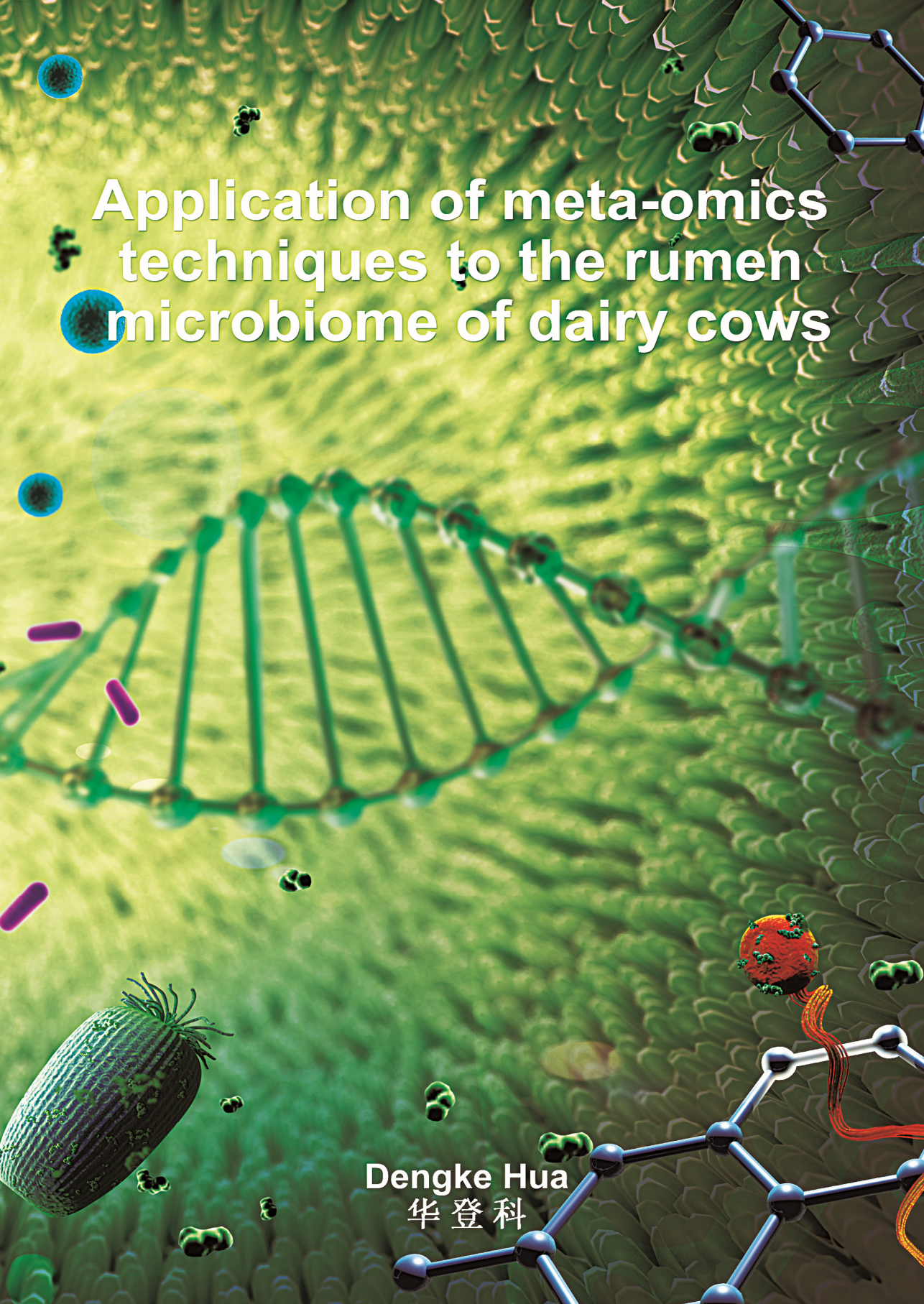
Application of meta-omics techniques to the rumen microbiome of dairy cows



Dengke Hua
华登科

Propositions

1. The recently proposed concept of 'core microbiome' in the rumen is of less interest in nutrition compared to the 'peripheral' microbiome.
   (this thesis)

2. The amylolytic bacterial community in the rumen is independent from differently processed corns.
   (this thesis)

3. Bioinformatics data analysis is hindering the application of omics in animal nutrition.

4. The quantity of the project funds has a positive correlation to the quality of the scientific output.

5. Writing a thesis is the real touchstone for a PhD candidate to enter the academia world.

6. One's perceived personality depends on one's language skills.

Propositions belonging to the thesis, entitled

Application of meta-omics techniques to the rumen microbiome of dairy cows

Dengke Hua

Wageningen, 25 October 2022

Application of meta-omics techniques to the rumen microbiome of dairy cows

Dengke Hua

Thesis committee

Promotor

Prof. Dr W. H. Hendriks

Professor of Animal Nutrition

Wageningen University & Research

Co-promotors

Prof. Dr B. Xiong

Professor of Animal Nutrition, Institute of Animal Sciences

Chinese Academy of Agricultural Sciences, Beijing, China

Dr W. F. Pellikaan

Assistant Professor of Animal Nutrition

Wageningen University & Research

Other members

Prof. Dr H. Smidt, Wageningen University & Research

Dr A. T. M. van Knegsel, Wageningen University & Research

Dr M. A. M. Rodrigues, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal

Dr F. Klevenhusen, German Federal Institute for Risk Assessment, Berlin, Germany

This research was conducted under the auspices of the Graduate School WIAS (Wageningen Institute of Animal Sciences).

Application of meta-omics techniques to the rumen microbiome of dairy cows

Dengke Hua

Thesis

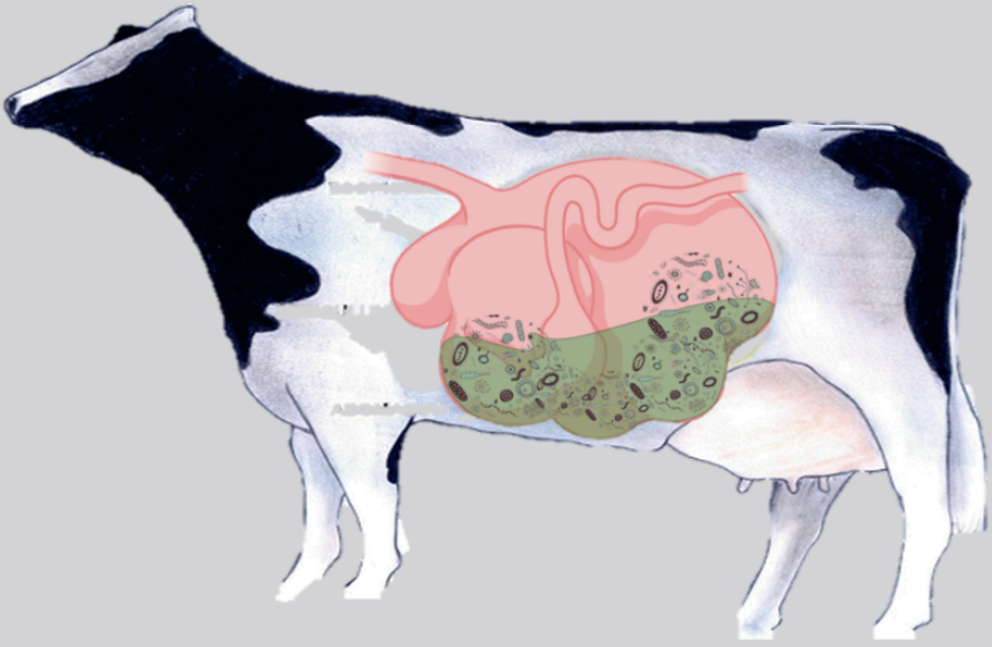
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 25 October 2022
at 4 p.m. in the Omnia Auditorium.

Dengke Hua
Application of meta-omics techniques to the rumen microbiome of dairy cows, 296 pages

PhD thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English

ISBN: 978-94-6447-300-1
DOI: https://doi.org/10.18174/572999

Table of contents



Chapter 1

General introduction

Ruminants, counting an estimated 200 species, are one of the most adapted mammals on earth, inhabiting environments from the arctic to the tropics (Hackmann and Spain, 2010). Over the approximately 50-million-year timespan of evolution, ruminants have developed a symbiotic relationship with prokaryotic (bacteria, archaea) and eukaryotic (fungi, protozoa) life forms, that has resulted in a complex rumen ecosystem which enables them to efficiently utilize low-quality feed resources (e.g., forages, food by-products and non-protein nitrogen) to produce energy-rich and high-quality protein-rich products (e.g., milk and meat). An efficient digestibility and consecutive conversion of nutrients into animal products relies on the symbiotic associations of microorganisms in the rumen (Gruninger et al., 2019). This chapter will provide a general overview of the rumen microbial composition, the application of omics techniques to assess ruminal microbiome responses to different dietary energy sources and the objective and outline of the research described in this thesis.

Rumen microbial ecosystem

The ruminal microbial ecosystem harbours billions of microorganisms including bacteria (10^{10}-10^{11}/g rumen content), archaea (10^{7}-10^{8}), protozoa (10^{4}-10^{6}) and fungi (10^{3}-10^{5}) (**Table 1.1**) (Agarwal et al., 2015), which have a symbiotic relationship with each other as well as the host.

Table 1.1. Composition of rumen microorganisms in domesticated ruminants

Microbe	Number/g rumen content	Mass (% of microbial mass)
Bacteria	10^{10}-10^{11}	40-50
Archaea	10^{7}-10^{8}	2-3
Protozoa	10^{4}-10^{6}	40-50
Fungi	10^{3}-10^{5}	3-4

Adapted from Agarwal et al. (2015).

Bacteria community

As the largest community, the bacteria are present in the rumen soon after birth and contribute most to carbohydrate and nitrogen digestion. The bacteria can degrade the substrates present in the feed (cellulose, hemicellulose, pectin, starch, protein, etc.) or utilize the degraded products of these compounds (Bryant, 1959). The bacterial population residing in the rumen can be subdivided into four categories: 1) liquid-

associated population, which contains the bacteria detached from the feed particles and the ones consuming soluble feed components from the rumen liquid (McAllister et al., 1994), 2) solid-associated population, including the ones that are loosely or tightly adhered to the feed particles (McAllister et al., 1994), 3) epithelium-associated population, which includes the bacteria attaching to the rumen epithelium (Cheng et al., 1979) and is more closely related to the host metabolic activities than the other subpopulations (Wallace et al., 1979); and 4) eukaryote-associated population, which is composed of the bacteria attaching to the surface of protozoa or fungal sporangia (Miron et al., 2001). So far, most of the research has focused on the bacteria in the liquid-associated and solid-associated communities (Zhou et al., 2015). Besides, concerning their functions, bacteria can be classified as fibre-degrading (cellulose, hemicelluloses and pectin) (fibrolytic), starch-degrading (amylolytic), protein-degrading (proteolytic), lactic acid utilizers, etc. (Choudhury et al., 2015). The number of active bacteria depends upon the animal species, type and chemical composition of the diet, frequency of feeding and many more identified or unidentified factors (Zhou et al., 2015). Although bacteria are better characterized compared to other microbes, only a small fraction of the total species have been cultured in the laboratory (Morgavi et al., 2013, Creevey et al., 2014), which indicates that a large number of novel microbes in the rumen still need to be characterized.

Archaea community

The ruminal archaea contribute for 0.3 to 3.3% to the microbial small subunit (16S and 18S) ribosomal RNA (rRNA, Sharp et al., 1998, Janssen and Kirs, 2008). A large part of the archaeal population in the rumen is made up of methanogens which can grow using H_2 and often formate as their energy source to produce methane with CO_2 and the electrons derived from H_2 (or formate) (Janssen and Kirs, 2008). The hydrogen is metabolized by the methanogenic archaea. Efficient H_2 removal leads to a nutritionally more favourable pattern of VFA formation and an increased rate of fermentation by eliminating the inhibitory effect of H_2 on microbial fermentation. Regarding the latter, methanogenic archaea have an essential role in rumen functioning and animal nutrition (Wolin, 1979, McAllister and Newbold, 2008).

Protozoa community

Rumen protozoa account for only a small fraction (10^{4-6}/ml) of the total number of microbes (10^{10-11}/ml). But in terms of protozoal mass, it is almost equal to that of bacteria present in the rumen (**Table 1.1**). Thus, rumen protozoa also play an important role in feed fermentation. Protozoa have been identified to have two types of functions, i.e., general functions of feed fermentation and the protection of easily fermentable carbohydrates (starch and sugar) from starch-/sugar-utilizing bacteria (Kamra, 2005). The majority of rumen protozoa are ciliates, and few are flagellates. The ciliates are very important in fibre digestion and the modulation of the fermentation profiles. In addition, between 20-45% of the ruminal amylolytic activity has been attributed to protozoa (Coleman, 1986). Fibre and starch are digested by engulfment of the protozoa with H_2, CO_2, acetate, butyrate and glycerol as metabolites (Williams and Coleman, 1992).

Fungi community

Rumen fungi constitute 5-8% of total microbial biomass. The rumen fungi community was identified only relatively late in the mid 70's of last century (Orpin, 1975, Bauchop, 1979) compared to other microbes because of its small amount, slow growth rate and difficulty to culture in the laboratory. When mode of action and enzyme profile were studied in detail, it was realized that rumen fungi were potentially effective fibre degraders (Akin et al., 1989, Akin et al., 1990). It is reported that the rumen bacteria together with anaerobic rumen fungi had a higher degrading ability of wheat straw than bacteria alone when using a semi continuous rumen simulation technique (Hillaire and Jouany, 1989). The rumen fungi secrete a range of enzymes including esterases, which cleave the ester bonds between hemicelluloses and lignin and release free celluloses and hemicelluloses for the other microbes to digest (Yue et al., 2009).

Application of omics on the rumen microbiome

Due to the microbial diversity of the rumen and the ever-improving analytical techniques, the community structure and metabolic pathways have been studied intensively during the past years and have shown to be of great interest and value to the animal nutritionist, in order to improve rumen functioning (Deusch et al., 2015,

Denman et al., 2018). The rumen microbiome has been shown to be significantly influenced by host species, diets and geographical location, with diet emerging as the most influential factor (Henderson et al., 2015). Great efforts have been made to explore the composition of the rumen microbial community and how it changes in response to different diets. Traditional methodology to characterize microbial communities has been based on the cultivation approach and pure culture characterization. However, only a small number of ruminal microbes is culturable (Morgavi et al., 2013). As new analytical techniques were developed, including metagenomics, metatranscriptomics, metaproteomics and metabolomics (**Figure 1.1**), what originally involved the isolation and detailed studies of single strains in the laboratory by cultivation approaches, has now evolved into large-scale sequencing of 'total' rumen microbiota (Denman et al., 2018, Gruninger et al., 2019).

Sequencing-based metagenomics has provided the collective genetic structure and functional composition at the DNA level of microbial communities in the rumen in a culture-independent manner, including previous labelled “uncharacterized” microbes (Svartström et al., 2017, Stewart et al., 2018). Since its first application to rumen material (Ferrer et al., 2005), metagenomics has been extensively utilized to study ruminal fibre degradation (Krause et al., 2003), identify new microbes (Attwood et al., 2008, Stewart et al., 2018), mine novel enzymes (Beloqui et al., 2006, Svartström et al., 2017) and identify functional dynamics in response to various diets (Li et al., 2011).

However, metagenome characterization is not able to show how the genetic information of a given microbiome is actually expressed. In this regard, metatranscriptomics can provide a comprehensive picture of the microbial messenger RNA (mRNA) transcript abundance, dynamics and regulation under various environmental conditions (Lim et al., 2013). So far, metatranscriptomics has been applied to the rumen microbiome, for instance, in order to detect carbohydrate-active enzymes and genes of the ruminal microbiome in dairy cows (Dai et al., 2015, Shinkai et al., 2016), to explore metabolic pathways between low and high feed intake cows (Shabat et al., 2016) and to identify novel methanogenic archaea species (Poulsen et al., 2013).

Metaproteomics offers a comprehensive characterization of the gene products (proteins) encoded in the metagenome and their posttranslational modifications and turnover. Limited studies have been published on its applications on the rumen microbiome. In a

recent metaproteomics study, 8,000 bacterial and 350 archaeal proteins were detected from different rumen samples including ruminal fluid, particle-associated liquid and solid matter in cows fed both forage- and grain-based diets (Deusch et al., 2017). However, only a relatively small fraction of the gene products in complex gut microbiota can be identified by metaproteomics, due to limitations in accurate detection and mass measurement of peptides and their annotation (Li, 2015).

Metabolomics emerged in the omics field only recently, which offers the possibility to extract a large amount of data related to metabolic phenotypes in mammals, plants and microbes (Vinayavekhin et al., 2010). Metabolomics has opened new avenues in the field of nutrition research, allowing scientists to explore the complex metabolic pathways in response to diets. This technique was applied to explore the rumen metabolite alterations of dairy cows receiving diets with different ratios of grains (Ametaj et al., 2010). In addition, data on ruminal metabolites facilitate the study of interactions between bacteria-specific metabolites and host proteins (Jacobsen et al., 2013). Recently, the comprehensive database called Bovine Metabolome Database (BMDB) was constructed containing all of the known chemical compounds that can be detected in bovine milk, blood, urine, rumen fluid, muscle, liver and testes as well as other biofluids and tissues (Foroutan et al., 2020).

As omic technologies develop, large numbers of data of high accuracy will be generated regarding the composition and functioning of rumen microbes. Through the integrations of these omic technologies, including metagenomics for DNA, metatranscriptomics for RNA, metaproteomics for proteins and peptides and metabolomics for metabolites, the relative abundance and shifts in microbial populations are now being related to gene transcripts and proteins that explain changes in detected metabolites. It can also be used to determine or map various genomic, proteomic and metabolic pathways that make it easier to modulate rumen functioning by just diet or other means.

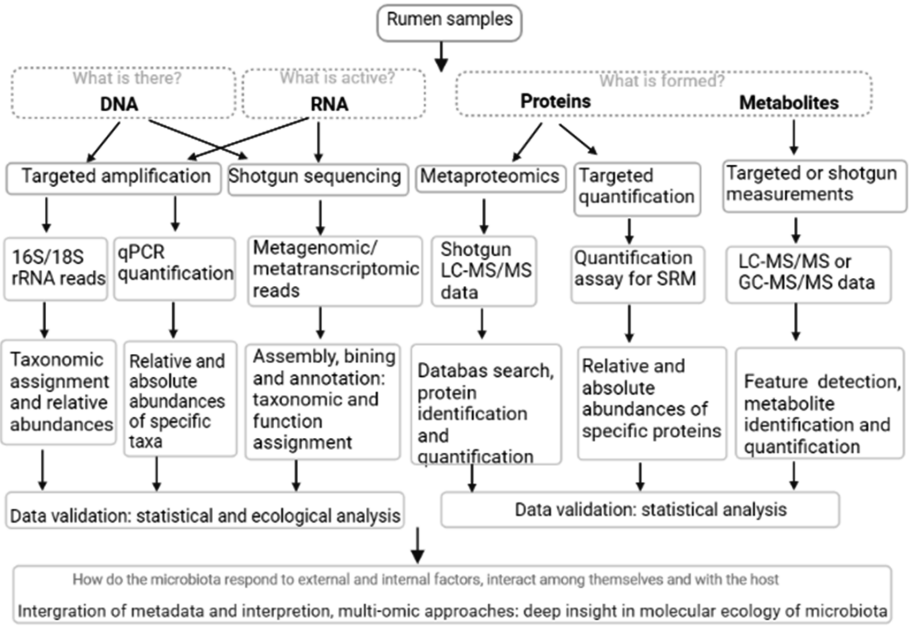


Figure 1.1. Workflow of possible methods to study the structure and function of the microbiota in the rumen. qPCR, quantitative polymerase chain reaction; SRM, selective reaction monitoring. Modified from Deusch et al. (2015) and created in BioRender.com.

Different dietary energy sources

The energy in the diets of dairy cows is mainly derived from the macronutrient carbohydrates, fat and protein, in which carbohydrates form the most important energy source. Corn is a major starch source in the diet of livestock under intensive farming systems. To improve the digestibility of corn starch, multiple processing methods are applied in practice including rolling, grinding, steam-flaking, etc. Steam-flaked corn is more readily digestible due to changes in the structure of the starch granules than ground ones (Cooper et al., 2002).

For ruminant animals, glucogenic nutrients in metabolism can originate from the ruminal fermentation of dietary starch to propionate, rumen bypass of dietary starch which is then digested in the small intestine and absorbed as glucose, or gluconeogenesis (van Knegsel et al., 2005). In addition, lipogenic nutrients in metabolism are supplied by acetate and butyrate from ruminal degradation of fibre or dietary fat, if not derived from the mobilization of body fat reserves (van Knegsel et al., 2005). Adjusting the glucogenic-to-lipogenic nutrient ratio in diets has been an

important strategy to improve the energy status of dairy cows (van Knegsel et al., 2007a, van Knegsel et al., 2007b). Previous research showed that glucogenic relative to lipogenic diets could improve the energy status and decrease the milk fat content of dairy cows, which was explained by a higher ruminal propionate production in animals receiving the glucogenic diet (van Knegsel et al., 2007a). The alterations of rumen microbial communities and functioning in response to a glucogenic vs lipogenic diet remain to be clarified although amylolytic- and fibrolytic- microbes will have a different response to dietary starch and fibre alterations in terms of community structure, enzymes and metabolic pathways.

Objective and outline of this thesis

The main objective of the research described in this thesis is to 1) assess the alterations in the community structure and functions of the ruminal microbiome when dairy cows were fed either a lipogenic diet or two different glucogenic corn-based diets, in which corn was subjected to contrasting processing conditions (grinding vs steam flaking), and 2) evaluate the applications of omics techniques in detecting the alterations of rumen microbiome in response to these diets.

Figure 1.2 visualizes the outline of the thesis. **Chapter 2** reviews the identified microbes and enzymes associated with amylolytic and cellulolytic activities in the rumen and the application of metagenomics in studying rumen functioning. This chapter provides key information on candidate microbes and enzymes, which is evaluated in the following chapters. **Chapters 3** and **4** study the changes in ruminal fermentation when the above-mentioned lipogenic and glucogenic diets were incubated with rumen fluid using an *in vitro* batch-culture technique. The parameters include the microbial communities, molecular metabolites, pH values, the extent and kinetics of gas production and other fermentation end-products such as volatile fatty acids, ammonia-nitrogen and lactic acid. Utilising the results of the *in vitro* fermentation studies, an animal trial was conducted to further investigate alterations in metabolic mechanisms of ruminal microbes. In **Chapter 5**, responses of ruminal microbes in terms of fermentation profiles, microbial community structure and metabolism pathways when dairy cows are fed the three diets are evaluated through a combination of 16S rRNA sequencing and metabolomics approaches. In **Chapter 6**, an integrated analysis of the metagenomics and metaproteomics techniques is made on the

community structure, metabolic pathways and enzymatic activities of the ruminal microbiome in dairy cows fed the experimental diets. **Chapter 7** provides a discussion of the major research findings, the rumen microbial structure through the metagenomics approach, the metabolic pathways involved in VFA synthesis and a future look about the applications of multi-omics techniques on rumen functioning studies.

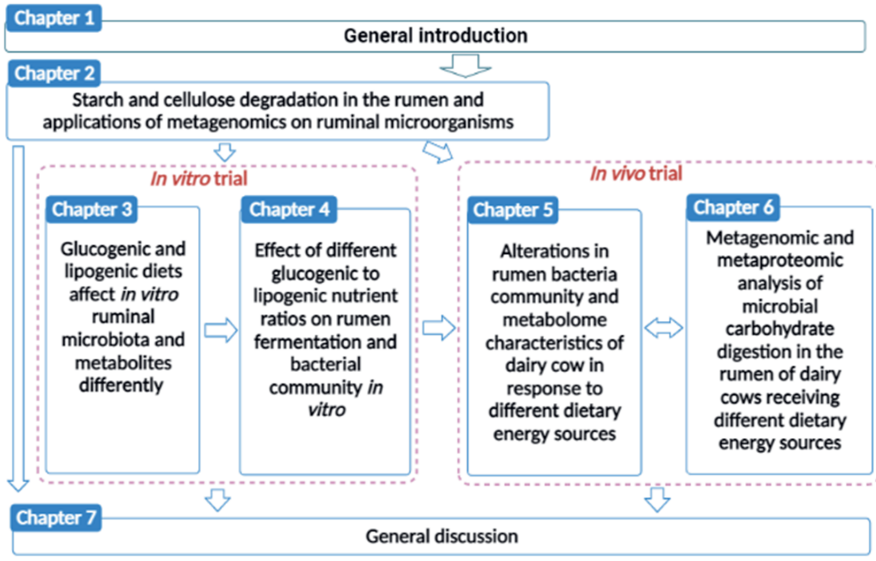


Figure 1.2. The structure frame of the thesis. Created in BioRender.com

Reference

Agarwal, N., Kamra, D. N., and Chaudhary, L. C. 2015. Rumen microbial ecosystem of domesticated ruminants, in Chapter 2, Page:22, Rumen microbiology:from evolution to revolution. Puniya, A. K., Singh, R., and Kamra, D. N., ed. Springer, India.

Akin, D. E., Borneman, W. S., and Lyon, C. E. 1990. Degradation of leaf blades and stems by monocentric and polycentric isolates of ruminal fungi. *Anim. Feed Sci. Technol.* 31(1990):205-221.

Akin, D. E., Lyon, C. E., Windham, W. R., and Rigsby, L. L. 1989. Physical degradation of lignified stem tissues by ruminal fungi. *Appl. Environ. Microbiol.* 55(3):611-616.

Ametaj, B. N., Zebeli, Q., Saleem, F., Psychogios, N., Lewis, M. J., Dunn, S. M., Xia, J., and Wishart, D. S. 2010. Metabolomics reveals unhealthy alterations in rumen metabolism with increased proportion of cereal grain in the diet of dairy cows. *Metabolomics* 6(4):583-594.

Attwood, G. T., Kelly, W. J., Altermann, E. H., Moon, C. D., Leahy, S., and Cookson, A. L. 2008. Application of rumen microbial genome information to livestock systems in the postgenomic era. *Aust. J. Exp. Agric.* 48(7):695.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. *Appl. Environ. Microbiol.* 38(1):148-158.

Beloqui, A., Pita, M., Polaina, J., Martínez-Arias, A., Golyshina, O. V., Zumárraga, M., Yakimov, M. M., García-Arellano, H., Alcalde, M., Fernández, V. M., Elborough, K., Andreu, J. M., Ballesteros, A., Plou, F. J., Timmis, K. N., Ferrer, M., and Golyshin, P. N. 2006. Novel polyphenol oxidase mined from a metagenome expression library of bovine rumen. *J. Biol. Chem.* 281(32):22933-22942.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bact. Rev.* 23:125-153.

Cheng, K., Bailey, C., and R, H. 1979. A technique for depletion of bacteria adherent to the epithelium of the bovine rumen. *Can. J. Anim. Sci.* 59:207-209.

Choudhury, P. K., Salem, A. Z. M., Jena, R., Kumar, S., Singh, R., and Puniya, A. K. 2015. Rumen microbiology: an overview, in Chapter 1, Page: 3, Rumen microbiology: from evolution to revolution. Puniya, A. K., Singh, R., and Kamra, D. N., ed. Springer, India.

Coleman, G. 1986. The metabolism of rumen ciliate protozoa. *FEMS Microbiol. Rev.* (39):321-344.

Cooper, R. J., Milton, C. T., Klopfenstein, T. J., Scott, T. L., Wilson, C. B., and Mass, R. A. 2002. Effect of corn processing on starch digestion and bacterial crude protein flow in finishing cattle1. *J. Anim. Sci.* 80:797-804.

Creevey, C. J., Kelly, W. J., Henderson, G., and Leahy, S. C. 2014. Determining the culturability of the rumen bacterial microbiome. *Microbiol. Biotechnol.* 7(5):467-479.

Dai, X., Tian, Y., Li, J., Su, X., Wang, X., Zhao, S., Liu, L., Luo, Y., Liu, D., Zheng, H., Wang, J., Dong, Z., Hu, S., and Huang, L. 2015. Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* 81(4):1375-1386.

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

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. 2017. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8:1605.

Deusch, S., Tilocca, B., Camarinha-Silva, A., and Seifert, J. 2015. News in livestock research-use of omics technologies to study the microbiota in the gastrointestinal tract of farm animals. *Comput. Struct. Biotechnol. J.* 13:55-63.

Ferrer, M., Golyshina, O. V., Chernikova, T. N., Khachane, A. N., Reyes-Duarte, D., Santos, V. A. P. M., Strompl, C., Elborough, K., Jarvis, G., Neef, A., Yakimov, M. M., Timmis, K. N., and Golyshin, P. N. 2005. Novel hydrolase diversity retrieved from a metagenome library of bovine rumen microflora. *Environ. Microbiol.* 7(12):1996-2010.

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo, A., Li, C., Guan, L. L., and Wishart, D. S. 2020. The bovine metabolome. *Metabolites* 10(6):233.

Gruninger, R. J., Ribeiro, G. O., Cameron, A., and McAllister, T. A. 2019. Invited review: Application of meta-omics to understand the dynamic nature of the rumen microbiome and how it responds to diet in ruminants. *Animal* 13(9):1843-1854.

Hackmann, T. J. and Spain, J. N. 2010. Invited review: ruminant ecology and evolution: perspectives useful to ruminant livestock research and production. *J. Dairy Sci.* 93(4):1320-1334.

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5(1):14567.

Hillaire, M. and Jouany, J. 1989. Effects of anaerobic rumen fungi on the digestion of wheat straw and the end products of microbial metabolism studies on a semi continues *in vitro* system, Page: 269-272, The roles of protozoa and fungi in ruminant digestion. Nolan, J. V., Leng, R. A., and Demeyer, D. I., ed. Penumble Books, Armidale.

Jacobsen, U. P., Nielsen, H. B., Hildebrand, F., Raes, J., Sicheritz-Ponten, T., Kouskoumvekaki, I., and Panagiotou, G. 2013. The chemical interactome space between the human host and the genetically defined gut metabotypes. *ISME J.* 7(4):730-742.

Janssen, P. H. and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74(12):3619-3625.

Kamra, D. N. 2005. Rumen microbial ecosystem. *Curr. Sci. India.* 89(1):124-135.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., and McSweeney, C. S. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5):663-693.

Li, R. W., Sparks, M. E., and Connor, E. E. 2011. Dynamics of the rumen microbiota, Page: 135-164, Metagenomics and its applications in agriculture. Li, R. W., ed. Nova Science Publishers, New York.

Lim, Y. W., Schmieder, R., Haynes, M., Willner, D., Furlan, M., Youle, M., Abbott, K., Edwards, R., Evangelista, J., Conrad, D., and Rohwer, F. 2013. Metagenomics and

metatranscriptomics: windows on CF-associated viral and microbial communities. *J. Cyst. Fibros*. 12(2):154-164.

McAllister, T. A. and Newbold, C. J. 2008. Redirecting rumen fermentation to reduce methanogenesis. *Aust. J. Exp. Agric*. 48(2):7.

McAllister, T., Bae, H., Jones, G., and Chen, K. J. 1994. Microbial attachment and feed digestion in the rumen. *J. Anim. Sci*. 72:3004-3018.

Miron, J., Ben-Ghedalia, D., and Morrison, M. 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. *J. Dairy Sci*. 84(6):1294-1309.

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

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol*. 91(2):249.

Poulsen, M., Schwab, C., Borg Jensen, B., Engberg, R. M., Spang, A., Canibe, N., Højberg, O., Milinovich, G., Fragner, L., Schleper, C., Weckwerth, W., Lund, P., Schramm, A., and Urich, T. 2013. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. *Nat. Commun*. 4:1428.

Shabat, S. K. B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., and Mizrahi, I. 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J*. 10(12):2958-2972.

Sharp, R., Ziemer, C. J., Stern, M. D., and Stahl, D. A. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol. Ecol*. 26(1998):71-78.

Shinkai, T., Mitsumori, M., Sofyan, A., Kanamori, H., Sasaki, H., Katayose, Y., and Takenaka, A. 2016. Comprehensive detection of bacterial carbohydrate-active enzyme coding genes expressed in cow rumen. *Anim. Sci. J*. 87(11):1363-1370.

Stewart, R. D., Auffret, M. D., Warr, A., Wiser, A. H., Press, M. O., Langford, K. W., Liachko, I., Snelling, T. J., Dewhurst, R. J., Walker, A. W., Roehe, R., and Watson, M. 2018. Assembly of 913 microbial genomes from metagenomic sequencing of the cow rumen. *Nat. Commun*. 9(1):870.

Svartström, O., Alneberg, J., Terrapon, N., Lombard, V., de Bruijn, I., Malmsten, J., Dalin, A., El Muller, E., Shah, P., Wilmes, P., Henrissat, B., Aspeborg, H., Andersson, A. F., and Sveriges, L. 2017. Ninety-nine *de novo* assembled genomes from the moose (*Alces alces*) rumen microbiome provide new insights into microbial plant biomass degradation. *ISME J*. 11(11):2538-2551.

van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., Tamminga, S., and Kemp, B. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev*. 45(6):665-688.

van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., van Straalen, W. M., Jorritsma, R., Tamminga, S., and Kemp, B. 2007a. Effect of glucogenic vs. lipogenic diets on energy balance, blood metabolites, and reproduction in primiparous and multiparous dairy cows in early lactation. *J. Dairy Sci*. 90(7):3397-3409.

van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., van Straalen, W. M., Heetkamp, M. J. W., Tamminga, S., and Kemp, B. 2007b. Dietary energy source in dairy cows in early lactation:energy partitioning and milk composition. *J. Dairy Sci*. 90(3):1467-1476.

Vinayavekhin, N., Homan, E. A., and Saghatelian, A. 2010. Exploring disease through metabolomics. *ACS Chem. Biol*. 5(1):91-103.

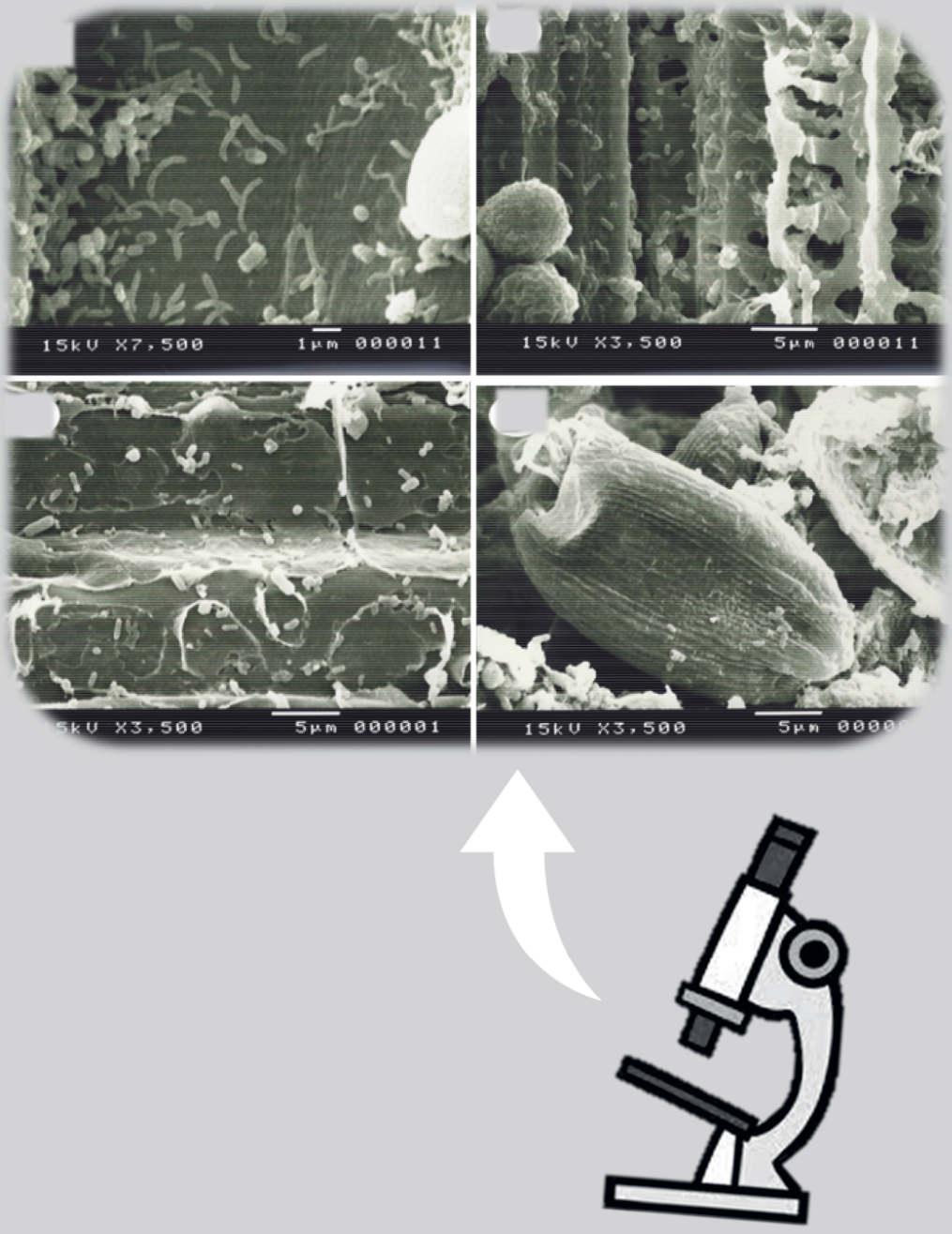
Wallace, R. J., Cheng, K. J., Dinsdale, D., and Ørskov, E. R. 1979. An independent microbial flora of the epithelium and its role in the ecomicrobiology of the rumen. *Nature* 279:424-426.

Williams, A. G. and Coleman, G. S. 1992. Metabolism of Entodiniomorphid protozoa, in Chapter 6, Page: 173, the rumen protozoa. Springer-Verlag, New York.

Wolin, M. J. 1979. The rumen fermentation: a model for microbial interactions in anaerobic ecosystems, in Chapter 3, Page: 49-77, Advances in microbial ecology. Alexander, M., ed. Plenum Press, New York.

Yue, Q., Yang, H. J., Cao, Y. C., Zhang, D. F., Jiang, Y. H., and Wang, J. Q. 2009. Feruloyl and acetyl esterase production of an anaerobic rumen fungus *Neocallimastix* sp. *YQ2* effected by glucose and soluble nitrogen supplementations and its potential in the hydrolysis of fibrous feedstuffs. *Anim. Feed Sci. Technol.* 153(3-4):263-277.

Zhou, M., Chen, Y., and Guan, L. L. 2015. Rumen bacteria, in Chapter 6, Page: 79, Rumen microbiology: from evolution to revolution. Puniya, A. K., Singh, R., and Kamra, D. N., ed. Springer, India.



The scanning electron microscopy picture is modified from Jesus et al. (2015)

Chapter 2

Starch and cellulose degradation in the rumen and applications of metagenomics on ruminal microorganisms

Dengke Hua[1,2], Wouter H. Hendriks[2], Benhai Xiong[1], Wilbert F. Pellikaan[2]

[1] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

[2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, 6708 WD, the Netherlands

Submitted to Animals

Abstract

Carbohydrates (e.g., starch and cellulose) are the main energy source in the diets of dairy cows. The ruminal digestion of starch and cellulose is achieved by microorganisms and digestive enzymes. In order to improve their digestibility, the microbes and enzymes involved in starch and cellulose degradation should be identified and their role(s) and activity known. As existing and new analytical techniques are continuously being developed, our knowledge of the amylolytic and cellulolytic microbial community in the rumen of dairy cows has been evolving rapidly.

Using traditional culture-based methods, the main amylolytic and cellulolytic bacteria, fungi and protozoa in the rumen of dairy cows have been isolated. These culturable microbes have been found to only account for a small fraction of the total population of microorganisms present in the rumen. A more recent application of the culture-independent approach of metagenomics has acquired a more complete genetic structure and functional composition of the rumen microbial community. Metagenomics can be divided into functional metagenomics and sequencing-based computational metagenomics. Both approaches have been applied in determining the microbial composition and function in the rumen. With these approaches, novel microbial species and as well as enzymes especially glycosyl hydrolases were discovered.

This review summarizes the current state of knowledge regarding the major amylolytic and cellulolytic microorganisms present in the rumen of dairy cows. The ruminal amylases and cellulases are briefly discussed. The application of metagenomics technology in investigating glycosyl hydrolases is provided and the novel enzymes are compared in terms of glycosyl hydrolase families related to amylolytic and cellulolytic activities.

Keywords: rumen, starch, cellulose, microbe, enzyme, metagenomics

Introduction

The rumen ecosystem harbours a vast number of microorganisms fermenting the ingested feedstuffs and producing various metabolites to meet the host's nutritional requirement. Nutritionists, microbiologists and physiologists among others, have been studying the rumen microbial ecosystem in order to improve productivity and health and reduce the environmental impact of dairy cows.

Unlike ruminants in the wild, starch and cellulose are the principal components in diets for ruminant livestock worldwide, providing the primary energy to the rumen microorganisms as well as the host. Starch and cellulose degradation in the rumen have always been of key importance for ruminant livestock with numerous studies investigating the ruminal microbes and enzymes involved in starch- and cellulose-degrading (Huntington, 1997, Krause et al., 2003). Most of this research is based on more traditional approaches which include culturing and microscopy (Huntington et al., 2006). Over the last decades, more and more knowledge has been generated as the advancement of existing and introduction of new analytical techniques occurred.

The exploration of the species and enzyme activities involved in ruminal cellulose and starch digestions has been hampered by the limited number of rumen bacteria that can be cultured (Edwards et al., 2004). Metagenomics, a culture-independent analysis technique, has emerged in recent years as a powerful tool for exploring the collective structure and functioning of microbial genomes within a complex ecosystem. The application of metagenomics on rumen samples was first published in 2005 by Ferrer et al. (2009) through functional screening technology. Since that, the metagenomic approach has been widely utilized to discover rumen microbial communities and enzymes. Li (2015) discussed the periodic progress prior to 2015 of the metagenomics technologies in mining novel enzymes from the rumen microbiome including fibrolytic and amylolytic enzymes. As high-throughput sequencing technologies developed, sequence-based metagenomics combined with a functional metagenomic approach has been used, through which additional novel enzymes and metabolic activities were identified by comparison with multiple databases. The purpose of this review is to describe: 1) our current understanding of the microbes and enzymes involved in starch and cellulose degradation in the rumen of dairy cows and 2) recent developments in

sequencing technology where sequence-based and functional metagenomics can contribute to our knowledge of the structure and function of amylolytic and cellulolytic microorganism in the rumen of dairy cows.

Starch and cellulose degradation in the rumen

Starch degradation

Starch-rich grain is the primary energy component used in the modern diet for dairy cows, accounting for 20-40% of the ration of high-yielding cows. Due to the relatively high price of starch-containing ingredients, dietary starch should be used wisely to achieve cost-effectiveness and efficient production. Starch is a heterogeneous polysaccharide containing two structurally distinct α-linked polymers of glucose: amylose and amylopectin. The former is a linear D-glucose polymer containing ~99% α-1,4-links and the latter is the most abundant component of starch with 95% α-1,4-links and 5% α-1,6-links (Parker and Ring, 2001).

Unlike non-ruminants, starch degradation mainly occurs in the rumen, partly in the small intestine with the remainder fermented in the hindgut of ruminants. Starch degradation in each segment of the gastrointestinal tract is influenced by starch sources (e.g., corn, wheat, sorghum, barley) and processing (moistening, heating, or mechanical pressure) of the grain (Huntington et al., 2006). Data from 87 studies across a wide range of starch intakes (1-5.7 kg/d) showed that, on average, 71% of the starch intake was digested in the rumen (Offner and Sauvant, 2004). Harmon et al. (2004) analysed data from 16 studies where the starch intake ranged from 1 to 5 kg/d and reported that ruminal starch digestion/fermentation was typically 75-80% of starch intake, with 35-60% of starch escaping rumen fermentation and digested in the small intestine. Between 35-50% of the starch that escapes small intestinal digestion was reported to be fermented in the large intestine. The starch digestion in the small intestine consists of three processes as reviewed previously (Harmon et al., 2004). Briefly, intestinal starch digestion starts in the lumen of the duodenum by the action of pancreatic α-amylase which hydrolyses amylose and amylopectin into maltose and other branched-chain products. The second process occurs at the brush border membrane via the action of the brush border carbohydrases (e.g., maltase, isomaltase) with the third process being

glucose transportation from the intestinal lumen to the portal circulation (Huntington et al., 2006).

Amylolytic organisms in the rumen

Amylolytic bacteria

The main starch-degrading microorganisms in the rumen are amylolytic bacteria, followed by protozoa and fungi (Huntington, 1997). Previous research has reported that bacterial digestion activities start with an attachment of bacteria to feed particles. The commonly reported amylolytic bacteria present include *Streptococcus bovis*, *Ruminobacter amylophilus*, *Succinimonas amylolytica*, *Selenomonas ruminantium* and *Bifidobacterium* spp. (**Table 2.1**).

Streptococcus bovis can be easily isolated from the rumen fluid but only account for a small number of the total bacteria present in the rumen (Hungate, 1966). *Streptococcus bovis*, producing lactate as the main end-product, is present only when a large amount of starch or sugar is available as a substrate and the pH of the rumen fluid is low (Dehority, 2004). When conditions are favourable with high availability of starch or sugar, this species can grow explosively which leads to the overwhelming production of lactate and can result in rumen acidosis. *Ruminobacter amylophilus* is strictly anaerobic and Gram-negative with multiple shapes, arrangements and sizes. This species is capable of utilizing three forms of starch: amylose (linear α-1,4-linked glucose polymer), amylopectin (α-1,6-linkage) and pullulan (linear polymer of maltotriose residues linked by α-1,6-bonds) (Kevin, 2000), mainly producing formate, acetate and succinate as end-products. The starch molecules bind to cell surface receptors and are transported into the cell and hydrolysed by intracellular amylase (Anderson, 1995). *Succinimonas amylolytica* is an anaerobic, Gram-negative, nonspore-forming and straight rod with rounded ends which can be motile with polar flagella. This species is less abundant among the ruminal bacteria when cattle are fed forage rations but is among the predominant bacteria when dietary starch is offered in the form of a grain mixture (Bryant et al., 1957). This species can hydrolyse starch producing succinate as the main product as well as a small amount of acetate and propionate. *Selenomonas ruminantium* is anaerobic and Gram-negative, and it consists of motile rods of 0.8-1.0 μm in width and 2-7 μm in length. This species was found to

be more abundant in the rumen when animals were fed cereal grains compared to that fed roughage (Caldwell and Bryant, 1966). Most strains can ferment a wide range of substrates (**Table 2.1**). Lactate is the major fermentation end-product when high concentrations of glucose are present, but this is replaced by acetate and propionate at low glucose concentrations (Dehority, 2004). Besides the abovementioned amylolytic bacteria, some strains of the cellulolytic bacteria like *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens* and *Clostridium* spp. are also capable of unitizing starch under certain conditions.

Amylolytic protozoa

The protozoa are also involved in degrading starch in the rumen. Between 20-45% of the amylolytic activities in the rumen have been attributed to protozoa (Coleman, 1986). The amylolytic protozoa digest starch through engulfment producing H_2, CO_2, acetate, butyrate and glycerol as products. However, the rate of uptake of starch grains varies greatly between species. The protozoa with high amylolytic activities include *Eremoplastron bovis*, *Diploplastron affine*, *Ophryoscolex caudatus* and *Polyplastron multiesiculatum*. The breakdown rate of starch by protozoa is by approximation determined by the initial starch or amylopectin concentration inside the protozoa (Coleman, 1986).

Protozoa also have the capacity of slowing down the ruminal starch-fermentation rate because, on one hand, protozoa ingest amylolytic bacteria resulting in a decrease in their population (Kurihara et al., 1978) while on the other hand, they need at most 36 h to metabolize the engulfed starch granules (Coleman, 1992).

Amylolytic fungi

Fungi account for a small proportion (~8%) of the rumen biomass where they are involved in degrading structural carbohydrates by producing a wide range of enzymes (Akin et al., 1983). *Neocallimastix frontalis* was reported to hydrolyse starch by generating an endo-hydrolytic α-amylase from which maltose, maltotriose and maltotetraose were the major products (Mountfort and Asher, 1988). Another three fungi species, *Orpinomyces joyonii*, *Neocallimastix patriciarum* and *Piromyces communis* were also observed to be capable of digesting cereal grains (McAllister et al., 1992).

Ruminal starch-degrading enzymes

Due to their small size, bacteria cannot directly ingest starch granules or high-molecular-weight starch (e.g., amylopectin), but generate enzymes which specifically cleave the α-1,4-or α-1,6-bonds of amylose and amylopectin. These amylases can be typically classified into three main categories of hydrolytic activity: endoamylases, exoamylases and debranching enzymes (**Table 2.2**).

Endoamylases cleave the α-1,4-glucosidic linkages in the interior of the starch polymer or oligosaccharides in a random manner leading to the production of linear and branched oligosaccharides. α-Amylase is the most popular bacterial endoamylase which mainly hydrolyses the internal α-1,4-bonds of amylose. A few types of α-amylases are also capable of hydrolysing the α-1,6-bonds of amylopectin (Kevin, 2000). α-Amylases have been classified into the glycosyl hydrolases (GH) superfamily 13 and 57 based on amino acid sequence similarity (Henrissat, 1991). Exoamylases hydrolyse the α-1,4-linkages at the nonreducing end of the starch molecule, of which the end-product is one predominant dextrin. β-Amylase which belongs to GH family 14 is an exoenzyme that liberates maltose by hydrolysing 1,4-bonds. Because it cannot bypass 1,6-linkages, there always remain some β-limit dextrins after β-amylolysis. α-Glucosidases are members of GH family 15 and 31 which hydrolyse the α-1,4-or α-1,6-linkages on the nonreducing end in short saccharides produced by other enzymes. Glucoamylases have the ability to degrade both 1,4- and 1,6-linkages, solely forming glucose as an end-product. Some debranching enzymes are also capable of cleaving the α-1,6-glucosyl link (Clark and Bauchop, 1977). Isoamylases can degrade various branched structures of amylopectin, glycogen and branched oligosaccharides and dextrins. The pullulanase cleaves the α-1,6-link of pullulan-producing maltotriose which can then be hydrolysed by isopullulanases yielding isopanose

Table 2.1. Fermentation characteristics of main ruminal amylolytic and cellulolytic bacteria

Microorganism	Substrate	Fermentation product	Gram stain
Amylolytic			
Streptococcus bovis	starch, maltose, cellobiose, sucrose, glucose, fructose, galactose, mannose, lactose (pectin, xylose, arabinose, mannitol, glycerol)	lactate, CO_2 (acetate, formate)	positive
Ruminobacter amylophilus	starch, maltose	formate, acetate, succinate (lactate)	negative
Ruminococcus bromii	starch, maltose, fructose (glucose, mannose)	acetate, H_2, CO_2 (lactate, formate, propionate, butyrate)	positive
Succinimonas amylolytica	starch, maltose, fructose	succinate (acetate, propionate)	negative
Selenomonas ruminantium	maltose, cellobiose, xylose, arabinose, glucose, fructose, galactose, mannose, lactose, mannitol (starch, sucrose, glycerol, lactate)	lactate, propionate, acetate, H_2, CO_2 (succinate)	negative
Bifidobacterium sp.	starch, sucrose, maltose, glucose, fructose, xylose, lactose, galactose	acetate, lactate	positive
Prevotella species	cellobiose, glucose, fructose, galactose, mannose, lactose (starch, xylan, pectin, maltose, sucrose, xylose, arabinose)	acetate, succinate, formate, propionate, isobutyrate (butyrate, isovalerate, lactate)	negative
Cellulolytic			
Ruminococcus albus	cellulose, xylan, cellobiose (sucrose, xylose, arabinose, glucose, fructose, mannose, lactose, mannitol)	acetate, ethanol H_2, CO_2 (formate, lactate)	positive
Ruminococcus flavefaciens	cellulose, xylan, cellobiose (sucrose, xylose, arabinose, glucose, mannose, lactose)	acetate, succinate, H_2, CO_2 (formate, lactate)	positive
Fibrobacter succinogenes	cellulose, cellobiose, glucose (starch, pectin, maltose, lactose)	acetate, succinate (formate, propionate, isovalerate)	negative
Butyrivibrio fibrisolvens	xylan, pectin, arabinose, glucose, fructose, galactose, mannose (starch, cellulose, maltose, cellobiose, sucrose, xylose, lactose)	formate, butyrate, acetate, H_2, CO_2 (lactate, succinate)	positive
Clostridium polysaccharolyticum	starch, cellulose, xylan, pectin, maltose, cellobiose, xylose, arabinose (fructose)	formate, butyrate, acetate, H_2, (propionate)	positive
Clostridium longisporum	cellulose, pectin, cellobiose, sucrose, glucose, fructose, galactose, mannose, xylose, arabinose	formate, butyrate, acetate	positive
Eubacterium cellulosolvens	cellulose, maltose, cellobiose, sucrose, glucose, fructose, lactose (xylan, pectin, galactose)	lactate, H_2 (formate, acetate, succinate, butyrate)	positive

Substrates or products in brackets indicate that they vary between strains. Modified from Stewart and Flint (1997).

Table 2.2. Enzymes involved in starch degradation in the rumen

Category	Link	Enzyme	Substrate	End-product
Endoamylase	endo-α-1,4-glycosyl	α-amylase	amylose, amylopectin, (granule, oligomer)	linear and branched oligosaccharides, glucose, maltose
Exoamylase	exo-α-1,4-glycosyl	β-amylase	amylose, amylopectin, oligomer (granule)	maltose, β-limit-dextrin
	exo-α-1,4-glycosyl	α-glucosidase	oligomer (amylose, amylopectin)	glucose
	exo-α -1,4 glycosyl, exo-α-1,6-glycosyl	glucoamylase	amylose, amylopectin, oligomer (granule)	glucose
Debranching enzyme	endo-α-1,6-glycosyl	isoamylase	amylopectin	linear oligosaccharide
	endo-α-1,6-glycosyl	pullulanase	amylopectin	maltotriose
	end-α-1,6-glycosyl	isopullulanase	amylopectin	isopanose

The substrate in brackets indicates that fermentation depends on the enzyme source. Modified from Kotarski et al. (1991).

Factors affecting ruminal starch degradation

Starch degradation in the rumen is influenced by intricate interrelations of multiple factors, including starch sources, diet composition, amount of feed consumed per unit time, mechanical alterations, chemical alterations and adapting degree of ruminal microbiota to the starch ratios in diet (Huntington, 1997).

The rate and content of ruminal starch degradation vary with the type of cereal grains. Usually, wheat and barley starch are degraded more rapidly in the rumen than corn or sorghum starch (Nordin and Campling, 1976). Ruminal digestion of starch in the ground, rolled, or cracked corn (50-90%) or sorghum (42-89%) is generally lower than that in similarly processed barley (87-90%) (Theurer, 1986). Starch granules within the grain endosperm are surrounded by a protein matrix. The protein matrix in corn is extremely resistant to the invasion of amylolytic bacteria and can only be penetrated by some fungi, while for barley and wheat the protein matrix is easily penetrated by a variety of proteolytic bacteria. In this regard, the combination of slowly and rapidly degraded grains was recommended (Mendoza et al., 1999).

Physical processing is another factor influencing ruminal starch degradation. Generally, processed grains are more digestible in the rumen (Huntington et al., 2006). With the rolling, cracking, or grinding of barley, a higher ratio of starch (87-90%) was digested in the rumen compared to the maize or sorghum (50-90%) (Kotarski et al., 1991). Steam-flacking as a processing technology increased the grain starch degradation in the rumen, resulting in less starch available for the post ruminal fermentation (Xiong et al., 1991).

Cellulose degradation

The rations for dairy cows are predominantly plant-based. The plant cell walls are primarily composed of cellulose which accounts for 20-30% of the dry weight of the primary cell wall. Cellulose is a homopolymer of glucose linked by linear 1,4-β-glycosidic bonds. Cellulose molecules associate with each other to form microfibrils in the form of crystalline formulations.

Cellulolytic organisms in the rumen

A large number of anaerobic bacteria, protozoa and fungi possess very efficient cellulolytic machinery which enables them to improve the feed conversion efficiency of cellulose. Cellulolytic organisms are those microbes predominantly digesting cellulose present in the diet, which were dominated by mainly bacteria, fungi and to a lesser extent the protozoa (Krause et al., 2003).

Cellulolytic bacteria

The *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Fibrobacter succinogenes* are the major cellulolytic bacteria (Krause et al., 2003). *Fibrobacter succinogenes* is one of the most widespread cellulolytic bacteria in the rumen, which contributes ~5-6% of the total prokaryotic 16S rRNA in the rumen contents of cattle (Briesacher et al., 1992). The species is strictly anaerobic and nonspore-forming with the cells Gram-negative. Their growth requires valerate and isobutyrate and partly need biotin and p-aminobenzoic acid (Bryant, 1959). *Fibrobacter succinogenes* strains were reported to degrade cellulose, glucose and cellobiose mainly producing acetate and succinate (Stewart and Flint, 1997). Some strains are capable of degrading some cellulose allomorphs which are not susceptible to degradation by *Ruminococcus flavefaciens*. *Ruminococcus flavefaciens* are usually Gram-positive or Gram-variable and often generate a characteristic yellow pigment, particularly when grown on cellulose. Most *Ruminococcus flavefaciens* strains are able to degrade quite recalcitrant forms of cellulose which is difficult to digest by other species (Stewart et al., 1990). Previous research showed that *Ruminococcus flavefaciens* mainly attach to the cut edges of the epidermis, sclerenchyma and phloem cells when incubated with ryegrass leaves (Latham et al., 1978), and the attachment occurred at the epidermis and parenchyma bundle-sheath when incubated with orchard grass and Bermuda grass (Akin and Rigsby, 1985). *Ruminococcus flavefaciens* mostly degrade cellulose and cellobiose, while some strains can also utilize glucose and other carbon compounds including maltose, lactose, xylose and starch (**Table 2.1**). The main end-products include acetate, succinate, formate and lactate, together with traces of hydrogen and CO_2. *Ruminococcus albus* cells are usually single or diplococcic, 0.8-2.0 μm in diameter and Gram-negative to Gram-variable. Generally, in the rumen, *Ruminococcus albus* is more abundant than *Ruminococcus flavefaciens* (Varel and Dehority, 1989). *Ruminococcus albus* strains are

able to degrade cellulose and cellobiose but cannot utilize glucose or other sugars. The main end-products of this degradation include acetate, ethanol, formate, lactate, hydrogen and CO_2 with different combinations and proportions as the major products. *Ruminococcus albus* can produce ethanol, while the *Ruminococcus flavefaciens* produce succinate instead. The abovementioned three cellulolytic bacteria share some common features: 1) their growth needs a strict pH range from 6 to 7, 2) they are all strictly anaerobic and cannot survive when exposed to oxygen, 3) they digest cellulose by attachment to the cell surface through an extracellular glycocalyx, and 4) these bacteria are majorly restricted to cellulose or the hydrolysed products of cellulose.

Apart from the above three major bacteria, some strains in *Butyrivibrio fibrisolvens*, *Eubacterium cellulosolvens* and *Clostridium* spp. have also been reported to be involved indirectly in cellulolytic activities (Krause et al., 2003). These cellulolytic bacteria degrade cellulose via adherence to an extracellular structure, the cellulosome. The processes for the adherence of bacteria to cellulose have been reviewed by Miron et al. (2001) and Krause et al. (2003). In short, the adherence could be defined in four steps: 1) non-motile bacteria are transported to the substrate, 2) bacteria adhere non-specifically to available sites on the plant cell wall, 3) the ligands or adhesins on the bacterial cell surface adhere specifically to the receptors on the substrate, and 4) the adhered bacteria proliferate to create colonies on potentially digestible sites of a substrate.

Cellulolytic fungi and protozoa

Cellulolytic activities have also been reported by fungal and protozoal populations in the rumen. The ruminal fungi with cellulolytic capacity include *Neocallimastix frontalis*, *Neocallimastix patriciarum* and *Neocallimastix joyonii*. The fungi also possess cellulosome-like machinery, which aids in the adherence process to cellulose (Steenbakkers et al., 2001). Furthermore, cellulolytic protozoa, such as *Eudiplodinium maggie*, *Ostracodinium album*, *Epidinium caudatum*, etc. degrade cellulose by engulfment (Castillo-Gonzalez et al., 2014).

Ruminal cellulose-degrading enzymes

Most cellulases are GH which are able to hydrolyse the glycosidic bonds within carbohydrate molecules (Henrissat and Bairoch, 1993). In general, the hydrolases

cleave the C-O, C-N, or C-C bonds of the glucosides producing sugar and another compound, while cellulases mainly cleave the 1,4-β-glycosidic bonds between glucosyl moieties in cellulose into its monomers.

Cellulose is hydrolysed to its monomeric glucose units by the synergistic action of three major types of cellulases: 1) endoglucanases (endo-1,4-β-D-glucan hydrolases), 2) exoglucanases (exo-1,4-β-D-glucan cellobiohydrolases) and 3) β-glucosidases (β-D-glucosidases) (Krause et al., 2003, **Table 2.3**). These three cellulases break down cellulose at different sites and work synergistically on cellulose hydrolysis (Lynd et al., 2002, **Figure 2.1**). Briefly, the endoglucanase firstly randomly breaks down the amorphous regions of cellulose creating new chain ends, then the exoglucanases attack the non-reducing ends of cellulose or cellotetraose produced by endoglucanase, yielding cellobiose and cellotriose as products. The products are finally hydrolysed to glucose by β-glucosidases.

Table 2.3. Information on cellulose-degrading enzymes in the rumen

Enzyme	Linkage	Substrate	Action
Endoglucanase	1,4-β-D-glucosidic linkage	cellulose	cleave internal bonds at amorphous sites creating new chain ends
Exoglucanase	1,4-β-D-glucosidic linkage	cellulose, cellotetraose	cleave two to four units from the non-reducing ends of the cellulose or cellotetraose molecules produced by endoglucanase
β-Glucosidase or cellobiase	1,4-β-D-glucosidic linkage	cellobiose, cellotriose	hydrolyse the exoglucanase products into individual monosaccharides (glucose)

All these abovementioned cellulases have been isolated from the ruminal cellulolytic microbes and classified into specific GH families, for example, the endoglucanases mainly belong to the GH family 5 and 9, whereas, exoglucanases are mostly present in the GH family 6, with the β-glucosidases mainly classified into GH family 3 (Vorgias and Antranikian, 2000).

Application of metagenomics on ruminal microorganisms

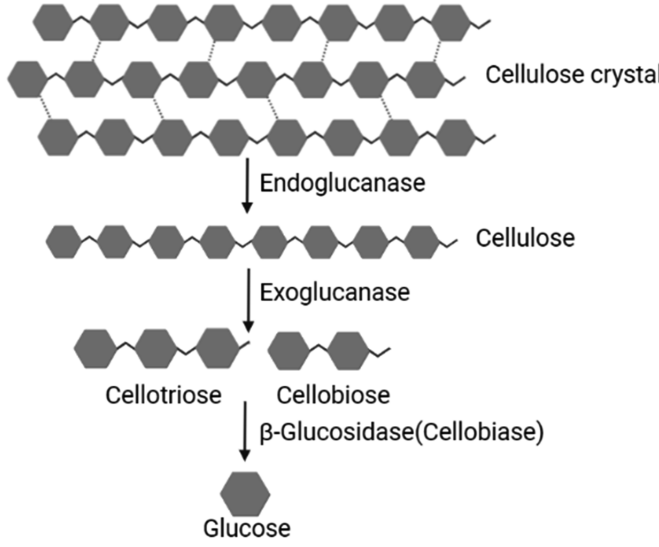


Figure 2.1. Structure cellulose breakdown by three cellulases. Created in BioRender.com

To date, only a relatively small fraction of rumen microorganisms has been successfully isolated and cultured. The largely unexplored microorganisms represent a huge untapped source of novel enzymes, especially those with multiple functions. Thanks to the development of next-generation sequencing technologies and bioinformatics tools together with the rapid progress in reference databases, metagenomics has become a powerful tool to study the rumen microbiome.

With metagenomics technologies, we can acquire the collective genetic structure and functional composition of rumen microorganisms without culturing their inhabitants. According to amino acid sequence similarity, GH and related enzymes are classified into specific families with all members in one family possessing the conserved catalytic mechanism. The public database of Carbohydrate Active enZyme (CAZy), which contains and updates all GH families, has been frequently used to mine enzymes in the rumen of dairy cows (Brulc et al., 2009). This section will summarize recent knowledge of the metagenomic insights into the starch- and cellulose-degrading enzymes in the rumen of dairy cows.

Rumen metagenomics analysis comprises two areas, including: 1) functional metagenomics, in which the high-throughput screening technique is used for investigating gene products out of cloned expression libraries established by rumen metagenome DNA and 2) sequencing-based metagenomics in which the genomes and genes present in rumen microbes are explored through high-throughput next-generation sequencing.

Functional metagenomics

Ferrer et al. (2005) first applied the functional metagenomics approach in identifying hydrolytic enzymes involved in the ruminal digestion of plant polysaccharides, from which nine endoglucanases and 12 esterases were detected from the metagenomic library of dairy cows. Since then, more research has been conducted to investigate specific polysaccharide-degrading enzymes from the rumen through metagenomic libraries. Morgavi et al. (2013) summarized the studies before 2012 about the applications of functional metagenomics for mining polysaccharide-degrading enzymes from the rumen (**Table 2.4**). In this review, the cellulose-degrading enzymes detected from the cow rumen by those studies mainly belonged to GH families 5, 3 and 26. Li (2015) reviewed the publications from 2012 to 2015, particularly on the lignocellulose-degrading enzymes mined from the rumen through functional metagenomic approaches (**Table 2.4**). They concluded that the new screened cellulases in the cow rumen mostly belonged to GH families 5, 8, 9 and 48. Even though the abovementioned studies have proven the applications of functional screening technique in characterizing ruminal enzymes, many challenges remain e.g., 1) the expression libraries can only show a small fraction of functional diversity because not all target genes are easy to be expressed in foreign host systems, and 2) the present techniques for detecting and screening desired functional activities need to be more efficient. To overcome these difficulties, new approaches have been developed. For instance, the habitat biasing methods were used to fractionate the microbial community in order to decrease the complexity of the microbiome or to enrich desired activities (Ekkers et al., 2012) or the combination of the *in vitro* compartmentalization and fluorescent-activated cell sorting was able to improve the functional screening of complex microbial ecosystems (Ferrer et al., 2009). With further evolutions of techniques, new enzymes

and metabolic activities will be characterized by the rumen microbiome with functional metagenomics.

Sequencing-based metagenomics

Sequencing-based metagenomics provides the collective genetic composition and functional activities of a microbial community at the DNA level. The first publication using next-generation sequencing-based rumen computational metagenomics for cataloguing the genes and activities involved in ruminal fibre degradation was reported in 2009 (Brulc et al., 2009). Later, Morgavi et al. (2013) compared the contributions of four rumen fibrolytic bacteria to the GH families involved in plant polysaccharide degradation. The cellulose-degrading GH families 3, 5, 8, 9 and 51 were represented in the bacteria species of *Fibrobacter succinogenes* S85 and *Ruminococcus albus*. The publications since 2015 on metagenomics application related to ruminal starch- and cellulose-degrading enzymes of cows are summarized in **Table 2.4**. Most of these studies were based on sequence-based metagenomics. Besides the cellulase GH families mentioned by Li (2015), more novel cellulose-degrading enzymes were detected from rumen microbiomes and mostly belonged to GH families 44, 45, 6, 7, 88, 10, 51 and 95. While the starch-degrading enzymes were mainly from 13, 97, 31, 57, 77 and 15. Gharechahi et al. (2021) compared the fibre-attached rumen-uncultured microbiota and CAZyme produced after incubation with six lignocellulosic substrates, in which they found the most abundant GH families containing the GH3, GH31 and GH97 glucosidases and the GH51 endoglucanases. They also identified proteins that were the main components of cellulosome complexes but also had the potential to encode the α-amylases (GH13, GH13_6, GH13_7, GH13_15, GH13_28 and GH97) and cellulases (GH5, GH5_2, GH5_4, GH9, GH124 and GH128). Literature shows most metagenomics studies mainly focus on the ruminal fibrolytic activities and the efforts on starch degradation were relatively less.

In total, with the assistance of metagenomics tools, comprehensive studies as illustrated above will broaden our knowledge of the ruminal microbial structure and enzymatic activities, which in turn would allow for rumen manipulations to achieve a more efficient fibre and starch degradation. For instance, 1) as more microbial amylases and cellulases are identified out of the ruminal microbiome, it will be foreseeable to regulate the ruminal microbial amylolytic and cellulolytic activities through supplementing

exogenous enzymes in the form of feed additive, 2) newly identified species will promote the process of isolating microbes out of the rumen and improve the development of microbe-culture techniques, and 3) it will facilitate the commercial applications of rumen enzymes in various industries including feed additives and biofuel production.

Conclusion

This review has summarized the microbes and enzymes involved in starch and cellulose degradation and discussed the state of metagenomics technology in mining novel cellulases and amylases GH families in the rumen of dairy cows. To date, a number of amylolytic and cellulolytic microorganisms, their characteristics and their metabolic mechanisms in the rumen of dairy cows have been described. But still, uncharacterized microbes and enzymes need to be identified. The recently emerging technologies like metagenomics have become more efficient in exploring new microbial species and strains, mining novel enzymes and monitoring microbial and enzymatic activities. This will improve the development of new culturing techniques. In turn, the advancement of our knowledge into the functioning of the microbiota of the rumen can facilitate the directed regulation of specific microbial activities or supplementation of exogenous enzymes.

Table 2.4. Amylolytic and cellulolytic enzymes mined from the rumen of dairy cows through metagenomics approach

Reference	Enzyme	Glycoside hydrolase family
Gharechahi et al. (2021)	amylase	13, 97
	cellulase	4, 5, 8, 9, 124, 128
	endocellulase	74
Shen et al. (2020)	amylase	13, 15, 31, 57, 77
	cellulase	97, 9, 5, 88, 45, 95, 44, 48
Zhao et al. (2020)	amylase	13, 15, 31, 4, 57, 63, 77, 97, 119
	cellulase	5, 6, 8, 9, 10, 11, 26, 44, 45, 48
Wang et al. (2020)	amylase	13, 57
	cellulase	5, 9, 88, 95
	endocellulase	5, 6, 7, 9, 44, 45
	β-glucosidase	13, 88
Bohra et al. (2019)	cellulase	5, 9, 44, 45
Terry et al. (2019)	endocellulase	5, 6, 7, 8, 9, 44, 45, 48
	exocellulase	5, 6, 9, 48
	β-glucosidase	5, 9
Jose et al. (2017)	cellulase	5
	endocellulase	6, 7, 9, 44, 45
	β-glucosidase	1, 3
Shinkai et al. (2016)	cellulase	5, 6, 8, 9, 44, 45, 48, 74
Pitta et al. (2016)	amylase	13, 27, 77, 88
	cellulase	5, 9, 48, 81
	oligosaccharide degrading	1, 2, 3, 4, 13, 27, 29, 31, 35, 37,
	enzymes	38, 42, 57, 59, 63, 65, 88
Kang et al. (2015)	cellulase	5, 6, 7, 8, 9, 12, 44, 45, 48
Ko et al. (2013)	exocellulase	48
Gong et al. (2012)	endoglucanase cellulases	5, 8, 9
Hess et al. (2011)	unspecified	5, 8, 9, 10, 26
Zhao et al. (2010)	α-amylase	57
Wang et al. (2009)	β-glucosidase	3
	endo-β-1,4-glucanase	5
Shedova et al. (2009)	endo-β-1,4-glucanase	5
Palackal et al. (2007)	glucanase/mannanase/xylanase	5, 26
Ferrer et al. (2005)	endo-glucanase	5, 26

Reference

Akin, D. E., Gordon, G. L. R., and Hogan, J. P. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. *Appl. Environ. Microbiol.* 46(3):738-748.

