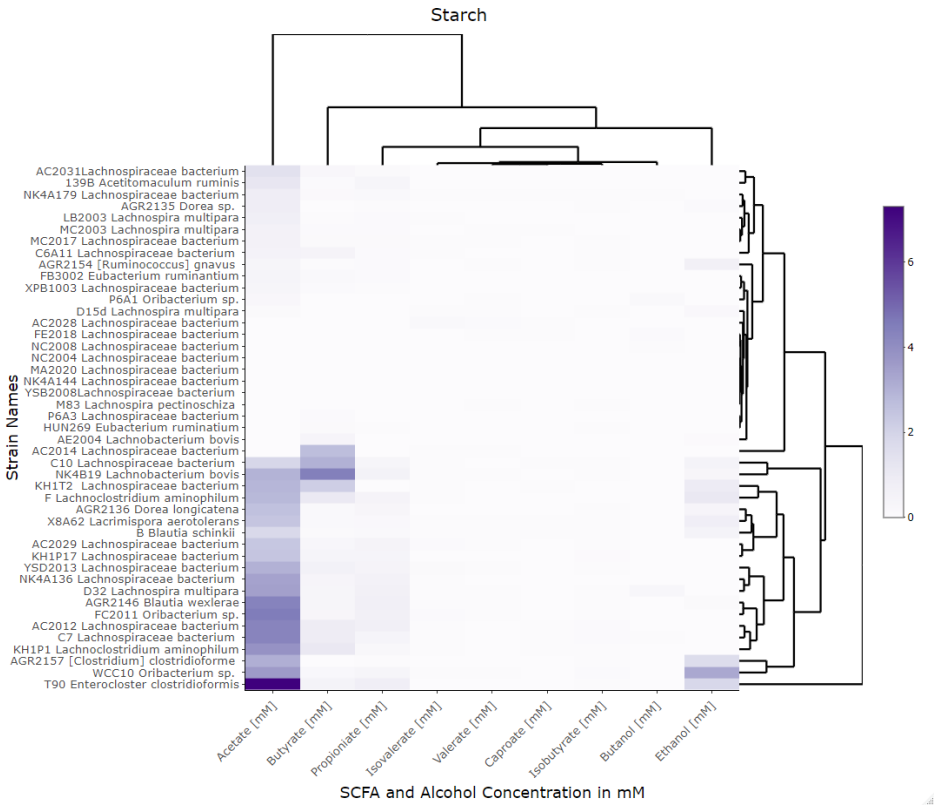
The SCFAs that were produced in the lowest concentration were caproate followed by valerate, *iso*-butyrate and *iso*-valerate. *Lachnospiraceae* bacterium AC2028 was the only strain to produce *iso*-valerate and valerate, whilst producing minimal amounts of the major SCFAs on all five substrates. For growth on cellulose, there was an increased number of strains producing the two *iso*-acids (*iso*-butyrate and *iso*-valerate) and valerate at low levels (Figure 3.7A).


Cellulose
MC2003 Lachnospira multipara
C6A11 Lachnospiraceae bacterium
LB2003 Lachnospira multipara
FB3002 Eubacterium ruminantium
NC2008 Lachnospiraceae bacterium
MA2020 Lachnospiraceae bacterium
YSB2008Lachnospiraceae bacterium
XPB1003 Lachnospiraceae bacterium
AE2004 Lachnobacterium bovis
P6A3 Lachnospiraceae bacterium
NC2004 Lachnospiraceae bacterium
NK4A144 Lachnospiraceae bacterium
HUN269 Eubacterium ruminatium
AC2028 Lachnospiraceae bacterium
KH1T2 Lachnospiraceae bacterium
AC2014 Lachnospiraceae bacterium
NK4A179 Lachnospiraceae bacterium
M83 Lachnospira pectinoschiza
D15d Lachnospira multipara
P6A1 Oribacterium sp.
AC2029 Lachnospiraceae bacterium
139B Acetitomaculum ruminis
AGR2135 Dorea sp.
MC2017 Lachnospiraceae bacterium
AGR2154 [Ruminococcus] gnavus
FE2018 Lachnospiraceae bacterium
F Lachnoclostridium aminophilum
B Blautia schinkii
X8A62 Lacrimispora aerotolerans
D32 Lachnospira multipara
AC2031Lachnospiraceae bacterium
KH1P17 Lachnospiraceae bacterium
C10 Lachnospiraceae bacterium
AGR2136 Dorea longicatena
YSD2013 Lachnospiraceae bacterium
NK4A136 Lachnospiraceae bacterium
C7 Lachnospiraceae bacterium
AC2012 Lachnospiraceae bacterium
KH1P1 Lachnoclostridium aminophilum
NK4B19 Lachnobacterium bovis
AGR2146 Blautia wexlerae
FC2011 Oribacterium sp.
AGR2157 [Clostridium] clostridioforme
WCC10 Oribacterium sp.
T90 Enterocloster clostridioformis
Strain Names
6
4
2
0
Acetate [mM]
Ethanol [mM]
Propionate [mM]
Butyrate [mM]
Butanol [mM]
Caproate [mM]
Isobutyrate [mM]
Valerate [mM]
Isovalerate [mM]
SCFA and Alcohol Concentration in mM

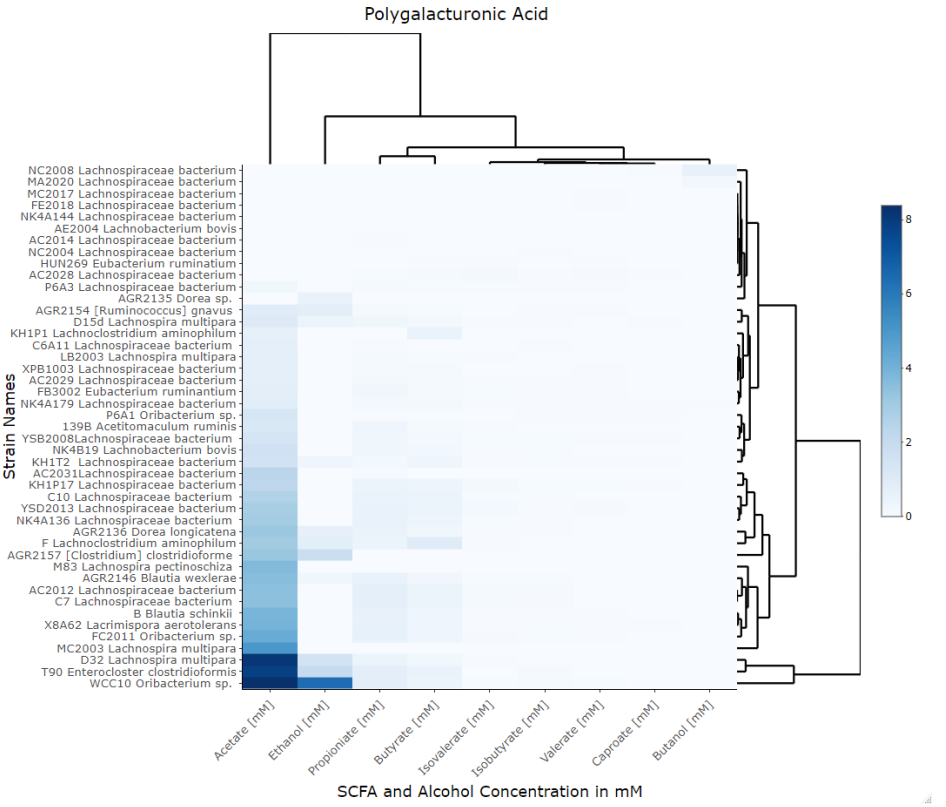
A


Pectin
M83 Lachnospira pectinoschiza
MC2003 Lachnospira multipara
AC2029 Lachnospiraceae bacterium
D15d Lachnospira multipara
MC2017 Lachnospiraceae bacterium
P6A3 Lachnospiraceae bacterium
FB3002 Eubacterium ruminantium
YSB2008Lachnospiraceae bacterium
C6A11 Lachnospiraceae bacterium
NC2008 Lachnospiraceae bacterium
MA2020 Lachnospiraceae bacterium
FE2018 Lachnospiraceae bacterium
AE2004 Lachnobacterium bovis
NK4A144 Lachnospiraceae bacterium
AC2014 Lachnospiraceae bacterium
AC2028 Lachnospiraceae bacterium
NK4A179 Lachnospiraceae bacterium
C7 Lachnospiraceae bacterium
AC2012 Lachnospiraceae bacterium
XPB1003 Lachnospiraceae bacterium
LB2003 Lachnospira multipara
P6A1 Oribacterium sp.
NC2004 Lachnospiraceae bacterium
AGR2154 [Ruminococcus] gnavus
AGR2135 Dorea sp.
AGR2157 [Clostridium] clostridioforme
T90 Enterocloster clostridioformis
AGR2136 Dorea longicatena
X8A62 Lacrimispora aerotolerans
WCC10 Oribacterium sp.
B Blautia schinkii
139B Acetitomaculum ruminis
AC2031Lachnospiraceae bacterium
NK4B19 Lachnobacterium bovis
C10 Lachnospiraceae bacterium
HUN269 Eubacterium ruminatium
NK4A136 Lachnospiraceae bacterium
F Lachnoclostridium aminophilum
KH1T2 Lachnospiraceae bacterium
KH1P17 Lachnospiraceae bacterium
YSD2013 Lachnospiraceae bacterium
KH1P1 Lachnoclostridium aminophilum
AGR2146 Blautia wexlerae
FC2011 Oribacterium sp.
D32 Lachnospira multipara
Strain Names
8
6
4
2
0
Acetate [mM]
Ethanol [mM]
Butyrate [mM]
Propionate [mM]
Isovalerate [mM]
Isobutyrate [mM]
Valerate [mM]
Caproate [mM]
Butanol [mM]
Methanol [mM]
SCFA and Alcohol Concentration in mM

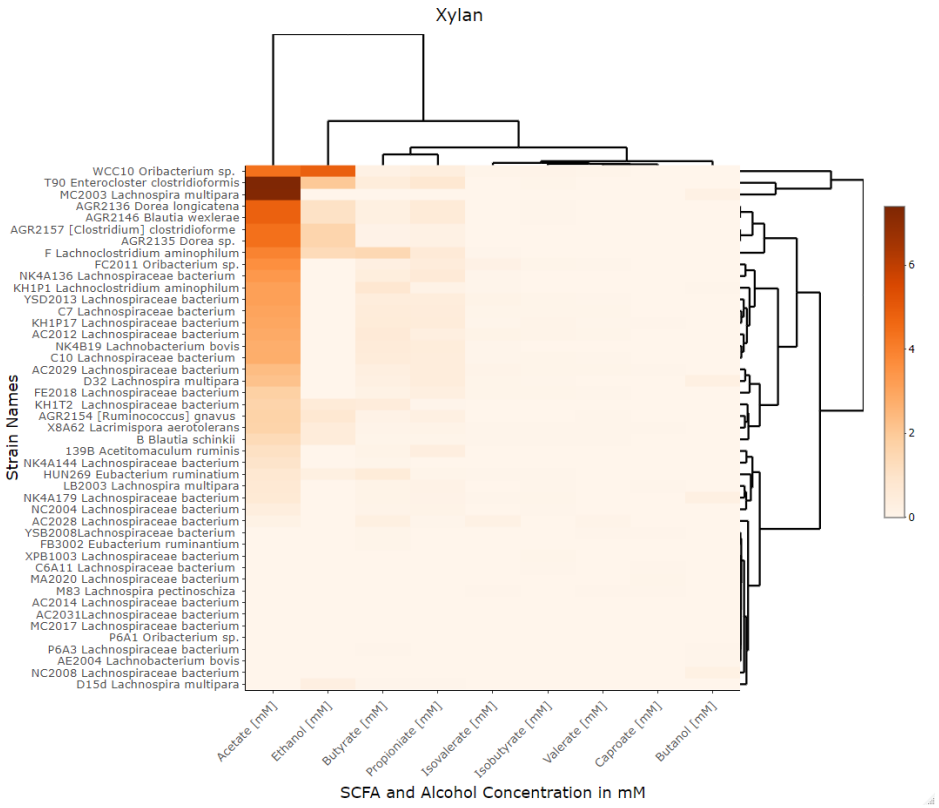
B


Starch
Strain Names
AC2031Lachnospiraceae bacterium
139B Acetitomaculum ruminis
NK4A179 Lachnospiraceae bacterium
AGR2135 Dorea sp.
LB2003 Lachnospira multipara
MC2003 Lachnospira multipara
MC2017 Lachnospiraceae bacterium
C6A11 Lachnospiraceae bacterium
AGR2154 [Ruminococcus] gnavus
FB3002 Eubacterium ruminantium
XPB1003 Lachnospiraceae bacterium
P6A1 Oribacterium sp.
D15d Lachnospira multipara
AC2028 Lachnospiraceae bacterium
FE2018 Lachnospiraceae bacterium
NC2008 Lachnospiraceae bacterium
NC2004 Lachnospiraceae bacterium
MA2020 Lachnospiraceae bacterium
NK4A144 Lachnospiraceae bacterium
YSB2008Lachnospiraceae bacterium
M83 Lachnospira pectinoschiza
P6A3 Lachnospiraceae bacterium
HUN269 Eubacterium ruminatium
AE2004 Lachnobacterium bovis
AC2014 Lachnospiraceae bacterium
C10 Lachnospiraceae bacterium
NK4B19 Lachnobacterium bovis
KH1T2 Lachnospiraceae bacterium
F Lachnoclostridium aminophilum
AGR2136 Dorea longicatena
X8A62 Lacrimispora aerotolerans
B Blautia schinkii
AC2029 Lachnospiraceae bacterium
KH1P17 Lachnospiraceae bacterium
YSD2013 Lachnospiraceae bacterium
NK4A136 Lachnospiraceae bacterium
D32 Lachnospira multipara
AGR2146 Blautia wexlerae
FC2011 Oribacterium sp.
AC2012 Lachnospiraceae bacterium
C7 Lachnospiraceae bacterium
KH1P1 Lachnoclostridium aminophilum
AGR2157 [Clostridium] clostridioforme
WCC10 Oribacterium sp.
T90 Enterocloster clostridioformis
Acetate [mM]
Butyrate [mM]
Propionate [mM]
Isovalerate [mM]
Valerate [mM]
Caproate [mM]
Isobutyrate [mM]
Butanol [mM]
Ethanol [mM]
SCFA and Alcohol Concentration in mM
0
2
4
6

C


Polygalacturonic Acid
Strain Names
NC2008 Lachnospiraceae bacterium
MA2020 Lachnospiraceae bacterium
MC2017 Lachnospiraceae bacterium
FE2018 Lachnospiraceae bacterium
NK4A144 Lachnospiraceae bacterium
AE2004 Lachnobacterium bovis
AC2014 Lachnospiraceae bacterium
NC2004 Lachnospiraceae bacterium
HUN269 Eubacterium ruminatium
AC2028 Lachnospiraceae bacterium
P6A3 Lachnospiraceae bacterium
AGR2135 Dorea sp.
AGR2154 [Ruminococcus] gnavus
D15d Lachnospira multipara
KH1P1 Lachnoclostridium aminophilum
C6A11 Lachnospiraceae bacterium
LB2003 Lachnospira multipara
XPB1003 Lachnospiraceae bacterium
AC2029 Lachnospiraceae bacterium
FB3002 Eubacterium ruminantium
NK4A179 Lachnospiraceae bacterium
P6A1 Oribacterium sp.
139B Acetitomaculum ruminis
YSB2008Lachnospiraceae bacterium
NK4B19 Lachnobacterium bovis
KH1T2 Lachnospiraceae bacterium
AC2031Lachnospiraceae bacterium
KH1P17 Lachnospiraceae bacterium
C10 Lachnospiraceae bacterium
YSD2013 Lachnospiraceae bacterium
NK4A136 Lachnospiraceae bacterium
AGR2136 Dorea longicatena
F Lachnoclostridium aminophilum
AGR2157 [Clostridium] clostridioforme
M83 Lachnospira pectinoschiza
AGR2146 Blautia wexlerae
AC2012 Lachnospiraceae bacterium
C7 Lachnospiraceae bacterium
B Blautia schinkii
X8A62 Lacrimispora aerotolerans
FC2011 Oribacterium sp.
MC2003 Lachnospira multipara
D32 Lachnospira multipara
T90 Enterocloster clostridioformis
WCC10 Oribacterium sp.
Acetate [mM]
Ethanol [mM]
Propionate [mM]
Butyrate [mM]
Isovalerate [mM]
Isobutyrate [mM]
Valerate [mM]
Caproate [mM]
Butanol [mM]
SCFA and Alcohol Concentration in mM
0
2
4
6
8

D



E

Figure 3.7. Heatmaps representing SCFA profiles of *Lachnospiraceae* strains tested on five different insoluble sugars; cellulose (**A**) pectin (**B**), polygalacturonic acid (**C**), starch (**D**), and xylan (**E**). Each data point represents the average SCFA and alcohol production (mM) of each strain in triplicate measured by GLC after a 48-hr growth period at 39 °C after subtraction of SCFAs from a media only control. The dendrogram on the right of the heat map depicts the similarity between the SCFA production profiles of each of the 45 strains. Euclidean Distance was used as a distance metric and Single Linkage was used as the hierarchical clustering method. The heatmap was produced in R Studio using the Heatmaply package

3.5.7. H_2 production analysis

The organisms that were the greatest H_2 producers were *[Clostridium] clostridioforme* AGR2157, *Lacrimispora aerotolerans* X8A62, *Lachnospiraceae* bacterium C10, *Lachnospiraceae* bacterium KH1P17*, Lachnospiraceae* bacterium MC2017, *Lachnospiraceae* bacterium KH1T2, and *Enterocloster clostridioformis* T90, which clustered together at the bottom of the dendrogram and *Oribacterium* sp. FC2011, which was located distal to this group (Figure 3.8).

The soluble substrates cellobiose, glucose, galactose, and sucrose showed the greatest amount of H_2 production when compared to the five insoluble substrates. In Figure 3.8 it can be observed that the substrates that showed the least amount of hydrogen production were D-glucuronic acid, D- galacturonic acid, rhamnose, xylose and mannose.

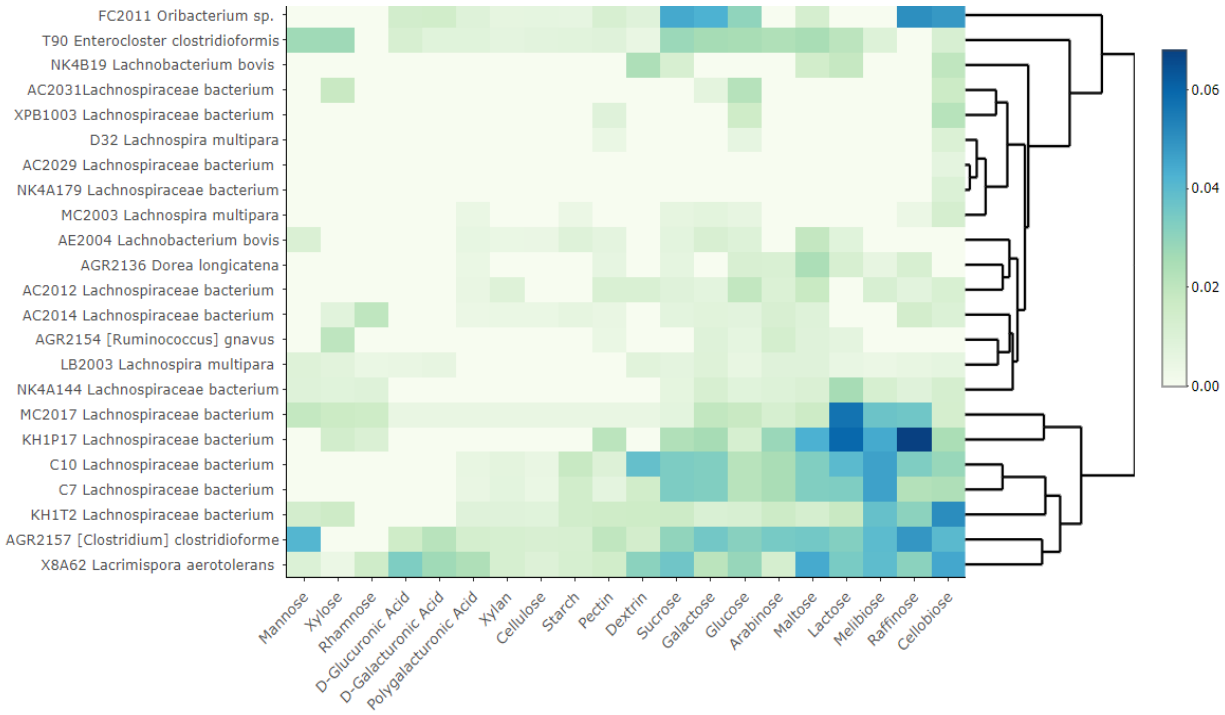


Figure 3.8. Heatmap showing total H_2 produced from 5mL of liquid culture as measured by gas chromatography. Each data point represents the average H_2 production in mM from triplicates of each of the *Lachnospiraceae* strains grown on 20 soluble and insoluble substrates for 48 hours at 39° C. Only the strains which produced H_2 are represented in the heat map. The dendrogram on the right of the heat map depicts the similarity between the H_2 profiles of each of the 23 strains. Euclidean Distance was used as a distance metric and single linkage was used as a hierarchical clustering method. Heatmap was produced in R Studio using the "Heatmaply" package.

3.6. Genomic prediction of phenotypic traits

Protologger was used as genome annotation tool to predict the potential phenotypic traits of all 45 strains.

3.6.1. Carbohydrate active enzyme summary

The most common CE families were CE4 and CE8, and *Lacrimispora aerotolerans* X8A62, contained the widest range and the largest number of CEs in its genome. Some strains only had one CE coding gene from the family CE4 in their genomes (*Lachnospiraceae* bacterium strains NC2008, NKA316 and AC2012).

The most common PL families seen across the 45 genomes were PL9 followed by PL1. *Lachnospira multipara* and *Lachnospira pectinoschiza* M83 had more PL coding genes than any other strains, specifically from the PL1, PL9, and PL11 families. Only *Enterocloster clostridioformis* T90, *[Clostridium] clostridioforme* AGR2157*, [Ruminococcus] gnavus* AGR2154 and *Blautia schinkii* B had genes coding for PL12 family enzymes. *Blautia schinkii* B was also the only strain that encoded PL27 family genes.

There were 30 CBM families encoded across the 45 *Lachnospiraceae* genomes the most common being CBM families CBM48, CBM50 and CBM34. *Lacrimispora aerotolerans* X8A62 had the greatest range and number of CBM genes and was the only strain encoding CBM family 16 genes.

None of the *Lachnospiraceae* strains encoded cohesins and only 11 strains encoded dockerins, (*Eubacterium ruminantium* HUN269, *Lachnospira multipara* D15d, *Lachnospira multipara* D32, *Lachnospira multipara* LB2003, *Lachnospira multipara* MC2003, *Lachnospira pectinoschiza* M83, *Lachnospiraceae* bacterium AC2014, *Lachnospiraceae* bacterium C6A11, *Lachnospiraceae bacterium* KH1P17, *Lachnospiraceae* bacterium NC2008, and *Lacrimispora aerotolerans* X8A62).

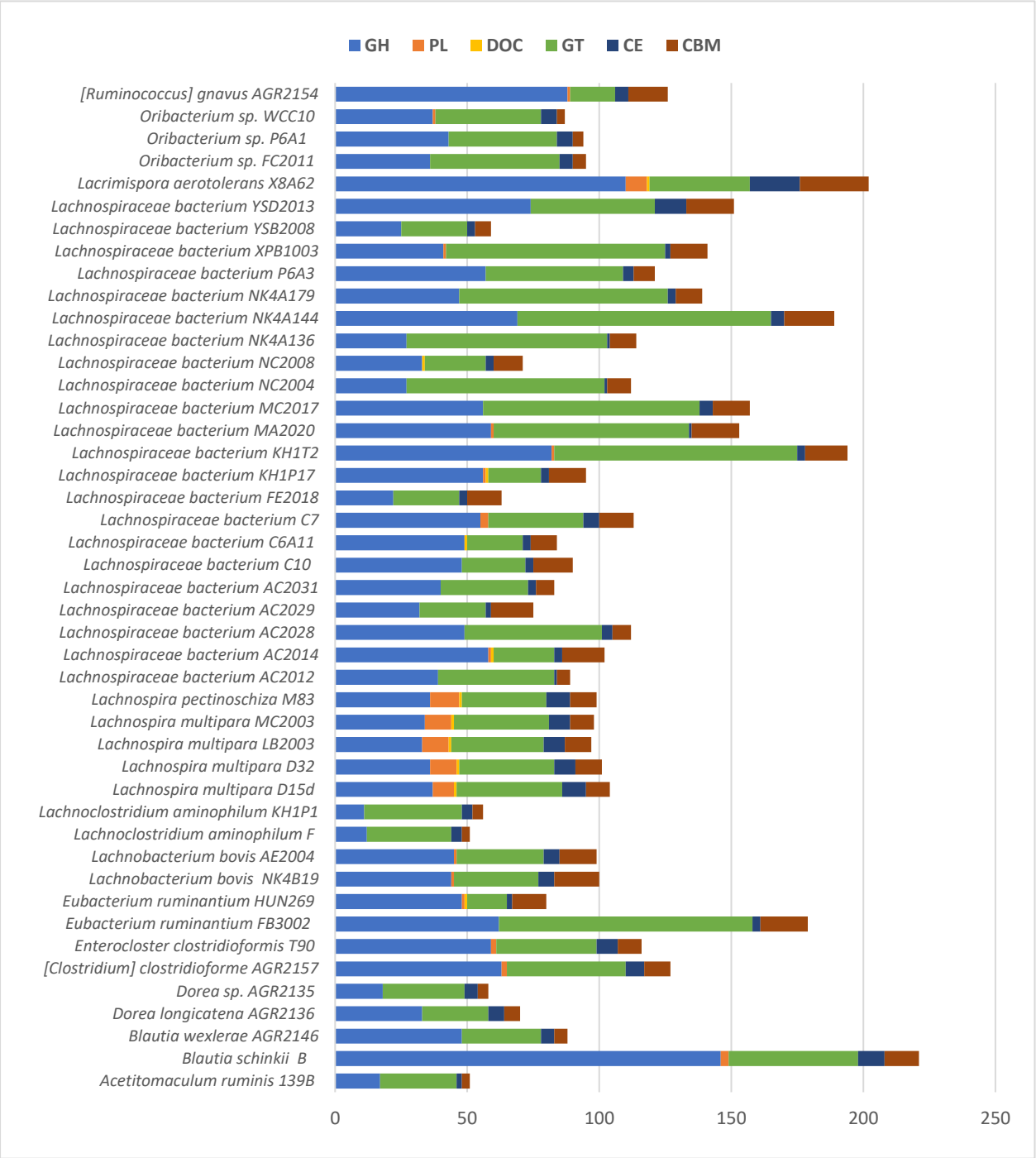


Figure 3.9. Bar graph summarising the various carbohydrate active enzymes in the genomes of the *Lachnospiraceae* strains. GH= Glycoside Hydrolases, PL = Polysaccharide Lysases, COH = Cohesins, DOC = Dockerins, GT= Glycosyl Transferases, CE= Carbohydrate Esterases, and CBM = Carbohydrate-binding Modules.

3.6.2. Protologger substrate utilisation predictions

Starch, followed by glucose and cellulose, were the most predicted carbon sources via Protologger analysis. *[Clostridium] clostridioforme* AGR2157 had the widest range of predicted substrates (glucose, arbutin, salicin, cellobiose, trehalose, and starch) and for many of the other strains only one or two substrates were predicted.

Table 3.3 Genomic predictions versus experimental findings in substrate utilisation assays

Strain Name	PC	PA	ST	XY	CL	RF	CLB	MB	SU	LA	ML	GL	GAL	AR	RH	XS	MN	DX	GLA	GLU
Acetitomaculum ruminis 139B	+/-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-
Blautia schinkii B	+/-	+/-	+	-	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-
Blautia wexlerae AGR2146	+	+/-	+	+	+	+	+	+	+	+	+	+	+	+	x	+/-	x	-	x	x
Dorea longicatena AGR2136	+	+/-	+/-	+	+/-	+	-	-	-	+	+	+	-	+/-	x	-	x	x	x	x
Dorea sp. AGR2135	-	x	-	+/-	-	+/-	x	x	x	x	+	+	+	-	-	x	-	x	x	-
[Clostridium] clostridioforme AGR2157	+/-	+/-	+/-	+/-	+/-	+	+	+	+/-	+	+	+/-	+/-	+/-	x	-	+/-	x	+/-	-
Enterocloster clostridioformis T90	+/-	+	+	+	+	-	-	-	+	+	+	+	+	+	-	+	+	-	+/-	+/-
Eubacterium ruminantium FB3002	-	-	-	-	x	-	+	+/-	+	-	x	+	x	x	x	-	x	+/-	-	x
Eubacterium ruminantium HUN269	+/-	x	-	-	-	x	+	x	x	x	+	+/-	+	+/-	x	+/-	x	+/-	-	x
Lachnobacterium bovis NK4B19	+/-	-	+	+/-	+	x	+	-	+	+	+	-	x	x	x	-	+/-	+	x	x
Lachnobacterium bovis AE2004	x	x	x	x	x	-	-	-	+/-	+/-	+	+/-	+/-	x	x	-	+/-	-	x	x
Lachnoclostridium aminophilum F	+/-	+/-	+/-	+	+/-	x	x	-	x	x	x	-	x	x	-	x	-	x	x	x
Lachnoclostridium aminophilum KH1P1	+	-	+	+	+/-	-	-	-	+/-	-	-	-	-	x	-	-	-	-	-	-
Lachnospira multipara D15d	-	-	-	x	-	-	+/-	-	+	x	-	+/-	-	x	x	-	-	-	x	-
Lachnospira multipara D32	+/-	+	+/-	+/-	+/-	-	+	x	+	x	x	+	x	-	x	x	x	-	x	-
Lachnospira multipara LB2003	-	x	+	-	x	-	+	-	+	-	-	+	-	+/-	-	+/-	+/-	-	+/-	+/-
Lachnospira multipara MC2003	+	+/-	-	x	x	-	+	-	+	x	-	+/-	-	-	-	x	-	-	-	-
Lachnospira pectinoschiza M83	+	+/-	x	x	-	-	+	-	+	-	-	+	-	x	x	-	-	-	-	-
Lachnospiraceae bacterium AC2012	-	+/-	+	+/-	+/-	-	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-	-	-	-	+/-	x	x
Lachnospiraceae bacterium AC2014	-	x	+/-	x	-	+	-	+	-	+	+/-	+/-	+/-	+/-	+/-	-	+/-	-	-	-
Lachnospiraceae bacterium AC2028	-	x	x	-	-	-	+/-	-	-	-	-	+/-	+/-	-	-	+/-	-	-	-	-
Lachnospiraceae bacterium AC2029	-	x	+/-	+/-	-	-	+/-	-	-	-	x	-	x	-	x	-	-	-	-	x
Lachnospiraceae bacterium AC2031	-	-	+/-	-	-	-	+	-	-	-	-	+	-	+/-	-	+/-	-	-	-	-
Lachnospiraceae bacterium C10	+/-	+/-	+	+/-	+/-	+	+	+	+/-	+/-	+/-	+/-	+/-	x	x	-	+/-	+/-	x	-
Lachnospiraceae bacterium C6A11	-	x	x	x	-	-	+	-	+	-	+	+/-	+/-	-	-	-	x	-	-	-
Lachnospiraceae bacterium C7	+/-	+/-	+	-	+/-	+/-	-	+	-	+/-	-	-	-	x	x	x	-	+/-	x	x
Lachnospiraceae bacterium FE2018	x	x	x	-	-	-	-	-	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
Lachnospiraceae bacterium KH1P17	+	+/-	+/-	+/-	+/-	+	+	+	+	+	+	+/-	+	+/-	-	+/-	x	x	x	x
Lachnospiraceae bacterium KH1T2	+	-	+	-	-	+	+	+	+	+	+/-	+/-	+	+/-	-	+	+	+/-	x	x
Lachnospiraceae bacterium MA2020	x	x	x	x	x	+	+	+	+	+	+	+/-	+/-	+/-	-	+	+/-	-	x	x
Lachnospiraceae bacterium MC2017	-	x	-	x	-	+	+/-	+	-	+	+/-	+/-	+/-	-	+/-	+/-	-	-	-	-
Lachnospiraceae bacterium NC2004	x	+/-	-	+/-	-	-	+/-	-	x	-	-	-	-	-	-	-	x	-	-	-
Lachnospiraceae bacterium NC2008	x	x	x	x	x	-	+	-	+/-	+	-	+	x	-	x	-	-	-	x	x
Lachnospiraceae bacterium NK4A136	+/-	+/-	+/-	+/-	+/-	-	+	-	+	-	-	+	-	-	+/-	-	-	-	-	-
Lachnospiraceae bacterium NK4A144	x	x	x	x	x	+/-	+	+	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-	-	x	x
Lachnospiraceae bacterium NK4A179	-	x	-	-	-	x	+/-	x	x	x	-	x	x	x	x	x	x	x	x	x
Lachnospiraceae bacterium P6A3	x	-	-	x	-	-	+	+	x	x	+	+/-	-	-	x	+/-	+/-	-	-	x
Lachnospiraceae bacterium XPB1003	-	-	-	-	x	x	+/-	x	x	x	x	+/-	x	x	x	+/-	x	x	-	x
Lachnospiraceae bacterium YSB2008	-	-	x	x	x	x	-	x	+/-	x	x	+/-	+/-	+/-	x	x	x	x	x	x
Lachnospiraceae bacterium YSD2013	+	+/-	+/-	+/-	+/-	x	+/-	-	-	-	-	-	-	-	x	-	-	x	x	-
Lacrimispora aerotolerans X8A62	+	+	-	-	+/-	+	+	+	+	+	+	+/-	+/-	+	-	+	+	+	+	-
Oribacterium sp. FC2011	+	+	+	+/-	+	+	+	+/-	+	+/-	-	+/-	+/-	-	x	x	x	-	+/-	+/-
Oribacterium sp. P6A1	x	x	x	+/-	-	+	+	+/-	+	+	+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
Oribacterium sp. WCC10	+	+	+/-	+	+	+	+/-	-	+	+/-	+/-	+/-	+/-	+/-	-	+/-	-	-	+/-	-
[Ruminococcus] gnavus AGR2154	-	-	-	-	-	x	x	-	x	+	+	+	+	+/-	+/-	+	x	-	x	-

SUBSTRATE ABBREVIATIONS			
Substrate	**Abbreviation**	**Substrate**	**Abbreviation**
Pectin	**PC**	Maltose	**ML**
Polygalacturonic Acid	**PA**	Glucose	**GL**
Starch	**ST**	Galactose	**GAL**
Xylan	**XY**	Arabinose	**AR**
Cellulose	**CL**	Rhamnose	**RH**
Raffinose	**RF**	Xylose	**XS**
Cellobiose	**CLB**	Mannose	**MN**
Melibiose	**MB**	Dextrin	**DX**
Sucrose	**SU**	D-Galacturonic Acid	**GLA**
Lactose	**LA**	D-Glucuronic Acid	**GLU**

Note: Table detailing the growth of the 45 *Lachnospiraceae* strains on all 20 substrates tested and whether the substrate utilisation matched the Protologger prediction. Total SCFA concentration was used as a measure of growth on the insoluble sugars (cellulose, pectin, polygalacturonic acid, starch, and xylan) and OD_{600} was used as a measure of growth on the soluble sugars. In the table above the following key is used to represent the quality of the growth of each of the strains on the various substrates: **'X'** = No Growth ($0D_{600}$< 0/ [SCFA] = 0) ,' **-**' = Poor Growth ($0D_{600}$ <0.1/ 0 mM < [SCFA]< 2.5 mM), ' **+/-**' = Fair Growth (OD_{600} > 0.1/ 2.5 mM < [SCFA]< 5 mM) ,' **+** ' Good Growth (OD_{600} > 0.5/ [SCFA] >5 mM). The cell colour corresponds to how the Protologger software was able to predict the phenotype of the strains: **matches genomic prediction from Protologger,** **not assessed by Protologger genomic prediction**, and **does not match Protologger genomic prediction.**

3.6.3. SCFA production prediction by Protologger

The Protologger predictions for SCFA production did not match very closely the observed end products determined from analysis of culture supernatants. Acetate and propionate were predicted as end products for all 45 strains due to detection of phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) as marker genes for acetate (from acetyl-CoA) and propionate (propanoyl-CoA) production. Butyrate production was only predicted for two strains *Lachnoclostridium aminophilum* F and *Lachnospiraceae* bacterium NC2008 due to their possession of butyryl CoA: acetate CoA transferase (EC. 2.8.3.8) gene, allowing butyrate production from butyryl-CoA.

Table 3.4. Table summarising SCFA production as predicted by Protologger.

Species name	SCFA production	Enzymes Markers for Pathways
Acetitomaculum ruminis 139B	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Blautia schinkii B	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Blautia wexlerae AGR2146	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Dorea longicatena AGR2136	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Dorea sp. AGR2135	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
[Clostridium] clostridioforme AGR2157	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Enterocloster clostridioformis T90	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Eubacterium ruminantium FB3002	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Eubacterium ruminantium HUN269	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnobacterium bovis NK4B19	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnobacterium bovis AE2004	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnoclostridium aminophilum F	Acetate/Propionate/Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)/ (EC:2.8.3.8)
Lachnoclostridium aminophilum KH1P1	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospira multipara D15d	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospira multipara D32	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospira multipara LB2003	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospira multipara MC2003	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospira pectinoschiza M83	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium AC2012	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium AC2014	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium AC2028	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium AC2029	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium AC2031	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium C10	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium C6A11	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium C7	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium FE2018	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium KH1P17	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium KH1T2	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium MA2020	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium MC2017	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium NC2004	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium NC2008	Acetate/Propionate/Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)/ (EC:2.8.3.8)
Lachnospiraceae bacterium NK4A136	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium NK4A144	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium NK4A179	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium P6A3	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium XPB1003	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium YSB2008	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lachnospiraceae bacterium YSD2013	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Lacrimispora aerotolerans X8A62	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Oribacterium sp. FC2011	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Oribacterium sp. P6A1	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
Oribacterium sp. WCC10	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)
[Ruminococcus] gnavus AGR2154	Acetate/ Propionate/ Butyrate/ Minor VFAs	(EC:2.3.1.8, 2.7.2.1)/(EC:2.3.1.8,2.7.2.1)

Did not match Protologger prediction/ Matched Protologger prediction

3.6.4 Genomic prediction of H_2 production

Protologger did not produce any data on H_2 as an end-product, therefore the genomes of the 45 strains were manually searched on the IMG database, for H_2 producing hydrogenases: [FeFe] hydrogenases and [NiFe] hydrogenases, hydrogenase maturation factors and expression proteins.

All the strains that produced H_2 as an end-product on any of the substrates tested had at least one gene coding for a [Fe Fe] hydrogenase/ [Ni Fe] hydrogenase (Table 3.5). ([FeFe] hydrogenase, group B1/B3, ferredoxin hydrogenase large subunit, Ni, Fe-hydrogenase III large subunit) and at least one maturation factor or an expression formation protein coding gene (*HypC*, *HydF, HypF, HypE, HypD, HydE*, and *HydG*).

There were six strains that did not produce H_2 despite having hydrogenase-coding genes and at least one maturation factor/ expression protein coding genes (*Blautia schinkii* B, *Blautia wexlerae* AGR2146, *Lachnospira multipara* D15d *Lachnospiraceae* bacterium FE2018, *Lachnospiraceae* bacterium MA2020, and *Lachnospiraceae* bacterium NC2004 (Table 3.5)).

Table 3.5. Summary of predicted H_2 production from genomic data taken from IMG database

Species	Total Hydrogen Production	Hydrogenase Genes
Acetitomaculum ruminis 139B	x	X (has hydrogenase maturation factors /expression proteins)
Blautia schinkii B	x	[FeFe] Hydrogenase
Blautia wexlerae AGR2146	x	[FeFe] Hydrogenase
Dorea longicatena AGR2136	-	[FeFe] Hydrogenase
Dorea sp. AGR2135	x	X (has hydrogenase maturation factors /expression proteins)
[Clostridium] clostridioforme AGR2157	+	[FeFe] Hydrogenase
Enterocloster clostridioformis T90	+/-	[FeFe] Hydrogenase
Eubacterium ruminantium FB3002	x	[FeFe] Hydrogenase
Eubacterium ruminantium HUN269	x	[FeFe] Hydrogenase
Lachnobacterium bovis NK4B19	-	[FeFe] Hydrogenase
Lachnobacterium bovis AE2004	-	[FeFe] Hydrogenase
Lachnoclostridium aminophilum F	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnoclostridium aminophilum KH1P1	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospira multipara D15d	x	[NiFe] Hydrogenase
Lachnospira multipara D32	-	[NiFe] Hydrogenase
Lachnospira multipara LB2003	+/-	[NiFe] Hydrogenase
Lachnospira multipara MC2003	-	[NiFe] Hydrogenase
Lachnospira pectinoschiza M83	x	[NiFe] Hydrogenase
Lachnospiraceae bacterium AC2012	+/-	[FeFe] Hydrogenase
Lachnospiraceae bacterium AC2014	+/-	[FeFe] Hydrogenase
Lachnospiraceae bacterium AC2028	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospiraceae bacterium AC2029	-	[FeFe] Hydrogenase
Lachnospiraceae bacterium AC2031	-	[FeFe] Hydrogenase
Lachnospiraceae bacterium C10	+	[FeFe] Hydrogenase
Lachnospiraceae bacterium C6A11	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospiraceae bacterium C7	+	[FeFe] Hydrogenase
Lachnospiraceae bacterium FE2018	x	[FeFe] Hydrogenase
Lachnospiraceae bacterium KH1P17	+	[FeFe] Hydrogenase
Lachnospiraceae bacterium KH1T2	+	[FeFe] Hydrogenase
Lachnospiraceae bacterium MA2020	x	[FeFe] Hydrogenase
Lachnospiraceae bacterium MC2017	+	[FeFe] Hydrogenase
Lachnospiraceae bacterium NC2004	x	[FeFe] Hydrogenase
Lachnospiraceae bacterium NC2008	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospiraceae bacterium NK4A136	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospiraceae bacterium NK4A144	+/-	[FeFe] Hydrogenase
Lachnospiraceae bacterium NK4A179	-	[FeFe] Hydrogenase
Lachnospiraceae bacterium P6A3	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Lachnospiraceae bacterium XPB1003	-	[FeFe] Hydrogenase
Lachnospiraceae bacterium YSB2008	x	X
Lachnospiraceae bacterium YSD2013	x	X
Lacrimispora aerotolerans X8A62	+	[FeFe] Hydrogenase
Oribacterium sp. FC2011	+	[FeFe] Hydrogenase
Oribacterium sp. P6A1	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
Oribacterium sp. WCC10	x	[FeFe] Hydrogenase but no maturation factors /expression proteins
[Ruminococcus] gnavus AGR2154	-	[FeFe] Hydrogenase

Note: Did Not Match Genomic Prediction/ Matched Genomic Prediction. (**'x'** = $[H_2]$ <0 / '- '= $[H_2]$> 0.005 mM/ **'+/- '**= $[H_2]$ > 0.1 mM / **'+'** = $[H_2]$> 0.3 mM)

Chapter 4. Discussion

As outlined in the review of the literature, studies using culture-independent techniques driven by high throughput sequencing technologies have far exceeded culture- based exploration of rumen microbial communities. However, culture-based studies involving pure and defined multi-organism assemblages are still required for accurate characterisation of bacterial phenotypes and for understanding the complex interactions that enable rumen microbial communities to function. The aim of this study was to phenotypically characterise *Lachnospiraceae* strains from the Hungate 1000 project to fill knowledge gaps around the function of this important group of rumen bacteria. The broader aim was to assess the utility of Protologger to predict characteristics of these strains compared to their phenotypic characteristics derived via culture-based assays conducted in the laboratory.

4.1. Fibre degrading capabilities of the *Lachnospiraceae* strains

The results of this study outline differences and similarities amongst individual members of the *Lachnospiraceae* family while also describing the fibre degrading and metabolic capabilities of individual strains which can be extrapolated back to their roles in the rumen environment. Plant polysaccharide components that enter the rumen environment are either plant storage polysaccharides or the structural components of plant cell walls. Structural components include celluloses, hemicelluloses, and pectin; whereas starch is a storage polysaccharide (Hobson & Stewart, 1988).

The insoluble substrate growth assay demonstrated that the 45 *Lachnospiraceae* strains are generally poor degraders of insoluble fibre. By evaluating the extent to which the strains were able to ferment the five insoluble substrates it is clear many of them are unable to fully ferment the provided substrates. The maximum concentration of total SCFAs produced by *Blautia wexlerae* AGR2146 on glucose was 26.06 mM (Appendix 1, Table A1) compared to 5.82 mM when grown on cellulose (Figure 3.3), therefore this strain is unlikely fermenting the cellulose completely. Cellulose, is made up of glucose subunits, so the potential total SCFAs produced from complete fermentation of the available substrate should fundamentally be similar (Lee & Rittmann, 2009). However, despite being tested at similar concentrations there is a clear preference for the soluble substrate glucose over cellulose. The average total SCFAs produced on any of the five insoluble substrates was between 3.06

mM (pectin) and 2.14 mM (cellulose), with most strains unable to produce more than 5 mM of total SCFAs. This supports the observation that many of the 45 *Lachnospiraceae* strains were poor complex fibre degraders.

In terms of substrate preference, overall, the 45 *Lachnospiraceae* strains preferred pectin, polygalacturonic acid and starch, rather than cellulose and xylan. Many of the strains preferred other insoluble sugars in comparison to microcrystalline cellulose and the results of the soluble substrate screen (Figure 3.2), suggest that the strains metabolise subunits sugars of cellulose rather than the intact polysaccharide. Protologger predicted that 15 of the 45 strains could use cellulose, but of these predictions, four strains were unable to utilise cellulose. There are 12 different GH families which contain cellulases: GH5, GH6, GH7, GH8, GH9, GH12, GH26, GH44, GH45, GH48, GH61, and GH74 (Sandgren et al., 2005). Of these GH families, only GH5, GH8, GH9 and GH26 were present in the genomes of the studied strains (Appendix 3, Figure A3.1). *Eubacterium ruminantium* (strains HUN269 and FB3002) had the greatest number of cellulolytic GH families encoded in their genomes, however, only *E. ruminantium* HUN269 was able to metabolise cellulose and produce SCFAs whereas FB3002 was unable to produce any SCFAs (Figure 3.3). This is an example of how an organism's possession of genes required to break down a certain substrate does not necessarily mean they can utilise it; phenotypic characterisation still needs to be carried out to confirm any genome-based prediction.

Similarly, xylan from oat spelt, was used poorly in comparison to starch, pectin and polygalacturonic acid. Many of the strains which could breakdown xylan (Figure 3.7D), were the same strains that were able to break down the hemicellulose associated sugars in the soluble substrate screen (arabinose, xylose, mannose, and D-glucuronic acid); *Entercloster clostridioformis* T90, *[Clostridium] clostridioforme* AGR2157 *and Blautia wexlerae* AGR2146 (Miyazaki et al., 1997) (Figure 3.2). The other strains that were unable to break down the monomer units of xylan may preferentially break down xylan as a whole polysaccharide, for example, *Dorea* sp. AGR2135, which did not show a preference for the xylan associated monomers in the soluble substrate screen was able to ferment some of the xylan to SCFAs in the insoluble substrate assay (Figure 3.2 and Figure 3.3). There was no information provided by Protologger for xylan utilisation. However, the GH families commonly associated with xylan degradation including GH10 and GH11 (endo -β-1,4-xylanses), GH31 (xylosidase),

GH43, GH51 and GH62 (α-arabinosidase) were found in all the strains apart from the *Dorea* species (Appendix 3, Figure A3.1) (Østby & Várnai, 2023). *Dorea* sp. AGR2153 interestingly, did not have any of these GHs in its genome despite being able to degrade xylan to some extent. However, *Dorea* sp. AGR2153 has genes for CE4 family enzymes (Appendix 3, Figure A3.3), which includes acetyl xylan esterases and this could explain how this organism is able to utilise xylan. These phenotypic results and genome together can be used to untangle the xylan degradation capabilities of these strains.

Starch is a storage polysaccharide and is mobilised when required by the plant, and this feature makes starch a more easily fermentable substrate (Hobson & Stewart, 1988). In the soluble substrate screen, many of the strains were able to break down glucose and maltose which are breakdown products of starch, (Figure 3.2). Dextrin, however, a more intact form of starch was utilised to a lesser extent. Studied strains which showed a preference for dextrin, also demonstrated a greater preference for starch in the insoluble substrate screen, e.g., *Lachnospiraceae* bacterium C7, *Lachnospiraceae* bacterium AC2012 and *Lachnobacterium bovis* (Figure 3.2 and Figure 3.3). This illustrates the amylolytic capabilities of these strains and experimentally validates the genomic predictions made by Protologger, where starch was predicted to be utilised by all the strains (Table 3.3). All the strains encoded GH13 α-amylase enzymes that can degrade starch (Appendix 3, Figure A3.1) (Svensson, 1994). Additionally, these GH13 CAZymes work together with CBM families known as starch binding domains (CBM34 and CBM48) and these were the most common CBM families found in the 45 strains (Appendix 3, Figure A3.3). The CBM34 and CBM48 families were found in all the strains apart from *Eubacterium ruminantium* FB2003 and *Oribacterium* sp. FC2011 (Appendix 3, Fig A3.3), while *Acetitomaculum ruminis* 139B only had a CBM48 coded for in its genome (Appendix 3, Fig A3.3). Many of the genomic predictions for starch utilisation were experimentally validated in the insoluble substrate assay.

Pectin is found in the middle lamellae between plant cell walls and allows for adhesion of cells together (Harholt et al., 2010). There were many *Lachnospiraceae* strains that were able to utilise pectin as a substrate (Figure 3.7B). The strains belonging to the *Lachnospira* genera were all able to break down pectin and produce methanol and acetate as the primary end products. The pectinolytic abilities of *Lachnospira multipara* and *Lachnospira*

pectinoschiza, have been described previously and the strains tested in this study align with this research (Cornick et al., 1994; Kelly et al., 2019). Other strains producing methanol as a product of pectin fermentation were *Oribacterium* species FC2011 and P6A1, *Eubacterium ruminantium* FB2003, and unclassified *Lachnospiraceae* bacterium strains AC2029, MC2017, P6A3, YSB2008, NC2004, C6A11 and XPB1003. The inability of all these strains to degrade rhamnose or galacturonic acid to a substantial degree suggests that they are using the whole pectin moiety or oligosaccharide breakdown products of pectin rather than breaking it down into its monomer units. This is also reaffirmed when looking at the results for growth on polygalacturonic acid, as these strains can metabolise this more complex form of pectin rather than the smaller subunits. Polygalacturonic acid forms the oligosaccharide backbone of pectin and, dependent on the type of pectin, these may have various side chains including other hexose and pentose sugars. The ability of these *Lachnospiraceae* strains to break down polygalacturonic acid supports the idea that they are attacking the whole pectin polysaccharide rather than its side chains. Protologger did not provide any information about pectin degradation or the ability of these organisms to produce methanol. By manually searching the genomes of these strains, several CAZyme families that are associated with pectinolytic activity were found (Kelly et al., 2019). For example, PLs belonging to families PL1 and PL9, and CEs belonging to CE8 and CE12 were found in the genomes of all the *Lachnospira* strains. Pectin is often esterified with methyl groups and CE8 contains the PME enzymes which cleave the methyl groups off the pectin chains, to produce methanol as a product (Goldberg et al., 1996). The PL families were less common amongst the remaining pectin degraders, with only *Eubacterium ruminantium* FB2003 having genes coding for enzymes belonging to PL9. PLs are important for attacking the glycosidic bonds of the polygalacturonic acid via an eliminase mechanism (Linhardt et al., 1986). This CAZyme information helps to reveal the possible mechanisms by which these strains are utilising pectin.

Feed material enters the rumen after processing via repeated mastication and rumination, however, at this stage, much of this material is still in a complex form (Hobson & Stewart, 1988). In order for direct transport into microbial cells these materials need to be broken down into smaller low molecular weight substances; for example, the substrates tested in the soluble substrate screen assay (Solden et al., 2018).

There was a large amount of variation in the ability of the 45 *Lachnospiraceae* strains to break down the 15 soluble substrates. Cellobiose was the most commonly utilised substrate, which is expected from bacteria isolated from the rumen environment, as cellobiose is released from the enzymatic hydrolysis of cellulose by cellulases. This may indicate that known cellulose degraders such as *Ruminococcus* and *Fibrobacter* species, break down cellulose and release free cellobiose units into the rumen milieu, which can be used by these cellobiose utilising *Lachnospiraceae* strains. The strains produced a larger total SCFA amount (mM) on glucose in comparison to the five insoluble sugars, which reiterates that these strains show a preference for simpler substrates in comparison to complex substrates. Furthermore, there appeared to be a preference for utilisation of hexose sugars rather than the pentose sugars tested (arabinose, xylose and rhamnose) which, may indicate that many of these strains feed off the di- and mono- saccharide products of cellulose degradation rather than hemicellulose degradation. However, it should be noted that only two hemicellulose-related pentose sugars were tested in this study: xylose and arabinose. This is further supported by the overall lack of growth on D-glucuronic acid, a common side chain of hemicelluloses like glucuronoxylan (York & O'Neill, 2008).

Strains that exhibited the best growth on the widest range of substrates in the soluble carbon source utilisation assay are strains that have larger genomes and a greater repertoire of CAZymes. For example, *Blautia schinkii* B, boasts the largest genome size of all strains (6.6Mbp) encoding 221 total CAZymes. This is followed by *Lacrimispora aerotolerans* XBA62 (4.7Mbp) with 202 CAZymes, *Lachnospiraceae* bacterium KH1T2 (3.8Mbp) with 194 CAZymes (Figure 3.9, Table 3.1). In contrast, the strains that had the least number of total CAZymes grew poorly or not at all on most of the 15 soluble substrates. For example, the two *Lachnoclostridium aminophilum* strains F (3.1 Mbp) and KH1P1 (3.2 Mbp) with only 51 and 56 CAZymes coded for in their genomes respectively, grew poorly if at all on the 15 soluble substrates screened. These organisms are known to be hyper ammonia producing peptide and amino acid degraders, and it is likely that they were metabolising the protein components of the medium that were used for this assay, i.e. casamino acids and peptone, (Table 2.7) (Whitehead & Cotta, 2004). Some strains were able to utilise a few similarly structured substrates with a limited repertoire of CAZymes, for example, *Acetitomaculum ruminis* 139B with a total of 51 CAZymes was only able to utilise two substrates: sucrose and

glucose. *Dorea* sp. AGR2135 with a total of 58 CAZymes was only able to utilise three types of substrates: glucose, maltose, and galactose.

4.2. End products of fermentation by *Lachnospiraceae* strains

The fermentation end products of growth of each of the strains on the 20 substrates illustrate the different metabolic pathways used, but also provides essential information about the complex cross feeding that occurs in the rumen. We can elucidate the potential commensal relationships *Lachnospiraceae* form with other rumen microorganisms from this data.