Akin, D. E. and Rigsby, L. L. 1985. Degradation of bermuda and orchard grass by species of ruminal bacteria. *Appl. Environ. Microbiol.* 50(4):825-830.

Anderson, K. L. 1995. Biochemical analysis of starch degradation by *Ruminobacter amylophilus* 70. *Appl. Environ. Microbiol.* 61(4):1488-1491.

Bohra, V., Dafale, N. A., and Purohit, H. J. 2019. Understanding the alteration in rumen microbiome and CAZymes profile with diet and host through comparative metagenomic approach. *Arch. Microbiol.* 201(10):1385-1397.

Briesacher, S. L., May, T., Grigsby, K. N., Kerley, M. S., Anthony, R. V., and Paterson, J. A. 1992. Use of DNA probes to monitor nutritional effects on ruminal prokaryotes and *Fibrobacter succinogenes S85*. *J. Anim. Sci.* 70(1):289-295.

Brulc, J. M., Antonopoulos, D. A., Miller, M. E. B., Wilson, M. K., Yannarell, A. C., Dinsdale, E. A., Edwards, R. E., Frank, E. D., Emerson, J. B., Wacklin, P., Coutinho, P. M., Henrissat, B., Nelson, K. E., and White, B. A. 2009. Gene-centric metagenomics of the fibre-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. *Proc. Natl. Acad. Sci.* 106(6):1948-1953.

Bryant, M. P. 1959. Bacterial species of the rumen. *Bacteriol. Rev.* 23:125-153.

Bryant, M. P., Small, N., Bouma, C., and Chu, H. 1957. *Bacteroides ruminicola n.* sp. and *Succinimonas amylolytica*; the new genus and species; species of succinic acid-producing anaerobic bacteria of the bovine rumen. *J. Bacteriol.* 76(1):15-23.

Caldwell, D. R. and Bryant, M. P. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. *Appl. Microbiol.* 14(5):794-801.

Castillo-Gonzalez, A. R., Burrola-Barraza, M. E., Dominguez-Viveros, J., and Chavez-Martinez, A. 2014. Rumen microorganisms and fermentation. *Arch. Med. Vet.* 46:349-361.

Clark, R., and Bauchop, T. 1977. Microbial ecology of the gut. Academic Press, New York, USA.

Coleman, G. 1986. The metabolism of rumen ciliate protozoa. *FEMS Microbiol. Rev.* (39):321-344.

Coleman, G. 1992. The rate of uptake and metabolism of starch grains and cellulose particles by Entodinium species, *Eudiplodinium maggii*, some other entodiniomorphid protozoa and natural protozoal populations taken from the ovine rumen. *J. Appl. Microbiol.* 73:507-513.

Dehority, B. A. 2004. Starch digestion, other less numerous species, and facultative anaerobes in the rumen, Page: 243-264, Rumen microbiology. Nottingham University Press, Nottingham.

Edwards, J. E., McEwan, N. R., Travis, A. J., and Wallace, R. J. 2004. 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie Van Leeuwenhoek* 86(3):263-281.

Ekkers, D. M., Cretoiu, M. S., Kielak, A. M., and van Elsas, J. D. 2012. The great screen anomaly-a new frontier in product discovery through functional metagenomics. *Appl. Microbiol. Biotechnol.* 93(3):1005-1020.

Ferrer, M., Beloqui, A., Vieites, J. M., Guazzaroni, M. E., Berger, I., and Aharoni, A. 2009. Interplay of metagenomics and *in vitro* compartmentalization. *Microb. Biotechnol.* 2(1):31-39.

Gharechahi, J., Vahidi, M. F., Bahram, M., Han, J., Ding, X., and Salekdeh, G. H. 2021. Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen. *ISME J.* 15(4):1108-1120.

Gong, X., Gruninger, R. J., Qi, M., Paterson, L., Forster, R. J., Teather, R. M., and McAllister, T. A. 2012. Cloning and identification of novel hydrolase genes from a dairy cow rumen metagenomic library and characterization of a cellulase gene. *BMC Res. Notes* 5:566.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280:309-316.

Henrissat, B. and Bairoch, A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293:781-788.

Hess, M., Sczyrba, A., Egan, R., Kim, T. W., Chokhawala, H., Schroth, G., Luo, S., Clark, D. S., Chen, F., Zhang, T., Mackie, R. I., Pennacchio, L. A., Tringe, S. G., Visel, A., Woyke, T., Wang, Z., and Rubin, E. M. 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. *Science* 331(6016):463-467.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York.

Huntington, G. B. 1997. Starch utilization by ruminants: from basics to the bunk. *J. Anim. Sci.* 75(3):852-867.

Huntington, G. B., Harmon, D. L., and Richards, C. J. 2006. Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle. *J. Anim. Sci.* 84:E14-E24.

Jesus, R. B. D., Omori, W. P., Lemos, E. G. D. M., and Souza, J. A. M. D. 2015. Bacterial diversity in bovine rumen by metagenomic 16S rDNA sequencing and scanning electron microscopy. *Acta Scientiarum* 3(37):251-257.

Jose, V. L., Appoothy, T., More, R. P., and Arun, A. S. 2017. Metagenomic insights into the rumen microbial fibrolytic enzymes in Indian crossbred cattle fed finger millet straw. *AMB Express* 7(1).

Kang, Y. M., Kim, M. K., An, J. M., Haque, M. A., and Cho, K. M. 2015. Metagenomics of un-culturable bacteria in cow rumen: construction of cel9E-xyn10A fusion gene by site-directed mutagenesis. *J. Mol. Catal. B Enzym.* 113:29-38.

Kevin, L. A. 2000. Degradation of cellulose and starch by anaerobic bacteria, Page: 359-386, Glycomicrobiology. Doyle, R. J., ed. Kluwer Academic Publishers, New York.

Ko, K., Han, Y., Cheong, D., Choi, J. H., and Song, J. J. 2013. Strategy for screening metagenomic resources for exocellulase activity using a robotic, high-throughput screening system. *J. Microbiol. Meth.* 94(3):311-316.

Kotarski, S. F., Waniska, R. D., and Thurn, K. K. 1991. Starch hydrolysis by the ruminal microflora. *J. Nutr.* 122(1):178-190.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., and McSweeney, C. S. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5):663-693.

Kurihara, Y., Takechi, T., and Shibata, F. 1978. Relationship between bacteria and ciliate protozoa in the rumen of sheep fed on a purified diet. *J. Agric. Sci.* 90(2):373-381.

Latham, M. J., Brooker, B. E., Pettipher, G. L., and Harris, P. J. 1978. Adhesion of *Bacteroides succinogenes* in pure culture and in the presence of *Ruminococcus flavefaciens* to cell walls in leaves of perennial ryegrass (Lolium perenne). *Appl. Environ. Microbiol.* 35(6):1166-1173.

Li, R. W. 2015. Rumen metagenomics, in Chapter 16, Page: 223, Rumen microbiology: From evolution to revolution. Puniya, A. K., Singh, R., and Kamra, D. N., ed. Springer, India.

Lynd, L. R., Weimer, P. J., Zyl, W. H., and Pretorius, I. S. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66(3):506-577.

McAllister, T. A., Dong, Y., Yanke, L. J., Bar, H. D., and Cheng, K. J. 1992. Cereal grain digestion by selected strains of ruminal fungi. *Can. J. Microbiol* 39:367-376.

Mendoza, G., Britton, R., and Stock, R. 1999. Effect of feeding mixtures of high moisture corn and dry-rolled grain sorghum on ruminal fermentation and starch digestion. *Small Ruminant Res.* 32:113-118.

Miron, J., Ben-Ghedalia, D., and Morrison, M. 2001. Invited review: adhesion mechanisms of rumen cellulolytic bacteria. *J. Dairy Sci.* 84(6):1294-1309.

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

Mountfort, D. O. and Asher, R. A. 1988. Production of alpha-amylase by the ruminal anaerobic fungus *Neocallimastix frontalis*. *Appl. Environ. Microbiol.* 54(9):2293-2299.

Nordin, M. and Campling, R. C. 1976. Digestibility studies with cows given whole and rolled cereal grains. *Anim. Sci.* 23(3):305-315.

Offner, A., and Sauvant, D. 2004. Prediction of *in vivo* starch digestion in cattle from *in situ* data. *Anim. Feed Sci. Technol.* 111(1-4):41-56.

Ortigues-Marty, I., Vernet, J., and Majdoub, L. 2003. Whole body glucose turnover in growing and non-productive adult ruminants: meta-analysis and review. *Reprod. Nutr. Dev.* 43(4):371-383.

Palackal, N., Lyon, C. S., Zaidi, S., Luginbühl, P., Dupree, P., Goubet, F., Macomber, J. L., Short, J. M., Hazlewood, G. P., Robertson, D. E., and Steer, B. A. 2007. A multifunctional hybrid glycosyl hydrolase discovered in an uncultured microbial consortium from ruminant gut. *Appl. Microbiol. Biotechnol.* 74(1):113-124.

Parker, R. and Ring, S. G. 2001. Aspects of the physical chemistry of starch. *J. Cereal Sci.* 34(1):1-17.

Pitta, D. W., Indugu, N., Kumar, S., Vecchiarelli, B., Sinha, R., Baker, L. D., Bhukya, B., and Ferguson, J. D. 2016. Metagenomic assessment of the functional potential of the rumen microbiome in Holstein dairy cows. *Anaerobe* 38:50-60.

Shedova, E. N., Berezina, O. V., Lunina, N. A., Zverlov, V. V., Schwartz, W. H., and Velikodvorskaia, G. A. 2009. Cloning and characterization of a large metagenomic DNA fragment containing glycosyl-hydrolase genes. *Mol. Gen. Mikrobiol. Virusol.* (1):11-15.

Shen, J., Zheng, L., Chen, X., Han, X., Cao, Y., and Yao, J. 2020. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of dairy goats fed different rumen degradable starch. *Front. Microbiol.* 11:1-9.

Shinkai, T., Mitsumori, M., Sofyan, A., Kanamori, H., Sasaki, H., Katayose, Y., and Takenaka, A. 2016. Comprehensive detection of bacterial carbohydrate-active enzyme coding genes expressed in cow rumen. *Anim. Sci. J.* 87(11):1363-1370.

Steenbakkers, J. M. P., Li, X., Ximenes, A. E., Arts, G. J., Chen, H., Ljungdahl, G. L., and Camp, J. M. O. D. 2001. Noncatalytic docking domains of cellulosomes of anaerobic Fungi. *J. Bacteriol.* 183(18):5325-5333.

Stewart, C. S., Duncan, S. H., McPherson, C. A., Richardson, A. J., and Flint, H. J. 1990. The implications of the loss and regain of cotton-degrading activity for the degradation of straw by *Ruminococcus flavefaciens strain 007*. *J. Microbiol. Bacteriol.* 68(4):349-356.

Stewart, S. C. and Flint, H. J. 1997. The rumen bacteria, in Chapter 1, Page: 16, The rumen microbial ecosystem. Hobson, P. N., and Stewart, C. S., ed. Blackie Academic Professional, London.

Terry, S. A., Badhan, A., Wang, Y., Chaves, A. V., and McAllister, T. A. 2019. Fibre digestion by rumen microbiota-a review of recent metagenomic and metatranscriptomics studies. *Can. J. Anim. Sci.* 99(4):678-692.

Theurer, C. B. 1986. Grain processing effects on starch utilization by ruminants. *J. Anim. Sci.* 63(5):1649-1662.

Varel, V. H. and Dehority, B. A. 1989. Ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids, and cattle fed three alfalfa-corn diets. *Appl. Environ. Microbiol.* 55(1):148-153.

Vorgias, C. and Antranikian, G. 2000. Glycosyl hydrolases from extremophiles, Glycomicrobiology. Doyle, R. J., ed. Kluwer Academic Publishers, New York.

Wang, F., Li, F., Chen, G., and Liu, W. 2009. Isolation and characterization of novel cellulase genes from uncultured microorganisms in different environmental niches. *Microbiol. Res.* 164(6):650-657.

Wang, L., Zhang, G., Li, Y., and Zhang, Y. 2020. Effects of high forage/concentrate diet on volatile fatty acid production and the microorganisms involved in VFA production in cow rumen. *Animals* 10(2):223.

Xiong, Y., Bartle, S. J., and Preston, R. L. 1991. Density of steam-flaked sorghum grain, roughage level, and feeding regimen for feedlot steer. *J. Anim. Sci.* 69:1707-1718.

Zhao, S., Wang, J., Bu, D., Liu, K., Zhu, Y., Dong, Z., and Yu, Z. 2010. Novel glycoside hydrolases identified by screening a Chinese holstein dairy cow rumen-derived metagenome library. *Appl. Environ. Microbiol.* 76(19):6701-6705.

Zhao, Y., Xue, F., Hua, D., Wang, Y., Pan, X., Nan, X., Sun, F., Jiang, L., and Xiong, B. 2020. Metagenomic insights into effects of thiamine supplementation on carbohydrate-active enzymes profile in dairy cows fed high-concentrate diets. *Animals* 10(2):304.


AGRS-III 微生物发酵微量产气自动记录仪
Automated Trace Gas Recording System for Microbial Fermentation

Chapter 3

Glucogenic and lipogenic diets affect *in vitro* ruminal microbiota and metabolites differently

Dengke Hua[1,2], Wouter H. Hendriks[2], Yiguang Zhao[1], Fuguang Xue[1], Yue Wang[1], Linshu Jiang[3], Benhai Xiong[1], Wilbert F. Pellikaan[2]

[1] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

[2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, 6708 WD, the Netherlands

[3] Beijing Key Laboratory for Dairy Cattle Nutrition, Beijing Agricultural College, Beijing, 102206, China

Submitted to Frontiers in Microbiology

Abstract

This study was conducted to evaluate the effects of two glucogenic diets (C: ground corn and corn silage; S: steam-flaked corn and corn silage) and a lipogenic diet (L: sugar beet pulp and alfalfa silage) on the ruminal bacterial and archaeal structures, the metabolomic products, *in vitro* rumen fermentation, gas production. Compared to the C and S diets, the L diet had a lower dry matter digestibility (DMD), propionate production and ammonia-nitrogen concentration. The two glucogenic diets performed worse in controlling methane and lactic acid production compared to the L diet. The S diet produced the greatest cumulative gas volume at any time point during incubation compared to the C and L diet. The metabolic analysis revealed that the lipid digestion especially the fatty acids was improved, but the amino acid digestion was decreased in the L diet than in other diets. Differences in rumen fermentation characteristics were associated with (or resulting from) changes in the relative abundance of bacterial and archaeal genera. The L diet had a significantly higher number of cellulolytic bacteria, including the genera of *Ruminococcus*, *Butyrivibrio*, *Eubacterium*, *Lachnospira*, unclassified *Lachnospiraceae* and unclassified *Ruminococcaceae*. The relative abundances of amylolytic bacteria genera including *Selenomonas_1*, *Ruminobacter* and *Succinivibrionaceae_UCG-002* were higher in diets C and S. The results indicated that the two glucogenic diets had a greater extent of gas production, a higher DMD and produced more propionate than diet L. The steam-flaked corn did not show a better performance on fermentation end-products than ground corn.

Keywords: glucogenic/lipogenic diet, rumen fermentation, microbiota, gas production, metabolomic, PICRUSt

Introduction

Dietary carbohydrates, such as starch and fibre, provide substrates for rumen microbes. Changes in carbohydrate composition and content in ruminant rations lead to the changes in microbial community and subsequently to changes in fermentation end-products, including the volatile fatty acids (VFAs), carbon dioxide (CO_2), methane (CH_4) and hydrogen (H_2) (Carberry et al., 2012). The major ruminal VFAs include acetate, propionate and butyrate. Acetate is the primary precursor of milk fatty acids and is termed a lipogenic nutrient, while propionate being the primary precursor of milk lactose is a glucogenic nutrient (van Knegsel et al., 2005). For ruminant animals, lipogenic nutrients in metabolism are supplied by acetate and butyrate from ruminal degradation of fibre or dietary fat, if not derived from the mobilization of body fat reserves. In contrast, glucogenic nutrients in metabolism can originate from the ruminal fermentation of dietary starch to propionate, rumen bypass of dietary starch which is then digested in the small intestine and absorbed as glucose, or gluconeogenesis (van Knegsel et al., 2007). Previous research showed that glucogenic nutrients increased plasma glucose and insulin concentrations, whereas lipogenic nutrients did not (van Knegsel et al., 2005). However, changes in microbial communities and their metabolic activities under lipogenic and glucogenic diets are also essential to investigate to unravel the production pathways of the affected metabolites and better understand how rumen functioning is regulated.

Ground corn is a major dietary energy source because of its high amount of readily fermentable starch. Steam-flaking can disintegrate the crystalline structure of cereal starch by gelatinisation (Ding et al., 2007), and subsequently, this can increase the accessibility to the starch granules of ruminal amylases and amylolytic microorganisms (Huntington, 1997). Previous studies showed that steam-flaked corn improved the ruminal degradability of starch, resulting in high production of ruminal propionate, and increased efficiency in microbial protein synthesis (Zhou et al., 2015).

The effects of dietary treatments on the ruminal microbes and microbial metabolism when incubated *in vitro* are rarely reported although the *in vitro* gas production technique is routinely used to evaluate dry matter (DM) degradation rate, amount and proportion of VFAs production and gas composition of various feeds and ingredients. This technique also yields valuable information on the effects of feedstuff on rumen microbial activity and predicts the kinetics of fermentation (Pellikaan et al., 2011a). With the 16S rRNA sequencing technology, a fast and

cost-effective way of microbial analysis and their correlations with environment factors coupled with liquid chromatography-mass spectrometry (LC-MS), an effective technique for metabolomics analysis, more knowledge on changes in ruminal microbiota metabolism can be generated.

Although studies on differences in rumen fermentation between glucogenic and lipogenic diets have been conducted, rumen bacterial community changes and functions are not yet fully understood. We hypothesized that glucogenic and lipogenic diets when evaluated using the *in vitro* gas production technique should lead to clear differences in bacterial communities and functions which affect intermediary metabolites besides the well-known differences in fermentation end-products and CH_4 production.

Materials and methods

Experimental design

Animal care followed the Chinese guidelines for animal welfare, and all protocols were approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (IAS2019-6).

In vitro incubation

Six lactating dairy cows (Holstein) were selected as rumen fluid donors for all three runs of this *in vitro* study, with different two cows for each run. The cows received a diet containing (% DM basis) a concentrated mixture (45%), alfalfa and oat hay (20%), corn silage (20%) and alfalfa silage (15%). The cows were fed three time daily at 7:00, 13:00 and 19:00, and they had free access to water and feed.

Three experimental diets were designed as fermenting substrates: two glucogenic diets including a ground corn diet (C, which used ground corn and corn silage as the primary energy sources) and a steam-flaked corn diet (S, which used steam-flaked corn and corn silage as the primary energy sources) and a lipogenic diet (L) mainly containing sugar beet pulp and alfalfa silage as the energy sources. In addition, other ingredients, including soybean meal, oat and alfalfa hay and calcium hydrogen phosphate were used to balance the ration to meet the nutritional requirements of dairy cattle (**Table 3.1**). Diets were isocaloric and were equal in digestible crude protein.

Table 3.1. Ingredient and nutritional composition of two glucogenic (C, S) and a lipogenic (L) diet

Item	Experimental diet		
	C	L	S
Ingredient, % of dry matter			
Ground corn	28.0	-	-
Sugar beet pulp	-	28.0	-
Steam flaked corn	-	-	28
Soybean meal	18.5	12.0	18.5
Oat hay	5.0	19.0	5
Alfalfa hay	10.0	10.0	10
Corn silage	38.0	-	38
Alfalfa silage	-	30.0	-
Dicalcium phosphate	0.5	1.0	0.5
Composition, g/kg of dry matter			
Crude protein	174.4	174.6	172.1
Ether extract	24.3	20.4	31.7
Starch	192.9	39.7	163.8
Neutral detergent fibre	326.0	562.2	320.2
Acid detergent fibre	197.9	348.9	199.1
Ash	47.6	98.7	47
Calcium	9.3	12.8	11.1
Phosphorus	10.4	4.9	11.9
NE_L, MJ/kg of dry matter	7.3	7.9	7.4

Nutrient composition of the experimental diets was calculated according to NRC (2001). Diets: C, corn and corn silage diet diet; L, sugar beet pulp and alfalfa silage diet; S, corn and steam-flaked corn diet. NE_L, net energy for lactation and calculated according to NRC (2001).

The fermentation substrates were the ground DM of each experimental diets. 0.5 g of substrates were firstly weighed into 150-ml serum bottles, with three replicate bottles for each dietary treatment within one fermentation run. A phosphate-bicarbonate buffer medium was anaerobically prepared as described by Menke and Steingass (Menke and Steingass, 1988).

Equal volumes of fresh ruminal fluid were collected through a stomach tube from cows, two hours after the first feeding (09:00 h), then poured into a sterilized and pre-warmed thermos flask (2,000 ml) leaving no headspace in the flask. After transportation to the laboratory, the rumen fluid was strained through four layers of cheesecloth and transferred into a flask placed in a water bath of 39 °C maintaining anaerobic conditions. The strained rumen fluid inoculum (25 ml) and anaerobic buffer (50 ml) were successively combined with substrates into each bottle, with the CO_2 continuously flushing in the headspace of bottles. After sealing with butyl rubber stoppers, the serum bottles were connected to the gas inlets of an automated gas production recording system (AGRS), as reported by Zhang and Yang (Zhang and Yang, 2011). Each fermentation run lasted for 48 h and were repeated for three runs within two weeks.

Sampling and chemical analysis

Calibrated gas volumes were automatically recorded and cumulative gas production was expressed against the time of incubation (Zhang and Yang, 2011). At 48 h of incubation, 20 µl of gas was collected through a 20 µl gastight syringe from each bottle to test the CH_4 concentration using gas chromatography (GC, 7890B, Agilent Technologies, USA). The GC was equipped with a capillary column (USF727432H, 30 m × 0.25 mm × 0.25 µm, Agilent, California, USA) and a flame ionization detector (FID). Nitrogen (N_2, 99.99%) was used as the carrier gas, with column settings as follows: the inlet pressure 18.85 psi, the total flow 30.2 ml/min, the column flow 1.7 ml/min, the linear speed 39.8 cm/s, the split ratio 15, the sweeping flow 3 ml/min and the cycling flow 8 ml/min. The hydrogen and airflow were 40 ml/min and 400 ml/min, respectively. Temperatures were set to 100 °C for the injection point, 80 °C for the column oven and 120 °C for the detector.

At 48 h, all bottles were transferred into an ice-water mixture to terminate the incubation. The pH value of the fermented substrates was determined using a portable pH meter (PHB-4, INESA, Shanghai, China). Then the substrates were filtered through a nylon bag (12 cm × 8 cm i.d. and 50 µm of pore size) and the residue left in the bag was used to analyse apparent dry matter digestibility (DMD) gravimetrically. A sample of 1 ml fluid was mixed with 0.25 ml of

25% meta-phosphoric acid to evaluate the VFA contents via the GC (7890B, Agilent Technologies, USA) (Mao et al., 2008). Also, 1 ml of fluid was collected to analyse the ammonia nitrogen (NH_3-N) according to the Berthelot reaction (Broderick and Kang, 1980). Another 1 ml fluid was used to determine the lactic acid concentration using an enzymatic method with the commercial kit (A019-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at 530 nm according to the manufacturer's instructions (Pan et al., 2016). Besides, two replicated 3 ml fluid samples were collected and stored under -80 °C for subsequent microbial and metabolomics analyses.

DNA extraction and amplification

The DNA of microbes was extracted from supernatant samples using the QIAamp DNA Stool Mini Kit (M5635-02, OMEGA, USA). The concentration of DNA was evaluated with the NanoDrop spectrophotometer (Thermo Scientific, USA), and then the agarose gel (1% w/v) electrophoresis was used to check the DNA quality.

The 16S rRNA gene of bacteria and archaea were separately amplified with the general primers (**Supplementary material**) based on the hypervariable region (V3-V4). The PCR was performed (**Supplementary material**) and the products were firstly extracted from an agarose gel (2% w/v), then purified with the commercial Extraction Kit (Axygen Biosciences, USA). The DNA products were finally quantified with QuantiFluor™-ST (Promega, USA).

Illumina miSeq sequencing and analysis

Purified amplicons were mixed in an equimolar ratio and paired-end sequenced (2 × 300 bp) through the MiSeq platform (Illumina, San Diego, USA) according to the manufacturer's standard (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China).

The raw fastq was quality-filtered with FLASH following the protocol previously reported by Pan et al. (2017). With a 97% sequence similarity cut-off, the operational taxonomic units (OTUs) were clustered through UPARSE. The taxonomy was calculated with the ribosomal database project (RDP) classifier against the SILVA (SSU123) 16S rRNA database with a confidence threshold of 70%. The principal coordinates analysis (PCoA) was analysed with the method of unweighted UniFrac distance to compare the interrelationships of bacterial communities between diets using the R software (3.4.4). The community richness and diversity

were analysed by the alpha diversity indexes including the OTU, Chao 1, ACE, Shannon and Simpson (Hua et al., 2021).

Inferred metagenomics analysis

The metagenome functions of ruminal bacteria were predicted using the analysis of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013). Firstly, the closed OTU table was normalized by the 16S rRNA copy number whereafter the results were exported into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The PCoA was conducted to calculate the similarities of the predicted functions among groups by the R software (3.4.4). The top ten abundant functions were further analysed to determine significant differences among diets using Welch's t-test in R software (3.4.4).

Metabolomics processing

The method was modified from the procedure described by Wang et al. (2021). The rumen fluid samples were firstly thawed under room temperature whereafter 200 µl supernatant of each sample was collected into a 1.5 ml centrifuge tube and mixed with 800 µl extracting solution (methanol:acetonitrile = 1:1 (v/v)). Each sample was then vortexed for 30 s and extracted ultrasonically (40 kHz) at 5 °C for 30 min before being treated under -20 °C for 30 min. All samples were centrifuged (13,000 × g, 4 °C, 15 min) and the supernatant transferred to a new tube, mixed with 100 µl acetonitrile solution (acetonitrile:water = 1:1), vortexed for 30 s, extracted ultrasonically (40 kHz) at 5 °C for 5 min, centrifuged (13,000 × g, 4 °C, 10 min), where after 200 µl of the supernatant was carefully transferred to sample vials for LC-MS/MS analysis. At the same time, 20 µl of supernatant was collected from each sample and mixed as the quality control sample (QC) in order to obtain information regarding system repeatability.

Chromatographic separation of the metabolites was performed on the ultra-performance liquid chromatography (UPLC) coupled with a triple time-of-flight (TOF) system (UPLC-Triple TOF, AB Sciex, USA). The system was equipped with the ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, Milford, USA). Mobile phase A consisted of 5% acetonitrile water and 0.1% formic acid, the mobile phase B contained 95% acetonitrile-isopropanol (1:1, v/v) and 0.1% formic acid. The injection volume was 10 µl, the flow rate was 0.4 ml/min, and the column temperature was 40 °C. The elution gradient of the mobile phases is shown in the supplementary material. After being treated with an electrospray ionization (ESI) source, the

signals of mass spectra were scanned in both positive mode and negative mode. The optimal conditions for mass spectra are shown in the supplementary material.

Metabolomics data analysis

After UPLC-TOF/MS analyses, the raw data were imported into Progenesis QI 2.3 (Waters Corporation, Milford, USA) for a series of pre-processing, including filtration of the baseline, identification and integration of the peak, correction of the retention time and alignment of the peak. After the pre-processing, a data matrix was generated consisting of the retention time (RT), mass-to-charge ratio (m/z) values and peak intensity. The MS and MS/MS information was searched in the Human metabolome database (HMDB) (http://www.hmdb.ca/) and Metlin database (https://metlin.scripps.edu/). Results were shown in the form of a data matrix.

After being pre-processed, the data matrix was analysed on the Majorbio Cloud Platform (https://cloud.majorbio.com). Using the R package of ROPLS (version1.6.2), a principal component analysis (PCA) was applied to obtain an overview of the metabolic data, general clustering, trends, or outliers whereafter orthogonal partial least squares discriminate analysis (OPLS-DA) was performed to observe the global difference of the metabolites between comparable groups. The variable importance in the projection (VIP) was calculated in the OPLS-DA model, and the *P*-value was estimated with paired Student's t-test. Statistically significant metabolites among groups were selected with VIP > 1 and $P \leq 0.05$. Differential metabolites between every two groups were summarized into different metabolic groups and mapped into their biochemical pathways through the KEGG database. The metabolic pathway enrichment analysis of the metabolic groups was conducted with the Fisher's exact test using the Python package of Scipy. stats (version1.0.0, SciPy.org).

Correlation between bacterial community and rumen metabolites

Correlation between the affected bacterial genera with a relative abundance > 0.5% and the rumen fermentation parameters, as well as the correlation between these affected bacterial genera and the differential metabolites (VIP > 1.5, fold change > 2 or < 0.5, $P \leq 0.05$), was separately assessed by Pearson’s correlation analysis in R (version 3.4.4). These correlations were visualized using the R package of Pheatmap.

Curve fitting and calculations

Data of the cumulative gas production curve was in accordance with the monophasic model using a non-linear least squares regression procedure NLIN in SAS 9.3 (SAS Institute Inc., Cary, NC) (Pellikaan et al., 2011b):

$$GP = A / (1 + (C / t)^B),$$

in which GP is the total gas produced (ml/g OM), A is the asymptotic gas production (ml/g OM), B equals the switching characteristic of the curve and C is the time at which half of the asymptote has been reached and t is the time (h). The maximum rate of gas production (R_{max}, ml/g OM/h) and the time when R_{max} appears (TR_{max}, h) were separately calculated using the equations below (Bauer et al., 2001):

$$R_{max} = (A \times C^B \times B \times TR_{max}^{(-B - 1)}) / (1 + (C^B \times TR_{max}^{(-B)}))^2,$$

$$TR_{max} = C \times (((B - 1) / (B + 1))^{(1 / B)}).$$

Statistical analysis

All fermentation end-products and gas kinetics data were analysed using PROC MIXED of SAS 9.3 (SAS Institute Inc., Cary, NC). The statistical model was

$$Y_{ij} = \mu + D_i + B_j + e_{ij},$$

where Y_{ij} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet (i = 1-3), B_j is the random effect of run (j = 1-3), e_{ij} is the random residual error. The Student-Newman-Keuls (SNK) multiple comparison procedure in the LSMEANS statement was used to test differences among treatments. Significance was considered at $P \leq 0.05$, and a trend was declared at $0.05 < P \leq 0.10$.

Data availability

All microbiota data were submitted to the NCBI (National Centre of Biotechnology Information, Bethesda, Maryland, USA) Sequence Read Archive (SRA) database (accession number, SUB8089454).

Results

Effect of treatments on gas production

The cumulative gas productions at 6, 12, 24 and 48 h of *in vitro* incubation showed the same direction of effects (**Table 3.2**), where the diet S had the highest gas production compared to the other two diets, while the diet L which composed of sugar beet pulp and alfalfa silage gave the lowest gas production ($P < 0.001$). The CH_4 production in diet S was higher than that in diet L ($P = 0.043$), but both diets did not differ from the glucogenic diet composed of ground corn and corn silage (C). The *in vitro* dry matter digestibility (DMD) of diet C and S was greater ($P < 0.001$) than that of diet L.

The cumulative gas production curve derived from the monophasic model is shown in **Supplementary Figure S3.1**. As for the curve fit parameter estimates (**Table 3.2**), the S diet had the highest asymptotic gas production (A) compared to the other diets ($P < 0.001$), the switching characteristic (B) of diet L was lower, while diet S had lower halftime (C), compared to the other two diets ($P < 0.001$). The S diet had the highest maximum gas production rate (R_{max}) followed by diet L and C diet ($P < 0.001$).

Effect of treatments on fermentation end-products

The concentration of fermentation end-products and pH at 48 h are shown in **Table 3.3**. Compared with the L diet, both C and S diets had greater DMD ($P < 0.001$) and higher lactic acid concentration ($P = 0.011$) but lower pH value ($P < 0.001$). The L diet had a significantly lower NH_3-N concentration ($P = 0.001$) and the lowest lactic acid level ($P = 0.011$). Both C and S diets had greater propionate ($P = 0.004$) and butyrate ($P = 0.015$) concentrations and lower acetate to propionate ratio ($P < 0.001$) compared to the L diet.

Table 3.2. Comparison of cumulative gas production at 6, 12, 24 and 48 h, curve fit parameters, head space methane concentration and dry matter digestibility at 48 h among two glucogenic (C, S) and a lipogenic (L) diet under *in vitro* fermentation with rumen fluid of dairy cows

Item	Experimental diet			SEM	*P*-value
	C	L	S		
Gas production (ml/g OM)					
6 h	94.37[b]	73.06[c]	106.08[a]	3.783	<0.001
12 h	118.14[b]	100.08[c]	132.45[a]	3.671	<0.001
24 h	125.67[b]	108.68[c]	139.29[a]	3.561	<0.001
48 h	128.24[b]	110.95[c]	141.40[a]	3.464	<0.001
Curve fit parameters					
A (ml/g OM)	139.77[b]	124.60[c]	155.84[a]	3.438	<0.001
B	1.41[a]	1.18[c]	1.36[b]	0.040	0.001
C (h)	3.91[b]	4.22[a]	3.57[c]	0.129	<0.001
Rmax (ml/h/g OM)	23.48[c]	24.49[b]	27.83[a]	0.936	<0.001
TRmax (h)	1.03[a]	0.51[c]	0.88[b]	0.100	<0.001
Methane (%, 48h)	11.64[ab]	9.23[b]	13.45[a]	0.844	0.043
DMD (%, 48h)	87.72[a]	75.82[b]	87.64[a]	0.979	<0.001

A, asymptotic gas production; B, switching characteristic of the curve; C, time at which half of the asymptote has been reached; Rmax, maximum rate of gas production; TRmax, time at which Rmax occurs. DMD, dry matter digestibility. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. OM, organic matter. SEM, standard error of the mean.

[a, b, c] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table 3.3. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the ruminal pH and end-products after 48 h *in vitro* fermentation with rumen fluid

Item	Experimental diet			SEM	*P*-value
	C	L	S		
pH	6.61[b]	6.74[a]	6.62[b]	0.011	<0.001
NH_3-N (mg/dl)	70.14[a]	52.98[b]	65.70[a]	1.817	0.001
Volatile fatty acids (mmol/l)					
Acetate	68.90	71.87	71.29	1.129	0.650
Propionate	27.58[a]	24.42[b]	29.34[a]	0.582	0.004
Acetate:Propionate	2.50[b]	2.94[a]	2.43[b]	0.037	<0.001
Butyrate	11.86[a]	10.42[b]	12.31[a]	0.258	0.015
Valerate	0.68	0.60	0.71	0.025	0.356
Isobutyrate	5.66	5.31	5.81	0.111	0.306
Isovalerate	7.34	6.66	7.39	0.165	0.208
Total VFA	124.5	122.2	129.3	2.10	0.492
Lactic acid (mmol/l)	0.51[a]	0.40[b]	0.50[a]	0.016	0.011

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean. Total VFA, total volatile fatty acid (acetate + propionate + butyrate + valerate + isobutyrate + isovalerate).

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Effect of treatments on ruminal bacteria and archaea

The alpha diversity measurements as influenced by the three diets are shown in **Table 3.4**. A total of 1,070,928 quality sequence reads across all samples were acquired with an average read length of 421 bp. The total number of reads from each sample varied from 28,949 to 70,861, with an average reads number of 38,919. The entire sequences were assigned to 2,042 operational taxonomic units (OTUs) using a cut-off of 97% sequence similarity. The richness and diversity estimators (**Table 3.4**) showed the total number of observed OTUs in the L diet was higher than in the other diets ($P = 0.031$). No differences in other diversity estimators (Chao 1, ACE, Shannon and Simpson indices) were observed among the three groups. The alpha diversity estimates of archaea (**Table 3.4**) showed that the total number of observed OTUs from the C diet was lower compared to the S and L diets ($P = 0.028$). Both the C and S diets had a significantly lower Shannon diversity index and a higher Simpson diversity index for archaea in comparison with the L diet ($P = 0.024$).

Table 3.4. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the alpha diversity indices of ruminal bacteria and archaea communities after 48 h *in vitro* fermentation with rumen fluid of dairy cows

Item	Experimental diet			SEM	*P*-value
	C	L	S		
Bacteria					
OTU	1403[b]	1493[a]	1408[b]	9.377	0.031
Chao 1	1652	1717	1655	6.734	0.241
ACE	1652	1717	1655	6.734	0.136
Shannon	5.58	5.78	5.56	0.026	0.339
Simpson	0.021	0.012	0.025	0.001	0.295
Archaea					
OTU	152[c]	182[a]	173[b]	4.114	0.028
Chao 1	306	352	364	8.095	0.056
ACE	518	533	590	10.201	0.427
Shannon	1.10[b]	1.57[a]	1.17[b]	0.068	0.018
Simpson	0.584[a]	0.357[b]	0.539[a]	0.032	0.024

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. ACE, abundance-based coverage estimator. OTU, operational taxonomic units. SEM, standard error of the mean.

[a, b, c] means within a row with different superscripts differ significantly ($P \leq 0.05$).

To visualise the impact of the diets on overall rumen bacteria and archaea communities, a PCoA was performed (**Figures 3.1a** and **3.1b**). The rumen bacterial community showed a clear separation between the S and L diet along PC1, explaining > 39% of the total variation, and the L diet was separated from the C diet along PC2, explaining > 20% of the total variation (**Figure 3.1a**). The C and S diets had a minimal separation. The archaea composition of the L diet was significantly different from the C and S diets, with approximately 76% of the variance explained along PC1 (**Figure 3.1b**).

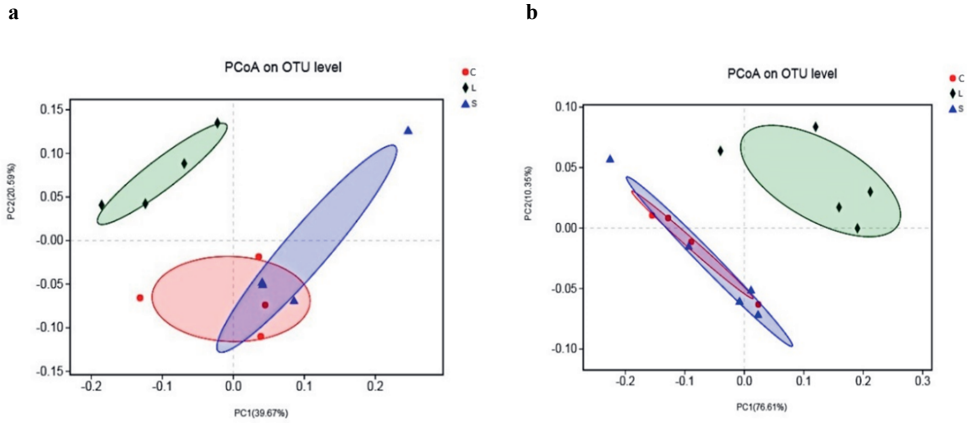


Figure 3.1. Principal coordinate analysis (PCoA) of the ruminal bacterial a) and archaea b) communities on OTU level among two glucogenic (C, S) and a lipogenic (L) diet after 48 h *in vitro* fermentation with rumen fluid of dairy cows. PCoA plots were constructed using the Bray-Curtis method. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

A total of 21 bacterial phyla were identified among all dietary treatments (**Supplementary Table S3.1**), with *Bacteroidetes*, *Firmicutes* and *Proteobacteria* being the top three predominant phyla, representing 45.0-49.4, 36.1-41.6 and 3.9-6.5% of all sequences, respectively. The L diet showed a higher relative abundance of *Tenericutes* than the other two diets ($P = 0.042$). The other predominant phyla were not affected by the treatments.

At the genus level, a total of 176 bacterial genera were identified which together accounted for 96% of all sequences. 89 of the identified genera which had a relative abundance of ≥ 0.1% in at least one sample were further analysed. Among all genera, 26 genera were affected by diet (**Supplementary Table S3.2**), with the top 20 of these genera listed in **Table 3.5**. 12 of these affected genera had higher relative abundances in the L diet compared to the other two diets, including *SP3-e08* ($P = 0.011$), *Christensenellaceae_R-7_group* ($P = 0.029$), *Ruminococcaceae_UCG-014* ($P = 0.026$), *Family_XIII-AD3011_group* ($P = 0.004$), unclassified_*o_Clostridiales* ($P = 0.010$), *Selenomonas_1* ($P = 0.005$), *Lachnospiraceae_ND3007_group* ($P = 0.025$), *[Eubacterium]_coprostanoligenes_group* ($P < 0.001$), unclassified_*f_Lachnospiraceae* ($P = 0.014$), unclassified_*f_Ruminococcaceae* ($P = 0.001$), *Ruminococcaceae_UCG_013* ($P = 0.006$), *Ruminococcus_1* ($P = 0.022$), *Butyrivibrio_2* ($P = 0.037$), *[Eubacterium]_oxidoreducens_group* ($P = 0.044$) and *Family_XIII_UCG-002* ($P = 0.026$). However, the relative abundances of *Ruminococcus_2* (*P*

= 0.018), *Ruminobacter* ($P < 0.001$) and *Succinivibrionaceae_UCG-002* ($P = 0.004$) were lower in the L diet. The S diet had a greater relative abundance of *Selenomonas_1* ($P = 0.005$), while the relative abundances of *Family_XIII_UCG-002* ($P = 0.026$) and *Family_XIII_AD3011_group* ($P = 0.004$) were lower compared to the other two diets. Compared with the S diet, the C diet had a higher relative abundance of the *Family_XIII_AD3011_group* ($P = 0.004$), *Family_XIII_UCG-002* ($P = 0.026$) and *Succinivibrionaceae_UCG-002* ($P = 0.004$).

In terms of the archaea community, the *Euryarchaeota* was the most predominant phylum. At the genus level, five archaeal genera were identified from all samples (**Supplementary Table S3.3**), and the affected ones by treatments are shown in **Table 3.5**. Compared to the L diet, both C and S diets had a significantly higher relative abundance of *Methanobrevibacter* ($P = 0.014$) but a lower relative abundance of *Candidatus_Methanomethylophilus* ($P = 0.001$) and tended to have a higher relative abundance of *Halostagnicola* ($P = 0.076$).

Predicted metagenomic functions of the ruminal bacteria

The functional prediction was conducted with PICRUSt in order to further understand the functioning of ruminal bacteria. Forty functional pathways (level 2) were predicted out of all samples (**Supplementary Table S3.4**), with amino acid metabolism, carbohydrate metabolism and membrane transport being the top three functions. The PCoA analysis showed that samples from the L diet clustered differently from those in the C and S diets (**Figure 3.2a**). The differences among groups of the top 15 most abundant functions are presented in **Figure 3.2**. Compared to the C diet, the S diet had a higher ($P = 0.044$) relative abundance in energy metabolism (**Figure 3.2b**), while the L diet had a higher ($P = 0.045$) relative abundance of membrane transport functions but lower relative abundances in amino acid metabolism ($P = 0.027$), replication and repair ($P = 0.01$), translation ($P = 0.015$), metabolisms of cofactors and vitamins ($P = 0.025$), nucleotide metabolism ($P = 0.034$) and cellular processes and signalling ($P = 0.003$) (**Figure 3.2c**). Compared to diet L, the S diet was higher in the relative abundances in amino acid metabolism ($P = 0.022$), translation ($P = 0.018$), replication and repair ($P = 0.01$) and cellular processes and signalling ($P = 0.003$) (**Figure 3.2d**).

Table 3.5. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of the top 20 affected bacteria and the top three differential archaea at the genus level after 48 h *in vitro* fermentation with rumen fluid of dairy cows

Domain/ Phylum	Genus/others	Experimental diet			SEM	*P*-value
		C	L	S		
Bacteria						
Bacteroidetes	*SP3-e08*	0.11^{b}	0.17^{a}	0.10^{b}	0.009	0.011
Firmicutes	*Christensenellaceae_R-7_group*	1.28^{b}	1.70^{a}	1.06^{b}	0.077	0.029
	Ruminococcus_2	0.87^{a}	0.55^{b}	1.0^{a}	0.055	0.018
	Ruminococcus_1	0.18^{b}	0.31^{a}	0.18^{b}	0.068	0.022
	Ruminococcaceae_UCG-014	0.74^{b}	1.05^{a}	0.70^{b}	0.045	0.026
	Ruminococcaceae_UCG-013	0.18^{b}	0.35^{a}	0.15^{b}	0.026	0.006
	Unclassified_f_Ruminococcaceae	0.19^{b}	0.35^{a}	0.20^{b}	0.021	0.001
	Butyrivibrio_2	0.24^{b}	0.62^{a}	0.29^{b}	0.076	0.037
	Selenomonas_1	0.38^{b}	0.26^{b}	0.88^{a}	0.077	0.005
	Family_XIII_AD3011_group	0.72^{b}	1.25^{a}	0.50^{c}	0.091	0.004
	Family_XIII_UCG-002	0.12^{a}	0.13^{a}	0.08^{b}	0.007	0.026
	Unclassified_o_Clostridiales	0.70^{b}	1.07^{a}	0.60^{b}	0.058	0.010
	[Eubacterium]_coprostanoligenes_group	0.63^{b}	0.91^{a}	0.50^{b}	0.050	<0.001
	[Eubacterium]_nodatum_group	0.19^{ab}	0.24^{a}	0.14^{b}	0.011	0.021
	[Eubacterium]_oxidoreducens_group	0.11^{b}	0.19^{a}	0.11^{b}	0.011	0.044
	Lachnospiraceae_ND3007_group	0.56^{b}	1.12^{a}	0.68^{b}	0.070	0.025
	Unclassified_f_Lachnospiraceae	0.36^{b}	0.78^{a}	0.36^{b}	0.057	0.014
Proteobacteria	*Ruminobacter*	1.90^{a}	0.16^{b}	1.14^{a}	0.206	<0.001
Saccharibacteria	*Candidatus_Saccharimonas*	0.75^{b}	1.16^{a}	1.01^{ab}	0.048	0.037
Archaea						
Euryarchaeota	*Methanobrevibacter*	78.3^{a}	57.6^{b}	74.0^{a}	3.382	0.014
	Candidatus_Methanomethylophilus	3.68^{b}	11.42^{a}	4.40^{b}	1.067	0.001
	Halostagnicola	0.04	0.02	0.04	0.003	0.076

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean. SEM, standard error of the mean. $^{a, b, c}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

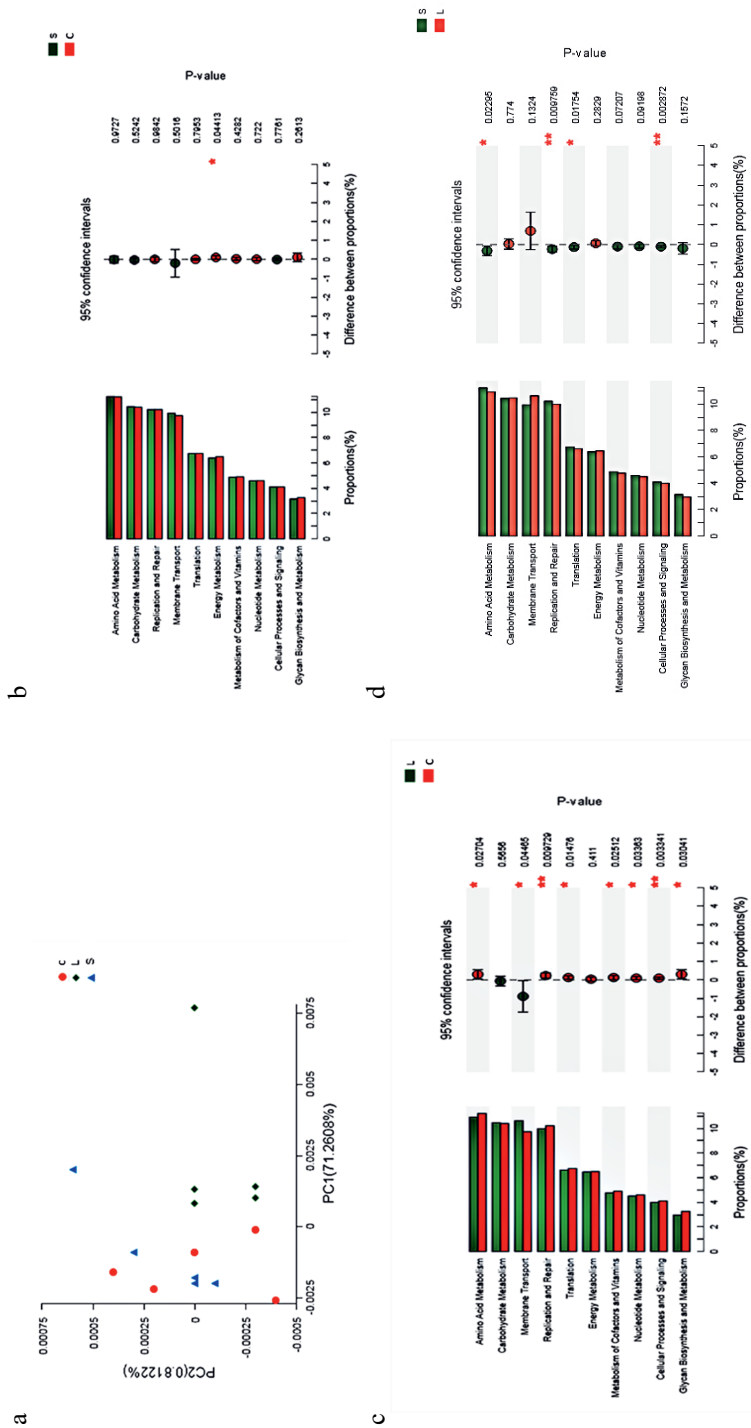


Figure 3.2. Principal coordinate analysis (a) and pairwise comparison (b, c, d) of the KEGG pathways of the bacteria in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic (C, S) and a lipogenic (L) diet. Panel b), C vs S; **c**), C vs L; **d**), S vs L. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. KEGG, the Kyoto Encyclopedia of Genes and Genomes.

Rumen metabolomics profiling

The total ion chromatogram of the QC samples in the positive and negative ion modes showed the reliable repeatability and precision of the data obtained in this analysis (**Supplementary Figure S3.2**). Metabolomic data were firstly examined by PCA in both positive and negative ion mode to obtain an overview of the differences among groups (**Figure 3.3a** and **b**). The results showed that samples of diet L could be separated from those of the other diets. The OPLS-DA score plots were conducted to verify the differentiated metabolites between every two groups showing a clear separation and discrimination between groups under both positive (**Supplementary Figure S3.3a, c** and **e**) and negative ion modes (**Supplementary Figure S3.4a, c** and **e**). Next, the response permutation test was to assess the OPLS-DA model in distinguishing the metabolite data between two groups, in which the cumulative values of R^2Y in the positive (0.9880, 0.8027 and 0.8598 for C vs S, C vs L and L vs S, respectively; **Supplementary Figure S3.3b, d,** and **f**) and negative (0.9856, 0.8697 and 0.8361 for C vs S, C vs L and L vs S, respectively; **Supplementary Figure S3.4b, d,** and **f**) ion models were all above 0.80 indicating the stability and reliability of the model.

A total of 801 metabolites (460 in positive ion mode and 341 in negative ion mode) were identified in the fermentation fluid from the three groups, containing 50.3% of the lipids and lipid-like molecules, 13.9% of the organoheterocyclic compounds, 10.9% of the organic acids and derivatives, 9.7% of organic oxygen compounds, 6.4% of both the benzenoids and the phenylpropanoids and polyketides in the superclass level of the human metabolome database (HMDB) classification (**Supplementary Figure S3.2a**).

Supplementary Table S3.5-S3.7 show that based on VIP > 1 and $P \leq 0.05$, a total of 272 significantly affected metabolites (168 positively and 104 negatively ionized metabolites) were obtained from the comparison of L vs. C (**Supplementary Table S3.5**); 260 (157 positively and 103 negatively ionized metabolites) from L vs. S (**Supplementary Table S3.6**); 89 (63 positively and 26 negatively ionized metabolites) from C vs. S (**Supplementary Table S3.7**). Most of these significantly affected metabolites belonged to the fatty acids and conjugates, the amino acids, peptides and analogues, the triterpenoids and the carbohydrates and carbohydrate conjugates in the subclass level. At the superclass level, compared to diets C and S, the diet L respectively up-regulated 68 out of 114 and 80 out of 118 metabolites in the lipids and lipid-like molecules; but separately down-regulated 18 out of 24 and 17 out of 23 metabolites in the organic acids and derivatives; separately down-regulated 11 out of 19 and 12 out of 20

metabolites in the organic oxygen compounds; separately down-regulated 14 out of 27 and 11 out of 22 metabolites in the organoheterocyclic compounds. At the subclass level, as for the metabolites belonging to fatty acids and conjugates, 12 out of 20 and 15 out of 20 were separately up-regulated in diet L in comparison to diets C and S; 16 out of 19 and 18 out of 18 metabolites belonging to triterpenoids were individually up-regulated in diet L than diets C and S; while15 out of 20 metabolites belonging to the amino acids, peptides and analogues were

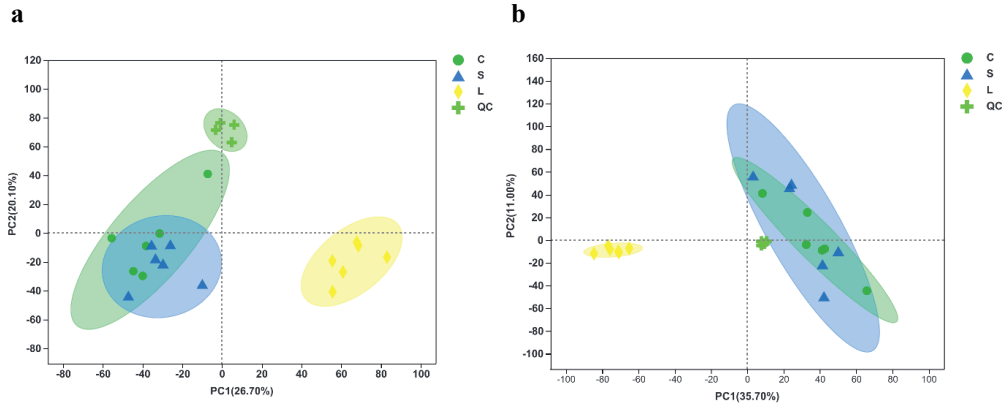


Figure 3.3. Principal component analysis (PCA) of metabolites following positive (a) and negative (b) mode ionization based on metabolomics analysis in the ruminal fluid samples of dairy cows after 48 h *in vitro* fermentation with two glucogenic and a lipogenic diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. QC, quality control samples.

down-regulated by the diet L compared to C and S. For the affected metabolites belonging to carbohydrates and carbohydrate conjugates, 10 out of 14 were down-regulated by diet L compared to C and S. Compared to diet S, the diet C significantly up-regulated 5 out of 6 metabolites belonging to the fatty acids and conjugates, 4 out of 4 metabolites belonging to the triterpenoids and 11 out of 12 metabolites belonging to the amino acids, peptides and analogues.

Figure 3.4 shows the metabolic KEGG pathway enrichment analysis of all different metabolites among all diets. The top three enriched pathways related to tryptophan metabolism, the cutin, suberin and wax biosynthesis and the biosynthesis of phenylpropanoids.

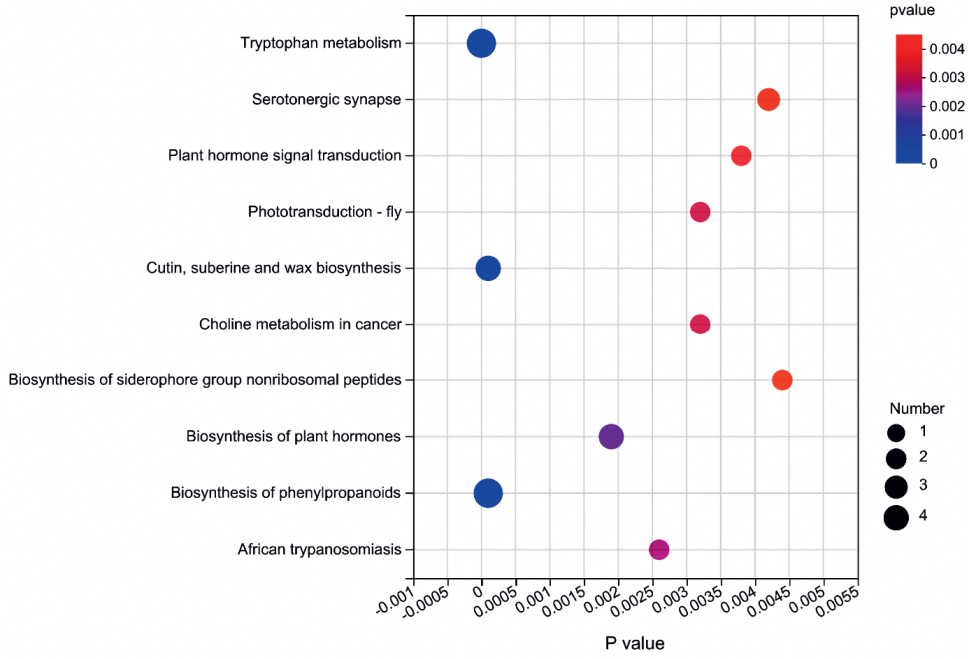


Figure 3.4. Metabolic pathway enrichment analysis of significant differential metabolites in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic and a lipogenic diet. The colour is to distinguish the enrichment significance (*P*-value), the darker the colour, the more significantly the metabolic pathway is enriched. The y axis indicates the name of the KEGG metabolic pathway (top 10). The x-axis indicates the *P*-value. A larger size dot indicates a higher pathway enrichment.

Correlation between bacteria and the fermentation parameters

The Pearson correlation analysis was conducted to assess the correlation between the different bacteria and the rumen fermentation parameters. As shown in **Figure 3.5**, the DMD was negatively correlated with the *Christensenellaceae_R-7_group*, *[Eubacterium]_coprostanoligenes_group*, *Ruminococcaceae_UCG-014*, *Family_XIII-AD3011_group* and *Lachnospiraceae_ND3007_group* but positively correlated with the *Selenomonas_1*, *Ruminobacter*, *Succinivibrionaceae_UCG-002* and *Ruminococcus_2*. The NH_3-N concentration was positively correlated with the *Succinivibrionaceae_UCG-002* and *Ruminococcus_2*. Moreover, the acetate concentration was negatively correlated with the *Succinivibrionaceae_UCG-002* and *Ruminobacter*, while the propionate concentration was positively correlated with the *Selenomonas_1*.

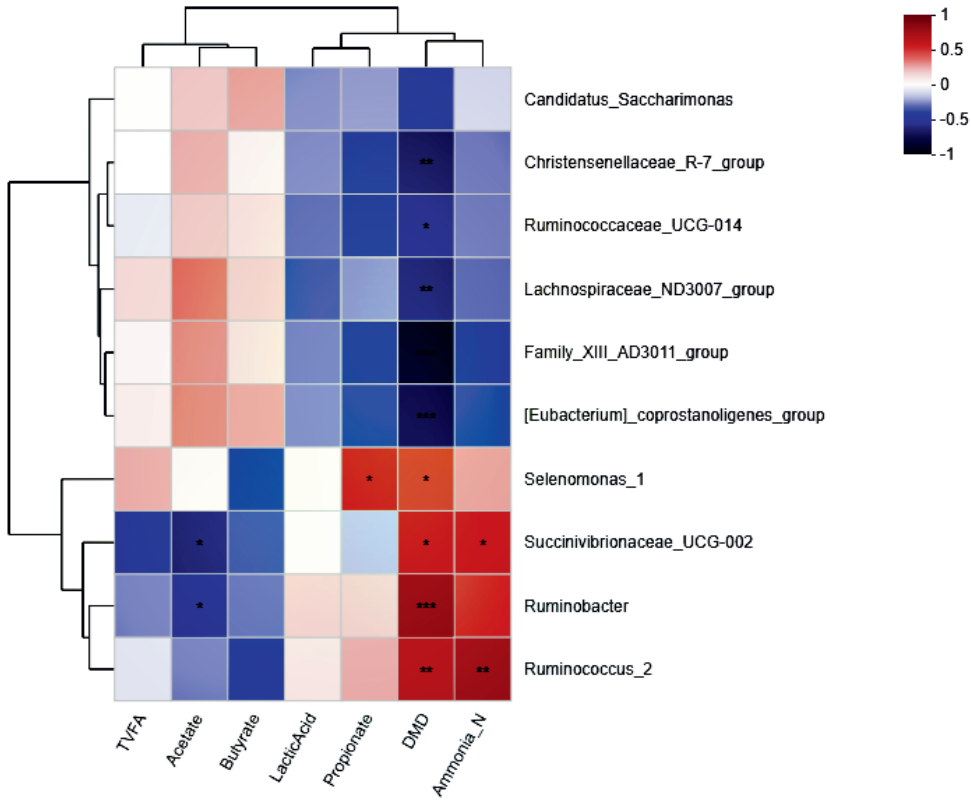


Figure 3.5. Correlation analysis between differential bacteria genus and differential fermentation parameters in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic and a lipogenic diet. Each row represents a bacteria genus, only the genera with a relative abundance > 0.5% are selected; each column represents a fermentation parameter. The colour blue means negative correlation, and the colour red means positive correlation. * $0.01 < P \leq 0.05$, ** $0.001 < P \leq 0.01$, *** $P \leq 0.001$.

Correlation between affected bacteria and the affected metabolites

As is shown in **Figure 3.6**, different abundant bacterial genera were closely correlated with the different metabolites in the fermentation fluid. Specifically, the *Family_XIII-AD3011_group*, *[Eubacterium]_coprostanoligenes_group*, *Christensenellaceae_R-7_group* and *Ruminococcaceae_UCG-010* were positively correlated to the dihydrocumambrin A, norpropoxyphene, D-urobilin, stearoyllactic acid, 15(R)-15-methyl prostaglandin A2,

ganoderic acid A, calenduloside E and 2-octenedioic acid, but negatively correlated with the noreleagnine, captopril-cysteine disulfide, 2-hepteneoylglycine, 3-propyl-1,2-cyclopentanedione, phenyl vinyl sulfide, N6-acetyl-5S-hydroxy-L-lysine and indoleacetic acid. Similarly, the *Lachnospiraceae_ND3007_group* was positively correlated to the dihydrocumambrin A, norpropoxyphene, stearoyllactic acid, 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E and 2-octenedioic acid, but negatively correlated with the noreleagnine, captopril-cysteine disulfide, 2-hepteneoylglycine, 3-propyl-1,2-cyclopentanedione, phenyl vinyl sulfide and N6-acetyl-5S-hydroxy-L-lysine. In addition, *Ruminobacter* was negatively correlated with stearoyllactic acid, 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E and 2-octenedioic acid, but positively correlated with noreleagnine, captopril-cysteine disulfide, 2-hepteneoylglycine and phenyl vinyl sulfide. The *Succinivibrionaceae_UCG-002* was negatively correlated with the stearoyllactic acid and 15(R)-15-methyl prostaglandin A2, but positively correlated with 2-hepteneoylglycine. *Ruminococcus_2* was negatively correlated with the 15(R)-15-methyl prostaglandin A2, ganoderic acid A, calenduloside E and 2-octenedioic acid, but positively correlated with the captopril-cysteine disulfide and 2-hepteneoylglycine. The *Selenomonas_1* was negatively correlated with the dihydrocumambrin A, D-urobilin, ganoderic acid A and 2-octenedioic acid, but positively correlated with the N6-acetyl-5S-hydroxy-L-lysine and indoleacetic acid. Moreover, *Candidatus_saccharimonas* was positively correlated with the stearoyllactic acid but negatively correlated with the 2-hepteneoylglycine and phenyl vinyl sulfide.

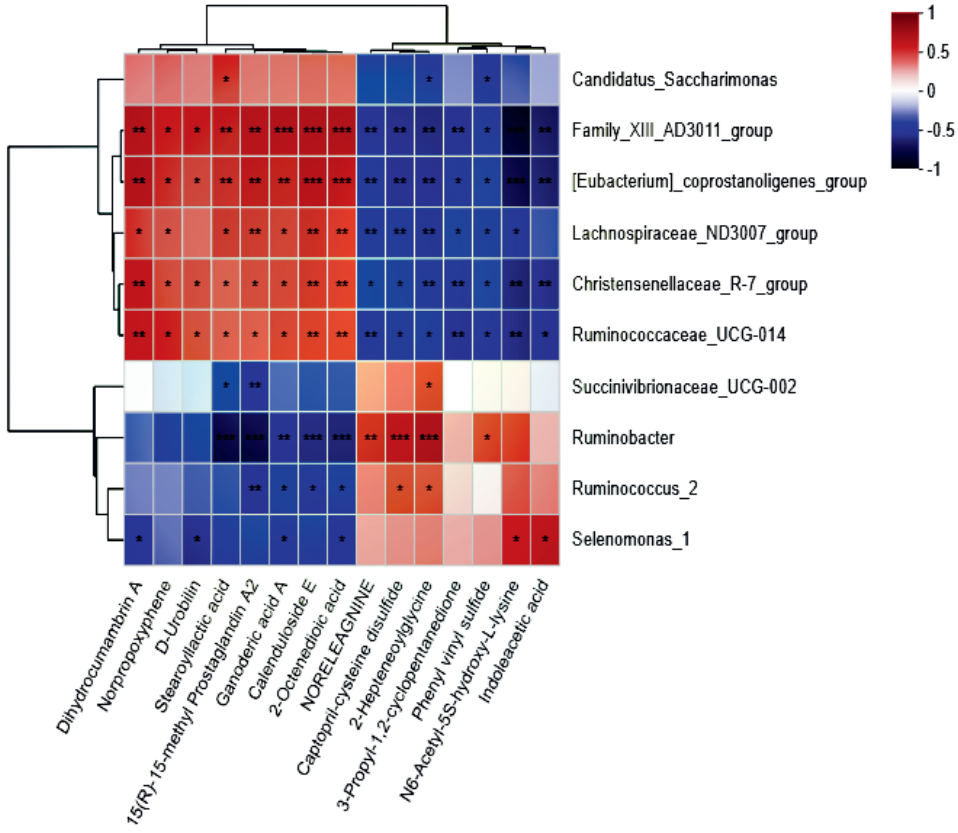


Figure 3.6. Correlation analysis of differential bacteria genus and differential metabolites in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic and a lipogenic diet. Each row represents a bacteria genus, only the genera with relative abundance > 0.5% are selected; each column represents a metabolite, only the affected metabolites with VIP > 1.5, FC < 0.5 and >2 are considered. The color blue means negative correlation, the color red means positive correlation. * $0.01 < P \leq 0.05$, ** $0.001 < P \leq 0.01$, *** $P \leq 0.001$.

Discussion

The present research reports the influence of two glucogenic diets and a lipogenic diet on ruminal fermentation end-products using an *in vitro* incubation system and provides the unknown information on metabolites formed and the bacterial communities in response to the glucogenic and lipogenic diets.

Influence on gas production

In the present study, the gas production of the ruminant feeds was highly correlated with their digestibility and available energetic contents, agreeing with the early work of Menke and Steingass (Menke and Steingass, 1988). The steam-flaked corn, compared to unprocessed corn, increased the gas production of the total mixed rations (TMR) incubated with buffered rumen liquor *in vitro* and increased the gas production rate, which agrees with the data of Qiao et al. (2015). The processing conditions (increased moisture content, pressure and temperature) involved in producing steam-flaking corn have been shown to improve the enzymatic hydrolysis of starch *in vitro*, thereby improving the digestibility of corn (de Peters et al., 2003). Starch digestibility was shown to be positively related to the percentage of starch that was gelatinized *in vitro* (Huntington, 1997). The gelatinization of the starch in the steam-flaking corn was highly likely the reason for their higher gas production.

Methanogenesis is an ancient metabolism of the methanogens belonging to the phylum *Euryarcheota*, domain archaea (Hook et al., 2010). All methanogens belong to seven euryarchaeal orders, including *Methanococcales*, *Methanopyrales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales* and *Thermoplasmatales* (Hook et al., 2010). Three classical CH_4-producing pathways were reported previously, including the hydrogenotrophic methanogenesis mainly using CO_2 and H_2 or formate as substrate, acetoclastic methanogenesis with acetate as substrate and methylotrophic methanogenesis with methylated C1 compounds as substrate (Hedderich and Whitman, 2006). Methanogens are known to grow better syntrophically *in vitro* (Sakai et al., 2009). For ruminants, *Methanobrevibacter* was recognised as the dominant genus producing CH_4 (Leahy et al., 2013), mainly through the CO_2 and H_2 pathway using CO_2 or formate as the elector acceptor and H_2 as the electron donor (Liu and Whitman, 2008). Our results are in line with the aforementioned observations and illustrate that the relative abundance of the dominant genus *Methanobrevibacter* followed the same trend as gas production and CH_4 proportion. The higher gas production of the S relative to the L diet might supply more substrates (CO_2 and H_2) for the

methanogenesis of *Methanobrevibacter*, which may lead to higher CH_4 production. The genus of *Candidatus_Methanomethylophilus* is also known as a CH_4-producing methanogen, which mainly depends on methanol as substrate via the methylotrophic methanogenesis pathway (Lino et al., 2013). In the present study, the L diet increased the genus of *Candidatus_Methanomethylophilus* significantly. Since this genus was newly defined, its methanogenesis pathway and its relationship with dietary ingredients deserve further research.

Influence on feed digestion

High starch concentration would decrease rumen pH (Nocek et al., 2002). The present study found that lactic acid concentration was negatively related to the pH value. The low pH value of the C and S diet is likely mainly attributed to their increase in lactic acid production. In addition, no difference between the S and C diets existed in the pH value, which is in line with the previous study (Cooper et al., 2002).

The L diet had a lower DMD than the other two diets, which is consistent with previous studies (Ruppert et al., 2003). The ruminal bacteria can be assigned to different functional groups, such as cellulolytic, amylolytic and proteolytic, based on their preferential use of energy. Starch digestion in the rumen is affected by dietary starch source, grain processing and adhering capacity of ruminal microbiota to the diet particles (Huntington, 1997). The main amylolytic bacteria included *Streptococcus bovis*, *Bacteroides amylophilus*, *Prevotella* spp., *Succinimonas amylolytica*, *Selenomonas ruminantium* and *Butyrivibrio* spp. (Xia et al., 2015). For the present study, the relative abundance of amylolytic bacteria genera, including *Selenomonas_1*, *Ruminobacter* and *Succinivibrionaceae_UCG-002*, were higher in diet C and S compared to diet L and also were significantly positively correlated with DMD. These increased genera may likely have contributed to the higher DMD in diets C and S.