The primary end products of microbial fermentation are VFAs, and these compounds are the major source of nutrition for ruminant mammals. The results of the GLC measurements of each of the strains grown on glucose or cellobiose (melibiose for *Lachnospiraceae* bacterium C7) show that all the strains can produce acetate, propionate, and butyrate as an end product. The greatest acetate producers were *Acetitomaculum ruminis* 139B and the two *Blautia* (B and AGR2146) strains. *Acetitomaculum ruminis* 139B is an acetogenic bacterium that is able to utilise H_2 and CO_2 to produce acetate, this reductive acetogenesis represents an alternative electron sink to methanogenesis (Greening & Leedle, 1989). Key enzymes responsible for reductive acetogenesis include carbon monoxide dehydrogenases, acetyl-CoA synthases, and pyruvate ferredoxin oxidoreductases, these genes are present in 24 of the 45 strains (Appendix 3, Table A3.2), indicating these strains could have the potential to carry out this type of metabolism via the Wood–Ljungdahl pathway (Ragsdale, 2008). Acetate formation was predicted by Protologger due to the presence of genes encoding phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1). There was no information however, on other modes of acetate production e.g., reductive acetogenesis. Acetate accounts for over 50% of the energy, provided by VFA metabolism by the host ruminant, so formation of this end product has important implications for the productivity of ruminant mammals (Aiello et al., 1989).

Propionate was also produced by all 45 strains, however at much lower concentrations than acetate. Propionate is an important fermentation product in the rumen system due to its role as a precursor for gluconeogenesis and has been associated with increased weight gain in Holstein heifers (Ren et al., 2019; Young, 1977). Glucose in the rumen is rapidly fermented

by microorganisms, therefore, ruminants rely on gluconeogenesis from propionate in the liver for their glucose requirements. Although all the strains were able to form propionate, it was produced at low concentrations, with the best producer, *Lacrimispora aerotolerans* X8A62 producing (2.65 mM, Figure 3.4A). Protologger correctly predicted that all the strains were able to produce propionate by the presence of genes encoding phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1). Protologger did not provide any information on other alternative pathways of propionate production (propanediol and acrylate pathways) (Reichardt et al., 2014).

Butyrate was produced by all 45 strains. Butyrate is known for its beneficial roles in supplying energy to gut epithelial cells maintaining the integrity of the gut epithelial wall of mammals, by increasing the assembly of tight junction proteins via the activation of AMP activated protein kinase (Zhu et al., 2021). Furthermore, microbial butyrate can act as an immunoregulatory agent by promoting the proliferation of regulatory T cells, mediating inflammatory pathways and regulating apoptosis in GIT epithelial cells (Plöger et al., 2012). It is apparent that butyrate produced by *Lachnospiraceae* in mammalian environments are key in maintaining a healthy GIT. The greatest producers of butyrate were *Lachnospiraceae* bacterium MA2020 (7.35 mM), *Lachnospiraceae* bacterium NK4A136 (6.97mM), *Lachnospiraceae* bacterium AC2014 (6.87 mM), *Lachnospiraceae* bacterium NC2008 (6.52 mM, Figure 3.4A). The results show that the *Lachnospiraceae* family are likely to be important butyrate producers in the rumen. Butyrate production was predicted for two strains of *Lachnoclostridium aminophilum* and *Lachnospiraceae* bacterium *NC2008.* The genome annotation only took into consideration butyrate formation via butyryl CoA transferase (EC 2.8.3.8). However, the butyrate kinase (EC 2.7.2.7) pathway can also result in the formation of butyrate, and this was overlooked in the Protologger genome annotation. Here, the pitfalls of relying solely on genomic methods of understanding functional phenotypes of bacteria are revealed.

The minor SCFAs were produced in much smaller quantities. The *iso*-acids (*iso*-valerate and *iso*-butyrate) and valerate were produced in the greatest quantities. These fermentation end products are produced from the degradation of amino acids and indicate that the strains are likely relying on proteolytic functions in addition to their fibrolytic capabilities. For example, *Lachnospiraceae* bacterium C7 struggled to grow on many of the soluble

substrates and produced the greatest concentration of minor SCFAs. This strain is likely breaking down the amino acids provided in the media specifically, leucine and valine via Stickland metabolism (Nisman, 1954), due to its limitations in degrading the carbohydrates provided. Additionally, *Oribacterium* sp. WCC10, *Lachnoclostridium aminophilum* strains KH1P1 and F and *Lachnospiraceae* bacterium AC2028 were in the top five producers of the *iso*-acids and valerate on all five of the insoluble sugars in addition to the major SCFAs. It may be that these strains are able to partially degrade the fibre, and once all the fermentable carbohydrates are depleted, they could be switching to proteolytic fermentation. This may explain the ability of these strains to produce the largest amounts of total SCFAs on all the insoluble substrates despite not having the greatest repertoire of CAZymes. Protologger did not produce any data on the ability of the 45 strains to produce these minor VFA end products.

Formate and non-volatile SCFAs were produced by many of the *Lachnospiraceae* strains. These products are often intermediary precursors for the formation of the major volatile fatty acids and are important interspecies electron carriers. Succinate was the least produced non-volatile fatty acid being produced by *Blautia wexlerae* AGR2146, *Dorea longicatena* AGR2136, *Dorea* sp. AGR2135, *[Clostridium] clostridioforme* AGR2157 and *Ruminococcus gnavus* AGR2154. It is likely that these succinate producers are either still in the process of converting succinate into propionate or are unable to use succinate as a precursor for propionate production, in the latter case it is released extracellularly to the rumen environment, where other succinate-using microorganisms would utilise it. Formate and lactate were produced at greater concentrations by the strains, and only three strains did not produce any (*Acetitomaculum ruminis* 139B, *Lachnospiraceae* bacterium AC2012 and *Lachnospiraceae* bacterium AC2029). Lactate, much like succinate is utilised to produce propionate and typically does not accumulate in the rumen environment. Formate is an important fermentation end product as it acts an interspecies electron transporter and can be used by certain hydrogenotrophic methanogens as an electron donor for methanogenesis. This means that bacteria such as *Lachnobacterium bovis* NK4B19*,* *Lachnospiraceae* bacterium MA2020, *Lachnospiraceae* bacterium NK4A136, and *Lachnospiraceae* bacterium NC2008 could form commensal relationships with hydrogenotrophic methanogens. Again, Protologger did not provide any information on the

production of these non-volatile SCFAs potentially because they act as intermediates and not end products of fermentation.

Short chain alcohols can be produced as a result of fibre fermentation. Many of the strains produced ethanol, the greatest alcohol producers being the *Oribacterium* sp. P6A1 (30 mM) and WCC10 (27.86 mM), (Figure 3.6). Interestingly, *Oribacterium* sp. FC2011 did not produce any ethanol, however this was the only *Oribacterium* strain that was able to produce H_2 and produced greater amounts of formate and lactate in comparison to the other strains. This demonstrates that Oribacterium sp. FC2011 may use H_2, formate and lactate as alternative electron sinks to dispose of electrons rather than producing ethanol. Ethanol is another example of an interspecies electron carrier and can act as an electron donor for methanogenesis specifically by *Methanobrevibacter boviskoreani* (Li et al., 2023).

As mentioned previously, some *Lachnospiraceae* strains were able to produce methanol from the demethoxylation of pectin via PME enzymes (Kelly et al., 2019). Methanol also acts as a substrate for methylotrophic methanogens, so it is possible that these pectinolytic *Lachnospiraceae* form symbiotic relationships with methylotrophic methanogens.

The H_2 released by fibre degrading bacteria is a major component of metabolic H_2 flows in the rumen fermentation system. H_2 production by rumen bacteria is facilitated by hydrogenases and requires the presence of a [FeFe] hydrogenase or a bidirectional [NiFe] hydrogenase. Not all the strains were able to produce H_2 when grown on 20 soluble and insoluble substrates. The genomes of the strains were searched for the presence of [FeFe] hydrogenases and [NiFe] hydrogenases, and 21 organisms possess genes for these hydrogenases (Table 3.6). Interestingly, there were strains that did not produce H_2 as a fermentation product despite having these genes in their genomes (Table 3.6). Some strains had genes for the hydrogenase subunits without the maturation factors and vice versa and these strains were unable to produce H_2 (Table 3.6) . In order for the hydrogenase proteins to be functional, maturation factors are required (Jacobi et al., 1992). It is also possible that the experimental conditions were not conducive to the production of H_2 or the media and substrates that were tested did not allow for these strains to produce H_2 as an end-product. The production of H_2 by these fibrolytic bacteria highlights the potential for commensal relationships between *Lachnospiraceae* and hydrogenotrophic members of the rumen microbial consortia.

4.3. Evaluation of the predictions made by Protologger

In this study we evaluated the ability of Protologger as a genome annotation tool to predict the metabolic functions of microbial strains. Much like with any other culture-independent functional analyses, this tool was unable to provide quantitative data on the metabolic capabilities of the strains, for example, how well they can degrade preferred substrates. Some of the *Lachnospiraceae* strains were predicted to utilise cellulose (Figure 3.4) however, the results of the insoluble substrate screen showed that although the strains had some ability to degrade cellulose, they were generally poor cellulose degraders (Figure 3.3). Without phenotypic data from culture dependent studies there is no way of discerning to what extent these organisms can produce metabolites or degrade various types of fibre.

There was a lot of missing information in the Protologger functional predictions, one example being that all the strains were able to produce butyrate, and this was overlooked as a predicted function for almost all the strains. Furthermore, when manually searching the strains using Meta Cyc rather than the Kyoto Encyclopedia of Genes and Genomics database (which is used by Protologger) it is apparent that some of these strains can use multiple pathways to produce butyrate as a metabolite (Appendix 3, Figure A3.5) (Kanehisa, 2019; Kanehisa et al., 2023; Kanehisa & Goto, 2000). Due to the non-standardised nomenclature of metabolic compounds, reactions, and pathways it is difficult to understand fully what these organisms could be capable of without manually searching each of the databases that are available (Altman et al., 2013). The ability for Protologger to provide a more comprehensive readout of the functional capabilities of the strains may be limited by the lack of integration of the various metabolic pathways and all their alternative names (Caspi et al., 2013).

Although there are inconsistencies from the Protologger predictions and the phenotypic data produced in the lab, there are benefits to using a tool that brings together functional data, ecological data, and taxonomic placement data from various databases. Protologger was able to provide valuable information on whether the strains tested were novel strains by comparing average nucleotide identities and percentage of conserved proteins values to other genomes in the GTDB (Appendix 2, Table A2) and by combining the three measures of taxonomic placement from two databases (GTDB and SILVA (Quast et al., 2012))

Ultimately, culture-independent approaches to understanding the metabolic roles of microorganisms does not provide the full story, there is a need to continue cultivation-based studies to appreciate the true metabolic potential of microbial species. There is a potential to use genomic tools such as Protologger to steer culturing efforts, by mining the genomes of strains to identify desirable traits.

4.4. Limitations of this study

There are fundamental differences in the way these *Lachnospiraceae* strains behave as pure cultures in the laboratory and their functional characteristics in *vivo* so this should be taken into consideration when interpreting the results of this study. The rumen is a complex environment where the metabolic functions of many species are intertwined within the rumen ecosystem. Therefore, the results seen in these pure culture experiments may not reflect the true physiology of these strains in the rumen. These results act as a guide to understand the capabilities of the 45 *Lachnospiraceae* strains to lead further research.

Protologger is an example of just one tool for genome annotation and metabolic prediction and may not be the best tool to metabolically characterise strains. It would have been interesting to compare different types of automated genomic prediction tools to provide a better picture of how the genome can be utilised for functional predictions, but this is outside of the scope for this present study.

Chapter 5. Conclusions and Future Directions

5.1. Conclusions

The rumen microbial community is a complex system of interdependent metabolic interactions. The results of this study have provided information to better describe the roles the *Lachnospiraceae* family has in the rumen. *Lachnospiraceae* are one of the most abundant microbial families in the rumen and previous knowledge has suggested a role in fibre degradation (Matthews et al., 2019). This study provides *Lachnospiraceae* bacterial strains with functional data adding to the knowledge of *Lachnospiraceae* functions.

The phenotypic characterisation of the 45 *Lachnospiraceae* strains in this study showed they have a diverse range of carbon source utilisation preferences. Soluble substrates were more readily fermented and there was a preference for cellulose and starch subunits, rather than xylan and pectin subunits. Additionally, pectin, polygalacturonic acid, and starch were the most fermented insoluble substrates, highlighting the pectinolytic and amylolytic capabilities of these strains. However, they were generally poor degraders of the insoluble fibres.

End product analysis illustrated the roles *Lachnospiraceae* strains play in formation of major SCFAs which are important for host nutrition and health. Additionally, the formation of non-volatile intermediary SCFAs, short chain alcohols, and H_2 , highlight the importance of the *Lachnospiraceae* family as producers of intermediate products with potential roles as interspecies electron carriers. These end products help propel rumen fermentation forward and provide substrates for organisms that occupy the lower trophic levels in the rumen, including the methanogenic archaea.

The comparison between the experimentally validated phenotypic traits and the genomic evaluation of the functional capabilities of these strains illustrates the gaps that remain in bacterial strain characterisation. This study highlights and emphasises the importance of continuing culture-dependent work on cultured strains of key species from the rumen environment and indeed other mammalian GIT environments. Although genomic approaches cover potential functional capabilities of bacterial strains, they can only act as a guide for the phenotypic validation of functional traits. Here in this study, it was demonstrated annotation tools such as Protologger provide a very basic outline of the

potential capabilities of bacterial strains and do not offer the necessary resolution to delineate novel isolates or strains to a species level. In conclusion, genomic data can be used to steer culturing efforts towards isolation and characterisation of more of the uncultured majority of rumen bacteria, and this will undoubtedly help in understanding the forage degradative capabilities of the rumen microbial consortia and their metabolic roles in rumen fermentation. Gaining such knowledge will potentially allow manipulation of the rumen microbiota to yield new animal phenotypes that are more productive and less environmentally damaging. Microbial culture collections, such as the Hungate1000, will be vital for understanding these processes and will act as repositories of agro-ecosystem biodiversity (Díaz-Rodríguez et al., 2021), from which new opportunities for research on functional characterisation of rumen microbes will emerge.

5.2. Future directions

Reducing enteric CH_4 production and increasing production from ruminant animals are important goals of the meat and dairy industries (Arora, 2019). Therefore, there is a need to discover novel biotechnological strategies to reduce enteric CH_4 production whilst maintaining animal productivity. The results of this study show that the *Lachnospiraceae* strains are likely to form commensal relationships with other microbial species in the rumen via their production of H_2, formate, succinate, lactate, ethanol, and methanol. Co-culture experimentation with *Lachnospiraceae* strains partnered with methanogenic archaea are likely to uncover how these organisms interact in the rumen and potentially identify ways in which these interactions can be manipulated to lead to lower CH_4 production. Similarly, the interactions between primary fibre-degrading rumen microorganisms and the *Lachnospiraceae* may be important in driving VFA production and enhancing animal growth and productivity.

The genomes of the acetate producing *Lachnospiraceae* strains indicate they may be able to utilise H_2 via the reductive acetogenesis pathways. This capacity could be further explored with the aim of finding alternative H_2-users that can compete with methanogens as electron sinks. There are also opportunities to mine the genomes of rumen bacterial strains and new isolates for useful characteristics for biotechnology applications, such as bacteriophages, plasmids, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 gene editing systems.

Chapter 6. References

Abdugheni, R., Wang, W.-Z., Wang, Y.-J., Du, M.-X., Liu, F.-L., Zhou, N., Jiang, C.-Y., Wang, C.-Y., Wu, L., Ma, J., Liu, C., & Liu, S.-J. (2022). Metabolite profiling of human-originated *Lachnospiraceae* at the strain level. *iMeta*, *1*(4), e58. https://doi.org/https://doi.org/10.1002/imt2.58

Aiello, R. J., Armentano, L. E., Bertics, S. J., & Murphy, A. T. (1989). Volatile fatty acid uptake and propionate metabolism in ruminant hepatocytes. *Journal of Dairy Science*, *72*(4), 942-949. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(89)79187-6

Allison, M. J., Mayberry, W. R., McSweeney, C. S., & Stahl, D. A. (1992). *Synergistes jonesii,* gen. nov., sp.nov.: A rumen bacterium that degrades toxic pyridinediols. *Systematic and Applied Microbiology*, *15*(4), 522-529. https://doi.org/https://doi.org/10.1016/S0723-2020(11)80111-6

Altman, T., Travers, M., Kothari, A., Caspi, R., & Karp, P. D. (2013). A systematic comparison of the MetaCyc and KEGG pathway databases. *BMC Bioinformatics*, *14*(1), 112. https://doi.org/10.1186/1471-2105-14-112

Arora, N. K. (2019). Impact of climate change on agriculture production and its sustainable solutions. *Environmental Sustainability*, *2*(2), 95-96. https://doi.org/10.1007/s42398-019-00078-w

Attwood, G. T., & Reilly, K. (1995). Identification of proteolytic rumen bacteria isolated from New Zealand cattle. *Journal of Applied Bacteriology*, *79*(1), 22-29. https://doi.org/10.1111/j.1365-2672.1995.tb03119.x

Attwood, G. T., Reilly, K., & Patel, B. K. C. (1996). *Clostridium proteoclasticum* sp. nov., a novel proteolytic bacterium from the bovine rumen. *International Journal of Systematic and Evolutionary Microbiology*, *46*(3), 753-758. https://doi.org/https://doi.org/10.1099/00207713-46-3-753

Bachochin, M. J., Venegas, J. C., Singh, G., Zhang, L., & Barber, R. D. (2020). Characterization of a butyrate kinase from *Desulfovibrio vulgaris* str. Hildenborough. *FEMS Microbiology Letters*, *367*(6). https://doi.org/10.1093/femsle/fnaa047

Balch, C. (1959). Structure of the ruminant stomach and the movement of its contents. *Proceedings of the Nutrition Society*, *18*(2), 97-102.

Bartholomew, J. W., & Mittwer, T. (1952). The Gram stain. *Bacteriological reviews*, *16*(1), 1-29. https://doi.org/10.1128/br.16.1.1-29.1952

Biddle, A., Stewart, L., Blanchard, J., & Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. *Diversity*, *5*(3), 627-640. https://www.mdpi.com/1424-2818/5/3/627

Birgen, C., Dürre, P., Preisig, H. A., & Wentzel, A. (2019). Butanol production from lignocellulosic biomass: revisiting fermentation performance indicators with exploratory data analysis. *Biotechnology for Biofuels*, *12*(1), 167. https://doi.org/10.1186/s13068-019-1508-6

Blaalid, R., Kumar, S., Nilsson, R. H., Abarenkov, K., Kirk, P. M., & Kauserud, H. (2013). ITS1 versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*, *13*(2), 218-224. https://doi.org/10.1111/1755-0998.12065

Brock, F. M., Forsberg, C. W., & Buchanan-Smith, J. G. (1982). Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Applied Environmental Microbiology*, *44*(3), 561-569. https://doi.org/10.1128/aem.44.3.561-569.1982

Bryant, M. P., & Small, N. (1956). Characteristics of two new genera of anaerobic curved rods isolated from the rumen of cattle. *Journal of Bacteriology*, *72*(1), 22-26.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., & Henrissat, B. (2009). The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Research*, *37*(Database issue), D233-238. https://doi.org/10.1093/nar/gkn663

Caspi, R., Billington, R., Keseler, I. M., Kothari, A., Krummenacker, M., Midford, P. E., Ong, W. K., Paley, S., Subhraveti, P., & Karp, P. D. (2020). The MetaCyc database of metabolic pathways and enzymes - a 2019 update. *Nucleic Acids Research*, *48*(D1), D445-d453. https://doi.org/10.1093/nar/gkz862

Caspi, R., Dreher, K., & Karp, P. D. (2013). The challenge of constructing, classifying, and representing metabolic pathways. *FEMS Microbiology Letters*, *345*(2), 85-93. https://doi.org/10.1111/1574-6968.12194

Chamkha, M., Garcia, J. L., & Labat, M. (2001). Metabolism of cinnamic acids by some *Clostridiales* and emendation of the descriptions of *Clostridium aerotolerans*, *Clostridium celerecrescens* and *Clostridium xylanolyticum*. *International Journal of*

Systematic and Evolutionary Microbiology, *51*(6), 2105-2111. https://doi.org/https://doi.org/10.1099/00207713-51-6-2105

Cord-Ruwisch, R., Seitz, H.-J., & Conrad, R. (1988). The capacity of hydrogenotrophic anaerobic bacteria to compete for traces of hydrogen depends on the redox potential of the terminal electron acceptor. *Archives of Microbiology*, *149*(4), 350-357. https://doi.org/10.1007/BF00411655

Cornick, N. A., Jensen, N. S., Stahl, D. A., Hartman, P. A., & Allison, M. J. (1994). *Lachnospira pectinoschiza* sp. nov., an anaerobic pectinophile from the pig Intestine. *International Journal of Systematic and Evolutionary Microbiology*, *44*(1), 87-93. https://doi.org/https://doi.org/10.1099/00207713-44-1-87

Cotta, M., & Forster, R. (2006). The family *Lachnospiraceae,* Including the genera *Butyrivibrio*, *Lachnospira* and *Roseburia*. In M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, & E. Stackebrandt (Eds.), *The Prokaryotes: Volume 4: Bacteria: Firmicutes, Cyanobacteria* (pp. 1002-1021). Springer US. https://doi.org/10.1007/0-387-30744-3_35

Creevey, C. J., Kelly, W. J., Henderson, G., & Leahy, S. C. (2014). Determining the culturability of the rumen bacterial microbiome. *Microbial Biotechnology*, *7*(5), 467-479. https://doi.org/10.1111/1751-7915.12141

Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S. H., Henrissat, B., Coutinho, P., Morrison, M., Mosoni, P., Yeoman, C. J., White, B. A., & Bayer, E. A. (2014). Rumen cellulosomics: Divergent fiber-degrading strategies revealed by comparative genome-wide analysis of six *Ruminococcal* Strains. *PLOS ONE*, *9*(7), e99221. https://doi.org/10.1371/journal.pone.0099221

Dehority, B. A. (1973, Jul). Hemicellulose degradation by rumen bacteria. Fed Proc,

Dehority, B. A. (2008). Improved in vitro procedure for maintaining stock cultures of three genera of rumen protozoa. *Journal of Animal Science*, *86*(6), 1395-1401. https://doi.org/10.2527/jas.2007-0238

Díaz-Rodríguez, A. M., Salcedo Gastelum, L. A., Félix Pablos, C. M., Parra-Cota, F. I., Santoyo, G., Puente, M. L., Bhattacharya, D., Mukherjee, J., & de los Santos-Villalobos, S. (2021). The current and future role of microbial culture collections in food security worldwide [Review]. *Frontiers in Sustainable Food Systems*, *4*. https://doi.org/10.3389/fsufs.2020.614739

Duncan, S. H., Barcenilla, A., Stewart, C. S., Pryde, S. E., & 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*(10), 5186-5190. https://doi.org/doi:10.1128/AEM.68.10.5186-5190.2002

Dušková, D., & Marounek, M. (2001). Fermentation of pectin and glucose, and activity of pectin-degrading enzymes in the rumen bacterium *Lachnospira multiparus*. *Letters in Applied Microbiology*, *33*(2), 159-163. https://doi.org/10.1046/j.1472-765x.2001.00970.x

Edwards, J. E., McEwan, N. R., Travis, A. J., & John Wallace, R. (2004). 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie van Leeuwenhoek*, *86*(3), 263-281. https://doi.org/10.1023/B:ANTO.0000047942.69033.24

Emerson, E. L., & 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*(10), 4269-4278. https://doi.org/10.1007/s00253-017-8150-7

Eram, M. S., & Ma, K. (2013). Decarboxylation of pyruvate to acetaldehyde for ethanol production by hyperthermophiles. *Biomolecules*, *3*(3), 578-596. https://doi.org/10.3390/biom3030578

Fox, D. K., Meadow, N. D., & Roseman, S. (1986). Phosphate transfer between acetate kinase and enzyme I of the bacterial phosphotransferase system. *Journal of Biological Chemistry*, *261*(29), 13498-13503. https://doi.org/https://doi.org/10.1016/S0021-9258(18)67046-2

Goldberg, R., Morvan, C., Jauneau, A., & Jarvis, M. C. (1996). Methyl-esterification, de-esterification and gelation of pectins in the primary cell wall. In J. Visser & A. G. J. Voragen (Eds.), *Progress in Biotechnology* (Vol. 14, pp. 151-172). Elsevier. https://doi.org/https://doi.org/10.1016/S0921-0423(96)80253-X

Gowtham, H. G., Priyanka, G., & Hariprasad, P. (2022). Untangling the structure and function of rumen microbes in relation to ruminant health and exploring their biotechnological applications. In S. Mahajan & A. Varma (Eds.), *Animal Manure: Agricultural and Biotechnological Applications* (pp. 61-106). Springer International Publishing. https://doi.org/10.1007/978-3-030-97291-2_5

Greening, C., Biswas, A., Carere, C. R., Jackson, C. J., Taylor, M. C., Stott, M. B., Cook, G. M., & Morales, S. E. (2016). Genomic and metagenomic surveys of hydrogenase distribution indicate H2 is a widely utilised energy source for microbial growth and survival. *The ISME Journal*, *10*(3), 761-777. https://doi.org/10.1038/ismej.2015.153

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., & Mackie, R. I. (2019). Diverse hydrogen production and consumption pathways influence methane production in ruminants. *The ISME Journal*, *13*(10), 2617-2632. https://doi.org/10.1038/s41396-019-0464-2

Greening, R. C., & Leedle, J. A. Z. (1989). Enrichment and isolation of *Acetitomaculum ruminis,* gen. nov., sp. nov.: acetogenic bacteria from the bovine rumen. *Archives of Microbiology*, *151*(5), 399-406. https://doi.org/10.1007/BF00416597

Haas, K. N., & Blanchard, J. L. (2020). Reclassification of the C*lostridium clostridioforme* and *Clostridium sphenoides* clades as *Enterocloster* gen. nov. and *Lacrimispora* gen. nov., including reclassification of 15 taxa. *International Journal of Systematic and Evolutionary Microbiology*, *70*(1), 23-34. https://doi.org/https://doi.org/10.1099/ijsem.0.003698

Hagarty, M. P., Court, R. D., Christie, G. S., & Lee, C. P. (1976). Mimosine in *Leucaena leucocephala* is metabolised to a goitrogen in ruminants. *Australian Veterinary Journal*, *52*(10), 490. https://doi.org/10.1111/j.1751-0813.1976.tb05416.x

Hagen, L. H., Brooke, C. G., Shaw, C. A., Norbeck, A. D., Piao, H., Arntzen, M. Ø., Olson, H. M., Copeland, A., Isern, N., & Shukla, A. (2021). Proteome specialization of anaerobic fungi during ruminal degradation of recalcitrant plant fiber. *The ISME Journal*, *15*(2), 421-434.

Harholt, J., Suttangkakul, A., & Vibe Scheller, H. (2010). Biosynthesis of pectin. *Plant Physiology*, *153*(2), 384-395. https://doi.org/10.1104/pp.110.156588

Harmon, D. L. (1993). Nutritional regulation of postruminal digestive enzymes in ruminants. *Journal of Dairy Science*, *76*(7), 2102-2111.

He, P., Duan, H., Han, W., Liu, Y., Shao, L., & Lü, F. (2019). Responses of *Methanosarcina barkeri* to acetate stress. *Biotechnology for Biofuels*, *12*(1), 289. https://doi.org/10.1186/s13068-019-1630-5

Hedderich, R., & Whitman, W. B. (2013). Physiology and biochemistry of the methane-producing archaea. In E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, & F. Thompson (Eds.), *The Prokaryotes: Prokaryotic Physiology and Biochemistry* (pp. 635-662). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-30141-4_81

Hegarty, R., & Klieve, A. (1999). Opportunities for biological control of ruminal methanogenesis. *Australian Journal of Agricultural Research*, *50*(8), 1315-1320.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., & 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. https://doi.org/10.1038/srep14567

Hespell, R. B., & Whitehead, T. R. (1990). Physiology and genetics of xylan degradation by gastrointestinal tract bacteria. *Journal of Dairy Science*, *73*(10), 3013-3022. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(90)78988-6

Hitch, T. C. A., Riedel, T., Oren, A., Overmann, J., Lawley, T. D., & Clavel, T. (2021). Automated analysis of genomic sequences facilitates high-throughput and comprehensive description of bacteria. *ISME Communications*, *1*(1), 16. https://doi.org/10.1038/s43705-021-00017-z

Hobson, P. N., & Stewart, C. S. (1988). *Rumen microbial ecosystem*. Springer Science & Business Media.

Hugouvieux-Cotte-Pattat, N., Condemine, G., & Shevchik, V. E. (2014). Bacterial pectate lyases, structural and functional diversity. *Environmental Microbiology Reports*, *6*(5), 427-440. https://doi.org/10.1111/1758-2229.12166

Hungate, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bacteriological reviews*, *14*(1), 1-49. https://doi.org/10.1128/br.14.1.1-49.1950

Huws, S. A. (2022, Feb 2022). *Flagship project ruminant cultures* https://globalresearchalliance.org/?s=flagship

Jacobi, A., Rossmann, R., & Böck, A. (1992). The hyp operon gene products are required for the maturation of catalytically active hydrogenase isoenzymes in *Escherichia coli*. *Archives of Microbiology*, *158*(6), 444-451. https://doi.org/10.1007/BF00276307

Jami, E., & Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. *PLOS ONE*, *7*(3), e33306. https://doi.org/10.1371/journal.pone.0033306

Janssen, P. H., & Kirs, M. (2008). Structure of the archaeal community of the rumen. *Applied and Environmental Microbiology*, *74*(12), 3619-3625. https://doi.org/doi:10.1128/AEM.02812-07

Joblin, K. (2005). Methanogenic archaea. In (pp. 47-53). https://doi.org/10.1007/1-4020-3791-0_4

Joshi, A., Lanjekar, V. B., Dhakephalkar, P. K., Callaghan, T. M., Griffith, G. W., & Dagar, S. S. (2018). *Liebetanzomycespolymorphus* gen. et sp. nov., a new anaerobic fungus (*Neocallimastigomycota*) isolated from the rumen of a goat. *MycoKeys*(40), 89.

Juturu, V., & Wu, J. C. (2013). Insight into microbial hemicellulases other than xylanases: a review. *Journal of Chemical Technology & Biotechnology*, *88*(3), 353-363. https://doi.org/https://doi.org/10.1002/jctb.3969

Kanehisa, M. (2019). Toward understanding the origin and evolution of cellular organisms. *Protein Science*, *28*(11), 1947-1951. https://doi.org/10.1002/pro.3715

Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M., & Ishiguro-Watanabe, M. (2023). KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Research*, *51*(D1), D587-d592. https://doi.org/10.1093/nar/gkac963

Kanehisa, M., & Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research*, *28*(1), 27-30. https://doi.org/10.1093/nar/28.1.27

Keegstra, K., Talmadge, K. W., Bauer, W. D., & Albersheim, P. (1973). The structure of plant cell walls: III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. *Plant Physiology*, *51*(1), 188-197. https://doi.org/10.1104/pp.51.1.188

Kelly, W. J., Leahy, S. C., Altermann, E., Yeoman, C. J., Dunne, J. C., Kong, Z., Pacheco, D. M., Li, D., Noel, S. J., Moon, C. D., Cookson, A. L., & Attwood, G. T. (2010). The glycobiome of the rumen bacterium *butyrivibrio proteoclasticus B316T* highlights adaptation to a polysaccharide-rich environment. *PLOS ONE*, *5*(8), e11942. https://doi.org/10.1371/journal.pone.0011942

Kelly, W. J., Leahy, S. C., Kamke, J., Soni, P., Koike, S., Mackie, R., Seshadri, R., Cook, G. M., Morales, S. E., Greening, C., & Attwood, G. T. (2019). Occurrence and expression of genes encoding methyl-compound production in rumen bacteria. *Animal Microbiome*, *1*(1), 15. https://doi.org/10.1186/s42523-019-0016-0

Kenters, N., Henderson, G., Jeyanathan, J., Kittelmann, S., & Janssen, P. H. (2011). Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *Journal of Microbiological Methods*, *84*(1), 52-60. https://doi.org/https://doi.org/10.1016/j.mimet.2010.10.011

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., & Janssen, P. H. (2013). Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLOS ONE*, *8*(2), e47879. https://doi.org/10.1371/journal.pone.0047879

Leahy, S. C., Kelly, W. J., Altermann, E., Ronimus, R. S., Yeoman, C. J., Pacheco, D. M., Li, D., Kong, Z., McTavish, S., Sang, C., Lambie, S. C., Janssen, P. H., Dey, D., & Attwood, G. T. (2010). The genome sequence of the rumen methanogen *Methanobrevibacter ruminantium* reveals new possibilities for controlling ruminant methane emissions. *PLOS ONE*, *5*(1), e8926. https://doi.org/10.1371/journal.pone.0008926

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., Altermann, E., & Attwood, G. T. (2013). Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal*, *7*, 235-243. https://doi.org/https://doi.org/10.1017/S1751731113000700

Lee, H. S., & Rittmann, B. E. (2009). Evaluation of metabolism using stoichiometry in fermentative biohydrogen. *Biotechnology and Bioengineering*, *102*(3), 749-758. https://doi.org/10.1002/bit.22107

Leedle, J. A., & Hespell, R. B. (1980). Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. *Applied Environmental Microbiology*, *39*(4), 709-719. https://doi.org/10.1128/aem.39.4.709-719.1980

Levy, S., York, W. S., Stuike-Prill, R., Meyer, B., & Staehelin, L. A. (1991). Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *The Plant Journal*, *1*(2), 195-215. https://doi.org/https://doi.org/10.1111/j.1365-313X.1991.00195.x

Li, Y., Crouzet, L., Kelly, W. J., Reid, P., Leahy, S. C., & Attwood, G. T. (2023). *Methanobrevibacter boviskoreani* JH1T growth on alcohols allows development of a high throughput bioassay to detect methanogen inhibition. *Current Research in*

Microbial Sciences, *4*, 100189. https://doi.org/https://doi.org/10.1016/j.crmicr.2023.100189

Lim, Y. W. (2022). *Isolation and characterisation of Faecalibacterium prausnitzii in New Zealand calves : a thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Microbiology at Massey University, Manawatū, New Zealand* [Masters, Massey University]. http://hdl.handle.net/10179/17584

Linhardt, R. J., Galliher, P. M., & Cooney, C. L. (1986). Polysaccharide lyases. *Biotechnology and Applied Biochemistry*, *12*(2), 135-176. https://doi.org/10.1007/bf02798420

Liu, L., Huang, W.-C., Liu, Y., & Li, M. (2021). Diversity of cellulolytic microorganisms and microbial cellulases. *International Biodeterioration & Biodegradation*, *163*, 105277. https://doi.org/https://doi.org/10.1016/j.ibiod.2021.105277

Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., Andrews, E., Ajami, N. J., Bonham, K. S., Brislawn, C. J., Casero, D., Courtney, H., Gonzalez, A., Graeber, T. G., Hall, A. B., Lake, K., Landers, C. J., Mallick, H., Plichta, D. R., . . . Investigators, I. (2019). Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature*, *569*(7758), 655-662. https://doi.org/10.1038/s41586-019-1237-9

Loerch, S. C., & Oke, B. O. (2018). Rumen protected amino acids in ruminant nutrition. In *Absorption and utilization of amino acids* (pp. 187-200). CRC Press.

Lombard, V., Bernard, T., Rancurel, C., Brumer, H., Coutinho, P. M., & Henrissat, B. (2010). A hierarchical classification of polysaccharide lyases for glycogenomics. *Biochemical Journal*, *432*(3), 437-444.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P. M., & Henrissat, B. (2014). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research*, *42*(Database issue), D490-495. https://doi.org/10.1093/nar/gkt1178

Louis, P., Duncan, S. H., McCrae, S. I., Millar, J., Jackson, M. S., & Flint, H. J. (2004). Restricted distribution of the butyrate kinase pathway among butyrate-producing bacteria from the human colon. *Journal of Bacteriology*, *186*(7), 2099-2106. https://doi.org/10.1128/jb.186.7.2099-2106.2004

Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, *294*(1), 1-8.

Makkar, H. P., & McSweeney, C. S. (2005). *Methods in gut microbial ecology for ruminants* (Vol. 10). Springer.

Matthews, C., Crispie, F., Lewis, E., Reid, M., O'Toole, P. W., & Cotter, P. D. (2019). The rumen microbiome: a crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes*, *10*(2), 115-132. https://doi.org/10.1080/19490976.2018.1505176

McDonough, M. A., Kadirvelraj, R., Harris, P., Poulsen, J. C., & Larsen, S. (2004). Rhamnogalacturonan lyase reveals a unique three-domain modular structure for polysaccharide lyase family 4. *FEBS Letters*, *565*(1-3), 188-194. https://doi.org/10.1016/j.febslet.2004.03.094

McSweeney, C., & Mackie, R. (2012). Commission on genetic resources for food and agriculture. *Micro-organisms and ruminant digestion: State of knowledge, trends and future prospects. Background Study Paper (FAO)*, *61*, 1-62.

Meehan, C. J., & Beiko, R. G. (2014). A phylogenomic view of ecological specialization in the *Lachnospiraceae*, a family of digestive tract-associated bacteria. *Genome biology and evolution*, *6*(3), 703-713.

Miyazaki, K., Martin, J. C., Marinsek-Logar, R., & Flint, H. J. (1997). Degradation and utilization of xylans by the rumen anaerobe *Prevotella bryantii* (formerly *P. ruminicola* subsp.*brevis)* B14. *Anaerobe*, *3*(6), 373-381. https://doi.org/https://doi.org/10.1006/anae.1997.0125

Moraïs, S., & Mizrahi, I. (2019). Islands in the stream: from individual to communal fiber degradation in the rumen ecosystem. *FEMS Microbiology Reviews*, *43*(4), 362-379. https://doi.org/10.1093/femsre/fuz007

Morgavi, D. P., Kelly, W. J., Janssen, P. H., & Attwood, G. T. (2013). Rumen microbial (meta)genomics and its application to ruminant production. *Animal*, *7 Suppl 1*, 184-201. https://doi.org/10.1017/s1751731112000419

Mosoni, P., Martin, C., Forano, E., & Morgavi, D. (2011). Long-term defaunation increases the abundance of cellulolytic *ruminococci* and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. *Journal of Animal Science*, *89*(3), 783-791.

Mudgil, D. (2017). Chapter 3 : The interaction between insoluble and soluble fiber. In R. A. Samaan (Ed.), *Dietary Fiber for the Prevention of Cardiovascular Disease* (pp. 35-59).

Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-805130-6.00003-3

Nakamura, A. M., Nascimento, A. S., & Polikarpov, I. (2017). Structural diversity of carbohydrate esterases. *Biotechnology Research and Innovation*, *1*(1), 35-51. https://doi.org/https://doi.org/10.1016/j.biori.2017.02.001

National Academies of Sciences, E., & Medicine. (2021). *Nutrient Requirements of Dairy Cattle: Eighth Revised Edition*. The National Academies Press. https://doi.org/doi:10.17226/25806

Newbold, C. J., de la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen [Review]. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01313

Nisman, B. (1954). The stickland reaction. *Bacteriological reviews*, *18*(1), 16-42.

Nissen, L. S., & Basen, M. (2019). The emerging role of aldehyde:ferredoxin oxidoreductases in microbially-catalyzed alcohol production. *Journal of Biotechnology*, *306*, 105-117. https://doi.org/https://doi.org/10.1016/j.jbiotec.2019.09.005

Nordberg, H., Cantor, M., Dusheyko, S., Hua, S., Poliakov, A., Shabalov, I., Smirnova, T., Grigoriev, I. V., & Dubchak, I. (2014). The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. *Nucleic Acids Research*, *42*(Database issue), D26-31. https://doi.org/10.1093/nar/gkt1069

Østby, H., & Várnai, A. (2023). Hemicellulolytic enzymes in lignocellulose processing. *Essays in Biochemistry*, *67*(3), 533-550. https://doi.org/10.1042/ebc20220154

Paggi, R. A., Rodríguez, C., & Fay, J. P. (2005). Growth and pectate-lyase activity of the ruminal bacterium *Lachnospira multiparus* in the presence of short-chain organic acids. *Letters in Applied Microbiology*, *41*(5), 434-439. https://doi.org/https://doi.org/10.1111/j.1472-765X.2005.01717.x

Palevich, N., Kelly, W. J., Ganesh, S., Rakonjac, J., & Attwood, G. T. (2019). *Butyrivibrio hungatei* MB2003 competes effectively for soluble sugars released by B*utyrivibrio proteoclasticus* B316 ^{T} during growth on xylan or pectin. *Applied and Environmental Microbiology*, *85*(3), e02056-02018. https://doi.org/doi:10.1128/AEM.02056-18

Palevich, N., Kelly, W. J., Leahy, S. C., Altermann, E., Rakonjac, J., & Attwood, G. T. (2017). The complete genome sequence of the rumen bacterium *Butyrivibrio hungatei*

MB2003. *Standards in Genomic Sciences*, *12*(1), 72. https://doi.org/10.1186/s40793-017-0285-8

Palevich, N., Kelly, W. J., Leahy, S. C., Denman, S., Altermann, E., Rakonjac, J., & Attwood, G. T. (2019). Comparative genomics of Rumen *Butyrivibrio* spp. uncovers a continuum of polysaccharide-degrading capabilities. *Applied and Environmental Microbiology*, *86*(1), e01993-01919. https://doi.org/doi:10.1128/AEM.01993-19

Parks, D. H., Chuvochina, M., Rinke, C., Mussig, A. J., Chaumeil, P.-A., & Hugenholtz, P. (2021). GTDB: an ongoing census of bacterial and archaeal diversity through a phylogenetically consistent, rank normalized and complete genome-based taxonomy. *Nucleic Acids Research*, *50*(D1), D785-D794. https://doi.org/10.1093/nar/gkab776

Parks, D. H., Chuvochina, M., Waite, D. W., Rinke, C., Skarshewski, A., Chaumeil, P.-A., & Hugenholtz, P. (2018). A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. *Nature Biotechnology*, *36*(10), 996-1004. https://doi.org/10.1038/nbt.4229

Patra, A., Park, T., Kim, M., & Yu, Z. (2017). Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *Journal of animal science and biotechnology*, *8*(1), 13. https://doi.org/10.1186/s40104-017-0145-9

Pelloux, J., Rustérucci, C., & Mellerowicz, E. J. (2007). New insights into pectin methylesterase structure and function. *Trends in Plant Science*, *12*(6), 267-277. https://doi.org/10.1016/j.tplants.2007.04.001

Peng, L., Li, Z. R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *Journal of Nutrition*, *139*(9), 1619-1625. https://doi.org/10.3945/jn.109.104638

Pfannkuche, H., Schellhorn, C., Schemann, M., & Gäbel, G. (2004). Intrinsic innervation patterns of the smooth muscle in the rumen and reticulum of lambs. *Journal of Anatomy*, *204*(4), 293-299. https://doi.org/10.1111/j.0021-8782.2004.00284.x

Pinnell, L., Reyes, A., Wolfe, C., Weinroth, M., Metcalf, J., Delmore, R., Belk, K., Morley, P., & Engle, T. (2022). Bacteroidetes and Firmicutes drive differing microbial diversity and community composition between micro-environments in the bovine rumen. *Frontiers in Veterinary Science*.

Plöger, S., Stumpff, F., Penner, G. B., Schulzke, J. D., Gäbel, G., Martens, H., Shen, Z., Günzel, D., & Aschenbach, J. R. (2012). Microbial butyrate and its role for barrier function in the gastrointestinal tract. *Annals of the New York academy of sciences*, *1258*(1), 52-59.

Prabhu, R., Altman, E., & Eiteman, M. A. (2012). Lactate and acrylate metabolism by *Megasphaera elsdenii* under batch and steady-state conditions. *Applied Environmental Microbiology*, *78*(24), 8564-8570. https://doi.org/10.1128/aem.02443-12

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219

Ragsdale, S. W. (2008). Enzymology of the Wood–Ljungdahl pathway of acetogenesis. *Annals of the New York academy of sciences*, *1125*(1), 129-136. https://doi.org/https://doi.org/10.1196/annals.1419.015

Reichardt, N., Duncan, S. H., Young, P., Belenguer, A., McWilliam Leitch, C., Scott, K. P., Flint, H. J., & Louis, P. (2014). Phylogenetic distribution of three pathways for propionate production within the human gut microbiota. *The ISME Journal*, *8*(6), 1323-1335. https://doi.org/10.1038/ismej.2014.14

Ren, H., Su, X., Bai, H., Yang, Y., Wang, H., Dan, Z., Lu, J., Wu, S., Cai, C., Cao, Y., Lei, X., & 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*(1), 209. https://doi.org/10.1186/s13568-019-0937-8

Roger, V., Fonty, G., Komisarczuk-Bony, S., & Gouet, P. (1990). Effects of physicochemical factors on the adhesion to cellulose avicel of the ruminal bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogene*s subsp. *succinogenes*. *Applied and Environmental Microbiology*, *56*(10), 3081-3087.

Russell, J. B., & Hespell, R. B. (1981). Microbial rumen fermentation. *Journal of Dairy Science*, *64*(6), 1153-1169. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(81)82694-X

Sandgren, M., Ståhlberg, J., & Mitchinson, C. (2005). Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes. *Progress in Biophysics and Molecular Biology*, *89*(3), 246-291. https://doi.org/https://doi.org/10.1016/j.pbiomolbio.2004.11.002

Sasaki, D., Morita, M., Sasaki, K., Watanabe, A., & Ohmura, N. (2012). Acceleration of cellulose degradation and shift of product via methanogenic co-culture of a cellulolytic bacterium with a hydrogenotrophic methanogen. *Journal of bioscience and bioengineering*, *114*(4), 435-439.

Scott, K. P., Martin, J. C., Campbell, G., Mayer, C.-D., & Flint, H. J. (2006). Whole-genome transcription profiling reveals genes up-regulated by growth on fucose in the human gut bacterium *Roseburia inulinivorans*. *Journal of Bacteriology*, *188*(12), 4340-4349. https://doi.org/doi:10.1128/jb.00137-06

Sengupta, K., Hivarkar, S. S., Palevich, N., Chaudhary, P. P., Dhakephalkar, P. K., & Dagar, S. S. (2022). Genomic architecture of three newly isolated unclassified *Butyrivibrio* species elucidate their potential role in the rumen ecosystem. *Genomics*, *114*(2), 110281. https://doi.org/https://doi.org/10.1016/j.ygeno.2022.110281

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., Palevich, N., Janssen, P. H., Ronimus, R. S., Noel, S., Soni, P., Reilly, K., Atherly, T., Ziemer, C., . . . Hungate project, c. (2018). Cultivation and sequencing of rumen microbiome members from the Hungate1000 collection. *Nature Biotechnology*, *36*(4), 359-367. https://doi.org/10.1038/nbt.4110

Shallom, D., & Shoham, Y. (2003). Microbial hemicellulases. *Current Opinion in Microbiology*, *6*(3), 219-228. https://doi.org/https://doi.org/10.1016/S1369-5274(03)00056-0

Shen, H., Xu, Z., Shen, Z., & Lu, Z. (2019). The regulation of ruminal short-chain fatty acids on the functions of rumen barriers [Original Research]. *Frontiers in Physiology*, *10*. https://doi.org/10.3389/fphys.2019.01305

Shevchik, V. E., & Hugouvieux-Cotte-Pattat, N. (2003). PaeX, a second pectin acetylesterase of *Erwinia chrysanthemi* 3937. *Journal of Bacteriology*, *185*(10), 3091-3100. https://doi.org/10.1128/jb.185.10.3091-3100.2003

Sidar, A., Albuquerque, E. D., Voshol, G. P., Ram, A. F., Vijgenboom, E., & Punt, P. J. (2020). Carbohydrate binding modules: diversity of domain architecture in amylases and

cellulases from filamentous microorganisms. *Frontiers in Bioengineering and Biotechnology*, *8*, 871.

Sissons, J. W. (1981). Digestive enzymes of cattle. *Journal of the Science of Food and Agriculture*, *32*(2), 105-114.

Solden, L. M., Naas, A. E., Roux, S., Daly, R. A., Collins, W. B., Nicora, C. D., Purvine, S. O., Hoyt, D. W., Schückel, J., Jørgensen, B., Willats, W., Spalinger, D. E., Firkins, J. L., Lipton, M. S., Sullivan, M. B., Pope, P. B., & Wrighton, K. C. (2018). Interspecies cross-feeding orchestrates carbon degradation in the rumen ecosystem. *Nature Microbiology*, *3*(11), 1274-1284. https://doi.org/10.1038/s41564-018-0225-4

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., & Urich, T. (2018). Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. *mSystems*, *3*(4), e00038-00018. https://doi.org/doi:10.1128/mSystems.00038-18

Solomon, R., Wein, T., Levy, B., Eshed, S., Dror, R., Reiss, V., Zehavi, T., Furman, O., Mizrahi, I., & Jami, E. (2022). Protozoa populations are ecosystem engineers that shape prokaryotic community structure and function of the rumen microbial ecosystem. *The ISME Journal*, *16*(4), 1187-1197. https://doi.org/10.1038/s41396-021-01170-y

Sorbara, M. T., Littmann, E. R., Fontana, E., Moody, T. U., Kohout, C. E., Gjonbalaj, M., Eaton, V., Seok, R., Leiner, I. M., & Pamer, E. G. (2020). Functional and genomic variation between human-derived isolates of *Lachnospiraceae* reveals inter- and intra-species diversity. *Cell Host & Microbe*, *28*(1), 134-146.e134. https://doi.org/https://doi.org/10.1016/j.chom.2020.05.005

Stams, A. J. M., Teusink, B., & Sousa, D. Z. (2019). Ecophysiology of acetoclastic methanogens. In A. J. M. Stams & D. Z. Sousa (Eds.), *Biogenesis of Hydrocarbons* (pp. 109-121). Springer International Publishing. https://doi.org/10.1007/978-3-319-78108-2_21

Steele, M. A., Penner, G. B., Chaucheyras-Durand, F., & Guan, L. L. (2016). Development and physiology of the rumen and the lower gut: Targets for improving gut health. *Journal of Dairy Science*, *99*(6), 4955-4966. https://doi.org/https://doi.org/10.3168/jds.2015-10351

Svensson, B. (1994). Protein engineering in the α-amylase family: catalytic mechanism, substrate specificity, and stability. *Plant Molecular Biology*, *25*(2), 141-157. https://doi.org/10.1007/BF00023233

Thibault, J.-F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P., & Crépeau, M.-J. (1993). Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. *Carbohydrate Research*, *238*, 271-286. https://doi.org/https://doi.org/10.1016/0008-6215(93)87019-O

Tseten, T., Sanjorjo, R. A., Kwon, M., & Kim, S.-W. (2022). Strategies to mitigate enteric methane emissions from ruminant animals. *Journal of Microbiology and Biotechnology*, *32*(3), 1-9.

Ungerfeld, E. M. (2015). Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: a meta-analysis. *Frontiers in Microbiology*, *6*, 37. https://doi.org/10.3389/fmicb.2015.00037

Vacca, M., Celano, G., Calabrese, F. M., Portincasa, P., Gobbetti, M., & De Angelis, M. (2020). The controversial role of human gut *Lachnospiraceae*. *Microorganisms*, *8*(4). https://doi.org/10.3390/microorganisms8040573

van Dijk, M., Morley, T., Rau, M. L., & Saghai, Y. (2021). A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. *Nature Food*, *2*(7), 494-501. https://doi.org/10.1038/s43016-021-00322-9

Vantcheva, Z. M., Pradhan, K., & Hemken, R. W. (1970). Rumen Methanol in vivo and in vitro. *Journal of Dairy Science*, *53*(10), 1511-1514. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(70)86424-4

Villarreal, F., Stocchi, N., & Ten Have, A. (2022). Functional classification and characterization of the fungal glycoside hydrolase 28 protein family. *Journal of Fungi*, *8*(3). https://doi.org/10.3390/jof8030217

Waghorn, G. C., Shelton, I. D., & Thomas, V. J. (1989). Particle breakdown and rumen digestion of fresh ryegrass (*Lolium perenne L.)* and lucerne (*Medicago sativa L.)* fed to cows during a restricted feeding period. *British Journal of Nutrition*, *61*(2), 409-423. https://doi.org/10.1079/BJN19890127

Wallace, R., Onodera, R., & Cotta, M. (1997). Metabolism of nitrogen-containing compounds. *The rumen microbial ecosystem*, 283-328.

Wallace, R. J., & McKain, N. (1991). A survey of peptidase activity in rumen bacteria. *Microbiology*, *137*(9), 2259-2264. https://doi.org/https://doi.org/10.1099/00221287-137-9-2259

Wallace, R. J., Sasson, G., Garnsworthy, P. C., Tapio, I., Gregson, E., Bani, P., Huhtanen, P., Bayat, A. R., Strozzi, F., Biscarini, F., Snelling, T. J., Saunders, N., Potterton, S. L., Craigon, J., Minuti, A., Trevisi, E., Callegari, M. L., Cappelli, F. P., Cabezas-Garcia, E. H., . . . Mizrahi, I. (2019). A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. *Science Advances*, *5*(7), eaav8391. https://doi.org/10.1126/sciadv.aav8391

Watanabe, H., & Tokuda, G. (2001). Animal cellulases. *Cellular and Molecular Life Sciences CMLS*, *58*(9), 1167-1178. https://doi.org/10.1007/PL00000931

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol*, *173*(2), 697-703. https://doi.org/10.1128/jb.173.2.697-703.1991

Whitehead, T. R., & Cotta, M. A. (2004). Isolation and identification of hyper-ammonia producing bacteria from swine manure storage pits. *Current Microbiology*, *48*, 20-26.