The fibre degradation in the rumen is mainly attributed to the ruminal cellulolytic bacteria (Jeyanathan et al., 2014). *Fibrobacter succinogenes* (belong to the genus *Fibrobacter*), *Ruminococcus flavefaciens* and *Ruminococcus albus* (belong to the genus *Ruminococcus*) were considered the dominant cellulolytic bacterial species due to their high capacity for cellulose digesting (Krause et al., 2003). In addition, the genera of *Butyrivibrio* and *Eubacterium* were also reported to be cellulolytic (Thoetkiattikul et al., 2013). Moreover, some unclassified taxa, including those assigned to *Ruminococcaceae*, *Lachnospiraceae*, *Christensenellaceae*, *Rikenellaceae*, *Prevotellaceae*, *Clostridium* and *Bacteroidales* were proven to have the capacity of adhering tightly to forages in the rumen, which indicates that these new taxa might play a significant role in forage digestion (Liu et al., 2016). In this study, the L diet had significantly

higher relative abundances of the cellulolytic bacterial genera, including *Ruminococcus*, *Eubacterium*, *Butyrivibrio_2* and *Lachnospira*, and the potential cellulolytic taxa, including unclassified_*Lachnospiraceae*, unclassified_*Ruminococcaceae* and unclassified_*o_Clostridiales*, but still resulting in a lower DMD, which illustrates that excess NDF and low starch in the L diet led to a lower degradation rate of the NDF.

Influence on NH_3-N

The NH_3-N concentration in the *in vitro* rumen fluid cultures is determined by the balance of the formation and utilization rate of NH_3-N by microorganisms (Shen et al., 2017). Due to the same level of crude protein among all treatments, the supplemented amount of nitrogen available for the microbiota can be considered equal. The efficiency of ruminal NH_3-N utilization is determined by the capacities of microbes to metabolise NH_3-N. Cellulolytic bacteria which degrade structural carbohydrates (e.g., NDF) grow slowly and mainly use NH_3-N as an N source, whereas amylolytic bacteria which degrade non-structural carbohydrates (e.g., starch) grow rapidly with higher requirements and use ammonia, peptides and amino acid as N sources (Fox et al., 1992). In our study, the NH_3-N concentration in diet L was significantly lower than in the other two diets. The L diet showed higher relative abundances of cellulolytic bacteria, among which the genera *Succinivibrionaceae_UCG-002* and *Ruminococcus_2* was proved to positively correlate with the NH_3-N concentration. These bacteria might use NH_3-N as the main N source leading to a lower NH_3-N concentration.

Influence on VFA

Ruminal fermentation of carbohydrates leads to the formation of VFA. The primary ruminal VFAs are acetate, propionate and butyrate, the molar proportions of which are mainly determined by the diet. Propionate and acetate are the main precursors of milk glucose and fatty acids, respectively. Cellulose ferments to acetate to a greater extent than propionate, whereas readily degradable starch is fermented less to acetate and more to propionate. Consistent with this, our data showed that both the C and S diets had a higher concentration of propionate and a lower acetate to propionate ratio compared to the L diet. The ruminal propionate is formed via two main pathways, the succinate pathway and the acrylate pathway (Jeyanathan et al., 2014). The former is known as the major pathway for propionate-production in the rumen and involves a large number of bacterial species, such as bacteria related to succinate production and utilization (*Fibrobacter succinogenes* and *Selenomonas ruminantium*), and lactate production and utilization (e.g., *Streptococcus Bovis* and *Selenomonas ruminantium*) (Jeyanathan et al., 2014). The genus *Selenomonas_1* had a positive correlation with the

propionate concentration, and the relative abundance of *Selenomonas_1* and *Succinivibrionaceae_UCG-002* in the L diet was lower than that in the other diets. The *Selenomonas_1* and *Succinivibrionaceae_UCG-002* may contribute to the higher propionate production in diets C and S by enhancing the succinate pathway according to current knowledge. These roles need to be confirmed by further research.

Influence on bacterial function

To further study the differences in the functional roles of rumen bacteria among dietary treatments, PICRUSt was used to estimate the potential functions of the bacteria. Compared with the C and S diets, the predicted pathway of amino acid metabolism was down-regulated in the L diet (**Figures 3.2c** and **3.2d**). The increased amino acid metabolism in diets S and C may lead to higher amino acid production with excessive amounts of amino acids contributing to the higher NH_3-N concentration via deamination (Petri et al., 2019). Also, the *Ruminobacter amylophilus* is known for its proteolytic activity (Whitman et al., 2015), which could explain that the diets C and S with a higher number of the genus *Ruminobacter* had enhanced function of amino acids metabolism. Moreover, the L diet reduced the relative abundance of the translation, replication and repair, as well as cellular processes and signalling, which is probably attributed to the rapid turnover rate of bacteria (Zhang et al., 2017a). As predicted by PICRUSt, the bacteria in the C diet had an enriched function for energy metabolism compared to the S diet, suggesting that the bacterial capacity of energy intake may be improved by the ground corn compared to the steam-flaked corn.

Influence on rumen metabolites

The metabolomics analysis provides direct evidence for changes in microbial activities among diets. The metabolomics data showed that the dietary treatments altered most metabolites related to lipid and protein digestion. The enriched metabolic pathways that were predicted by PICRUSt, such as 'amino acid metabolism and cellular processes and signalling', were similar to the enriched metabolic pathways through the metabolome functions analysis, such as the 'tryptophan metabolism and sphingolipid signalling pathways.

Most metabolites in the lipids and lipid-like molecules were higher in the diet L compared to the other two diets, indicating that the L diet could promote lipid utilisation to some degree. Most metabolites belonging to fatty acids and conjugates were also higher in the diet L. Previous *in vitro* bacterial culturing experiments have shown that fatty acids had a negative effect on bacterial growth (Henderson, 1973). The bacterial communities were modified

differently by the fatty acid supplements, where cellulolytic strains of bacteria showed to be more sensitive to fatty acids than the amylolytic ones (Doreau and Ferlay, 1995). The present contribution also observed a strong correlation between the cellulolytic bacteria and metabolites associated with fatty acid. The initial step of lipid metabolism in the rumen is the hydrolysis of the ester linkages, which is predominantly controlled by rumen bacteria (Bauman et al., 2003). The strains of *Butyrivibrio fibrisolvens* have been reported to play an important role in the degradation of polyunsaturated fatty acids in the rumen (Latham et al., 1972), including hydrolysing phospholipids and glycolipids (Harfoot and Hazlewod, 1997). Besides, some strains of *Borrelia* (Yokoyama and Davis, 1971), a strain in each of *Ruminocccus* and *Eubacterium* (White and Kemp, 1971) and two strains of cellulolytic *Clostridium* spp. (Viviani et al., 1968) have also been reported to participate in biohydrogenation. The higher abundance of genera *Butyrivibrio_2*, *Ruminococcus_1*, *Ruminococcaceae_UCG-013*, *Ruminococcaceae_UCG-014*, *Unclassified_o_Clostridiales* in diet L is in line with the higher level of the metabolites related to fatty acids and conjugates. Correlation analysis proved that the different fatty acid metabolites had significant relations with several cellulolytic bacteria, including the *Ruminococcaceae_UCG-014* and *[Eubacterium]_coprostanoligenes_group*. These cellulolytic bacteria might contribute to the higher fatty acid production in the L diet.

Most metabolites associated with 'amino acids, peptides and analogues' were decreased in the L compared to the C and S diets, which was also in line with the PICRUSt result. The ruminal amino acids mainly arise from the dietary protein degradation and protein produced by microbiota. A large number of microbial species contribute to the ruminal proteolysis, with starch-degrading bacteria contributing more to the protein degradation in the rumen than the cellulolytic bacteria (Zhang et al., 2017b). This could explain the higher level of amino acids, peptides and analogues in the C and S diets. Besides, in the *de novo* synthesis of ruminal amino acids, acetate and propionate can be used as carbon sources and the compounds like ammonia as the nitrogen sources by the microbes (Zhang et al., 2017b). The high concentrations of propionate, butyrate and NH_3-N in diet C and S also agrees with their higher levels of amino acids.

In conclusion, the glucogenic diet had greater effects than the lipogenic diet in terms of improving the dry matter digestibility, increasing propionate concentration and promoting amino acid metabolism. The improvement in propionate production may be attributed to the increased number of bacterial spp. functioning in the succinate pathway. Compared to ground corn, steam-flaked corn didn't show more differences in fermentation end-products except for

increasing gas production and down-regulating the production of some fatty acids and amino acids. Several amylolytic and cellulolytic bacteria were sensitive to the dietary changes, while most highly abundant bacteria were stable or minorly affected. The affected bacteria showed to have high associations with certain metabolites. This study has offered a deeper understanding of ruminal microbial functions which could assist the improvement of rumen functions and thereby in the ruminant production. Moreover, these findings provide essential references for future *in vivo* studies.

Acknowledgement

The study was financially supported by the National Key R&D Program of China (2021YFE 2000800, 2019YFE0125600) and the Science and Technology Innovation Project of the Institute of Animal Sciences (cxgc-ias-09). We appreciate the Breeding Farm of Beijing Centre for Dairy Cows, Beijing, China, for offering the experimental cows. We appreciate the Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture, China, for providing the experimental devices.

Supplementary material

DNA extraction and amplification

For the bacteria, the primers amplifying the V3-V4 hypervariable regions of the bacterial 16S rRNA gene: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). PCR reactions were performed in a triplicate 20 μl mixture containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu Polymerase, 0.2 μl of BSA, and 10 ng of template DNA. The PCR program contains 3 min of denaturation at 95 °C; 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, 45 s for elongation at 72 °C; a final extension at 72 °C for 10 min.

For the archaea, in the first PCR circle, the primers amplifying the V3-V4 hypervariable regions of the archaeal 16S rRNA gene were 340F (CCCTAYGGGGYGCASCAG) and 1000R (GGCCATGCACYWCYTCTC). PCR reactions were performed in a triplicate 30 μl mixture containing 15 μl of 2 × Taq master Mix (P111-03, Vazyme), 1 μl of bar-PCR primer F (10 μM), 1 μl of primer R (10 μM), 10-20 ng of template DNA. The PCR program contains 3 min of denaturation at 94 °C; 5 cycles of 30 s at 94 °C, 20 s for annealing at 45 °C, 30 s for elongation at 65 °C; 20 cycles of 20 s at 94 °C, 20 s for annealing at 55 °C, 30 s for elongation at 72 °C; a final extension at 72 °C for 5 min.

In the second PCR circle, the primers were 349F (5'- CCCTACACGACGCTCTTCCGATCTN (barcode) GYGCASCAGKCGMGAAW -3') and 806R (5'-GACTGGAGTTCCTTGGCACCCGAGAATTCCAGGACTACVSGGGTATCTAAT-3').

PCR reactions were performed in a triplicate 30 μl mixture containing 15 μl of 2 × Taq master Mix (P111-03, Vazyme), 1 μl of bar-PCR primer F (10 μM), 1 μl of primer R (10 μM), 10-20 ng of PCR products from the first circle. The PCR program contains 3 min of denaturation at 94 °C; 5 cycles of 30 s at 94 °C, 20 s for annealing at 45 °C, 30 s for elongation at 65 °C; 20 cycles of 20 s at 94 °C, 20 s for annealing at 55 °C, 30 s for elongation at 72 °C; a final extension at 72 °C for 5 min.

Table S3.1. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of ruminal bacterial phyla after 48 h *in vitro* fermentation

Phylum	Experimental diet			SEM	*P*-value
	C	L	S		
Bacteroidetes	49.4	45.0	48.1	1.704	0.424
Firmicutes	36.1	41.6	39.0	2.065	0.411
Proteobacteria	6.54	5.16	3.87	0.777	0.181
Verrucomicrobia	4.08	3.25	4.36	0.444	0.638
Spirochaetae	1.50	1.64	2.09	0.281	0.762
Saccharibacteria	0.81	1.16	1.01	0.085	0.092
Synergistetes	0.41	0.54	0.33	0.050	0.312
Lentisphaerae	0.40	0.47	0.36	0.042	0.688
Tenericutes	0.24^{b}	0.49^{a}	0.25^{b}	0.039	0.042
SR1__Absconditabacteria	0.14	0.28	0.22	0.027	0.084
Actinobacteria	0.14	0.14	0.14	0.014	0.996

Only the bacterial phyla that account for ≥ 0.1% in at least one of the samples are listed; Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S3.2. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of ruminal bacterial genera after 48 h *in vitro* fermentation with rumen fluid of dairy cows

Phylum	Genus/others	Experimental diet			SEM	*P*-value
		C	L	S		
Bacteroidetes	*SP3-e08*	0.11^{b}	0.17^{a}	0.10^{b}	0.009	0.011
Firmicutes	*Christensenellaceae_R-7_group*	1.28^{b}	1.70^{a}	1.06^{b}	0.077	0.029
	Ruminococcaceae_UCG-014	0.74^{b}	1.05^{a}	0.70^{b}	0.045	0.026
	Family_XIII_AD3011_group	0.72^{b}	1.25^{a}	0.50^{b}	0.091	0.004
	Ruminococcus_2	0.87^{a}	0.55^{b}	1.00^{a}	0.055	0.018
	Unclassified_o_Clostridiales	0.70^{b}	1.07^{a}	0.60^{b}	0.058	0.010
	Lachnospiraceae_ND3007_group	0.56^{b}	1.12^{a}	0.68^{b}	0.070	0.025
	[Eubacterium]_coprostanoligenes_group	0.63^{b}	0.91^{a}	0.50^{b}	0.050	<0.001
	Selenomonas_1	0.38^{b}	0.26^{b}	0.88^{a}	0.077	0.005
	unclassified*_f_Lachnospiraceae*	0.36^{b}	0.78^{a}	0.36^{b}	0.057	0.014
	Butyrivibrio_2	0.24^{b}	0.62^{a}	0.29^{b}	0.076	0.037
	unclassified*_f_Ruminococcaceae*	0.19^{b}	0.35^{a}	0.20^{b}	0.021	0.001
	Ruminococcaceae_UCG-013	0.18^{b}	0.35^{a}	0.15^{b}	0.026	0.006
	[Eubacterium]_nodatum_group	0.19^{ab}	0.24^{a}	0.14^{b}	0.011	0.021
	[Eubacterium]_hallii_group	0.14^{ab}	0.18^{a}	0.11^{b}	0.011	0.031
	[Eubacterium]_oxidoreducens_group	0.11^{b}	0.19^{a}	0.11^{b}	0.011	0.044
	Family_XIII_UCG-002	0.12^{ab}	0.13^{a}	0.08^{b}	0.007	0.026
	Lachnospiraceae_UCG-006	0.08^{b}	0.15^{a}	0.08^{b}	0.011	0.015
	Tyzzerella_3	0.04^{b}	0.19^{a}	0.04^{b}	0.022	0.006
	Anaerotruncus	0.06^{b}	0.13^{a}	0.06^{b}	0.011	0.006
	[Eubacterium]_ventriosum_group	0.06^{b}	0.13^{a}	0.05^{b}	0.013	0.043
	Lachnospira	0.01^{b}	0.17^{a}	0.01^{b}	0.023	0.022
Proteobacteria	*Ruminobacter*	1.90^{a}	0.16^{c}	1.14^{b}	0.206	<0.001
	Succinivibrionaceae_UCG-002	1.34^{a}	0.08^{c}	0.54^{b}	0.151	0.004
Saccharibacteria	*Candidatus_Saccharimonas*	0.75^{b}	1.16^{a}	1.01^{b}	0.048	0.037
SR1_Absconditabacteria	*norank_p_SR1_Absconditabacteria*	0.13^{b}	0.28^{a}	0.22^{a}	0.027	0.006

Only bacterial genera (accounting for ≥ 0.1% in at least one of the samples) affected by treatments are listed. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean. $^{a, b, c}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S3.3. Effect of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of ruminal archaea after 48 h *in vitro* fermentation with rumen fluid of dairy cows

Phylum	Genus/others	Experimental diet			SEM	*P*-value
		C	L	S		
Euryarchaeota	*Methanobrevibacter*	78.3[a]	57.6[b]	74.0[a]	3.382	0.014
	norank_f__Thermoplasmatales_Incertae_Sedis	17.7	30.8	21.4	2.348	0.125
	Candidatus_Methanomethylophilus	3.68[b]	11.4[a]	4.40[b]	1.067	0.001
	Methanosphaera	0.45	0.39	0.38	0.032	0.798
	Halostagnicola	0.04	0.02	0.04	0.003	0.076

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S3.4. Effects of two glucogenic (C, S) and a lipogenic (L) diet on the relative abundance (%) of the KEGG pathways of ruminal bacteria predicted by PICRUSt after 48 h *in vitro* fermentation with rumen fluid of dairy cows

Category/pathway	Experimental diet			SEM	*P*-value
	C	L	S		
Metabolism					
Amino Acid Metabolism	10.67	10.39	10.67	0.053	0.053
Carbohydrate Metabolism	9.90	9.97	9.93	0.766	0.766
Energy Metabolism	6.18	6.15	6.08	0.104	0.104
Metabolism of Cofactors and Vitamins	4.66	4.54	4.63	0.055	0.055
Nucleotide Metabolism	4.38	4.29	4.37	0.080	0.080
Glycan Biosynthesis and Metabolism	3.10	2.82	3	0.054	0.054
Lipid Metabolism	2.79	2.80	2.77	0.750	0.750
Enzyme Families	2.25	2.21	2.25	0.165	0.165
Metabolism of Terpenoids and Polyketides	1.80	1.76	1.79	0.250	0.250
Metabolism of Other Amino Acids	1.63	1.57	1.63	0.063	0.063
Xenobiotics Biodegradation and Metabolism	1.49	1.49	1.51	0.222	0.222
Biosynthesis of Other Secondary Metabolites	1.07	1.03	1.07	0.170	0.170
Environmental Information Processing					
Membrane Transport	9.26	10.12	9.45	0.092	0.092
Signal Transduction	1.42^{b}	1.50^{a}	1.42^{b}	0.027	0.027
Signalling Molecules and Interaction	0.16	0.16	0.16	0.168	0.168
Genetic Information Processing					
Replication and Repair	9.72^{a}	9.51^{b}	9.71^{a}	0.020	0.020
Translation	6.42^{a}	6.30^{b}	6.41^{a}	0.031	0.031
Folding, Sorting and Degradation	2.68	2.63	2.63	0.329	0.329
Transcription	2.28	2.40	2.32	0.075	0.075
Unclassified					
Cellular Processes and Signaling	3.90^{a}	3.81^{b}	3.91^{a}	0.004	0.004
Genetic Information Processing	2.68	2.68	2.69	0.843	0.843
Metabolism	2.44	2.41	2.45	0.107	0.107
Cellular Processes					
Cell Motility	1.71^{b}	2.21^{a}	1.74^{b}	0.030	0.030
Cell Growth and Death	0.59	0.58	0.59	0.628	0.628
Transport and Catabolism	0.41	0.38	0.38	0.195	0.195
Organismal Systems					
Endocrine System	0.31	0.30	0.31	0.739	0.739

Table S3.4 (continued).

Category/pathway	Experimental diet			SEM	*P*-value
	C	L	S		
Environmental Adaptation	0.14[b]	0.15[a]	0.14[b]	0.026	0.026
Nervous System	0.10	0.10	0.1	0.587	0.587
Immune System	0.089	0.092	0.087	0.399	0.399
Digestive System	0.051[a]	0.044[b]	0.055[a]	0.045	0.045
Excretory System	0.039	0.033	0.037	0.094	0.094
Circulatory System	0.004	0.007	0.001	0.154	0.154

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. KEGG = Kyoto Encyclopedia of Genes and Genomes. PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S3.5 Significantly differential metabolites between the lipogenic diet L and glucogenic diet C after 48 h *in vitro* fermentation with rumen fluid of dairy cows

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
Lipids and lipid-like molecules	Fatty acids and conjugates	Myristoleic acid	191.18	9.14	2.77	1.65	< 0.001	pos
		Goshuyic acid	247.17	9.13	2.16	1.57	< 0.001	pos
		2-Octenoic acid	302.23	6.36	1.71	1.33	< 0.001	pos
		Stearic acid	307.26	7.14	1.46	1.14	< 0.001	pos
		5-Hexyl-2-furanhexanoic acid	289.18	5.85	1.19	1.09	< 0.001	pos
		13-hydroxyoctadecanoic acid	283.26	9.43	1.63	0.69	0.005	pos
		10-Hydroxy-2,8-decadiene-4,6-diynoic acid	194.08	3.22	1.31	0.85	< 0.001	pos
		Pentadecanoic acid	275.26	8.76	1.18	0.89	< 0.001	pos
		Petroselinic acid	283.26	10.95	1.18	0.93	< 0.001	pos
		Dioscoretine	242.17	4.93	1.17	1.12	0.003	pos
		Floionolic acid	315.25	9.73	1.10	0.94	< 0.001	pos
		2-Octenedioic acid	217.07	2.24	2.12	2.78	< 0.001	neg
		2-hydroxyhexadecanoic acid	271.23	9.10	1.34	1.10	< 0.001	neg
		5-Tetradecenoic acid	271.19	7.16	1.27	1.33	< 0.001	neg
		2-Hydroxymyristic Acid	243.20	8.45	1.14	1.10	< 0.001	neg
		(S)-10,16-Dihydroxyhexadecanoic acid	287.22	7.97	1.07	1.13	0.001	neg
		3,3-Dimethylglutaric acid	159.07	3.81	1.38	0.81	< 0.001	neg
		(9S,10S)-9,10-dihydroxyoctadecanoate	315.25	8.59	1.24	1.27	0.019	neg
		3b,15b,17a-Trihydroxy-pregnenone	329.21	8.09	1.23	0.54	0.039	neg
		(R)-3-Hydroxy-Octadecanoic acid	345.26	9.04	1.09	0.61	0.034	neg
	Fatty acid esters	Butyl 2-decenoate	191.18	9.22	2.85	1.85	< 0.001	pos
		4,8 dimethylnonanoyl carnitine	330.26	8.46	1.05	0.92	< 0.001	pos
		Dipropyl hexanedioate	213.15	4.52	1.03	0.95	< 0.001	pos
		Stearoyllactic acid	355.28	9.88	2.31	2.57	< 0.001	neg
		Ethyl (Z, Z)-5,8-tetradecadienoate	297.21	6.53	1.22	1.17	< 0.001	neg
		Oleoylcarnitine	462.30	8.73	1.06	0.86	< 0.001	neg
	Fatty acyl glycosides	1-Octen-3-yl primeveroside	445.20	7.93	2.23	0.71	< 0.001	pos
		4-Hydroxyproline galactoside	258.10	3.23	1.86	0.79	< 0.001	pos
		6S,9R-Dihydroxy-4,7E-megastigmadien-3-one 9-[apiosyl-(1->6)-glucoside]	563.23	8.41	1.26	1.14	< 0.001	neg
	Fatty alcohols	1-Undecanol	367.36	10.50	1.64	1.90	< 0.001	pos
		Avocadene	304.29	6.62	1.42	1.12	< 0.001	pos
		Avocadyne	329.23	8.91	1.24	1.32	< 0.001	neg
		Ursolic acid	457.37	8.90	3.00	1.56	< 0.001	pos
	Triterpenoids	Tomentosolic acid	437.34	9.48	3.02	1.55	< 0.001	pos

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Camelledionol	423.33	8.71	2.84	1.52	< 0.001	pos
		Oleanolic acid	457.37	9.05	2.59	1.61	< 0.001	pos
		Sandosapogenol	439.36	8.90	2.55	1.31	< 0.001	pos
		(3beta,17alpha,23S,24S)-17,23-Epoxy-3,24,29-trihydroxy-27-norlanost-8-en-15-one	457.33	7.07	2.37	1.48	< 0.001	pos
		Asperagenin	449.33	7.19	1.79	1.18	< 0.001	pos
		Gamma-Taraxastane-3,20-diol	427.39	10.37	1.34	1.10	0.001	pos
		Lucidenic acid E2	539.26	9.24	1.33	1.07	< 0.001	pos
		Ganoderenic acid E	551.27	8.85	1.01	1.04	< 0.001	pos
		3beta-Acetoxy-19alpha-hydroxy-12-ursene	502.42	10.48	1.71	0.57	0.019	pos
		Glyuranolide	530.34	10.24	1.29	1.12	0.012	pos
		Melilotoside D	527.29	7.52	1.21	0.91	< 0.001	pos
		Medicoside C	461.25	7.59	1.04	0.94	< 0.001	pos
		Glabric acid	531.33	6.84	2.31	1.65	< 0.001	neg
		Ganoderic acid A	497.29	8.39	2.27	1.98	< 0.001	neg
		Ganoderiol I	501.36	9.14	2.22	1.34	< 0.001	neg
		Medicagenic acid	547.33	7.39	1.59	1.35	< 0.001	neg
		(3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid	539.33	6.96	1.54	1.19	< 0.001	neg
	Sesquiterpenoids	Ipomeatetrahydrofuran	239.20	7.87	2.27	1.42	< 0.001	pos
		Auberganol	241.22	8.03	1.73	1.28	< 0.001	pos
		4,7-Megastigmadien-9-ol	411.33	8.63	1.22	1.08	< 0.001	pos
		Sterebin A	343.25	5.87	1.06	1.05	< 0.001	pos
		7(14)-Bisabolene-2,3,10,11-tetrol	255.19	6.43	2.09	0.64	< 0.001	pos
		Curcumol	269.21	7.04	1.26	0.85	< 0.001	pos
		7-Hydroxytrichodermol	267.16	4.49	1.03	0.94	< 0.001	pos
		Deoxynivalenol 3-glucoside	491.21	9.33	1.02	0.90	< 0.001	pos
		7(14)-Farnesene-9,12-diol	287.22	7.80	1.26	1.35	< 0.001	neg
	Terpene lactones	8-Deoxy-11,13-dihydroxygrosheimin	298.18	4.86	1.76	0.76	< 0.001	pos
		Isoalantolactone	233.15	6.28	1.43	0.90	< 0.001	pos
		Tatridin B	229.12	6.15	1.30	0.90	< 0.001	pos
		Dihydrocumambrin A	307.16	7.83	2.29	3.19	< 0.001	neg
		2alpha-Hydroxyalantolactone	229.12	7.92	1.73	0.67	< 0.001	neg
		Tavulin	245.12	6.17	1.28	0.85	< 0.001	neg
		Crispolide	261.11	4.48	1.18	0.83	< 0.001	neg
		4,11,13,15-Tetrahydroridentin B	249.15	7.03	1.39	0.76	< 0.001	neg

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
	Monoterpenoids	Withangulatin A	549.25	8.69	2.48	1.32	< 0.001	pos
		Linalyl propionate	243.20	5.46	1.13	1.17	< 0.001	pos
		Piperitone	170.15	5.71	1.44	1.34	< 0.001	pos
		Alpha-Terpineol propanoate	211.17	7.24	1.01	0.86	< 0.001	pos
		3-(5,6,6-Trimethylbicyclo [2.2.1] hept-1-yl) cyclohexanol	281.21	7.58	1.64	1.46	< 0.001	neg
		Soyasapogenol B 3-O-b-D-glucuronide	671.35	7.95	1.28	1.24	< 0.001	neg
		Valechlorin	457.17	8.02	1.38	1.13	< 0.001	neg
	Steroid lactones	Withaperuvin H	611.29	8.17	2.73	1.55	< 0.001	pos
		Physagulin F	567.26	8.21	1.80	1.19	< 0.001	pos
		Physagulin C	565.24	8.41	1.25	1.07	< 0.001	pos
		Physapubenolide	565.25	8.23	1.43	1.42	< 0.001	neg
		(5alpha,6beta,14alpha,20R,22R)-5,6,14,20,27-Pentahydroxy-1-oxowith-24-enolide	505.28	5.66	1.35	1.20	< 0.001	neg
	Diterpenoids	Phytocassane B	317.21	6.59	1.29	1.14	< 0.001	pos
		Austroinulin	355.28	6.68	1.13	1.06	< 0.001	pos
		Beta-Tocopheryl quinone	433.37	10.23	1.96	1.90	< 0.001	pos
		(13R,14R)-7-Labdene-13,14,15-triol	357.30	8.36	1.01	0.95	< 0.001	pos
	Lineolic acids and derivatives	Punicic acid	243.21	7.35	1.51	0.85	< 0.001	pos
		13S-hydroxyoctadecadienoic acid	329.27	9.87	1.31	0.87	< 0.001	pos
		2-Hydroxylinolenic acid	317.21	7.35	1.22	0.88	< 0.001	pos
		Cibaric acid	369.19	6.18	1.27	1.22	< 0.001	neg
	Diradylglycerols	DG(16:0/16:0/0:0)	591.50	10.92	1.34	1.23	< 0.001	pos
		DG(15:0/18:0/0:0)	627.52	11.53	1.31	0.77	< 0.001	neg
		DG(20:1(11Z)/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	677.55	9.98	1.17	0.82	< 0.001	neg
	Terpene glycosides	Melilotin	549.30	7.50	1.33	0.87	< 0.001	pos
		Calenduloside E	677.39	7.77	2.64	2.72	< 0.001	neg
		L-Citronellol glucoside	317.20	5.63	1.00	1.15	< 0.001	neg
	Glycerophosphocholines	PC(16:0/18:2(9Z,12Z))	802.56	11.16	1.24	0.84	< 0.001	neg
		LysoPC(16:0)	540.33	8.36	1.16	0.86	< 0.001	neg
	Glycerophosphoethanolamines	PE(14:1(9Z)/16:1(9Z))	660.46	10.33	2.29	0.64	< 0.001	pos
		LysoPE(0:0/16:0)	436.28	8.40	1.54	0.80	< 0.001	pos
	Hydroxysteroids	Tetrahydrodeoxycorticosterone	379.22	8.98	1.58	1.19	< 0.001	pos
		Prednisone	379.15	8.92	1.13	0.85	< 0.001	neg
		Halobetasol Propionate	521.13	7.58	1.97	1.86	< 0.001	neg
	Pregnane steroids	17a-Hydroxypregnenolone	333.24	5.75	2.83	1.77	< 0.001	pos

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
	Steroidal glycosides	Corchoroside B	541.28	9.01	1.48	1.12	< 0.001	pos
		Torvoside D	747.39	7.73	2.25	1.86	< 0.001	neg
	Bile acids, alcohols and derivatives	5beta-Cholestane-3alpha,7alpha,24,26-tetrol	401.34	7.36	1.16	0.92	< 0.001	pos
	Glycerophosphates	1-(9Z-tetradecenoyl)-glycero-3-phosphate	379.19	8.11	1.31	0.66	< 0.001	neg
	Cholestane steroids	5a-Cholest-8-en-3b-ol	409.35	10.61	1.01	0.89	< 0.001	pos
	Fatty amides	Palmitoyl Serinol	330.30	8.94	1.89	1.36	< 0.001	pos
	Monoradylglycerols	MG(i-12:0/0:0/0:0)	255.20	8.07	1.40	1.18	< 0.001	neg
	Quinone and hydroquinone lipids	13'-Carboxy-alpha-tocopherol	493.39	11.52	1.74	0.69	< 0.001	pos
	Steroid esters	Physapubescin	553.28	7.95	1.76	1.17	< 0.001	pos
	Stigmastanes and derivatives	6-Deoxohomodolichosterone	463.38	10.59	1.11	1.12	< 0.001	pos
	Eicosanoids	Carboprost Tromethamine	367.25	7.62	1.27	0.81	< 0.001	neg
	Ergostane steroids	Delta 8,14 -Sterol	393.35	12.76	1.08	0.91	< 0.001	pos
Organic acids and derivatives	Amino acids, peptides, and analogues	N6-Acetyl-5S-hydroxy-L-lysine	243.08	4.68	2.31	0.42	< 0.001	pos
		N-[[3-Hydroxy-2-(2-pentenyl) cyclopentyl]acetyl]isoleucine	308.22	7.67	1.93	1.27	< 0.001	pos
		Prolyl-Tyrosine	323.10	5.66	1.48	1.27	0.007	pos
		5-Hydroxyindoleacetylglycine	213.07	3.25	1.04	1.13	0.004	pos
		Cytidine 2',3'-cyclic phosphate	350.01	5.50	2.13	0.68	0.001	pos
		Valyl-Glutamate	229.12	1.00	1.75	0.83	< 0.001	pos
		Isoleucyl-Glutamate	243.13	1.41	1.72	0.85	< 0.001	pos
		Tetrahydrodipicolinate	204.09	1.57	1.66	0.80	< 0.001	pos
		Phenylalanylproline	245.13	3.54	1.29	0.91	< 0.001	pos
		Glutamylleucine	243.13	0.76	1.02	0.95	< 0.001	pos
		4-Hydroxystachydrine	158.08	3.22	1.60	1.61	0.001	neg
		N-Acetylproline	138.06	2.85	1.34	1.46	0.001	neg
		N-Arachidonoyl glycine	406.26	8.05	1.24	0.71	0.002	neg
		2-Hepteneoylglycine	230.10	3.07	2.25	0.35	< 0.001	neg
		Captopril-cysteine disulfide	371.05	8.00	1.96	0.37	< 0.001	neg
		(1R)-Glutathionyl-(2R)-hydroxy-1,2-dihydronaphthalene	472.12	5.47	1.78	0.52	< 0.001	neg
		N-(4-Hydroxycinnamoyl) tyrosine	362.08	5.41	1.54	0.75	< 0.001	neg
		Gamma-Glutamyl-S-methylcysteine sulfoxide	279.07	4.76	1.20	0.82	0.043	neg
		Indolylacryloylglycine	225.07	3.35	1.41	0.89	< 0.001	neg

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		3-Hydroxymugineic acid	357.09	5.55	1.16	0.81	0.027	neg
	Hybrid peptides	Beta-Alanyl-L-lysine	476.32	8.31	1.77	0.80	< 0.001	pos
	Carboxylic acid derivatives	Dihydroceramide	374.29	8.95	1.53	1.40	< 0.001	neg
	Medium-chain hydroxy acids and derivatives	12-Hydroxydodecanoic acid	258.21	5.72	1.47	0.85	< 0.001	pos
	Organic carbonic acids	Moracin L	342.13	5.06	1.39	0.78	< 0.001	pos
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	3,4,5-trihydroxy-6-[(4-methoxy-1-benzofuran-6-yl) oxy]oxane-2-carboxylic acid	363.07	6.81	1.89	1.35	< 0.001	pos
		4-Methoxybenzyl O-(2-sulfoglucoside)	345.06	6.38	1.87	1.71	0.002	pos
		Arbutin	317.06	9.96	1.48	1.19	0.001	pos
		Indican	296.11	2.97	2.84	0.66	< 0.001	pos
		N-Acetylneuraminic acid	274.09	2.80	2.10	0.63	< 0.001	pos
		6-(5-ethyl-2,3-dihydroxyphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid	295.08	3.42	1.85	1.47	0.001	pos
		(2S)-2-Butanol O-[b-D-Apiofuranosyl-(1->6)-b-D-glucopyranoside]	410.20	8.36	1.22	0.90	< 0.001	pos
		Lotaustralin	226.11	2.79	1.17	0.94	< 0.001	pos
		Dihydromaleimide beta-D-glucoside	260.08	1.40	2.79	0.24	< 0.001	neg
		Tyramine glucuronide	294.10	2.97	2.35	0.68	< 0.001	neg
		Pisatoside	260.08	1.63	2.26	0.57	< 0.001	neg
		3-oxo-3-[(3,4,5,6-tetrahydroxyoxan-2-yl) methoxy] propanoic acid	247.05	2.90	1.57	0.74	< 0.001	neg
		Fluoxetine glucuronide	520.14	7.36	1.55	0.52	0.003	neg
		6-Sinapoylglucoraphenin	640.08	0.83	1.08	0.91	< 0.001	neg
	Carbonyl compounds	3-Propyl-1,2-cyclopentanedione	182.12	4.51	1.99	0.48	0.006	pos
		4-Heptanone	115.11	3.45	1.01	1.11	0.003	pos
		3-Methylcyclopentadecanone	283.23	8.91	1.14	1.20	0.001	neg
		2-Nonanone	187.13	4.81	1.07	1.17	< 0.001	neg
		1-Hydroxyepiacorone	289.12	3.60	1.33	0.77	< 0.001	neg
Organoheterocyclic compounds	1-benzopyrans	Theaflagallin	365.06	7.11	2.15	1.45	0.010	pos
		9'-Carboxy-gamma-chromanol	421.26	7.53	1.19	1.20	< 0.001	neg
		Gamma-CEHC	263.13	5.26	1.30	0.75	0.001	neg
		2-Hydroxypyridine	140.04	2.51	1.46	1.58	< 0.001	neg
	Hydropyridines	(E)-5-(3,4,5,6-Tetrahydro-3-pyridylidenemethyl)-2-furanmethanol	209.13	2.85	1.09	0.88	0.015	pos

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		5-(2-Furanyl)-1,2,3,4,5,6-hexahydro-7H-cyclopenta[b]pyridin-7-one	248.09	3.96	1.24	0.72	0.001	neg
	Gamma butyrolactones	5-Nonyltetrahydro-2-oxo-3-furancarboxylic acid	237.15	6.26	1.07	1.16	0.002	neg
		Artabsinolide A	261.11	6.13	1.16	0.73	0.012	neg
		3-Hydroxyadipic acid 3,6-lactone	143.04	1.62	1.15	0.68	0.009	neg
	Indolyl carboxylic acids and derivatives	L-Tryptophan	426.22	2.88	1.43	1.19	0.017	pos
		Indoleacetic acid	220.06	3.70	1.16	0.56	0.029	neg
		5-Hydroxyindoleacetic acid	190.05	3.53	1.39	0.70	0.005	neg
	Dibenzoxepines	Doxepin N-oxide glucuronide	507.17	7.28	2.11	2.19	0.011	neg
	Benzoxazinones	(R)-2,7-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one	380.11	5.58	1.62	0.81	< 0.001	pos
	Benzylisoquinolines	8-Propanoylneosolaniol	473.16	7.24	1.92	0.64	0.001	neg
	Bilirubins	D-Urobilin	609.27	8.19	2.16	1.79	< 0.001	neg
	Hydroxyindoles	11-beta-Hydroxyandrosterone-3-glucuronide	463.24	8.09	1.57	2.22	0.041	neg
	Indoles	Indoleacrylic acid	188.07	3.41	1.62	0.81	< 0.001	pos
	Methylpyridines	2-Ethyl-5-methylpyridine	139.12	1.87	1.51	1.41	0.020	pos
	Pyranones and derivatives	Erinapyrone A	187.06	2.69	1.17	1.21	< 0.001	neg
	Quinolones and derivatives	Quinoline-4,8-diol	206.05	2.91	1.15	0.85	0.007	neg
	Unclassified	Canescein	611.25	8.45	2.77	1.84	< 0.001	pos
	Unclassified	(+)-2,3-Dihydro-3-methyl-1H-pyrrole	125.11	1.14	1.43	1.16	0.018	pos
	Unclassified	(S)-N-Methylsalsolinol	158.10	3.24	1.42	0.80	0.001	pos
	Unclassified	Isoquinoline	130.06	4.87	1.21	0.90	< 0.001	pos
	Unclassified	3-Pyridinebutanoic acid	210.08	4.75	1.17	1.26	< 0.001	neg
	Unclassified	N-Carbamoyl glucuronide lorcaserin	454.17	8.36	1.42	0.74	< 0.001	neg
Phenylpropanoids and polyketides	Furanocoumarins	Edulisin I	525.15	8.03	1.27	1.21	0.006	neg
		(R)-Heraclenol 2'-(3-methyl-2-butenoate)	385.13	7.70	1.11	0.75	0.008	neg
	Hydroxycinnamic acids and derivatives	Sinapinic acid-O-glucuronide isomer	365.09	4.79	1.23	1.22	0.034	pos
		N-(p-Hydroxyphenyl) ethyl p-hydroxycinnamide	318.09	4.63	1.45	1.91	0.025	neg
	Anthocyanidins	2-{2-[(6-carboxy-3,4,5-trihydroxyoxan-2-yl) oxy] phenyl}-1lambda⁴-chromen-1-ylium	434.08	5.84	1.90	1.79	0.025	neg
	Cyclic diarylheptanoids	Myricatomentoside II	571.16	8.02	1.37	1.31	0.010	neg
	Coumarin glycosides	Aesculin	363.07	7.11	1.94	1.32	0.012	pos

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
	Flavans	2-phenyl-3,4-dihydro-2H-1-benzopyran-3,5,7-triol	259.10	3.88	1.03	0.83	0.044	pos
	Flavonoid glycosides	Delphinidin 3-rutinoside	594.16	7.01	1.06	0.85	0.008	pos
	Furanoisoflavonoids	Kanzonol F	443.18	7.09	1.52	1.43	0.040	pos
	Pyranocoumarins	Trans-O-Methylgrandmarin	327.09	3.20	1.06	0.91	0.001	neg
	Pyranoflavonoids	Cycloartocarpin	479.17	7.66	1.56	1.41	0.002	neg
	Stilbene glycosides	3,4,5-trihydroxy-6-(2-hydroxy-1,2-diphenylethoxy) oxane-2-carboxylic acid	408.17	4.48	1.29	0.89	< 0.001	pos
	Unclassified	3-(1,2-dihydroxybutyl)-7-hydroxy-1H-isochromen-1-one	268.12	4.98	1.84	0.58	0.001	pos
		Neosaxitoxin	280.12	4.14	1.60	0.79	< 0.001	pos
		Yuzu lactone	197.15	6.77	1.29	0.88	< 0.001	pos
		Alpha-methylphenylalanine	180.10	3.65	1.23	0.91	< 0.001	pos
		Coriandrone C	264.09	3.02	1.19	0.90	< 0.001	pos
		Norpropoxyphene	370.20	7.44	2.07	2.55	< 0.001	neg
		6-[(1Z)-2-hydroxy-3-oxobut-1-en-1-yl]-7-methoxy-2H-chromen-2-one	305.07	4.32	1.24	1.22	0.011	neg
Benzenoids	Benzoic acids and derivatives	Antibiotic SB 202742	393.24	9.47	1.97	1.62	< 0.001	pos
		3,5-Bis(1,1-dimethylethyl)-4-hydroxy-benzoic acid ethyl ester	573.34	8.00	1.59	1.66	0.001	neg
		Salicylic acid	137.02	4.65	1.04	1.07	< 0.001	neg
	Aniline and substituted anilines	OR-1855	204.11	2.94	1.51	0.64	0.013	pos
	Anisoles	1-(4-methoxyphenyl)-4-methylpentan-3-ol	461.29	8.43	1.12	0.76	0.004	neg
	Benzenediols	(Z, Z)-2-Methyl-5-(8,11,14-pentadecatrienyl)-1,3-benzenediol	392.25	7.10	1.09	1.14	0.023	pos
		Pyrocatechol	109.03	3.14	2.08	0.54	0.004	neg
	Indanones	Pterosin N	279.12	3.41	1.09	0.81	0.004	neg
	Methoxybenzenes	Homoveratric acid	195.07	3.72	1.06	1.26	0.007	neg
	Unclassified	2-Phenoxyethanol	180.10	3.16	1.19	0.93	< 0.001	pos
		1-Phenylethanol	121.07	4.20	1.74	1.43	< 0.001	neg
		5-Phenyl-1,3-oxazinane-2,4-dione	226.03	4.40	1.30	1.11	0.003	neg
Alkaloids and derivatives	Unclassified	Harmalol	181.08	3.35	1.24	0.89	< 0.001	neg
Organosulfur compounds	Aryl thioethers	Phenyl vinyl sulfide	181.03	6.43	1.91	0.46	0.001	neg

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
Hydrocarbon derivatives	Tropolones	Beta-Thujaplicin	209.08	3.69	1.04	0.77	0.043	neg
Others		15(R)-15-methyl Prostaglandin A2	349.24	4.87	3.16	2.17	< 0.001	pos
		(3beta,5xi,9xi,18xi) -Olean-12-en-28-oic acid,3-hydroxy-	439.36	9.22	2.90	1.43	< 0.001	pos
		2-aminohexadecanoic acid	272.26	7.49	1.92	1.42	< 0.001	pos
		2-oxo-pentadecanoic acid	239.20	8.05	1.78	1.21	< 0.001	pos
		Oleoyl dopamine	418.33	8.58	1.71	1.19	< 0.001	pos
		4-formyl Indole	146.06	2.98	1.68	1.32	0.001	pos
		7alpha,12alpha,24-trihydroxycholest-4-en-3-one	433.33	7.78	1.60	1.21	< 0.001	pos
		16,16-dimethyl-PGE1	383.28	7.32	1.59	1.19	< 0.001	pos
		3,5,7-Trimethyl-2E,4E,6E,8E-decatetraene	177.16	8.85	1.58	1.17	< 0.001	pos
		Hydroxy-gamma-sanshool	290.21	4.42	1.55	0.78	0.034	pos
		1alpha,25-dihydroxy-26,27-ethanovitamin D3	443.35	8.68	1.49	1.11	< 0.001	pos
		PG (16:0/0:0) [U]	467.28	10.18	1.46	1.09	0.001	pos
		1,8-Diazacyclotetradecane-2,9-dione	227.17	2.88	1.43	1.18	0.022	pos
		Nuatigenin	431.32	8.03	1.35	1.12	< 0.001	pos
		Pfaffic acid	441.34	9.13	1.33	1.08	0.001	pos
		Vitamin D2 (Ergocalciferol)	397.35	9.13	1.32	1.10	0.001	pos
		9-hydroxy-10-oxo-12(Z)-octadecenoic acid	335.22	6.68	1.26	1.05	< 0.001	pos
		Coumestrol	269.04	5.34	1.25	1.18	0.002	pos
		Orcinol	125.06	2.67	1.25	1.16	0.005	pos
		Alpha-9(10)-EpODE	295.23	6.49	1.22	1.06	< 0.001	pos
		(±)12,13-DiHOME	337.23	6.76	1.20	1.05	< 0.001	pos
		Amprotropine	308.22	4.74	1.20	1.08	0.001	pos
		Tetranor-12(R)-HETE	267.19	5.89	1.11	1.07	< 0.001	pos
		9,10-epoxy-13-hydroxy-11-octadecenoic acid	313.24	6.49	1.04	1.04	< 0.001	pos
		TG (12:0/13:0/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]	755.61	11.02	1.03	1.15	0.009	pos
		13Z-Docosenamide	338.34	10.15	1.00	1.04	0.035	pos
		6-hydroxysphingosine	316.28	5.81	2.52	0.68	< 0.001	pos
		NORELEAGNINE	173.11	3.22	2.50	0.50	< 0.001	pos
		8-Isoquinoline methanamine (hydrochloride)	158.10	3.33	2.42	0.71	< 0.001	pos
		Docosahexaenoyl Glycine	386.27	9.00	2.06	0.53	< 0.001	pos
		3-Methyloxyindole	148.08	4.43	1.62	0.85	0.009	pos
		1-ACETYLPIPERIDINE	128.11	3.56	1.60	1.28	0.027	pos

Table S3.5 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Quadrone	249.15	6.22	1.58	0.81	< 0.001	pos
		Cis-5-dodecenoic acid	199.17	6.74	1.48	0.84	< 0.001	pos
		(±)-Equol	243.10	5.11	1.44	0.88	< 0.001	pos
		Malvidin	331.08	5.08	1.42	1.18	0.008	pos
		2,3,5-Trimethacarb	194.12	3.51	1.41	0.89	< 0.001	pos
		R-Palmitoyl-(2-methyl) Ethanolamide	314.30	7.48	1.37	1.13	0.001	pos
		Lumichrome	526.18	4.17	1.34	1.15	0.001	pos
		PE (16:1(5Z)/16:1(5Z))	688.49	10.81	1.27	0.91	0.001	pos
		Confertifoline	235.17	6.64	1.23	0.91	< 0.001	pos
		Farnesyl acetone	263.24	11.53	1.16	0.91	0.002	pos
		9S,10R-Epoxy-6Z-octadecene	570.54	12.18	1.14	0.92	0.001	pos
		PG (17:1(9Z)/0:0)	497.29	10.39	1.10	1.10	0.018	pos
		9,10-Epoxy-18-hydroxystearate	315.25	8.36	1.10	0.93	< 0.001	pos
		PE (16:0/0:0)	454.29	8.59	1.07	1.06	0.007	pos
		(+)-Prosopinine	314.27	4.51	1.06	0.93	< 0.001	pos
		C-2 Ceramide	342.30	9.05	1.03	0.93	< 0.001	pos
		Bifemelane (M4)	269.12	5.13	1.00	0.96	< 0.001	pos
		P-Salicylic acid	137.02	4.20	1.14	1.09	< 0.001	neg
		MEDICA 16	341.27	8.94	1.02	1.08	< 0.001	neg
		Leu-Trp-OH	424.15	6.84	1.43	1.82	0.010	neg
		SIMMONDSIN-2'-FERULATE	550.19	8.11	1.18	0.86	< 0.001	neg

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; HMDB, human metabolome database; M/Z, mass-to-charge ratio; FC, fold change; VIP, variable importance in the projection; pos, in positive ion mode; neg, in negative ion mode.

Table S3.6 Significantly differential metabolites between the lipogenic diet L and glucogenic diet S after 48 h *in vitro* fermentation with rumen fluid of dairy cows

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
Lipids and lipid-like molecules	Fatty acids and conjugates	13-hydroxyoctadecanoic acid	283.26	9.43	1.62	0.71	0.005	pos
		Petroselinic acid	283.26	10.95	1.13	0.94	< 0.001	pos
		Myristoleic acid	191.18	9.14	2.89	1.69	< 0.001	pos
		Goshuyic acid	247.17	9.13	2.36	1.68	< 0.001	pos
		12,15-Epoxy-13,14-dimethyleicosa-12,14,16-trienoic acid	349.27	8.08	1.79	1.58	0.003	pos
		Stearic acid	307.26	7.14	1.50	1.13	< 0.001	pos
		2-Octenoic acid	302.23	6.36	1.47	1.26	0.002	pos
		5-Hexyl-2-furanhexanoic acid	289.18	5.85	1.41	1.12	0.000	pos
		Dioscoretine	242.17	4.93	1.35	1.14	0.002	pos
		Docosatrienoic acid	357.28	8.56	1.24	1.26	0.014	pos
		3,3-Dimethylglutaric acid	159.07	3.81	1.39	0.84	< 0.001	neg
		(R)-3-Hydroxy-Octadecanoic acid	345.26	9.04	1.20	0.65	0.003	neg
		6-Ketomyristic acid	241.18	7.68	1.09	0.90	< 0.001	neg
		2-Octenedioic acid	217.07	2.24	2.29	3.12	< 0.001	neg
		2-hydroxyhexadecanoic acid	271.23	9.10	1.52	1.11	< 0.001	neg
		Nonate	233.10	2.88	1.23	1.19	< 0.001	neg
		5-Tetradecenoic acid	271.19	7.16	1.18	1.24	< 0.001	neg
		2-Hydroxymyristic Acid	243.20	8.45	1.11	1.09	0.001	neg
		7Z,10Z-Hexadecadienoic acid	297.21	6.94	1.08	1.15	< 0.001	neg
		(S)-10,16-Dihydroxyhexadecanoic acid	287.22	7.97	1.07	1.11	0.001	neg
	Fatty acid esters	4,8 dimethylnonanoyl carnitine	330.26	8.46	1.10	0.92	< 0.001	pos
		Butyl 2-decenoate	191.18	9.22	3.03	1.94	< 0.001	pos
		Propyl 2,4-decadienoate	211.17	3.86	1.01	1.05	< 0.001	pos
		Oleoylcarnitine	462.30	8.73	1.02	0.89	0.019	neg
		Stearoyllactic acid	355.28	9.88	2.27	2.39	< 0.001	neg
		Ethyl (Z, Z)-5,8-tetradecadienoate	297.21	6.53	1.19	1.14	< 0.001	neg
	Triterpenoids	Tomentosolic acid	437.34	9.48	3.22	1.60	< 0.001	pos
		Ursolic acid	457.37	8.90	3.10	1.54	< 0.001	pos
		Camelledionol	423.33	8.71	2.98	1.52	< 0.001	pos
		Oleanolic acid	457.37	9.05	2.86	1.66	< 0.001	pos
		Sandosapogenol	439.36	8.90	2.72	1.33	< 0.001	pos
		Asperagenin	449.33	7.19	1.83	1.17	< 0.001	pos
		(3beta,17alpha,23S,24S)-17,23-Epoxy-3,24,29-trihydroxy-27-norlanost-8-en-15-one	457.33	7.07	2.51	1.50	< 0.001	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Lucidenic acid E2	539.26	9.24	1.57	1.10	< 0.001	pos
		Gamma-Taraxastane-3,20-diol	427.39	10.37	1.43	1.11	0.008	pos
		Glyuranolide	530.34	10.24	1.32	1.11	0.005	pos
		Ganoderic acid F	593.28	9.06	1.23	1.06	0.002	pos
		Ganoderenic acid E	551.27	8.85	1.20	1.05	< 0.001	pos
		Glabric acid	531.33	6.84	2.61	1.77	< 0.001	neg
		Ganoderic acid A	497.29	8.39	2.58	2.06	< 0.001	neg
		Ganoderiol I	501.36	9.14	2.43	1.37	< 0.001	neg
		(3beta,19alpha)-3,19,23,24-Tetrahydroxy-12-oleanen-28-oic acid	539.33	6.96	1.69	1.21	< 0.001	neg
		Medicagenic acid	547.33	7.39	1.48	1.32	0.004	neg
		Camellenodiol	463.34	9.97	1.16	1.20	0.001	neg
	Sesquiterpenoids	7(14)-Bisabolene-2,3,10,11-tetrol	255.19	6.43	1.99	0.68	< 0.001	pos
		Deoxynivalenol 3-glucoside	491.21	9.33	1.09	0.90	0.018	pos
		S-Japonin	378.21	9.79	1.01	0.91	0.006	pos
		Ipomeatetrahydrofuran	239.20	7.87	2.30	1.42	< 0.001	pos
		Auberganol	241.22	8.03	1.79	1.26	< 0.001	pos
		4,7-Megastigmadien-9-ol	411.33	8.63	1.38	1.08	< 0.001	pos
		Sterebin A	343.25	5.87	1.20	1.06	< 0.001	pos
		(1(10)E,4a,5E)-1(10),5-Germacradiene-12-acetoxy-4,11-diol	295.19	7.05	1.77	1.80	0.001	neg
		7(14)-Farnesene-9,12-diol	287.22	7.80	1.32	1.32	< 0.001	neg
		Guaidiol	283.19	6.38	1.03	1.13	0.001	neg
	Terpene lactones	Isoalantolactone	233.15	6.28	1.35	0.92	0.000	pos
		8-Deoxy-11,13-dihydroxygrosheimin	298.18	4.86	1.35	0.84	0.010	pos
		Tatridin B	229.12	6.15	1.32	0.90	< 0.001	pos
		2alpha-Hydroxyalantolactone	229.12	7.92	1.81	0.68	< 0.001	neg
		4,11,13,15-Tetrahydroridentin B	249.15	7.03	1.52	0.76	< 0.001	neg
		Tavulin	245.12	6.17	1.33	0.86	< 0.001	neg
		Crispolide	261.11	4.48	1.24	0.84	< 0.001	neg
		Dihydrocumambrin A	307.16	7.83	2.94	5.71	0.001	neg
	Monoterpenoids	Withangulatin A	549.25	8.69	2.64	1.33	< 0.001	pos
		Piperitone	170.15	5.71	1.70	1.34	< 0.001	pos
		3-(5,6,6-Trimethylbicyclo [2.2.1] hept-1-yl)cyclohexanol	281.21	7.58	1.71	1.43	< 0.001	neg
		Linalyl propionate	243.20	5.46	1.28	1.25	0.012	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Valechlorin	457.17	8.02	1.63	1.15	0.002	neg
		Soyasapogenol B 3-O-b-D-glucuronide	671.35	7.95	1.11	1.14	0.001	neg
	Steroid lactones	Withaperuvin H	611.29	8.17	2.64	1.45	0.001	pos
		Physagulin F	567.26	8.21	1.92	1.19	< 0.001	pos
		Physagulin C	565.24	8.41	1.54	1.11	< 0.001	pos
		Physapubenolide	565.25	8.23	1.53	1.44	0.001	neg
	Steroid lactones	(5alpha,6beta,14alpha,20R,22R)-5,6,14,20,27-Pentahydroxy-1-oxowith-24-enolide	505.28	5.66	1.43	1.20	< 0.001	neg
	Fatty alcohols	1-Undecanol	367.36	10.50	1.23	1.47	0.036	pos
		Avocadene	304.29	6.62	1.02	1.06	0.012	pos
		Avocadyne	329.23	8.91	1.14	1.22	0.002	neg
		Oleyl alcohol	313.27	9.57	1.13	1.10	< 0.001	neg
	Glycerophosphoethanolamines	PE (14:1(9Z)/16:1(9Z))	660.46	10.33	1.62	0.75	0.008	pos
		LysoPE (0:0/16:0)	436.28	8.40	1.12	0.87	0.026	pos
		PE (15:0/22:1(13Z))	804.58	11.72	1.28	0.81	0.016	neg
		1-Heptadecanoylglycerophosphoethanolamine	512.30	7.63	1.09	1.15	0.007	neg
	Lineolic acids and derivatives	Punicic acid	243.21	7.35	1.51	0.85	< 0.001	pos
		13S-hydroxyoctadecadienoic acid	329.27	9.87	1.24	0.89	< 0.001	pos
		2-Hydroxylinolenic acid	317.21	7.35	1.15	0.90	< 0.001	pos
		Cibaric acid	369.19	6.18	1.29	1.20	< 0.001	neg
	Diradylglycerols	DG (16:0/16:0/0:0)	591.50	10.92	1.20	1.15	0.048	pos
		DG (20:1(11Z)/22:5(4Z,7Z,10Z,13Z,16Z)/0:0)	677.55	9.98	1.31	0.78	0.040	neg
		DG (15:0/18:0/0:0)	627.52	11.53	1.21	0.81	0.006	neg
	Diterpenoids	Beta-Tocopheryl quinone	433.37	10.23	1.60	1.47	0.043	pos
		Austroinulin	355.28	6.68	1.33	1.07	< 0.001	pos
		Phytocassane B	317.21	6.59	1.31	1.14	< 0.001	pos
	Fatty acyl glycosides	1-Octen-3-yl primeveroside	445.20	7.93	1.75	0.80	0.015	pos
		4-Hydroxyproline galactoside	258.10	3.23	1.51	0.86	< 0.001	pos
		6S,9R-Dihydroxy-4,7E-megastigmadien-3-one 9-[apiosyl-(1->6)-glucoside]	563.23	8.41	1.40	1.16	< 0.001	neg
	Bile acids, alcohols and derivatives	5beta-Cholestane-3alpha,7alpha,24,26-tetrol	401.34	7.36	1.14	0.92	0.008	pos
	Cholestane steroids	5a-Cholest-8-en-3b-ol	409.35	10.61	1.27	0.86	0.003	pos
		5alpha-Cholestanone	811.68	11.16	1.22	1.20	0.014	pos
	Glycerophosphates	LysoPA(P-16:0e/0:0)	417.24	9.12	1.55	1.25	0.001	pos
		27-Nor-5b-cholestane-3a,7a,12a,24,25-pentol	480.37	9.34	1.14	0.90	0.012	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		1-(9Z-tetradecenoyl)-glycero-3-phosphate	379.19	8.11	1.80	0.56	< 0.001	neg
	Glycerophosphocholines	PC (16:0/18:2(9Z,12Z))	802.56	11.16	1.25	0.86	0.008	neg
		LysoPC (16:0)	540.33	8.36	1.06	0.88	0.011	neg
	Hydroxysteroids	Tetrahydrodeoxycorticosterone	379.22	8.98	1.47	1.15	< 0.001	pos
		Prednisone	379.15	8.92	1.36	0.83	0.001	neg
	Pregnane steroids	17a-Hydroxypregnenolone	333.24	5.75	3.02	1.80	< 0.001	pos
		Halobetasol Propionate	521.13	7.58	2.13	1.72	0.001	neg
	Steroidal glycosides	Corchoroside B	541.28	9.01	1.64	1.14	< 0.001	pos
		Torvoside D	747.39	7.73	2.24	1.67	< 0.001	neg
	Terpene glycosides	Lansioside A	642.44	11.64	1.24	1.14	0.019	pos
		Calenduloside E	677.39	7.77	2.83	2.77	< 0.001	neg
	Ceramides	N-[(4E,8E)-1,3-dihydroxyoctadeca-4,8-dien-2-yl]hexadecanamide	580.49	11.88	1.15	0.86	0.018	neg
	Eicosanoids	Carboprost Tromethamine	367.25	7.62	1.06	0.87	< 0.001	neg
	Ergostane steroids	Delta 8,14 -Sterol	393.35	12.76	1.38	0.88	< 0.001	pos
	Estrane steroids	4-hydroxyestradiol	289.18	5.25	1.15	1.08	< 0.001	pos
	Monoradylglycerols	MG(i-12:0/0:0/0:0)	255.20	8.07	1.46	1.17	< 0.001	neg
	Quinone and hydroquinone lipids	13'-Carboxy-alpha-tocopherol	493.39	11.52	1.62	0.71	0.001	pos
	Steroid esters	Physapubescin	553.28	7.95	2.09	1.22	< 0.001	pos
	Stigmastanes and derivatives	6-Deoxohomodolichosterone	463.38	10.59	1.63	1.17	0.003	pos
Organic acids and derivatives	Amino acids, peptides, and analogues	N6-Acetyl-5S-hydroxy-L-lysine	243.08	4.68	2.85	0.37	< 0.001	pos
		Cytidine 2',3'-cyclic phosphate	350.01	5.50	1.68	0.75	0.014	pos
		Valyl-Glutamate	229.12	1.00	1.63	0.86	< 0.001	pos
		Frangulanine	483.34	9.62	1.55	0.75	0.010	pos
		Isoleucyl-Glutamate	243.13	1.41	1.44	0.90	< 0.001	pos
		Tetrahydrodipicolinate	204.09	1.57	1.24	0.88	< 0.001	pos
		Phenylalanylproline	245.13	3.54	1.07	0.93	< 0.001	pos
		N-[[3-Hydroxy-2-(2-pentenyl) cyclopentyl] acetyl] isoleucine	308.22	7.67	2.39	1.41	< 0.001	pos
		5-Hydroxyindoleacetylglycine	213.07	3.25	1.61	1.24	< 0.001	pos
		2-Hepteneoylglycine	230.10	3.07	2.04	0.43	< 0.001	neg
		N-(4-Hydroxycinnamoyl) tyrosine	362.08	5.41	1.73	0.74	< 0.001	neg
		(1R)-Glutathionyl-(2R)-hydroxy-1,2-dihydronaphthalene	472.12	5.47	1.76	0.55	0.001	neg

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Captopril-cysteine disulfide	371.05	8.00	1.61	0.48	< 0.001	neg
		Indolylacryloylglycine	225.07	3.35	1.47	0.89	< 0.001	neg
		N-Arachidonoyl glycine	406.26	8.05	1.25	0.77	< 0.001	neg
		3-Hydroxymugineic acid	357.09	5.55	1.24	0.80	0.034	neg
		Arginyl-Gamma-glutamate	283.15	7.15	1.16	0.80	0.001	neg
		4-Hydroxystachydrine	158.08	3.22	1.19	1.31	0.039	neg
		Oleoyl glycine	384.27	8.85	1.08	1.20	0.003	neg
		N-Acetylproline	138.06	2.85	1.08	1.22	0.003	neg
	Hybrid peptides	Beta-Alanyl-L-lysine	476.32	8.31	1.48	0.86	< 0.001	pos
		Astin I	516.20	2.93	1.17	1.10	0.002	pos
	Medium-chain hydroxy acids and derivatives	12-Hydroxydodecanoic acid	258.21	5.72	1.34	0.88	< 0.001	pos
Organoheterocyclic compounds	Gamma butyrolactones	3-Hydroxyadipic acid 3,6-lactone	143.04	1.62	1.58	0.61	0.001	neg
		Artabsinolide A	261.11	6.13	1.32	0.69	0.013	neg
		5-Nonyltetrahydro-2-oxo-3-furancarboxylic acid	237.15	6.26	1.17	1.14	< 0.001	neg
	1-benzopyrans	Gamma-CEHC	263.13	5.26	1.33	0.76	0.001	neg
		9'-Carboxy-gamma-chromanol	421.26	7.53	1.22	1.20	0.000	neg
	Hydropyridines	5-(2-Furanyl)-1,2,3,4,5,6-hexahydro-7H-cyclopenta[b]pyridin-7-one	248.09	3.96	1.10	0.75	0.015	neg
		2-Hydroxypyridine	140.04	2.51	1.64	1.69	< 0.001	neg
	Indolyl carboxylic acids and derivatives	Indoleacetic acid	220.06	3.70	1.79	0.46	0.002	neg
		5-Hydroxyindoleacetic acid	190.05	3.53	1.14	0.77	0.029	neg
	Benzoxazinones	(R)-2,7-Dihydroxy-2H-1,4-benzoxazin-3(4H)-one	380.11	5.58	1.76	0.81	< 0.001	pos
	Benzylisoquinolines	8-Propanoylneosolaniol	473.16	7.24	1.71	0.68	0.018	neg
	Bilirubins	D-Urobilin	609.27	8.19	2.50	2.26	0.002	neg
	Dibenzoxepines	Doxepin N-oxide glucuronide	507.17	7.28	2.28	2.29	0.016	neg
	Indoles	Indoleacrylic acid	188.07	3.41	1.46	0.85	0.000	pos
	Pterins and derivatives	2-Amino-4-oxo-6-(1',2'-dioxoprolyl)-7,8-dihydroxypteridine	288.03	1.24	1.16	0.87	< 0.001	neg
	Pyranones and derivatives	Erinapyrone A	187.06	2.69	1.27	1.20	< 0.001	neg
		(+)-2,3-Dihydro-3-methyl-1H-pyrrole	125.11	1.14	1.27	1.10	0.022	pos
	Unclassified	Canescein	611.25	8.45	2.75	1.76	0.001	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		3alpha-Hydroxyoreadone	285.17	3.39	1.00	1.05	< 0.001	pos
		N-Carbamoyl glucuronide lorcaserin	454.17	8.36	1.28	0.78	0.003	neg
		Jasmine ketolactone	253.11	5.43	1.40	1.37	< 0.001	neg
		3-Pyridinebutanoic acid	210.08	4.75	1.28	1.26	< 0.001	neg
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Indican	296.11	2.97	2.76	0.69	< 0.001	pos
		N-Acetylneuraminic acid	274.09	2.80	1.90	0.68	0.001	pos
		(2S)-2-Butanol O-[b-D-Apiofuranosyl-(1->6)-b-D-glucopyranoside]	410.20	8.36	1.18	0.91	< 0.001	pos
		Ethyl glucuronide	187.06	3.10	1.01	0.92	0.002	pos
		6-(5-ethyl-2,3-dihydroxyphenoxy)-3,4,5-trihydroxyoxane-2-carboxylic acid	295.08	3.42	2.02	1.52	0.001	pos
		4-Methoxybenzyl O-(2-sulfoglucoside)	345.06	6.38	1.57	1.48	0.022	pos
		Arbutin	317.06	9.96	1.42	1.13	0.000	pos
		3,4,5-trihydroxy-6-[(4-methoxy-1-benzofuran-6-yl) oxy] oxane-2-carboxylic acid	363.07	6.81	1.39	1.23	0.048	pos
		Dihydromaleimide beta-D-glucoside	260.08	1.40	2.87	0.25	< 0.001	neg
		Tyramine glucuronide	294.10	2.97	2.32	0.71	< 0.001	neg
		Pisatoside	260.08	1.63	2.22	0.61	< 0.001	neg
		Fluoxetine glucuronide	520.14	7.36	2.14	0.44	< 0.001	neg
		3-oxo-3-[(3,4,5,6-tetrahydroxyoxan-2-yl) methoxy] propanoic acid	247.05	2.90	1.57	0.76	< 0.001	neg
		6-Sinapoylglucoraphenin	640.08	0.83	1.21	0.91	< 0.001	neg
	Carbonyl compounds	3-Propyl-1,2-cyclopentanedione	182.12	4.51	1.66	0.57	0.032	pos
		1H-Pyrrole-2-carboxaldehyde	96.04	0.71	1.29	1.11	< 0.001	pos
		(3R,8E)-3-Hydroxy-5,8-megastigmadien-7-one	209.15	4.35	1.02	1.06	0.002	pos
		1-Hydroxyepiacorone	289.12	3.60	1.00	0.85	0.007	neg
		3-Methylcyclopentadecanone	283.23	8.91	1.15	1.16	0.001	neg
	Ethers	Digoxigenin monodigitoxoside	565.28	8.94	1.42	1.09	< 0.001	pos
Phenylpropanoids and polyketides	Furanocoumarins	(R)-Heraclenol 2'-(3-methyl-2-butenoate)	385.13	7.70	1.06	0.76	0.016	neg
		Edulisin I	525.15	8.03	1.43	1.21	0.004	neg
	Flavonoid glycosides	Delphinidin 3-rutinoside	594.16	7.01	1.24	0.83	0.001	pos
	Pyranoflavonoids	Cycloartocarpin	479.17	7.66	1.90	1.49	< 0.001	neg
	Cyclic diarylheptanoids	Myricatomentoside II	571.16	8.02	1.56	1.31	0.003	neg

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
	Flavans	2-phenyl-3,4-dihydro-2H-1-benzopyran-3,5,7-triol	259.10	3.88	1.48	0.76	0.007	pos
	Unclassified	3-(1,2-dihydroxybutyl)-7-hydroxy-1H-isochromen-1-one	268.12	4.98	1.30	0.68	0.049	pos
		Coriandrone C	264.09	3.02	1.21	0.90	< 0.001	pos
		Yuzu lactone	197.15	6.77	1.20	0.90	< 0.001	pos
		Alpha-methylphenylalanine	180.10	3.65	1.15	0.92	< 0.001	pos
		3-(3,4-Dihydroxyphenyl)-2-methylpropionic acid	195.07	5.42	1.36	0.87	< 0.001	neg
		Norpropoxyphene	370.20	7.44	1.90	1.92	0.003	neg
		6-[(1Z)-2-hydroxy-3-oxobut-1-en-1-yl]-7-methoxy-2H-chromen-2-one	305.07	4.32	1.68	1.32	0.002	neg
Benzenoids	Lineolic acids and derivatives	Antibiotic SB 202742	393.24	9.47	1.92	1.49	< 0.001	pos
		3,5-Bis(1,1-dimethylethyl)-4-hydroxy-benzoic acid ethyl ester	573.34	8.00	1.67	1.69	0.001	neg
		Salicylic acid	137.02	4.65	1.07	1.06	< 0.001	neg
	Benzenediols	(Z, Z)-2-Methyl-5-(8,11,14-pentadecatrienyl)-1,3-benzenediol	392.25	7.10	1.17	1.12	0.002	pos
		3-(3,4-dihydroxyphenyl)-N-[2-(4-hydroxyphenyl) ethyl] propanimidic acid	338.08	1.28	1.01	0.89	< 0.001	neg
	Indanones	Pterosin N	279.12	3.41	1.03	0.83	0.011	neg
	Unclassified	1-Phenylethanol	121.07	4.20	1.78	1.38	< 0.001	neg
Organosulfur compounds	Aryl thioethers	Phenyl vinyl sulfide	181.03	6.43	1.55	0.56	0.015	neg
Nucleosides, nucleotides, and analogues	Unclassified	1-Methylinosine	606.22	10.46	1.11	1.16	0.020	pos
Alkaloids and derivatives	Unclassified	Harmalol	181.08	3.35	1.32	0.89	< 0.001	neg
Others		6-hydroxysphingosine	316.28	5.81	2.55	0.70	< 0.001	pos
		8-Isoquinoline methanamine (hydrochloride)	158.10	3.33	2.38	0.73	< 0.001	pos
		Docosahexaenoyl Glycine	386.27	9.00	2.20	0.53	< 0.001	pos
		NORELEAGNINE	173.11	3.22	2.09	0.58	0.002	pos
		Hydroxy-gamma-sanshool	290.21	4.42	1.71	0.78	0.014	pos
		Quadrone	249.15	6.22	1.51	0.84	< 0.001	pos
		Cis-5-dodecenoic acid	199.17	6.74	1.57	0.84	< 0.001	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		(±)-Equol	243.10	5.11	1.41	0.89	< 0.001	pos
		2,3,5-Trimethacarb	194.12	3.51	1.38	0.90	< 0.001	pos
		9S,10R-Epoxy-6Z-octadecene	570.54	12.18	1.29	0.92	0.001	pos
		12-SAHSA	589.52	13.60	1.27	0.93	0.003	pos
		PE (16:1(5Z)/16:1(5Z))	688.49	10.81	1.26	0.91	0.007	pos
		Cer(d14:2(4E,6E)/16:0)	480.44	10.40	1.23	0.91	0.004	pos
		Confertifoline	235.17	6.64	1.21	0.92	< 0.001	pos
		(+)-Prosopinine	314.27	4.51	1.12	0.93	< 0.001	pos
		1-Palmitoyl-2-linoleoyl PE	716.52	11.28	1.11	0.94	0.008	pos
		Bifemelane (M5)	287.13	5.24	1.08	0.83	0.020	pos
		15(R)-15-methyl Prostaglandin A2	349.24	4.87	3.30	2.11	< 0.001	pos
		(3beta,5xi,9xi,18xi)-Olean-12-en-28-oic acid,3-hydroxy-	439.36	9.22	3.05	1.43	< 0.001	pos
		Fenirofibrate	321.09	4.08	2.51	2.60	0.017	pos
		2-aminohexadecanoic acid	272.26	7.49	2.12	1.48	< 0.001	pos
		3,5,7-Trimethyl-2E,4E,6E,8E-decatetraene	177.16	8.85	1.79	1.20	< 0.001	pos
		2-oxo-pentadecanoic acid	239.20	8.05	1.77	1.19	< 0.001	pos
		Amprotropine	308.22	4.74	1.70	1.15	< 0.001	pos
		16,16-dimethyl-PGE1	383.28	7.32	1.69	1.20	< 0.001	pos
		1alpha,25-dihydroxy-26,27-ethanovitamin D3	443.35	8.68	1.68	1.13	< 0.001	pos
		Lumichrome	526.18	4.17	1.67	1.20	< 0.001	pos
		Oleoyl dopamine	418.33	8.58	1.67	1.19	0.002	pos
		7alpha,12alpha,24-trihydroxycholest-4-en-3-one	433.33	7.78	1.54	1.18	< 0.001	pos
		Pfaffic acid	441.34	9.13	1.52	1.09	< 0.001	pos
		Vitamin D2 (Ergocalciferol)	397.35	9.13	1.51	1.11	< 0.001	pos
		4-formyl Indole	146.06	2.98	1.48	1.22	0.001	pos
		Malvidin	331.08	5.08	1.46	1.18	0.008	pos
		PG(16:0/0:0)[U]	467.28	10.18	1.42	1.08	0.001	pos
		Alpha-Vetivone	219.17	10.05	1.40	1.15	0.049	pos
		Alpha-9(10)-EpODE	295.23	6.49	1.38	1.08	< 0.001	pos
		Nuatigenin	431.32	8.03	1.38	1.11	< 0.001	pos
		9-hydroxy-10-oxo-12(Z)-octadecenoic acid	335.22	6.68	1.35	1.05	< 0.001	pos
		1-ACETYLPIPERIDINE	128.11	3.56	1.35	1.19	0.040	pos
		Orcinol	125.06	2.67	1.28	1.12	< 0.001	pos
		(±)12,13-DiHOME	337.23	6.76	1.34	1.06	< 0.001	pos

Table S3.6 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		Coumestrol	269.04	5.34	1.33	1.18	0.001	pos
		Tetranor-12(R)-HETE	267.19	5.89	1.25	1.08	< 0.001	pos
		R-Palmitoyl-(2-methyl) Ethanolamide	314.30	7.48	1.17	1.09	0.008	pos
		Stigmasta-5,7-dien-3beta-ol	413.38	10.27	1.17	1.12	0.009	pos
		9,10-epoxy-13-hydroxy-11-octadecenoic acid	313.24	6.49	1.15	1.04	< 0.001	pos
		TG(12:0/13:0/20:5(5Z,8Z,11Z,14Z,17Z))[iso6]	755.61	11.02	1.06	1.16	0.021	pos
		7S,8S-DiHOTrE	311.22	5.61	1.04	1.04	< 0.001	pos
		Glycerophospho-N-Palmitoyl Ethanolamine	454.29	9.49	1.01	1.07	0.001	pos
		PE (16:0/0:0)	454.29	8.59	1.01	1.05	0.004	pos
		PG (17:1(9Z)/0:0)	497.29	10.39	1.01	1.07	0.008	pos
		SIMMONDSIN-2'-FERULATE	550.19	8.11	1.11	0.89	0.001	neg
		P-Salicylic acid	137.02	4.20	1.17	1.08	< 0.001	neg
		MEDICA 16	341.27	8.94	1.17	1.09	< 0.001	neg
		Coumestrol	269.04	5.34	1.33	1.18	0.001	pos
		Tetranor-12(R)-HETE	267.19	5.89	1.25	1.08	< 0.001	pos
		R-Palmitoyl-(2-methyl) Ethanolamide	314.30	7.48	1.17	1.09	0.008	pos
		Stigmasta-5,7-dien-3beta-ol	413.38	10.27	1.17	1.12	0.009	pos

Diets: S, steam-flaked corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; HMDB, human metabolome database; M/Z, mass-to-charge ratio; FC, fold change; VIP, variable importance in the projection; pos, in positive ion mode; neg, in negative ion mode.