Williams, C. L., Thomas, B. J., McEwan, N. R., Rees Stevens, P., Creevey, C. J., & Huws, S. A. (2020). Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. *Frontiers in Microbiology*, *11*, 720-720. https://doi.org/10.3389/fmicb.2020.00720

Woodfield, D. (1999). Genetic improvements in New Zealand forage cultivars Proceedings of the New Zealand Grassland Association,

Xie, Y., Sun, H., Xue, M., & Liu, J. (2022). Metagenomics reveals differences in microbial composition and metabolic functions in the rumen of dairy cows with different residual feed intake. *Animal Microbiome*, *4*(1), 19. https://doi.org/10.1186/s42523-022-00170-3

Yang, T., Datsomor, O., Jiang, M., Ma, X., Zhao, G., & Zhan, K. (2022). Protective roles of sodium butyrate in lipopolysaccharide-induced bovine ruminal epithelial cells by activating g protein-coupled receptors 41. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.842634

Yao, Q., Li, Y., Meng, Q., & Zhou, Z. (2017). The effect of calcium propionate on the ruminal bacterial community composition in finishing bulls. *Asian-Australasian journal of animal sciences*, *30*(4), 495-504. https://doi.org/10.5713/ajas.16.0469

York, W. S., & O'Neill, M. A. (2008). Biochemical control of xylan biosynthesis — which end is up? *Current Opinion in Plant Biology*, *11*(3), 258-265. https://doi.org/https://doi.org/10.1016/j.pbi.2008.02.007

Young, J. W. (1977). Gluconeogenesis in cattle: Significance and methodology. *Journal of Dairy Science*, *60*(1), 1-15. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(77)83821-6

Zhu, F., Wu, R., Zhang, H., & Wu, H. (2013). Structural and biochemical analysis of a bacterial glycosyltransferase. *Methods in Molecular Biology*, *1022*, 29-39. https://doi.org/10.1007/978-1-62703-465-4_3

Zhu, L. B., Zhang, Y. C., Huang, H. H., & Lin, J. (2021). Prospects for clinical applications of butyrate-producing bacteria. *World Journal of Clinical Pediatrics*, *10*(5), 84-92. https://doi.org/10.5409/wjcp.v10.i5.84

Zhu, Z., Difford, G. F., Noel, S. J., Lassen, J., Løvendahl, P., & Højberg, O. (2021). Stability assessment of the rumen bacterial and archaeal communities in dairy cows within a single lactation and its association with host phenotype [Original Research]. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.636223

Appendix 1: Concentration of SCFAs produced from growth of 45 *Lachnospiraceae* strains on soluble substrates

Table A1. Concentration of SCFA on soluble substrates in mM

Species	Acetate	Butyrate	Proprionate	Caproate	Isobutyrate	Isovalerate	Valerate	Formate	Lactate	Succinate	Ethanol	Butanol	Methanol
***Acetitomaculum ruminis* 139B**	19.88	0.32	0.65	0.00	0.00	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00
***Blautia Schinkii* B**	16.66	0.46	0.85	0.00	0.02	0.05	0.01	0.00	0.21	0.00	7.73	0.00	0.00
***Blautia wexlerae* AGR2146**	24.55	0.49	0.89	0.00	0.05	0.05	0.03	0.72	2.60	2.14	1.19	0.00	0.00
***Dorea longicatena* AGR2136**	9.52	0.37	0.65	0.00	0.03	0.01	0.01	3.60	0.00	1.05	13.51	0.00	0.00
***Dorea* sp. AGR2135**	8.30	0.43	0.74	0.00	0.00	0.00	0.01	0.97	1.22	0.66	11.36	0.00	0.00
***[Clostridium] clostridioforme* AGR2157**	11.09	0.32	0.59	0.00	0.00	0.04	0.01	0.59	2.91	0.66	16.74	0.00	0.00
***Enterocloster clostridioformis* T90**	10.01	0.43	0.83	0.00	0.00	0.04	0.00	0.66	14.45	0.00	13.31	0.00	0.00
***Eubacterium ruminantium* FB3002**	4.69	2.61	0.89	0.01	0.03	0.09	0.06	6.46	5.02	0.00	0.26	0.00	0.00
***Eubacterium ruminantium* HUN269**	4.46	2.51	0.85	0.01	0.02	0.19	0.14	5.63	2.39	0.00	0.00	0.00	0.00
Lachnobacterium bovis* NK4B19	4.15	3.64	0.73	0.01	0.01	0.04	0.04	16.66	16.55	0.00	0.62	0.00	0.00
***Lachnobacterium bovis* AE2004**	4.70	1.71	1.03	0.01	0.03	0.10	0.04	0.66	14.45	0.00	0.39	0.50	0.00
Lachnoclostridium aminophilum* F *	4.73	2.32	0.74	0.00	0.00	0.03	0.00	0.00	4.57	0.00	1.37	0.00	0.00
Lachnoclostridium aminophilum* KH1P1 *	8.99	2.51	0.94	0.00	0.05	0.10	0.01	0.00	0.01	0.00	0.00	0.00	0.00
***Lachnospira multipara* D15d**	5.29	0.47	0.74	0.01	0.04	0.13	0.07	3.24	0.53	0.00	10.81	0.00	0.00
***Lachnospira multipara* D32**	9.54	0.44	0.82	0.00	0.02	0.05	0.01	5.98	3.66	0.00	19.52	0.00	0.00
***Lachnospira multipara* LB2003**	10.49	3.42	1.50	0.02	0.07	0.10	0.05	10.71	0.17	0.00	0.32	0.00	0.00
***Lachnospira multipara* MC2003**	6.41	0.09	0.38	0.00	0.08	0.04	0.00	7.20	1.49	0.00	1.26	0.00	0.00
***Lachnospira pectinoschiza* M83**	6.90	0.47	0.76	0.01	0.05	0.09	0.05	8.67	0.34	0.00	15.33	0.00	0.00
***Lachnospiraceae* bacterium AC2012**	4.63	0.71	0.94	0.00	0.04	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium AC2014**	6.22	6.86	0.76	0.02	0.00	0.06	0.03	8.53	0.00	0.00	0.13	0.00	0.00
***Lachnospiraceae* bacterium AC2028**	4.08	1.82	0.85	0.00	0.00	0.16	0.10	10.26	1.98	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium AC2029 ***	4.37	2.31	0.73	0.02	0.04	0.08	0.06	0.00	0.00	0.00	0.39	0.00	0.00
Lachnospiraceae* bacterium AC2031	5.99	3.47	0.87	0.00	0.00	0.00	0.01	2.80	0.00	0.00	0.13	0.00	0.00
***Lachnospiraceae* bacterium C10**	5.35	2.37	1.05	0.01	0.04	0.15	0.04	0.08	8.72	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium C6A11**	4.71	2.91	0.72	0.00	0.00	0.09	0.02	7.42	0.97	0.00	0.00	0.00	0.00
Lachnospiraceae* bacterium C7 **	5.28	0.94	1.08	0.01	0.05	0.20	0.10	0.00	13.11	0.00	0.00	0.00	0.00
Lachnospiraceae* bacterium FE2018	4.76	0.38	0.86	0.00	0.08	0.06	0.01	0.00	7.50	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium KH1P17**	5.93	3.12	0.85	0.01	0.07	0.20	0.06	8.57	3.48	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium KH1T2**	6.19	2.43	0.99	0.00	0.04	0.16	0.08	6.26	6.57	0.00	0.50	0.00	0.00
***Lachnospiraceae* bacterium MA2020**	7.17	7.35	0.72	0.00	0.00	0.10	0.04	14.98	0.01	0.00	0.38	0.00	0.00
***Lachnospiraceae* bacterium MC2017**	5.56	3.82	0.79	0.00	0.00	0.14	0.06	0.00	0.13	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium NC2004 ***	5.96	2.17	0.94	0.00	0.08	0.08	0.01	8.42	0.22	0.00	0.20	0.00	0.00
***Lachnospiraceae* bacterium NC2008**	5.60	6.52	0.78	0.00	0.00	0.08	0.03	13.20	0.00	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium NK4A136**	6.69	6.97	0.72	0.00	0.01	0.00	0.00	13.67	0.00	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium NK4A144**	5.93	2.61	0.74	0.00	0.02	0.04	0.00	5.49	6.55	0.00	1.33	0.00	0.00
***Lachnospiraceae* bacterium NK4A179 ***	4.54	1.96	0.72	0.00	0.01	0.06	0.01	1.88	0.00	0.00	0.11	0.00	0.00
***Lachnospiraceae* bacterium P6A3**	3.99	2.86	0.40	0.00	0.02	0.04	0.02	4.66	1.64	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium XPB1003 ***	3.26	1.53	0.22	0.00	0.07	0.03	0.02	4.02	6.70	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium YSB2008**	5.42	3.38	0.41	0.00	0.02	0.07	0.00	1.85	2.36	0.00	0.00	0.00	0.00
***Lachnospiraceae* bacterium YSD2013**	4.53	2.44	0.83	0.01	0.04	0.25	0.13	6.05	0.27	0.00	0.00	0.00	0.00
***Lacrimispora aerotolerans* X8A62**	11.52	2.37	2.65	0.00	0.00	0.10	0.21	5.60	0.00	0.00	15.51	0.00	0.00
***Oribacterium* sp. FC2011**	8.59	0.42	0.84	0.00	0.00	0.07	0.04	7.07	1.56	0.00	0.00	0.00	0.00
***Oribacterium* sp. P6A1**	11.46	0.71	1.46	0.02	0.07	0.10	0.05	4.66	0.00	0.00	31.50	0.00	0.00
***Oribacterium* sp. WCC10**	6.16	0.32	0.82	0.00	0.00	0.20	0.14	2.74	0.48	0.00	27.86	0.00	0.00
***[Ruminococcus] gnavus* AGR2154**	8.10	0.48	0.85	0.00	0.01	0.05	0.03	3.88	1.22	0.66	15.18	0.00	0.00

Note: there were six strains grown on cellobiose (*) two strains that did not grow to satisfactory OD_{600} on any of the sugars, therefore, the growth on glucose was used despite poor growth (**). Finally, one strain was grown on melibiose (***) another hexose disaccharide, as it did not not grow on either cellobiose or glucose to an OD_{600} greater than 0.1.

Appendix 2. Protologger taxonomic placement information

Table A2. Taxonomic placement readout provided from Protologger using 16S rRNA percentage distances, ANI, and POCP values.

Strain Name	16S rRNA % identity	Genome match (Average Nucleotide Identity, ANI)	Genome Match (POCP, Percentage of Conserved Proteins)
***Acetitomaculum ruminis* DSM 5522 (139B)**	*Acetitomaculum ruminis 100.0%*	*ANI identified the input genome as being a strain of Acetitomaculum_ruminis due to an ANI value of 100.0%*	The input genome was assigned to *Acetitomaculum* with a POCP value of 99.9808098254%
***Blautia schinkii* DSM 10518 (B)**	*Blautia schinkii 100.0%*	ANI identified the input genome as being a strain of *Blautia_schinkii* due to an ANI value of 100.0%	The input genome was assigned to *Blautia* with a POCP value of 99.9913874774%
***Blautia wexlerae* AGR2146**	*Blautia wexlerae 99.1373112868%*	ANI identified the input genome as being a strain of *Blautia_wexlerae* due to an ANI value of 99.8514%	The input genome was assigned to *Blautia* with a POCP value of 60.0642673522%
***[Clostridium] clostridioforme* AGR2157**	*Dorea longicatena 99.7718631179%*	*ANI identified the input genome as novel, with the best match being to Dorea_longicatena with a value of 90.0123%*	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Dorea longicatena* AGR2136**	*Dorea formicigenerans 96.1832061069%*	ANI identified the input genome as being a strain of *Dorea_formicigenerans* due to an ANI value of 97.0177%	The input genome was assigned to *Coprococcus* with a POCP value of 52.5622466705%
***Dorea* sp. AGR2135**	*Clostridium bolteae 97.5%*	ANI identified the input genome as novel, with the best match being to *Clostridium_clostridioforme* with a value of 93.6071%	The input genome was assigned to *Clostridium* with a POCP value of 50.1658925017%
***Enterocloster clostridioformis* ATCC 25537 (T90)**	*Clostridium clostridioforme 100.0%*	ANI identified the input genome as being a strain of *Clostridium_clostridioforme* due to an ANI value of 100.0%	The input genome was assigned to *Clostridium* with a POCP value of 51.0326137744%
***Eubacterium ruminantium* FB3002**	*Desulfotomaculum carboxydivorans 86.4661654135%*	Due to the low similarity of the input genome to its validly named close relatives, no ANI values were able to be calculated.	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Eubacterium ruminantium* HUN269**	*Eubacterium ruminantium 99.4787788533%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_ruminantium* with a value of 80.4094%	The input genome was assigned to *Eubacterium* with a POCP value of 61.1079599168%
***Lachnobacterium bovis* AE2004**	*Lachnobacterium bovis 99.1741225052%*	ANI identified the input genome as novel, with the best match being to *Lachnobacterium_bovis* with a value of 85.2776%	The input genome was assigned to *Lachnobacterium* with a POCP value of 86.0632497274%
***Lachnobacterium bovis* NK4B19**	*Lachnobacterium bovis 99.3233082707%*	ANI identified the input genome as novel, with the best match being to *Lachnobacterium_bovis* with a value of 85.1976%	The input genome was assigned to *Lachnobacterium* with a POCP value of 84.1308298001%
***Lachnoclostridium aminophilum* F**	*Clostridium aminophilum 100.0%*	ANI identified the input genome as novel, with the best match being to *Clostridium_aminophilum* with a value of 93.6326%	The input genome was assigned to *Clostridium* with a POCP value of 86.0744985673%
***Lachnoclostridium aminophilum* KH1P1**	*Clostridium aminophilum 100.0%*	ANI identified the input genome as being a strain of *Clostridium_aminophilum* due to an ANI value of 100.0%	The input genome was assigned to *Clostridium* with a POCP value of 99.9812065401%
***Lachnospira multipara* ATCC 19207 (D32)**	*Lachnospira multipara 99.0291262136%*	ANI identified the input genome as being a strain of *Lachnospira_multipara* due to an ANI value of 98.004%	The input genome was assigned to *Lachnospira* with a POCP value of 89.6538002388%
***Lachnospira multipara* D15d**	*Lachnospira multipara 100.0%*	ANI identified the input genome as being a strain *of Lachnospira_multipara* due to an ANI value of 100.0%	The input genome was assigned to Lachnospira with a POCP value of 100.0%
***Lachnospira multipara* LB2003**	*Lachnospira multipara 98.6657303371%*	ANI identified the input genome as being a strain of *Lachnospira_multipara* due to an ANI value of 100.0%	The input genome was assigned to *Lachnospira* with a POCP value of 100.0%
***Lachnospira multipara* MC2003**	*Lachnospira multipara 98.5964912281%*	ANI identified the input genome as novel, with the best match being to *Lachnospira_multipara* with a value of 85.2113%	The input genome was assigned to *Lachnospira* with a POCP value of 83.0944963655%
***Lachnospira pectinoschiza* M83**	*Lactobacillus rogosae 95.2083333333%*	ANI identified the input genome as being a strain of *Lachnospira_multipara* due to an ANI value of 95.5522%	The input genome was assigned to *Lachnospira* with a POCP value of 82.2990420658%
***Lachnospiraceae* bacterium AC2012**	*Eubacterium oxidoreducens 94.2857142857%*	ANI identified the input genome as novel, with the best match being to *Pseudobutyrivibrio_ruminis* with a value of 79.8828%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium AC2014**	*Butyrivibrio hungatei 88.7585532747%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_oxidoreducens* with a value of 78.784%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium AC2028**	*Clostridium aminophilum 92.5847457627%*	No validly named species with a sequenced genome within the GTDB-Tk database was identified to have an ANI value >95% with the studied genome	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium AC2029**	*Eubacterium ruminantium 94.5492662474%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_ruminantium* with a value of 78.7099%	No POCP values >50% were identified, indicating the input genome represents a novel genus
Lachnospiraceae* bacterium *AC2031	*Eubacterium ruminantium 92.4515235457%*	No POCP values >50% were identified, indicating the input genome represents a novel genus	No POCP values >50% were identified, indicating the input genome represents a novel genus
Lachnospiraceae* bacterium *C10	*Eubacterium oxidoreducens 92.0%*	ANI identified the input genome as novel, with the best match being to *Lachnospira_multipara* with a value of 80.622%	No POCP values >50% were identified, indicating the input genome represents a novel genus

Strain Name	16S rRNA % identity	Genome match (Average Nucleotide Identity, ANI)	Genome Match (POCP, percentage of conserved proteins)
***Lachnospiraceae* bacterium C6A11**	*Eubacterium ruminantium 96.7517401392%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_ruminantium* with a value of 80.6182%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium C7**	*Lachnobacterium bovis 92.8057553957%*	ANI identified the input genome as novel, with the best match being to *Lachnobacterium_bovis* with a value of 81.0423%	The input genome was assigned to Lachnobacterium with a POCP value of 74.7770172163%
***Lachnospiraceae* bacterium FE2018**	*Syntrophococcus sucromutans 91.3879003559%*	ANI identified the input genome as novel, with the best match being to *Fusicatenibacter_saccharivorans* with a value of 76.3455%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium KH1P17**	*Anaerosporobacter mobilis (Valid) (89.84375%)*	ANI identified the input genome as novel, with the best match being to *Eubacterium_eligens* with a value of 76.6471%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium KH1T2**	*Roseburia intestinalis 89.1366906475%*	ANI identified the input genome as novel, with the best match being to *Lachnospira_multipara* with a value of 79.0983%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium MA2020**	*Clostridium aldenense 89.201183432%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 77.6504%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium MC2017**	*Coprococcus eutactus 90.618336887%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 78.6444%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium NC2004**	*Coprococcus eutactus 92.1469740634%*	ANI identified the input genome as being a strain of *Clostridium_aerotolerans* due to an ANI value of 100.0%	
***Lachnospiraceae* bacterium NC2008**	*Dorea longicatena 92.5212027756%*	ANI identified the input genome as novel, with the best match being to *Coprococcus_eutactus* with a value of 77.8362%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium NK4A136**	*Lachnospira multipara 92.1462423832%*	ANI identified the input genome as novel, with the best match being to *Lachnospira_multipara* with a value of 77.9429%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium NK4A144**	*Lachnobacterium bovis 99.1741225052%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 77.504%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium NK4A179**	*Kineothrix alysoides 90.863668808%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_ruminantium* with a value of 77.479%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium P6A3**	*Falcatimonas natans 89.349112426%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 76.6511%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium XPB1003**	*Clostridium fimetarium 91.6726489591%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 77.0714%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lachnospiraceae* bacterium YSB2008**	*Eubacterium ruminantium 93.8013442868%*	ANI identified the input genome as novel, with the best match being to *Eubacterium_ruminantium* with a value of 78.8953%	The input genome was assigned to *Eubacterium* with a POCP value of 51.5369261477%
***Lachnospiraceae* bacterium YSD2013**	*Faecalimonas umbilicata 93.074204947%*	Due to the low similarity of the input genome to its validly named close relatives, no ANI values were able to be calculated.	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Lacrimispora aerotolerans* DSM 5434 (X8A62)**	*Clostridium aerotolerans 100.0%*	ANI identified the input genome as being a strain of *Clostridium_aerotolerans* due to an ANI value of 100.0%	The input genome was assigned to *Clostridium* with a POCP value of 60.9296734121%
***Oribacterium* sp. FC2011**	*Oribacterium sinus 96%*	Due to the low similarity of the input genome to its validly named close relatives, no ANI values were able to be calculated.	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Oribacterium* sp. P6A1**	*Oribacterium sinus 96%*	ANI identified the input genome as novel, with the best match being to *Butyrivibrio_fibrisolvens* with a value of 77.5393%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***Oribacterium* sp. WCC10**	*Roseburia inulinivorans 91.4942528736%*	ANI identified the input genome as novel, with the best match being to *Clostridium_aminophilum* with a value of 79.774%	No POCP values >50% were identified, indicating the input genome represents a novel genus
***[Ruminococcus] gnavus* AGR2154**	*Ruminococcus gnavus 99.1338582677%*	ANI identified the input genome as being a strain of *Ruminococcus_gnavus* due to an ANI value of 96.1489%	The input genome was assigned to Ruminococcus with a POCP value of 72.8286304198%

Appendix 3. Further relevant genes and pathways

Note: Information detailed in the figures and tables below were taken from the IMG database and Meta Cyc (Caspi et al., 2020; Nordberg et al., 2014).

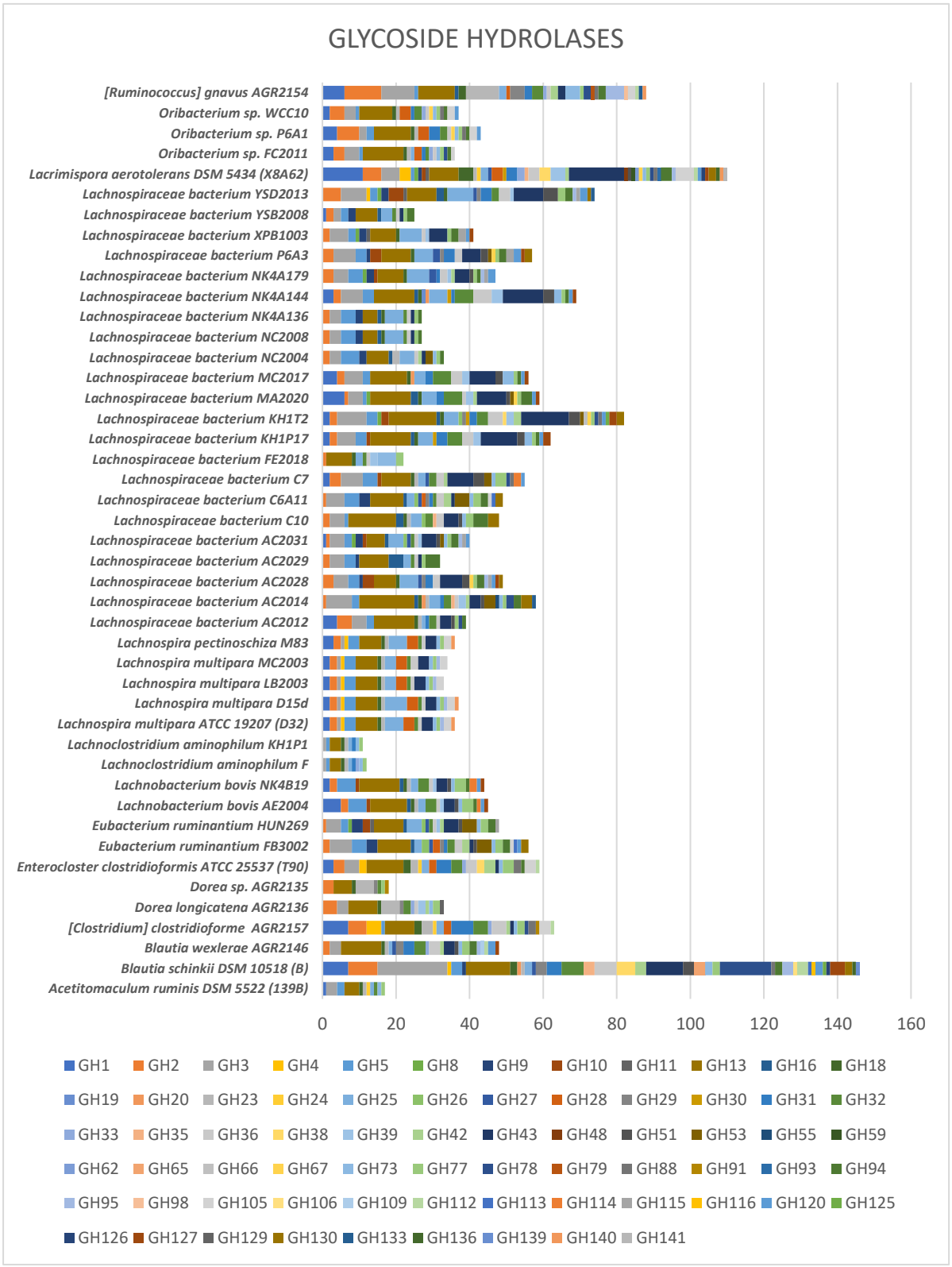


Figure A3.1 Summary of GH genes found in the genomes of 45 *Lachnospiraceae* strains.

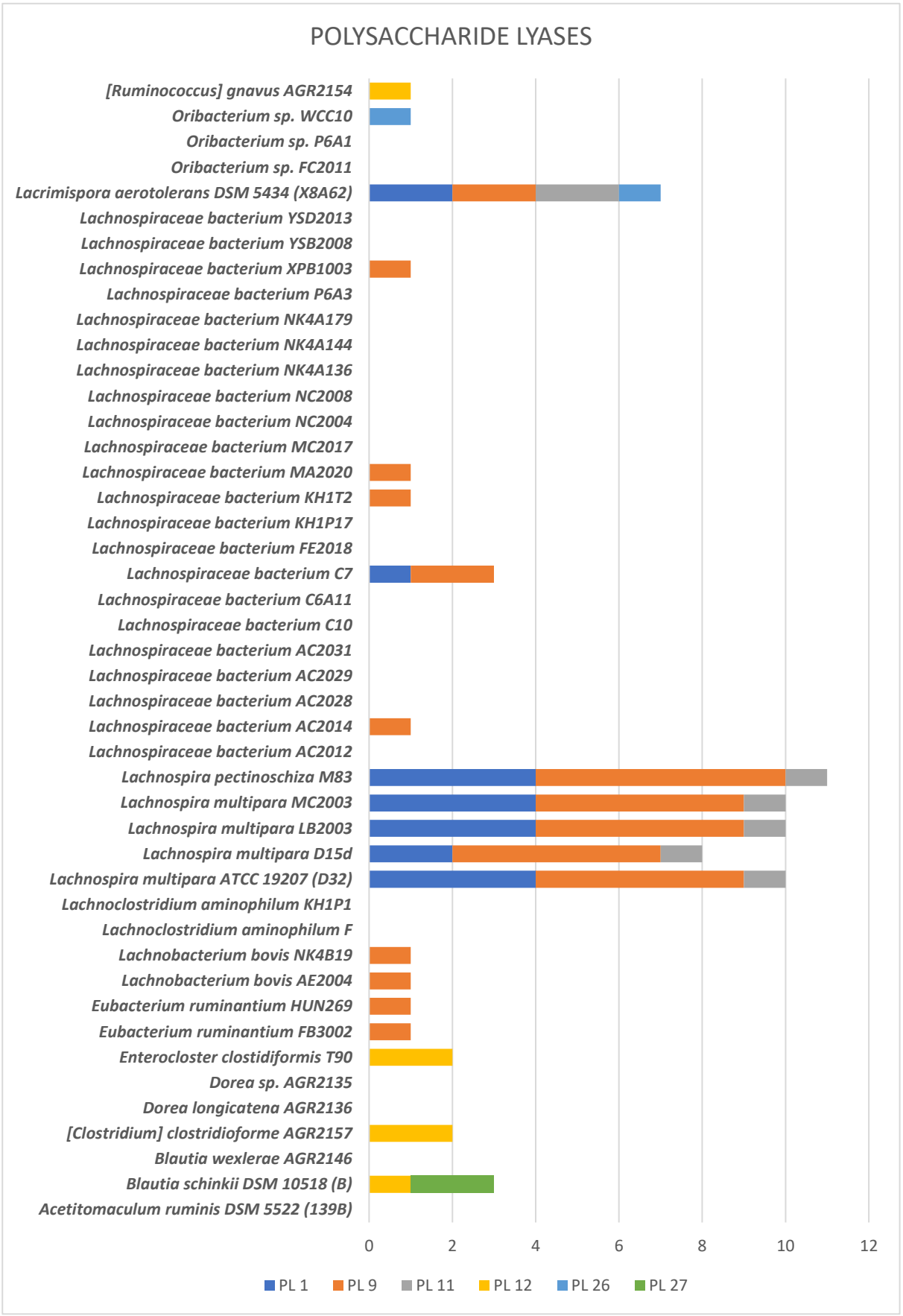


Figure A3.2 Summary of PL genes found in the genomes of 45 *Lachnospiraceae* strains

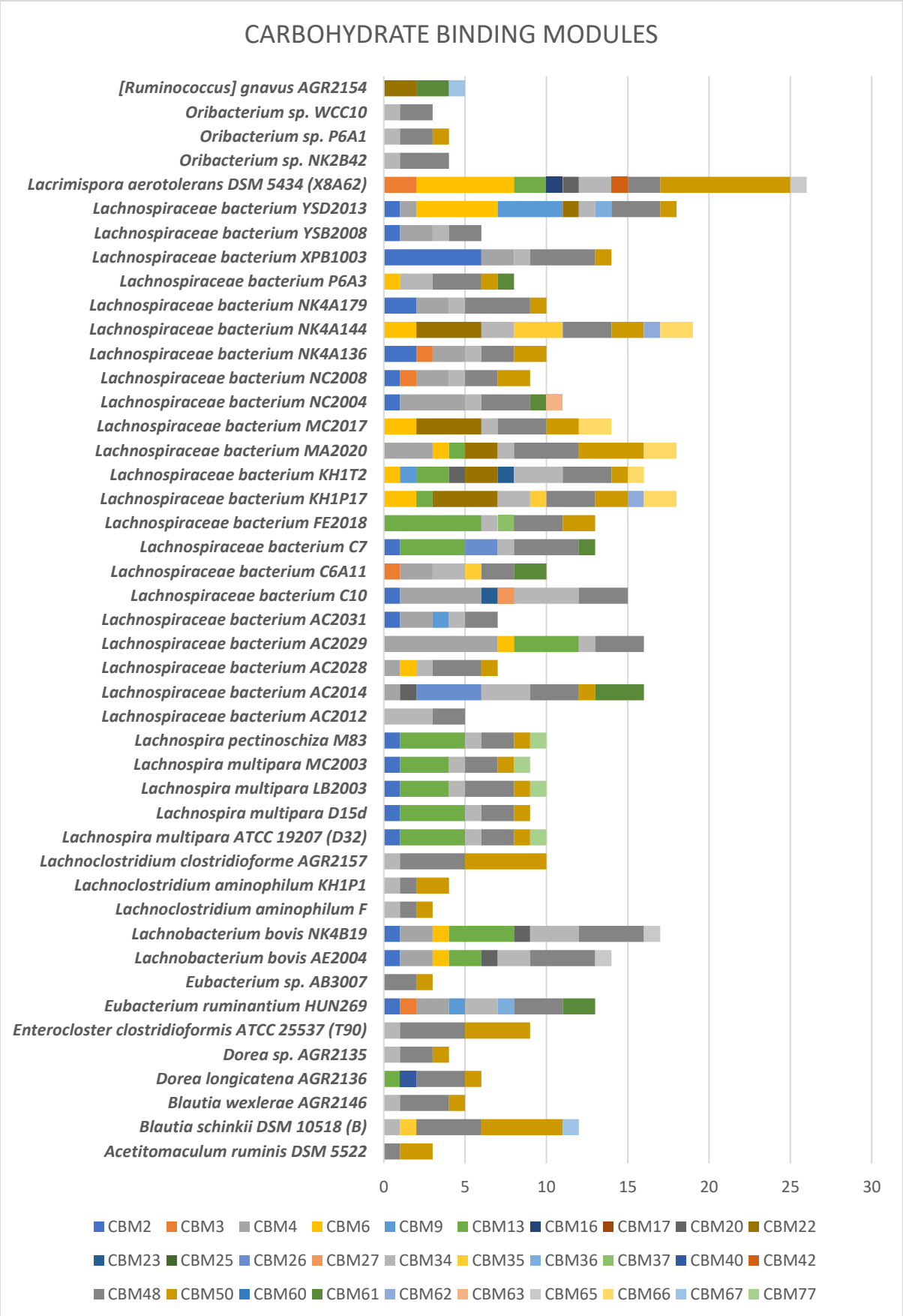


Figure A3.3 Summary of CBM genes found in the genomes of 45 *Lachnospiraceae* strains

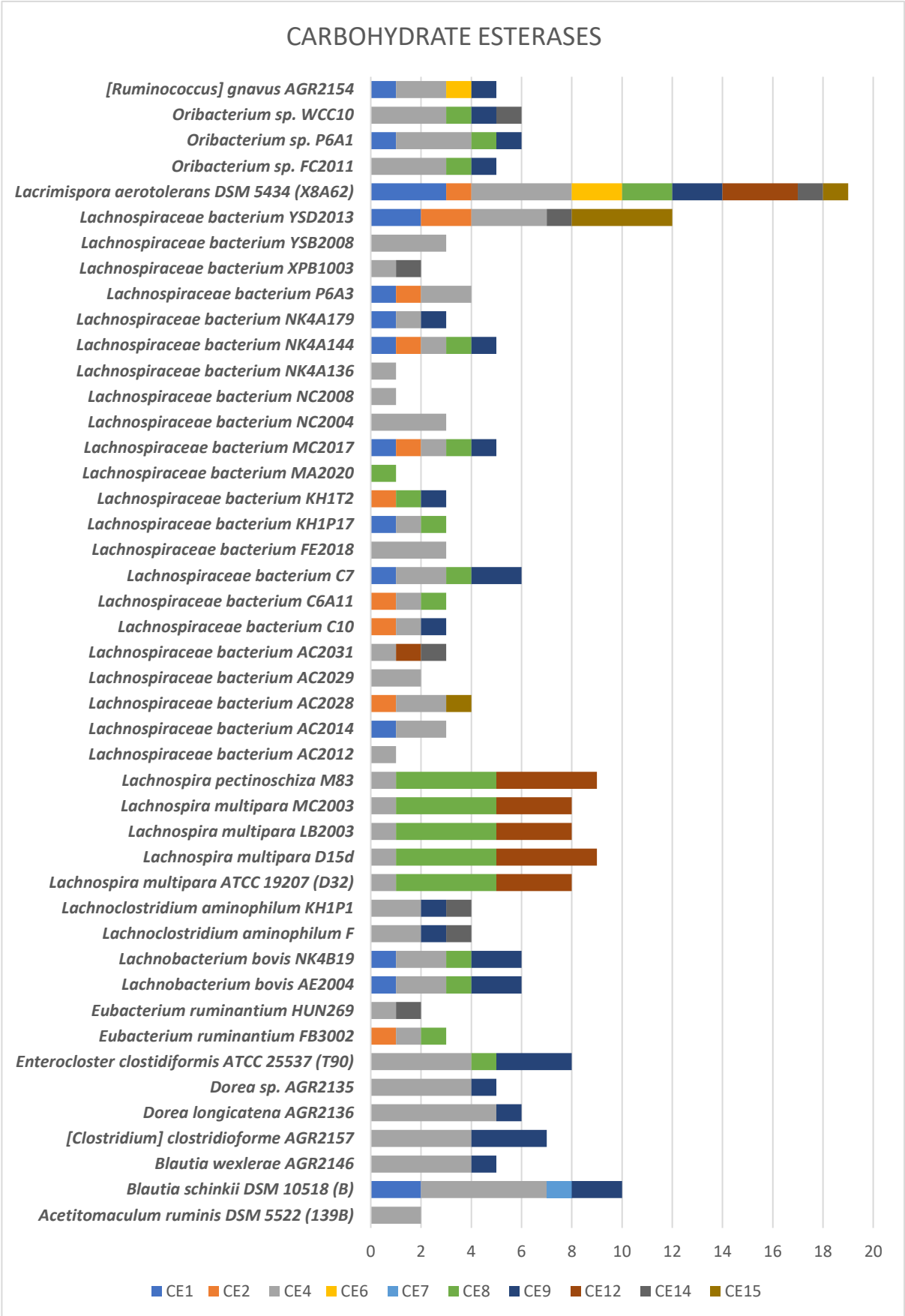


Figure A3.4 Summary of CE genes found in the genomes of 45 *Lachnospiraceae* strains

Table A3.1 Hydrogenase genes present in the genomes of the 45 *Lachnospiraceae* strains

Gene Product Name	Genome Name
Hydrogenase maturation protein HypC	*Acetitomaculum ruminis* DSM 5522
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Acetitomaculum ruminis* DSM 5522
Hydrogenase maturation protein, carbamoyl transferase HypF	*Acetitomaculum ruminis* DSM 5522
Hydrogenase maturation protein, carbamoyl dehydratase HypE	*Acetitomaculum ruminis* DSM 5522
Hydrogenase maturation protein HypD	*Acetitomaculum ruminis* DSM 5522
iron-only hydrogenase maturation protein HydE	*Acetitomaculum ruminis* DSM 5522
Hydrogenase maturation factor	*Acetitomaculum ruminis* DSM 5522
iron-only hydrogenase maturation protein HydE	*Blautia schinkii* DSM 10518
[FeFe] hydrogenase, group B1/B3	*Blautia schinkii* DSM 10518
iron-only hydrogenase maturation protein HydG	*Blautia schinkii* DSM 10518
iron-only hydrogenase maturation protein HydF	*Blautia schinkii* DSM 10518
ferredoxin hydrogenase	*Blautia schinkii* DSM 10518
Hydrogenase maturation factor	*Blautia schinkii* DSM 10518
iron-only hydrogenase maturation protein HydE	*Blautia wexlerae* AGR2146
[FeFe] hydrogenase, group B1/B3	*Blautia wexlerae* AGR2146
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Blautia wexlerae* AGR2146
iron-only hydrogenase maturation protein HydG	*Blautia wexlerae* AGR2146
Hydrogenase maturation factor	*Blautia wexlerae* AGR2146
[FeFe] hydrogenase, group A	*Clostridium clostridioforme* AGR2157
iron-only hydrogenase maturation protein HydE	*Clostridium clostridioforme* AGR2157
[FeFe] hydrogenase, group B1/B3	*Clostridium clostridioforme* AGR2157
iron-only hydrogenase maturation protein HydF	*Clostridium clostridioforme* AGR2157
[FeFe] hydrogenase, group B1/B3	*Clostridium clostridioforme* AGR2157
iron-only hydrogenase maturation protein HydG	*Clostridium clostridioforme* AGR2157
iron-only hydrogenase maturation protein HydE	*Dorea longicatena* AGR2136
hydrogenase expression/formation protein HypC	*Dorea longicatena* AGR2136
hydrogenase expression/formation protein HypD	*Dorea longicatena* AGR2136
hydrogenase expression/formation protein HypE	*Dorea longicatena* AGR2136
hydrogenase maturation protein HypF	*Dorea longicatena* AGR2136
[FeFe] hydrogenase, group B1/B3	*Dorea longicatena* AGR2136
iron-only hydrogenase maturation protein HydF	*Dorea longicatena* AGR2136
Hydrogenase maturation factor	*Dorea longicatena* AGR2136
iron-only hydrogenase maturation protein HydE	*Dorea* sp. AGR2135
hydrogenase expression/formation protein HypD	*Dorea* sp. AGR2135
hydrogenase expression/formation protein HypC	*Dorea* sp. AGR2135
hydrogenase expression/formation protein HypE	*Dorea* sp. AGR2135
hydrogenase maturation protein HypF	*Dorea* sp. AGR2135
iron-only hydrogenase maturation protein HydF	*Dorea* sp. AGR2135
Hydrogenase maturation factor	*Dorea* sp. AGR2135
iron-only hydrogenase maturation protein HydF	*Enterocloster clostridioformis* ATCC 25537
iron-only hydrogenase maturation protein HydG	*Enterocloster clostridioformis* ATCC 25537
[FeFe] hydrogenase, group A	*Enterocloster clostridioformis* ATCC 25537
[FeFe] hydrogenase, group B1/B3	*Enterocloster clostridioformis* ATCC 25537
[FeFe] hydrogenase, group B1/B3	*Enterocloster clostridioformis* ATCC 25537
ferredoxin hydrogenase large subunit	*Eubacterium ruminantium* FB3002
Hydrogenase maturation factor	*Eubacterium ruminantium* FB3002
ferredoxin hydrogenase large subunit	*Eubacterium ruminatium* HUN269
Hydrogenase maturation factor	*Eubacterium ruminatium* HUN269
ferredoxin hydrogenase large subunit	*Lachnobacterium bovis* AE2004
iron-only hydrogenase maturation protein HydE	*Lachnobacterium bovis* AE2004
iron-only hydrogenase maturation protein HydF	*Lachnobacterium bovis* AE2004
Hydrogenase maturation factor	*Lachnobacterium bovis* AE2004
iron-only hydrogenase maturation protein HydE	*Lachnobacterium bovis* NK4B19
iron-only hydrogenase maturation protein HydF	*Lachnobacterium bovis* NK4B19
ferredoxin hydrogenase large subunit	*Lachnobacterium bovis N*K4B19
Hydrogenase maturation factor	*Lachnobacterium bovis* NK4B19
ferredoxin hydrogenase large subunit	*Lachnoclostridium aminophilum* F
Iron hydrogenase small subunit	*Lachnoclostridium aminophilum* KH1P1
ferredoxin hydrogenase large subunit	*Lachnoclostridium aminophilum* KH1P1
hydrogenase maturation protein HypF	*Lachnospira multipara* ATCC 19207
hydrogenase expression/formation protein HypE	*Lachnospira multipara* ATCC 19207
hydrogenase expression/formation protein HypE	*Lachnospira multipara* ATCC 19207
hydrogenase expression/formation protein HypD	*Lachnospira multipara* ATCC 19207
Ni,Fe-hydrogenase III large subunit	*Lachnospira multipara* ATCC 19207

hydrogenase expression/formation protein HypC	*Lachnospira multipara* ATCC 19207
hydrogenase expression/formation protein HypE	*Lachnospira multipara* D15d
hydrogenase expression/formation protein HypD	*Lachnospira multipara* D15d
hydrogenase maturation protein HypF	*Lachnospira multipara* D15d
hydrogenase expression/formation protein HypC	*Lachnospira multipara* D15d
Ni,Fe-hydrogenase III large subunit	*Lachnospira multipara* D15d
hydrogenase expression/formation protein HypE	*Lachnospira multipara* D15d
hydrogenase expression/formation protein HypE	*Lachnospira multipara* LB2003
hydrogenase expression/formation protein HypE	*Lachnospira multipara* LB2003
hydrogenase expression/formation protein HypD	*Lachnospira multipara* LB2003
Ni,Fe-hydrogenase III large subunit	*Lachnospira multipara* LB2003
hydrogenase expression/formation protein HypC	*Lachnospira multipara* LB2003
hydrogenase maturation protein HypF	*Lachnospira multipara* LB2003
hydrogenase expression/formation protein HypE	*Lachnospira multipara* MC2003
hydrogenase maturation protein HypF	*Lachnospira multipara* MC2003
hydrogenase expression/formation protein HypE	*Lachnospira multipara* MC2003
Ni,Fe-hydrogenase III large subunit	*Lachnospira multipara* MC2003
hydrogenase expression/formation protein HypD	*Lachnospira multipara* MC2003
hydrogenase expression/formation protein HypC	*Lachnospira multipara* MC2003
hydrogenase expression/formation protein HypD	*Lachnospira pectinoschiza* M83
hydrogenase expression/formation protein HypC	*Lachnospira pectinoschiza* M83
hydrogenase maturation protein HypF	*Lachnospira pectinoschiza* M83
Ni,Fe-hydrogenase III large subunit	*Lachnospira pectinoschiza* M83
hydrogenase expression/formation protein HypE	*Lachnospira pectinoschiza* M83
hydrogenase expression/formation protein HypE	*Lachnospira pectinoschiza* M83
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium AC2012
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium AC2012
Hydrogenase maturation factor	*Lachnospiraceae* bacterium AC2012
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium AC2014
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium AC2014
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium AC2014
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium AC2014
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium AC2014
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium AC2014
Hydrogenase maturation factor	*Lachnospiraceae* bacterium AC2014
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium AC2028
iron-only hydrogenase maturation protein HydE	*Lachnospiraceae* bacterium AC2029
iron-only hydrogenase maturation protein HydF	*Lachnospiraceae* bacterium AC2029
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium AC2029
Hydrogenase maturation factor	*Lachnospiraceae* bacterium AC2029
iron-only hydrogenase maturation protein HydE	*Lachnospiraceae* bacterium AC2031
iron-only hydrogenase maturation protein HydF	*Lachnospiraceae* bacterium AC2031
Hydrogenase maturation protein HypC	*Lachnospiraceae* bacterium AC2031
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium AC2031
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium AC2031
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium AC2031
Hydrogenase maturation factor	*Lachnospiraceae* bacterium AC2031
iron-only hydrogenase maturation protein HydF	*Lachnospiraceae* bacterium C10
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium C10
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium C10
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium C10
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium C10
Hydrogenase maturation factor	*Lachnospiraceae* bacterium C10
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium C6A11
Hydrogenase maturation factor	*Lachnospiraceae* bacterium C6A11
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium C7
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium C7
Hydrogenase maturation factor	*Lachnospiraceae* bacterium C7
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium FE2018
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium FE2018
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium FE2018
iron-only hydrogenase maturation protein HydE	*Lachnospiraceae* bacterium FE2018
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium FE2018
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium FE2018
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium FE2018
Hydrogenase maturation factor	*Lachnospiraceae* bacterium FE2018
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium KH1P17

[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium KH1P17
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium KH1P17
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium KH1P17
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium KH1P17
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium KH1P17
Hydrogenase maturation factor	*Lachnospiraceae* bacterium KH1P17
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium KH1T2
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium KH1T2
iron-only hydrogenase maturation protein HydE	*Lachnospiraceae* bacterium KH1T2
Hydrogenase maturation protein, carbamoyl dehydratase HypE	*Lachnospiraceae* bacterium KH1T2
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium KH1T2
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium KH1T2
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium KH1T2
Hydrogenase maturation factor	*Lachnospiraceae* bacterium KH1T2
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium MA2020
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium MA2020
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium MA2020
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium MA2020
Hydrogenase maturation factor	*Lachnospiraceae* bacterium MA2020
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium MC2017
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium MC2017
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lachnospiraceae* bacterium MC2017
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium MC2017
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium MC2017
Hydrogenase maturation factor	*Lachnospiraceae* bacterium MC2017
iron-only hydrogenase maturation protein HydF	*Lachnospiraceae* bacterium NC2004
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium NC2004
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium NC2004
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium NC2004
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium NC2004
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium NC2004
Hydrogenase maturation factor	*Lachnospiraceae* bacterium NC2004
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium NC2008
Hydrogenase maturation factor	*Lachnospiraceae* bacterium NC2008
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium NK4A136
Hydrogenase maturation factor	*Lachnospiraceae* bacterium NK4A136
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium NK4A144
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium NK4A144
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium NK4A144
iron-only hydrogenase maturation protein HydF	*Lachnospiraceae* bacterium NK4A144
iron-only hydrogenase maturation protein HydE	*Lachnospiraceae* bacterium NK4A144
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium NK4A144
Hydrogenase maturation factor	*Lachnospiraceae* bacterium NK4A144
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium NK4A179
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium NK4A179
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium NK4A179
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium NK4A179
Hydrogenase maturation factor	*Lachnospiraceae* bacterium *NK4A179*
[FeFe] hydrogenase, group B1/B3	*Lachnospiraceae* bacterium P6A3
hydrogenase expression/formation protein HypC	*Lachnospiraceae* bacterium XPB1003
hydrogenase expression/formation protein HypD	*Lachnospiraceae* bacterium XPB1003
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium XPB1003
hydrogenase expression/formation protein HypE	*Lachnospiraceae* bacterium XPB1003
hydrogenase maturation protein HypF	*Lachnospiraceae* bacterium XPB1003
ferredoxin hydrogenase large subunit	*Lachnospiraceae* bacterium YSB2008
Hydrogenase maturation factor	*Lachnospiraceae* bacterium YSB2008
hydrogenase maturation protein HypF	*Lacrimispora aerotolerans* DSM 5434
hydrogenase expression/formation protein HypE	*Lacrimispora aerotolerans* DSM 5434
hydrogenase expression/formation protein HypD	*Lacrimispora aerotolerans* DSM 5434
ferredoxin hydrogenase large subunit	*Lacrimispora aerotolerans* DSM 5434
hydrogenase expression/formation protein HypC	*Lacrimispora aerotolerans* DSM 5434
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Lacrimispora aerotolerans* DSM 5434
[FeFe] hydrogenase H-cluster maturation GTPase HydF	*Oribacterium* sp. FC2011
[FeFe] hydrogenase, group B1/B3	*Oribacterium* sp. FC2011
[FeFe] hydrogenase, group B1/B3	*Oribacterium* sp. P6A1
[FeFe] hydrogenase, group B1/B3	*Oribacterium* sp. WCC10
iron-only hydrogenase maturation protein HydF	*Ruminococcus gnavus* AGR2154

[FeFe] hydrogenase, group B1/B3	*Ruminococcus gnavus* AGR2154
iron-only hydrogenase maturation protein HydE	*Ruminococcus gnavus* AGR2154
iron-only hydrogenase maturation protein HydG	*Ruminococcus gnavus* AGR2154
Hydrogenase maturation factor	*Ruminococcus gnavus* AGR2154

Table A3.2 Reductive acetogenesis pathway genes present in the genomes of *Lachnospiraceae* strains

Gene Product Name	Genome Name
acetyl-CoA synthase	*Acetitomaculum ruminis* DSM 5522
acetyl-CoA synthase	*Acetitomaculum ruminis* DSM 5522
carbon-monoxide dehydrogenase small subunit	*Blautia schinkii* DSM 10518
carbon-monoxide dehydrogenase small subunit	*Blautia schinkii* DSM 10518
carbon-monoxide dehydrogenase small subunit	*Blautia wexlerae* AGR2146
carbon-monoxide dehydrogenase small subunit	*Blautia wexlerae* AGR2146
carbon-monoxide dehydrogenase small subunit	*Clostridium clostridioforme* AGR2157
carbon-monoxide dehydrogenase small subunit	*Clostridium clostridioforme* AGR2157
carbon-monoxide dehydrogenase small subunit	*Clostridium clostridioforme* AGR2157
carbon-monoxide dehydrogenase small subunit	*Dorea longicatena* AGR2136
carbon-monoxide dehydrogenase small subunit	*Dorea* sp. AGR2135
carbon-monoxide dehydrogenase small subunit	*Enterocloster clostridioformis* ATCC 25537
carbon-monoxide dehydrogenase small subunit	*Enterocloster clostridioformis* ATCC 25537
carbon-monoxide dehydrogenase small subunit	*Enterocloster clostridioformis* ATCC 25537
carbon-monoxide dehydrogenase small subunit	*Enterocloster clostridioformis* ATCC 25537
carbon-monoxide dehydrogenase small subunit	*Lachnoclostridium aminophilum* F
carbon-monoxide dehydrogenase small subunit	*Lachnoclostridium aminophilum* KH1P1
carbon-monoxide dehydrogenase small subunit	*Lachnospiraceae* bacterium KH1P17
carbon-monoxide dehydrogenase small subunit	*Lachnospiraceae* bacterium MC2017
carbon-monoxide dehydrogenase small subunit	*Lachnospiraceae* bacterium NK4A144
carbon-monoxide dehydrogenase small subunit	*Ruminococcus gnavus* AGR2154
carbon-monoxide dehydrogenase small subunit	*Ruminococcus gnavus* AGR2154
pyruvate ferredoxin oxidoreductase alpha subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase alpha subunit	*Lachnospiraceae* bacterium NC2004
pyruvate ferredoxin oxidoreductase alpha subunit	*Blautia schinkii* DSM 10518
pyruvate ferredoxin oxidoreductase alpha subunit	*Lachnospiraceae* bacterium P6A3
pyruvate ferredoxin oxidoreductase alpha subunit	*Eubacterium ruminatium* HUN269
pyruvate ferredoxin oxidoreductase beta subunit	*Dorea* sp. AGR2135
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium NK4A136
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium YSB2008
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium NC2008
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium NC2004
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium NC2004
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium C6A11
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium AC2028
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium AC2029
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium AC2014
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium P6A3
pyruvate ferredoxin oxidoreductase beta subunit	*Lachnospiraceae* bacterium FE2018
pyruvate ferredoxin oxidoreductase beta subunit	*Eubacterium ruminatium* HUN269
pyruvate ferredoxin oxidoreductase beta subunit	*Eubacterium ruminantium* FB3002
pyruvate ferredoxin oxidoreductase delta subunit	*Dorea* sp. AGR2135
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium NK4A136
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium YSB2008
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium NC2008
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium NC2004
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium C6A11
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium AC2028
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium AC2029
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium AC2014
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium P6A3
pyruvate ferredoxin oxidoreductase delta subunit	*Lachnospiraceae* bacterium FE2018
pyruvate ferredoxin oxidoreductase delta subunit	*Eubacterium ruminatium H*UN269
pyruvate ferredoxin oxidoreductase delta subunit	*Eubacterium ruminantium* FB3002
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium AC2031
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium YSB2008
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium NC2004

pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium C6A11
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium AC2028
pyruvate ferredoxin oxidoreductase gamma subunit	*Blautia schinkii* DSM 10518
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium AC2029
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium AC2014
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium P6A3
pyruvate ferredoxin oxidoreductase gamma subunit	*Lachnospiraceae* bacterium FE2018
pyruvate ferredoxin oxidoreductase gamma subunit	*Eubacterium ruminatium* HUN269
pyruvate ferredoxin oxidoreductase gamma subunit	*Eubacterium ruminantium* FB3002

CARBOHYDRATE METABOLISM	139B	B	AGR2146	AGR2136	AGR2135	AGR2157	T90	FB3002	HUN269	NK4B19	AE2004	F	KH1P1	D15d	D32	LB2003	MC2003	M83	AC2012	AC2014	AC2028	AC2029	AC2031	C10	C6A11	C7	FE2018	KH1P17	KH172	MA2020	MC2017	NC2004	NC2008	NK4A136	NK4A144	NK4A179	P6A3	XPB1003	YSB2008	YSD2013	XBA62	FC2011	P6A1	WCC10	AGR2154
glycolysis III (from glucose)	8	26	13	17	13	11	23	11	10	10	11	10	10	12	12	12	12	12	11	10	11	10	9	11	10	12	11	13	11	12	11	11	12	10	12	8	10	7	9	9	17	13	14	14	12
Entner-Doudoroff shunt	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycolysis I (from glucose 6-phosphate)	9	27	14	19	14	13	25	12	12	11	12	11	11	13	13	13	13	13	11	11	11	10	10	11	10	13	12	14	11	13	12	11	13	11	13	9	10	8	11	9	18	14	15	15	13
pentose phosphate pathway (non-oxidative branch) I	4	18	5	15	9	4	16	4	4	6	6	7	7	5	5	7	7	5	7	6	6	4	7	9	4	8	14	8	5	10	6	3	5	5	7	4	5	5	6	4	14	9	7	9	10
Entner-Doudoroff pathway II (non-phosphorylative)	2	8	5	5	3	7	6	8	6	4	4	3	3	4	3	4	4	5	4	6	4	7	11	4	6	4	9	4	5	5	3	10	6	5	4	2	4	1	5	2	5	3	3	3	5
pentose phosphate pathway (oxidative branch) I	0	5	1	4	0	0	5	0	0	0	0	1	1	1	1	1	1	1	1	0	1	0	0	1	0	0	1	1	0	0	0	0	1	1	0	1	1	1	0	1	1	2	1	1	0
pentose phosphate pathway (partial)	3	13	3	11	3	2	12	2	2	3	3	4	4	2	2	4	4	2	3	4	4	2	5	6	2	5	13	6	4	7	5	2	4	4	6	3	4	4	4	3	9	4	3	6	8
glycolysis V (Pyrococcus)	5	19	7	9	7	6	14	6	7	5	6	5	5	6	6	6	6	6	6	5	4	6	5	6	5	6	7	10	7	8	6	6	7	6	7	4	4	2	5	5	9	6	6	6	7
pentose phosphate pathway	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycolysis IV	7	24	12	16	12	10	22	10	9	10	10	9	9	11	11	11	11	11	10	9	8	9	8	10	9	10	11	12	10	11	10	10	11	10	11	9	8	7	7	8	16	12	13	13	12
Entner-Doudoroff pathway III (semi-phosphorylative)	2	5	5	3	3	4	5	3	3	3	3	2	2	3	3	3	3	3	3	2	1	3	3	3	2	3	3	3	3	4	2	3	4	3	3	3	1	2	1	3	6	3	3	3	7
glycolysis II (from fructose 6-phosphate)	8	25	13	18	13	12	24	11	11	11	11	10	10	12	12	12	12	12	10	10	10	9	9	10	9	12	11	13	10	12	11	10	12	10	12	9	9	8	10	8	17	13	14	14	13
pentose phosphate pathway (oxidative branch) II	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Entner-Doudoroff pathway I	3	8	6	7	5	4	9	4	4	4	4	4	4	5	5	5	5	5	5	3	5	3	3	5	3	5	5	5	3	3	3	4	6	4	4	4	4	4	4	5	8	7	6	6	5
pentose phosphate pathway (non-oxidative branch) II	5	32	8	21	11	6	27	7	6	8	8	8	8	8	8	10	10	8	8	8	9	6	9	11	7	10	17	13	9	14	10	6	9	9	11	7	8	7	8	6	15	12	9	12	12