Table S3.7 Significantly differential metabolites between the glucogenic diet C and S after 48 h *in vitro* fermentation with rumen fluid of dairy cows

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
Lipids and lipid-like molecules	Fatty acids and conjugates	Ethylmalonic acid	131.04	0.78	1.10	0.96	0.034	neg
		11Z-Eicosenoic acid	355.28	9.19	1.12	1.03	0.007	neg
		Docosatrienoic acid	357.28	8.56	2.52	1.26	0.034	pos
		Elaidic Acid	283.26	8.50	1.34	1.02	0.005	pos
		Floionolic acid	315.25	9.73	1.19	1.02	0.045	pos
		5-Hexyl-2-furanhexanoic acid	289.18	5.85	1.03	1.02	0.026	pos
	Triterpenoids	Camellenodiol	463.34	9.97	2.30	1.16	< 0.001	neg
		Ganoderic acid beta	545.31	8.51	1.27	1.02	0.017	neg
		Lucidenic acid E2	539.26	9.24	1.33	1.02	0.003	pos
		Medicoside C	461.25	7.59	1.13	1.02	0.010	pos
	Glycerophosphates	1-(9Z-tetradecenoyl)-glycero-3-phosphate	379.19	8.11	2.13	0.84	0.007	neg
		1-tetradecanoyl-sn-glycero-3-phosphate	365.21	8.67	2.45	0.89	0.005	pos
		LysoPA(P-16:0e/0:0)	417.24	9.12	2.56	1.19	0.021	pos
	Monoterpenoids	(1beta,2beta,5beta) -p-Menth-3-ene-1,2,5-triol	231.12	4.67	1.04	0.97	0.004	neg
		Piperitol	155.14	6.10	3.11	1.54	0.016	pos
		Alpha-Terpineol propanoate	211.17	7.24	1.59	1.10	0.037	pos
	Sesquiterpenoids	(1(10)E,4a,5E)-1(10),5-Germacradiene-12-acetoxy-4,11-diol	295.19	7.05	2.40	1.45	0.043	neg
		7(14)-Bisabolene-2,3,10,11-tetrol	255.19	6.43	1.52	1.06	0.018	pos
		S-Japonin	378.21	9.79	1.23	0.96	0.049	pos
	Ergostane steroids	(24R)-Ergost-4-ene-3,6-dione	457.33	9.53	1.27	1.06	0.014	neg
		Delta 8,14 -Sterol	393.35	12.76	1.16	0.97	0.049	pos
	Glycerophosphoethanolamines	PE (14:1(9Z)/16:1(9Z))	660.46	10.33	2.56	1.17	0.040	pos
		LysoPE (0:0/16:0)	436.28	8.40	1.85	1.09	0.009	pos
	Diterpenoids	(13R,14R)-7-Labdene-13,14,15-triol	357.30	8.36	1.39	1.02	0.001	pos
	Eicosanoids	Carboprost Tromethamine	367.25	7.62	1.57	1.07	0.011	neg
	Fatty acyl glycosides	4-Hydroxyproline galactoside	258.10	3.23	2.07	1.08	0.002	pos
	Fatty alcohols	13-Heptadecyn-1-ol	253.25	8.48	1.21	1.03	0.042	pos
	Fatty amides	Palmitoyl Serinol	330.30	8.94	3.04	0.80	0.004	pos
	Steroid lactones	Physagulin C	565.24	8.41	1.41	1.03	0.040	pos
	Terpene lactones	Isoalantolactone	233.15	6.28	1.04	1.02	0.040	pos
	Unclassified	11,13-Dihydrotaraxinic acid glucosyl ester	407.17	7.10	3.30	0.54	0.029	neg
	Steroid esters	Physapubescin	553.28	7.95	1.45	1.04	0.026	pos

Table S3.7 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
Organic acids and derivatives	Amino acids, peptides, and analogues	Captopril-cysteine disulfide	371.05	8.00	2.38	1.30	0.014	neg
		2-Hepteneoylglycine	230.10	3.07	2.66	1.22	< 0.001	neg
		Tetrahydrodipicolinate	204.09	1.57	2.29	1.11	< 0.001	pos
		Valyl-Proline	197.13	3.02	1.90	1.09	0.008	pos
		Isoleucyl-Glutamate	243.13	1.41	1.85	1.05	0.004	pos
		Dynorphin B (10-13)	459.30	3.72	1.76	0.93	0.028	pos
		5-Hydroxyindoleacetylglycine	213.07	3.25	1.65	1.10	0.033	pos
		Glutamylleucine	243.13	0.76	1.44	1.03	0.002	pos
		Alanyl-Phenylalanine	219.11	2.68	1.38	1.07	0.039	pos
		5-hydroxyhexanoylglycine	212.09	0.75	1.36	1.03	0.003	pos
		D-Pipecolic acid	130.09	0.68	1.36	1.03	0.005	pos
		Phenylalanylproline	245.13	3.54	1.21	1.03	0.031	pos
	Hybrid peptides	Beta-Alanyl-L-lysine	476.32	8.31	1.87	1.07	0.003	pos
		Astin I	516.20	2.93	1.45	1.05	0.038	pos
	Carboxylic acid derivatives	Dihydroceramide	374.29	8.95	2.76	0.78	0.017	neg
	Organic carbonic acids	Moracin L	342.13	5.06	2.02	1.17	0.037	pos
	Fumonisins	Fumonisin B1	722.40	5.05	1.28	1.02	0.005	pos
Organic oxygen compounds	Carbohydrates and carbohydrate conjugates	Dihydromaleimide beta-D-glucoside	260.08	1.40	1.43	1.06	0.049	neg
		Pisatoside	260.08	1.63	1.85	1.08	0.008	neg
		Tyramine glucuronide	294.10	2.97	1.82	1.05	0.006	neg
		N-Acetylneuraminic acid	274.09	2.80	1.72	1.08	0.047	pos
		Lotaustralin	226.11	2.79	1.20	1.02	0.014	pos
		Indican	296.11	2.97	1.92	1.05	0.003	pos
	Ethers	Digoxigenin monodigitoxoside	565.28	8.94	1.84	1.05	0.001	pos
		Heptaethylene glycol	327.20	7.69	1.09	1.02	0.024	pos
	Carbonyl compounds	1-Hydroxyepiacorone	289.12	3.60	1.90	1.10	0.008	neg
Organoheterocyclic compounds	1,3-dioxolanes	Valeraldehyde propyleneglycol acetal	125.10	4.29	1.23	1.03	0.050	neg
	Hydroxyindoles	11-beta-Hydroxyandrosterone-3-glucuronide	463.24	8.09	3.93	0.49	0.003	neg
	Gamma butyrolactones	3-Hydroxyadipic acid 3,6-lactone	143.04	1.62	1.62	0.89	0.018	neg
	Pterins and derivatives	2-Amino-4-oxo-6-(1',2'-dioxoprolyl)-7,8-dihydroxypteridine	288.03	1.24	1.23	0.96	0.018	neg
	Tryptamines and derivatives	N-Methyl-1H-indole-3-propanamide	203.12	4.31	1.25	1.03	0.016	pos
		Jasmine ketolactone	253.11	5.43	1.79	1.17	0.027	neg
		Isoquinoline	130.06	4.87	1.94	1.07	0.008	pos

Table S3.7 (continued)

HMDB Superclass	HMDB Subclass	Metabolite	M/Z	Retention time	VIP	FC(L/C)	*P*-value	Mode
		3alpha-Hydroxyoreadone	285.17	3.39	1.63	1.04	0.002	pos
Phenylpropanoids and polyketides	Flavonoid glycosides	6"-Malonylcosmosiin	517.10	4.90	1.21	0.97	0.007	neg
		1-Hydroxyibuprofen	221.12	5.74	1.79	0.89	0.026	neg
		3-(3,4-Dihydroxyphenyl)-2-methylpropionic acid	195.07	5.42	1.63	0.95	0.016	neg
		(Z)-7-Hexadecen-1,16-olide	294.24	11.13	1.61	0.94	0.046	pos
		Neosaxitoxin	280.12	4.14	2.76	1.19	0.003	pos
Benzenoids	Benzenediols	3-(3,4-dihydroxyphenyl)-N-[2-(4-hydroxyphenyl) ethyl] propanimidic acid	338.08	1.28	1.31	0.95	0.014	neg
		3-Phenylpropyl acetate	179.11	3.66	1.15	0.97	0.024	pos
		1,1-Dimethoxy-2-phenylethane	167.11	3.59	1.19	1.04	0.034	pos
Alkaloids and derivatives		Dextrorphan O-glucuronide	398.20	8.45	1.37	1.03	0.003	pos
		C-2 Ceramide	342.30	9.05	1.41	1.04	0.008	pos
Others		3-Methyl-1-phenyl-1-butanone	163.11	5.69	1.41	0.94	0.030	pos
		Amprotropine	308.22	4.74	1.84	1.06	0.037	pos
		L-Homotyrosine	196.10	1.69	1.70	1.05	0.000	pos
		3-Indolepropionic acid	190.09	4.87	1.65	1.04	0.017	pos
		Indole-3-carboxaldehyde	354.15	3.15	1.57	1.04	0.002	pos
		Pyridoxine (Vitamin B6)	170.08	0.73	1.46	1.03	0.004	pos
		Trans-EKODE-(E)-Ib	311.22	7.41	1.39	1.03	0.012	pos
		PHENACYLAMINE	136.07	4.08	1.39	0.96	0.048	pos
		PE (18:1(9Z)/0:0)	480.31	8.69	1.36	1.03	0.031	pos
		Polyoxyethylene (600) mono- ricinoleate	341.30	8.44	1.27	1.02	0.024	pos
		9-hydroxy-12Z-octadecenoic acid	299.26	8.47	1.10	1.01	0.016	pos
		9,10-Epoxy-18-hydroxystearate	315.25	8.36	1.03	1.03	0.033	pos
		12-OPDA	293.21	7.02	1.02	1.02	0.020	pos

Diets: S, steam-flaked corn and corn silage diet; C, corn and corn silage diet; HMDB, human metabolome database; M/Z, mass-to-charge ratio; FC, fold change; VIP, variable importance in the projection; pos, in positive ion mode; neg, in negative ion mode.

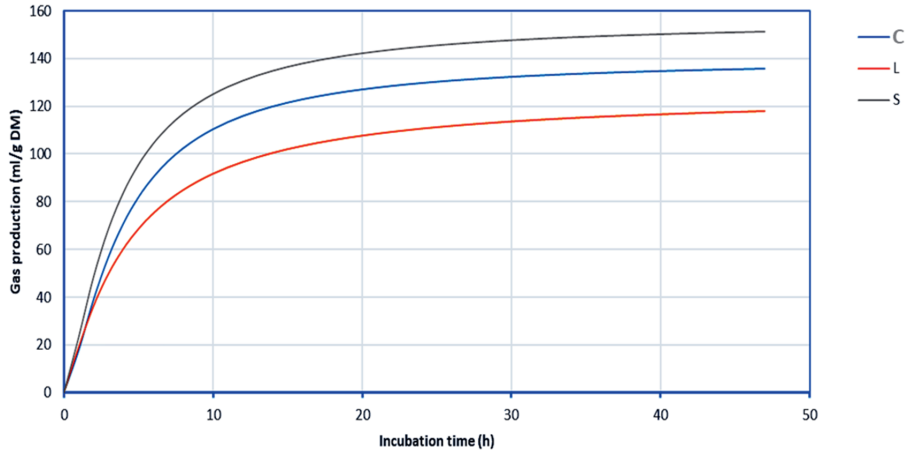
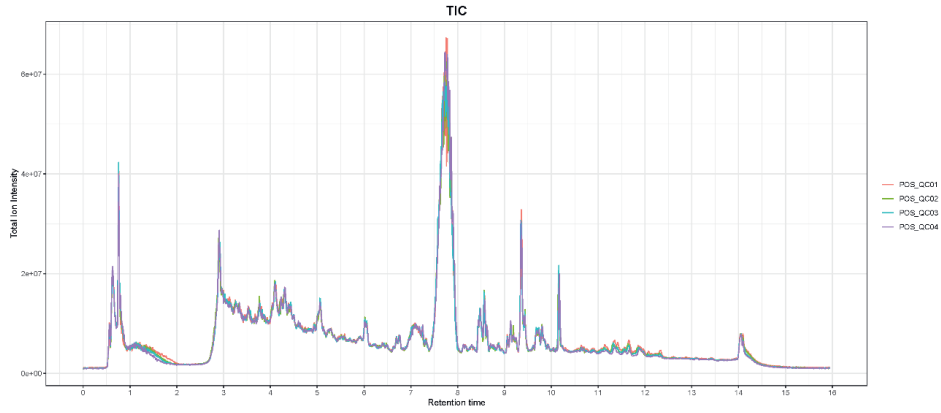


Figure S3.1. Effects of two glucogenic (C, S) and a lipogenic (L) diet on the gas production curve after 48 h *in vitro* fermentation with rumen fluid of dairy cows. The cumulative gas production curve was fitted to the monophasic model: $GP = A / (1 + (C / t)^{B})$, where GP = total gas produced (ml/g DM); A = asymptotic gas production (ml/g DM); B = switching characteristic of the curve; C = time at which half of the asymptote has been reached; t = time (h). Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

A



B

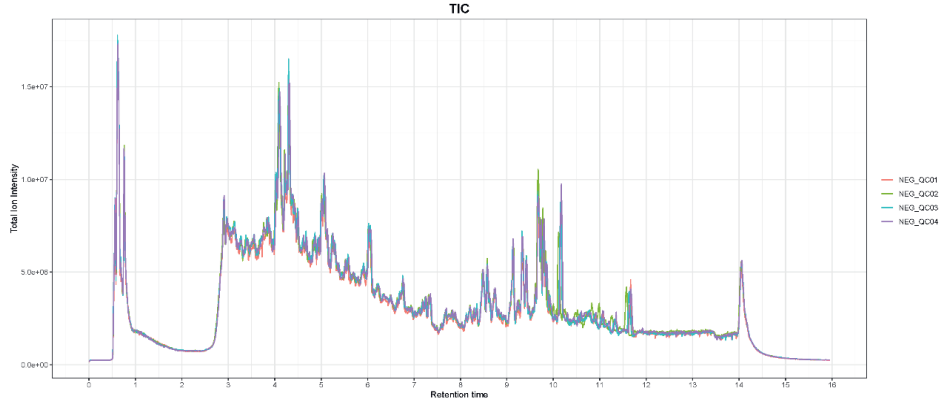


Figure S3.2. LC-MS/MS total ion chromatogram (TIC) of the QC samples in (A) the positive ion mode and (B) the negative ion mode. QC, quality control samples

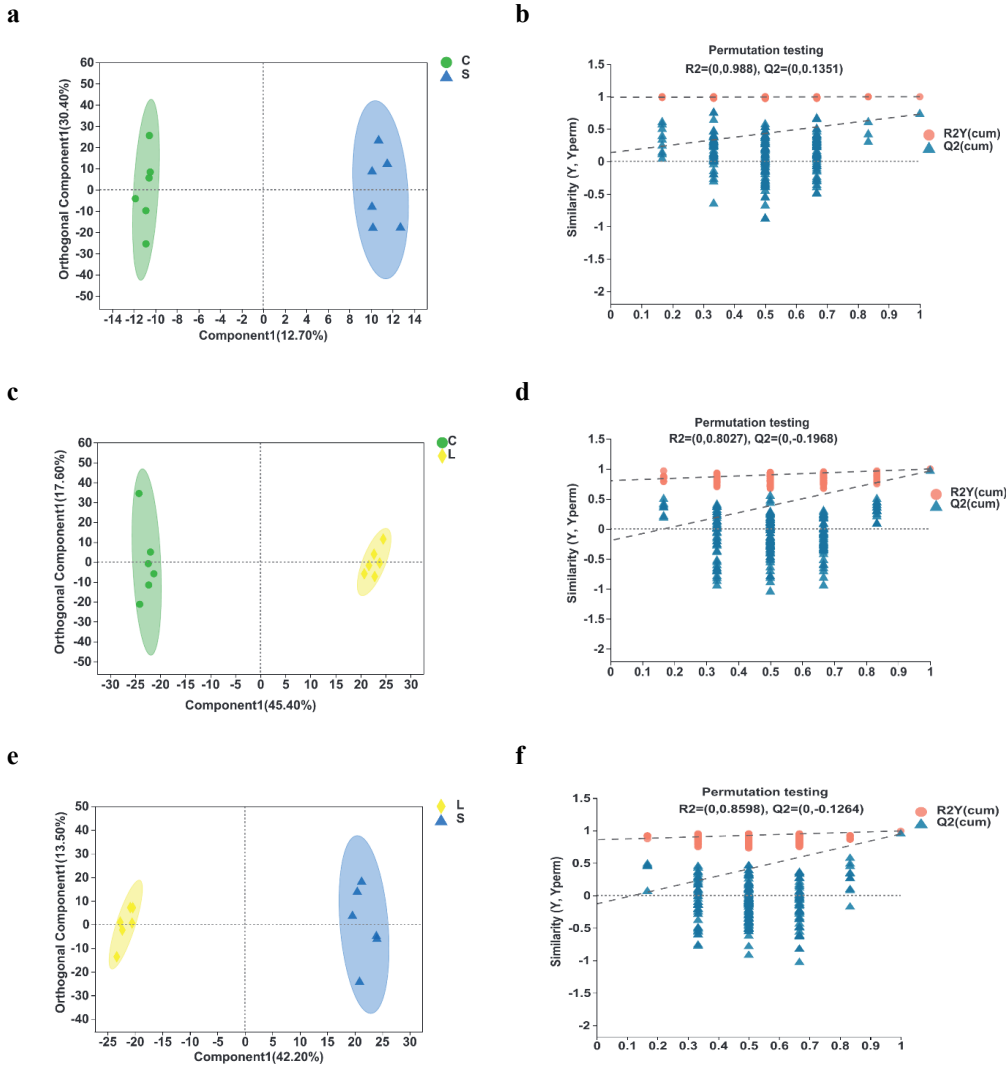


Figure S3.3. Orthogonal partial least squares discriminant analysis (OPLS-DA) (a, c, e) and corresponding permutation test (b, d, f) of the affected metabolites derived from the metabolomics analysis following positive mode ionization in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. R^2Y (cum) indicates the cumulative interpretation power. Q^2 indicates the predictive power of the model.

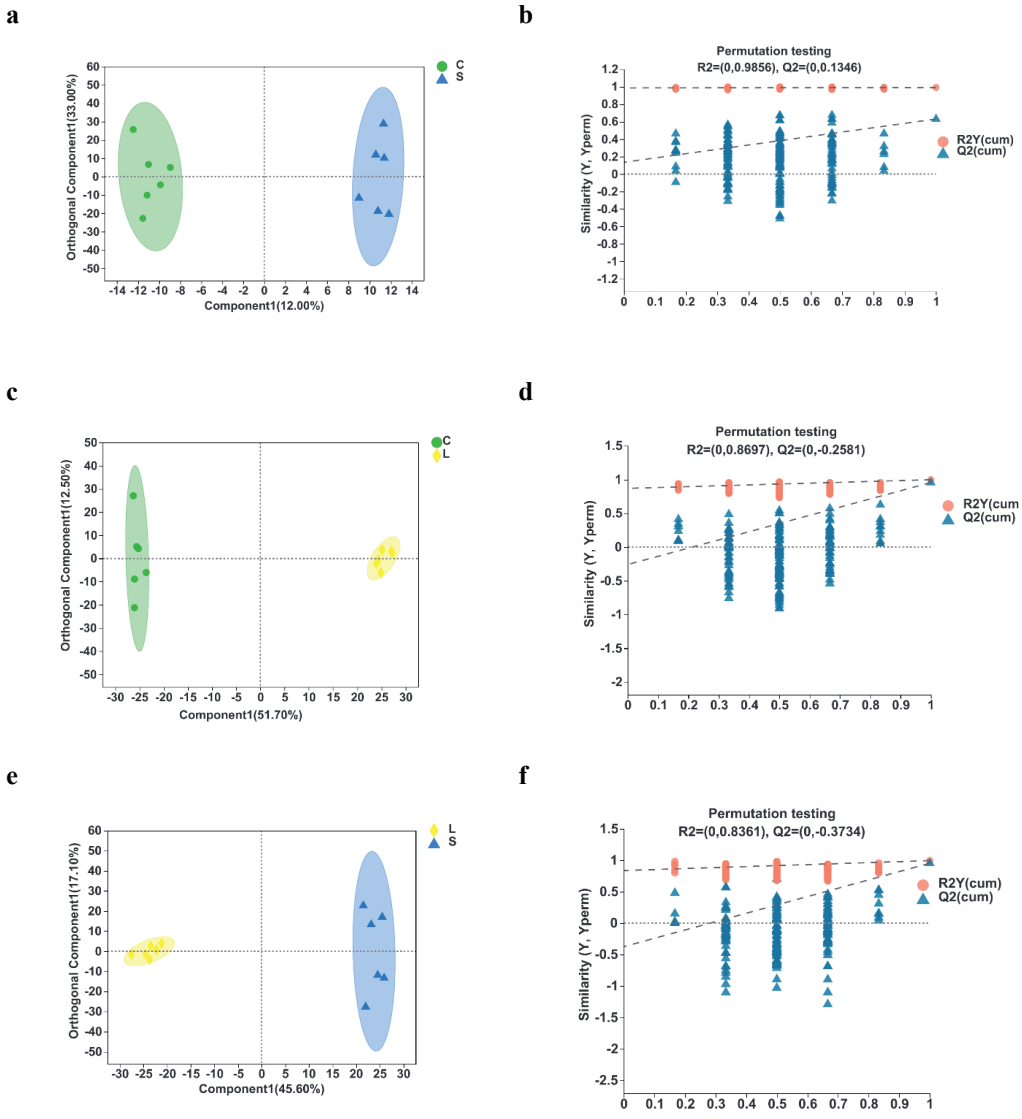


Figure S3.4 Orthogonal partial least squares discriminant analysis (OPLS-DA) (a, c, e) and corresponding permutation test (b, d, f) of the affected metabolites derived from the metabolomics analysis following negative mode ionization in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

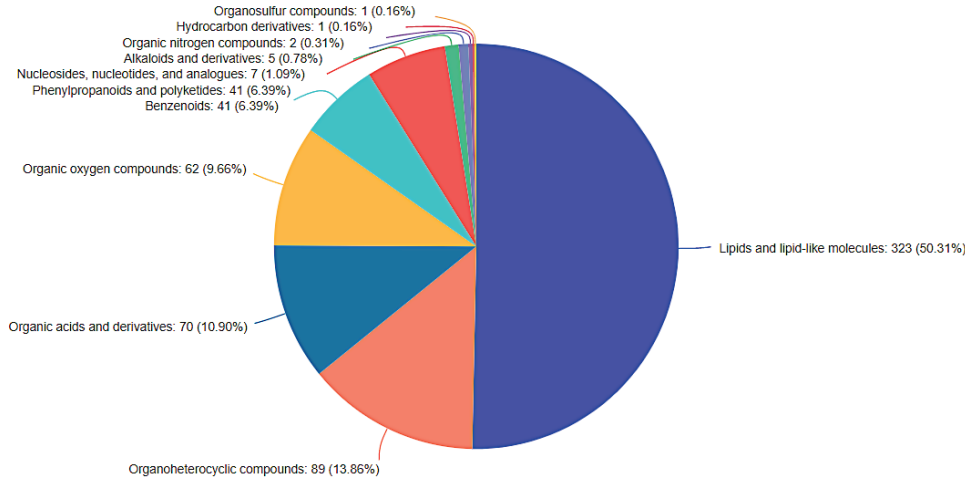


Figure S3.5. The HMDB compound classification of 801 identified metabolites (in the superclass level) detected by metabolomics in the rumen fluid of dairy cows after 48 h *in vitro* fermentation with two glucogenic and a lipogenic diet. HMDB, the human metabolome database.

Reference

Bauer, E., Williams, B. A., Voigt, C., Mosenthin, R., and Verstegen, M. W. A. 2001. Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Anim. Sci.* 2(73):313-322.

Bauman, D. E., Perfield, J. W. I., De Veth, M. J., and Lock, A. L. New perspectives on lipid digestion and metabolism in ruminants. *Proceedings of the Cornell Nutrition Conference* 2003:175-189.

Borrel, G., O Toole, P. W., Harris, H. M. B., Peyret, P., Brugère, J., and Gribaldo, S. 2013. Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. *Genome Biol. Evol.* 5(10):1769-1780.

Broderick, G. A. and Kang, J. H. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *J. Dairy Sci.* 63(1):64-75.

Carberry, C. A., Kenny, D. A., Han, S., Mccabe, M. S., and Waters, S. M. 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. *Appl. Environ. Microbiol.* 78:4949-4958.

Cooper, R. J., Milton, C. T., Klopfenstein, T. J., Scott, T. L., Wilson, C. B., and Mass, R. A. 2002. Effect of corn processing on starch digestion and bacterial crude protein flow in finishing cattle. *J. Anim. Sci.* 80(3):797-804.

de Peters, E. J., Getachew, G., Fadel, J. G., Zinn, R. A., Taylor, S. J., Pareas, J. W., Hinders, R. G., and Aseltine, M. S. 2003. *In vitro* gas production as a method to compare fermentation characteristics of steam-flaked corn. *Anim. Feed Sci. Technol.* 105(1-4):109-122.

Ding, J., Zhang, L., Wan, R., Ren, L. P., and Meng, Q. X. 2007. Disintegration of starch crystal structure by steam flaking may be responsible for the improvement of *in vitro* ruminal fermentation of steam flaked sorghum grains. *J. Anim. Feed Sci.* 16(Suppl 2):448-453.

Doreau, M., and Ferlay, A. 1995. Effect of dietary lipids on nitrogen metabolism in the lumen: a review. *Livest Proc. Sci.* 43:97-110.

Fox, D. G., Sniffen, C. J., O'Connor, J. D., Russell, J. B., and Van Soest, P. J. 1992. A net carbohydrate and protein system for evaluating cattle diets: III. Cattle requirements and diet adequacy. *J. Anim. Sci.* 70(11):3578-3596.

Harfoot, C. G., and Hazlewod, G. P. 1997. Lipid metabolism in the rumen, in Chapter 9, Page: 382-426, The rumen microbial ecosystem. Hobson, P. N., and Stewart, C. S., ed. Chapman & Hall, London, UK.

Hedderich, R., and Whitman, W. 2006. Physiology and biochemistry of the methane-producing archaea, Page: 1050-1079, Prokaryotes: a handbook on the biology of bacteria. Falkow, S. , Rosenberg, E. , Schleifer, K. , Stackebrandt, E., and Dworkin, M., ed. Springer, New York.

Henderson, C. 1973. The effects of fatty acids on pure cultures of rumen bacteria. *J. Agric. Sci.* 81:107-112.

Hook, S. E., Wright, A. G., and McBride, B. W. 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010:1-11.

Hua, D., Zhao, Y., Nan, X., Xue, F., Wang, Y., Jiang, L., and Xiong, B. 2021. Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community *in vitro*. *J. Appl. Microbiol.* 130(6):1868-1882.

Huntington, G. B. 1997. Starch utilization by ruminants: from basics to the bunk. *J. Anim. Sci.* 75(3):852-867.

Jeyanathan, J., Martin, C., and Morgavi, D. P. 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8(2):250-261.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., and McSweeney, C. S. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5):663-693.

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., and Huttenhower, C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* 31(9):814-821.

Latham, M. J., Storry, J. E., and Elisabeth Sharpe, M. 1972. Effect of low-roughage diets on the microflora and lipid metabolism in the rumen. *Appl. Microbiol.* 6(24):871-877.

Leahy, S. C., Kelly, W. J., Ronimus, R. S., Wedlock, N., and Altermann, E. 2013. Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. *Animal* 7:235-243.

Lino, T., Tamaki, H., Tamazawa, S., Ueno, Y., Ohkuma, M., Suzuki, K., Igarashi, Y., and Haruta, S. 2013. Candidatus methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of Methanomassiliicoccaceae fam. Nov. and Methanomassiliicoccales ord. Nov., for a methanogenic lineage of the class Thermoplasmata. *Microbes Environ*. 28(2):244-250.

Liu, J., Zhang, M., Xue, C., Zhu, W., and Mao, S. 2016. Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J. Dairy Sci*. 99(12):9668-9681.

Liu, Y., and Whitman, W. B. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. *Ann. Ny. Acad. Sci*. 1125(1):171-189.

Mao, S. Y., Zhang, G., and Zhu, W. Y. 2008. Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *Anim. Feed Sci. Technol.* 140(3-4):293-306.

Menke, K. H., and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim. Res. Dev.* 28:7-55.

Nocek, J. E., Allman, J. G., and Kautz, W. P. 2002. Evaluation of an indwelling ruminal probe methodology and effect of grain level on diurnal pH variation in dairy cattle. *J. Dairy Sci*. 85(2):422-428.

NRC, 2001. Nutrient requirements of dairy cattle, seventh ed. National Academy Press, Washington, DC.

Pan, X. H., Yang, L., Xue, F. G., Xin, H. R., Jiang, L. S., Xiong, B. H., and Beckers, Y. 2016. Relationship between thiamine and subacute ruminal acidosis induced by a high-grain diet in dairy cows. *J. Dairy Sci*. 99(11):8790-8801.

Pan, X. H., Yang, L., Beckers, Y., Xue, F. G., Tang, Z. W., Jiang, L. S., and Xiong, B. H. 2017. Thiamine supplementation facilitates thiamine transporter expression in the rumen epithelium and attenuates high-grain-induced inflammation in low-yielding dairy cows. *J. Dairy Sci*. 100(7):5329-5342.

Pellikaan, W. F., Hendriks, W. H., Uwimana, G., Bongers, L. J. G. M., Becker, P. M., and Cone, J. W. 2011a. A novel method to determine simultaneously methane production during *in vitro* gas production using fully automated equipment. *Anim. Feed Sci. Technol.* 168(3-4):196-205.

Pellikaan, W. F., Stringano, E., Leenaars, J., Bongers, D. J. G. M., Schuppen, S. V. L., Plant, J., and Mueller-Harvey, I. 2011b. Evaluating effects of tannins on extent and rate of *in vitro* gas and CH4 production using an automated pressure evaluation system (APES). *Anim. Feed Sci. Technol.* 166-167:377-390.

Petri, R. M., Mickdam, E., Klevenhusen, F., Beyer, B., and Zebeli, Q. 2019. Effects of the supplementation of plant-based formulations on microbial fermentation and predicted metabolic function *in vitro*. *Anaerobe* 57:19-27.

Qiao, F., Wang, F., Ren, L., Zhou, Z., Meng, Q., and Bao, Y. 2015. Effect of steam-flaking on chemical compositions, starch gelatinization, *in vitro* fermentability, and energetic values of maize, wheat and rice. *J. Integr. Agr.* 14(5):949-955.

Ruppert, L. D., Drackley, J. K., Bremmer, D. R., and Clark, J. H. 2003. Effects of tallow in diets based on corn silage or alfalfa silage on digestion and nutrient use by lactating dairy cows 1. *J. Dairy Sci*. 86(2):593-609.

Sakai, S., Imachi, H., Sekiguchi, Y., Tseng, I., Ohashi, A., Harada, H., and Kamagata, Y. 2009. Cultivation of methanogens under low-hydrogen conditions by using the coculture method. *Appl. Environ. Microbiol.* 75(14):4892-4896.

Shen, J., Zhuang, L., Zhongtang, Y., and Weiyun, Z. 2017. Monensin and nisin affect rumen fermentation and microbiota differently *in vitro*. *Front. Microbiol*. 8:1111.

Thoetkiattikul, H., Mhuantong, W., Laothanachare, T., Eurwilaichitr, L., and Champreda, V. 2013. Comparative analysis of microbial profiles in cow rumen fed with different dietary fibre by tagged 16S rRNA gene pyrosequencing. *Curr. Microbiol*. 67(2):130-137.

van Knegsel, A., Brand, H., Dijkstra, J., Tamminga, S., and Kemp, B. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod. Nutr. Dev*. 45(6):665-688.

van Knegsel, A., van den Brand, H., Graat, E. A., Dijkstra, J., Jorritsma, R., Decuypere, E., Tamminga, S., and Kemp, B. 2007. Dietary energy source in dairy cows in early lactation: metabolites and metabolic hormones. *J. Dairy Sci*. 90(3):1477-1485.

Viviani, R., Borgatti, A. R., and Matteuzzi, D. 1968. Isolation of typical rumen bacteria active in unsaturated fatty acid biohydrogenation. *Boll. Soc. Ital. Biol. Sper*. 44:2185-2189.

Wang, Y., Nan, X., Zhao, Y., Wang, Y., Jiang, L., and Xiong, B. 2021. Ruminal degradation of rumen-protected glucose influences the ruminal microbiota and metabolites in early-lactation dairy cows. *Appl. Environ. Microbiol*. 87(2):e1908-e1920.

White, R. W., and Kemp, P. 1971. Sheep rumen bacterial isolates which biohydrogenate unsaturated dietary fatty acids. *J. Gen. Microbiol*. 68:vi.

Whitman, W. B., Rainey, F., Kampfer, P., Trujillo, M., Chun, J., DeVos, P., Hedlund, B., and Dedysh, S. 2015. Bergey's manual of systematics of archaea and bacteria: Ruminobacter. 205:1-6.

Xia, Y., Kong, Y., Seviour, R., Yang, H., Forster, R., Vasanthan, T., and McAllister, T. 2015. In situ identification and quantification of starch-hydrolyzing bacteria attached to barley and corn grain in the rumen of cows fed barley-based diets. *FEMS Microbiol. Ecol*. 91(8):v77.

Yokoyama, M. T., and Davis, C. L. 1971. Hydrogenation of unsaturated fatty acids by Treponema (Borrelia) strain $B_2$5, a rumen spirochete. *J. Bacteriol*. 107:519-527.

Zhang, D. F., and Yang, H. J. 2011. *In vitro* ruminal methanogenesis of a hay-rich substrate in response to different combination supplements of nitrocompounds; pyromellitic diimide and 2-bromoethanesulphonate. *Anim. Feed Sci. Technol.* 163(1):20-32.

Zhang, R., Ye, H., Liu, J., and Mao, S. 2017a. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* 101(18):6981-6992.

Zhang, R., Zhu, W., Jiang, L., and Mao, S. 2017b. Comparative metabolome analysis of ruminal changes in Holstein dairy cows fed low- or high-concentrate diets. *Metabolomics* 13(6).

Zhou, X. Q., Zhang, Y. D., Zhao, M., Zhang, T., Zhu, D., Bu, D. P., and Wang, J. Q. 2015. Effect of dietary energy source and level on nutrient digestibility, rumen microbial protein synthesis, and milk performance in lactating dairy cows. *J. Dairy Sci*. 98(10):7209-7217.

Glucogenic diet 1

Glucogenic diet 2

Lipogenic diet

Chapter 4

Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community *in vitro*

Dengke Hua[1,2], Yiguang Zhao[1], Xuemei Nan[1], Fuguang Xue[1], Yue Wang[1], Linshu Jiang[3], and Benhai Xiong[1]

[1] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

[2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, 6708 WD, the Netherlands

[3] Beijing Key Laboratory for Dairy Cattle Nutrition, Beijing Agricultural College, Beijing, 102206, China

Published in

Journal of Applied Microbiology (2020) 130: 1868-1882

https://doi.org/10.1111/jam.14873

Abstract

The objective of this study was to investigate the effect of different ratios of glucogenic to lipogenic nutrients on rumen fermentation and the corresponding ruminal bacterial communities on an isocaloric basis. Four dietary diets including glucogenic diet (G), lipogenic diet (L) and two mixed diets: GL1 (G:L = 2:1) and GL2 (G:L = 1:2), served as substrates and were incubated with rumen fluid of dairy cows *in vitro*. The results revealed that the gas production, dry matter digestibility and propionate proportion were significantly increased by the G diet than others, while the diet L significantly increased the acetate proportion. The bacterial genera of *Succinivibrionaceae_UCG_002*, *Succinivibrio*, *Selenomonas_1* and *Ruminobacter* were significantly increased by the G diet compared to others. The GL1, GL2 and L diets significantly increased the relative abundance of certain cellulolytic bacteria than the diet G, including the *Eubacterium* and several genera in the family *Ruminococcaceae*. The GL1 and GL2 diets produced a higher number of *Ruminococcaceae_NK4A214_group* and *Ruminococcus_gauvreauii_group* but a lower number of *Ruminococcaceae_UCG_group* and *Lachnospiraceae_group* than diet L. The relative abundance of bacterial functions including the cofactors and vitamins metabolism, replication and repair, and cellular processes and signalling, were enriched by diet G than others. When the glucogenic nutrient was above 1/3 of the dietary energy source among the four diets, the *in vitro* incubation had a higher feed digestibility and lower acetate to propionate ratio. Bacterial genera including *Selenomonas*, *Succinivibrio*, *Ruminobacter*, certain genera in *Ruminococcaceae*, *Christensenellaceae_R-7_group*, *Eubacterium* and some unclassified taxa were more sensitive to the glucogenic to lipogenic nutrients ratio.

Keywords: glucogenic/lipogenic nutrients, ruminal bacteria, *in vitro*, PICRUSt, gas production

Introduction

Carbohydrate is the dominating nutrition source for the ruminants, providing the major source of energy for the host animal metabolism and rumen microbial growth (Zhao et al., 2017). It has been reported that diets with high lipogenic nutrients, such as forages, CaLCFA, tallow or prilled fat, are expected to increase the plasma β-hydroxybutyrate and the partitioning of metabolic energy into milk and consequently decrease the partitioning of metabolic energy into body reserves (Knegsel et al., 2013). In contrast, glucogenic nutrients, such as grain, non-fibre carbohydrates, concentrates, starch, glucose infusion and propylene glycol, are expected to decrease the plasma non-esterified fatty acid level, elevate plasma insulin (Miyoshi et al., 2001) and reduce milk fat concentration indicating that glucogenic nutrients stimulate body fat deposition and the partitioning of metabolic energy into body tissue (Ruppert et al., 2003). For the ruminants, glucogenic nutrients originated either from rumen fermentable starch that promotes the production of propionate which is an intermediary precursor for gluconeogenesis or from starch escaping from rumen degradation which is then absorbed as glucose in the small intestine. Lipogenic nutrients stimulate the ruminal production of acetate and butyrate (Knegsel et al., 2005). These findings indicate that different glucogenic and lipogenic nutrients lead to different ruminal fermentation products. Another study demonstrated that the complete mix of glucogenic and lipogenic contents made it impossible to ascribe changes in the fermentation products to the concentration changes of specific carbohydrate fractions (Armentano and Pereira, 1997). Thus, the confounding effects of different glucogenic to lipogenic nutrient ratios on the rumen fermentation products are still not clear.

The *in vitro* technique which is more convenient and time-saving than the *in vivo* is widely used to estimate the feed digestibility using the dry matter digestibility (DMD) (Tilley and Terry, 1963) and gas production (Menke and Steingass, 1988), respectively. Ruminal microbiota plays a key role in the feed digestion and the production of gas, volatile fatty acid (VFA) and ammonia-nitrogen (NH_3-N) in the rumen (Patra and Yu, 2014). Ruminants hold a large variety of microorganisms in their rumen including bacteria, protozoa, fungi and archaea (Kim et al., 2011). Although they are the smallest in size, bacteria account for approximately 50% of total microbial volume and are the most investigated population (Fernando et al., 2010). In accordance with their main metabolic activity, rumen bacteria are classified into different groups, including amylolytic (e.g., *Selenomonas ruminantium*, *Streptococcus bovis*), fibrolytic (e.g., *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*

and *Ruminococcus albus*), proteolytic (e.g., *Prevotella* spp.), lipolytic (e.g., *Anaerovibrio lipolytica*), lactate producers (e.g., *S. bovis and S. ruminantium*) and lactate consumers (e.g., *Megasphaera elsdenii*) (Belanche et al., 2012). In addition, it was also reported that the bacterial functions were influenced by the type of feed, rumen environment and interaction with other bacteria (Sawanon and Kobayashi, 2006). Some nonfibrolytic bacteria can activate fibrolytic bacteria through an interaction termed "cross-feeding", such as *Treponema bryantii* (Kudo et al., 1987), *Prevotella ruminicola* (Fondevila and Dehority, 1996) and *Selenomonas ruminantium* (Koike et al., 2003), which means both fibrolytic bacteria and nonfibrolytic bacteria are important for fibre degradation in the rumen (Wolin et al., 1997). Based on these previous studies, the fermentation end-products under different ratios of glucogenic to lipogenic nutrients might be attributed to the changes of bacteria as well as the interaction between bacteria. Thus, a comprehensive characterization of the bacterial community is essential to understand the effects of glucogenic to lipogenic nutrient ratios on the rumen fermentation end-products.

Therefore, we hypothesized that different ratios of glucogenic to lipogenic ingredients might impact the rumen bacteria composition, thereby resulting in different fermentation products. To test this hypothesis, the present study, by integrating Illumina sequencing of 16S rRNA gene amplicons, investigated the changes in the rumen bacterial community and their fermentation profiles in response to various ratios of glucogenic to lipogenic ingredients via an *in vitro* model.

Materials and methods

Animal care and procedures were operated following the Chinese guidelines for animal welfare and approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (approval number: IAS2019-6). Six rumen-cannulated Holstein dairy cows served as ruminal fluid donors for all three trial runs. The cows were fed a total mixed ration containing (DM basis) 45% concentrate, 20% grass hay and 35% corn silage, three times daily, and had free access to water.

The experimental diets were designed as follows: the glucogenic diet (G) using corn and corn silage as main energy sources, the lipogenic diet (L) using sugar beet pulp and alfalfa silage

Table 4.1. Composition and nutrient levels of experimental diets including glucogenic diet (G), glucogenic:lipogenic nutrient = 2:1 (GL1), glucogenic:lipogenic nutrient = 1:2 (GL2) and lipogenic (L) diet

Items	G	GL1	GL2	L
Ingredient（% of dry matter）				
corn	28.0	20.0	10.0	-
sugar beet pulp	-	12.6	20.8	28.0
soybean meal	18.5	16.8	14.6	12.0
oat hay	5.0	7.1	14.2	19.0
alfalfa hay	10.0	10.0	10.0	10.0
corn silage	38.0	23.5	10.0	-
alfalfa silage	-	10.0	19.0	30.0
calcium hydrogen phosphate	0.5	-	1.4	1.0
Composition (g/kg dry matter)				
CP	174.4	177.7	175.4	174.6
EE	24.3	22.3	20.6	20.4
starch	280.0	207.6	121.0	41.1
NDF	326.0	402.8	482.5	562.2
ADF	197.9	243.9	294.1	348.9
NE_L MJ/kg of dry matter	7.3	7.7	7.6	7.9

Nutrient composition of the experimental diets was calculated according to NRC (2001); ADF = acid detergent fibre; CP = crude protein; EE = ether extract; NDF = neutral detergent fibre; NE_L = net energy for lactation, calculated according to NRC (2001).

as main energy sources, mixed diet one (GL1): 2/3 of the energy source originated from corn and corn silage and 1/3 from sugar beet pulp and alfalfa silage, mixed diet two (GL2): 1/3 of the energy source originated from corn and corn silage and 2/3 from sugar beet pulp and alfalfa silage. Besides, soybean meal, oat and alfalfa hay and calcium hydrogen phosphate were used to balance the nutritional requirement. All diets were on an isocaloric basis, and their composition and chemical analysis of the experimental diets were shown in **Table 4.1**.

***In vitro* incubation**

A ground dry matter (1.0 mm) of each diet was used as the substrate in the incubation. Fresh ruminal fluid from two cows (two different cows for each run) was collected through rumen fistula separately one hour after morning feeding, combined in equal portions and strained through four layers of cheesecloth. The inoculation and incubation procedures were operated as described by Shen et al. (2017). Briefly, 0.5 g substrate was preloaded into a 150 ml serum vial. The buffered medium was prepared anaerobically at 39 °C according to Menke and Steingass (1988). The anaerobic buffer medium (50 ml per vial) and rumen fluid inoculum (25 ml per vial) were added to the vials successively. All the inoculating procedures were conducted in a water bath of 39 °C under a stream of CO_2. Each serum vial was sealed with a butyl rubber stopper and secured with an aluminium crimp seal. Three replicate vials were prepared for each diet treatment in each run. All the incubation vials were individually connected to the gas inlet of an automated gas production recording system (AGRS, **Supplementary Figure S4.1**) and then incubated under 39 °C for 48 h, as described by Zhang and Yang (2011). The *in vitro* incubation was repeated for triple runs with different cows as ruminal fluid donors.

Sample collection and processing

After 48 h of incubation, the total gas produced by fermentation in each vial was recorded by the AGRS. All vials were withdrawn from the incubator and transferred into an ice-water mixture to terminate the incubation. The pH of the whole contents was measured using a portable pH-meter (PHB-4, INESA, Shanghai, China). Then the fermented substrates were filtered through a nylon bag (50 μm of the pore size, weighed after drying at 65 °C for 48 h before use). The bag together with filtered residual was washed under running water until the effluent was clear and then dried at 65 °C for 48 h. Bags and contents were weighed to estimate the DMD. 1 ml of supernatant were preserved by adding 0.2 ml of 25% metaphosphoric acid for VFA measurement by gas chromatography (7890B, Agilent

Technologies, USA) according to the method described by Mao et al. (Mao et al., 2008). Another 1 ml of supernatant was used to determine the NH_3-N concentration by the phenol-hypochlorite method (Shen et al., 2017). Finally, five supernatant samples per diet of all three runs were randomly chosen to do DNA extractions and subsequent microbial analysis.

DNA extraction

Microbial DNA was extracted from 5 ml supernatant using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with the addition of a bead-beating step as described in a previous study (Pan et al., 2017). Briefly, the supernatant sample was homogenized with 0.5 g zirconium beads (0.5 mm diameter) and 800 ml CTAB buffer using a Mixer Mill MM 400 (Retsch, Haan, Germany) with a vibrational frequency of 1,800 rpm and grinding time of 60 s. Then the mixture was incubated at 70 °C for 20 min to increase DNA yield. The supernatant was further processed using QIAamp kits according to the manufacturer's instructions. The integrity and length of the extracted DNA were assessed by agarose gel (1%) electrophoresis on gels containing 0.5 mg/ml ethidium bromide and quantified using a NanoDrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA). DNA was stored at -80 °C until analysis.

Sequencing data processing and analysis

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA) (Ye et al., 2016, Pan et al., 2017), where the barcode was an eight-base sequence unique to each sample. PCR reactions were performed in a triplicate 20 µl mixture containing 4 µl of 5 × FastPfu Buffer, 2 µl of 2.5 mmol dNTPs, 0.8 µl of each primer (5 µmol), 0.4 µl of FastPfu Polymerase, and 10 ng of rumen microbial DNA. PCR amplification started with a 3 min of pre-denaturation at 95 °C, followed by 27 cycles of denaturation (95 °C for 30 s), annealing (55 °C for the 30 s), and elongation (72 °C for 45 s) steps, and a final extension at 72 °C for 10 min. The PCR amplicons were extracted from 2% agarose gels and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according

to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) (Jin et al., 2017).

Raw fastq files were quality-filtered using Trimmomatic (Bolger et al., 2014) and merged using FLASH (Magoc and Salzberg, 2011), based on the following criteria: 1) the reads were truncated at any site receiving an average quality score of < 20 over a 50 bp sliding window, 2) sequences of each sample were separated according to barcodes (exactly matching) with primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases removed, and 3) only sequences whose overlaps were longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. Operational taxonomic units (OTUs) were clustered with a cutoff of 0.03 (97% similarity) using UPARSE (Edgar 2011) with a novel greedy algorithm that performs chimaera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was aligned with the ribosomal database project (RDP) classifier algorithm and compared with the Silva (SSU123) 16S rRNA database (Pruesse et al., 2007) with a confidence threshold of 70% (Amato et al., 2013). Alpha diversity was estimated with the normalized reads using the based coverage estimator Shannon, Simpson, ACE, Chao1 and Coverage indices. The principal coordinates analysis (PCoA) was performed based on the Bray-Curtis dissimilarity (Mitter et al., 2017), and the significant differences between samples were tested by an analysis of similarity (ANOSIM) in QIIME with 999 permutations (R Core Team, 2013). Tabular representation of the relative abundance of microbial diversity at phylum and genus levels were counted depending on the taxonomic data.

In addition to bacterial community structure analysis, the method of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was also used to predict the metagenomic potential functions of ruminal bacteria based on 16S rRNA data. Firstly, the closed OTU table was performed using the sampled reads against the Greengenes database (13.5) with QIIME (Liu et al., 2016). Next, the table was normalized by the 16S rRNA copy number. Then, the metagenome functions were predicted, and the data were exported into the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using PICRUSt (Langille et al., 2013). The difference in the predicted functions among diets was determined by a one-way analysis of variance with SAS 9.3 (SAS Institute Inc., Cary, NC).

Statistical analysis

Data were checked for normal distribution and homogeneity by Shapiro-Wilk's and Levene's tests by SAS 9.3 (SAS Institute Inc., Cary, NC). Rumen fermentation parameters, alpha diversity index and bacterial relative abundance were analysed using PROC MIXED by SAS 9.3 with Tukey host-test (SAS Institute Inc., Cary, NC) with the following model:

$$Y_{ij} = \mu + D_i + R_j + e_{ij},$$

where Y_{ij} is the dependent variable, μ is the overall mean, D_i is the fixed effect of diet ($i = 1$-4), R_j is the random effect of the run ($j = 1$-3), e_{ij} is the random residual error. Significance was declared at $P \leq 0.05$ and a trend was considered at $0.05 < P \leq 0.10$. Pearson correlation coefficients between the relative abundances of bacterial genera and the ruminal fermentation variables were calculated using SAS 9.3 (SAS Institute Inc., Cary, NC). Only the top 25 genera in the relative abundance were included in this analysis. A significant correlation was considered at $P \leq 0.05$.

Results

Effect of glucogenic to lipogenic nutrient ratios on rumen fermentation parameters

The fermentation characteristics were shown in **Table 4.2**. As lipogenic ingredients increased, gas production had a significantly decreasing trend and the DMD showed a similar trend ($P < 0.001$). The pH of the G and GL1 diet was significantly lower than that of the diet GL2 and L ($P < 0.001$). The NH_3-N concentration of the G diet was significantly higher than that of the GL2 and L diet ($P < 0.001$). For VFA contents, the L diet significantly increased the proportion of acetate to the other three diets ($P < 0.001$), while diet G significantly increased the propionate proportion to others ($P < 0.001$). Consequently, the acetate to propionate ratio in diet G was the lowest and was the highest in the diet L ($P < 0.001$).

Table 4.2. Effects of glucogenic to lipogenic nutrient ratios on rumen fermentation parameters in rumen fluid of dairy cows after 48h *in vitro* fermentation

Item	G	GL1	GL2	L	SEM	*P*-value
Gas production (ml g^{-1} DM)	135.43^{a}	116.10^{b}	106.73^{c}	92.24^{d}	2.885	<0.001
DMD (%)	87.64^{a}	83.22^{b}	81.39^{b}	75.82^{c}	0.823	<0.001
pH	6.60^{b}	6.61^{b}	6.68^{a}	6.72^{a}	0.011	<0.001
NH_3-N (mmol l^{-1})	38.97^{a}	33.64^{ab}	31.77^{b}	29.34^{b}	1.218	<0.001
tVFA (mmol l^{-1})	129.29	129.36	128.03	119.28	1.569	0.100
VFA contents (% of tVFA)						
Acetate	55.64^{c}	57.93^{b}	58.93^{b}	60.25^{a}	0.309	<0.001
Propionate	23.62^{a}	21.33^{b}	21.32^{b}	20.47^{b}	0.261	<0.001
A/P	2.36^{c}	2.74^{b}	2.76^{b}	2.94^{a}	0.044	<0.001
Isobutyrate	4.60	4.47	4.36	4.45	0.059	0.580
Butyrate	9.74	9.80	9.57	9.61	0.102	0.687
Isovalerate	5.84	5.93	5.67	5.59	0.069	0.304
Valerate	0.56	0.55	0.51	0.50	0.020	0.658

A/P = acetate/propionate. Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet. DMD = dry matter digestibility. SEM = standard error of the mean. tVFA = total volatile fatty acid.

$^{a, b, c}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

Effect of glucogenic to lipogenic nutrient ratios on rumen bacterial communities

Across all samples, 1,064,890 qualified sequence reads were acquired with an average read length of 418 bases, all reads were assigned to 2089 OTUs using a cutoff of 97% sequence similarity. The total number of reads from each sample varied from 28,702 to 49,765 with an average of 36,951. Among the bacterial community, 21 phyla were identified across all samples. The top predominant phyla with relative abundance above 0.01 in at least one sample were shown in **Figure 4.1**. *Bacteroidetes*, *Firmicutes* and *Kiritimatiellaeota* were the three dominant phyla, representing 46.94, 39.19 and 3.55% of the total sequences, respectively. *Epsilonbacteraeota*, *Proteobacteria*, *Spirochaetes* and *Patescibacteria* represented an average of 2.81, 2.83, 1.65 and 1.09%, separately, of the total sequences. The other phyla, such as *Synergistetes*, *Lentisphaerae* and *Actinobacteria* were not consistently present in all ruminal samples.

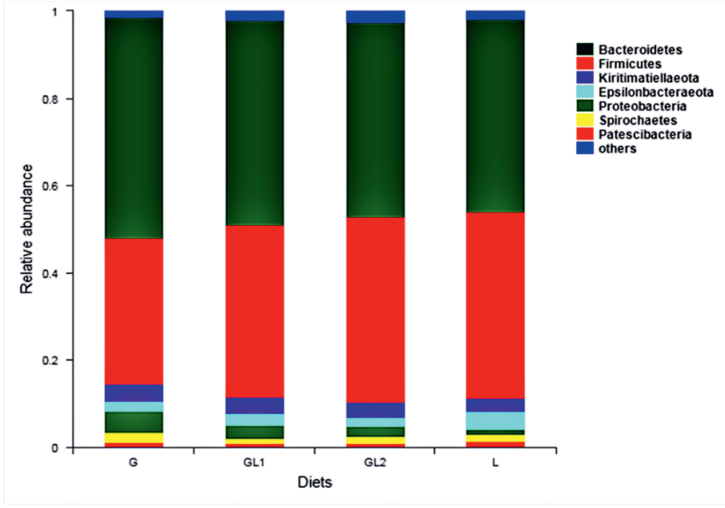


Figure 4.1. Composition of the top predominant bacteria phyla (%) in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipgenic diets. Only the phyla with a relative abundance above 0.01 in at least one sample are shown in the figure. Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet.

Table 4.3. Effects of glucogenic to lipogenic nutrient ratios on the alpha diversity of ruminal bacteria in rumen fluid of dairy cows after 48h *in vitro* fermentation

Estimators	G	GL1	GL2	L	SEM	*P*-value
OTU	1467^{b}	1527^{ab}	1586^{a}	1553^{a}	13.960	0.006
Shannon	5.75	5.79	5.85	5.78	0.032	0.694
Simpson	0.015	0.010	0.010	0.013	0.001	0.419
ACE	1760^{b}	1803^{ab}	1850^{a}	1817^{ab}	12.328	0.005
Chao1	1797^{b}	1831^{ab}	1866^{a}	1848^{ab}	11.108	0.013
Coverage	0.9895	0.9909	0.9912	0.9912	0.0003	0.172

Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet. SEM = standard error of the mean.

$^{a, b}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

As for the alpha diversity estimates (**Table 4.3**), the G diet significantly decreased the number of OTUs compared with GL2 and L diets. The ACE and Chao 1 estimates of richness in the GL2 diet were significantly higher than that of the G diet.

The PCoA result was performed in **Figure 4.2**. The diets GL1 and GL2 were separated from the diets G and L along PC1, which explained 30.21% of the total variation, while G was

separated from the other diets along PC2, which explained 24.57% of the total variation. The separation between GL1 and GL2 was not significant.

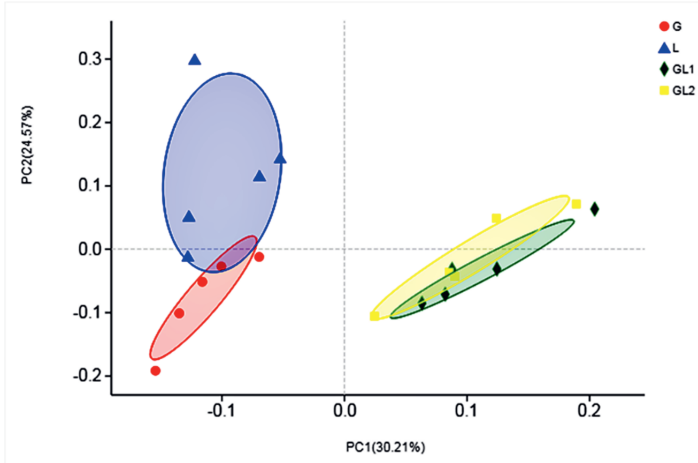


Figure 4.2. Principal coordinate analysis (PCoA) of bacteria community structure in rumen fluid samples of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipgenic diets. Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet.

At the phylum level, the top five phyla which were influenced by the treatments are listed in **Table 4.4**. The G diet significantly increased the relative abundance of *Bacteroidetes* and *Proteobacteria*, while the L diet significantly increased the relative abundance of *Firmicutes* and *Patescibacteria* (P = 0.039 and 0.007 separately).

At the genus level, a total of 260 bacteria genera were identified. The top 25 of the influenced genera ($P \leq 0.05$) with a relative abundance of ≥ 0.1% in at least one sample are listed in **Table 4.4**. Specifically, the L diet significantly increased the proportions of seven genera compared to others, including *Ruminococcaceae_UCG_group*, *Lachnospiraceae_group*, *Oribacterium*, *Anaerovorax*, *Saccharofermentans*, *SP3-e08* and *Candidatus_Saccharimonas*, while significantly decreased the relative abundance of *Ruminococcus_2* and *Ruminobacter*. Compared to the GL1, GL2 and L diets, four genera were increased by the G diet, including *Selenomonas_1*, *Ruminobacter*, *Succinivibrionaceae_UCG_002* and *Succinivibrio*. In addition, compared to the diets G and L, the GL1 and GL2 diets increased the relative abundance of *Ruminococcus_2* and *Ruminobacter*.

Table 4.4. Effect of glucogenic to lipogenic nutrient ratios on the relative abundance (%) of bacterial phyla and genera in rumen fluid of dairy cows after 48h *in vitro* fermentation

Phyla	Family	Genus/other	G	GL1	GL2	L	SEM	*P*-value
Bacteroidetes		Total	50.88	47.37	45.05	44.48	0.948	0.089
	Rikenellaceae	*SP3-e08*	0.11^{b}	0.07^{c}	0.08^{bc}	0.17^{a}	0.011	<0.001
	unclassified	unclassified_*o__Bacteroidales*	0.05^{b}	0.09^{a}	0.10^{a}	0.10^{a}	0.007	0.007
Firmicutes		Total	33.19^{b}	39.19^{ab}	42.12^{a}	42.27^{a}	1.272	0.039
	Ruminococcaceae	*Ruminococcaceae_UCG_group*	3.70^{b}	2.81^{c}	2.98^{bc}	4.63^{a}	0.236	0.001
		Ruminococcaceae_NK4A214_group	2.33^{b}	4.30^{a}	4.14^{a}	2.73^{b}	0.231	<0.001
		Ruminococcus_2	0.96^{b}	1.37^{a}	1.06^{b}	0.55^{c}	0.078	<0.001
		Ruminococcus_1	0.17^{c}	0.27^{bc}	0.41^{a}	0.35^{ab}	0.030	0.016
		[Ruminococcus]_gauvreauii_group	0.33^{b}	1.04^{a}	1.06^{a}	0.42^{b}	0.085	<0.001
		Saccharofermentans	0.42^{b}	0.29^{c}	0.35^{bc}	0.56^{a}	0.030	0.001
		unclassified_*f__Ruminococcaceae*	0.24^{c}	0.27^{bc}	0.34^{ab}	0.37^{a}	0.017	0.009
	Lachnospiraceae	*Lachnospiraceae__group*	1.66^{b}	1.65^{b}	1.81^{b}	3.12^{a}	0.212	0.007
		unclassified_*f__Lachnospiraceae*	0.38^{b}	0.54^{ab}	0.62^{a}	0.71^{a}	0.040	0.010
		Oribacterium	0.55^{b}	0.61^{b}	0.86^{b}	1.51^{a}	0.126	0.013
		Eubacterium	1.34^{c}	2.50^{b}	3.02^{a}	2.01^{b}	0.172	<0.001
		Acetitomaculum	0.18^{b}	0.78^{a}	0.84^{a}	0.21^{b}	0.074	<0.0001
	Christensenellaceae	*Christensenellaceae_R-7_group*	1.24^{b}	4.99^{a}	4.65^{a}	1.62^{b}	0.452	<0.0001
	Family_XIII	*Family_XIII_AD3011_group*	0.773^{b}	2.006^{a}	2.359^{a}	1.291^{b}	0.164	<0.0001
		Anaerovorax	0.47^{b}	0.31^{c}	0.35^{bc}	0.64^{a}	0.038	0.002
	Veillonellaceae	*Selenomonas_1*	0.55^{a}	0.29^{b}	0.27^{b}	0.26^{b}	0.035	0.001
Patescibacteria		Total	1.06^{b}	0.92^{b}	0.93^{b}	1.46^{a}	0.067	0.007
	Saccharimonadaceae	*Candidatus_Saccharimonas*	0.89^{b}	0.66^{b}	0.65^{b}	1.16^{a}	0.060	0.001
Proteobacteria		Total	4.720^{a}	2.951^{b}	2.311^{b}	1.321^{c}	0.333	<0.001
	Succinivibrionaceae	*Ruminobacter*	1.65^{a}	0.79^{b}	0.59^{b}	0.16^{c}	0.129	<0.001
		Succinivibrionaceae_UCG_002	1.29^{a}	0.32^{b}	0.16^{b}	0.08^{b}	0.134	<0.001
		Succinivibrio	0.52^{a}	0.28^{b}	0.15^{c}	0.13^{c}	0.039	<0.001
		Pseudomonas	0.07^{b}	0.48^{a}	0.36^{a}	0.01^{b}	0.051	<0.001
Actinobacteria		Total	0.13^{b}	0.61^{a}	0.69^{a}	0.14^{b}	0.073	<0.001
	Eggerthellaceae	*DNF00809*	0.02^{b}	0.17^{a}	0.19^{a}	0.03^{b}	0.021	<0.001
	Atopobiaceae	*Atopobium*	0.04^{b}	0.13^{a}	0.18^{a}	0.04^{b}	0.017	0.002

Only the top 25 influenced genera with a relative abundance of ≥ 0.1% in at least one sample are listed. Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet. SEM = standard error of the mean. $^{a, b, c}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

Compared to the GL1, GL2 and L diets, four genera were increased by the G diet, including *Selenomonas_1*, *Ruminobacter*, *Succinivibrionaceae_UCG_002* and *Succinivibrio*. In addition, compared to the diets G and L, the GL1 and GL2 diets increased the relative abundance of *Ruminococcaceae_NK4A214_group*, *[Ruminococcus]_gauvreauii_group*, *Christensenellaceae_R-7_group*, *Acetitonmaculum*, unclassified_*o_Bacteroidales*, *Pseudomonas*, *DNF00809*, *Family_XIII_AD3011_group* and *Atopobium*.

Correlation analysis between the relative abundance of bacterial genera and the fermentation parameters

As shown in **Figure 4.3**, the genus of *Ruminobacter* was positively correlated with the gas production, DMD and propionate proportion, but negatively correlated with the pH, acetate proportion and acetate to propionate ratio. The genera of *Prevotella_1*, *Sphaerochaeta*, *Prevotellaceae_UCG_003* and *Prevotellaceae_UCG_001* were negatively correlated with the pH but positively correlated with the concentrations of NH_3-N. The *prevotella_1* was negatively correlated with the acetate proportion. The *Oribacterium* was positively correlated with the pH, acetate proportion and acetate to propionate ratio, but negatively correlated with the gas production, DMD and propionate proportion. The *[Eubacterium]_coprostanoligenes_group* was positively correlated with the acetate proportion and acetate to propionate ratio, but negatively correlated with the gas production, DMD and propionate proportion. The *Lachnospiraceae_ND3007_group* was positively correlated with the pH, acetate proportion and acetate to propionate ratio but negatively correlated with the NH_3-N concentration, DMD and propionate proportion. The *Candidatus_Saccharimonas* was negatively correlated with the DMD, while the *Ruminococcaceae_UCG_010* was positively correlated with the pH.