FERMENTATION TO SCFAs	139B	B	AGR2146	AGR2136	AGR2135	AGR2157	T90	FB3002	HUN269	NK4B19	AE2004	F	KH1P1	D15d	D32	LB2003	MC2003	M83	AC2012	AC2014	AC2028	AC2029	AC2031	C10	C6A11	C7	FE2018	KH1P17	KH172	MA2020	MC2017	NC2004	NC2008	NK4A136	NK4A144	NK4A179	P6A3	XPB1003	YSB2008	YSD2013	XBA62	FC2011	P6A1	WCC10	AGR2154
homolactic fermentation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to butanoate	1	3	1	1	1	6	1	5	4	1	1	1	1	1	1	1	1	1	1	5	5	5	10	1	5	1	9	2	3	2	2	8	4	4	2	1	5	1	5	0	1	1	2	2	1
mixed acid fermentation	9	13	11	11	11	9	11	9	6	5	5	8	8	7	7	7	7	7	7	11	5	8	7	7	9	5	7	9	10	9	10	6	7	6	11	8	6	6	6	6	18	6	11	9	9
L-glutamate degradation VII (to butanoate)	1	3	1	1	1	5	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
pyruvate fermentation to propanoate I	2	3	2	4	3	4	3	4	2	0	0	3	3	2	2	2	2	2	2	4	3	4	2	2	4	1	3	4	4	5	5	2	2	2	5	3	3	2	2	3	6	2	6	4	2
heterolactic fermentation	12	20	16	18	15	8	20	11	9	8	9	12	10	11	11	12	12	12	10	12	10	8	9	12	9	11	10	14	12	12	11	9	12	10	11	8	11	9	9	12	25	12	13	13	14
Bifidobacterium shunt	10	26	13	22	18	10	24	11	12	11	12	15	13	12	12	15	15	13	13	12	11	11	13	16	10	15	21	15	12	19	11	10	11	10	13	9	11	9	11	10	25	16	15	16	19
pyruvate fermentation to acetate I	1	3	1	1	1	5	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
acetylene degradation (anaerobic)	5	7	5	5	5	2	5	2	1	1	1	2	2	2	2	2	2	2	1	4	1	1	1	2	2	1	2	2	2	1	2	1	1	1	2	2	2	2	1	1	10	2	3	3	4
L-glutamate degradation V (via hydroxyglutarate)	1	5	1	2	3	2	1	1	1	1	1	9	9	1	1	1	1	1	1	4	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	2
L-lysine fermentation to acetate and butanoate	2	7	3	5	1	1	5	2	1	1	1	2	2	1	1	1	1	1	1	4	1	1	1	1	2	1	1	2	3	1	3	1	1	1	3	1	1	1	1	1	10	2	2	2	2
pyruvate fermentation to acetate and (S)-lactate I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hexitol fermentation to lactate, formate, ethanol and acetate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
superpathway of L-alanine fermentation (Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4-aminobutanoate degradation V	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
L-glutamate degradation VIII (to propanoate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to acetate and alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to acetate and lactate II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
propanoate fermentation to 2-methylbutanoate	0	1	0	1	0	0	2	1	1	1	1	3	1	0	0	0	0	0	1	1	1	2	1	1	1	2	1	1	1	1	1	1	2	2	1	1	1	1	1	1	1	0	0	0	0
pyruvate fermentation to (S)-lactate	1	0	1	1	1	0	1	1	1	0	0	3	1	1	1	2	2	2	0	0	1	1	0	1	1	0	0	0	1	3	0	1	0	0	0	0	1	0	1	0	3	0	1	0	2
pyruvate fermentation to acetate II	2	4	3	3	1	1	3	2	1	1	1	2	2	1	1	1	1	1	1	3	1	1	1	2	2	1	2	1	1	1	2	1	1	1	2	1	1	1	1	1	5	1	1	1	2
pyruvate fermentation to acetate III	1	4	1	1	1	5	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
pyruvate fermentation to acetate IV	2	5	4	4	2	2	4	3	2	2	2	3	3	2	2	2	2	2	2	4	2	2	2	3	2	1	3	3	2	3	2	2	2	1	3	2	2	2	2	2	6	2	2	2	3
pyruvate fermentation to ethanol II	2	2	2	1	4	1	2	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	4	1	2	2	2
pyruvate fermentation to propanoate II (acrylate pathway)	0	1	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
acetate and ATP formation from acetyl-CoA II	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
acetate formation from acetyl-CoA (succinate)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to acetate V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to acetate VI	1	3	1	1	1	5	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
pyruvate fermentation to acetate VII	1	3	1	1	1	5	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
acetyl-CoA fermentation to butanoate II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	1	1	1	1	1	1	0	0	0	0	0	0	0
succinate fermentation to butanoate	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0
pyruvate fermentation to acetate VIII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycerol degradation III	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycerol degradation to butanol	5	8	7	7	7	6	8	6	6	6	6	5	5	6	6	6	6	6	6	5	4	6	5	6	5	6	7	6	5	6	5	6	6	5	6	5	4	4	4	5	8	7	7	7	8
(S)-propane-1,2-diol degradation	2	4	3	3	4	1	4	0	0	0	0	2	2	1	1	1	1	1	0	2	0	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	6	1	2	2	4
pyruvate fermentation to isobutanol	8	12	9	9	10	7	9	5	5	6	6	7	8	5	5	5	5	6	6	8	7	5	6	7	5	6	6	6	6	5	4	5	6	7	8	6	4	6	11	8	9	10	9	10	9
1,3-propanediol biosynthesis	3	18	6	10	6	5	15	5	4	4	5	5	5	6	6	6	6	6	5	5	5	4	4	5	5	5	4	7	6	6	6	5	5	5	6	3	5	2	4	3	8	6	7	7	4
butanol and isobutanol biosynthesis	3	3	3	2	5	2	3	1	1	1	1	1	1	2	2	2	2	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	2	2	2	1	1	5	2	3	3	3
1,2-propanediol biosynthesis from lactate	0	2	1	2	0	0	4	0	0	0	0	3	2	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	2	0	0	0	1
glycolate degradation II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(S)-lactate fermentation to propanoate, acetate and hydrogen	6	9	8	10	7	8	9	9	6	3	3	9	7	6	6	7	7	7	5	9	6	8	5	6	9	4	6	8	9	11	9	6	5	4	9	6	7	5	6	6	16	5	10	7	8
L-alanine degradation VI (reductive Stickland reaction)	0	3	2	0	1	0	2	1	1	1	1	2	1	0	0	0	0	0	1	0	0	1	3	0	1	1	2	1	1	2	2	1	1	1	2	2	1	0	0	0	1	1	0	0	0
acetate and ATP formation from acetyl-CoA I	2	4	3	3	1	1	3	2	1	1	1	2	2	1	1	1	1	1	1	3	1	1	1	1	2	1	1	2	2	1	2	1	1	1	2	1	1	1	1	1	5	1	1	1	2

FERMENTATION TO ALCOHOLS	139B	B	AGR2146	AGR2136	AGR2135	AGR2157	T90	FB3002	HUN269	NK4B19	AE2004	F	KH1P1	D15d	D32	LB2003	MC2003	MB3	AC2012	AC2014	AC2028	AC2029	AC2031	C10	C6A11	C7	FE2018	KH1P17	KH1T2	MA2020	MC2017	NC2004	NC2008	NK4A136	NK4A144	NK4A179	P6A3	XPB1003	YSB2008	YSD2013	XBA62	FC2011	P6A1	WCC10	AGR2154
mixed acid fermentation	9	13	11	11	11	9	11	9	6	5	5	8	8	7	7	7	7	7	7	11	5	8	7	7	9	5	7	9	10	9	10	6	7	6	11	8	6	6	6	6	18	6	11	9	9
heterolactic fermentation	12	20	16	18	15	8	20	11	9	8	9	12	10	11	11	12	12	12	10	12	10	8	9	12	9	11	10	14	12	12	11	9	12	10	11	8	11	9	9	12	25	12	13	13	14
acetylene degradation (anaerobic)	5	7	5	5	5	2	5	2	1	1	1	2	2	2	2	2	2	2	1	4	1	1	1	2	2	1	2	2	2	1	2	1	1	1	2	2	2	2	1	1	10	2	3	3	4
hexitol fermentation to lactate, formate, ethanol and acetate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to ethanol I	3	4	3	3	5	2	3	1	1	1	1	1	1	2	2	2	2	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	0	1	2	2	2	1	1	6	2	3	3	3
pyruvate fermentation to ethanol II	2	2	2	1	4	1	2	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	1	1	0	0	4	1	2	2	2
glycerol degradation III	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
pyruvate fermentation to butanol I	1	4	1	1	1	6	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	2	2	2	2	8	4	4	2	1	4	1	4	0	2	1	1	1	2
pyruvate fermentation to ethanol III	4	6	3	3	5	6	3	5	4	1	1	1	1	2	2	2	2	2	1	6	4	5	9	2	5	1	9	1	2	2	1	8	4	4	1	2	5	2	4	0	6	2	3	3	3
pyruvate fermentation to hexanol (engineered)	1	4	2	1	1	6	1	5	4	1	1	1	1	1	1	1	1	1	1	5	4	5	9	1	5	1	8	1	2	2	1	8	4	4	1	1	4	1	4	0	1	1	1	1	1
pyruvate fermentation to butanol II (engineered)	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1-butanol autotrophic biosynthesis (engineered)	3	5	5	4	4	4	5	4	4	4	4	3	3	4	4	4	4	4	4	3	2	4	3	4	3	4	5	4	3	4	3	4	4	3	4	3	2	2	2	3	5	4	4	4	6
glycerol degradation to butanol	5	8	7	7	7	6	8	6	6	6	6	5	5	6	6	6	6	6	6	5	4	6	5	6	5	6	7	6	5	6	5	6	6	5	6	5	4	4	4	5	8	7	7	7	8
pyruvate fermentation to isobutanol (engineered)	8	12	9	9	10	7	9	5	5	6	6	7	8	5	5	5	5	6	6	8	7	5	6	7	5	6	6	6	6	6	6	5	4	5	6	7	8	6	4	6	11	8	9	10	9
1,3-propanediol biosynthesis (engineered)	3	18	6	10	6	5	15	5	4	4	5	5	5	6	6	6	6	6	5	5	5	4	4	5	5	5	4	7	6	6	6	5	5	5	6	3	5	2	4	3	8	6	7	7	4
butanol and isobutanol biosynthesis (engineered)	3	3	3	2	5	2	3	1	1	1	1	1	1	2	2	2	2	2	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	2	2	2	1	1	5	2	3	3	3
1,2-propanediol biosynthesis from lactate (engineered)	0	2	1	2	0	0	4	0	0	0	0	3	2	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	1	1	0	1	1	1	0	0	0	0	0	2	0	0	0	1

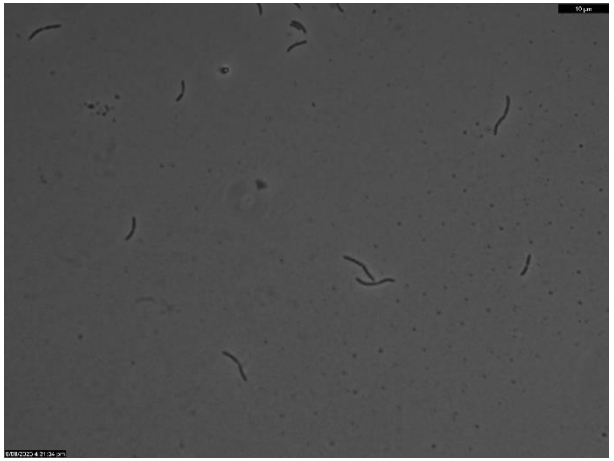
PROTEIN FERMENTATION	139B	B	AGR2146	AGR2136	AGR2135	AGR2157	T90	FB3002	HUN269	NK4B19	AE2004	F	KH1P1	D15d	D32	LB2003	MC2003	MB3	AC2012	AC2014	AC2028	AC2029	AC2031	C10	C6A11	C7	FE2018	KH1P17	KH1T2	MA2020	MC2017	NC2004	NC2008	NK4A136	NK4A144	NK4A179	P6A3	XPB1003	YSB2008	YSD2013	XBA62	FC2011	P6A1	WCC10	AGR2154
superpathway of L-alanine fermentation (Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-arginine degradation XIV (oxidative Stickland reaction)	1	1	1	1	1	1	2	1	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	2	1	1	1	2	1	1	2	1	2	1	1	1	1	1	1	1	1	1	1	1	2
L-leucine degradation IV (reductive Stickland reaction)	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-phenylalanine degradation VI (reductive Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
glycine degradation (reductive Stickland reaction)	0	0	0	6	3	9	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0
L-tyrosine degradation V (reductive Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-tryptophan degradation XIII (reductive Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-valine degradation III (oxidative Stickland reaction)	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1
L-isoleucine degradation III (oxidative Stickland reaction)	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	1	1
L-leucine degradation V (oxidative Stickland reaction)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-proline degradation II (reductive Stickland reaction)	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
L-arginine degradation XIII (reductive Stickland reaction)	2	2	2	2	2	2	2	2	2	2	3	3	2	2	2	2	2	2	2	2	2	1	3	1	2	2	3	2	3	2	3	2	3	2	2	3	2	2	2	2	2	2	2	2	2
L-alanine degradation VI (reductive Stickland reaction)	0	3	2	0	1	0	2	1	1	1	1	2	1	0	0	0	0	0	1	0	0	1	3	0	1	1	2	1	1	0	1	1	2	2	1	0	0	0	1	1	1	0	0	0	0
L-alanine degradation V (oxidative Stickland reaction)	1	4	2	1	2	5	1	6	5	1	1	1	1	1	1	1	1	1	1	5	4	6	10	1	6	1	9	1	2	2	1	9	4	4	1	1	4	1	5	0	2	1	1	1	1
L-glutamate degradation XI (reductive Stickland reaction)	1	5	1	2	3	1	1	1	1	1	1	9	9	1	1	1	1	1	1	4	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	2

HYDROGEN METBOLISM	139B	B	AGR2146	AGR2136	AGR2135	AGR2157	T90	FB3002	HUN269	NK4B19	AE2004	F	KH1P1	D15d	D32	LB2003	MC2003	MB3	AC2012	AC2014	AC2028	AC2029	AC2031	C10	C6A11	C7	FE2018	KH1P17	KH1T2	MA2020	MC2017	NC2004	NC2008	NK4A136	NK4A144	NK4A179	P6A3	XPB1003	YSB2008	YSD2013	XBA62	FC2011	P6A1	WCC10	AGR2154
hydrogen production I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hydrogen production II	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hydrogen production III	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hydrogen production IV	3	5	0	4	1	1	4	1	0	0	0	1	1	0	0	0	0	0	0	0	2	1	2	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	3	1	1	1	0
hydrogen production V	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
hydrogen production VI	2	2	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	1
hydrogen production VIII	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
superpathway of hydrogen production	0	0	0	0	0	1	0	1	1	0	0	0	0	2	1	1	1	3	0	0	0	2	1	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0

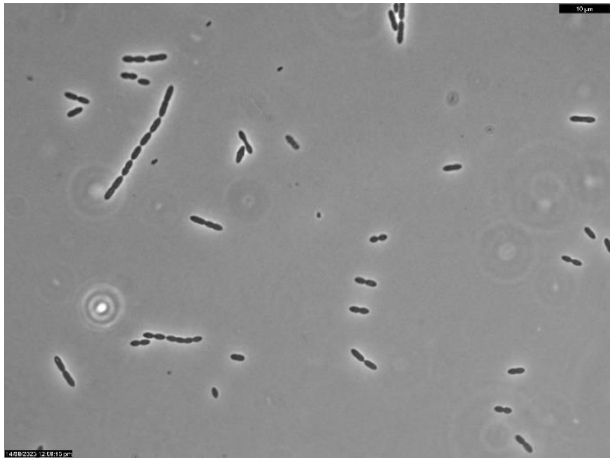
Figure A3.5 Meta Cyc pathway genes found in the genomes of 45 *Lachnospiraceae* strains. Metabolic pathway information taken from the Meta Cyc Database (Caspi et al., 2020), through the IMG portal.

Appendix 4. Agarose gel slide pictures of 45 *Lachnospiraceae* strains

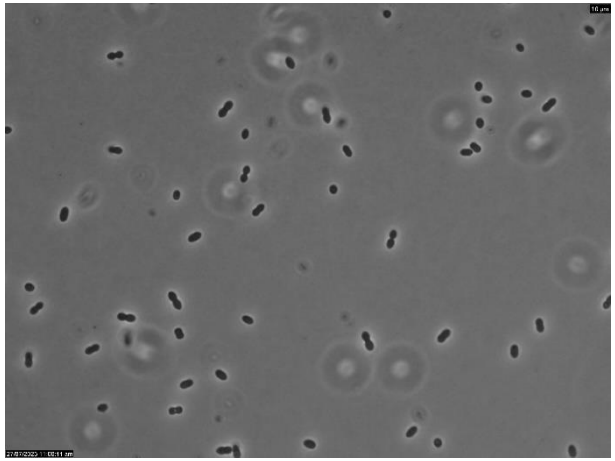
Figure A4. Phase contrast microscope pictures of 45 *Lachnospiraceae* strains taken at 100x magnification using a light microscope