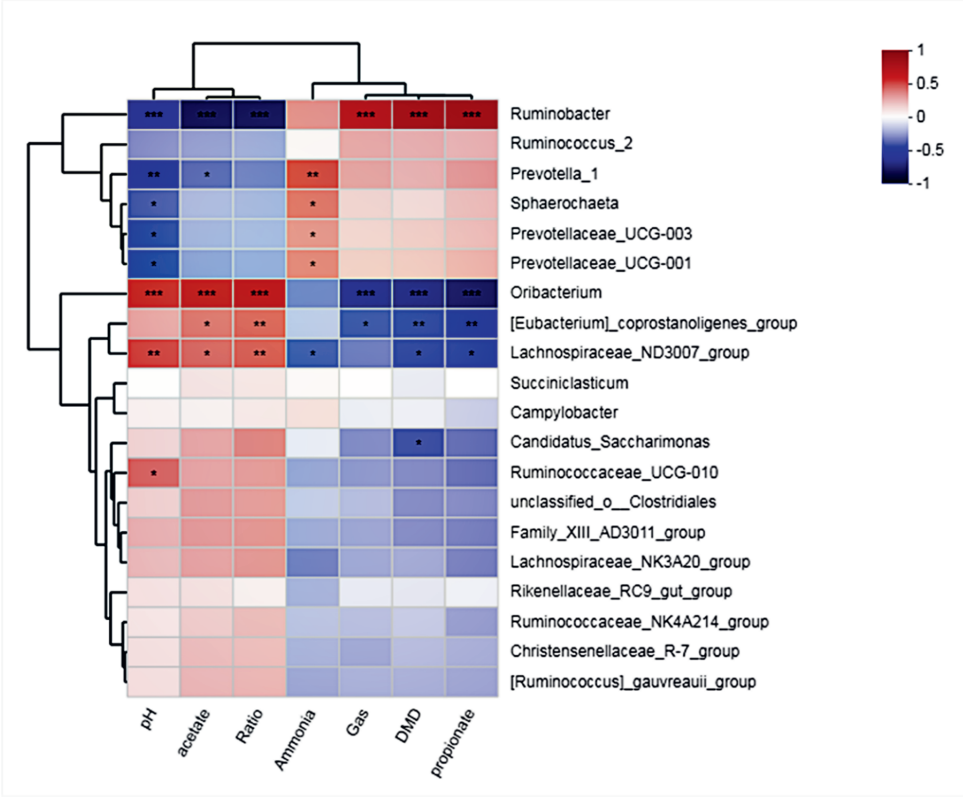


Figure 4.3. Correlation analyses between the relative abundance of the top 20 bacterial genera and influenced ruminal fermentation parameters, including pH, acetate proportion, acetate/propionate ratio (labeled as ratio in the figure), ammonia-nitrogen (labeled as ammonia in the figure), gas volume, dry matter digestibility (labeled as DMD in the figure) and propionate proportion in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipgenic diet. The red represents a positive correlation, the blue represents a negative correlation. * means the correlation is at a significant level ($P \leq 0.05$), ** means the correlation is at an extremely significant level ($P < 0.01$).

Functional analysis

To characterise the functional alterations of ruminal bacteria among different diets, the functional composition profiles were predicted from 16S rRNA sequencing data with PICRUSt (**Supplementary Table S4.1**). The top 10 out of 40 KEGG pathways of level 2 were illustrated in **Figure 4.4**. Amino acid metabolism, carbohydrate metabolism, membrane transport and replication and repair were the most abundant functions in all samples. Multiple KEGG categories were disturbed by diets. Compared with other diets, diet G had a significantly higher relative abundance of translation, metabolism of cofactors and vitamins

and cellular processes and signalling, but had a lower relative abundance of membrane transport ($P = 0.005$). Compared to the diet GL2 and L, the G diet could significantly increase the relative abundance of replication and repair ($P = 0.005$) as well as nucleotide metabolism ($P = 0.015$).

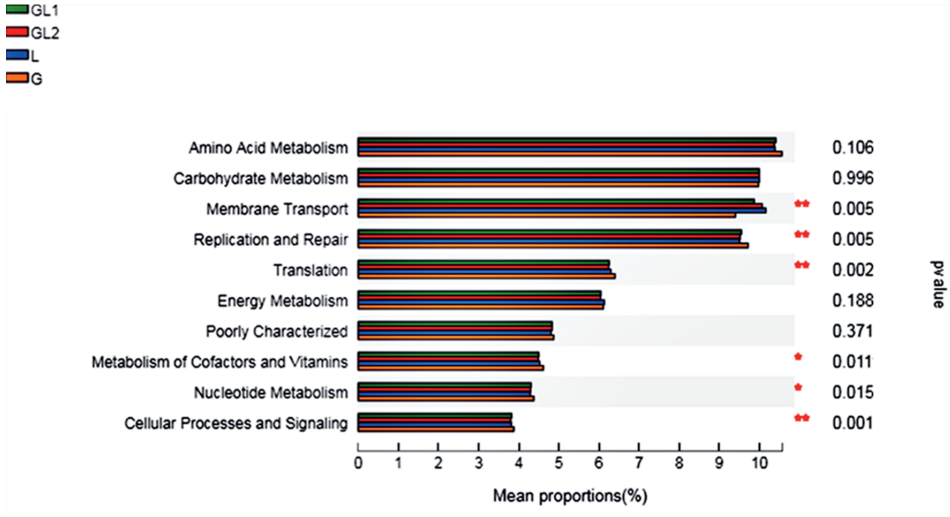


Figure 4.4. Effect of different glucogenic to lipogenic nutrient ratios on the relative abundance (%) of KEGG pathways of the bacteria in the rumen fluid of dairy cows after 48h *in vitro* fermentation with two glucogenic and a lipgenic diet. Only the top 10 relative abundance of the inferred functions are presented. * means the difference is at a significant level ($P \leq 0.05$), ** means the correlation is at an extremely significant level ($P < 0.01$). Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet. KEGG = Kyoto Encyclopedia of Genes and Genomes. The proportion in the figure equals the relative abundance.

Discussion

Effects of glucogenic to lipogenic nutrient ratios on major bacterial communities involved in feed digestion

According to the preferential utilization of energy, rumen microorganisms can be classified into different functional groups, such as amylolytic, cellulolytic and proteolytic, which either digest the dietary components or further degrade products produced by microbes. The rumen metagenome is highly dominated by the phyla *Bacteroidetes* and *Firmicutes*, the interaction of which contributes to the catabolism of organic matter of simpler form (Thomas et al., 2011, Gruninger et al., 2014). In the present study, from diet G to L, as the ratio of lipogenic ingredients increased, the relative abundance of *Bacteroidetes* had a decreasing trend which was in line with the DMD, while the relative abundance of *Firmicutes* increased gradually (**Table 4.4**). Recent studies also reported that the fibre enhanced *Firmicutes* and reduced *Bacteroidetes* (Parmar et al., 2014, Parnell and Reimer, 2014). Probably because the phyla *Firmicutes* contain a large amount of cellulose-degrading bacteria (Naas et al., 2014), while the ruminal *Bacteroidetes* contain a large number of amylolytic bacteria (Dodd et al., 2010).

The rate and extent of starch digestion in the rumen were determined by several factors, including the source of dietary starch, diet composition, grain processing and degree of adaptation of ruminal microbiota to the diet (Huntington, 1997). The rumen amylolytic bacteria convert starch to glucose, which is then used for growth and provides energy for the synthesis of microbial proteins. Reported amylolytic bacteria included *Streptococcus bovis*, *Bacteroides amylophilus*, *Prevotella* spp., *Succinimonas amylolytica*, *Selenomonas ruminantium* and *Butyrivibrio* spp. (Giraud et al., 1994, Huntington, 1997), some of whose amylolytic activities have been demonstrated *in vitro* previously (Minato and Suto, 1979, Miura et al., 1983, Cotta, 1988, Xia et al., 2015). Pure culture studies have demonstrated that most of these starch-degrading bacteria have more energy supply sources not only from starch but also from other nutrients (Kotarski et al., 1992, Klieve et al., 2007). Thus, their dominant presence in ruminants fed diets with high starch may not be necessarily associated with their starch-hydrolyzing capacity (Klieve et al., 2012). This might explain that the most dominant amylolytic bacteria didn't differ among all diets in the present study. However, the relative abundance of *Selenomonas_1*, *Ruminobacter*, *Succinivibrionaceae_UCG_002* and *Succinivibrio*, were significantly higher in the G diet than in the other three diets. These

increased bacteria genera might be recognized as being sensitive to dietary glucogenic nutrients.

Generally, the apparent digestibility of starch was nearly twice as high as that of neutral detergent fibre (NDF) (Firkins et al., 2001). The cellulolytic bacteria were the dominating contributors to fibre degradation. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are recognized as the most active cellulolytic bacteria (Wanapat et al., 2014). *Butyrivibrio*, *Oscillibacter*, *Pseudobutyrivibrio* and *Eubacterium* were also known as cellulolytic bacterial genera (Thoetkiattikul et al., 2013). Besides, some unclassified groups, such as the taxa assigned to *Lachnospiraceae*, *Christensenellaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Prevotellaceae* and *Bacteroidales*, had been proved tightly attaching to fibre in the rumen, suggesting that they might play a significant role in the ruminal digestion of fibre (Liu et al., 2016). In the present study, the GL1, GL2 and L diets compared to the G diet significantly increased the relative abundance of the fibrolytic bacterial genera, including *Ruminococcus_2*, *Ruminococcaceae_UCG_group*, *Ruminococcaceae_NK4A214_group*, *Ruminococcus_gauvreauii_group*, *Ruminococcus_1* (Krause et al., 2003), some unclassified taxa *(*unclassified*_f_Lachnospiraceae*, unclassified*_f_Ruminococcaceae*, unclassified*_o_Bacteroidales)* (Liu et al., 2016) and the genus of *[Eubacterium]_group* (Thoetkiattikul et al., 2013). In addition, compared to the diet L, the two mixed diets gained a higher number of the *Ruminococcaceae_NK4A214_group*, *Ruminococcus_2*, *Christensenellaceae_R-7_group* and *Ruminococcus_gauvreauii_group*, but gained a lower number of *Ruminococcaceae_UCG_group* and *Lachnospiraceae_group*. These changes illustrated that when the dietary lipogenic nutrients were higher than 2/3 of the dietary energy source, some bacteria in the genera *Ruminococcaceae_NK4A214_group*, *Ruminococcus_gauvreauii_group*, *Ruminococcus_2* and *Christensenellaceae_R-7_group* would rapidly decrease, while other bacteria in the genera *Ruminococcaceae_UCG_group* and *Lachnospiraceae_group* would increase.

Furthermore, according to the correlated analysis (**Figure 4.3**), the DMD and gas production were positively correlated with the genus of *Ruminobacter*. A previous study also reported that bacteria related to *Ruminobacter* would dominate in the ruminal ecosystem when cows were introduced to a high grain diet (Klieve et al., 2012). The genus *Ruminobacter* might play an important role in leading to the difference in fermentation end-products.

In summary, these sensitive amylolytic and cellulolytic bacteria might lead to the difference in feed digestion. In addition, some genera whose function was not clear were also influenced

by the diets, including *SP3-e08*, *Pseudomonas*, *DNF00809* and *Atopobium*. Their function and contribution to fermentation products still need further research.

Effects of glucogenic to lipogenic nutrient ratios on VFA and related bacteria

The dietary carbohydrate was finally fermented to VFA by microbes in the rumen. The major ingredients of VFA contain acetate, propionate and butyrate, whose proportions are mainly affected by the NDF to the starch ratio in the diet. Ruminants fed a high proportion of dietary starch produced proportionally more propionate than those fed a high forage diet which produced more acetate (Wu et al., 1994, Marounek and Bartos, 2010, Wang et al., 2016). Propionate is produced in the ruminal ecosystem by two major pathways. One is the succinate pathway in which the propionate is produced directly by decarboxylating of succinate (Scheifinger and Wolin, 1973, Jeyanathan et al., 2014). This pathway involves a large number of microbes, such as fumarate reducers (e.g., *Wolinella succinogenes*), succinate producers (e.g., *Fibrobacter succinogenes*) and succinate utilizers (e.g., *Selenomonas ruminantium*) (Scheifinger and Wolin, 1973, Jeyanathan et al., 2014). Succinate is produced by the members in the genus *Succinivibrio* as their key fermentation end-product (Pope et al., 2011), which is then digested to propionate by the members of *Selenomonas* (e.g., *Selenomonas ruminantium*) via the succinate pathway (Scheifinger and Wolin, 1973). The other one is the acrylate pathway which starts indirectly from lactate via dehydration to acrylate and turns to propionate via reduction reaction (Puniya et al., 2015, Zhao et al., 2020). Starch is degraded by *Streptococcus bovis* and *Lactobacillus* spp. to lactic acid (Hutton et al., 2012) which is then utilized by *Megasphaera elsdenii*, the major bacteria involved in the acrylate pathway (Hino et al., 1994). Other lactate-utilizing bacteria such as *Selenomonas ruminantium and Propionibacterium* spp. (Klieve et al., 2003), and some strains of the bacterium *Prevotella ruminicola* also play important roles in the acrylate pathway (Wallnofer and Baldwin, 1967). In the present study, the greatly increased relative abundance of *Succinivibrio* members (*Succinivibrionaceae_UCG_002* and *Succinivibrio*), *Selenomonas* member (*Selenomonas_1*) and the *Ruminobacter* in the G diet probably contributed to the increased propionate production via the succinate pathway.

The decreased acetate in the G diet can be explained by the reduction of some Gram-positive fibrolytic bacteria, such as *Ruminococcus* spp., which is recognized as the main acetate-producing bacteria (Jeyanathan et al., 2014). The *Anaerosporobacter* and *Saccharofermentans* are also known for producing acetate as the main end-products (Ziemer, 2014). In addition, some unclassified bacteria, such as unclassified bacteria in

Ruminococcaceae, *Lachnospiraceae* and *Christensenellaceae* were reported to be correlated with acetate concentration (Shen et al., 2017). In the present study, the increased populations of *Saccharofermentans*, *Anaerovorax*, *Lachnospiraceae_ND3007_group* and the unclassified groups in *Ruminococcaceae*, *Lachnospiraceae* and *Christensenellaceae* might have also contributed to the improvement of acetate production in the L diet.

The genus *Oribacterium* was positively correlated with acetate proportion and negatively correlated with the DMD and propionate proportion. This was a newly classified genus proposed by Carlier et al. (2004), which was latterly reported to be identified in the rumen of cows fed forage-based diets (Kong et al., 2010, Zened et al., 2013) and capable to degrade pectin from plant cell walls in the rumen environment (Zened et al., 2013, Kang et al., 2019). This could explain their high population in the diet L. To our knowledge, its function related to acetate production was not reported yet, thus it needs further research.

Effects of glucogenic to lipogenic nutrient ratios on NH_3-N and related microbes

The NH_3-N concentration was consistent with the DMD trend, which was towards a lower NH_3-N concentration as the lipogenic nutrient ratio increased. This result was in line with the study of Beckman and Weiss (Beckman and Weiss, 2005). Dietary protein is degraded in the rumen to peptides and amino acids, and eventually deaminated into NH_3-N or incorporated into microbial protein (Bach et al., 2005). When the rumen-digested protein exceeds the requirement of ruminal microorganisms, the protein is degraded to NH_3-N which is then metabolized to urea in the liver and finally excreted in urine (Tamminga, 1996). The NH_3-N accounts for about 34% of the protein requirement for ruminal microorganisms. The NH_3-N concentration in the rumen depends on the balance between the rate of formation and utilization of NH_3-N by microbes. Amylolytic bacteria tended to be more proteolytic than fibrolytic bacteria (Siddons and Paradine, 1981, Wallace et al., 1997, Ferme et al., 2004). It was also reported that amylases had positive effects on protein degradation in the rumen (Tománková and Kopečný, 1995). In addition, the cellulolytic microbes grow slowly with low maintenance requirements and solely take NH_3-N as their nitrogen source; while the amylolytic microbial communities grow fast, require more nitrogen for maintenance and have multiple nitrogen sources including NH_3-N, peptides and AA (Bach et al., 2005). This preferential use of nitrogen sources by ruminal bacteria was in agreement with the difference in NH_3-N concentrations in the present study. To summarize, the G diet tended to increase protein degradation and decrease the nitrogen utilization by ruminal bacteria, which might partially explain the increased ruminal NH_3-N concentration.

In addition, some species in the genus *Prevotella* were considered ammonia-producing bacteria, such as *Prevotella ruminantium* and *Prevotella bryantii* (Ferme et al., 2004). This could explain the positive correlations between the NH_3-N concentration and the genus *Prevotella*.

Effects of glucogenic to lipogenic nutrient ratios on metagenomic functions

Diets can reshape the bacterial communities in the rumen, consequently, the functions of ruminal bacteria may be altered along with the changes. A tool of PICRUSt is developed for inferring the functional potential of microbial communities based on 16S data, which needs little extra skill or cost compared to the metagenomics and metatranscriptomics technologies (Wilkinson et al., 2018). In the present study, the PICRUSt was carried out to predict the functional alterations of rumen bacteria associated with different ratios of glucogenic to lipogenic ingredients. In the results, the most abundant functional categories contained amino acid metabolism, carbohydrate metabolism, replication and repair, membrane transport and translation, which were proved to be fundamental for the growth and reproduction of bacteria (Seddik et al., 2019). The G diet was predicted to lower the pathway of membrane transport than other diets. The membrane transport function is significant for microbes in the communication with the rumen environment, such as capturing nutrients and secreting functional proteins or substrates (Konishi et al., 2015, Zhang et al., 2017). The relation between bacterial membrane transport function and their digesting capacity in the rumen deserves further research. In addition, several functions, such as translation, cofactors and vitamins metabolism, replication and repair, and cellular processes and signalling, were enriched by diet G compared to other diets. These results were partly in line with the previous report (Zhang et al., 2017). These improved functions in diet G might relate to the high feed digestion. However, further studies are required to enhance our understanding of bacterial functions and their relation to dietary nutrients.

Conclusion

The present study confirmed the hypothesis that the bacteria community and fermentation products *in vitro* could be altered by feeding isocaloric diets that differed in glucogenic and lipogenic nutrient content. When the glucogenic nutrient was above 1/3 of the energy source, the best feed digestion traits, as well as a lower acetate to propionate ratio, were obtained. The amylolytic bacteria including *Selenomonas*, *Succinivibrio* and *Ruminobacter*, as well as some cellulolytic bacteria including genera within the family *Ruminococcaceae*, the *Christensenellaceae_R-7_group*, the *Eubacterium* and some unclassified taxa were more sensitive to the ratio of glucogenic to lipogenic nutrients.

Acknowledgement

This research was funded by the National Key Research and Development Plan (2016YFD0700205). We appreciate the technical support from Wouter Hendriks and Wilbert Pellikaan, the Animal Nutrition group of Wageningen University and Research. We appreciate the Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture, China, for providing the experimental devices, and the Yanqing Research Station of Beijing Dairy Centre for providing experimental animals.

Supplementary material

Figure S4.1. The *in vitro* gas production machine with Automated Gas Production Recording System.

Table S4.1. Effect of glucogenic to lipogenic nutrient ratios on the relative abundance (%) of bacterial phyla in rumen fluid of dairy cows after 48h *in vitro* fermentation

Phyla	G	GL1	GL2	L	SEM	*P*-value
Bacteroidetes	50.88	47.37	45.05	44.48	0.948	0.089
Firmicutes	33.19^{b}	39.19^{ab}	42.12^{a}	42.27^{a}	1.272	0.039
Kiritimatiellaeota	3.99	3.68	3.59	3.27	0.302	0.904
Proteobacteria	4.72^{a}	2.95^{b}	2.31^{bc}	1.32^{c}	0.333	0.001
Epsilonbacteraeota	2.34	2.75	2.27	3.88	0.620	0.910
Spirochaetes	2.42	1.15	1.57	1.64	0.233	0.392
Patescibacteria	1.06^{b}	0.92^{b}	0.93^{b}	1.46^{a}	0.066	0.010
Synergistetes	0.32	0.43	0.50	0.54	0.048	0.314
Lentisphaerae	0.42	0.33	0.46	0.48	0.035	0.299
Actinobacteria	0.13^{b}	0.61^{a}	0.69^{a}	0.14^{b}	0.073	0.002
Tenericutes	0.29	0.27	0.32	0.5	0.028	0.094
unclassified_k_d__Bacteria	0.05	0.14	0.17	0.05	0.020	0.088
Elusimicrobia	0.06	0.04	0.09	0.09	0.009	0.101
Cyanobacteria	0.05	0.09	0.06	0.05	0.007	0.440
Chloroflexi	0.02	0.08	0.10	0.02	0.012	0.052
Fibrobacteres	0.06	0.02	0.02	0.04	0.006	0.082
WPS-2	0.013^{bc}	0.031^{a}	0.019^{b}	0.007^{c}	0.002	<0.001
Armatimonadetes	0.008	0.010	0.013	0.013	0.0017	0.757
Planctomycetes	0.002	0.002	0.001	0.003	0.0006	0.763
Verrucomicrobia	0.004	0.001	0.001	0.002	0.0007	0.465

Diets: G, glucogenic diet; GL1, glucogenic:lipogenic nutrient = 2:1; GL2, glucogenic:lipogenic nutrient = 1:2; L, lipogenic diet. SEM = standard error of the mean.

$^{a, b, c}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

Reference

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Gaskins, H. R., Stumpf, R. M., Yildirim, S., Torralba, M., Gillis, M., Wilson, B. A., Nelson, K. E., White, B. A., and Leigh, S. R. 2013. Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. *ISME J*. 7(7):1344-1353.

Bach, A., Calsamiglia, S., and Stern, M. D. 2005. Nitrogen metabolism in the rumen. *J. Dairy Sci*. 88:E9-21.

Beckman, J. L., and Weiss, W. P. 2005. Nutrient digestibility of diets with different fibre to starch ratios when fed to lactating dairy cows. *J. Dairy Sci*. 88(3):1015-1023.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., and Pinloche, E. 2012. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J. Nutr*. 142(9):1684-1692.

Bolger, A. M., Lohse, M., and Usadel, B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114-2120.

Carlier, J., K'Ouas, G., Bonne, I., Lozniewski, A., and Mory, F. 2004. Oribacterium sinus gen. nov., sp. nov., within the family "*Lachnospiraceae*" (phylum *Firmicutes*). *Int. J. Syst. Evol. Micr*. 54(5):1611-1615.

Cotta, M. 1988. Amylolytic activity of selected species of ruminal bacteria. *Appl. Environ. Microbiol*. 54(3):772-776.

Dodd, D., Moon, Y., Swaminathan, K., Mackie, R. I., and Cann, I. K. O. 2010. Transcriptomic analyses of xylan degradation by Prevotella bryantii and insights into energy acquisition by xylanolytic bacteroidetes. *J. Biol. Chem*. 285(39):30261-30273.

Ferme, D., Banjac, M., Calsamiglia, S., Busquet, M., and Kamel, C. 2004. The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia. Microbiol*. 49:151-155.

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., and Krehbiel, C. R. 2010. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl. Environ. Microbiol*. 76(22):7482-7490.

Firkins, J. L., Eastridge, M. L., St-Pierre, N. R., and Noftsger, S. M. 2001. Effects of grain variability and processing on starch utilization by lactating dairy cows. *J. Anim. Sci*. 79(Suppl E):E218-E238.

Fondevila, M., and Dehority, B. A. 1996. Interactions between Fibrobacter succinogenes, Prevotella ruminicola, and Ruminococcus flavefaciens in the digestion of cellulose from forages. *J. Anim. Sci*. 74(3):678-684.

Giraud, E., Champailler, A., and Raimbault, M. 1994. Degradation of raw starch by a wild amylolytic strain of Lactobacillus plantarum. *Appl. Environ. Microbiol*. 60(12):4319-4323.

Gruninger, R. J., Sensen, C. W., McAllister, T. A., and Forster, R. J. 2014. Diversity of rumen bacteria in canadian cervids. *PLoS One* 9(2):e89682.

Hino, T., Shimada, K., and Maruyama, T. 1994. Substrate preference in a strain of *Megasphaera elsdenii*, a ruminal bacterium, and its implications in propionate production and growth competition. *Appl. Environ. Microbiol.* 60(6):1827-1831.

Huntington, G. B. 1997. Starch utilization by ruminants: from basics to the bunk. *J. Anim. Sci.* 75(3):852-867.

Hutton, P. G., Durmic, Z., Ghisalberti, E. L., Flematti, G. R., Duncan, R. M., Carson, C. F., Riley, T. V., and Vercoe, P. E. 2012. Inhibition of ruminal bacteria involved in lactic acid metabolism by extracts from Australian plants. *Anim. Feed Sci. Technol.* 176(1-4):170-177.

Jeyanathan, J., Martin, C., and Morgavi, D. P. 2014. The use of direct - fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8(2):250-261.

Jin, D., Zhao, S., Zheng, N., Bu, D., Beckers, Y., Denman, S. E., McSweeney, C. S., and Wang, J. 2017. Differences in ureolytic bacterial composition between the rumen digesta and rumen wall based on ureC gene classification. *Front. Microbiol.* 8.

Kang, S., Denman, S., and McSweeney, C. 2019. Draft genome sequence and annotation of oribacterium sp. strain C9, isolated from a cattle rumen. *Microbiol. Resour. Announc.* 8(13):e1562.

Kim, M., Morrison, M., and Yu, Z. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiol. Ecol.* 76(1):49-63.

Klieve, A. V., Hennessy, D., Ouwerkerk, D., Forster, R. J., Mackie, R. I., and Attwood, G. T. 2003. Establishing populations of *Megasphaera elsdenii YE 34* and *Butyrivibrio fibrisolvens YE 44* in the rumen of cattle fed high grain diets. *J. Appl. Microbiol.* 95(3):621-630.

Klieve, A. V., McLennan, S. R., and Ouwerkerk, D. 2012. Persistence of orally administered *Megasphaera elsdenii* and *Ruminococcus bromii* in the rumen of beef cattle fed a high grain (barley) diet. *Anim. Prod. Sci.* 52(5):297.

Klieve, A. V., O'Leary, M. N., McMillen, L., and Ouwerkerk, D. 2007. *Ruminococcus bromii*, identification and isolation as a dominant community member in the rumen of cattle fed a barley diet. *J. Appl. Microbiol.* 103(6):2065-2073.

Koike, S., Yoshitani, S., Kobayashi, Y., and Tanaka, K. 2003. Phylogenetic analysis of fibre-associated rumen bacterial community and PCR detection of uncultured bacteria. *FEMS Microbiol. Lett.* 229(1):23-30.

Kong, Y., Ronald, T., and Robert, F. 2010. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. *FEMS Microbiol. Ecol.* 74:612-622.

Konishi, H., Fujiya, M., and Kohgo, Y. 2015. Host-microbe interactions via membrane transport systems. *Environ. Microbiol.* 17(4):931-937.

Kotarski, S. F., Waniska, R. D., and Thurn, K. K. 1992. Starch hydrolysis by the ruminal microflora. *J. Nutr.* 122(1):178-190.

Krause, K. M., Combs, D. K., and Beauchemin, K. A. 2003. Effects of increasing levels of refined cornstarch in the diet of lactating dairy cows on performance and ruminal pH. *J. Dairy Sci.* 86(4):1341-1353.

Kudo, H., Cheng, K. J., and Costerton, J. W. 1987. Interactions between *Treponema bryantii* and cellulolytic bacteria in the *in vitro* degradation of straw cellulose. *Can. J. Microbiol.* 33(3):244-248.

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., and Huttenhower, C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat*. *Biotechnol*. 31(9):814-821.

Liu, J., Zhang, M., Xue, C., Zhu, W., and Mao, S. 2016. Characterization and comparison of the temporal dynamics of ruminal bacterial microbiota colonizing rice straw and alfalfa hay within ruminants. *J*. *Dairy Sci*. 99(12):9668-9681.

Magoc, T., and Salzberg, S. L. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27(21):2957-2963.

Mao, S. Y., Zhang, G., and Zhu, W. Y. 2008. Effect of disodium fumarate on ruminal metabolism and rumen bacterial communities as revealed by denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA. *Anim*. *Feed Sci*. *Technol.* 140(3-4):293-306.

Marounek, M., and Bartos, S. 2010. Interactions between rumen amylolytic and lactate-utilizing bacteria in growth on starch. *J*. *Appl*. *Bacteriol*. 63:233-238.

Menke, K. H., and Steingass, H. 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. *Anim*. *Res*. *Dev*. 28:7-55.

Minato, H., and Suto, T. 1979. Technique for fractionation of bacteria in rumen microbial ecosystem III attachment of bacteria isolated from bovine rumen to starch granules *in vitro* and elution of bacteria attached therefrom. *J*. *Gen*. *Appl*. *Microbiol*. 25(2):71-93.

Mitter, E. K., de Freitas, J. R., and Germida, J. J. 2017. Bacterial root microbiome of plants growing in oil sands reclamation covers. *Front*. *Microbiol*. 8:849.

Miura, H., Horiguchi, M., Ogimoto, K., and Matsumoto, T. 1983. Nutritional interdependence among rumen bacteria during cellulose digestion *in vitro*. *Appl*. *Environ*. *Microbiol*. 45(2):726-729.

Naas, A. E., Mackenzie, A. K., Mravec, J., Schückel, J., Willats, W. G. T., Eijsink, V. G. H., and Pope, P. B. 2014. Do rumen bacteroidetes utilize an alternative mechanism for cellulose degradation? *MBio* 5(4):e1401-e1414.

NRC, 2001. Nutrient requirements of dairy cattle, seventh ed. National Academy Press, Washington, DC.

Pan, X., Fuguang, X., Xuemei, N., Zhiwen, T., and Kun, W. 2017. Illumina sequencing approach to characterize thiamine metabolism related bacteria and the impacts of thiamine supplementation on ruminal microbiota in dairy cows fed high-grain diets. *Front*. *Microbiol*. 8:1818.

Parmar, N. R., Solanki, J. V., Patel, A. B., Shah, T. M., Patel, A. K., Parnerkar, S., Kumar J. I., N., and Joshi, C. G. 2014. Metagenome of mehsani buffalo rumen microbiota: an assessment of variation in feed-dependent phylogenetic and functional classification. *J*. *Mol*. *Microb*. *BioTechnol.* 24(4):249-261.

Parnell, A. J., and Reimer, A. R. 2014. Prebiotic fibre modulation of the gut microbiota improves risk factors for obesity and the metabolic syndrome. *Gut Microbes* 3(1):29-34.

Pope, P. B., Smith, W., Denman, S. E., Tringe, S. G., and Barry, K. 2011. Isolation of succinivibrionaceae implicated in low methane emissions from tammar wallabies. *Science* 333:646-648.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., and Glockner, F. O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res*. 35(21):7188-7196.

Puniya, A. K., Singh, R., and Kamra, D. N. 2015. Rumen microbiology: from evolution to revolution. Springer, New Delhi.

Sawanon, S. and Kobayashi, Y. 2006. Synergistic fibrolysis in the rumen by cellulolytic *Ruminococcus flavefaciens* and non-cellulolytic *Selenomonas ruminantium*: Evidence in defined cultures. *Anim. Sci. J.* 77(2):208-214.

Scheifinger, C. C., and Wolin, M. J. 1973. Propionate formation from cellulose and soluble sugars by combined cultures of *Bacteroides succinogenes* and *Selenomonas ruminantium*. *J. Appl. Microbiol.* 26(5):789-795.

Seddik, H., Xu, L., Wang, Y., and Mao, S. Y. 2019. A rapid shift to high-grain diet results in dynamic changes in rumen epimural microbiome in sheep. *Animal* 13(8):1614-1622.

Shen, J., Zhuang, L., Zhongtang, Y., and Weiyun, Z. 2017. Monensin and nisin affect rumen fermentation and microbiota differently *in vitro*. *Front. Microbiol.* 8:1111.

Siddons, R. C., and Paradine, J. 1981. Effect of diet on protein degrading activity in the sheep rumen. *J. Sci. Food Agr.* 32(10):973-981.

Tamminga, S. 1996. A review on environmental impacts of nutritional strategies in ruminants. *J. Anim. Sci.* 74:3112-3124.

Thoetkiattikul, H., Mhuantong, W., Laothanachare, T., Eurwilaichitr, L., and Champreda, V. 2013. Comparative analysis of microbial profiles in cow rumen fed with different dietary fibre by tagged 16S rRNA gene pyrosequencing. *Curr. Microbiol.* 67(2):130-137.

Thomas, F., Hehemann, J., Rebuffet, E., Czjzek, M., and Michel, G. 2011. Environmental and gut bacteroidetes: the food connection. *Front. Microbiol.* 2:93.

Tománková, O., and Kopečný, J. 1995. Prediction of feed protein degradation in the rumen with bromelain. *Anim. Feed Sci. Technol.* 53(1):71-80.

Wallace, R. J., Onodera, R., and Cotta, M. A. 1997. Metabolism of nitrogen-containing compounds, Page: 283-328, The rumen microbial ecosystem. 2 ed. Hobson, P. N., and Stewart, C. S., ed. Chapman & Hall, London.

Wallnofer, P., and Baldwin, R. L. 1967. Pathway of propionate formation in *Bacteroides ruminicola*. *J. Bacteriol.* 93(1):504-505.

Wanapat, M., Gunun, P., Anantasook, N., and Kang, S. 2014. Changes of rumen pH, fermentation and microbial population as influenced by different ratios of roughage (rice straw) to concentrate in dairy steers. *J. Agric. Sci.* 152(4):675-685.

Wang, M., Wang, R., Xie, T. Y., Janssen, P. H., Sun, X. Z., Beauchemin, K. A., Tan, Z. L., and Gao, M. 2016. Shifts in rumen fermentation and microbiota are associated with dissolved ruminal hydrogen concentrations in lactating dairy cows fed different types of carbohydrates. *J. Nutr.* 146(9):1714-1721.

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

Wolin, M., Miller, T., and Stewart, C. 1997. Microbe-microbe interactions, Page: 467-491, The rumen microbial ecosystem. Hobson, P., and Stewart, C., ed. Blackie Academic and Professional, London.

Wu, Z., Sleiman, F. T., Theurer, C. B., Santos, F., Simas, J. M., Francolin, M., and Huber, J. T. 1994. Effect of isocalorlc infusion of glucose in the rumen or propionate in the duodenum. *J. Dairy Sci*. 77:1556-1562.

Xia, Y., Kong, Y., Seviour, R., Yang, H., Forster, R., Vasanthan, T., and McAllister, T. 2015. *In situ* identification and quantification of starch-hydrolyzing bacteria attached to barley and corn grain in the rumen of cows fed barley-based diets. *FEMS Microbiol*. *Ecol*. 91(8):v77.

Ye, H., Liu, J., Feng, P., Zhu, W., and Mao, S. 2016. Grain-rich diets altered the colonic fermentation and mucosa-associated bacterial communities and induced mucosal injuries in goats. *Sci*. *Rep*. 6:20329.

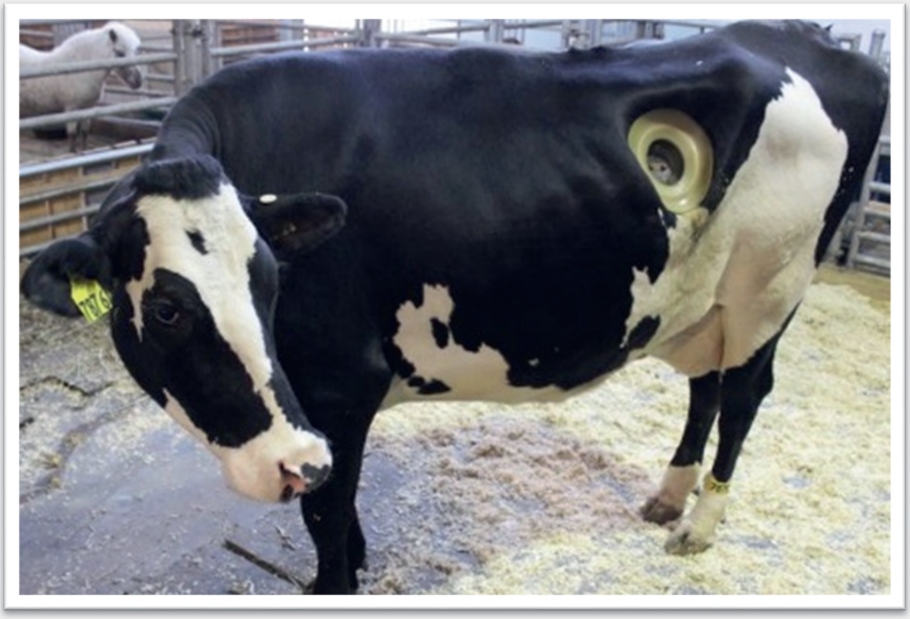
Zened, A., Combes, S., Cauquil, L., Mariette, J., Klopp, C., Bouchez, O., Troegeler-Meynadier, A., and Enjalbert, F. 2013. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. *FEMS Microbiol*. *Ecol*. 83(2):504-514.

Zhang, D., and Yang, H. 2011. Combination effects of nitrocompounds, pyromellitic diimide, and 2-bromoethanesulfonate on *in vitro* ruminal methane production and fermentation of a grain-rich feed. *J*. *Agr*. *Food Chem*. 60(1):364-371.

Zhang, R., Ye, H., Liu, J., and Mao, S. 2017. High-grain diets altered rumen fermentation and epithelial bacterial community and resulted in rumen epithelial injuries of goats. *Appl. Microbiol. Biotechnol.* 101(18):6981-6992.

Zhao, Y., Xue, F., Hua, D., Wang, Y., Pan, X., Nan, X., Sun, F., Jiang, L., and Xiong, B. 2020. Metagenomic insights into effects of thiamine supplementation on carbohydrate-active enzymes' profile in dairy cows fed high-concentrate diets. *Animals* 10(2):304.

Ziemer, C. J. 2014. Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. *Appl. Environ. Microbiol.* 80(2):574-585.



Chapter 5

Alterations in rumen bacteria community and metabolome characteristics of dairy cows in response to different dietary energy sources

Dengke Hua[1,2], Wouter H. Hendriks[2], Zihao Liu[1], Yapin Wang[1], Yue Wang[1], Linshu Jiang[3], Benhai Xiong[1], Wilbert F. Pellikaan[2]

[1] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

[2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, 6708 WD, the Netherlands

[3] Beijing Key Laboratory for Dairy Cattle Nutrition, Beijing Agricultural College, Beijing, 102206, China

Submitted to Frontiers in Nutrition

Abstract

In order to optimize rumen functioning, more research on the functioning of ruminal microorganisms and metabolites under different feeding conditions is needed. This study was conducted to investigate the influence of different dietary energy sources on the changes and interactions of ruminal bacteria and metabolites. Six rumen-cannulated Holstein Friesian dairy cows were randomly distributed and allocated to their dietary treatments into two replicated 3 × 3 Latin squares. During each of the three 21-d periods, cows were offered one of the three chemically similar diets but varied in energy source (two glucogenic type diets, C: corn and corn silage, S: steam-flaked corn and corn silage and a lipogenic type diet, L: sugar beet pulp and alfalfa silage). Compared to the C and S diets, the L diet resulted in significantly lower ruminal concentrations of lactic acid and ammonia-nitrogen, higher proportions of propionate, isobutyrate, isovalerate and higher proportions of acetate and butyrate ($P \leq 0.05$). As for the bacterial community at the genus level, diet L had significantly higher relative abundances of *Prevotella_1*, *Prevotellaceae_Ga6A1_group*, *Ruminococcus_1*, *Eubacterium_coprostanoligenes_group*, *Ruminiclostridium_6*, *Ruminococcaceae_UCG-013* and *Tyzzerella_3* but lower relative abundances of *Ruminococcus_2*, *CAG-352*, *Ruminococcaceae_UCG-005*, *Papillibacter*, *Lachnobacterium* and *Selenomonas* ($P \leq 0.05$), and tended to lower relative abundances of *Prevotella_7* and *Prevotellaceae_YAB2003_group* ($0.05 < P \leq 0.1$), compared to those in the diet C and S. A total of 188 significantly differential metabolites were obtained, most of these differential metabolites belong to the triterpenoids (16), the amino acids, peptides and analogues (11) and the fatty acid and conjugates (10). Ten out of 11 metabolites in amino acids, peptides and analogues were significantly more abundant in diets C and S than that in diet L. The results indicated that protein digestion was improved when starch was the main energy source in the diet, which might be attributed to the bacterial genera *Prevotella_7* and *Selenomonas*. The genera of *Ruminococcus_2*, *Prevotella_7*, *CAG_352* and *Ruminococcaceae_UCG-005* might contribute to the amylolytic activities in glucogenic diets, while *Ruminococcus_1*, *Prevotella_1* and *Eubacterium_coprostanoligenes_group* might contribute to the fibrolytic activities in the lipogenic diet. This suggests the presence of new starch-fermenting and cellulose-fermenting ruminal bacteria that need to be confirmed and classified.

Keywords: rumen, diet, bacteria, metabolomics, amylolytic, fibrolytic

Introduction

During early lactation, the increasing energy demand for milk production together with a reduced appetite and reduction in dry matter intake can lead to a negative energy balance (NEB) status of dairy cows. Previous studies have demonstrated dietary energy sources to be a significant factor affecting NEB and associated metabolic alterations. Sequential studies by van Knegsel and co-workers have shown that starch-rich glucogenic diets compared to fibre-rich lipogenic diets, were effective in increasing the ruminal production of propionate, improving the energy balance and decreasing plasma β-hydroxybutyrate and liver triacylglycerides concentrations (van Knegsel et al., 2005, van Knegsel et al., 2007a, van Knegsel et al., 2007b). However, the details of the rumen metabolic mechanisms underlying those differences remain to be clarified.

The ruminal ecosystem harbours a wide diversity of microorganisms including bacteria, archaea, fungi and protozoa, which are coexisting in a symbiotic relationship. The most abundant among these ruminal microorganisms are the bacteria with an estimated population density of $10^{10\text{-}11}$ /ml of rumen fluid. According to their preference for certain substrates, the bacteria can be defined as fibrolytic, amylolytic and proteolytic with the composition of the ruminal bacteria and the formation of fermentation end-products being affected by diet composition (Castillo-Gonzalez et al., 2014). Approximately 55%-60% of metabolites in the rumen have been linked to the rumen microorganism (Saleem et al., 2013). Glucogenic diets can be formulated using high starch content ingredients such as corn grain which then form the main and functional energy source for ruminants yielding increased amounts of propionate in the rumen or glucose for small intestinal absorption (van Knegsel et al., 2007a). Compared with finely ground corn, steam-flaked corn is more readily digestible due to changes in the structure of the starch granules. The starch digestibility for the steam-flaked corn in both the rumen and post-rumen was reported to be greater than that for ground corn (Cooper et al., 2002). Lipogenic diets are predominantly formulated using high levels of fat and fibre resulting in the main fermentation products being acetate and butyrate in the rumen and absorption of fat.

The current study aimed to fully understand the metabolic mechanisms underlying the observed effects of three dietary energy sources on the ruminal bacteria and their metabolites in dairy cows through a combination of 16S rRNA sequencing and metabolomic analysis. Moreover,

the relationships between ruminal microbiota abundance and metabolites were also analysed in the present study.

Materials and methods

Animals and experimental design

Animal care and experimental handlings were done according to the Chinese Guidelines for Animal Welfare and the study was approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (IAS2019-6).

Six rumen-cannulated, first-parity, Chinese Holstein Friesian dairy cows, 108 ± 10 DIM (days in milk, mean ± SD) with an average milk yield of 28 ± 1.7 kg/d and weighing 578 ± 33.5 kg at the start were used in a replicated 3 × 3 Latin square design. They were paired by initial weight, DIM and milk production with an animal within pairs being randomly assigned to one of the two squares. The trial consisted of three 21-d experimental periods, where each period contained a 14-d feed adaptation period, followed by a 7-d period of data and sample collection. Diet treatments included a diet with alfalfa silage and sugar beet pulp as the main energy source (L), two glucogenic diets separately containing ground corn and corn silage (C) and steam-flaked corn and corn silage (S) as main energy sources. The diets were formulated to be isoenergetic and to meet the energy requirements of Holstein dairy cows yielding 25 kg/d of milk with 3.5% of milk fat and 3.0% of milk protein according to the NRC (2001). Dietary composition and chemical analyses are shown in **Table 5.1**. Experimental animals were fed three times daily (07:00, 14:00 and 20:00) with free access to water.

Rumen fluid sampling and parameters measurement

On d 21 of each period, rumen contents of cranial, caudal, dorsal and ventral sites were collected by hand using a sterile glove through the rumen fistula 1 h after morning feeding from each cow. Collected contents were immediately squeezed tightly through four layers of cheesecloth (250 μm for mesh size) into a sterilized container (around 100 ml fluid from each site, 400 ml fluid per cow) before the squeezed material was returned to the rumen. Two aliquots of 5 ml rumen fluid were quickly collected into two cryogenic vials and immediately frozen in liquid nitrogen until being stored at -80 °C. One sample was used for later DNA extraction and the other for metabolomics analysis.

Table 5.1. Ingredient and chemical composition of the two glucogenic (C, S) and a lipogenic (L) diet

Item	Experimental diet		
	C	L	S
Ingredient composition, % of dry matter			
Corn, ground	28.3	-	-
Steam-flaked corn	-	-	28.3
Sugar beet pulp	-	30.1	-
Soybean meal	11.5	10.4	11.5
Rapeseed meal	7.2	3.9	7.2
Cottonseed meal	7.2	3.9	7.2
Alfalfa hay	7.4	6.7	7.4
Oat hay	4.5	4.1	4.5
Alfalfa silage	-	39.5	-
Corn silage	32.2	-	32.2
Dicalcium phosphate	1.7	1.5	1.7
Composition, % of dry matter			
Crude protein	20.6	20.8	20.4
Ether extract	2.3	2.1	3.0
Starch	18.6	4.4	15.6
Neutral detergent fibre	33.3	54.7	32.7
Acid detergent fibre	18.7	34.6	18.8
Calcium	0.8	1.3	1.0
Phosphorus	0.9	0.4	1.1
NE_L, MJ/kg of dry matter	7.1	8.7	7.2

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

NE_L, net energy for lactation, calculated based on NRC (2001).

Three aliquots of 5 ml fluid samples were collected into freezing vials and stored at -20 °C until further index measurements including volatile fatty acids (VFA), ammonia-nitrogen (NH_3-N) and lactic acid.

The pH value of the rumen fluid was determined using a portable pH meter (PHB-4, INESA, Shanghai, China) immediately when the rumen fluid was squeezed. Before all measurements, the rumen fluid samples were firstly thawed in a 39 °C water bath and then centrifuged for 15 min at 10,000 × g at 4 °C. The total and individual VFA concentrations were measured by gas chromatography (GC, 7890B, Agilent, USA) according to the method described by Hua et al. (2020). The NH_3-N concentration was determined based on the Berthelot reaction (Broderick and Kang, 1980). The lactic acid concentration was determined using an enzymatic method with the commercial kit (A019-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) at 530 nm according to the manufacturer's instructions (Pan et al., 2016).

DNA extraction, sequencing processing and analysis

DNA was extracted using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). The DNA concentration was checked on a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of extracted DNA was assessed on 1% agarose gel electrophoresis.

The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA) (Pan et al., 2017). The PCR reactions were performed in triplicate with a total reaction volume of 20 μl. The amplified products were detected using 2% agarose gel electrophoresis, further purified using the Ax yPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and then quantified using QuantiFluorTM-ST (Promega, USA) according to the manufacturer's protocols.

Following amplification, paired-end sequencing libraries were constructed by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Subsequently, purified amplicons were pooled in equimolar amounts and sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) for paired-end reads of 300 bp at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw FASTQ files were quality-filtered by Trimmomatic (version 0.36) and merged by FLASH software (version 1.2.11) (**Supplementary material**).

Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using UPARSE (version 7.1) with a novel "greedy" algorithm that performs chimaera-filtering and OTU-clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analysed using the RDP classifier algorithm against the Silva (SSU123) 16S rRNA database with a confidence threshold of 70% (Amato et al., 2013).

Analysis was performed using the online platform, Majorbio I-Sanger Cloud Platform (http://www.i-sanger.com). Alpha diversity indexes were calculated using Mothur (version 1.30.1). The rarefaction curve was generated using the vegan package in R (version 1.6.2, Wang et al., 2021a). Beta-diversity was estimated by computing the unweighted UniFrac distance and visualized using principal coordinate analysis (PCoA), and the results were plotted using GUniFrac and ape packages in R (version 1.6.2).

Metabolomics processing

The rumen fluid samples were prepared based on the procedures described by Liu et al. (2019). Briefly, 0.1 ml of each sample was mixed with 0.4 ml of methanol:water (4:1, v/v) solution to extract the metabolites. The mixture was allowed to settle at -20 °C and treated by high throughput tissue crusher Wonbio-96c (Shanghai wanbo biotechnology co., LTD) at 50 Hz for 6 min, then followed by vortex for 30 s and ultrasound at 40 kHz for 30 min at 5 °C. The samples were placed at -20 °C for 30 min to precipitate proteins. After centrifugation at 13,000 × g at 4 °C for 15 min, the supernatant was carefully transferred to sample vials for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Meanwhile, the quality control (QC) sample was prepared by pooling equal volumes of extracted metabolites from each sample. The QC samples were inserted into the queue after every 10 samples in the entire sample run to monitor the analyses.

Metabolomics data analysis

Raw mass spectrometry data were transferred into Progenesis QI 2.3 (Waters Corporation, Milford, USA) for preprocessing including peak picking, detection, deconvolution, alignment and missing values filling. After these initial preprocessing steps, a data matrix with retention time, M/Z and peak intensity was acquired. The variables were then normalized with the sum normalization method in order to eliminate errors caused by sample treatments and equipment instability. Only variables with a relative standard deviation ≤ 30% in the QC samples were retained in the final data matrix. The values were log-transformed before further analysis. The

mass spectrometry information was matched in the human metabolome database (HMDB) (http://www.hmdb.ca/) and Metlin database (https://metlin.scripps.edu/) to acquire metabolite information (Wang et al., 2021b).

The pre-processed data were further analysed on the Majorbio Cloud Platform (https://cloud.majorbio.com). The principal component analysis (PCA) and the orthogonal partial least squares discriminate analysis (OPLS-DA) were calculated through the R package of Ropls (version 1.6.2) to observe the global difference of the metabolites between every two diets. To avoid overfitting, the default 7-fold cross-validation was used with one-seventh of the samples being eliminated from the model in each running round. The PROC MIXED model (see below) was used to evaluate the significant differences in individual metabolites among the three diets. The significantly influenced metabolites were further screened based on the variable important in projection (VIP) from the OPLS-DA model and the *P*-value from the PROC MIXED model. The metabolites meeting the criteria of VIP > 1 and $P \leq 0.05$ were recognized as the significantly affected metabolites. The metabolic pathways where the significantly affected metabolites clustered were annotated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The enrichment analysis of the annotated pathways was achieved by Fisher's exact test and the *P*-values were further adjusted by the Benjamini and Hochberg method through Scipy.stats package in Python (version 1.0.0).

Statistical analyses

The data of rumen fermentation parameters, the relative abundances of bacteria and abundances of metabolites were analysed as a replicated 3 × 3 Latin square design using PROC MIXED combined with *post-hoc* Tukey by SAS version 9.3 (SAS Institute Inc., Cary, NC). The statistical model for the trial was as follows:

$$Y_{ijkl}=u + D_i + P_j + S_k + C_l\,(S_k) + e_{ijkl}$$

where Y_{ijkl} is the dependent variable, u is the overall mean, D_i is the fixed effect of diet (i=1-3), P_j is the random effect of the period (j=1-3), S_k is the random effect of Latin square (k=1-2), C_l (S_k) is the random effect of cow nested within a square (l=1-6), and e_{ijkl} is the random residual error. Significance was declared at $P \leq 0.05$ and a trend was considered at $0.05 < P < 0.1$.

Results

Rumen fermentation parameters

Data about the influence of three dietary energy sources on rumen fermentation is shown in **Table 5.2**. Compared to the C and S diet, the L diet led to a lower lactic acid concentration (P = 0.023), higher pH value (P = 0.017) and lower NH_3-N concentration (P = 0.006) in the rumen fluid of dairy cows. The total VFA concentration was not influenced by the three diets (P = 0.407), but the molecular proportions of individual VFAs were affected by diet. Compared to diets C and S, the rumen fluid from cows fed diet L had higher proportions of acetate (P = 0.008) and butyrate (P = 0.042), a higher acetate to propionate ratio (P < 0.001) but lower proportions of propionate (P < 0.001), isobutyrate (P = 0.019), isovalerate (P = 0.006) and valerate (P = 0.095).

Table 5.2. Ruminal fermentation parameters of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Item	Experimental diet			SEM	*P*-value
	C	L	S		
pH	6.48[b]	6.63[a]	6.40[b]	0.057	0.017
Lactic acid (mmol/l)	0.80[a]	0.70[b]	0.88[a]	0.045	0.023
Ammonia-N (mg/dl)	19.3[a]	12.2[b]	19.1[a]	1.290	0.006
Volatile fatty acids (% of total)					
Acetate	60.8[b]	64.9[a]	58.6[b]	1.176	0.008
Butyrate	13.4[b]	16.6[a]	14.7[b]	0.519	0.042
Acetate/propionate	2.92[b]	4.29[a]	2.75[b]	0.223	<0.001
Propionate	21.7[a]	15.2[b]	22.4[a]	1.116	<0.001
Isobutyrate	1.02[a]	0.90[b]	0.97[a]	0.032	0.019
Isovalerate	1.62[a]	1.22[b]	1.72[a]	0.076	0.006
Valerate	1.37[ab]	1.16[b]	1.63[a]	0.098	0.095
Total (mmol/l)	129.2	147.1	134.4	5.415	0.407

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Ruminal microbial composition

Based on the bacteria 16S rRNA sequencing, a total of 872,181 reads were acquired after quality-filtering from the 18 rumen samples. After each sample was sub-sampled to an average depth (21,373 reads on average) and clustered, 2,107 OTUs were obtained at an identity of 97%. According to the microbial diversity index of all samples at different sequencing depths, the Shannon rarefaction curves were calculated to reflect the microbial diversity at different sequencing numbers. As is shown in the **Supplementary Figure S5.1**, the curves asymptotically became flat, indicating that sequencing depth was sufficient to reflect the ruminal bacterial community. According to the alpha indexes including the Shannon, Simpson, abundance-based coverage estimator (ACE), Chao 1 and Coverage values (**Table 5.3**), no significant differences were observed in the microbiota diversity and richness among the three diets.

Table 5.3. Bacterial alpha diversity parameters in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Parameter	Experimental diet			SEM	*P*-value
	C	L	S		
Sobs	1242	1097	1112	38.03	0.269
Shannon	5.03	4.86	4.86	0.168	0.902
Simpson	0.04	0.06	0.05	0.015	0.870
ACE	1463	1326	1339	36.10	0.199
Chao 1	1479	1347	1361	37.57	0.256
Coverage	0.99	0.99	0.99	0.001	0.884

ACE, abundance-based coverage estimator. Sobs, species observed. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

The taxonomic analysis showed that the rumen bacterial community contained 20 identified phyla, dominated by the following three bacterial phyla (the values indicated the average relative abundance across the diets): *Firmicutes* (43.8%), *Bacteroidetes* (40.2%) and *Proteobacteria* (9.2%) (**Supplementary S5.2a**). In terms of bacterial genera, the analysis showed that the classified genera across all samples were dominated by *Prevotella_1* (16.2%), *Ruminococcus_1* (14.7%), *Succinivibrionaceae_UCG-001* (8.0%), *Ruminococcus_2* (6.7%) and *Prevotella_7* (5.1%) (**Supplementary Figure S5.2b**).

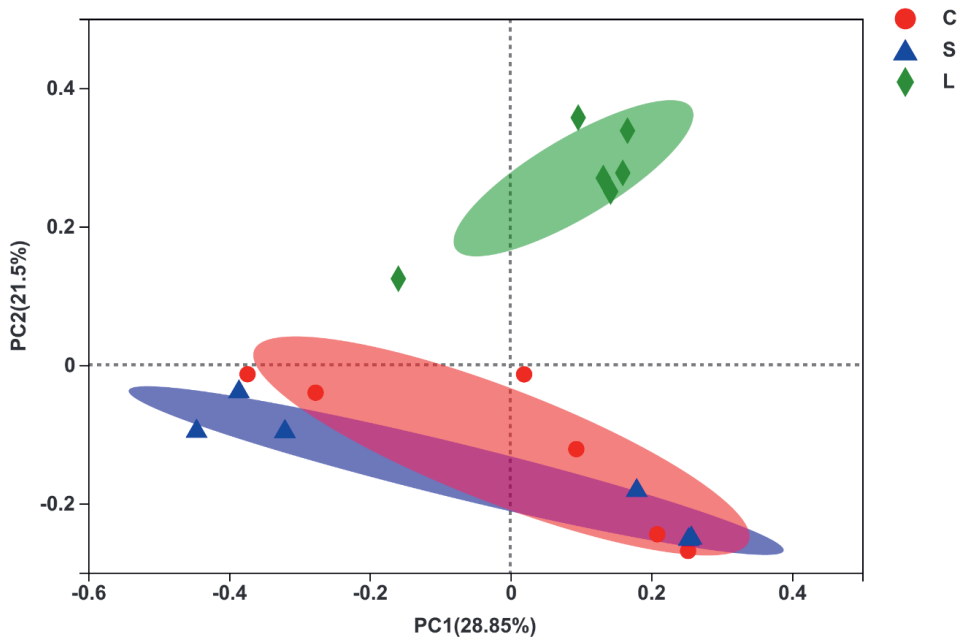


Figure 5.1. Principal coordinate analysis of bacteria community in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

The PCoA figure indicated the samples in diet L were distinguished from those in diets C and S (**Figure 5.1**). The bacteria with significantly different relative abundances are shown in **Tables 5.4** and **5.5**. At a phylum level (**Table 5.4**), there was a trend for the relative abundance of *Proteobacteria* for diet S to be higher than that for diet L (P = 0.088) as well as for *Tenericutes* for diet C to be higher than that for other two diets (P = 0.067). Diet L resulted in a higher relative abundance of *Cyanobacteria* (P = 0.018) and *Verrucomicrobia* (P = 0.016) compared to diets C and S. In addition, there was a trend for diet L to have a higher relative abundance of *Kiritimatiellaeota* compared to diet C (P = 0.085) and a lower relative abundance of *WPS-2* compared to diet S (P = 0.079). The top 20 significantly differential bacterial genera affected by diets are listed in **Table 5.5**. Diet L had significantly higher relative abundances of *Ruminococcus_1* (P = 0.004), *Prevotella_1* (P = 0.044), *Eubacterium_coprostanoligenes_group* (P = 0.023), *Ruminiclostridium_6* (P = 0.003), *Ruminococcaceae_UCG-013* (P = 0.018), *Tyzzerella_3* (P = 0.006) and potentially higher relative abundance of *Prevotellaceae_Ga6A1_group* (P = 0.067), but significantly lower

relative abundances of *Ruminococcus_2* (P = 0.011), *CAG-352* (P = 0.023), *Ruminococcaceae_UCG-005* (P = 0.043), *Papillibacter* (P = 0.008), *Lachnobacterium* (P = 0.004) and *Selenomonas* (P = 0.046), and had a trend towards a lower relative abundances of *Prevotella_7* (P = 0.1) and *Prevotellaceae_YAB2003_group (P* = 0.099*)*, compared to diet C and S.

Table 5.4. Comparison of the relative abundance (%) of bacteria phyla with $P \leq 0.1$ in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Phyla	Experimental diet			SEM	*P*-value
	C	L	S		
Proteobacteria	7.42	0.62	18.8	3.730	0.088
Tenericutes	1.63	1.02	1.04	0.123	0.067
Cyanobacteria	0.54[b]	1.26[a]	0.56[b]	0.131	0.018
Kiritimatiellaeota	0.17	0.35	0.28	0.052	0.085
WPS-2	0.08	0.03	0.18	0.027	0.079
Verrucomicrobia	0.03[b]	0.06[a]	0.01[b]	0.008	0.016

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table 5.5. Comparison of the relative abundance (%) of bacterial genera with $P \leq 0.1$ in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Genus	Experimental diet			SEM	*P*-value
	C	L	S		
Ruminococcus_1	6.92[b]	30.8[a]	4.39[b]	3.755	0.004
Prevotella_1	12.8[b]	24.4[a]	12.3[b]	2.255	0.044
Ruminococcus_2	12.8[a]	0.78[b]	8.51[a]	2.112	0.011
Prevotella_7	6.93	0.21	6.34	1.930	0.100
CAG-352	5.13[a]	0.01[b]	3.74[a]	1.004	0.023
Ruminococcaceae_UCG-005	1.45[a]	0.77[b]	1.72[a]	0.170	0.043
Eubacterium_coprostanoligenes_group	1.46[b]	3.59[a]	1.11[b]	0.402	0.023
Prevotellaceae_YAB2003_group	0.59	0.09	0.55	0.129	0.099
Papillibacter	0.15[a]	0.05[b]	0.12[a]	0.020	0.008
Selenomonas	0.12[a]	0.01[b]	0.16[a]	0.036	0.046
Lachnobacterium	0.18[a]	0.01[b]	0.15[a]	0.026	0.004
Ruminiclostridium_6	0.15[b]	0.66[a]	0.06[b]	0.081	0.003
Ruminococcaceae_UCG-013	0.11[b]	0.62[a]	0.07[b]	0.091	0.018
Tyzzerella_3	0.16[b]	0.64[a]	0.10[b]	0.088	0.006
Prevotellaceae_Ga6A1_group	0.15	0.33	0.14	0.040	0.067
unclassified_*o_Bacteroidales*	0.14[ab]	0.19[a]	0.07[b]	0.025	0.040
Eubacterium_ventriosum_group	0.25[a]	0.04[b]	0.10[b]	0.040	0.033
Erysipelotrichaceae_UCG-004	0.31	0.07	0.21	0.043	0.053
Anaeroplasma	0.43[a]	0.08[b]	0.21[b]	0.064	0.039
Alloprevotella	0.18	0.09	0.31	0.045	0.056

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Correlation between rumen fermentation indexes and ruminal bacteria

Correlations between the top significantly affected ruminal bacterial genera (with a relative abundance > 0.1% in at least one of the samples) and the rumen fermentation end-products are shown in **Figure 5.2**. The NH_3-N concentration was positively correlated to *Prevotella_7*, *Ruminococcaceae_UCG-005* and *Selenomonas*. The lactic acid concentration was positively correlated to *Ruminococcaceae_UCG-005*. The acetate proportion was positively correlated to *Prevotella_1* and negatively correlated to *Selenomonas*. The propionate proportion was positively correlated to *Prevotella_7* and *Selenomonas* but negatively correlated to *Ruminiclostridium_6* and *Tyzzerella_3*. The butyrate proportion was negatively correlated to

CAG-352 and *Ruminococcus_2* whereas the valerate proportion was positively correlated to *Prevotella_7*.

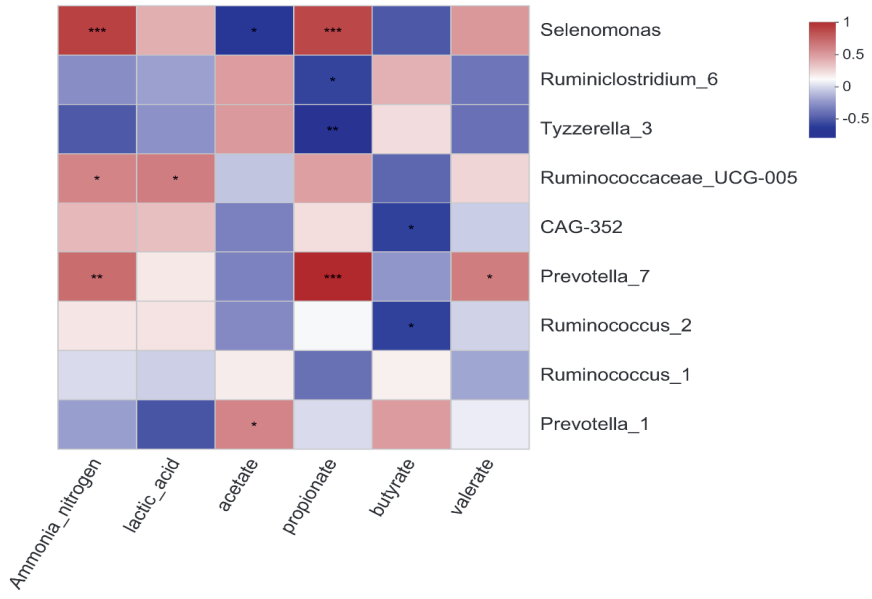


Figure 5.2. Correlation analysis between affected fermentation end-products and affected bacterial genera (relative abundance > 0.1% in at least one sample) in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Colours indicate correlation direction and strength as represented in the legend; blue, a negative correlation; red, a positive correlation; darker colours indicate a stronger correlation. *, $P \leq 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Rumen metabolomics profiling

The total ion chromatograms of QC samples under both positive and negative ion modes are shown in the **Supplementary Figure S5.3,** in which the overlap of QC samples showed the high repeatability and accuracy of the detected data. All samples were first examined by PCA to determine the differences in metabolites among diets and the degree of variation within diets following positive and negative mode ionization (**Figure 5.3**). The PCA results showed samples from diet L could be significantly separated from those in the other two diets in both positive and negative ionization modes. The OPLS-DA score plots and response permutation test between two groups under both positive and negative ion modes are shown in **Figures S4** and **S5**. All samples presented in the score plots were within the 95% Hotelling T^2 ellipse and clear separations were observed between every two groups (**Figure S5.4 a, c, e** and **Figure S5.5 a,**

c, e). The permutation tests showed the corresponding R^2Y values were 0.634, 0.610 and 0.737 for the positive (**Figure S5.4 b, d and f**) and negative ion modes 0.676, 0.741 and 0.720 for C vs. L, S vs. L and C vs. S, respectively **(Figure S5.5 b, d and f**), which were all above 0.5 indicating the effectivenees of this model in distinguishing the differences between diets.

After searching against the HMDB, a total of 504 metabolites were quantified in all rumen fluid samples from cows fed the three diets, with 52.3% originating from lipids and lipid-like molecules, 12.6% from organic acids and derivatives and 11.7% from organoheterocyclic compounds at the superclass level (**Supplementary Figure S5.6**). At the subclass level, the top three metabolite categories were amino acids, peptides and analogues (10.6% of all metabolites), fatty acids and conjugates (9.3%) and triterpenoids (6.4%) (**Supplementary Figure S5.7**).

Based on VIP > 1 and $P \leq 0.05$, a total of 188 significantly differential metabolites (109 in positive and 79 in negative ion mode) were identified based on the feeding of the three diets, of which 81 metabolites belong to the lipids and lipid-like molecules, 18 to organoheterocyclic compounds, 14 to organic acids and derivatives and 11 to phenylpropanoids and polyketides at a superclass level (**Supplementary Table S5.1**). At a subclass level, the top three categories with the most differential metabolites were triterpenoids (16, metabolite amount), amino acids, peptides and analogues (11) and fatty acids and conjugates (10) (**Supplementary Table S5.1**). The top 20 most abundant metabolites are shown in **Table 5.6**.

In addition, the top 10 enriched KEGG pathways of the differential metabolites set are shown in **Figure 5.4**. The pathways of protein digestion and absorption, biosynthesis of plant secondary metabolites, aminoacyl-tRNA biosynthesis and tryptophan metabolism were the top pathways that influenced by the dietary treatments.

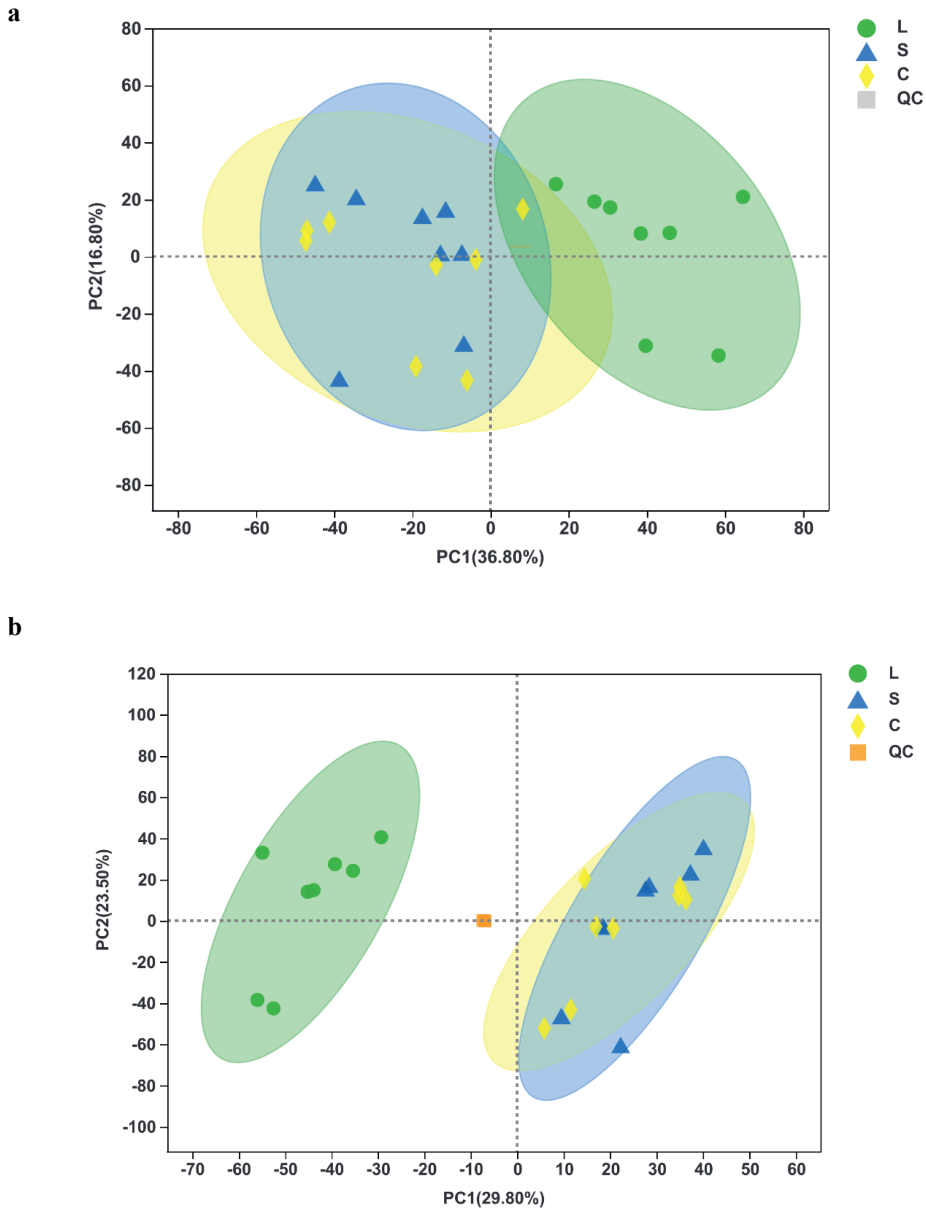


Figure 5.3. Principal component analysis (PCA) of metabolites based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet following (a) positive and (b) negative mode ionization. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. QC: quality control sample.