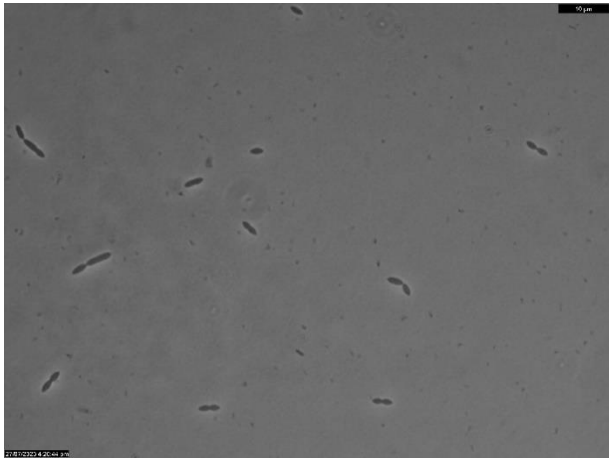
Acetitomaculum ruminis 139B



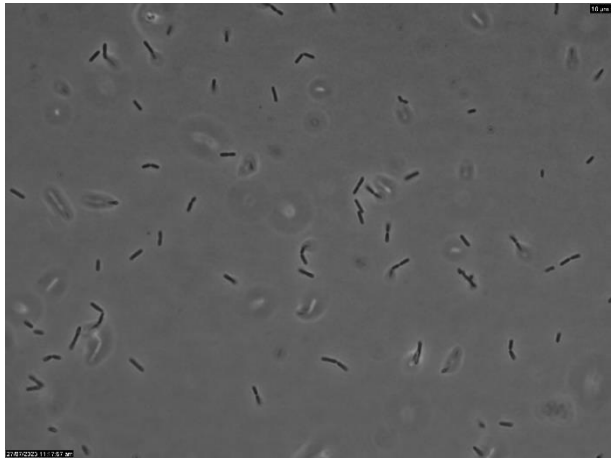
Blautia schinkii B



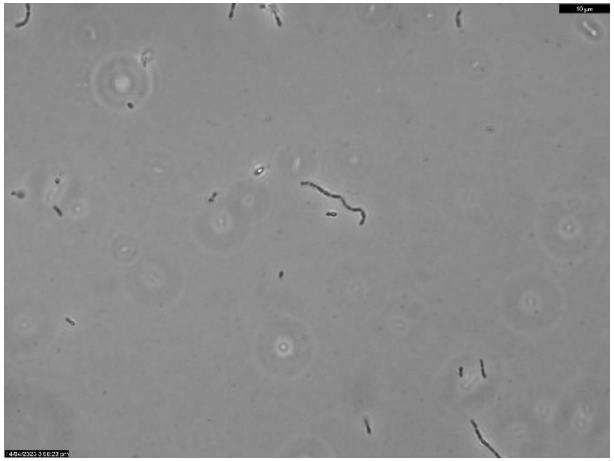
Blautia wexlerae AGR2146



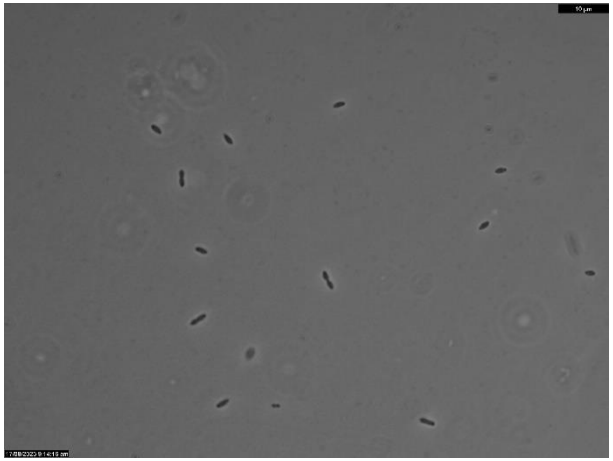
[Clostridium] clostridioforme AGR2157



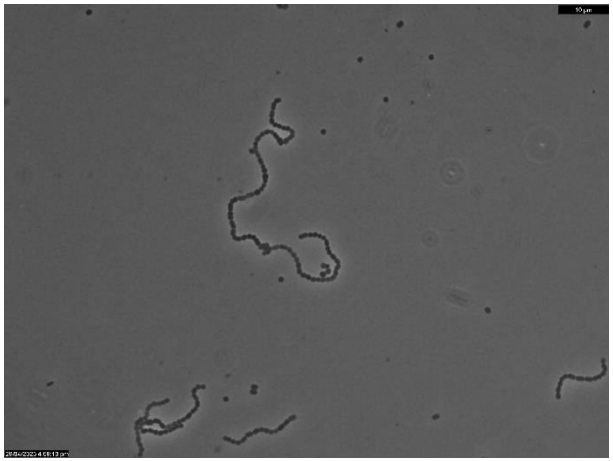
Dorea longicatena AGR2136



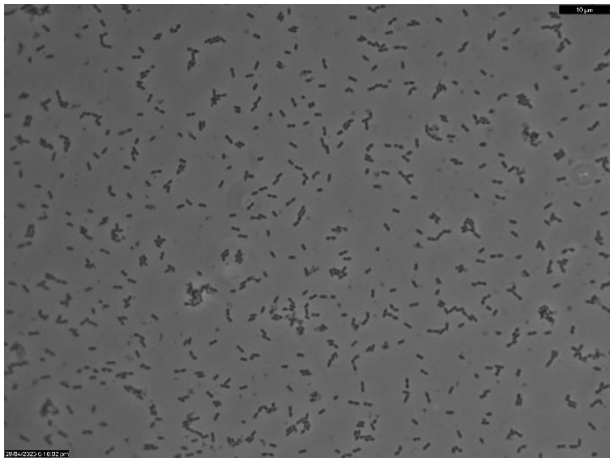
Dorea sp. AGR2135



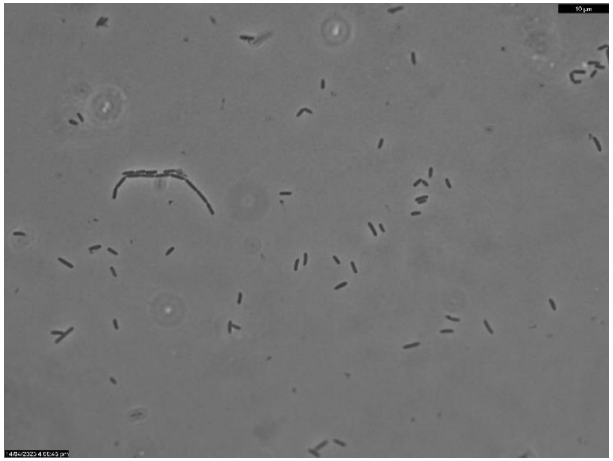
Enterocloster clostridioformis T90



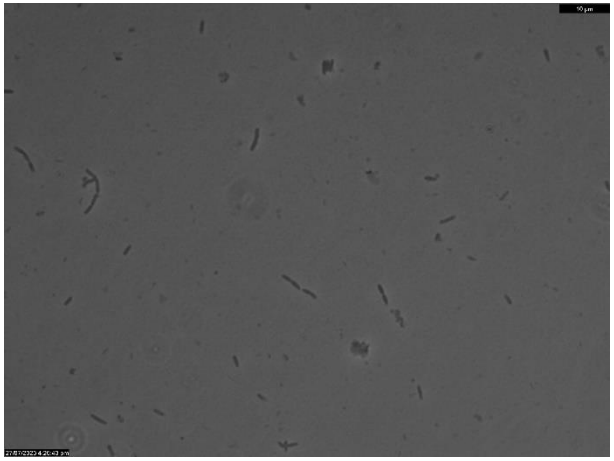
Eubacterium ruminantium FB3002



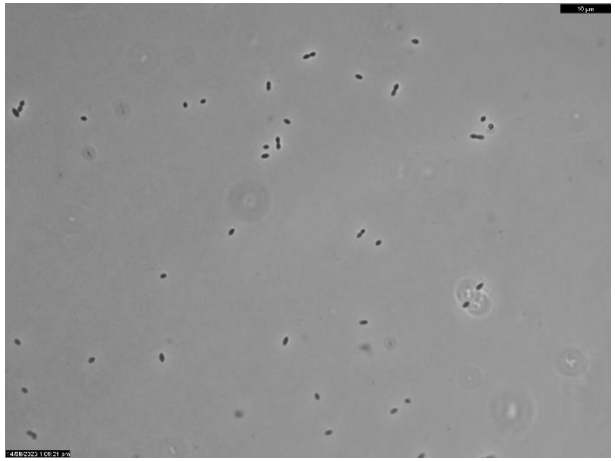
Eubacterium ruminantium HUN269



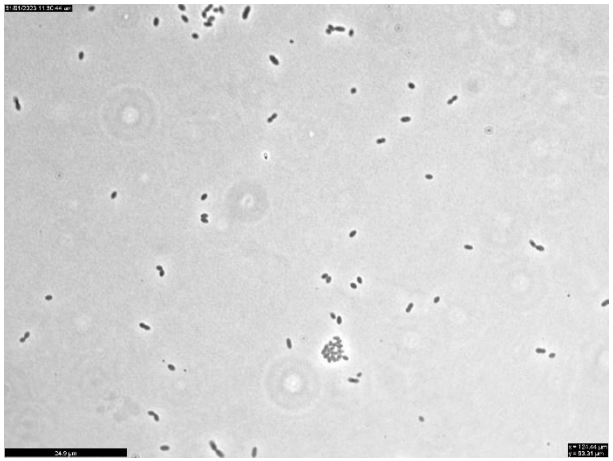
Lachnobacterium bovis AE2004



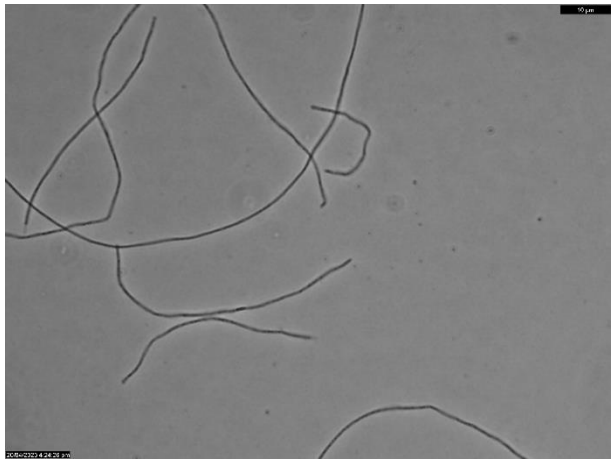
Lachnobacterium bovis NK4B19



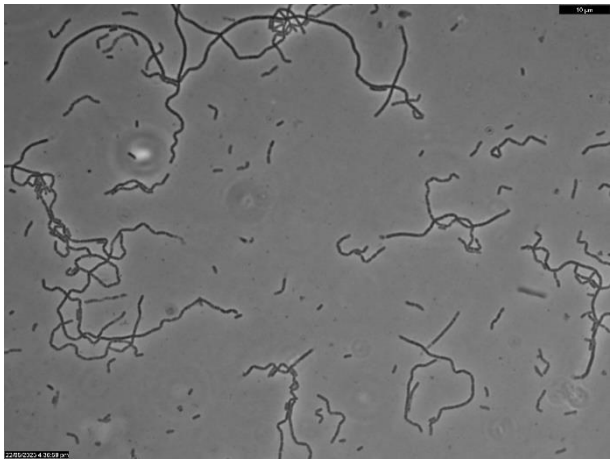
Lachnoclostridium aminophilum F



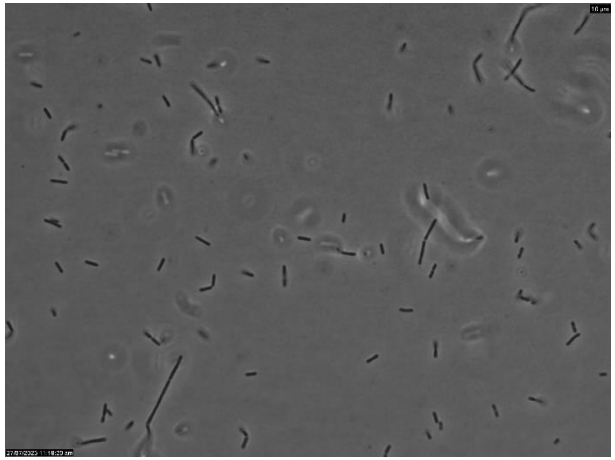
Lachnoclostridium aminophilum KH1P1



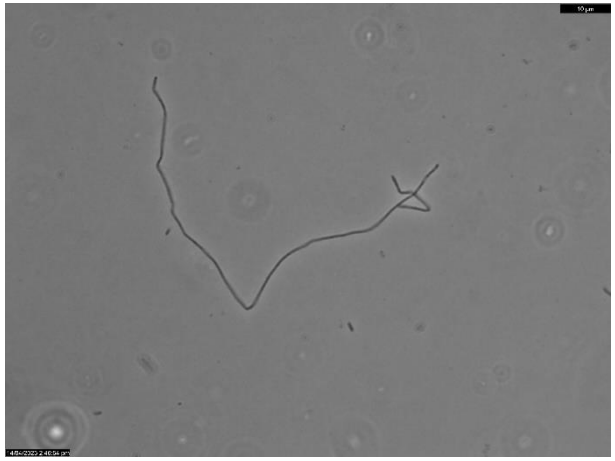
Lachnospira multipara D32



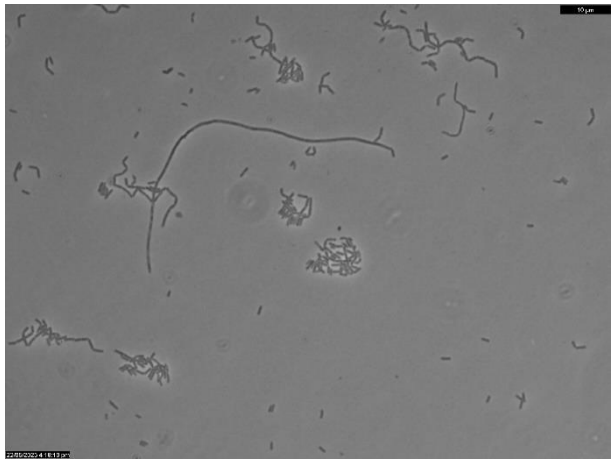
Lachnospira multipara D15d



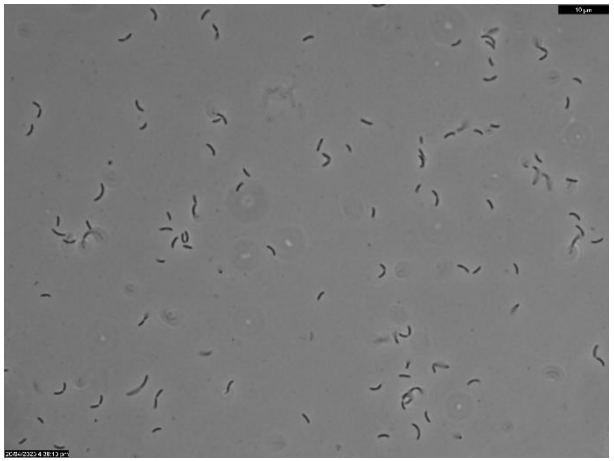
Lachnospira multipara LB2003



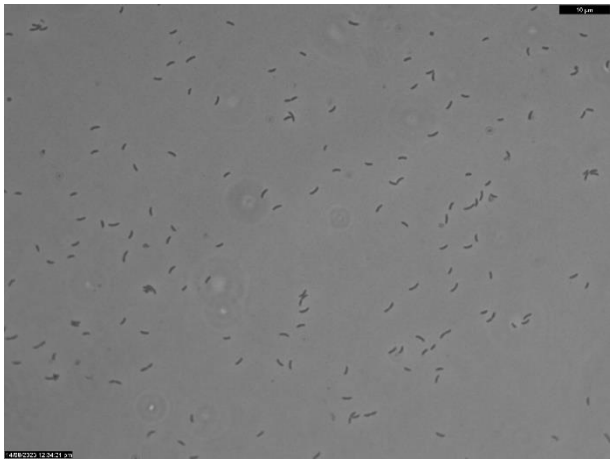
Lachnospira multipara MC2003



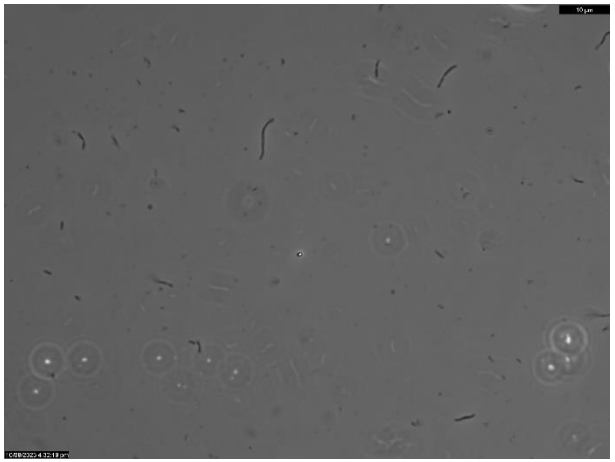
Lachnospira pectinoschiza M83



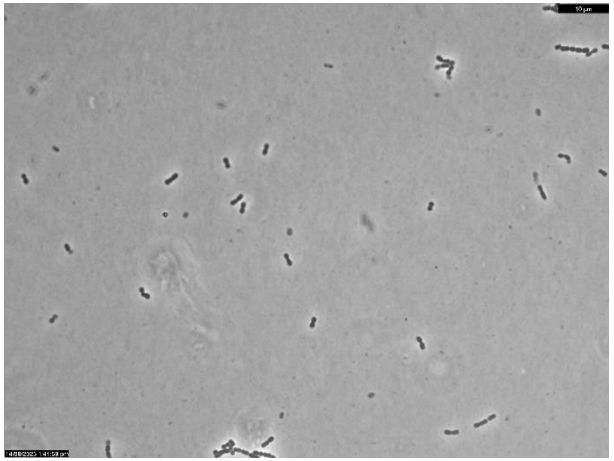
Lachnospiraceae bacterium AC2012



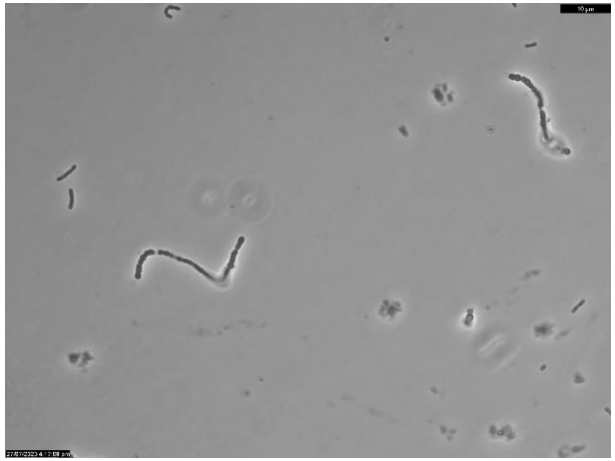
Lachnospiraceae bacterium AC2014



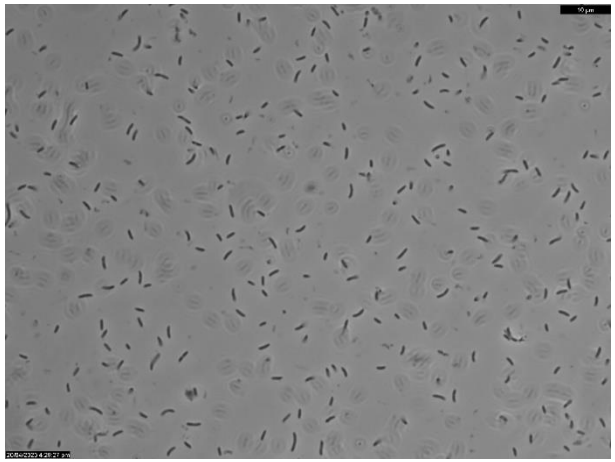
Lachnospiraceae bacterium AC2028



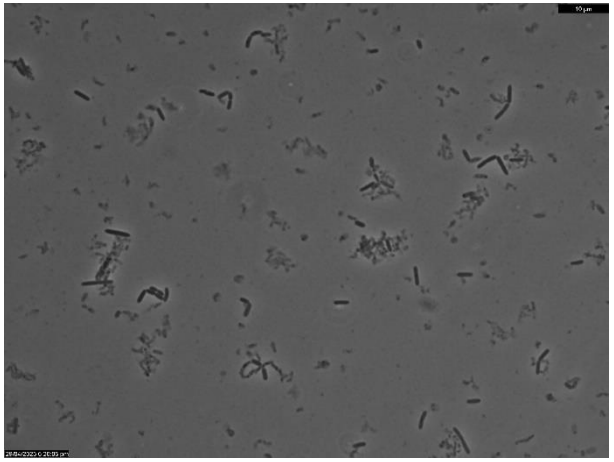
Lachnospiraceae bacterium AC2029



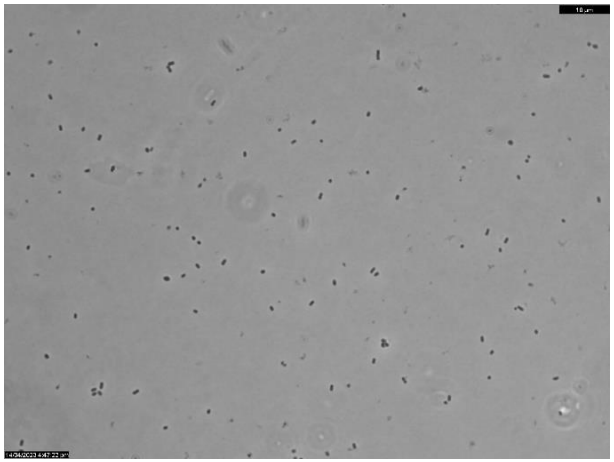
Lachnospiraceae bacterium AC2031



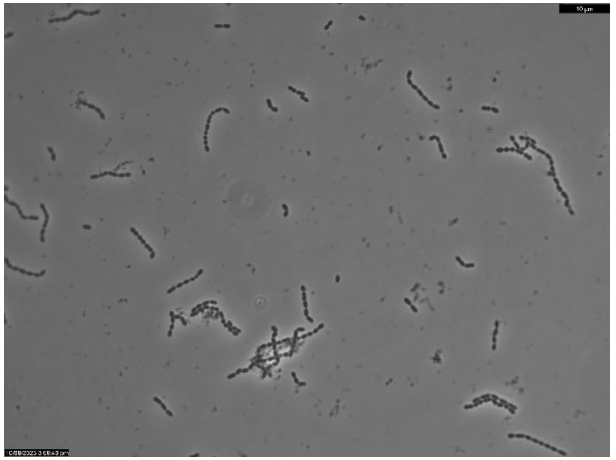
Lachnospiraceae bacterium C10



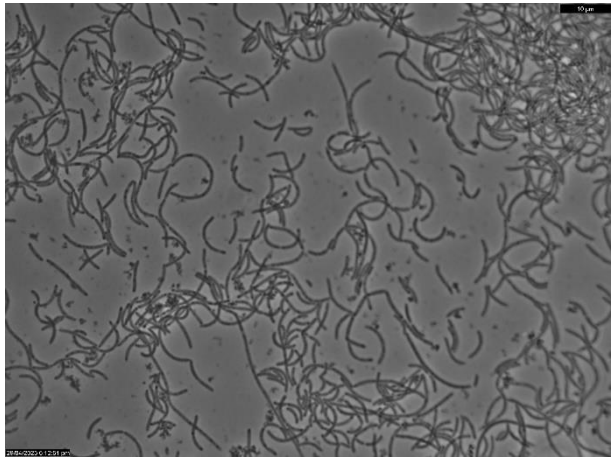
Lachnospiraceae bacterium C7



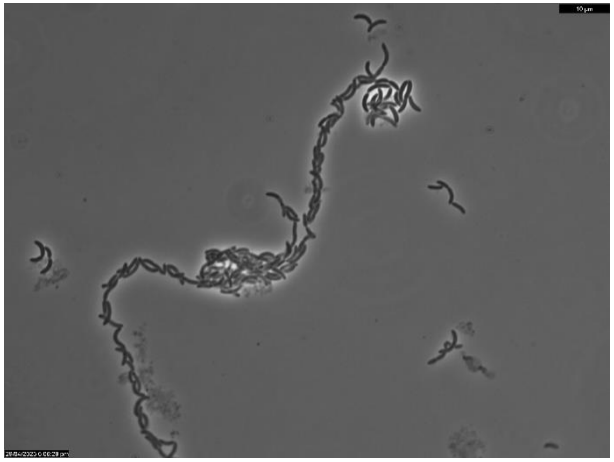
Lachnospiraceae bacterium C6A11



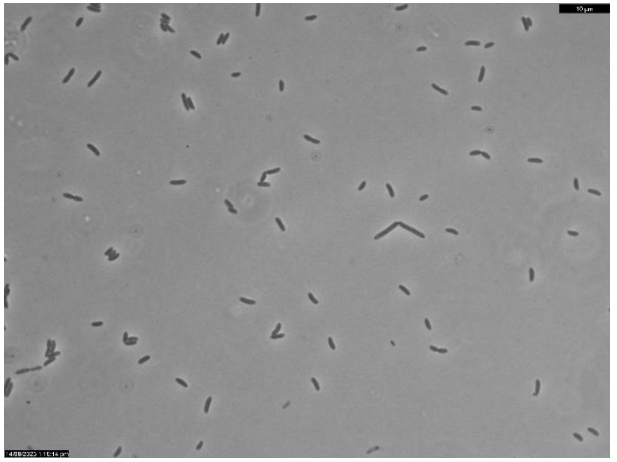
Lachnospiraceae bacterium FE2018



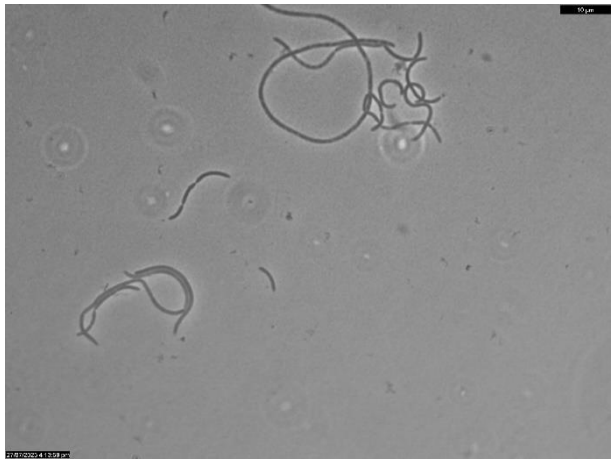
Lachnospiraceae bacterium KH1P17



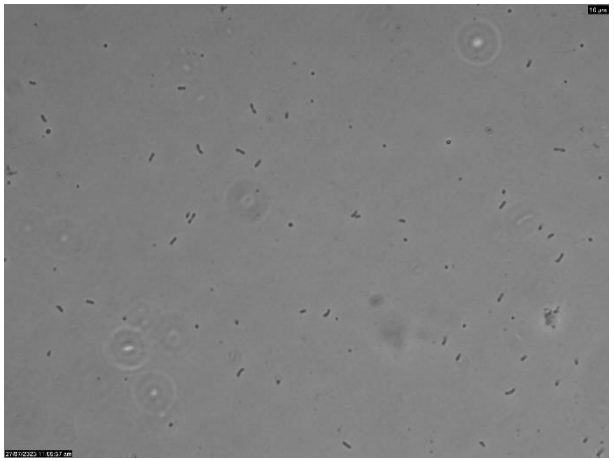
Lachnospiraceae bacterium KH1T2



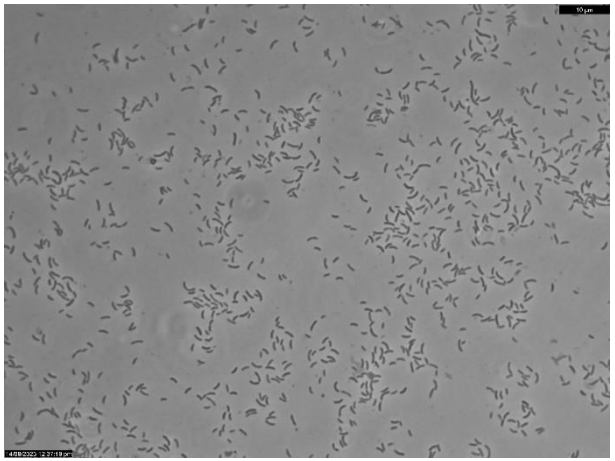
Lachnospiraceae bacterium MA2020



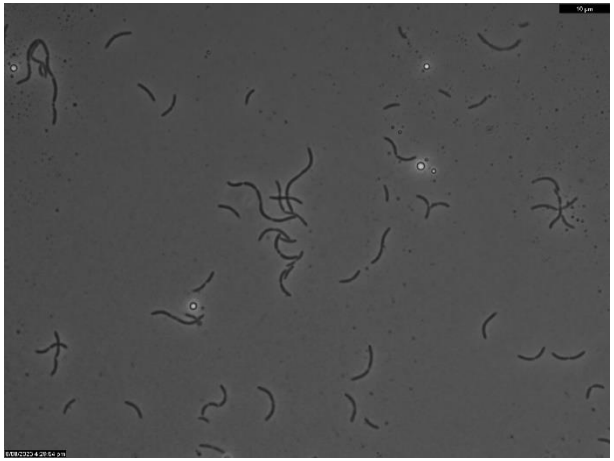
Lachnospiraceae bacterium MC2017



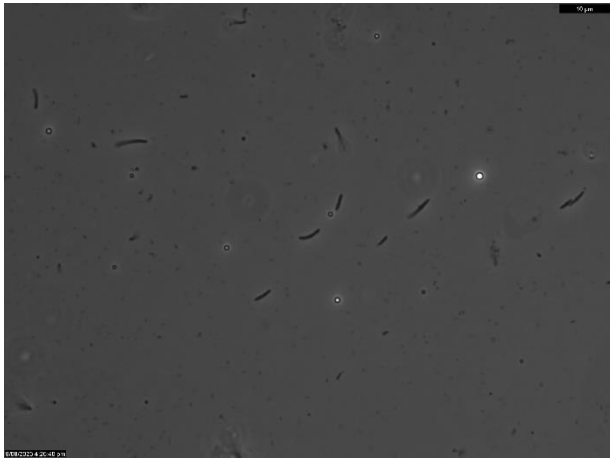
Lachnospiraceae bacterium NC2004



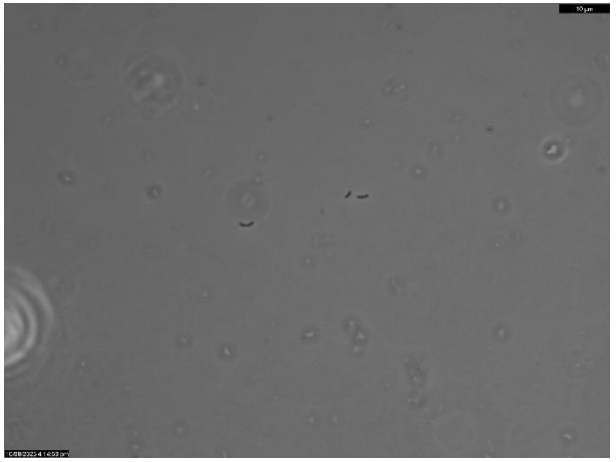
Lachnospiraceae bacterium NC2008



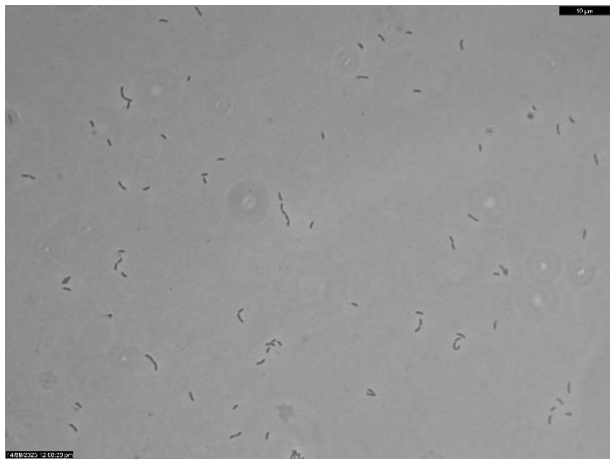
Lachnospiraceae bacterium NK4A144



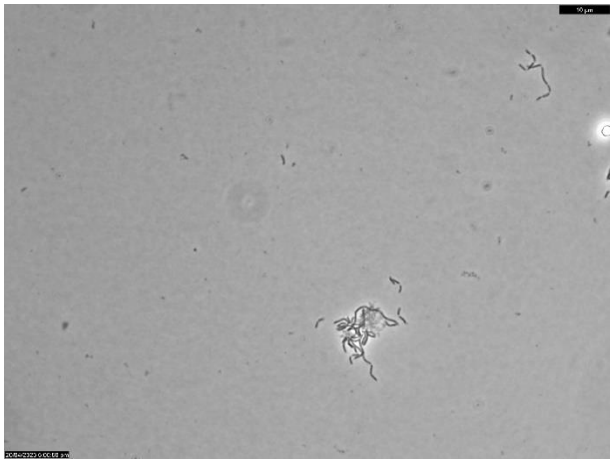
Lachnospiraceae bacterium NK4A136



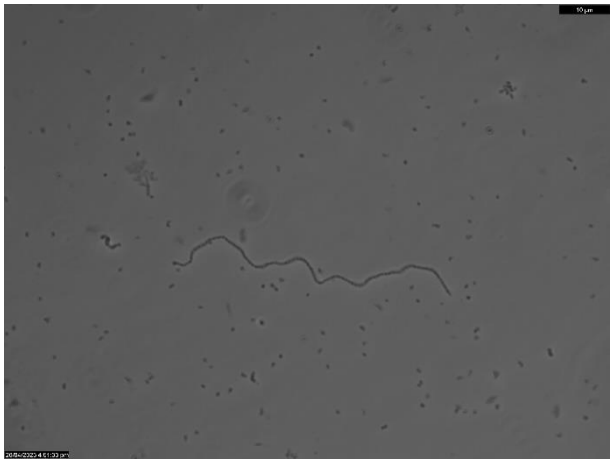
Lachnospiraceae bacterium NK4A179



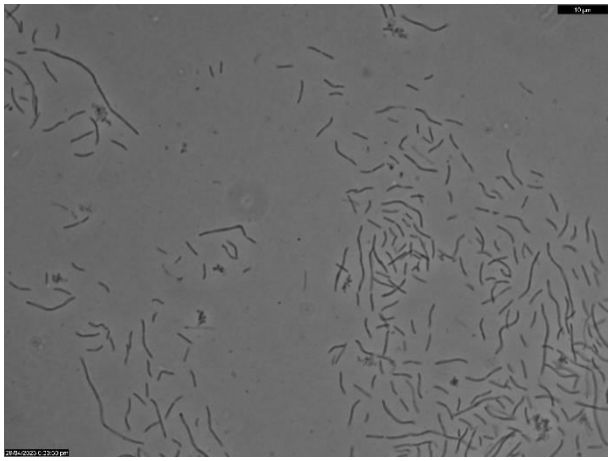
Lachnospiraceae bacterium P6A3



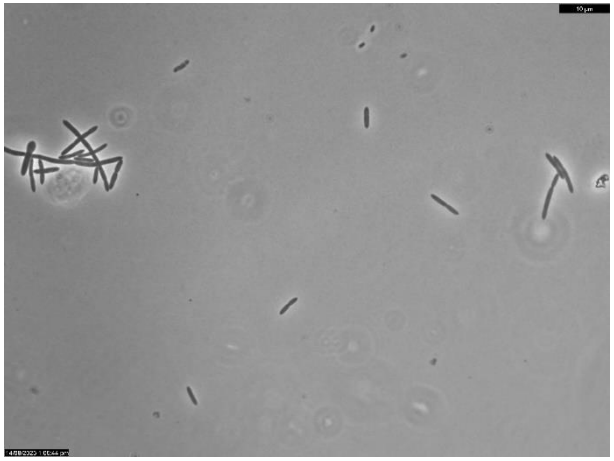
Lachnospiraceae bacterium XPB1003



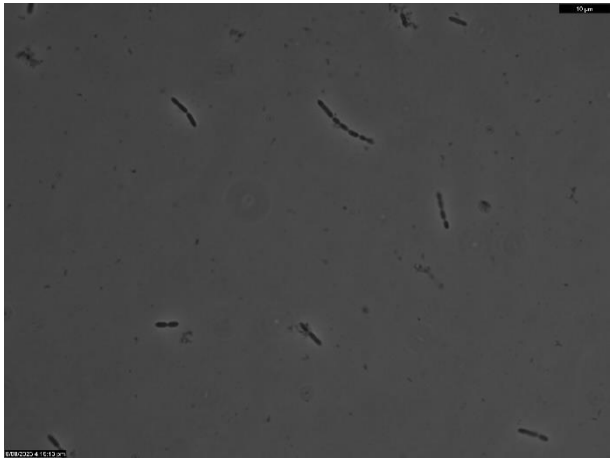
Lachnospiraceae bacterium YSD2013



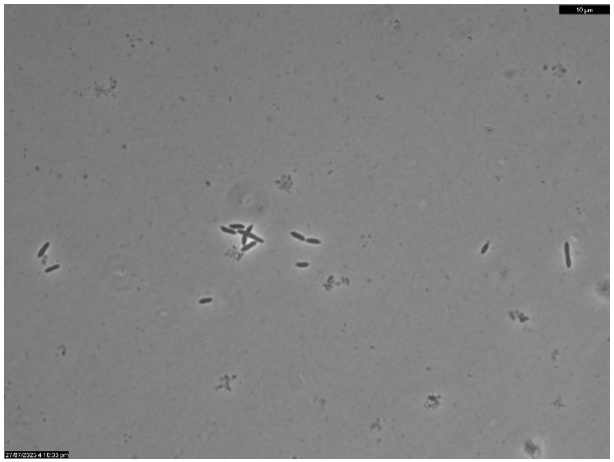
Lachnospiraceae bacterium YSD2013



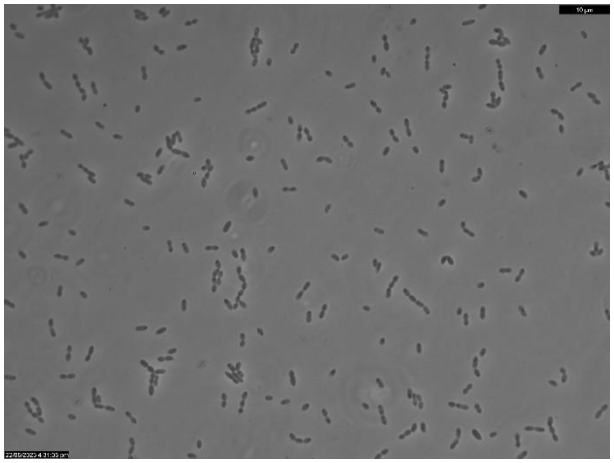
Lacrimispora aerotolerans X8A62



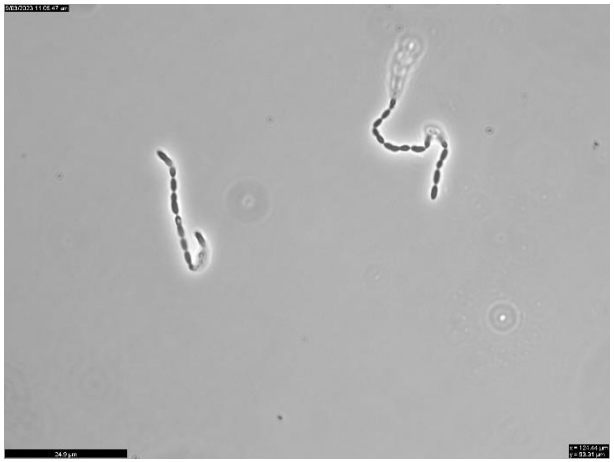
Oribacterium sp. FC2011



Oribacterium sp. P6A1



Oribacterium sp. WCC10



[Ruminococcus] gnavus AGR2154