Table 5.6. Comparison of the abundance of top 20 differential metabolites based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Subclass	Metabolite	Experimental diet			SEM	*P*-value
		C	L	S		
Triterpenoids	Ganoderic acid F	5.18[b]	5.96[a]	5.26[b]	0.097	<0.001
	Medicagenic acid	3.80[b]	4.39[a]	3.83[b]	0.094	0.001
	Camelliagenin B	3.62[a]	3.20[b]	3.66[a]	0.069	0.001
	(3beta,17alpha,23S)-17,23-Epoxy-3,29-dihydroxy-27-norlanost-8-en-24-one	3.37[b]	4.19[a]	3.44[b]	0.118	<0.001
Fatty acids and conjugates	Petroselinic acid	4.38[a]	3.95[b]	4.41[a]	0.058	<0.001
	Xi-10-Hydroxyoctadecanoic acid	3.67[b]	4.23[a]	3.72[b]	0.083	0.005
	2-Hydroxyhexadecanoic acid	3.39[b]	4.17[a]	3.34[b]	0.110	0.001
Amino acids, peptides and analogues	Indolylacryloylglycine	4.19[a]	2.86[b]	4.28[a]	0.164	<0.001
	Asparaginyl-aspartic acid	3.52[a]	2.82[b]	3.57[a]	0.085	<0.001
	L-Methionine	2.54[a]	2.07[b]	2.50[a]	0.073	<0.001
Sesterterpenoids	Gossypol	3.79[b]	2.76[a]	3.98[b]	0.152	<0.001
Linoleic acids and derivatives	Dimorphecolic acid	4.05[b]	4.69[a]	4.07[b]	0.081	<0.001
Glycerophosphoethanolamines	LysoPC (18:0)	3.35[a]	2.99[b]	3.32[a]	0.044	<0.001
Cycloartanols and derivatives	Cyclopassifloic acid B	3.45[b]	4.24[a]	3.07[c]	0.127	<0.001
Hybrid peptides	Anserine	4.30[b]	3.53[c]	4.44[a]	0.098	<0.001
Tetrapyrroles and derivatives	Bilirubin	3.37[b]	4.05[a]	3.43[b]	0.096	<0.001
Amines	Dehydrophytosphingosine	4.06[b]	4.64[a]	4.08[b]	0.081	0.003
Indoles	Indole-3-carbinol	3.50[a]	2.94[b]	3.49[a]	0.071	<0.001
Indolecarboxylic acids and derivatives	2-Indolecarboxylic acid	4.04[a]	0.91[b]	4.04[a]	0.375	<0.001

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.
[a, b, c] means within a row with different superscripts differ significantly ($P \leq 0.05$).

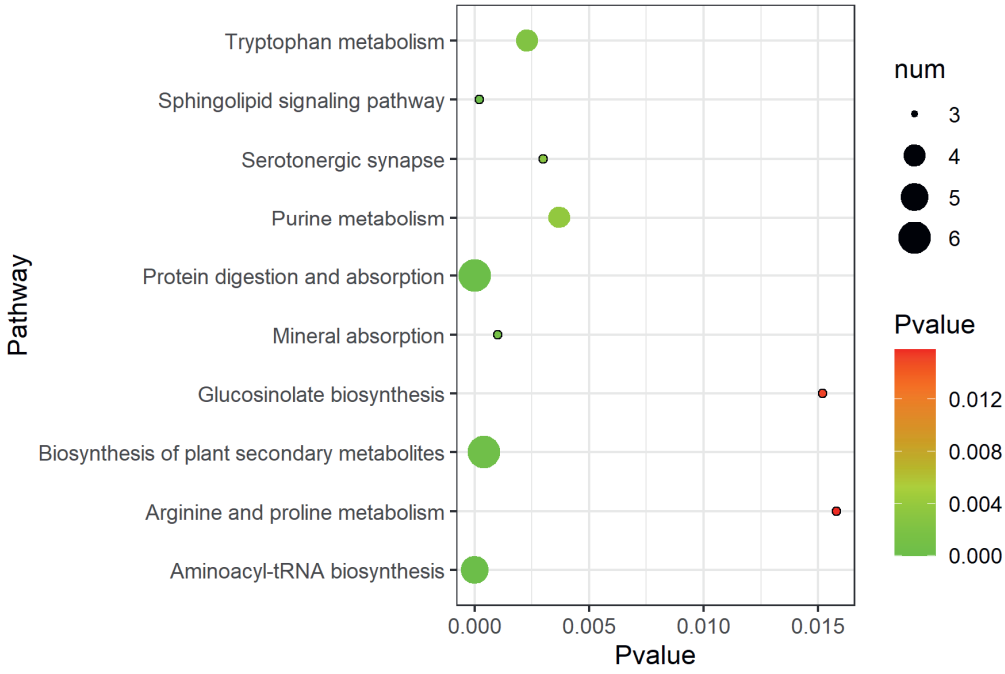


Figure 5.4. The top 10 enriched KEGG pathways of all significantly affected metabolites based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. The colour shows enrichment significance; bubble size represents the number of metabolites enriched in the pathway in the legend. KEGG = Kyoto Encyclopedia of Genes and Genomes.

Discussion

The study aimed to fully understand the effects of glucogenic and lipogenic diets on the ruminal bacterial community of dairy cows and the resulting fermentation products through 16S rRNA sequencing and metabolomic analysis. The ruminal fermentation end-products, as well as other metabolites, were significantly different between the diet with starch as an energy source and the diet with fibre as an energy source. Previous studies have shown that when feeding rations high in starch, amylolytic microorganisms are present in larger percentages of the total microbial population in the rumen, including *Streptococcus bovis*, *Ruminobacter amylophilus*, *Ruminococcus bromii*, *Selenomonas ruminantium*, *Prevotella ruminocola*, *Eubacterium ruminantium*, *Succinimonas amylolytica*, *Lactobacillus* sp. and *Bacteriodes ruminicola* (Cerrilla and Martinez, 2003, Castillo-Gonzalez et al., 2014). In the present study, the rumen fluid of cows fed diets C and S (high starch content) had greater abundances of the genera *Prevotella_7*, *Ruminococcus_2*, *CAG_352*, *Ruminococcaceae_UCG-005*, *Lachnobacterium* and *Selenomonas*. The members in these bacterial genera might be more sensitive to dietary starch content compared to the abovementioned amylolytic bacteria. This result indicates that when fed high starch rations, the classical amylolytic bacteria may not always be present as the dominant species with amylolytic activities. Similarly, Stevenson and Weimer (2007) showed that the ruminal degradation of starch from high-grain dairy rations was only partly attributed to the typical starch-fermenting bacteria (viz., *Streptococcus bovis*, *Selenomonas ruminantium* and *Ruminococcus bromii*), indicating the presence of alternative starch-fermenting species (especially *Prevotella* species) that await isolation by pure culture and/or the involvement of eukaryotic species. In addition, Liu et al. (2019) observed that a high starch concentrate diet stimulated the growth of *Ruminococcus_2* suggesting its characteristic amylolytic activity, which agreed with the present study. Since *Prevotella_7* and *Ruminococcus_2* was also observed to be highly related to amylolytic activities in the present study, alternative starch-fermenting species likely exist in the genus *Prevotella_7* and *Ruminococcus_2* that would need to be isolated in pure culture for further identification.

Besides, the literature shows that the fibrolytic bacteria in the rumen include *Fibrobacter succinogenes* (cellulose, hemicellulose and pectin digesters), *Ruminococcus albus* (cellulose and hemicellulose digesters), *Ruminococcus flavefaciens* (cellulose and hemicellulose digesters), *Butyrivibrio fibrisolvens* (cellulose, hemicellulose and pectin digesters), *Prevotella ruminicola* (hemicellulose and pectin digesters), *Lachnospira multiparus* (pectin digester),

Succinivibrio dextrinosolvens (pectin digester) and *Eubacterium ruminantium* (cellobiose digester) (Stewart et al., 1997, Krause et al., 2003, Castillo-Gonzalez et al., 2014, Zhou et al., 2015). Among the most abundant genera in the rumen fluid of cows fed diet L, *Ruminococcus_1*, *Prevotella_1* and *Eubacterium_coprostanoligenes_group* were significantly higher in relative abundance than diets C and S. These bacterial genera are likely to have contributed mainly to ruminal fibre digestion when diet L was fed to the cows. This result agrees with Stevenson and Weimer's research which indicated that the ruminal fibrolytic activities are attributed to the combination of fibrolytic eucaryotes (protozoa or fungi) and novel uncultured fibrolytic bacterial species (Stevenson and Weimer, 2007).

Compared to diet C which contained ground corn, diet S with steam-flaked corn did not lead to a higher production of VFAs, nor did it reveal changes in the bacterial community typical for these diets. Ren et al. (2019) reported that feeding 7-month-old Heifers a steam-flaking diet resulted in a higher relative abundance of amylolytic bacteria genera including *Succinivibrio*, *Roseburia* and *Blautia*, but lower fibrolytic bacteria including *Ruminococcaceae_UCG-014* and *Ruminococcaceae_UCG-013* compared to Heifers fed ground corn diet. The differences compared to our study might be caused by the ages of the cows, which then indicates that the ruminal bacteria community of Heifers was more sensitive to the dietary corn differing in processing methods compared to adult dairy cows.

As the techniques of molecular microbial ecology have been developing over the past decades, the taxonomy of bacteria has become more comprehensive. For instance, we detected multiple genera within the family of *Prevotellaceae*, including *Prevotella_1*, *Prevotella_7*, *Prevotellaceae_YAB2003_group* and *Prevotellaceae_Ga6A1_group*. The genus of *Prevotella*, the most abundant genus of the phyla *Bacteroidetes* (Thoetkiattikul et al., 2013), has been known as a highly active hemicellulolytic bacteria (Matsui et al., 2000) and is also known to be involved in the digestion of starch, xylan and pectin (Jami and Mizrahi, 2012). Sugar beet pulp contains approximately 40% neutral detergent fibre and has a high proportion of neutral-detergent soluble fibre, especially pectic substances (250 g/kg of dry matter) (Voelker and Allen, 2003). Among the multiple *Prevotella* genera, *Prevotella_1* was the most abundant and its abundance was significantly greater in diet L, while *Prevotella_7* was abundant in diets C and S. This indicates that the genus *Prevotella_1* likely contains more hemicellulolytic and pectinolytic species, while *Prevotella_7* possesses more amylolytic species.

Nagaraja and Titgemeyer (2007) observed that a diet higher in starch increases lactic acid production resulting in a lower rumen pH. The higher concentration of lactic acid in the rumen fluid when diets C and S were fed might have contributed to the observed decrease in pH as total VFAs were not different among the three diets. When lactic acid production exceeds the uptake capacity of the lactate utilizing microbes, accumulation of rumen lactic acids occurs (Mills et al., 2014). The *Streptococcus bovis*, *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens* and *Lactobacillus* spp. are reported as lactate producers, while the *Megasphaera elsdenii* was the major lactate utilizer of the ruminal bacterium (Hutton et al., 2012). Among the ruminal bacteria detected in the current study, the abovementioned bacteria were all present at a relatively low abundance < 0.01%. The difference in lactic acid production was more likely caused by the different dominant bacteria genera, including the amylolytic and fibrolytic bacteria mentioned above. Correlation analysis showed that *Ruminococcaceae_UCG-005* might play an important role in lactic acid production. In addition, sugar beet pulp contains a high level of pectin which is degraded in the rumen more rapidly than cellulose and hemicellulose (Mojtahedi and Danesh Mesgaran, 2011). Pectin fermentation in the rumen, which does not inhibit cellulose and hemicellulose digestion, can produce less lactate and propionate than starch fermentation, primarily because pectinolytic bacteria are also inhibited at low pH in treatments C and S.

The substrate for rumen fermentation determines not only the abundance of microbes in the ecosystem but also the metabolites profile (Abecia et al., 2018). Amino acid metabolism in rumen is significant for its nutritional implications. In ruminants, the amino acids available in the rumen originate from dietary and ruminal microbial protein degradation and then they are either incorporated into microbial protein or deaminated to ammonia. The metabolomic data revealed that the abundances of most metabolites in the subclass of amino acids, peptides and analogues were higher when diets C and S were fed than diet L. *Prevotella* spp. are considered to be among the prominent ruminal proteolytic bacteria (Wallace and Cotta, 1988) and there is substantial evidence to support the role of *Prevotella* spp. in protein (Wallace and Brammall, 1985) and peptide (Wallace and McKain, 1991) metabolism in the rumen. The relative abundance of *Prevotella_7* was in line with the results of the metabolites related to amino acid metabolism. In addition, early bacterial culture studies have shown that most rumen bacteria need amino acids for growth (Bryant and Robinson, 1962) with methionine being important for *Prevotella* spp. (Pittman and Bryant, 1964). In the present study, the higher relative abundance of the genera of *Prevotella_1* and *Prevotellaceae_Ga6A1_group* in the treatment L might lead

to higher consumption of L-methionine, which result in its lower abundance compared with the treatments C and S.

The extent of ammonia production in the rumen is an important factor to be considered in the nitrogen utilization of the ruminant. The rate of amino acid degradation is usually greater than that of amino acid utilization by the ruminal microorganisms, and hence, excess amino acids are being broken down, and amino acids are therefore the most important source of ammonia in the rumen (Al-Rabbat et al., 1971, Chalupa, 1976). According to the metabolomics data, the metabolites belonging to the amino acid, peptides and analogues had higher abundances when cows received diets C and S, which could offer more metabolites for the production of NH_3-N. Previous studies also proved that ammonia metabolism was closely linked to starch metabolism as the energy supplied from starch degradation is required for the incorporation of ammonia into microbial cells, while conversely, insufficient ammonia may limit microbial growth and microbial enzyme production. According to Russell et al. (1992), the bacterial community can be divided into two categories, 1) fibrolytic bacteria which mainly use ammonia as a nitrogen source for microbial protein synthesis, and 2) amylolytic bacteria which use amino acids to grow next to ammonia for an important part. The fibrolytic bacteria that ferment cellulose grow slowly because of the resistant structure of cellulose, which suggested that the presence of amino acids did not provide a selective advantage over the presence of ammonia for the growth of fibrolytic bacteria. The amylolytic bacteria have been estimated to derive 66% of their N from preformed amino acids and the remaining 34% from ammonia when both are available (Russell et al., 1992). A previous study showed that the *Prevotella* were capable of reducing nitrogen losses in the rumen (Liu et al., 2019). Other species of probable significance include *Selenomonas ruminantium*, *Peptostreptococcus elsdenii* and some strains of the genus *Butyrivibrio* (Bladen et al., 1961). In addition, the genera *Prevotella_7*, *Ruminococcaceae_UCG-005* and *Selenomonas* were positively correlated with the NH_3-N concentration in the present study. Thus, the *Prevotella_7* and *Selenomonas* might play an important role in ammonia utilization.

Conclusion

Combining microbiome and metabolomics analysis provided detailed information on the associations between bacterial genera and fermentation metabolites in the rumen of dairy cows fed two glucogenic and a lipogenic diet. Glucogenic diets with either ground corn or steam-flaked corn in combination with corn silage as the main energy sources resulted in higher ruminal lactic acid, NH_3-N and propionate productions, but lower acetate production compared to the lipogenic diet with sugar beet pulp and alfalfa silage as main energy sources. The glucogenic diets with starch as an energy source would improve protein digestion thereby resulting in increased availability of amino acids and NH_3-N in the rumen, which might be attributed to species in genera *Prevotella_7* and *Selenomonas*. The typical amylolytic or cellulolytic bacteria were not observed to be highly abundant in the rumen fluid of cows fed diets high in starch or fibre, respectively. Bacteria belonging to the genera of *Ruminococcus_2* and *Prevotella_7* might contribute to the ruminal amylolytic activities when glucogenic diets are fed while *Ruminococcus_1* and *Prevotella_1* might contribute to the fibrolytic activities of the lipogenic diet. This may lead to new perspectives for the exploration of alternative species of amylolytic and fibrolytic bacteria.

Acknowledgement

We appreciate the Breeding Farm of Beijing Centre for Dairy Cows, Beijing, China, for offering the experimental cows. The study was financially supported by the National Key R&D Program of China (2021YFE 2000800, 2019YFE0125600) and Project of the National Nature Science Foundation of China (grant No. 31572435).

Supplementary material

DNA extraction and amplification

The primers amplifying the V3-V4 hypervariable regions of the bacterial 16S rRNA gene: 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3').

PCR reactions were performed in a triplicate 20 μl mixture containing 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM dNTPs, 0.8 μl of each primer (5 μM), 0.4 μl of FastPfu Polymerase, 0.2 μl of BSA and 10 ng of template DNA. The PCR program contains 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, 45 s for elongation at 72 °C and a final extension at 72 °C for 10 min.

Sequence processing and analysis

Raw FASTQ files were quality-filtered by Trimmomatic and merged by FLASH according to the following criteria: (i) The reads were truncated at any site receiving an average quality score < 20 over a 50-bp sliding window, (ii) Sequences with overlaps longer than 10-bp were merged according to their overlap with mismatches ≤ 2 bp and (iii) Sequences of each sample were separated according to barcodes (exactly matching) and primers (allowing 2 nucleotide mismatches), and reads containing ambiguous bases were removed.

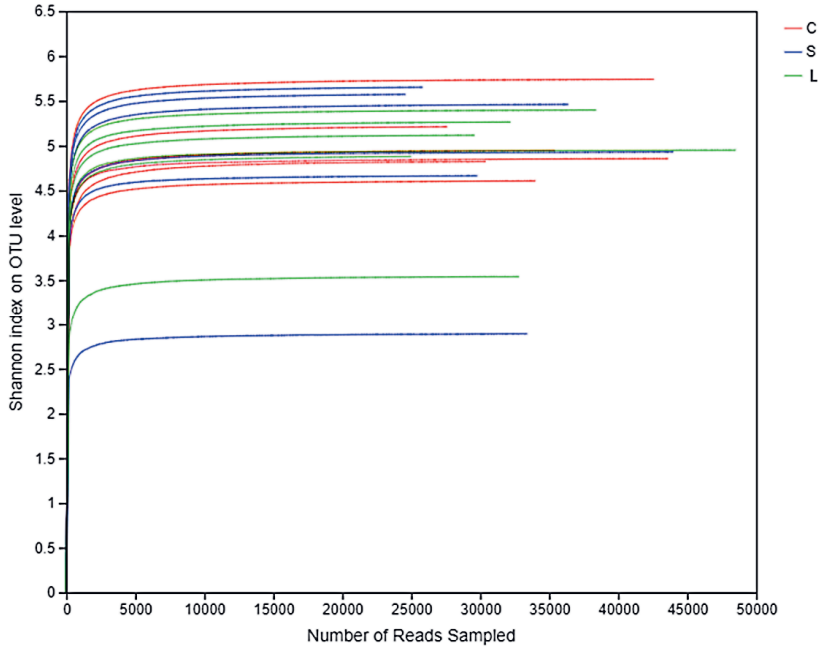
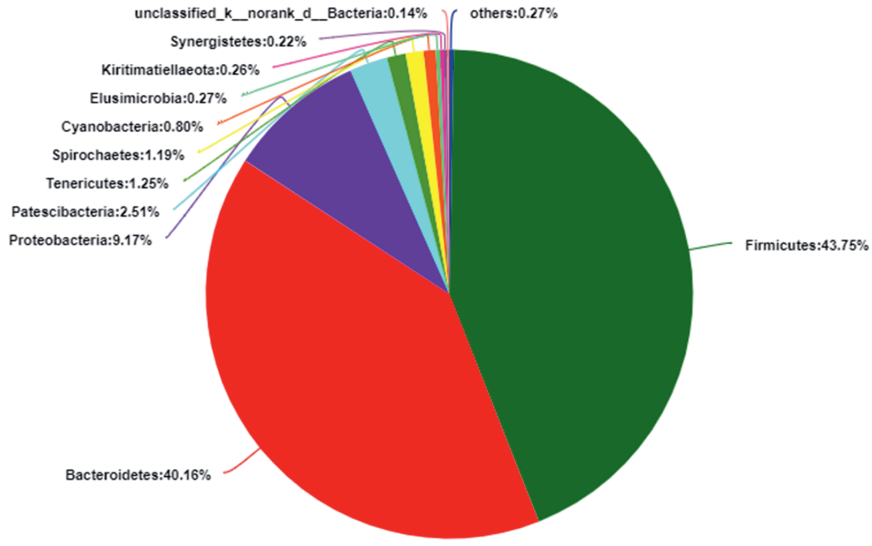


Figure S5.1. Rarefaction curves of Shannon index based on 16S rRNA sequencing technique in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

a



b

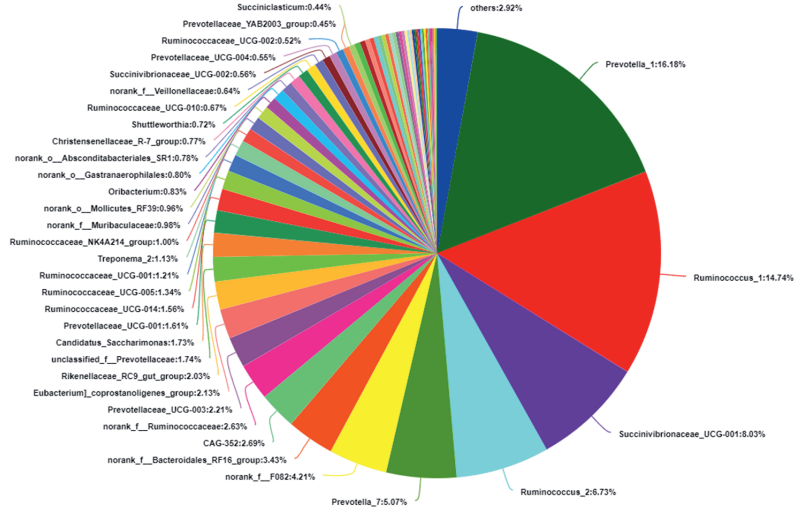


Figure S5.2. Pieplot analysis of ruminal bacterial community at (a) phylum and (b) genus levels across all rumen fluid samples of dairy cows fed two glucogenic and a lipogenic diet. The values indicate the average relative abundance across all samples.

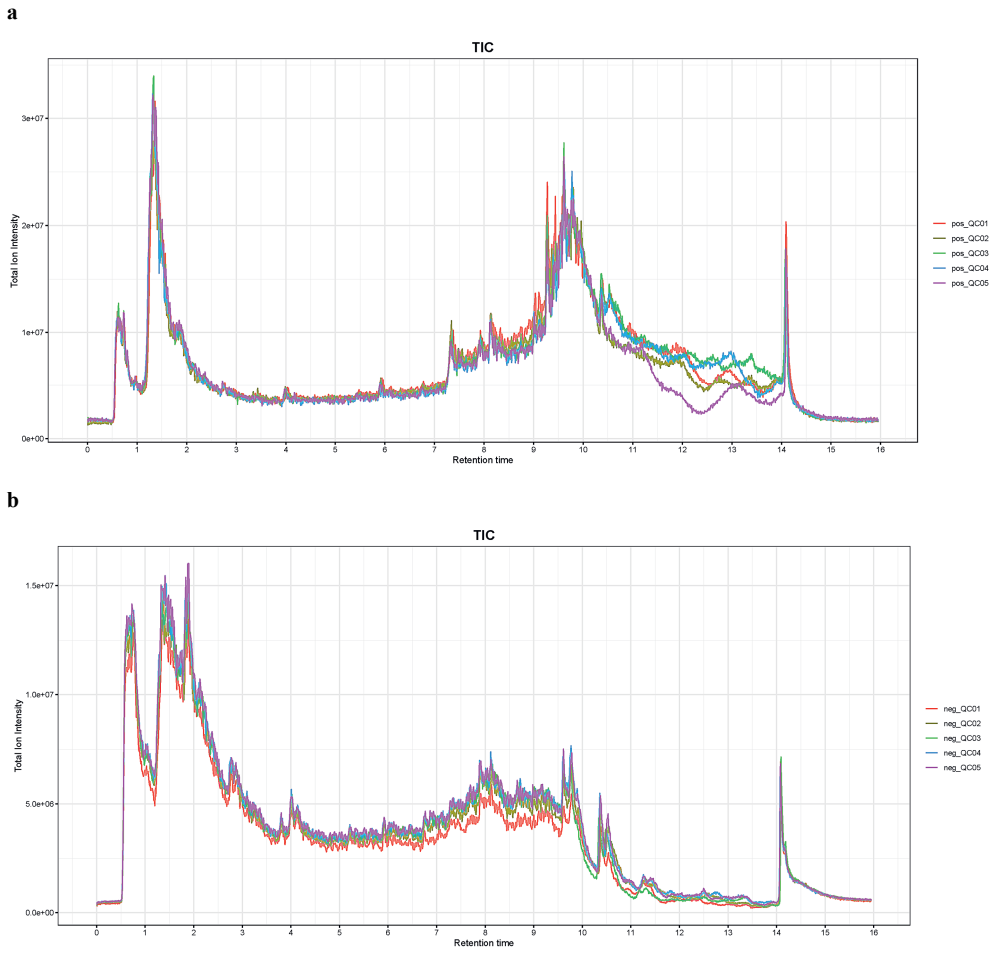


Figure S5.3. Total ion chromatogram (TIC) of the quality control samples under both positive (a) and negative (b) ion modes in LC-MS/MS analysis for the rumen fluid samples of dairy cows fed two glucogenic and a lipogenic diet. LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry.

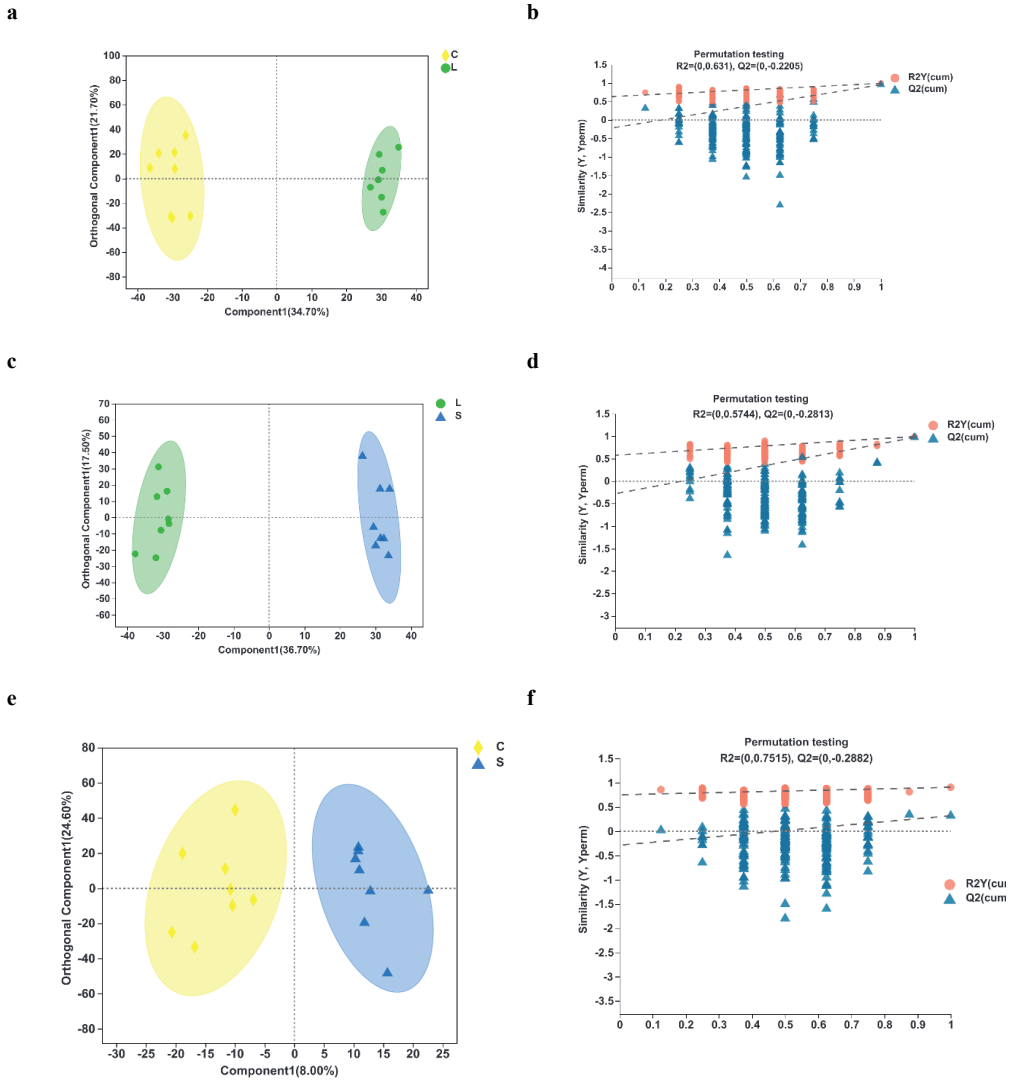


Figure S5.4. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots and corresponding permutation test following positive mode ionization based on the metabolomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. a, c and e, separately represent the score plots of C vs L, S vs L and C vs S; b, d and f, separately represent the permutation test of C vs L, S vs L and C vs S. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

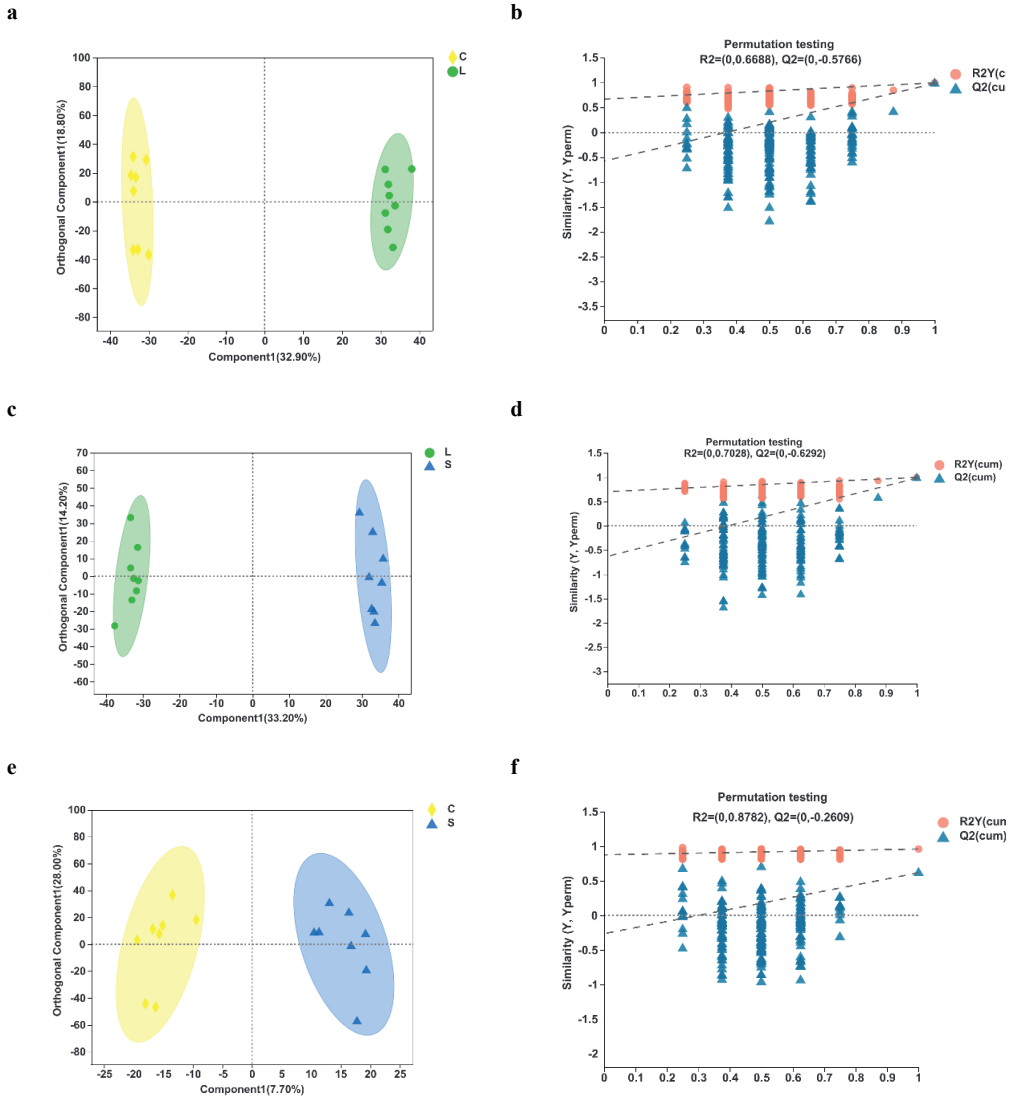


Figure S5.5. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plots and corresponding permutation test following negsitive mode ionization based on the metabolomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. a, c and e, separately represent the score plots of C vs L, S vs L and C vs S; b, d and f, separately represent the permutation test of C vs L, S vs L and C vs S. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

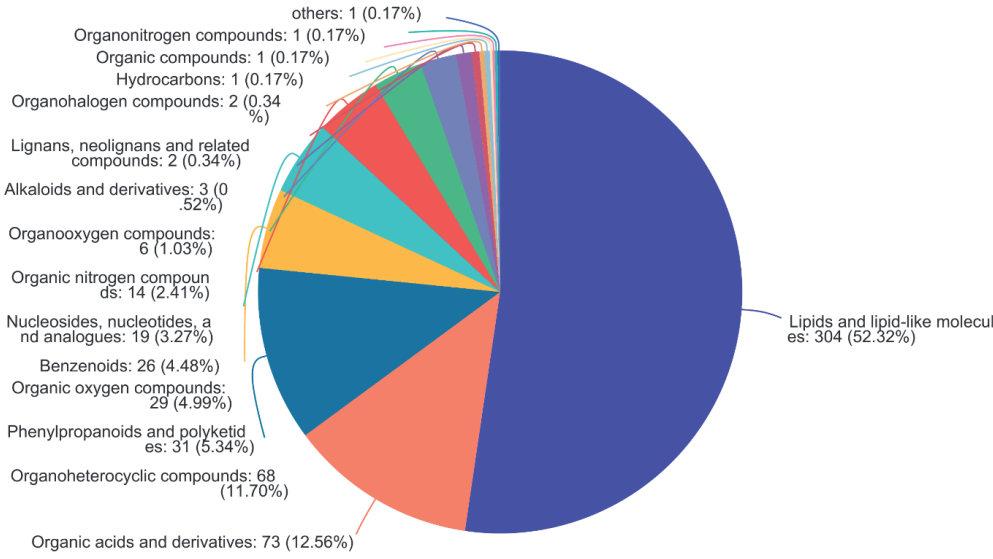


Figure S5.6. The metabolites classification at the superclass level based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet.

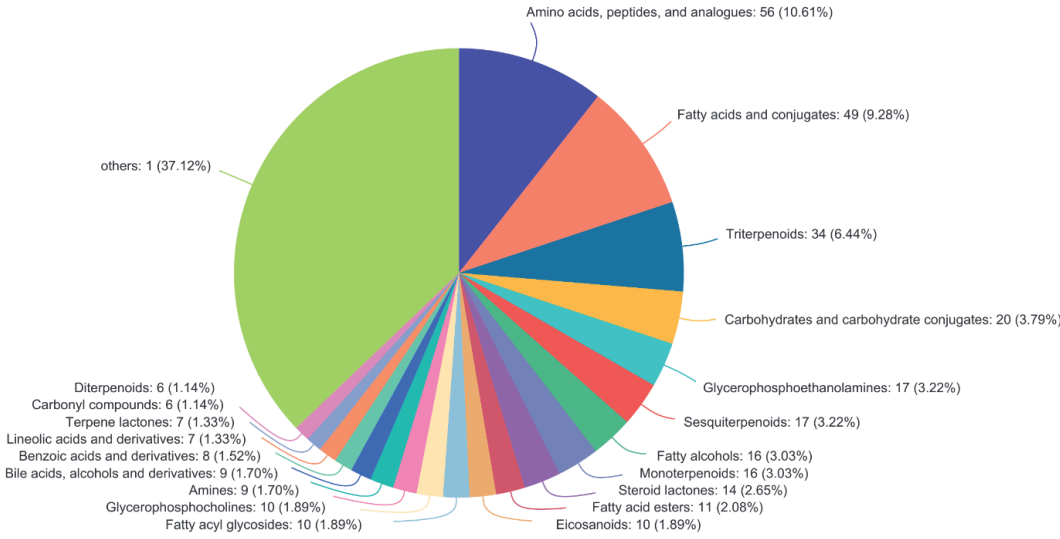


Figure S5.7. The metabolites classification at the subclass level based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet.

Table S5.1. Affected metabolites with a VIP > 1 and $P \leq 0.05$ based on metabolomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
Lipids and lipid-like molecules (Superclass level)										
Triterpenoids	Ganoderic acid F	593.28	pos	9.447	5.680	5.18	5.96	5.26	0.097	<0.001
	Medicagenic acid	501.32	neg	6.757	2.336	3.80	4.39	3.83	0.094	<0.001
	(3beta,17alpha,23S)-17,23-Epoxy-3,29-dihydroxy-27-norlanost-8-en-24-one	459.35	pos	5.911	-0.557	3.37	4.19	3.44	0.118	<0.001
	Oleanolic acid	439.36	pos	6.107	-0.796	2.74	4.11	2.56	0.180	<0.001
	Sandosapogenol	439.36	pos	8.152	-1.318	3.31	3.88	3.18	0.078	<0.001
	Ursolic acid	457.37	pos	8.714	-2.675	2.96	3.75	2.68	0.114	<0.001
	Ganodermic acid Jb	515.34	neg	6.990	0.584	2.84	3.50	3.00	0.093	<0.001
	(3beta,17alpha,23S)-17,23-Epoxy-3,28,29-trihydroxy-27-norlanost-8-en-24-one	519.33	neg	5.338	0.674	2.56	3.24	2.63	0.080	<0.001
	Camelliagenin B	487.34	neg	7.963	1.592	3.62	3.20	3.66	0.069	<0.001
	3b,18b-3-Methoxy-11-oxo-12-oleanen-30-oic acid	517.39	pos	6.761	-2.597	1.95	2.98	2.02	0.158	<0.001
	(3beta,17alpha,23S,24S)-17,23-Epoxy-3,24,29-trihydroxy-27-norlanost-8-en-15-one	519.33	neg	7.022	2.937	2.12	2.97	2.16	0.116	<0.001
	Ganoderiol I	547.36	neg	5.548	-4.099	2.04	2.73	2.17	0.087	<0.001
	Camelledionol	423.32	pos	5.590	-3.595	2.12	2.65	2.04	0.073	<0.001
	Camelliagenin A	475.38	pos	6.172	-4.048	1.29	2.61	1.50	0.156	<0.001
	Tsugaric acid B	529.39	pos	8.661	0.179	2.29	1.85	2.31	0.068	<0.001
	Ganoderic acid H	595.29	pos	8.692	4.319	0.77	1.81	1.07	0.138	<0.001
Terpene lactones	Armexifolin	283.10	neg	2.811	9.508	3.02	2.24	3.15	0.104	<0.001
	Hydroxyisonobilin	407.17	neg	4.101	1.270	2.86	1.89	2.90	0.123	<0.001
	2beta,9xi-Dihydroxy-8-oxo-1(10),4,11(13)-germacratrien-12,6alpha-olide	259.10	neg	5.969	-0.787	1.25	0.45	1.67	0.165	<0.001
	Cynaroside A	409.19	pos	3.691	1.123	1.82	0.09	1.86	0.204	<0.001
Terpene glycosides	Betavulgaroside VII	791.39	neg	6.218	8.218	2.85	4.42	2.40	0.229	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
Terpene glycosides	Tragopogonsaponin C	955.46	neg	4.338	-4.900	2.60	3.08	2.62	0.061	<0.001
Sesterterpenoids	Apo-10'-violaxanthal	450.30	pos	8.847	-7.614	3.20	2.64	3.26	0.072	<0.001
	Gossypol	517.19	neg	8.468	2.030	3.79	2.76	3.98	0.152	<0.001
	Valerenolic acid	251.16	pos	2.235	-1.128	2.70	1.89	2.70	0.095	<0.001
	Auberganol	241.22	pos	6.684	-2.205	0.06	1.64	0.40	0.173	<0.001
	Epioxylubimin	217.16	pos	4.140	-4.046	0.74	1.59	1.05	0.119	<0.001
	3-Acetoxyscirpene-4,15-diol	307.15	pos	1.936	-0.226	2.12	1.53	2.08	0.069	<0.001
	Procurcumadiol	295.15	neg	3.450	-2.183	1.69	1.32	1.76	0.055	<0.001
	Pisumic acid	263.13	neg	4.474	-1.854	1.74	0.64	1.87	0.143	<0.001
Monoterpenoids	(+/-)-trans- and cis-4,8-Dimethyl-3,7-nonadien-2-ol	359.29	pos	8.125	-3.522	1.85	2.22	1.80	0.067	<0.001
	(4S,8R)-8,9-Dihydroxy-p-menth-1(6)-en-2-one	229.11	neg	1.724	0.217	0.80	2.22	1.08	0.184	<0.001
	5-Isopropyl-2-(2-methylpropyl)-2-cyclohexen-1-one	177.16	pos	7.967	-1.769	1.97	0.93	1.93	0.150	<0.001
Diterpenoids	Carnosic acid	333.21	pos	2.063	-0.096	2.34	1.04	2.35	0.156	<0.001
	Ent-15-Kaurene-17,19-dioic acid	333.21	pos	4.130	-0.771	1.96	0.15	1.94	0.211	<0.001
Fatty acids and conjugates	Xi-10-Hydroxyoctadecanoic acid	345.26	neg	7.891	0.480	3.67	4.23	3.72	0.083	<0.001
	2-hydroxyhexadecanoic acid	271.23	neg	9.783	0.712	3.39	4.17	3.34	0.110	<0.001
	Petroselinic acid	283.26	pos	9.776	1.557	4.38	3.95	4.41	0.058	<0.001
	16-Hydroxy hexadecanoic acid	317.23	neg	8.670	0.650	2.22	3.04	2.13	0.113	<0.001
	(Z)-13-Octadecenoic acid	324.29	pos	7.908	-0.853	1.94	2.89	1.99	0.135	<0.001
	10,20-Dihydroxyeicosanoic acid	386.33	pos	6.275	-2.517	0.74	2.07	0.73	0.175	<0.001
	(2E,11Z)-Wyerone acid	289.07	neg	1.877	0.364	2.74	1.80	2.91	0.153	<0.001
	12-hydroxyheptadecanoic acid	321.22	neg	9.202	-0.636	2.41	1.78	2.34	0.087	<0.001
	2(R)-hydroxyicosanoic acid	363.27	neg	10.352	0.465	2.66	1.76	2.31	0.124	<0.001
	Goshuyic acid	269.18	neg	4.977	-0.218	1.81	0.22	1.71	0.258	<0.001
Lineolic acids and derivatives	Dimorphecolic acid	297.24	pos	7.633	-0.392	4.05	4.69	4.07	0.081	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
	6Z,9Z-octadecadienoic acid	245.23	pos	8.125	-0.419	1.10	2.38	1.19	0.176	<0.001
Fatty alcohols	Momordol	421.33	neg	11.044	2.479	0.08	2.28	0.05	0.257	<0.001
Fatty alcohols	1-Acetoxy-2-hydroxy-16-heptadecyn-4-one	345.21	neg	6.328	9.062	2.13	1.65	2.06	0.065	<0.001
Fatty alcohol esters	Geranyl 3-methylbutanoate	239.20	pos	6.978	-1.276	0.58	1.62	0.73	0.122	<0.001
Fatty alcohol esters	Cis-4-Decenyl acetate	395.32	neg	11.133	3.524	0.08	1.46	0.15	0.170	<0.001
Fatty acyl glycosides	Capsianoside IV	625.32	neg	4.486	-7.142	2.81	3.54	2.81	0.109	<0.001
Fatty acyl glycosides	1-Octen-3-yl primeveroside	445.20	pos	7.249	-9.063	2.20	1.87	2.46	0.071	<0.001
Fatty acid esters	Tetracosatetraenoyl carnitine	504.40	pos	6.588	-0.890	0.33	2.36	0.16	0.252	<0.001
Fatty acid esters	Isobutyl 10-undecenoate	241.22	pos	6.858	-2.352	1.30	1.83	1.36	0.071	<0.001
Eicosanoids	11-Dehydro-thromboxane B2	349.20	neg	3.277	-0.304	3.23	1.54	3.31	0.203	<0.001
Vitamin D and derivatives	Paricalcitol	439.32	pos	8.481	7.679	1.09	2.40	1.26	0.175	<0.001
Steroidal glycosides	Goyaglycoside g	855.48	neg	4.768	7.976	1.69	0.89	1.74	0.152	<0.001
Steroid lactones	Withangulatin A	549.25	pos	8.460	6.165	3.16	3.96	3.30	0.102	<0.001
	Pubesenolide	491.34	pos	7.227	-1.581	0.92	2.37	1.30	0.178	<0.001
Ergostane steroids	(3beta,5alpha,9alpha,22E,24R)-3,5,9-Trihydroxy-23-methylergosta-7,22-dien-6-one	459.35	pos	7.237	1.302	2.63	3.42	2.58	0.121	<0.001
	(3beta,5alpha,6beta,22E,24R) -23-Methylergosta-7,22-diene-3,5,6-triol	486.39	pos	8.314	-3.655	1.17	1.72	0.84	0.110	<0.001
	Canescein	601.25	neg	9.402	5.592	2.02	1.29	1.98	0.140	<0.001
	(3beta,5alpha,6alpha,7alpha,22E,24R)-5,6-Epoxyergosta-8,14,22-triene-3,7-diol	471.31	neg	7.646	2.216	2.10	3.20	2.12	0.196	<0.001
Cycloartanols and derivatives	Cyclopassifloic acid B	501.36	neg	8.588	0.415	3.45	4.24	3.07	0.127	<0.001
	Ambonic acid	501.39	pos	8.661	-7.929	2.97	2.58	2.94	0.058	<0.001
Cholestane steroids	5a-Cholestane-3a,7a,12a,25-tetrol	435.35	neg	11.503	2.877	1.83	2.33	1.85	0.074	<0.001
	5a-Cholesta-8,24-dien-3-one	403.30	neg	10.773	2.960	0.85	2.20	0.37	0.221	<0.001
Bile acids, alcohols and derivatives	7alpha-hydroxy-3-oxochol-4-en-24-oic Acid	421.29	pos	9.535	-0.427	3.17	2.78	3.24	0.055	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
Bile acids, alcohols and derivatives	Polyporusterone A	523.33	neg	4.745	6.195	2.67	2.34	2.73	0.048	<0.001
	Glycocholic acid	446.29	neg	3.461	0.003	1.17	2.02	1.29	0.123	<0.001
	3a,6b,7b,12a-Tetrahydroxy-5b-cholanoic acid	407.28	pos	8.629	3.907	2.22	1.71	2.24	0.072	<0.001
Androstane steroids	Polyporusterone D	461.33	pos	9.535	-0.835	3.29	2.95	3.38	0.054	<0.001
	Testosterone	289.22	pos	5.654	-0.559	2.15	0.42	2.32	0.216	<0.001
Glycosphingolipids	Ganglioside GA2 (d18:1/12:0)	989.63	neg	9.591	8.963	0.92	1.58	0.94	0.087	<0.001
Ceramides	Ceramide (d18:1/12:0)	514.48	pos	12.028	-1.048	1.44	2.35	1.57	0.144	<0.001
Glycerophosphoethanolamines	PE (15:0/22:4(7Z,10Z,13Z,16Z))	736.53	pos	11.814	3.597	0.86	2.33	1.02	0.191	<0.001
	LysoPC (18:0)	568.36	neg	8.308	0.359	3.35	2.99	3.32	0.044	<0.001
	PC (16:0/P-18:1(11Z))	764.55	neg	12.376	-9.808	1.60	2.32	1.61	0.131	<0.001
Diradylglycerols	3-(Acetyloxy)-2-hydroxypropyl icosanoate	409.33	neg	11.441	2.345	0.01	1.96	0.04	0.224	<0.001
Monoradylglycerols	MG (0:0/15:0/0:0)	361.26	neg	6.581	-2.885	1.52	0.33	1.39	0.178	<0.001
Organic acids and derivatives (Superclass level)										
Amino acids, peptides and analogues	Indolylacryloylglycine	227.08	pos	1.393	-1.256	4.19	2.86	4.28	0.164	<0.001
	Asparaginyl-Aspartic acid	311.09	pos	1.686	8.198	3.52	2.82	3.57	0.085	<0.001
	Gluten exorphin B5	623.25	pos	6.739	6.219	0.91	2.44	1.19	0.184	<0.001
	Pyridinoline	449.16	neg	2.618	-7.813	3.02	2.30	3.21	0.107	<0.001
	L-Methionine	150.06	pos	0.731	-5.712	2.54	2.07	2.50	0.073	<0.001
	N-acetyl-L-2-aminoadipate (2-)	184.06	neg	1.327	-2.441	2.20	1.54	2.09	0.098	<0.001
	N-Acetylproline	138.06	neg	1.234	1.399	2.33	1.46	2.20	0.114	<0.001
	Citrulline	176.10	pos	1.060	-1.396	1.82	1.16	1.91	0.125	<0.001
	N-Acetylvanilalanine	548.23	pos	7.031	7.266	2.09	0.74	2.27	0.176	<0.001
	Tryptophyl-Asparagine	353.10	neg	2.890	-3.091	2.29	0.52	2.38	0.210	<0.001
	Vignatic acid B	554.27	neg	5.120	2.494	1.57	0.29	1.66	0.177	<0.001
Carboxylic acid derivatives	2,4-Hexadienyl acetate	121.07	neg	1.974	0.677	2.01	2.68	2.03	0.081	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
Hybrid peptides	Anserine	285.11	neg	2.902	4.379	4.30	3.53	4.44	0.098	<0.001
Medium-chain hydroxy acids and derivatives	8-Hydroxy-5,6-octadienoic acid	201.08	neg	1.410	-3.700	1.41	1.97	0.98	0.108	<0.001
Phenylpropanoids and polyketides (Superclass level)										
Stilbene glycosides	4-Hydroxytamoxifen-O-glucuronide	596.29	pos	9.274	2.958	3.15	3.90	3.26	0.093	<0.001
	(E)-Oxyresveratrol 3'-O-b-D-glucoside	470.15	pos	2.224	7.157	0.77	1.62	0.91	0.114	<0.001
O-methylated flavonoids	Diosmetin	301.07	pos	3.005	0.572	0.92	2.62	0.91	0.195	<0.001
	4'-Hydroxy-5,7-dimethoxyflavan	331.12	neg	2.991	-1.050	2.61	1.05	2.69	0.206	<0.001
Isoflavans	Cajaisoflavone	451.18	pos	2.598	3.413	1.92	0.42	2.19	0.214	<0.001
Furanoisoflavonoids	Neodunol	281.08	pos	2.224	1.504	1.84	0.58	2.04	0.169	<0.001
Coumestans	Coumestrol	267.03	neg	2.879	-0.147	2.00	2.77	2.09	0.150	<0.001
Hydroxycinnamic acids and derivatives	Prenyl cis-caffeate	293.10	neg	3.200	0.446	2.24	1.89	2.24	0.049	<0.001
-	3-[4-Hydroxy-3-(3-methyl-2-butenyl) phenyl]-2-propenal	217.12	pos	0.731	-3.939	2.59	2.19	2.54	0.053	<0.001
-	5,6-Dihydro-5-hydroxy-6-methyl-2H-pyran-2-one	273.08	neg	1.701	1.551	2.25	0.77	2.28	0.173	<0.001
-	11-Methoxynoryangonin	295.06	neg	2.228	-4.565	1.86	0.62	1.98	0.152	<0.001
Organoheterocyclic compounds (Superclass level)										
Quinolones and derivatives	Quinoline-4,8-diol	206.05	neg	1.374	-1.426	3.05	2.65	3.11	0.066	<0.001
Pyridinecarboxylic acids and derivatives	4-Pyridoxic acid	182.05	neg	1.234	0.164	3.11	2.45	3.01	0.086	<0.001
Hydropyridines	1,2,3,4,5,6-Hexahydro-5-(1-hydroxyethylidene)-7H-cyclopenta[b]pyridin-7-one	224.09	neg	1.701	-2.220	2.25	1.73	2.20	0.085	<0.001
Pterins and derivatives	7,8-Dihydropteroic acid	295.10	neg	4.945	8.665	1.86	1.13	2.00	0.120	<0.001
-	Jasmine ketolactone	253.11	neg	3.287	0.183	1.53	2.10	1.30	0.105	<0.001
Gamma butyrolactones	Neocnidilide	239.13	neg	5.559	1.194	1.57	1.01	1.58	0.096	<0.001
Tryptamines and derivatives	N-Methyl-1H-indole-3-propanamide	203.12	pos	2.063	0.137	1.03	1.84	1.22	0.164	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
	N6-cis-p-Coumaroylserotonin	361.10	pos	1.470	2.149	2.08	1.43	2.15	0.093	<0.001
Indolyl carboxylic acids and derivatives	5-Hydroxyindoleacetic acid	192.07	pos	1.502	-2.174	3.11	2.48	3.14	0.097	<0.001
Indolines	3-Methyldioxyindole	146.06	pos	1.641	-2.482	3.10	2.69	3.18	0.064	<0.001
	7-Hydroxyetodolac	304.15	pos	2.870	-0.454	3.00	1.97	3.15	0.133	<0.001
Indoles	INDOLE-3-CARBINOL	148.08	pos	1.948	-2.229	3.50	2.94	3.49	0.071	<0.001
Indolecarboxylic acids and derivatives	2-Indolecarboxylic acid	162.05	pos	1.470	-2.212	4.04	0.91	4.04	0.375	<0.001
-	Adrenochrome	160.04	neg	2.151	-0.501	2.70	2.15	2.70	0.081	<0.001
-	3-Methylene-indolenine	130.06	pos	2.074	-1.275	2.18	1.80	2.20	0.054	<0.001
-	Asteltoxin	399.19	neg	7.891	9.086	0.86	2.40	1.18	0.190	<0.001
-	Marasmone	259.10	neg	3.341	1.236	1.33	0.51	1.44	0.173	<0.001
Imidazolidines	Phenytoin methylcatechol	279.08	pos	3.573	-0.946	2.04	0.65	2.24	0.191	<0.001
Organic oxygen compounds (Superclass level)										
Carbohydrates and carbohydrate conjugates	4-Methoxybenzyl glucoside	345.10	pos	1.536	-8.473	3.23	2.52	3.21	0.089	<0.001
	Urolithin B 3-O-glucuronide	423.05	neg	1.783	1.438	2.73	2.27	2.85	0.101	<0.001
	Cotinine glucuronide	317.11	pos	1.360	-0.598	2.21	1.33	2.34	0.127	<0.001
	5-(3',5'-Dihydroxyphenyl)-gamma-valerolactone-O-glucuronide-O-methyl	363.11	pos	1.618	4.448	2.57	1.25	2.58	0.181	<0.001
Ethers	Cadabicine	456.19	neg	3.906	-8.639	2.09	1.43	2.12	0.096	<0.001
Alcohols and polyols	Piperdial	233.15	pos	4.461	-1.026	2.59	1.18	2.69	0.169	<0.001
Benzenoids (Superclass level)										
Methoxyphenols	Norcapsaicin	290.18	neg	5.085	1.761	1.22	0.52	1.46	0.117	<0.001
Hydrophenanthrenes	Solanolone	301.11	pos	2.656	8.752	1.92	1.48	1.95	0.060	<0.001
Indanones	Pteroside Z	395.21	pos	4.108	3.097	2.19	0.70	2.24	0.177	<0.001
Phenylbutylamines	Didemethylcitalopram	317.10	neg	3.633	-7.828	1.72	1.24	1.79	0.097	<0.001
Anthraquinones	Emodin	269.05	neg	2.851	0.046	0.99	2.68	0.91	0.212	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
Organooxygen compounds (Superclass level)										
Acyloins	1-Hydroxyacorenone	249.15	neg	4.464	-0.985	2.06	1.72	2.17	0.050	<0.001
Glycosyl compounds	Osmundalin	325.07	neg	1.783	9.299	3.23	2.65	3.32	0.076	<0.001
Nucleosides, nucleotides and analogues (Superclass level)										
-	7-Methylinosine	347.12	pos	1.502	0.698	3.20	2.61	3.25	0.075	<0.001
-	Arabinofuranosylguanine	320.08	neg	2.228	2.467	2.61	1.54	2.60	0.148	<0.001
Organic nitrogen compounds (Superclass level)										
Amines	Dehydrophytosphingosine	316.28	pos	8.125	-0.420	4.06	4.64	4.08	0.081	<0.001
Organic compounds (Superclass level)										
Tetrapyrroles and derivatives	Bilirubin	605.24	neg	8.199	8.045	3.37	4.05	3.43	0.096	<0.001
Hydrocarbons (Superclass level)										
Alkanes	Pentane	114.13	pos	0.698	0.877	2.05	1.53	2.03	0.084	<0.001
Alkaloids and derivatives (Superclass level)										
-	Sampangine	277.06	neg	3.621	2.648	1.31	0.47	1.53	0.152	<0.001
Unclassified (Superclass level)										
Unclassified	9R,10S-epoxy-stearic acid	299.26	pos	8.125	-0.468	4.93	5.41	4.93	0.069	<0.001
	Betulonic acid	455.35	pos	9.120	-0.058	3.00	3.86	2.50	0.147	<0.001
	Cer(d14:1(4E)/20:0(2OH))	554.51	pos	13.164	-0.188	3.24	3.84	3.15	0.118	<0.001
	(22S)-1alpha,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydrovitamin D3	457.33	pos	6.761	-0.130	3.08	3.73	3.04	0.107	<0.001
	Cer(d14:1(4E)/20:1(11Z) (2OH))	534.49	pos	12.929	-1.204	2.39	3.63	2.58	0.167	<0.001
	1alpha-hydroxy-26,27-dimethylvitamin D3	429.37	pos	10.357	-2.633	2.78	3.33	2.81	0.084	<0.001
	25-hydroxy-26,27-dimethylvitamin D3	429.37	pos	11.100	-2.152	2.74	3.32	2.83	0.088	<0.001
	Malvidin	331.08	pos	2.951	0.169	2.09	3.54	2.20	0.168	<0.001
	Cer(d16:2(4E,6E)/20:1(11Z) (2OH))	578.51	pos	11.752	0.813	2.06	3.11	1.32	0.197	<0.001
	Amprotropine	308.22	pos	6.662	0.423	2.18	2.76	2.24	0.076	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
	(20S)-1alpha,20,25-trihydroxy-26,27-dimethyl-24a-homovitamin D3	461.36	pos	7.675	-2.507	0.93	2.75	0.78	0.242	<0.001
	(22R)-1alpha,22,25-trihydroxy-26,27-dimethyl-20-epivitamin D3	461.36	pos	8.767	-0.152	1.22	2.68	1.13	0.207	<0.001
	Dihydroceramide C2	344.32	pos	7.151	-0.015	2.30	2.71	2.29	0.065	<0.001
	1alpha,25-dihydroxy-2beta-(2-hydroxyethoxy) vitamin D3	477.36	pos	7.151	-0.454	1.11	2.66	1.02	0.201	<0.001
	Ent-Corey PG-Lactone Diol	269.17	pos	2.155	-1.941	2.96	2.59	2.91	0.047	<0.001
	PS (15:0/22:2(13Z,16Z))	800.55	neg	12.216	8.262	1.52	2.55	1.89	0.154	<0.001
	2-Phenylbutyramide	164.11	pos	1.259	-3.160	1.08	2.37	0.75	0.206	<0.001
	Cer(d18:0/13:0)	498.49	pos	12.577	-1.055	2.72	2.36	2.88	0.088	<0.001
	Bifemelane (M4)	269.12	pos	2.870	-0.439	3.53	2.35	3.66	0.148	<0.001
	MG (18:1(9Z)/0:0/0:0) [rac]	357.30	pos	9.311	0.030	3.27	2.34	3.25	0.126	<0.001
	Fusaric acid	180.10	pos	1.618	-1.136	2.48	2.06	2.57	0.067	<0.001
	TOFA	325.24	pos	6.674	-4.933	2.40	2.01	2.46	0.065	<0.001
	PS (14:0/22:5(4Z,7Z,10Z,13Z,16Z))	746.48	pos	5.911	9.999	0.70	1.99	1.24	0.145	<0.001
	5-Cholestene-3beta,7alpha,12alpha,24,25-pentol	451.34	pos	7.270	-2.845	0.80	1.94	0.62	0.149	<0.001
	(20S)-14alpha,20,25-trihydroxy-26,27-dimethylvitamin D3	461.36	pos	5.804	-1.875	1.15	1.90	1.14	0.123	<0.001
	PE (12:0/0:0)	398.23	pos	5.128	0.677	3.17	1.78	3.20	0.176	<0.001
	Penaresidin A	330.30	pos	7.813	-1.897	0.45	1.72	0.82	0.170	<0.001
	6,8-dimethyl-2-phenyl-3,4-dihydro-2H-1-benzopyran-4,5,7-triol	321.09	neg	2.991	0.662	2.92	1.69	3.02	0.153	<0.001
	2-(phenylmethylidene) heptane-1,3-diol	203.14	pos	7.010	-1.527	2.10	1.68	2.04	0.056	<0.001
	Prostaglandin lactone-diol	313.17	neg	2.957	-0.383	2.26	1.63	2.15	0.091	<0.001
	Nigakilactone M	395.21	pos	3.809	3.680	2.50	1.48	2.56	0.122	<0.001
	Talatizamine	422.29	pos	7.569	5.119	2.18	1.45	2.10	0.123	<0.001
	17-Methyl-5-alpha-androst-2-en-17-beta-ol	289.25	pos	6.934	-1.217	1.68	1.29	1.90	0.076	<0.001

Table S5.1 (continued).

HMDB category	Metabolite	Ion (m/z)	Mode	RT (min)	Mass Error	C	L	S	SEM	*P*-value
	Micropine	266.21	pos	5.697	-1.521	1.69	1.21	1.81	0.075	<0.001
	Quadrone	249.15	pos	3.459	-0.282	2.07	1.15	2.13	0.114	<0.001
	Testolic acid	321.21	pos	4.553	1.584	1.52	1.08	1.63	0.084	<0.001
	Trilobinone	361.20	neg	8.178	-0.023	1.91	1.04	2.27	0.156	<0.001
	Mikanolide	291.09	pos	1.813	0.898	1.64	0.92	1.94	0.156	<0.001
	4-hydroxy-3-(4-hydroxy-3-methylbut-2-en-1-yl) benzoic acid	221.08	neg	2.778	0.092	1.69	0.90	1.59	0.106	<0.001
	Sinaticin	435.11	pos	2.097	1.049	2.61	0.88	2.85	0.228	<0.001
	(+)-Prosopinine	314.27	pos	2.431	-0.996	2.80	0.82	2.81	0.232	<0.001
	Parthenin	261.11	neg	2.083	1.244	2.25	0.79	2.27	0.177	<0.001
	Seco-isolariciresinol diglucoside	685.27	neg	5.936	2.254	1.86	0.69	1.76	0.150	<0.001
	Calligonine	187.12	pos	1.492	-2.290	1.78	0.08	1.51	0.253	<0.001

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. HMDB, human metabolome database. in positive/negative ion mode. RT, retention time. - unclassified. Pos/Neg,

Reference

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J., and Yáñez-Ruiz, D. R. 2018. Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9:2227.

Al-Rabbat, M. F., Baldwin, R. L., and Weir, W. C. 1971. *In vitro* nitrogen-tracer technique for some kinetic measures of ruminal ammonia. *J. Dairy Sci.* 54:1150-1161.

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., Gaskins, H. R., Stumpf, R. M., Yildirim, S., Torralba, M., Gillis, M., Wilson, B. A., Nelson, K. E., White, B. A., and Leigh, S. R. 2013. Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J.* 7(7):1344-1353.

Bladen, H. A., Bryant, M. P., and Doetsch, R. N. 1961. A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. *Appl. Microbiol.* 9(2):175-180.

Broderick, G. A. and Kang, J. H. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *J. Dairy Sci.* 63(1):64-75.

Bryant, M. P. and Robinson, I. M. 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. *J. Bacteriol.* 84:605-614.

Castillo-Gonzalez, A. R., Burrola-Barraza, M. E., Dominguez-Viveros, J., and Chavez-Martinez, A. 2014. Rumen microorganisms and fermentation. *Arch. Med. Vet.* 46:349-361.

Cerrilla, M. E. O. and Martinez, G. M. 2003. Starch digestion and glucose metabolism in the ruminant: a review. *Interciencia* 28:7.

Chalupa, W. 1976. Degradation of amino acids by the mixed rumen microbial population. *J. Anim. Sci.* 43(4):828-834.

Cooper, R. J., Milton, C. T., Klopfenstein, T. J., Scott, T. L., Wilson, C. B., and Mass, R. A. 2002. Effect of corn processing on starch digestion and bacterial crude protein flow in finishing cattle. *J. Anim. Sci.* 80(3):797-804.

Hua, D., Zhao, Y., Nan, X., Xue, F., Wang, Y., Jiang, L., and Xiong, B. 2020. Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community *in vitro*. *J. Appl. Microbiol.* 130(6):1868-1882.

Hutton, P. G., Durmic, Z., Ghisalberti, E. L., Flematti, G. R., Duncan, R. M., Carson, C. F., Riley, T. V., and Vercoe, P. E. 2012. Inhibition of ruminal bacteria involved in lactic acid metabolism by extracts from Australian plants. *Anim. Feed Sci. Technol.* 176(1-4):170-177.

Jami, E. and Mizrahi, I. 2012. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 7(3):e33306.

Krause, D., Denman, S., Mackie, R., Morrison, M., Rae, A., Attwood, G., and McSweeney, C. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5):663-693.

Liu, C., Wu, H., Liu, S., Chai, S., Meng, Q., and Zhou, Z. 2019. Dynamic alterations in Yak rumen bacteria community and metabolome characteristics in response to feed type. *Front. Microbiol.* 10:1-19.

Matsui, H., Ogata, K., Tajima, K., Nakamura, M., Nagamine, T., Aminov, R. I., and Benno, Y. 2000. Phenotypic characterization of polysaccharidases produced by four *Prevotella* type strains. *Curr. Microbiol.* 41(1):45-49.

Mills, J. A. N., Crompton, L. A., Ellis, J. L., Dijkstra, J., Bannink, A., Hook, S., Benchaar, C., and France, J. 2014. A dynamic mechanistic model of lactic acid metabolism in the rumen. *J. Dairy Sci.* 97(4):2398-2414.

Mojtahedi, M. and Danesh Mesgaran, M. 2011. Effects of the inclusion of dried molassed sugar beet pulp in a low-forage diet on the digestive process and blood biochemical parameters of Holstein steers. *Livest. Sci.* 141(2-3):95-103.

Nagaraja, T. G. and Titgemeyer, E. C. 2007. Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook. *J. Dairy Sci.* 90:E17-E38.

NRC, 2001. Nutrient requirements of dairy cattle, seventh ed. National Academy Press, Washington, DC.

Pan, X. H., Yang, L., Xue, F. G., Xin, H. R., Jiang, L. S., Xiong, B. H., and Beckers, Y. 2016. Relationship between thiamine and subacute ruminal acidosis induced by a high-grain diet in dairy cows. *J. Dairy Sci.* 99(11):8790-8801.

Pan, X., Xue, F., Nan, X., Tang, Z., Wang, K., Beckers, Y., Jiang, L., and Xiong, B. 2017. Illumina sequencing approach to characterize thiamine metabolism related bacteria and the impacts of thiamine supplementation on ruminal microbiota in dairy cows fed high-grain diets. *Front. Microbiol.* 8:1818.

Pittman, K. A. and Bryant, M. P. 1964. Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. *J. Bacteriol.* 88:401-410.

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

Russell, J. B., O'Connor, J. D., Fox, D. G., Van Soest, P. J., and Sniffen, C. J. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *J. Anim. Sci.* 70(11):3551-3561.

Saleem, F., Bouatra, S., Guo, A. C., Psychogios, N., Mandal, R., Dunn, S. M., Ametaj, B. N., and Wishart, D. S. 2013. The bovine ruminal fluid metabolome. *Metabolomics* 9(2):360-378.

Stevenson, D. M. and Weimer, P. J. 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75(1):165-174.

Stewart, S. C., Flint, H. J., and Bryant, M. P. 1997. The rumen bacteria, in Chapter 1, Page: 10, The rumen microbial ecosystem. Hobson, P. N., and Stewart, C. S., ed. Blackie Acadamic and Professional, London.

Thoetkiattikul, H., Mhuantong, W., Laothanachareon, T., Tangphatsornruang, S., Pattarajinda, V., Eurwilaichitr, L., and Champreda, V. 2013. Comparative analysis of microbial profiles in cow rumen

fed with different dietary fibre by tagged 16S rRNA gene pyrosequencing. *Curr*. *Microbiol*. 67(2):130-137.

van Knegsel, A. T. M., van den Brand, H., Dijkstra, J., Tamminga, S., and Kemp, B. 2005. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. *Reprod*. *Nutr*. *Dev*. 45(6):665-688.

van Knegsel, A. T., van den Brand, H., Dijkstra, J., van Straalen, W. M., Heetkamp, M. J., Tamminga, S., and Kemp, B. 2007a. Dietary energy source in dairy cows in early lactation: energy partitioning and milk composition. *J*. *Dairy Sci*. 90(3):1467-1476.

van Knegsel, A. T., van den Brand, H., Graat, E. A., Dijkstra, J., Jorritsma, R., Decuypere, E., Tamminga, S., and Kemp, B. 2007b. Dietary energy source in dairy cows in early lactation: metabolites and metabolic hormones. *J*. *Dairy Sci*. 90(3):1477-1485.

Voelker, J. A. and Allen, M. S. 2003. Pelleted beet pulp substituted for high-moisture corn: 3. Effects on ruminal fermentation, pH, and microbial protein efficiency in lactating dairy cows. *J*. *Dairy Sci*. 86(11):3562-3570.

Wallace, R. J. and Brammall, M. L. 1985. The role of different species of bacteria in the hydrolysis of protein in the rumen. *J*. *Gen*. *Microbiol*. 131:821-832.

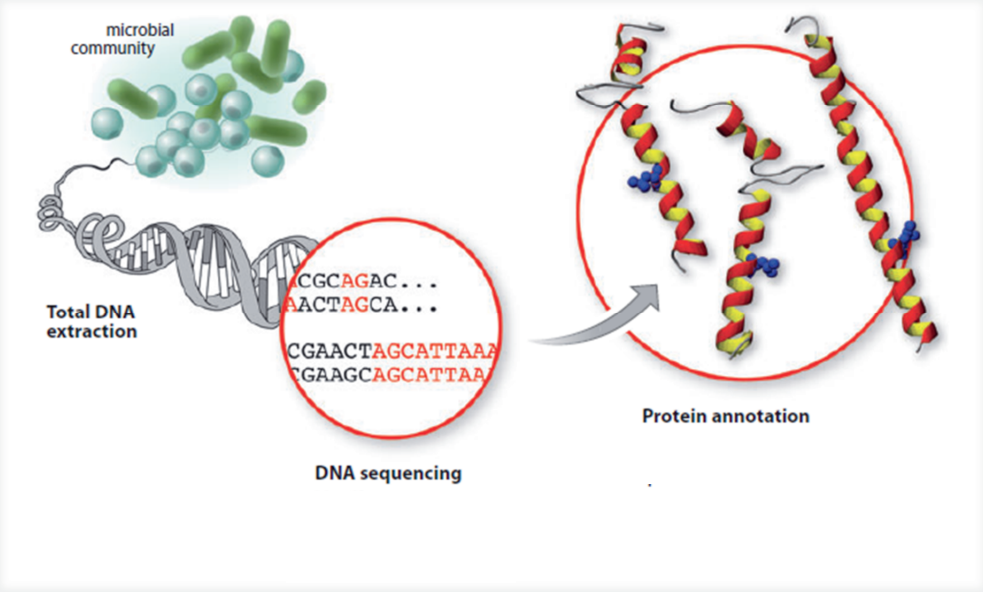
Wallace, R. J. and Cotta, M. A. 1988. Metabolism of nitrogen-containing compounds, Page: 217-249, The rumen microbial ecosystem. Hobson, P. N., and Stewart, C. S., ed. Blackie Academic and Professional, London.

Wallace, R. J. and McKain, N. 1991. A survey of peptidase activity in rumen bacteria. *J*. *Gen*. *Microbiol*. 137(9):2259-2264.

Wang, Y., Nan, X., Zhao, Y., Jiang, L., Wang, H., Hua, D., Zhang, F., Wang, Y., Liu, J., Yao, J., and Xiong, B. 2021b. Dietary supplementation with inulin improves lactation performance and serum lipids by regulating the rumen microbiome and metabolome in dairy cows. *Anim*. *Nutr*. 7(4):1189-1204.

Wang, Y., Nan, X., Zhao, Y., Wang, Y., Jiang, L., and Xiong, B. 2021a. Ruminal degradation of rumen-protected glucose influences the ruminal microbiota and metabolites in early-lactation dairy cows. *Appl*. *Environ*. *Microbiol*. 87(2):e1908-e1920.

Zhou, M., Chen, Y., and Guan, L. L. 2015. Rumen bacteria, in Chapter 6, Page: 79, Rumen microbiology: From evolution to revolution. Puniya, A. K., and Singh, R., ed. Springer, India.


microbial
community
Total DNA
extraction
CGCAGAC...
AACTAGCA...
CGAACTAGCATTAAA
CGAAGCAGCATTAA
DNA sequencing
Protein annotation

Chapter 6

Metagenomics and metaproteomics analysis of microbial carbohydrate digestion in the rumen of dairy cows receiving different dietary energy sources

Dengke Hua[1,2], Wouter H. Hendriks[2], Zihao Liu[1], Yue Wang[1], Yapin Wang[1], Linshu Jiang[3], Benhai Xiong[1], Wilbert F. Pellikaan[2]

[1] State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

[2] Animal Nutrition Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, 6708 WD, the Netherlands

[3] Beijing Key Laboratory for Dairy Cattle Nutrition, Beijing Agricultural College, Beijing, 102206, China

To be submitted to Microbiology Spectrum

Abstract

This study aimed to explore 1) the alterations of the amylolytic and fibrolytic microbial communities and 2) the associated enzymes involved in the carbohydrate metabolism in the rumen of dairy cows receiving three different dietary energy sources through a combination of metagenomics and metaproteomics approaches. Two glucogenic (ground corn and corn silage (diet C) and steam-flaked corn and corn silage (diet S)) and a lipogenic diet composed of sugar beet pulp and alfalfa silage (diet L) were fed to six rumen-cannulated Holstein Friesian dairy cows, paired by initial parameters of body weight, day in milk and milk production with an animal within pairs randomly assigned to one of two replicated 3 × 3 Latin squares. The trial consisted of three 21-d periods with rumen fluid samples collected on the last day of each period for metagenomics and metaproteomics analysis. The metagenomics data show that the amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* had higher relative abundances when the cows were fed diets C and S compared to L. The fibrolytic bacteria of *Ruminococcus flavefaciens* and *Lachnospira multipara* had higher relative abundance when cows were fed diet L compared to diets C and S. The starch and sucrose metabolism pathway were significantly down-regulated while the galactose metabolism and the pentose and glucuronate interconversions were significantly up-regulated when cows were fed diet L relative to diets C and S. For the enzymes involved in the starch and sucrose metabolism, the relative abundances of α-amylase, pullulanase and maltose α-D-glucosyltransferase were higher but the cellulose, glucokinase and isoamylase were lower in samples of diets C and S compared to L. For the metaproteomics data, the taxonomic analysis showed that the relative abundances of the amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* were higher but the relative abundance of the fibrolytic bacteria *Prevotella ruminicola* was lower in the samples of diets C and S compared to L. Among the differential affected enzymes, cellulase (derived from *Ruminococcus* sp.), α-amylase (*Prevotella buccae*) and glucose-1-phosphate adenylyltransferase (*Treponema* sp.) were up-regulated in the rumen fluid of cows fed diet L. While the α-amylase (*Aeromonas enteropelogenes* and *Trichomonas vaginalis G3*), pullulanase (*Succinimonas amylolytica*, *Photorhabdus australis*, *Photobacterium marinum* and *Photorhabdus asymbiotica*) and amylopullulanase (*Selenomonas bovis*) were up-regulated in the rumen fluid samples from diets C and S compared to L. Most amylolytic and fibrolytic bacterial communities were unaffected by the starch and fibre alterations in diets, but the amylolytic bacteria of *Succinimonas amylolytica* and *Ruminococcus bromii* were observed to

be sensitive to starch as an energy source relative to fibre. The starch and sucrose metabolism were up-regulated when cows were fed diets C and S compared to L, which was attributed to the enzyme pullulanase originated from *Succinimonas amylolytica*. The integration of metagenomics and metaproteomics showed to be efficient to detect the shifts of the rumen microbes in response to diets.

Keywords: rumen, diet, metagenomics, metaproteomics, bacteria, CAZyme, KEGG

Introduction

As an ecosystem, the rumen harbours a huge number of microorganisms which contribute to the fermentative degradation of coarse vegetation, grass and other dietary ingredients. The ruminal microorganism produces an array of enzymes to degrade and utilize different plant constituents. Carbohydrates form the main energy source in the dariy cows' diet of which starch and fibre are the most widely used. Adjusting the source and ratio of dietary starch and fibre is a popular practical method for nutritionists to improve the energy status of dairy cows.

Previous studies have reported the amylolytic and fibrolytic activities in the rumen including the communities and their related enzymes (Stewart et al., 1997, Kevin, 2000, Miron et al., 2001). Although updated insight into the structure of the rumen microbiota has been reported, there exists an incomplete understanding of the microbial mechanisms of starch and fibre digestion when jointly offered in composite diets, for instance, the sensitivity and interactions of amylolytic and fibrolytic microbes, the diversity of enzymes and the metabolism pathways.

Nowadays, the widely used 'omics' technologies have enhanced the exploration of the structure, diversity and function of the microbial community in the rumen, such as metagenomics, metatranscriptomics, metaproteomics and metabolomics. Metagenomics is frequently used to assess the community structure and gene function potential of the rumen microbiome (Stewart et al., 2019, Shen et al., 2020). However, the gene expression patterns are not always directly translatable to their biological functions, thus the functionality needs to be confirmed at the protein level. The metaproteomics analysis aims at characterising the entire complement of proteins that are expressed by the microbiome in a given environment pool at a certain time point. The application of metaproteomics on ruminal microorganisms is still limited due to the difficulties for rumen samples in separating the prokaryotic cells from the residual matter before protein extraction and the low availability of accurate reference databases (Deusch et al., 2015, Deusch et al., 2017). Technical progress in mass spectrum and the establishment of more databases with high-quality reference sequences promote the application of metaproteomics analysis. Although challenging, the application of meta-proteomics has the potential for a more complete understanding of the rumen (Hart et al., 2018). The combination of metagenomics and metaproteomics has been applied in studying rumen functioning (Zhu et al., 2016), but to the authors' knowledge, such studies are still very limited.

The aim of the present study was to explore 1) the alterations of the amylolytic and fibrolytic microbial communities and 2) the associated enzymes involved in the carbohydrate metabolism in the rumen of dairy cows receiving three different dietary energy sources through the combination of metagenomics and metaproteomics approaches, then to enlarge our understandings of the omics application on rumen function studies.

Materials and methods

Animals and experimental design

All animals involved in this experiment were cared according to the Chinese Guidelines for Animal Welfare and the study was approved by the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences (IAS2019-6).

Six rumen-cannulated, first parity Chinese Holstein Friesian dairy cows were allocated to a replicated 3 × 3 Latin square design. At the start of the experiment, cows were (mean ± SD) 108 ± 10 days in milk (DIM) with an average milk yield of 28 ± 1.7 kg/d and a bodyweight of 578 ± 33.5 kg. Cows were paired by initial weight, DIM and milk production and subsequently randomly assigned to one of the two Latin squares. The trial consisted of three 21-d experimental periods, where each period contained a 20-d feed adaptation period, followed by a 1-d period of data and sample collection. Diet treatments included a lipogenic diet (L, sugar beet pulp and alfalfa silage as energy sources), glucogenic diet one (C, ground corn and corn silage as energy sources) and glucogenic diet two (S, steam-flaked corn and corn silage as energy sources). The diets were formulated to be isoenergetic according to NRC (2001) to meet or exceed the energy requirements of Holstein dairy cows yielding 25 kg of milk/d with 3.5% milk fat and 3.0% milk protein. Diet composition and its chemical analysis are shown in **Table 6.1**. Experimental animals were fed their respective diet three times (07:00, 14:00 and 20:00) and had free access to water.

Sample collection

On d 21 of each period, rumen content was collected from cranial, caudal, dorsal and ventral locations through the rumen fistula of each animal approximately 1 h after morning feeding. Rumen contents were pooled per animal and immediately strained through 4 layers of cheesecloth (400 ml rumen fluid per cow). Two cryogenic vials of rumen fluid (5 ml/vial) per

cow were separately collected and directly snap frozen and kept in liquid nitrogen until stored at -80 °C, for further metagenomics and metaproteomics analysis.

Table 6.1. Ingredient and chemical composition of the two glucogenic (C, S) and a lipogenic (L) diet

Item	Experimental diet		
	C	L	S
Ingredient composition, % of dry matter			
Corn, ground	28.3	-	-
Steam-flaked corn	-	-	28.3
Sugar beet pulp	-	30.1	-
Soybean meal	11.5	10.4	11.5
Rapeseed meal	7.2	3.9	7.2
Cottonseed meal	7.2	3.9	7.2
Alfalfa hay	7.4	6.7	7.4
Oat hay	4.5	4.1	4.5
Alfalfa silage	-	39.5	-
Corn silage	32.2	-	32.2
Dicalcium phosphate	1.7	1.5	1.7
Composition, % of dry matter			
Crude protein	20.6	20.8	20.4
Ether extract	2.3	2.1	3.0
Starch	18.6	4.4	15.6
Neutral detergent fibre	33.3	54.7	32.7
Acid detergent fibre	18.7	34.6	18.8
Calcium	0.8	1.3	1.0
Phosphorus	0.9	0.4	1.1
NE_L, MJ/kg of dry matter	7.1	8.7	7.2

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. NE_L, net energy for lactation, calculated based on NRC (2001).

Metagenomic analysis

DNA extraction and metagenomic sequencing

A total of 18 rumen fluid samples (6 samples for each diet) were used for metagenomics analysis. DNA was extracted using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany). The DNA concentration was checked on a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the purity was monitored on 1% agarose gel electrophoresis. Extracted DNA was stored at -80 °C until further processing.

DNA extract was fragmented to an average size of about 300 bp using Covaris M220 (Gene Company Limited, China) for paired-end library construction using the TruSeq^{TM} DNA Sample Prep Kit (Illumina, San Diego, CA, USA). Adapters containing the full complement of sequencing primer hybridization sites were ligated to the blunt end of fragments. Paired-end sequencing was performed on the Illumina HiSeq4000 platform (Illumina Inc., San Diego, CA, United States) at Majorbio Bio-Pharm Technology Co., Ltd., (Shanghai, China) using the HiSeq 3,000/4,000 PE Cluster Kit and the HiSeq 3,000/4,000 SBS Kit according to the manufacturer's instructions (www.illumina.com).

Sequencing data analysis and genome assembly

Raw sequences were firstly filtered for reads with the adapter contamination at the end of the reads by SeqPrep (Version 1.1). Subsequently, reads with low quality (quality score < 20, or reads length < 50 bp, or having ambiguous N bases) were removed by the program Sickle (Version 1.33). Then, the quality-passed reads were aligned to the bovine genome by the Burrows Wheeler Aligner (Version 0.7.9a), and all hits associated with the reads or their mated reads were finally removed. Only high-quality pair-end reads and single-end reads were further analysed. The data was analysed on the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com). The resulting cleaned sequences were *de novo* assembled into contigs using Megahit (Version 1.1.2). Only contigs longer than 300 bp were used for further analysis.

Gene prediction and functional annotation

Open reading frames (ORFs) within contigs were used for gene prediction by MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/). The predicted ORFs with a length being or over 100 bp were retrieved and translated into amino acid sequences using the NCBI translation table.

Subsequently, the predicted genes were clustered using CD-HIT (Version 4.6.1) with a standard of 95% nucleotide identity and 90% of length coverage. The longest sequences of each cluster were chosen as representative sequences to construct the non-redundant gene catalogues. Quality-controlled reads from each sample were mapped to the non-redundant gene catalogues with 95% identity using SOAPaligner (version 2.2.1), and the gene abundance in each sample was calculated by the reads per kilobase per million mapped (RPKM). Representative sequences of the non-redundant gene catalogue were aligned to a Non-redundant (NR) database in the National Center for Biotechnology Information (NCBI) with an e-value cutoff of $1e^{-5}$ using Blastp (Version 2.3.0) for taxonomic annotations. The metabolism pathway annotation was conducted using Blastp (Version 2.2.28+) against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/keeg/) with an e-value cutoff of $1e^{-5}$. Carbohydrate-active enzymes (CAZymes) annotation was performed by hmmscan (Version 3.1b2) against the CAZy database (http://www.cazy.org) version 6.0 with an e-value cutoff of $1e^{-5}$ (Wang et al., 2019).

Metaproteomic analysis

Protein extraction and quality control

Among the 18 rumen fluid samples, the samples from the cows fed the same diet within each period were pooled yielding a total of nine (pooled) rumen fluid samples which were used for protein extracting. These samples were freeze-dried and ground using liquid nitrogen, mixed and vortexed with lysis buffer (8 M urea, 1% SDS, protease inhibitor), and then lysed on ice for 30 min with the mixed solutions being vortexed every 5 min. Pure acetone (purity ⩾ 99.5%, Guoyao, Shanghai, China) was added to the solution with a ratio of 1:4 (v/v), vortexed at 4 °C, and then stored overnight at -20 °C to obtain protein precipitation. The solution was centrifuged at 12,000 × g for 20 min at 4 °C, the pellet was collected and washed three times by being resuspensed in 90% pre-cooled acetone and centrifugated at 12,000 × g for 20 min under 4 °C. After washing, the precipitate was re-suspended in lysis buffer (8 M urea + proteinase inhibitor cocktail), sonicated for 2 min on ice, whereafter the lysate was centrifuged at 12,000 × g for 20 min under 4 °C, with the supernatant subsequently collected. Protein concentration in the supernatant was determined by the Bicinchoninic acid (BCA) method using a BCA Protein Assay Kit (Beyotime Biotechnology). The protein quality was evaluated by SDS-PAGE.

Protein digestion and peptide quantification

Protein was firstly digested with trypsin. Briefly, 100 μg protein was mixed in a sample tube with the triethylammonium bicarbonate buffer (TEAB) to a final concentration of 100 mM, whereafter the tris-2-carboxyethyl phosphine (TCEP) was added to a final concentration of 10 mM before incubating the tube at 37 °C for 60 min. Iodoacetamide (IAM) was added to a final concentration of 40 mM and the solution was allowed to react in the dark. After 40 min, pre-cooled acetone was added (v/v = 6:1) to the sample and the solution incubated at -20 °C for 4 h to precipitate protein which was collected after centrifugation at 10,000 × g for 20 min. The pellet was resuspended in 100 μl TEAB (100 mM). Then, the trypsin was added into the protein solution in a ratio of 1:50 (trypsin:protein, m/m). The protein was digested at 37 °C overnight. Upon completion of digestion, the hydrolysed peptide was dried using a vacuum pump.

Then, the dried peptide was resuspended in 0.1% trifluoroacetic acid and desalted with the Oasis® HLB 96-well plate (Waters, US) and Oasis® MCX μElution plate (Waters, US) and dried using a vacuum pump. The concentration of peptide was determined using a quantitative colorimetric peptide assay (NO. 23275, ThermoFisher Scientific, USA). Loading buffer (2% acetonitrile and 0.1% formic acid) was added to each tube to a peptide concentration of 0.25 μg/μl before each sample was analysed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analysis

The mass spectrometry analysis was performed on a Q Exactive HF-X mass spectrometer (Thermo, USA) coupled with Easy-nLC 1200 (Thermo, USA). Each peptide sample was injected onto a C18-reversed column (75 μm × 25 cm, Thermo, USA) and separated for 120 min at a flow rate of 300 nL/min, then eluted in buffer A (2% acetonitrile and 0.1% formic acid) and a 90 min gradient of 5-100% buffer B (80% acetonitrile and 0.1% formic acid). Q Exactive HF-X mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z, 300-1,500) were acquired for selecting precursor ions with a mass resolution of 60 K, followed by high energy collisional dissociation (HCD)-MS/MS scan with a resolution of 15 K. The dynamic exclusion parameter was set as 18 s.

Data processing and analysis

For protein identification, the raw MS/MS spectra were searched against the customized database constructed by the metagenomic-derived nonredundant protein sequence using Proteome Discoverer^{TM} Software 2.4 (Thermo Fisher Scientific, San Jose, CA). The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. The parameters for protein searching were set as follows: tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines as fixed modification and oxidation of methionines and protein N-terminal acetylation as variable modifications. Peptide spectral matches were validated based on a false discovery rate (FDR) ≤ 1%.

Annotation of all identified proteins was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathway analysis. The protein-derived taxonomic analysis was determined by comparing the sequence against the NR database in NCBI.

The differentially expressed proteins (DEPs) in each group set (A vs B) were determined with the thresholds of fold change (FC) < 0.5 (this protein is down-regulated in group A than in group B) or > 2 (up-regulated) and $P \leq 0.05$. Volcano plots were created in R (version 3.3.1) with the FC (log2 value) as the abscissa and the *P*-value as the ordinate to summerize the DEPs information. DEPs were further performed for KEGG pathway enrichment analysis using Fisher's exact test adjusted by FDR with Scipy.stats package in Python (version1.0.0), significantly enriched pathways were considered with a value of $P \leq 0.05$.

Data analysis

The comparison of the microbes and enzymes annotated from both metagenomics and metaproteomics data were analysed by the one-way analysis of variance (ANOVA) adjusted with FDR using stats package in R software (version 3.3.1) and scipy package in Python (version1.0.0). The $P \leq 0.05$ was considered as a significant level and $0.05 < P < 0.1$ as a potentially significant level (trend). The DEPs in each group set of A vs B (control group vs experimental group) were analysed with Student's *t*-test in R (version 3.3.1).

Results

Rumen metagenome data statistics

Approximately 1.65 billion raw reads comprising 249 gigabases of raw data were generated from the 18 rumen fluid samples, with 91,640,810 ± 8,260,725 (mean ± SD) reads per sample (**Supplementary Table S6.1**). About 1.21 billion optimized reads, out of raw reads were generated after eliminating low-quality reads and removing the represented bovine genome. Then, *de novo* assembly was performed as described above, resulting in a total of 18,627,023 contigs, with an N50 of 673 bp (range 578-808 bp). For gene prediction, 24,620,061 ORFs were predicted using the program MetaGene. 8,114,000 genes were detected for constructing the non-redundant gene catalogue.

Microbial composition

The taxonomic analysis of all protein-coding genes in the metagenome at the domain level revealed that the bacteria were the predominant microorganisms (96.5% of total sequences) present among all samples, followed by viruses (1.2%), archaea (1.1%), eukaryotes (0.9%) and unclassified (0.3%) (**Supplementary Figure S6.1a**). For bacteria, 16 phyla with a relative abundance of all bacteria above 0.1% were identified among all samples (**Supplementary Figure S6.1b**). The rumen fluid from cows fed diets C and S were dominated by *Bacteroidetes* (49.2 and 49.8%, respectively), *Firmicutes* (31.5 and 31.8%) and *Proteobacteria* (4.1 and 5.9%), while the rumen fluid from cows fed diet L was dominated by *Firmicutes* (45.5%), *Bacteroidetes* (36.2%) and *Proteobacteria* (3.2%). At the genus level, a total of 109 bacterial genera were identified with a relative abundance of bacteria above 0.1% in all samples (**Supplementary Figure S6.1c**). For samples from the treatment of diet L, the five dominant genera included *Prevotella* (19.7%), *Bacteriodes* (8.9%), *Clostridium* (8.3%), unclassified tax (4.3%) and *Ruminococcus* (4.1%). For samples from the treatments of diets C and S, *Prevotella* (29.7 and 32.3%, respectively), *Bacteriodes* (9.2 and 8.8%), *Clostridium* (5.9 and 5.5%) and two unclassified taxa (7.9 and 5.7%). A total of 294 bacterial spp. was detected with a relative abundance within the bacterial community above 0.1%. The most abundant species for diet C, L and S were *Prevotella* sp. CCMP3155 (2.8, 2.1 and 3.0%, respectively), *Prevotella ruminicola* (2.7, 1.9 and 2.7%) and *Prevotella brevis* (2.7, 1.9 and 2.6%) (**Supplementary Figure S6.1d**).

The bacteria genera were compared between treatments and the top 20 genera in relative abundance that were significantly affected are shown in the **Supplementary Figure S6.2a**. The rumen fluid had higher relative abundances of *Alloprevotella* ($P = 0.017$) and *Lachnobacterium* ($P = 0.009$), but lower unclassified *f_Lachnospiraceae* ($P = 0.024$) when the cows were fed diets C and S compared to diet L. The bacteria species were also compared between treatments and the top 20 relative abundant bacterial species significantly affected by the diets are shown in the **Supplementary Figure S6.2b**. Diets C and S resulted in a significantly higher relative abundances of *Prevotella albensis* ($P = 0.049$), *Succinimonas amylolytica* ($P = 0.003$), *Ruminococcus bromii* ($P = 0.002$), *Eubacterium* sp. CAG:202 ($P = 0.002$), *Ruminococcus* sp. CAG:108 ($P = 0.003$), *Succinivibrionaceae* bacterium WG-1 ($P = 0.003$), *Ruminobacter* sp. RM87 ($P = 0.031$), *Lachnobacterium bovis* ($P = 0.009$), *Alloprevotella rava* ($P = 0.013$), *Sutterella wadsworthensis* ($P = 0.013$), *Eubacterium* sp CAG:603 ($P = 0.042$), *Bacteroides* sp. CAG:530 ($P = 0.046$), *Prevotella* sp. P4-65 ($P = 0.019$) and *Bacteroides graminisolvens* ($P = 0.021$) in the rumen fluid of the cows compared to when diet L was fed. Feeding diet L resulted in higher relative abundances of *Ruminococcus flavefaciens* ($P = 0.041$), *Parabacteroides distasonis* ($P = 0.019$), *Clostridium* sp. CAG:433 ($P = 0.017$), *Clostridium* sp. CAG:678 ($P = 0.010$), *Eubacterium plexicaudatum*, ($P = 0.026$), *Lachnospira multipara* ($P = 0.019$) and *Clostridium* sp. CAG:492 ($P = 0.019$) in the rumen fluid compared to the two glucogenic diets. Feeding the cows diet S resulted in a higher relative abundance of *Selenomonas ruminantium* compared to the other diets ($P = 0.041$).

The comparisons for the typical amylolytic and fibrolytic bacteria are shown in **Figure 6.1**. The amylolytic bacteria of *Succinimonas amylolytica* ($P = 0.003$) and *Ruminococcus bromii* ($P = 0.002$) had higher relative abundances in the cows receiving diets C and S than diet L. The relative abundance of *Selenomonas ruminantium* was higher when feeding diet S than diet L ($P \leq 0.05$). As for the fibrolytic bacteria, the relative abundances of *Ruminococcus flavefaciens* ($P = 0.041$) and *Lachnospira multipara* ($P = 0.019$) were significantly higher in the cows when diet L was fed compared to when diet C and S was fed. Although not significant, the relative abundance of *Ruminococcus albus* and *Lachnospira multipara* was numerically higher and *[Eubacterium] cellulosolvens* numerically lower when the cows were fed diet L compared to diet C and S. In addition, *Butyrivibrio fibrisolvens* had a higher relative abundance when the cows were fed diet S.

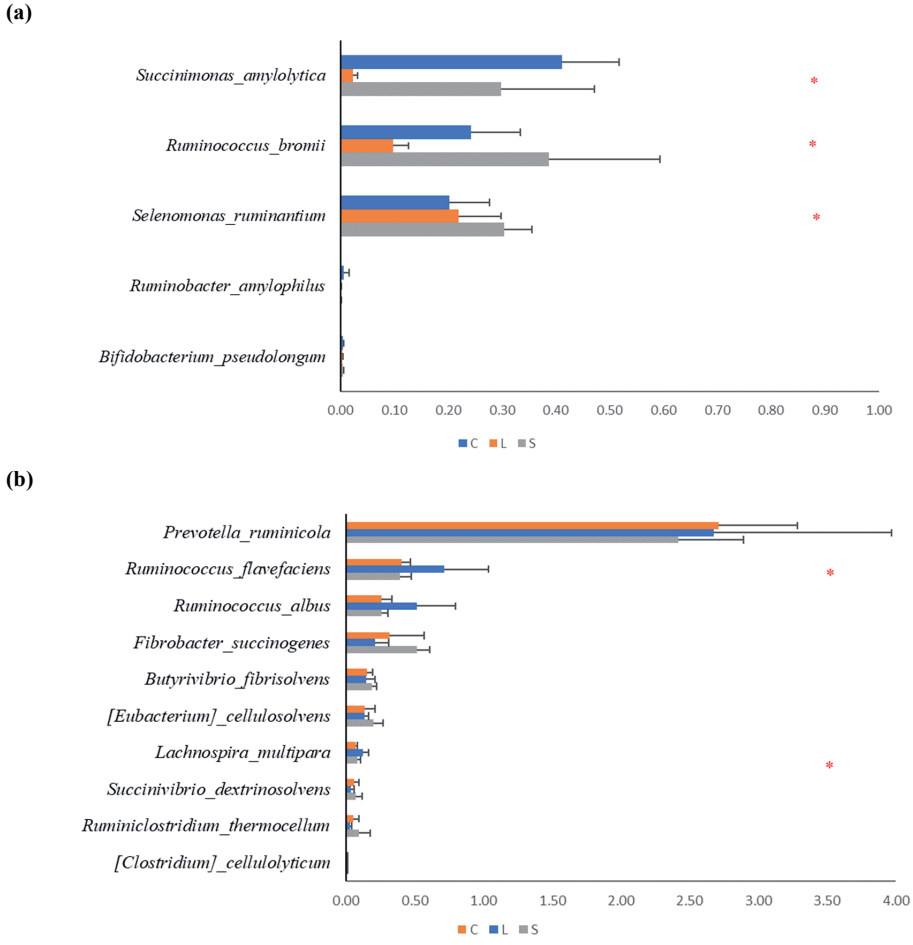


Figure 6.1. Comparison of amylolytic bacteria (a) and fibrolytic bacteria (b) in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets, C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. * $P \leq 0.05$.

CAZymes of the rumen microbiome

Enzymes associated with the degradation of dietary carbohydrates, also referred as the Carbohydrate active enzymes (CAZymes), mainly include starch degrading enzymes like glucosidases and amylases and fibrolytic enzymes like cellulases and hemicellulases. CAZymes comprise six classes including glycoside hydrolases (GHs), glycosyl transferases (GTs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), polysaccharide lyases (PLs) and auxiliary activities (AAs).

The non-redundant contigs with 8,114,000 genes were blasted against the CAZymes database (V6.0) using the hmmscan tool with an e-value cutoff of $1e^{-5}$. A total of 264,952 genes were annotated to the CAZymes. Among the six classes of CAZymes, the GHs had the highest relative abundance (52.6%), followed by GTs (26.4%), CEs (11.4%) CBMs (5.2%) and PLs and AAs (4.4%) (**Figure 6.2a**). No significant differences between the top six classes were observed among the three dietary groups (**Supplementary Figure S6.3**).

According to the phylogenetic origin of core microbial populations which primarily contribute to the CAZymes, the genera of *Prevotella*, *Bacteroides* and an unclassified genus contributed the most of the CAZyme encoding gene fragments of the GH, GT, CE, CBM, PL and AA families (**Figure 6.2b**).

In order to evaluate the alterations in carbohydrate biodegradation, the GH families functioning in the cellulose, hemicellulose and starch degradation were further compared (**Supplementary Table S6.2**). After comparison, the GH families with $P \leq 0.1$ are listed in **Table 6.2**. For the GH families related to fibre degradation, the relative abundances of GH16 ($P = 0.034$), GH5_13 ($P = 0.032$), GH5_26 ($P = 0.042$) and GH5_25 ($P = 0.012$) were higher when the cows were fed diet C compared to L. The relative abundances of GH5_28 and GH48 were higher when the cows were fed diet L than the other two diets. Feeding diet S resulted in a higher relative abundance of GH13_14 ($P = 0.017$) in rumen fluid than diet L. In addition, 15 GH families related to amylolytic functions were obtained. The relative abundances of GH13_36 ($P = 0.015$), GH13_13 ($P = 0.022$), GH13_28 ($P = 0.003$), GH13_15 ($P = 0.022$), GH13_2 ($P = 0.003$), GH13_37 ($P = 0.004$), GH13_42 ($P = 0.006$), GH13_16 ($P = 0.015$) and GH13_27 ($P = 0.079$) were higher when the cows were fed diets C and S compared to L. Diet S resulted in a higher relative abundance of GH13_20 ($P = 0.090$) and GH13_14 ($P = 0.017$) and the diet C led to a higher relative abundance of GH13_6 ($P = 0.059$) when separately compared to diet L.

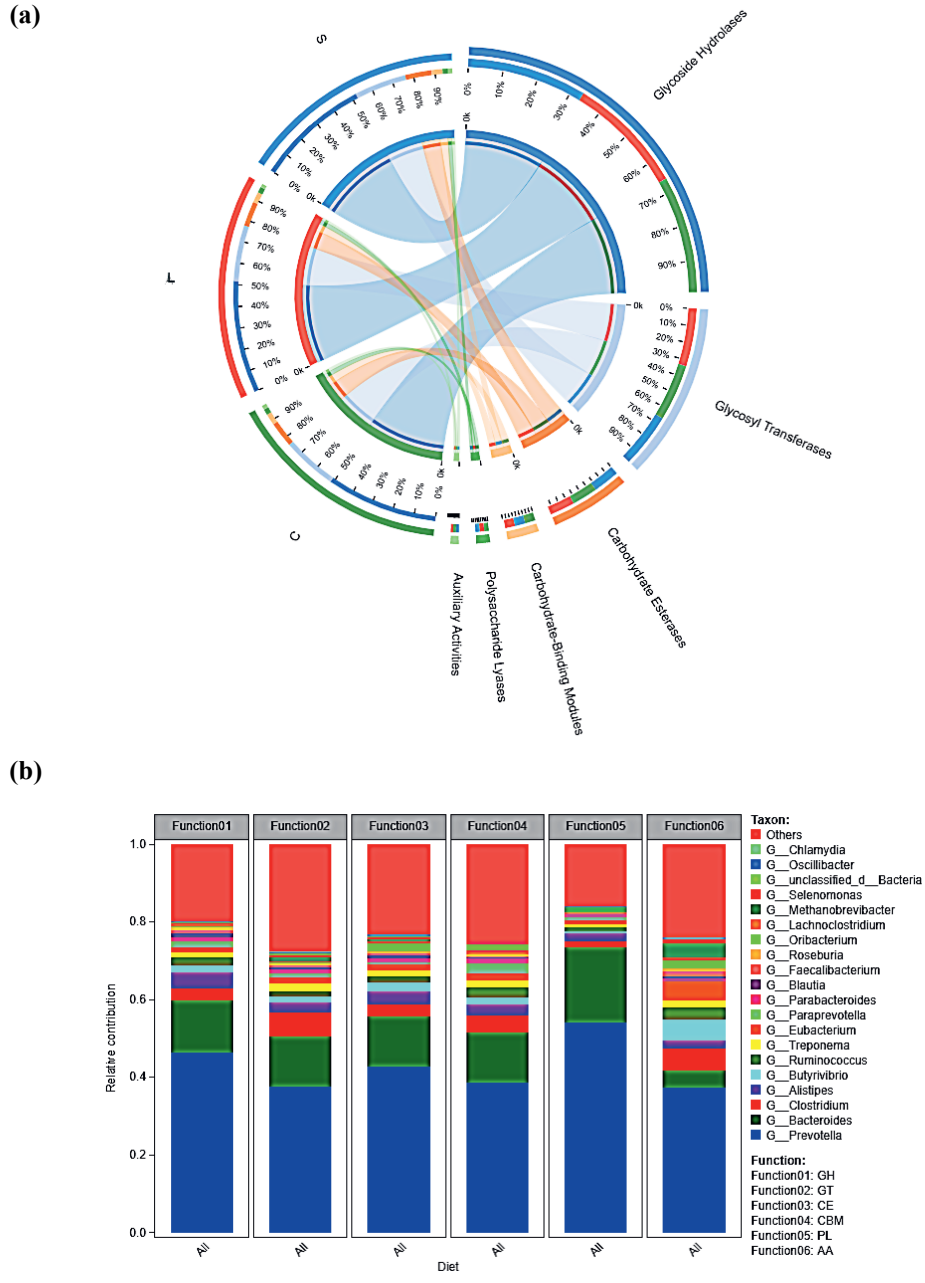


Figure 6.2. **Circos map of CAZyme classes (a) and percent contributions of CAZymes from the major microbial genera (b) based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet**. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. GH, glycoside hydrolase. GT, glycosyltransferase. PL, polysaccharide lyase. CE, carbohydrate esterases. CBM, carbohydrate-binding module. AA, auxiliary activity.

Table 6.2. Glycoside hydrolase (GH) families involved in fibre and starch degradation identified in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

GH family category	Representative enzyme	Experimental diet			SEM	*P*-value
		C	L	S		
Fibre degrading enzymes						
GH16	Xyloglucanases	0.88^{a}	0.70^{b}	0.80^{ab}	0.030	0.034
GH5_13	β-D-galactofuranosidase	0.14^{a}	0.08^{b}	0.12^{ab}	0.011	0.032
GH5_26	Endo-β-1,4-glucanase	0.03^{a}	0.01^{b}	0.02^{ab}	0.003	0.042
GH5_25	Endo-β-1,4-glucanase	0.02^{a}	0.01^{b}	0.02^{b}	0.002	0.012
GH5_28	Endoglycosylceramidase	<0.01	0.02	0.01	0.003	0.080
GH48	Cellobiohydrolases	<0.01^{b}	0.02^{a}	0.00^{b}	0.002	0.045
Starch degrading enzymes						
GH13_36	α-Amylase	0.65^{a}	0.37^{b}	0.63^{a}	0.048	0.015
GH13_20	Cyclic α-1,6-maltosyl-maltose hydrolase	0.53	0.43	0.68	0.041	0.090
GH13_13	Pullulanase	0.50^{a}	0.34^{b}	0.55^{a}	0.033	0.022
GH13_6	α-Amylase	0.41	0.30	0.37	0.020	0.059
GH13_14	α-Glycosidase	0.16^{ab}	0.09^{b}	0.21^{a}	0.023	0.017
GH13_28	α-Amylase	0.16^{a}	0.04^{b}	0.13^{a}	0.016	0.003
GH13_15	α-Amylase	0.11^{a}	0.05^{b}	0.12^{a}	0.012	0.022
GH13_2	α-Amylase	0.08^{a}	0.03^{b}	0.07^{a}	0.007	0.003
GH13_37	α-Amylase	0.07^{a}	0.00^{b}	0.07^{a}	0.014	0.004
GH13_42	α-Amylase	0.04^{a}	0.01^{b}	0.03^{a}	0.004	0.006
GH13_16	Maltose glucosylmutase	0.03^{a}	0.01^{b}	0.02^{a}	0.003	0.015
GH13_27	α-Amylase	0.03	0.01	0.02	0.004	0.079

Diets: C, corn and corn silage diet; GH, glycoside hydrolase; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

$^{a, b}$ means within a row with different superscripts differ significantly ($P \leq 0.05$).

Carbohydrate metabolism pathways

To better understand the alterations of the carbohydrate metabolism relative to the three diets, the carbohydrate metabolism KEGG pathways were compared (**Supplementary Table S6.3**), in which the starch and sucrose metabolism was significantly down-regulated while the galactose metabolism and the pentose and glucuronate interconversions were significantly up-regulated when diet L was fed compared to diets C and S. The enzymes involved in these affected pathways were further analysed (**Supplementary Figure S6.4**). The affected enzymes in starch and sucrose metabolism are shown in **Figure 6.3a**. The relative abundances of α-

amylase (Enzyme commission number, EC3.2.1.1, P = 0.005), pullulanase (EC3.2.1.41, P = 0.020) and maltose α-D-glucosyltransferase (EC5.4.99.16, P = 0.026) were higher, while the cellulose (EC3.2.1.4, P = 0.030), glucokinase (EC2.7.1.2, P = 0.008) and isoamylase (EC3.2.1.68, P = 0.033) were lower in the rumen fluid of dair cows when diets C and S were fed compared to diet L. The differentially expressed enzymes involved in pentose and glucuronate interconversions including pectinesterase (EC3.1.1.11, P = 0.033), L-arabinose isomerase (EC5.3.1.4, P = 0.013), tagaturonate reductase (EC1.1.1.58, P = 0.002), oligogalacturonide lyase (EC4.2.2.6, P = 0.014) and polygalacturonase (EC3.2.1.15, P = 0.043) were all up-regulated when cows were fed diet L compared to diets C and S (**Figure 6.3b**). Only two enzymes in the galactose metabolism (**Figure 6.3c**) were affected by dietary treatments, 6-phospho-beta-galactosidase (EC3.2.1.85) and lactose-specific IIA component (EC2.7.1.207), both of which were up-regulated (P = 0.030 and 0.021, respectively) when cows were fed diet S relative to the other two diets.

Metaproteomic data statistics

Protein identification and taxonomic analysis

A total of 11,676 peptides and 5,328 protein from 9 pooled rumen fluid samples were identified (**Figure 6.4**). As for each dietary treatment, a total of 4,199 proteins were detected from all rumen fluid samples of the cows fed diet C, 3,800 for diet L and 4,284 proteins for diet S (**Supplementary Figure S6.5**).

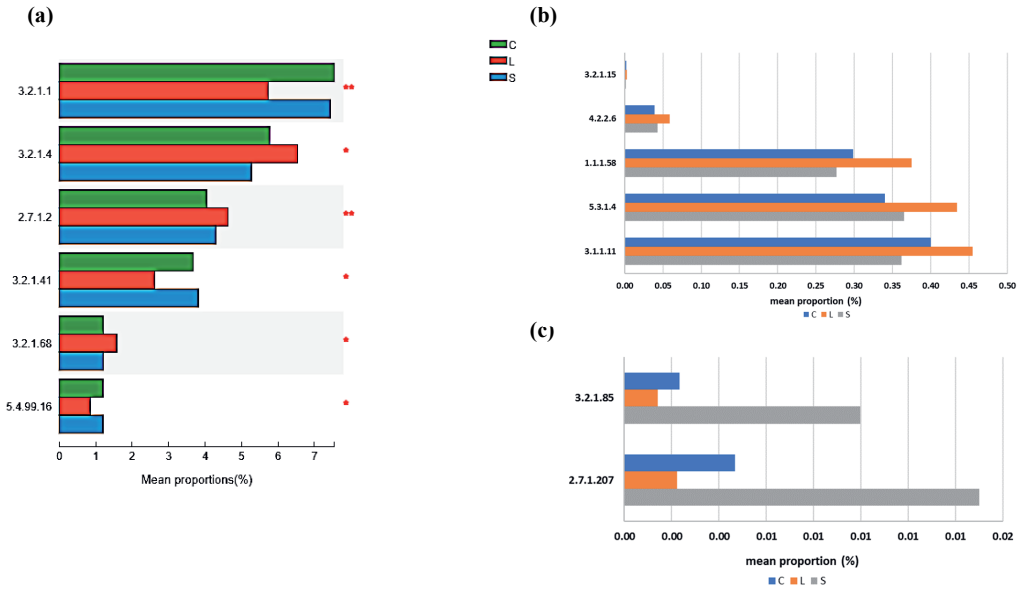


Figure 6.3. **Affected enzymes involved in a) starch and sucrose metabolism, b) pentose and glucuronate interconversions and c) galactose metabolism pathways based on metagenomics analysis in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet**. a) 3.2.1.1, α-Amylase; 3.2.1.4, Cellulase; 2.7.1.2, Glucokinase; 3.2.1.41, Pullulanase; 3.2.1.68, Isoamylase; 5.4.99.16, Maltose α-D-glucosyltransferase. b) 3.1.1.11, Pectinesterase; 5.3.1.4, L-arabinose isomerase; 1.1.1.58, Tagaturonate reductase; 4.2.2.6, Oligogalacturonide lyase; 3.2.1.15, Polygalacturonase. c) 3.2.1.85, 6-Phospho-beta-galactosidase; 2.7.1.207, Lactose-specific IIA component. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

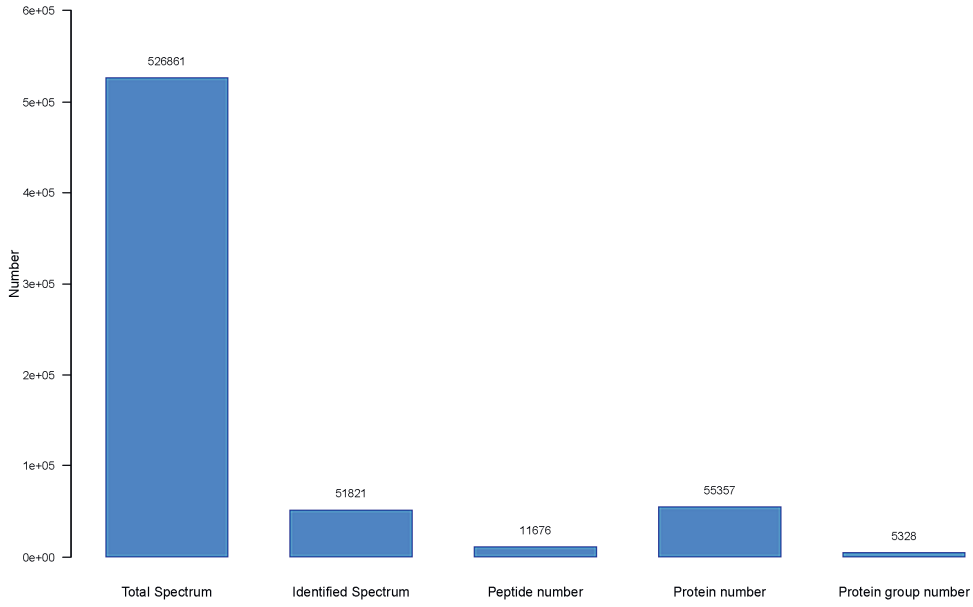


Figure 6.4. **Information for protein identification based on metaproteomics analysis in rumen fluid samples of dairy cows fed two glucogenic and a lipogenic diet**.

The protein-based taxonomic classification of the ruminal microbes is shown in **Figure 6.5**. The detected proteins among all rumen fluid samples were mainly assigned to *Bacteriodetes* (on average 28.9, 39.3 and 33.3% for diet C, L and S, respectively), *Firmicutes* (24.8, 31.1 and 29.3%), *Proteobacteria* (22.9, 4.6 and 24.0%), *Ciliophora* (9.0, 7.1 and 4.5%) and *Spirochaetes* (2.2, 5.4 and 1.3%) in the phyla level (**Figure 6.5a**). The dominant genera contained *Prevotella* (22.7, 32.1 and 27.3% for diet C, L and S, respectively), *Clostridium* (1.9, 5.5 and 1.8%), *Treponema* (2.1, 5.3 and 1.2%), *Bacteroides* (2.3, 2.8 and 2.4%) and *Aeromona* (3.6, 0.8 and 3.2%) (**Figure 6.5b**). At a species level, the top five abundant species when the cows were fed diet L contained *Prevotella ruminicola* (3.5%), *Prevotella* sp. bacterium (3.2%), *Prevotella* sp. tc2-28 (2.6%), *Clostridiales* bacterium (2.3%) and *Stylonychia lemnae* (2.1%). When cows were fed diet C, the top five abundant species were *Stylonychia lemnae* (3.0%), *Succinimonas amylolytica* (2.9%), *Succinivibrionaceae* bacterium WG-1 (2.4%), *Prevotella ruminicola* (2.4%) and *Clostridiales* bacterium (2.2%). For diet S, the top five abundant species were *Succinimonas amylolytica* (3.0%), *Succinivibrionaceae* bacterium WG-1 (2.4%), *Prevotella ruminicola* (2.0%), *Clostridiales* bacterium (1.7%) and *Prevotella* sp. bacterium (1.6%) (**Supplementary Figure S6.6**).

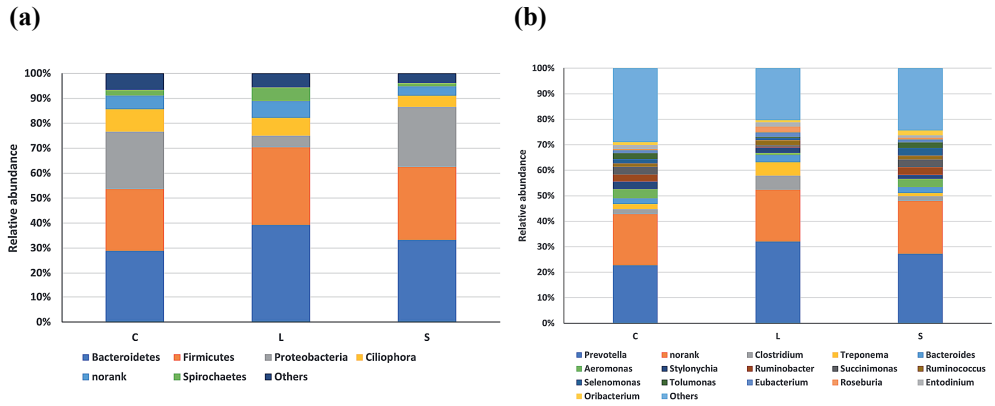


Figure 6.5. **Protein-based taxonomic profiles at the phyla (a) and genus (b) level based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet**. C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

The top 50 annotated bacterial species were compared among the three dietary treatments (**Supplementary Table S6.4**). The comparisons of amylolytic and fibrolytic bacteria are shown in **Figure 6.6**. For annotated amylolytic bacteria, the relative abundances of *Succinimonas amylolytica* and *Ruminococcus bromii* were higher when the cows were fed diets C and S than diet L ($P < 0.001$ and $P = 0.016$, respectively). When the cows were fed diet S, a higher relative abundance of *Selenomonas ruminantium* was observed compared to the other diets ($P = 0.028$) (**Figure 6.6a**). As for the annotated fibrolytic bacteria, diet L resulted in a higher relative abundance of *Prevotella ruminicola* ($P = 0.016$) but a lower *Succinivibrio dextrinosolvens* ($P = 0.002$) and a trend towards a lower relative abundance of *[Eubacterium] cellulosolvens* ($P = 0.057$) compared to the other diets (**Figure 6.6b**). When the cows were fed diet S, a higher relative abundance of *Butyrivibrio fibrisolvens* ($P = 0.009$) was observed compared to diets L and C. Although not significantly different, *Ruminococcus flavefaciens* and *Lachnospira multipara* were all highly abundant in when diet L was fed compared to diets C and S.

Analysis of DEPs

Using a threshold of $P \leq 0.05$ and FC > 2 (up-regulated) or < 0.5 (down-regulated), 865 proteins were identified as DEPs, with 539 proteins up-regulated and 326 proteins down-regulated when cows were fed diet L than diet S. For the L vs C comparison, 706 DEPs were identified with 446 proteins up-regulated and 260 proteins down-regulated for the L diet. The S vs C

comparison showed 139 DEPs with 89 proteins up-regulated and 50 protein down-regulated for the S diet (**Figure 6.7** and **Supplementary Table S6.5**).

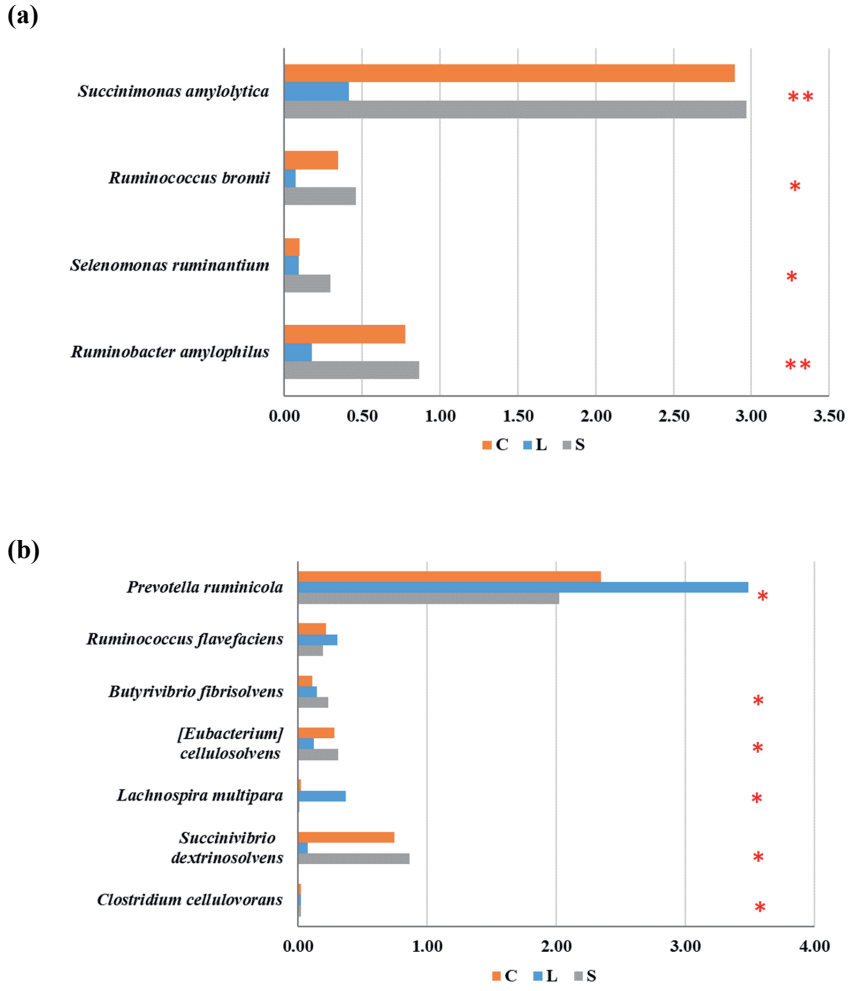


Figure 6.6. Comparison of the amylolytic (a) and fibrolytic (b) bacteria based on metaproteomics analysis in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. *, $P \leq 0.05$; **, $P < 0.01$.

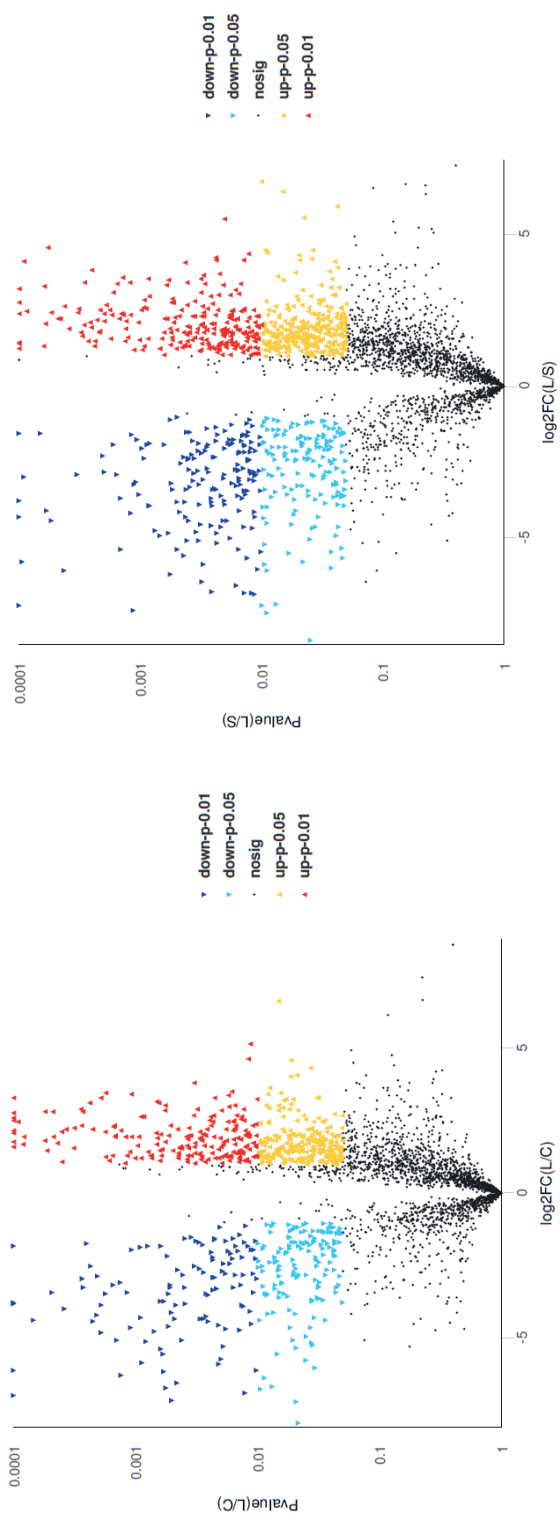


Figure 6.7. Pairwise comparison of proteins based on metaproteomics analysis in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. The abscissa represents the fold change (FC, log2 value) of differentially expressed protein between two treatments and the ordinate represents *P*-value. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. *P*-value (diet1/diet2), comparison of the relative abundance of one protein between diet1 and diet2. A dark blue downward-pointing triangle, down-regulated protein ($P \leq 0.01$); a light blue downward-pointing triangle, down-regulated protein ($P \leq 0.05$); a yellow triangle, up-regulated protein ($P \leq 0.05$); a red triangle, up-regulated protein ($P \leq 0.01$); a black rhombus, no significance.

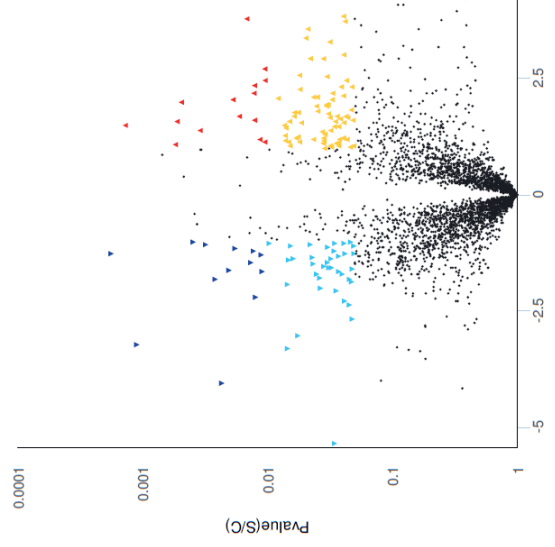


Figure 6.7 (continued). Pairwise comparison of proteins based on metaproteomics analysis in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. The abscissa represents the fold change (FC, log2 value) of differentially expressed protein between two treatments and the ordinate represents *P*-value. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. *P*-value (diet1/diet2), comparison of the relative abundance of one protein between diet1 and diet2; A dark blue downward-pointing triangle, down-regulated protein ($P \leq 0.01$); a light blue downward-pointing triangle, down-regulated protein ($P \leq 0.05$); a yellow triangle, up-regulated protein ($P \leq 0.05$); a red triangle, up-regulated protein ($P \leq 0.05$); a black rhombus, no significance.

KEGG pathways analysis

To understand the functions of the DEPs, the KEGG pathway enrichment analysis was performed. The top 10 KEGG pathways that enriched the most DEPs in the three pairwise comparisons included the biosynthesis of antibiotics, the carbon metabolism, the ribosome, the biosynthesis of amino acids, the glycolysis/gluconeogenesis, the carbon fixation pathways in prokaryotes, the carbon fixation in photosynthetic organisms, the two-component system, the starch and sucrose metabolism and the 2-oxocarboxylic acid metabolism (**Table 6.3**). As for the starch and sucrose metabolism pathway, 35 proteins were down-regulated and 7 were up-regulated when the cows were fed diet L compared to diet C, 42 proteins were down-regulated and 12 were up-regulated when the cows were fed diet L compared to diet S, while this pathway was not significantly enriched in DEPs between dietary treatments S and C.

Table 6.3. Significance values of the top 10 down- and up-regulated KEGG pathways according to the differential expressed proteins based on metaproteomics analysis in rumen fluid samples of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Enriched KEGG pathways	Down-regulated			Up-regulated		
	L vs C	L vs S	S vs C	L vs C	L vs S	S vs C
Biosynthesis of antibiotics	0.018	0.001	0.001	0.014	0.003	0.054
Carbon metabolism	0.032	0.036	0.055	0.043	0.034	0.056
Ribosome	0.001	0.001	0.050	0.001	0.001	0.005
Biosynthesis of amino acids	0.040	0.005	0.000	0.028	0.007	0.061
Glycolysis/Gluconeogenesis	0.003	0.022	0.008	0.060	0.056	0.034
Carbon fixation pathways in prokaryotes	0.038	0.041	0.107	0.049	0.015	0.048
Carbon fixation in photosynthetic organisms	0.002	0.003	0.053	0.059	0.054	0.048
Two-component system	0.007	0.003	0.070	0.081	0.071	0.101
Starch and sucrose metabolism	0.001	0.001	0.081	0.001	0.009	0.128
2-Oxocarboxylic acid metabolism	0.007	0.003	0.104	0.026	0.011	0.112

Diet: C, corn and corn silage; L, sugar beet pulp and alfalfa silage; S, steam-flaked corn and corn silage as the main energy source. KEGG, Kyoto Encyclopedia of Genes and Genomes. $P \leq 0.05$ indicates a pathway significantly enriched among differential affected proteins.

To compare the results with metagenomics analysis, the KEGG pathways of starch and sucrose metabolism, pentose and glucuronate interconversions, and galactose metabolism were further analysed. 92 proteins were detected to be involved in the starch and sucrose metabolism pathway (**Supplementary Table S6.6**), which were annotated into 29 enzymes with EC

numbers (**Table 6.4** and **Supplementary Figure S6.7a**). Among the differential affected enzymes, the cellulase (derived from *Ruminococcus* sp.), α-amylase (*Prevotella buccae*) and glucose-1-phosphate adenylyltransferase (*Treponema* sp.) were up-regulated when the cows were fed diet L. While the α-amylase (*Aeromonas enteropelogenes* and *Trichomonas vaginalis* G3), Pullulanase (*Succinimonas amylolytica*, *Photorhabdus australis*, *Photobacterium marinum* and *Photorhabdus asymbiotica*) and Amylopullulanase (*Selenomonas bovis*) were up-regulated when the cows were fed diets C and S compared to diet L. The amylopullulanase (*Selenomonas ruminantium*) was up-regulated when cows were fed diet S compared to C. There were 39 proteins annotated into the pentose and glucuronate interconversions pathway, of which 20 proteins were differentially expressed among three dietary groups (**Table 6.4** and **Supplementary Figure S6.7b**). Consistent with the results of the metagenomic analysis, the L-arabinose isomerase derived from *Lachnobacterium bovis* and *Prevotella* sp. tc2-28, the tagaturonate reductase originating from *Bacteroides pectinophilus* and *Prevotella* sp. (*Prevotella* sp. BP1-145, *Prevotella* sp. tc2-28, etc.) were up-regulated when cows were fed diet L compared to C and S. As for the galactose metabolism pathway, 24 out of 44 detected proteins were significantly regulated by dietary treatments (**Table 6.4** and **Supplementary Figure S6.7c**), but these proteins were not significantly affected by diets based on the metagenomics analysis.

Table 6.4. Pairwise comparison of affected enzymes involved in the pathways of starch and sucrose metabolism, pentose and glucuronate interconversions, and galactose metabolism based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Enzyme in different pathways	Taxonomy	L vs C	L vs S	S vs C
Starch and sucrose metabolism				
Cellulase	*Ruminococcus* sp.	+	+	NS
α-Amylase	*Aeromonas enteropelogenes*	-	-	NS
α-Amylase	*Prevotella buccae*	+	+	NS
α-Amylase	*Trichomonas vaginalis* G3	-	-	NS
Pullulanase	*Succinimonas amylolytica*, *Photorhabdus australis*, *Photobacterium marinum* *Photorhabdus asymbiotica*	-	-	NS
Pullulanase precursor	*Vibrio quintilis*	-	-	NS
Amylopullulanase	*Selenomonas bovis*	-	-	NS
Amylopullulanase	*Selenomonas ruminantium*	NS	NS	+
4-α-glucanotransferase	*Aeromonas jandaei*	-	-	NS
4-α-glucanotransferase	*Ruminococcaceae* bacterium	+	NS	+
4-α-glucanotransferase	*Clostridiales* bacterium	+	+	NS
6-phospho-α-glucosidase	*Selenomonas bovis*	-	-	NS
α catalytic domain containing protein	*Stylonychia lemnae*	-	-	-
α-glucan family phosphorylase	*Alloprevotella rava*	+	NS	+
Carbohydrate kinase	*Prevotella* sp. lc2012	-	-	NS
Glucokinase	*Escherichia coli*	-	-	NS
Glucosamine-6-phosphate deaminase	*Prevotella ruminicola*	+	+	NS
Glucose-1-phosphate adenylyltransferase	*Oribacterium* sp. P6A1	-	-	NS
Glucose-1-phosphate adenylyltransferase	*Treponema* sp.	+	+	NS
Glucose-1-phosphate adenylyltransferase Subunit GlgD	*Lachnospiraceae* bacterium	-	-	NS
Glycogen synthase GlgA	*Lachnospiraceae* bacterium	-	-	NS
Glycogen debranching protein GlgX	*Succinimonas amylolytica*	-	-	NS
Glucose-6-phosphate isomerase	*Lachnospiraceae* bacterium, *Lachnospiraceae* bacterium NK4A144, *Anaerobiospirillum succiniciproducens*	-	-	NS
Glycogen/starch/α-glucan family phosphorylase	*Stomatobaculum longum*	-	-	NS
Glycogen/starch/α-glucan phosphorylase	*Aeromonas simiae*	-	-	NS
Hypothetical protein	*Treponema porcinum*	-	-	NS

Table 6.4 (continued).

Enzymes	Taxonomy	L vs C	L vs S	S vs C
1,4-α-Glucan branching protein GlgB	*Ruminobacter*	-	-	NS
α-D-glucose phosphate-specific phosphoglucomutase	*Ruminobacter*	-	-	NS
Glucose-6-phosphate isomerase	*Aeromonas*	-	-	NS
Phospho-sugar mutase	*Prevotella ruminicola*	-	-	NS
PKD domain-containing protein	*Succinivibrionaceae* bacterium WG-1, *Ruminobacter* sp. RM87	-	-	NS
ROK family protein	*Prevotellaceae* bacterium LKV-178-WT-2A	-	-	NS
UTP-glucose-1-phosphate uridylyltransferase GalU	*Anaerobiospirillum succiniciproducens*	-	-	NS
Pentose and glucuronate interconversions				
L-arabinose isomerase	*Lachnobacterium bovis*	+	+	NS
L-arabinose isomerase	*Prevotella* sp. tc2-*28*	+	+	NS
Arabinose isomerase	*Prevotella* sp. tc2-28	+	+	NS
Pectinesterase	*Prevotella* sp. PMUR	NS	-	+
Tagaturonate reductase	*Bacteroides pectinophilus*	+	+	NS
	Prevotella sp. BP1-145	+	+	NS
	Prevotella sp. tc2-28	+	+	NS
	Prevotella (multiple species)	+	+	NS
4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase	*Prevotella ruminicola*	+	+	NS
5-dehydro-4-deoxy-D-glucuronate isomerase	*Lachnospiraceae* bacterium NK3A20	+	+	-
Mannonate dehydratase	unclassified *Prevotella*	NS	+	-
Glucuronate isomerase	*Prevotella* sp. tf2-5	+	-	+
Glucuronate isomerase	*Prevotella* sp.	+	+	NS
Hypothetical protein PIROE2DRAFT_67719	*Piromyces* sp. E2	-	-	NS
L-ribulose-5-phosphate 4-epimerase	*Prevotella* sp. AGR2160	-	-	NS
L-ribulose-5-phosphate 4-epimerase	*Prevotella* sp. tc2-28	NS	+	NS
Lysophospholipase L1	*Treponema berlinense*	+	+	NS
UTP-glucose-1-phosphate Uridylyltransferase GalU	*Anaerobiospirillum succiniciproducens*	-	-	NS
Xylose isomerase	*Prevotella*	-	-	NS
Xylose isomerase	*Bacteroidales* bacterium	NS	-	+
Galactose metabolism				
6-Phosphofructokinase	*Lachnospiraceae* bacterium	NS	-	NS
6-Phosphofructokinase	*Oribacterium* sp. FC2011	-	-	NS
6-Phosphofructokinase	*Treponema saccharophilum*	-	-	NS

Table 6.4 (continued).

Enzymes	Taxonomy	L vs C	L vs S	S vs C
Alpha-galactosidase	*Prevotella* sp. P4-98	NS	-	+
DUF4982 domain-containing protein	*Prevotella* sp. 885	-	NS	-
Galactokinase	*Prevotella brevis*	NS	+	NS
Galactokinase	*Prevotella oris*	NS	NS	-
Galactokinase	*Prevotella* sp. tf2-5	+	+	NS
Galactokinase	*Prevotellaceae* bacterium LKV-178-WT-2A	NS	-	+
Galactose mutarotase	*Prevotella* sp. PMUR	+	+	NS
Galactose mutarotase	*Prevotella* sp. tf2-5	+	NS	NS
Glucokinase	*Escherichia coli*	-	-	NS
Hypothetical protein FGO68_gene11367	*Halteria grandinella*	NS	+	-
Multispecies: alpha-D-glucose Phosphate-specific Phosphoglucomutase	*Ruminobacter*	-	-	NS
Phosphoglucomutase	*Bacteroidales* bacterium	NS	+	NS
Phosphoglucomutase	*Prevotella* sp. tf2-5	NS	-	+
Phospho-sugar mutase	*Prevotella ruminicola*	-	-	NS
ROK family protein	*Prevotellaceae* bacterium LKV-178-WT-2A	-	-	NS
RpiB/LacA/LacB family sugar-phosphate isomerase	*Lachnospiraceae* bacterium oral taxon 500	+	+	NS
UDP-glucose 4-epimerase GalE	*Bacteroides graminisolvens*	+	+	NS
UDP-glucose 4-epimerase GalE	*Prevotella brevis*	NS	+	NS
UDP-glucose 4-epimerase GalE	*Prevotella* sp. P6B4	+	+	NS
UDP-glucose 4-epimerase GalE	*Prevotellaceae* bacterium LKV-178-WT-2A	-	-	NS
UTP--glucose-1-phosphate Uridylyltransferase GalU	*Anaerobiospirillum succiniciproducens*	-	-	NS

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. + and - indicate up- and down-regulated proteins significantly ($P \leq 0.05$), respectively in group 1 compared to 2. NS = not significant ($P > 0.05$).

Discussion

"Omics" techniques have opened new avenues in nutrition research, offering advanced approaches for exploring the complex metabolic pathways in response to diet. Rumen metagenomics addresses the collective genetic structure and functional composition of the ruminal microbes without the bias or necessity for culturing individual inhabitants. Rumen metaproteomic reflects the entire complement of proteins that is actually expressed by the rumen microbiome. The combination of these two omics technologies provides a more complete picture of how the microbiome reacts to dietary changes at both the gene and protein level. The present study aimed to show determining alterations in the ruminal microbial community and functions of dairy cows receiving three different dietary energy sources through a combination of the metagenomics and metaproteomics analysis.

Metagenomic analysis

Microbial community composition

The rumen microbial community plays a vital role in rumen digestion of feed and has been shown to vary significantly according to the animal's breed, gender, age, diet and other ecological factors (Jami et al., 2012, Morgavi et al., 2013, Jose et al., 2017). Nonetheless, most of the studies have confirmed the predominant role of bacterial phyla *Bacteroidetes* and *Firmicutes* in the rumen microbial community despite the diversity in diet, breed, or gender (Stevenson and Weimer, 2007, Henderson et al., 2015, Jose et al., 2017). In this study, the whole metagenome of 18 rumen fluid samples from dairy cows receiving two glucogenic diets and a lipogenic diet was sequenced, resulting in a total of 249 Gbps of sequencing data. According to the taxonomic analysis, the average composition of the rumen microbial community among all samples was dominated by 45.1% of *Bacteriodetes* and 36.3% of *Firmicutes*. However, the relative abundances of these predominant microbiomes were diverse among diets, which agrees with the statement that the ruminal microbial community structure is affected by changes of dietary composition (Morgavi et al., 2013). More specifically, when diet L was fed, bacterial *Firmicutes* at the phyla level predominated, while with diets C and S, the *Bacteroidetes* was the most abundant. *Prevotella* was the dominant genus among all samples, indicating its important role in rumen digestion. This numerical dominance of *Prevotella* observed here is in accordance with the study of Stevenson and Weimer (2007) who used relative quantification real-time PCR and Jose et al. (2017) who used a metagenomics approach.

The analysis of the taxonomic distribution also indicated that the genera *Prevotella* contributed the most to all CAZymes.

Alterations of amylolytic and fibrolytic microbes

In order to detect the sensitivity and preference of the typical amylolytic and fibrolytic bacteria to dietary starch and fibre alteration, these bacteria were specifically compared. The amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* were higher when the cows were fed diets C and S, indicating their high sensitivity to dietary starch alteration. Similarly, the fibrolytic bacteria of *Ruminococcus flavefaciens* and *Lachnospira multipara* might be more sensitive to dietary fibre content. Besides the abovementioned bacteria, the *Prevotella albensis*, *Eubacterium* sp. CAG:202, *Ruminococcus* sp. CAG:108, *Succinivibrionaceae* bacterium WG-1, *Ruminobacter* sp. RM87, *Lachnobacterium bovis*, *Alloprevotella rava*, *Sutterella wadsworthensis*, *Eubacterium* sp CAG:603, *Bacteroides* sp. CAG:530, *Prevotella* sp. P4-65 and *Bacteroides graminisolvens* might contribute to the amylolytic activities of diets C and S. The bacteria of *Parabacteroides distasonis*, *Clostridium* sp. CAG:433, *Clostridium* sp. CAG:678 and *Prevotella albensis* might play important roles in the fibre digestion in diet L. In total, the typical reported amylolytic and fibrolytic microbes showed different sensitivities to dietary starch and fibre changes. Some of them are very stable to the dietary starch and fibre alteration, which indicates other yet identified species or microbial interactions might be involved in amylolytic and fibrolytic activities in the rumen. The abovementioned species detected in the present study may provide candidate species for future identification of new amylolytic and fibrolytic microbes.

CAZymes and function analysis

The CAZy database (http://www.cazy.org/) includes the diverse enzyme groups that degrade, modify, or create glycosidic bonds, which has been widely applied in ruminal enzymatic research (Cantarel et al., 2009). This database possesses manually curated information for all CAZyme families so that it can be used to evaluate the known enzymes or families involved in certain activities, such as amylolysis, cellulolysis and hemicellulolysis. It is widely reported that the GH family enzymes are the most abundant catalytic enzymes accounting for more than 50% of all enzymes classified into the CAZy database. The GH level (52.6%) of all CAZymes classes in the present study is in line with a previous study (Gharechahi et al., 2021), which explains the capacity of GHs in breaking down plant polysaccharides.

Starch is degraded in the rumen by a series of enzymes mainly including α-amylase, β-amylase, α-glucosidase, glucoamylase, isoamylase and pullulanase, etc., produced by rumen microorganisms (Kotarski et al., 1992). The GH13, GH57 and their subfamilies are known as the main α-amylase families (Shen et al., 2020). The GH14 comprises mostly β-amylase and the GH15 and GH31 contain mainly α-glucosidase (Vorgias and Antranikian, 2002). In the present study, they showed different sensitivities to dietary starch in the present study. Among these amylolytic enzymes, only enzymes in GH13 subfamilies were significantly influenced by diets (**Table 6.2**). As a review of the classification of glycosidase in families showed (Henrissat, 1991), the majority of the enzymes acting on starch, glycogen and related oligo- and polysaccharides belong to the family GH13. The current GH13 family contains 44 subfamilies (April 2022), 32 of which were detected in the present study. When diets C and S compared to L were fed, more amylolytic enzymes including α-amylase (GH13_36, GH13_28, GH13_15, GH13_2, GH13_37, GH13_42 and GH13_27), pullulanase (GH13_13 and GH13_14) and maltose glucosylmutase (GH13_16) were observed. Among the affected enzymes, GH13_15, GH13_16, GH13_27, GH13_28 and GH13_37 are monospecific subfamilies, while the remaining subfamilies contain more than one reported activity (Stam et al., 2006). Nonetheless, the other reported amylolytic GH families were not observed as being sensitive to the three diets containing different energy sources.

Similarly, fibrolytic activity has been reported (Wang et al., 2019, Shen et al., 2020) to be mainly associated with five GH families (GH5, GH9, GH45, GH88 and GH97). The endoglycosylceramidase (GH5_28) and cellobiohydrolases (GH48) were observed to be higher when diet L was fed compared to diets C and S (**Table 6.2**). When cows received diet C, the rumen fluid contained more cellulase (GH5_25 and GH5_26), a-L-arabinofuranosidase (GH5_13) and xyloglucanases (GH16) compared to when cows received diet L (**Table 6.2**). GH16 is a polyspecific family of β-glycanases involved in the degradation or remodelling of cell wall polysaccharides in biomass (Viborg et al., 2019). The GH5 family was the first cellulase family described (Aspeborg et al., 2012) within which a diversity of enzyme activities was observed (e.g., cellulases, mannanases, xylanases, galactanases and xyloglucanases). Subfamily GH5_25 (derived mainly from thermophiles) and GH5_26 (derived mainly from uncultured microorganisms) possess multiple enzymatic activities besides endo-β-1,4-glucanase, such as exhibition of β-mannan-based and β-glucan-based polymers and activity against lichenan (Voget et al., 2006). GH48 was reported as a critical component of numerous natural lignocellulose-degrading systems. Even though GH48 was not highly abundant in the

genomes of cellulose-degrading bacteria compared to GH5 and GH9 in the present study, GH48 cellulases often act in synergy with GH9 cellulases which could increase their catalytic activity significantly (Sukharnikov et al., 2012). The present study indicates that the fibrolytic GH families show diverse preferences to fibre sources, such as corn silage (in diets C and S), alfalfa silage and sugar beet pulp (diet L).

In addition, according to the KEGG pathway analysis, the pathway of starch and sucrose metabolism was up-regulated by diets C and S. By comparing the enzymes involved in this pathway, we found diets C and S had higher abundances of α-amylase, pullulanase and maltose α-D-glucosyltransferase. All these enzymes belong to the amylolytic enzyme family GH13. In addition, diet L had higher abundances of cellulase (GH5) and isoamylase (GH13). Thus, this KEGG pathway analysis was largely in line with the CAZy results.

Metaproteomics analysis

Predictions based on gene sequences from culture-independent metagenomic techniques have shown that there was considerable diversity in the rumen ecosystem of the dairy cows when receiving the three dietary energy sources. However, it is difficult to determine the microbial functionality only through analysing the predicted expression of key enzymes. In this regard, a metaproteomics approach was performed to assess the relationship between the expressed proteins and the predicted proteins by gene expression patterns in the derived organisms from metagenomics data.

Taxonomic analysis

Taxonomic analysis from metaproteomics data was conducted by searching the identified proteins against the NCBI NR database. By comparing the taxonomic structures detected from both metagenomics and metaproteomics analysis, the results from metaproteomics were in line with the taxonomic profiles from metagenomics which showed that *Bacteriodetes*, *Firmicutes* and *Proteobacteria* were the most highly abundant phyla, which is also consistent with a previous study (Hart et al., 2018). However, the relative abundances of phyla *Proteobacteria* and *Ciliophora* became higher in metaproteomic analysis relative to metagenomic analysis. Similarly, *Prevotella* was the predominant genera in both metagenomics and metaproteomics analysis, the rest genera varied in their relative abundances.

The comparison of amylolytic and fibrolytic microbes showed that when the cows were fed the two starchy diets (C and S), rumen fluid contained a higher abundance of the amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* which was inconsistent with the metagenomics data. In addition, the relative abundance of *Selenomonas ruminantium* was higher in diet S than diets C and L, in both the metagenomics and metaproteomics analysis. For the fibrolytic bacteria, the results were also in line with the gene prediction results by the metagenomics analysis. *Ruminococcus flavefaciens* and *Lachnospira multipara* has a significantly higher abundance with the fibrous diet L while they also had a higher abundance in diet L though not to a significant level with the protein-based taxonomic analysis. In addition, comparing the two starchy diets C and S, *Selenomonas ruminantium* was observed to have a higher relative abundance in diet S compared to C both at the gene and protein level.

Functions and enzymes

One of the major advantages of metaproteomics is the ability to assess the pathway alterations by directly profiling the expressed proteins. To better understand the biological functions of the DEPs, they were blasted against the KEGG database. We further analysed the enzymes involved in starch and sucrose metabolism, pentose and glucuronate interconversions, and galactose metabolism which were detected as being significantly regulated in the metagenomics analysis. Similar to the gene function prediction results of the metagenomics analysis, the starch and sucrose metabolism pathway was also observed to be up-regulated at the protein level by diets C and S. For the affected enzymes, the cellulase, α-amylase, isoamylase and pullulanase were observed to have the same results with metagenomics analysis. Therefore, based on these observations it can be concluded that the starchy diets of C and S promoted the number of amylolytic bacteria of *Succinimonas amylolytica*, leading to a higher production of the amylolytic enzyme pullulanase, which contributes to the up-regulated pathway of the starch and sucrose metabolism. Similarly, the L-arabinose isomerase and tagaturonate reductase in the pentose and glucuronate interconversions pathway were up-regulated in the rumen fluid samples of diet L compared to C and S both at a gene and protein level, which might be attributed to the higher abundances of *Prevotella* sp. tc2-28 and *Bacteroides pectinophilus*. Amylopullulanase produced by *Selenomonas ruminantium* was up-regulated in diet S than diet C, which agrees with a higher relative abundance of this organism in diet S. Amylopullulanse has the enzymatic activities of both alpha-amylase and pullulanase and is classified into the GH13 and GH57 families, which is mostly produced by *Lactobacillus* spp. (Vishnu et al., 2006). This result offers new insight for studying the enzymatic activities of *Selenomonas ruminantium*.

Conclusion

According to the gene predictions by metagenomics analysis and taxonomic analysis of the secreted proteins by metaproteomics, we found that most amylolytic and fibrolytic bacterial communities were unaffected by the starch or fibre alterations in the concentrate diets. The amylolytic bacteria of *Succinimonas amylolytica* and *Ruminococcus bromii* were observed to be sensitive to starch as an energy source relative to fibre in the diet, of which the higher amount of *Succinimonas amylolytica* led to increased production of pullulanase, thereby, contributing to the upregulation of the starch and sucrose metabolism. The processing of corn by steam-flaking, resulted in a higher proportion of the *Selenomonas ruminantium* compared to ground corn both at the metagenomic and metaproteomic level, which would suggest a higher production of amylopullulanase. The combination of metagenomics and metaproteomics analysis showed to be a powerful approach for future research with an aim of investigating the activities of certain microbial communities in response to diet changes in the rumen of dairy cows.

Acknowledgement

We appreciate the Breeding Farm of Beijing Centre for Dairy Cows, Beijing, China, for offering the experimental cows. The study was financially supported by the National Key R&D Program of China (2021YFE 2000800, 2019YFE0125600) and Project of the National Nature Science Foundation of China (grant No. 31572435).

Supplementary materials

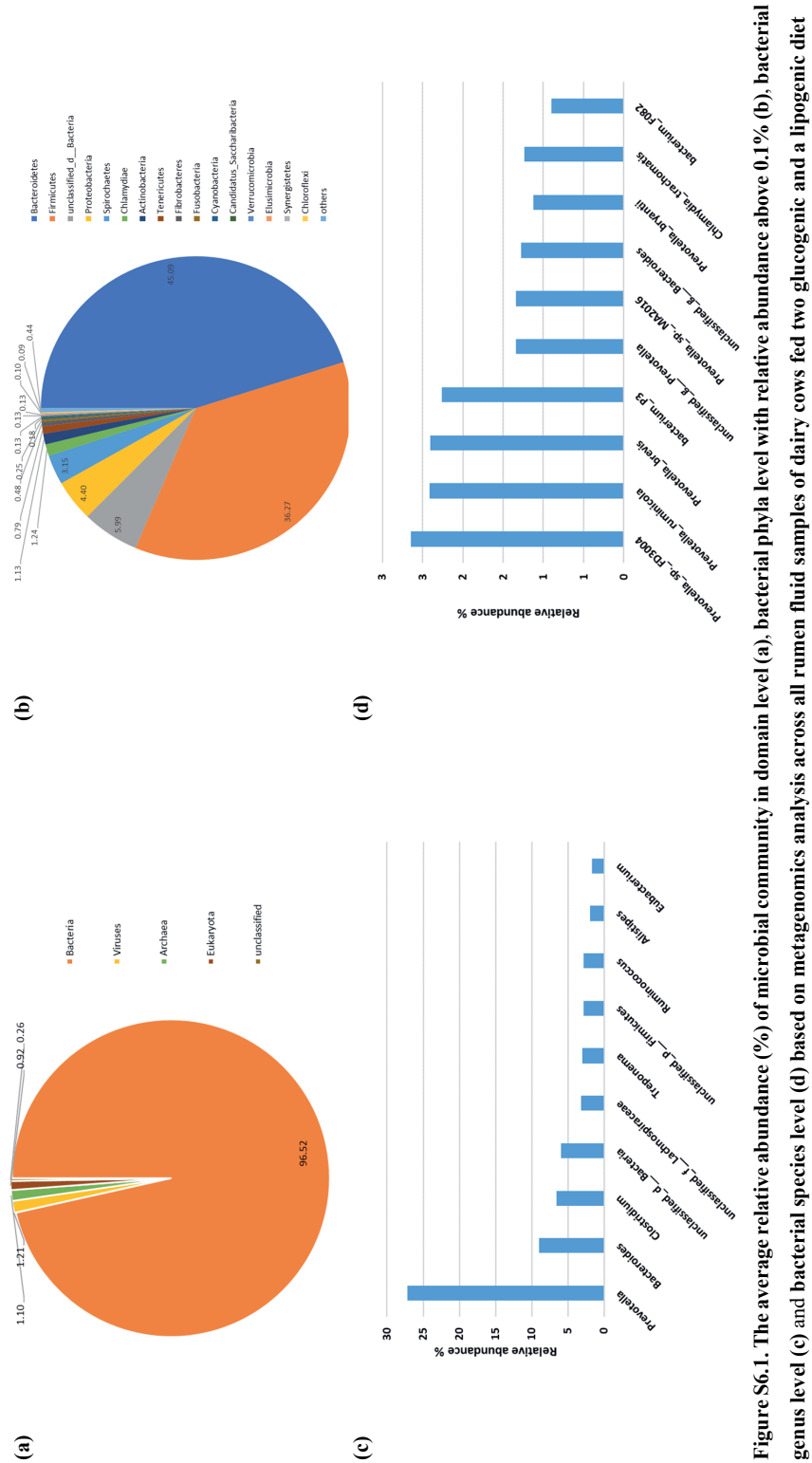


Figure S6.1. The average relative abundance (%) of microbial community in domain level (a), bacterial phyla level with relative abundance above 0.1% (b), bacterial genus level (c) and bacterial species level (d) based on metagenomics analysis across all rumen fluid samples of dairy cows fed two glucogenic and a lipogenic diet

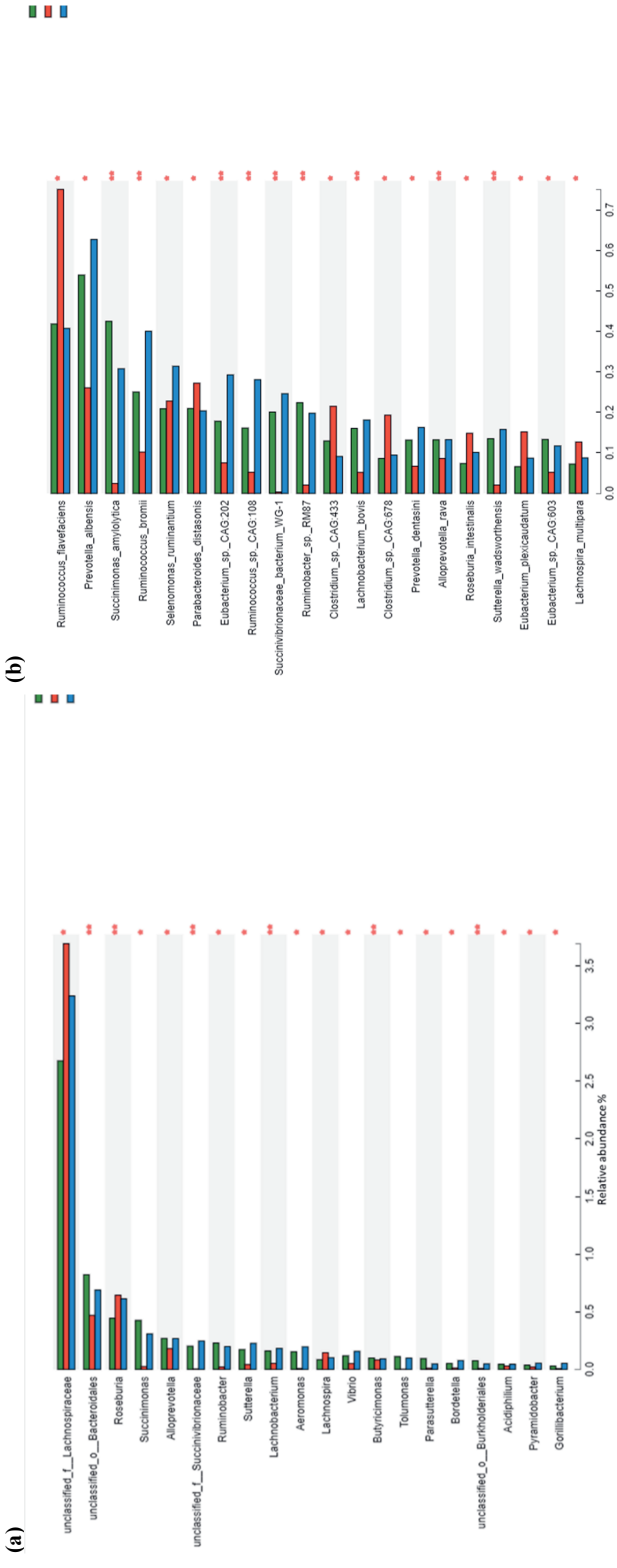


Figure S6.2. Comparison of top 20 affected bacterial genera (a) and species (b) based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. *, $P \leq 0.05$; **, $P \leq 0.001$.

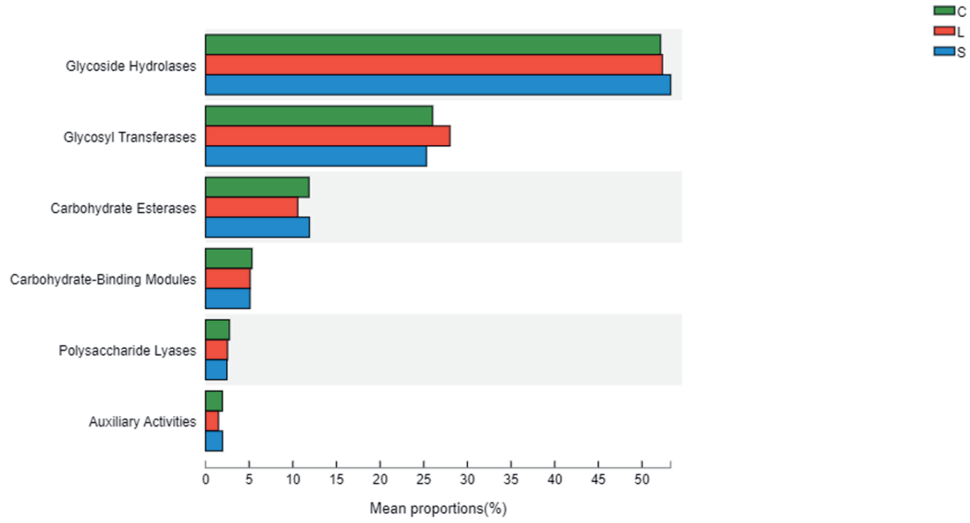


Figure S6.3. Comparison of the CAZyme classes based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

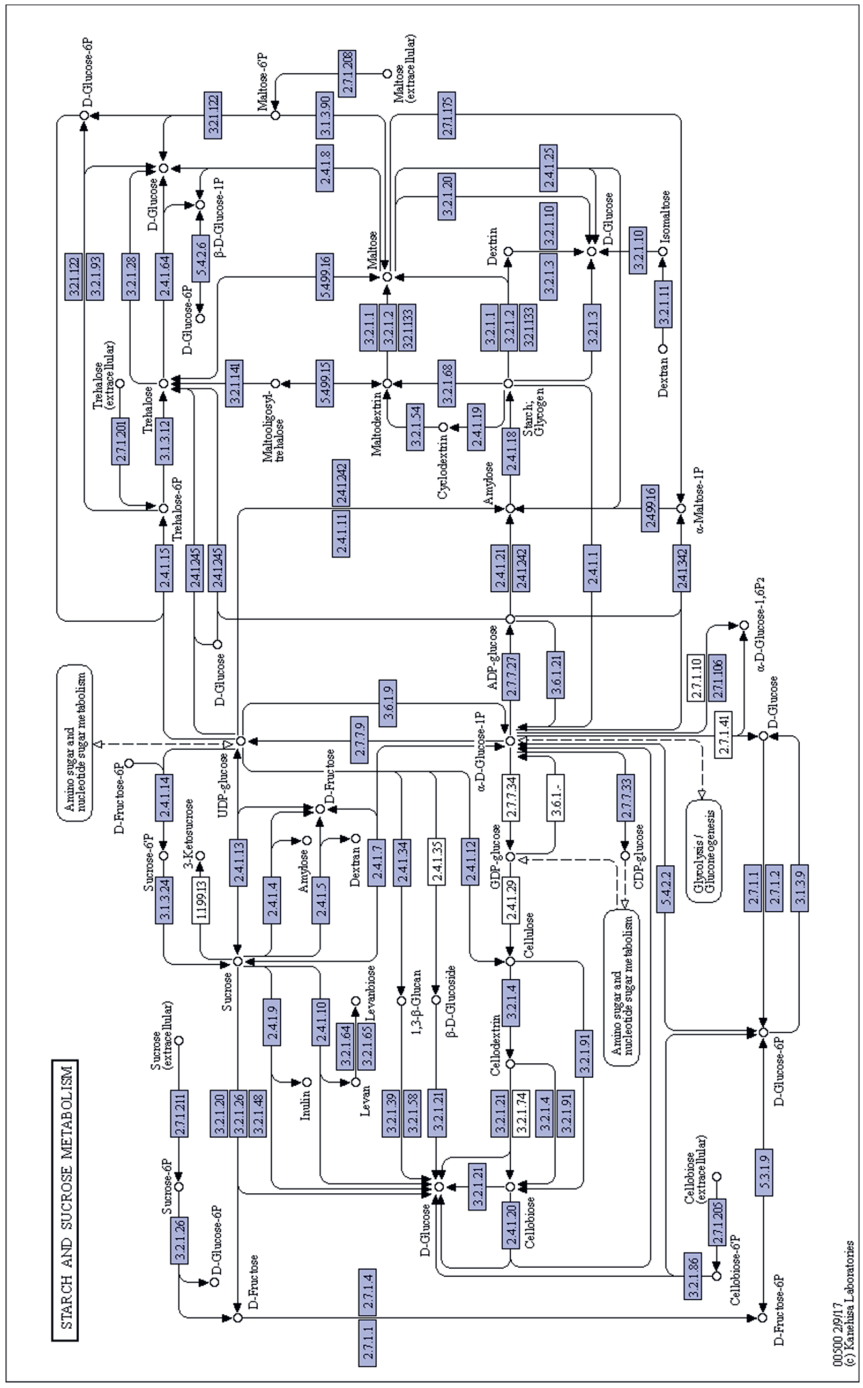


Figure S6.4a. KEGG pathway map of the starch and sucrose metabolism based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, detected enzymes; box in white, no protein was detected.

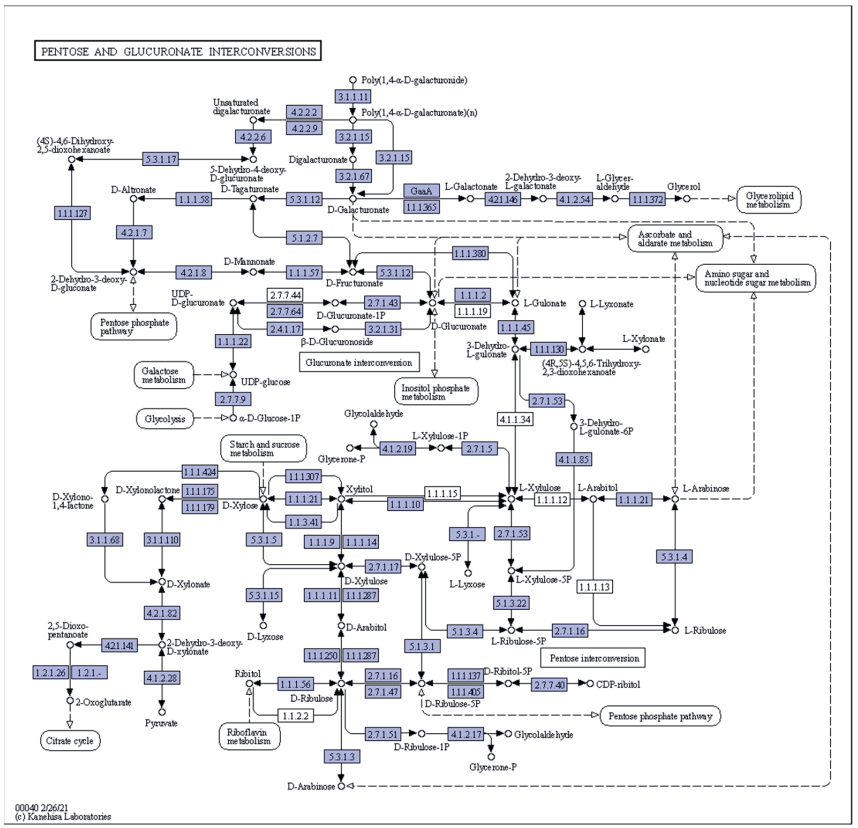


Figure S6.4b. KEGG pathway map of petose and glucuronate interconversions based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, detected enzymes; box in white, no protein was detected.

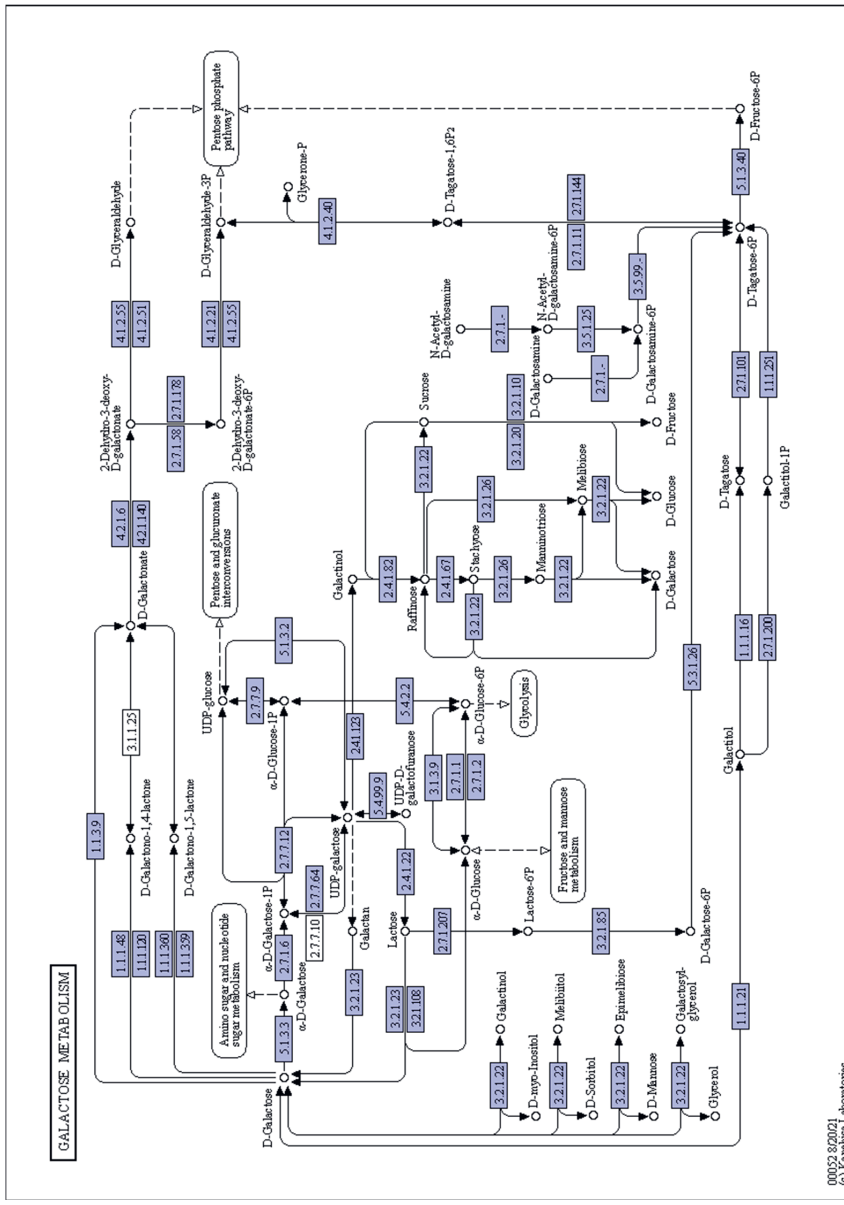


Figure S6.4c. KEGG pathway map of galactose metabolism based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, detected enzymes; box in white, no protein was detected.

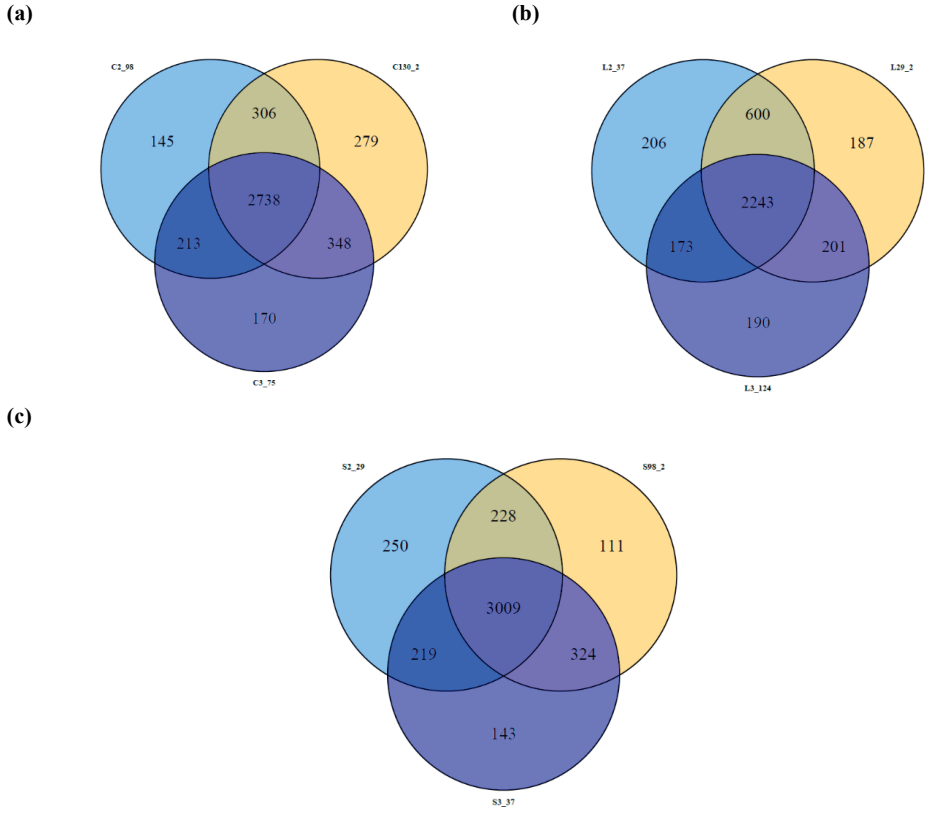


Figure S6.5. Protein numbers identified by metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. a) three rumen fluid samples from dairy cows fed diet C; b) three rumen fluid samples from dairy cows fed diet L; c) three rumen fluid samples from dairy cows fed diet S. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

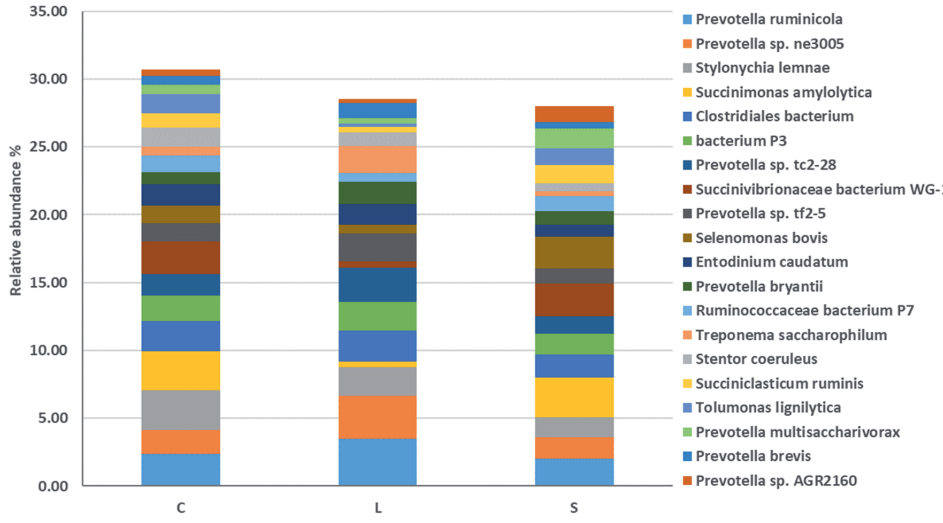


Figure S6.6. Top 20 bacterial species derived from metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet.

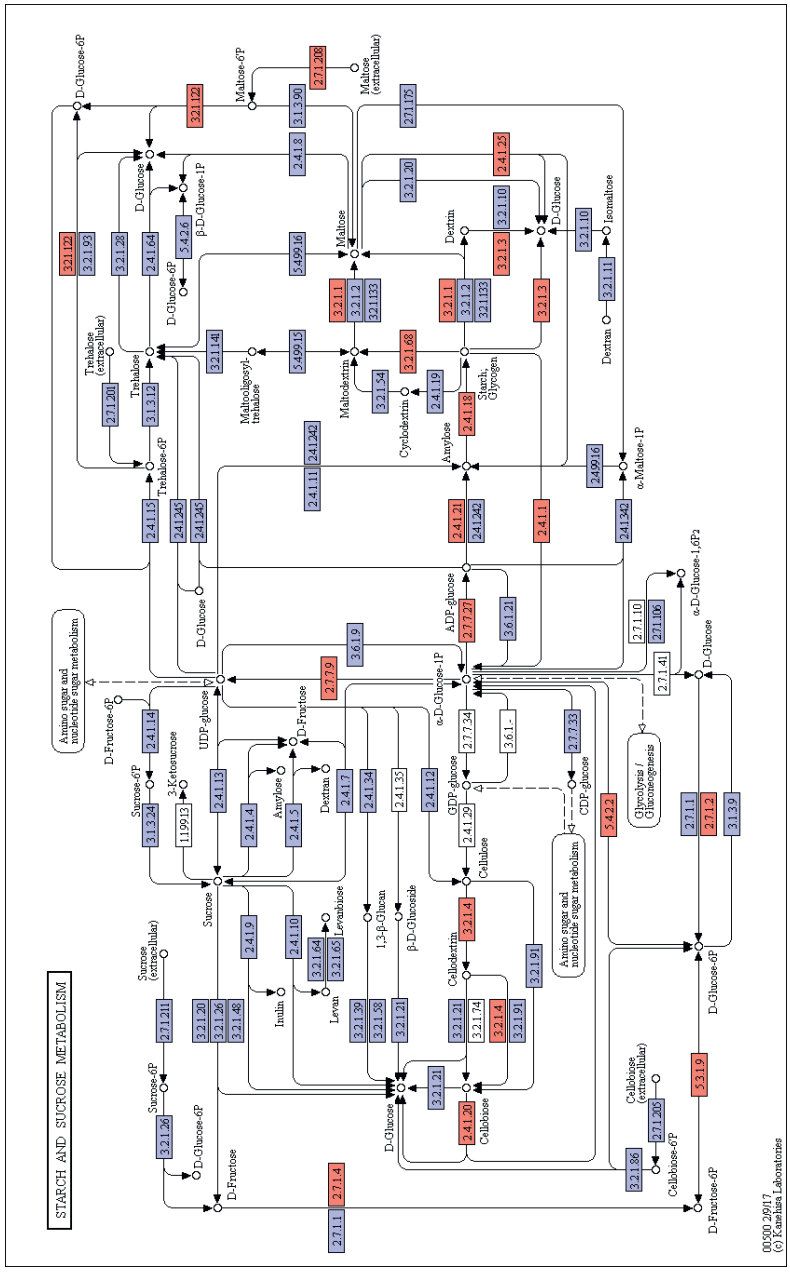


Figure S6.7a. Pathway map of starch and sucrose metablism annotated by metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, enzymes annotated with the detected proteins that are not affected by treatments; the white box, no proteins were detected; box in red, enzymes annotated with the detected proteins that were significantly influenced by treatments.

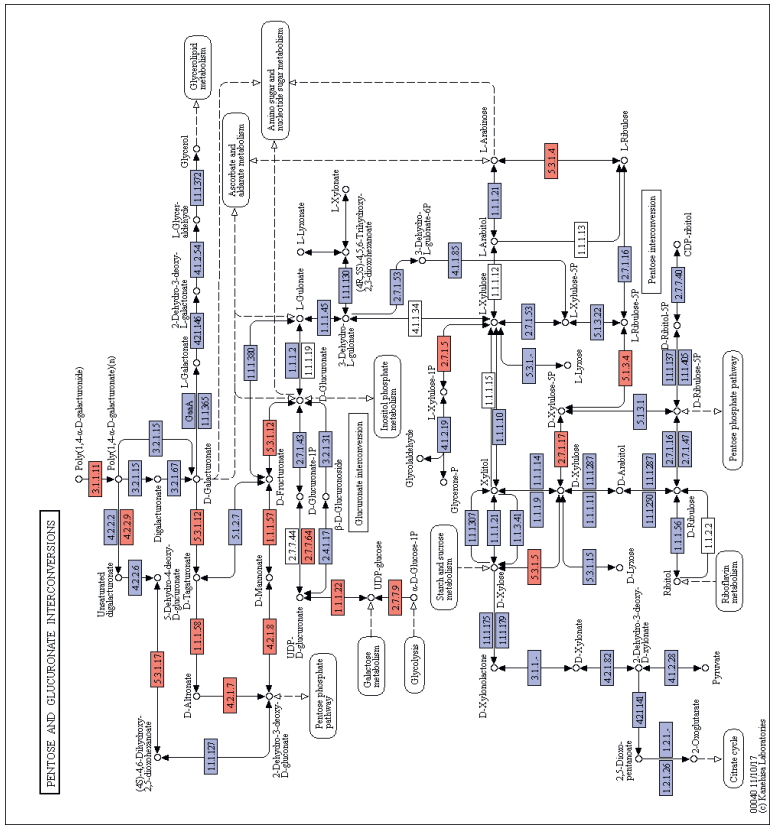


Figure S6.7b. Pathway map of pentose and glucuronate interconversions annotated by metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, enzymes annotated with the detected proteins that are not affected by treatments; the white box, no proteins were detected; box in red, enzymes annotated with the detected proteins that were significantly influenced by treatments.

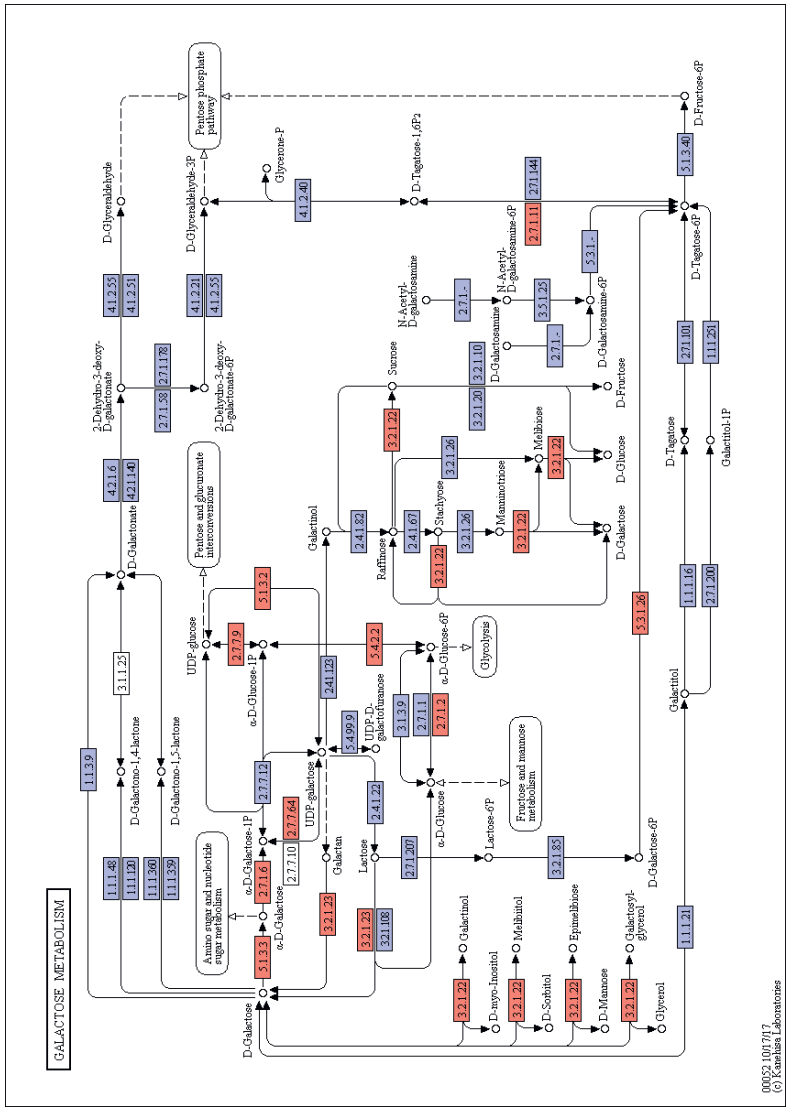


Figure S6.7c. Pathway map of galactose metabolism annotated by metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet. Box in blue, enzymes annotated with the detected proteins that are not affected by treatments; the white box, no proteins were detected; box in red, enzymes annotated with the detected proteins that were significantly influenced by treatments.

Table S6.1 The sequence information for metagenomics analysis from all rumen fluid samples of dairy cows fed two glucogenic and a lipogenic diet

Raw reads	Optimized reads	Contig number	N50(bp)	ORFs	Catalog genes
1,649,534,584	1,207,764,624	18,627,023	673.4	24,620,061	8,114,000

N50, the sum of the lengths of all contigs of size N50 or longer contain at least 50% of the total genome sequence; ORFs, open reading frames.

Table S6.2. Comparison of putative GH families (% of each taxon relative to total GHs) involved in fibre and starch degradation based on the metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Enzyme/GH taxa	Major activity	C	L	S	SEM	*P*-value
Starch degrading enzymes						
GH1	β-Glucosidases	0.30	0.26	0.40	0.044	0.203
GH2	β-galactosidases	10.20	8.39	9.36	0.431	0.484
GH3	β-Glucosidases	4.00	3.16	3.87	0.231	0.386
GH13	α-Amylase	1.80	1.26	1.76	0.105	0.191
GH13_1	α-Amylase	0.09	0.09	0.10	0.015	0.983
GH13_10	α-Amylase	0.07	0.05	0.07	0.009	0.587
GH13_11	4-α-glucanotransferase	0.30	0.28	0.29	0.020	0.977
GH13_13	Pullulanase	0.50^{a}	0.34^{b}	0.55^{a}	0.033	0.022
GH13_14	α-Glycosidase	0.16^{ab}	0.09^{b}	0.21^{a}	0.023	0.017
GH13_15	α-Amylase	0.10^{a}	0.05^{b}	0.12^{a}	0.012	0.022
GH13_16	Maltose glucosylmutase	0.03^{a}	0.01^{b}	0.02^{a}	0.001	0.015
GH13_17	α-Glucosidase	0.01	0.01	0.01	0.001	0.236
GH13_18	Sucrose 6(F)-phosphate phosphorylase	0.03	0.02	0.03	0.001	0.529
GH13_19	α-Amylase	0.17	0.09	0.14	0.017	0.372
GH13_2	α-Amylase	0.08^{a}	0.03^{b}	0.07^{a}	0.007	0.003
GH13_20	Cyclic a-1,6-maltosyl-maltose hydrolase	0.53^{ab}	0.43^{b}	0.68^{a}	0.041	0.090
GH13_21	α-Amylase	0.06	0.05	0.06	0.008	0.567
GH13_24	α-Amylase	0.02	0.02	0.02	0.001	0.810
GH13_25	4-α-Glucanotransferase	0.01	0.01	0.01	0.001	0.932
GH13_27	α-Amylase	0.03	0.01	0.02	0.001	0.078
GH13_28	α-Amylase	0.16^{a}	0.04^{b}	0.13^{a}	0.016	0.003
GH13_29	α-Glucosidase	0.03	0.02	0.04	0.001	0.372
GH13_3	α-1,4-glucan:phosphate a-maltosyltransferase	0.01	0.01	0.01	0.000	0.751
GH13_31	α-Glucosidase	0.32	0.24	0.34	0.044	0.368
GH13_32	α-Amylase	0.01	0.01	0.01	0.001	0.983
GH13_36	α-Amylase	0.64^{a}	0.37^{b}	0.63^{a}	0.048	0.015
GH13_37	α-Amylase	0.06^{a}	0.00^{b}	0.07^{a}	0.014	0.004
GH13_38	α-Glucosidase	0.75	0.68	0.72	0.018	0.372
GH13_4	Amylosucrase	0.16	0.11	0.17	0.027	0.505
GH13_40	Oligo-a-1,6-glucosidase	0.08	0.07	0.08	0.007	0.755
GH13_42	α-Amylase	0.04^{a}	0.01^{b}	0.03^{a}	0.001	0.006

Table S6.2 (continued).

Enzyme/GH taxa	Major activity	C	L	S	SEM	*P*-value
GH13_5	α-Amylase	0.11	0.10	0.13	0.014	0.676
GH13_6	α-Amylase	0.41	0.30	0.37	0.020	0.059
GH13_7	α-Amylase	0.29	0.24	0.29	0.015	0.191
GH13_8	α-1,4-Glucan branching enzyme	0.44	0.36	0.45	0.023	0.236
GH13_9	α-1,4-Glucan branching enzyme	0.37	0.30	0.45	0.034	0.402
GH18	β-Glucosidases	0.52	0.38	0.57	0.036	0.130
GH20	β-Hexosaminidase	1.17[a]	0.76[b]	1.16[a]	0.094	0.091
GH27	α-Galactosidase	0.52	0.41	0.46	0.031	0.309
GH29	α-L-fucosidosis	1.57	1.22	1.52	0.095	0.220
GH31	α-Glucosidase	2.69	2.28	2.53	0.130	0.484
GH32	Invertase, endo-inulinase	1.24	1.00	1.27	0.082	0.325
GH35	β-Galactosidases	0.83	0.80	0.70	0.038	0.198
GH38	α-Mannosidases	0.12	0.10	0.13	0.010	0.493
GH39	β-Xylosidases	0.19	0.17	0.17	0.014	0.778
GH42	β-Galactosidases	0.18	0.18	0.21	0.015	0.700
GH43_16	β-Glycosidase	0.01	0.01	0.01	0.000	0.834
GH57	α-Amylase	0.64	0.48	0.57	0.032	0.139
GH92	α-1,2-Mannosidase	1.89	1.24	1.86	0.149	0.114
GH94	Cellobiose phosphorylase	1.63	1.55	1.62	0.080	0.895
GH97	α-Glucosidase	3.91	3.21	3.72	0.171	0.557
GH130	β-1,4-Mannosylglucose phosphorylase	0.68[a]	0.49[b]	0.63[a]	0.034	0.090
Cellulose degrading enzymes						
GH95	α-L-Fucosidase	2.39	1.98	2.31	0.092	0.459
GH9	Endoglucanase	0.75	0.62	0.71	0.040	0.359
GH88	β-Glucuronyl hydrolase	0.34	0.23	0.31	0.025	0.128
GH44	Endoglucanase	0.02	0.02	0.01	0.001	0.470
GH45	Endoglucanase	0.02	0.01	0.02	0.001	0.581
GH48	Cellobiohydrolases	0.00[b]	0.02[a]	0.00[b]	0.001	0.045
GH5	Cellulases	0.20	0.17	0.19	0.012	0.264
GH13_14	α-Glycosidase	0.16[ab]	0.09[b]	0.21[a]	0.023	0.017
GH5_13	β-D-galactofuranosidase	0.14[a]	0.08[b]	0.12[ab]	0.011	0.032
GH5_26	β-Glycosidase	0.03[a]	0.01[b]	0.02[ab]	0.003	0.042
GH5_25	Endo-β-1,4-glucanase	0.02[a]	0.01[b]	0.02[b]	0.002	0.012

Table S6.2 (continued).

Enzyme/GH taxa	Major activity	C	L	S	SEM	*P*-value
GH5_28	Endoglycosylceramidase	0.00[b]	0.02[a]	0.01[b]	0.003	0.080
Hemicellulose degrading enzymes						
GH8	Endoxylanses,	0.38	0.30	0.31	0.019	0.135
GH53	Endo-1,4-b-galactanases	1.52	1.32	1.50	0.064	0.587
GH28	Polygalacturonase	2.56	2.57	2.41	0.101	0.331
GH26	β-mannanase and xylanases	1.07	1.03	1.00	0.043	0.296
GH12	Xyloglucanases	0.00	0.00	0.00	0.000	0.119
GH11	Xylanases	0.03	0.01	0.03	0.001	0.139
GH10	Endo-1,4-b-xylanases	1.61	1.32	1.39	0.069	0.209
GH43_1	β-Xylosidase	0.46	0.35	0.39	0.026	0.203
GH43_2	α-L-arabinofuranosidase	0.18	0.20	0.17	0.011	0.444
GH43_3	β-D-galactofuranosidase	0.21[a]	0.16[b]	0.20[a]	0.009	0.078
GH43_4	Exo-a-1,5-L-arabinanase	0.56	0.47	0.47	0.031	0.343
GH43_5	Exo-a-1,5-L-arabinanase	0.44	0.40	0.38	0.027	0.557
GH43_6	Exo-a-1,5-L-arabinanase	0.00	0.00	0.00	0.000	0.283
GH43_7	Endo-b-1,4-xylanase	0.33	0.35	0.30	0.022	0.476
GH43_8	β-D-galactofuranosidase	0.02	0.01	0.02	0.000	0.119
GH43_9	α-L-arabinofuranosidase	0.08	0.05	0.06	0.008	0.529
GH43_10	Xylan 1,4-b-xylosidase	1.11	1.02	1.01	0.051	0.688
GH43_11	β-1,3-Xylosidase	0.03	0.02	0.03	0.001	0.676
GH43_12	Xylan 1,4-β-xylosidase	0.34	0.30	0.33	0.016	0.960
GH43_13	Exo-a-1,5-L-arabinanase	0.00	0.00	0.00	0.000	0.184
GH43_14	Xylan 1,4-β-xylosidase	0.05	0.04	0.04	0.001	0.312
GH43_15	Exo-a-1,5-L-arabinanase	0.00	0.00	0.00	0.000	0.949
GH43_17	α-L-arabinofuranosidase	0.18	0.17	0.19	0.014	0.796
GH43_18	α-L-arabinofuranosidase	0.11	0.10	0.09	0.006	0.343
GH43_19	α-L-arabinofuranosidase	0.57	0.50	0.46	0.028	0.109
GH43_20	α-L-arabinofuranosidase	0.01	0.01	0.01	0.000	0.359
GH43_21	α-L-arabinofuranosidase	0.00	0.01	0.00	0.000	0.135
GH43_22	α-L-arabinofuranosidase	0.02	0.01	0.02	0.000	0.386
GH43_23	α-L-arabinofuranosidase	0.00	0.01	0.00	0.000	0.261
GH43_24	Exo-β-1,3-galactanase	0.28	0.22	0.23	0.018	0.884
GH43_25	Exo-α-1,5-L-arabinanase	0.01	0.00	0.00	0.000	0.802
GH43_26	Exo-α-1,5-L-arabinofuranosidase	0.06	0.06	0.06	0.001	0.960

Table S6.2 (continued).

Enzyme/GH taxa	Major activity	C	L	S	SEM	*P*-value
GH43_27	Xylan 1,4-β-xylosidase	0.02	0.02	0.02	0.001	0.359
GH43_28	Exo-α-1,5-L-arabinanase	0.13	0.08	0.13	0.020	0.587
GH43_29	α-1,2-L-arabinofuranosidase	0.55	0.50	0.51	0.024	0.476
GH43_30	β-D-galactofuranosidase	0.00	0.00	0.00	0.000	0.162
GH43_31	β-D-galactofuranosidase	0.10	0.08	0.10	0.006	0.421
GH43_32	β-D-galactofuranosidase	0.00	0.00	0.00	0.000	0.651
GH43_33	α-L-arabinofuranosidase	0.07	0.04	0.05	0.006	0.160
GH43_34	β-D-galactofuranosidase	0.02	0.02	0.02	0.001	0.470
GH43_35	Xylan 1,4-β-xylosidase	0.54	0.50	0.53	0.027	0.688
GH43_36	α-L-arabinofuranosidase	0.01	0.00	0.01	0.000	0.653
GH43_37	β-D-galactofuranosidase	0.03[b]	0.04[a]	0.03[b]	0.001	0.075
GH16	Xyloglucanases	0.88[a]	0.70[b]	0.79[a]	0.030	0.034
GH74	Xyloglucanases	0.29	0.39	0.31	0.025	0.347
GH43	Arabino/xylosidases	0.67	0.57	0.59	0.032	0.372

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.
[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S6.3. Comparison of the carbohydrate metabolism KEGG pathways based on the metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Pathways in carbohydrate metabolism	C	L	S	SEM	*P*-value
Starch and sucrose metabolism	13.51[a]	12.64[b]	13.65[a]	0.175	0.036
Amino sugar and nucleotide sugar metabolism	13.01	13.78	13.09	0.228	0.475
Glycolysis/Gluconeogenesis	11.68	11.54	11.74	0.048	0.274
Pyruvate metabolism	9.29	9.17	9.18	0.087	0.834
Galactose metabolism	9.22[b]	9.57[a]	9.04[b]	0.079	0.039
Fructose and mannose metabolism	6.91	6.83	6.98	0.085	0.796
Glyoxylate and dicarboxylate metabolism	6.70	6.72	6.68	0.043	0.959
Pentose phosphate pathway	6.50	6.70	6.54	0.069	0.604
Citrate cycle (TCA cycle)	6.36	5.81	6.28	0.148	0.488
Butanoate metabolism	5.55	5.34	5.51	0.098	0.738
Pentose and glucuronate interconversions	4.20[b]	4.60[a]	4.21[b]	0.077	0.044
Propanoate metabolism	3.59	3.65	3.59	0.040	0.819
C5-Branched dibasic acid metabolism	1.69	1.61	1.75	0.058	0.595
Inositol phosphate metabolism	0.93	1.02	0.93	0.033	0.597
Ascorbate and aldarate metabolism	0.86	0.96	0.84	0.027	0.324

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S6.4. Comparison of top 50 bacteria species based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Species	C	L	S	SEM	*P*-value
Prevotella ruminicola	2.35[b]	3.49[a]	2.02[b]	0.256	0.016
Prevotella sp. ne3005	1.79[b]	3.17[a]	1.56[b]	0.27	0.002
Stylonychia lemnae	2.92	2.12	1.46	0.373	0.315
Succinimonas amylolytica	2.89[a]	0.41[b]	2.97[a]	0.429	<0.001
Clostridiales bacterium	2.20	2.30	1.71	0.213	0.542
Bacterium P3	1.90	2.08	1.53	0.106	0.066
Prevotella sp. tc2-28	1.57[b]	2.56[a]	1.29[b]	0.198	<0.001
Succinivibrionaceae bacterium WG-1	2.42[a]	0.46[b]	2.38[a]	0.332	<0.001
Prevotella sp. tf2-5	1.34[b]	2.05[a]	1.15[b]	0.144	0.001
Selenomonas bovis	1.29[ab]	0.61[b]	2.33[a]	0.303	0.034
Entodinium caudatum	1.56	1.56	0.85	0.237	0.414
Prevotella bryantii	0.88	1.61	1.00	0.194	0.290
Ruminococcaceae bacterium P7	1.24	0.66	1.15	0.207	0.541
Treponema saccharophilum	0.68[b]	1.97[a]	0.35[b]	0.262	0.001
Stentor coeruleus	1.38	1.01	0.55	0.184	0.190
Succiniclasticum ruminis	1.05[a]	0.43[b]	1.38[a]	0.145	0.001
Tolumonas lignilytica	1.38	0.24	1.21	0.26	0.148
Prevotella multisaccharivorax	0.75	0.37	1.47	0.221	0.104
Prevotella brevis	0.64[b]	1.16[a]	0.50[b]	0.111	0.007
Prevotella sp. AGR2160	0.44[ab]	0.29[b]	1.14[a]	0.152	0.017
Prevotella copri	0.35	0.44	1.03	0.148	0.105
Ruminobacter amylophilus	0.78[a]	0.18[b]	0.87[a]	0.118	0.004
Ruminobacter sp. RM87	0.84[a]	0.09[b]	0.85[a]	0.135	0.002
Prevotellaceae bacterium HUN156	0.51	0.84	0.40	0.092	0.111
Succinivibrio dextrinosolvens	0.75[a]	0.08[b]	0.87[a]	0.132	0.002
Pseudocohnilembus persalinus	0.70	0.68	0.30	0.094	0.138
Treponema bryantii	0.47[b]	0.90[a]	0.29[b]	0.094	0.001
Aeromonas simiae	0.96	0.10	0.54	0.287	0.534
Prevotellaceae bacterium MN60	0.49	0.62	0.45	0.04	0.182
Schwartzia succinivorans	0.40	0.59	0.51	0.043	0.220
Sarcina sp. DSM 11001	0.44	0.58	0.41	0.04	0.164
Tolumonas auensis	0.64[a]	0.10[b]	0.63[a]	0.094	0.001
Clostridia bacterium	0.50	0.38	0.47	0.046	0.629
Vitrella brassicaformis	0.54	0.53	0.24	0.085	0.281
Succinatimonas hippei	0.51[a]	0.12[b]	0.67[a]	0.092	0.009
Aeromonas sp. RU39B	0.46	0.29	0.52	0.056	0.236
Halteria grandinella	0.53	0.36	0.33	0.055	0.316
Paramecium tetraurelia	0.61	0.32	0.27	0.081	0.184
Tetrahymena thermophila	0.51	0.41	0.27	0.079	0.532
Prevotella sp. BP1-145	0.32	0.41	0.46	0.031	0.157
Prevotella sp. P2-180	0.28	0.60	0.26	0.072	0.073
Prevotella sp. lc2012	0.40	0.42	0.31	0.031	0.361
Bacterium F083	0.42	0.37	0.33	0.031	0.559
Succinatimonas sp. CAG:777	0.47[a]	0.03[b]	0.55[a]	0.088	0.006
Prevotella sp. MA2016	0.33[ab]	0.42[a]	0.27[b]	0.024	0.011
Lchthyophthirius multifiliis	0.44	0.34	0.21	0.055	0.261
Bacteroidales bacterium	0.31	0.37	0.27	0.029	0.449
Ruminococcus bromii	0.35[ab]	0.07[b]	0.46[a]	0.067	0.016
Prevotella aff. ruminicola Tc2-24	0.29	0.31	0.24	0.016	0.155
[Bacteroides] pectinophilus	0.03[b]	0.18[a]	0.01[b]	0.034	0.007

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. SEM, standard error of the mean.

[a, b] means within a row with different superscripts differ significantly ($P \leq 0.05$).

Table S6.5. Pairwise comparison of protein numbers based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Group set (A vs B)	Protein identified in both group	DEPs	Up-regulated protein	Down-regulated protein
L vs C	2777	706	446	260
L vs S	2751	865	539	326
S vs C	3277	139	89	50

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. DEPs, differentially expressed proteins. Up/down-regulated means group A relative to B.

Table S6.6. Pairwise comparison of protein information involved in KEGG pathways of starch and sucrose metabolism, pentose and glucuronate interconversions, and galactose metabolism based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
Starch and sucrose metabolism						
multispecies: 1,4-alpha-glucan branching protein GlgB [*Ruminobacter]*	0.05	0	0.04	0	1.27	0.21
pullulanase-type alpha-1,6-glucosidase [*Succinimonas amylolytica*]	–	–	–	–	1.51	0.27
pullulanase-type alpha-1,6-glucosidase [*Photorhabdus australis*]	0.11	0.03	0.08	0.01	1.32	0.26
glycogen/starch/alpha-glucan phosphorylase [*Aeromonas simiae*]	0.04	0	0.03	0.04	1.3	0.37
PKD domain-containing protein [*Succinivibrionaceae* bacterium WG-1]	0.04	0	0.03	0	1.22	0.35
type I pullulanase [*Lachnospiraceae* bacterium]	2.81	0.15	0.39	0.56	7.16	0.43
multispecies: alpha-D-glucose phosphate-specific phosphoglucomutase [*Ruminobacter*]	0.1	0	0.07	0	1.47	0.12
pullulanase-type alpha-1,6-glucosidase [*Photorhabdus asymbiotica*]	–	–	–	–	0.99	0.98
glycogen/starch/alpha-glucan phosphorylase [*Succinimonas amylolytica*]	0.52	0.2	0.47	0.22	1.11	0.61
pullulanase-type alpha-1,6-glucosidase [*Succinimonas amylolytica*]	–	–	–	–	1.34	0.29
4-alpha-glucanotransferase [*Aeromonas jandaei*]	0.09	0	0.08	0.01	1.12	0.58
alpha-glucan family phosphorylase [*Prevotella* sp. *khp7*]	2.01	0.18	1.97	0.11	1.02	0.97
PKD domain-containing protein [*Ruminobacter* sp. kh1p2]	0.1	0.01	0.1	0.05	1.07	0.86
Pathways/enzymes [related microbe]						
clathrin heavy chain 1 [*Stylonychia lemnae*]	1.08	0.85	2.57	0.11	0.42	0.2
Pullulanase precursor [*Vibrio quintilis*]	–	–	–	–	1.43	0.29
phosphoglucomutase [*Lachnospiraceae* bacterium]	0.55	0.26	0.47	0.14	1.17	0.73
hypothetical protein FGO68-gene7773 [*Halteria grandinella*]	0.81	0.53	1.68	0.38	0.48	0.23
phospho-sugar mutase [*Prevotella ruminicola*]	0.25	0.01	0.19	0	1.35	0.1
alpha-amylase [*Aeromonas enteropelogenes*]	0.06	0	0.05	0.01	1.25	0.38
glycogen phosphorylase [*Stylonychia lemnae*]	0.78	0.63	1.53	0.54	0.51	0.28
glycogen phosphorylase [*Stylonychia lemnae*]	0.74	0.58	1.38	0.62	0.54	0.32
multispecies: glucose-6-phosphate isomerase [*Aeromonas*]	0.09	0	0.07	0	1.23	0.17
alpha-amylase [*Prevotella* sp. tc2-28]	1.1	0.58	1.39	0	0.8	0.32
glucokinase [*Escherichia coli*]	0.19	0.01	0.17	0.01	1.17	0.55
ubiquitin family [*Ruminococcus albus* 8]	1.4	0.2	2.79	0.03	0.5	0.08
phosphoglucomutase [*Prevotella* sp. tf2-5]	–	–	–	–	–	–
alpha catalytic domain containing protein [*Stylonychia lemnae*]	0.67	0.45	1.34	0.7	0.5	0.31
glycogen phosphorylase [*Stylonychia lemnae*]	0.84	0.8	–	–	–	–
alpha catalytic domain containing protein [*Stylonychia lemnae*]	0.49	0.01	–	–	–	–
phospho-sugar mutase [*Prevotella brevis*]	0.82	0.73	2.6	0.14	0.31	0.34

Table S6.6 (continued).

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
amylopullulanase [*Selenomonas bovis*]	–	–	–	–	2.44	0.13
clathrin heavy chain 1 [*Stylonychia lemnae*]	1.00	0.99	3.53	0.07	0.28	0.07
UTP--glucose-1-phosphate uridylyltransferase GalU [*Anaerobiospirillum succiniciproducens*]	–	–	–	–	2.98	0.11
glucosamine-6-phosphate deaminase [*Prevotella ruminicola*]	2.16	0.04	3.39	0.02	0.64	0.07
alpha catalytic domain containing protein [*Stylonychia lemnae*]	–	–	–	–	–	–
glucose-1-phosphate adenylyltransferase subunit GlgD [*Lachnospiraceae* bacterium]	–	–	–	–	0.69	0.61
alpha-amylase [*Prevotella buccae*]	3.33	0.02	2.79	0.02	1.19	0.43
clathrin heavy chain 1 [*Stylonychia lemnae*]	–	–	–	–	–	–
alpha amylase C-terminal domain-containing protein [*Prevotella* sp. bacterium]	0.63	0.21	1.59	0.15	0.4	0.06
cellodextrinase [uncultured microorganism]	1.81	0.01	3.09	0.01	0.59	0.12
glucose-6-phosphate isomerase [*Coprococcus comes*]	1.63	0.43	2.11	0.3	0.77	0.7
ROK family protein [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	0.9	0.9
4-alpha-glucanotransferase [*Clostridiales* bacterium]	0.51	0.32	1.28	0.76	0.4	0.32
alpha-glucan family phosphorylase [*Alloprevotella rava*]	–	–	1.27	0.62	–	–
amylopullulanase [*Selenomonas bovis*]	–	–	–	–	1.24	0.67
maltose phosphorylase [*Lachnospiraceae* bacterium]	–	–	–	–	–	–
4-alpha-glucanotransferase [*Clostridiales* bacterium]	–	–	–	–	–	–
putative glucokinase [*Prevotella* sp. *CAG:1185*]	0.93	0.8	1.35	0.61	0.69	0.35
glycosyl hydrolase family 5 [*Ruminococcus* sp.]	–	–	–	–	–	–
hypothetical protein SteCoe-20439 [*Stentor coeruleus*]	2.17	0.15	2.84	0.1	0.76	0.69
amylase [*Eudiplodinium maggii*]	1.46	0.28	3.31	0.03	0.44	0.09
Alpha amylase, catalytic domain containing protein [*Trichomonas vaginalis* G3]	–	–	–	–	0.4	0.15
glucose-6-phosphate isomerase [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	–	–
glycogen/starch/alpha-glucan family phosphorylase [*Stomatobaculum longum*]	–	–	–	–	1.87	0.09
glycogen synthase GlgA [*Lachnospiraceae* bacterium]	–	–	–	–	1.27	0.72
glucose-6-phosphate isomerase [*Anaerobiospirillum succiniciproducens*]	–	–	–	–	1.42	0.38
glycogen debranching protein GlgX [*Succinimonas amylolytica*]	0.43	0.06	0.47	0.01	0.91	0.68
pullulanase-type alpha-1,6-glucosidase [*Photobacterium marinum*]	–	–	–	–	1.24	0.13
multispecies: glucose-6-phosphate isomerase [unclassified *Prevotella*]	2.26	0.11	3.16	0.07	0.72	0.45
glycosyl transferase [*Prevotella* sp. tc2-28]	–	–	–	–	–	–
glucose-1-phosphate adenylyltransferase [*Lachnospiraceae* bacterium]	1.05	0.89	0.91	0.8	1.16	0.76
hypothetical protein [*Ruminococcaceae* bacterium]	0.77	0.59	1.64	0.52	0.47	0.26
phosphoglucomutase [*Bacteroidales* bacterium]	1.99	0.05	2.97	0	0.67	0.38

Table S6.6 (continued).

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
4-alpha-glucanotransferase [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	–	–
hypothetical protein [*Treponema porcinum*]	–	–	–	–	0.53	0.15
4-alpha-glucanotransferase [*Prevotella* sp. lc2012]	0.73	0.35	1.45	0.57	0.5	0.3
carbohydrate kinase [*Prevotella* sp. lc2012]	–	–	–	–	1.33	0.82
6-phospho-alpha-glucosidase [*Selenomonas bovis*]	–	–	–	–	1.58	0.28
alpha-glucan family phosphorylase, partial [*Bacteroidetes* bacterium HGW-Bacteroidetes-20]	4.77	0.11	0.63	0.21	7.61	0.02
glycogen debranching protein GlgX [*Succinimonas amylolytica*]	–	–	–	–	0.82	0.51
glucose-6-phosphate isomerase [*Lachnospiraceae* bacterium]	–	–	–	–	0.84	0.27
hypothetical protein FGO68-gene11367 [*Halteria grandinella*]	1.35	0.56	–	–	–	–
pullulanase [*Vibrio xuii*]	0.44	0.32	0.3	0.13	1.44	0.46
glycoside hydrolase family 97 catalytic domain-containing protein [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	1.61	0.33
glucose-1-phosphate adenylyltransferase subunit GlgD [*Clostridiales* bacterium]	1.31	0.61	5.71	0.02	0.23	0.19
endonuclease I [Bacterium F083]	0.51	0.28	0.57	0.16	0.89	0.69
4-alpha-glucanotransferase [*Ruminococcaceae* bacterium]	–	–	0.56	0.47	–	–
glycogen/starch/alpha-glucan phosphorylase [*Selenomonas bovis*]	2.35	0.54	0.5	0.34	4.72	0.14
glycoside hydrolase family 97 catalytic domain-containing protein [*Prevotellaceae* bacterium LKV-178-WT-2A]	1.14	0.25	1.07	0.53	1.06	0.67
PTS transporter subunit EIIC [*Selenomonas bovis*]	–	–	–	–	–	–
glucose-1-phosphate adenylyltransferase [*Oribacterium* sp. P6A1]	–	–	–	–	0.73	0.43
glucose-6-phosphate isomerase [*Selenomonas bovis*]	1.91	0.41	1.21	0.7	1.58	0.25
clathrin heavy chain [*Ascobolus immersus* RN42]	–	–	–	–	0.79	0.07
phosphorylase [*Clostridium* sp. CAG:230]	6.03	0.12	3.85	0.17	1.57	0.51
ROK family protein [*Prevotella* sp. P6B4]	2.53	0.21	4.62	0.16	0.55	0.64
unnamed protein product [*Vitrella brassicaformis* CCMP3155]	0.84	0.55	1.55	0.2	0.54	0.15
1,4-alpha-glucan branching enzyme [*Lachnospiraceae* bacterium]	–	–	–	–	–	–
cellobiose phosphorylase [*Prevotella* sp. Sc00066]	0.73	0.48	0.88	0.67	0.83	0.51
multispecies: chitobiase/beta-hexosaminidase C-terminal domain-containing protein [*Bacteroidales*]	–	–	–	–	–	–
glucose-1-phosphate adenylyltransferase [*Treponema* sp.]	–	–	–	–	–	–
Pentose and glucuronate interconversions						
clathrin heavy chain 1 [*Stylonychia lemnae*]	1.08	0.85	2.57	0.11	0.42	0.2
glucuronate isomerase [*Prevotella* sp.]	2.22	0.02	3.88	0.01	0.57	0.15
L-arabinose isomerase [*Prevotella* sp. tc2-28]	3.66	0.01	2.65	0.01	1.38	0.53

Table S6.6 (continued).

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
glucuronate isomerase [*Prevotella* sp. tf2-5]	–	–	1.8	0.49	–	–
ubiquitin family [*Ruminococcus albus* 8]	1.4	0.2	2.79	0.03	0.5	0.08
clathrin heavy chain 1 [*Stylonychia lemnae*]	1	0.99	3.53	0.07	0.28	0.07
UTP-glucose-1-phosphate uridylyltransferase GalU [*Anaerobiospirillum succiniciproducens*]	–	–	–	–	2.98	0.11
hypothetical protein PIROE2DRAFT-67719 [*Piromyces* sp. E2]	–	–	–	–	0.81	0.63
tagaturonate reductase [*Prevotella* sp. BP1-145]	2.58	0	2.69	0	0.96	0.9
clathrin heavy chain 1 [*Stylonychia lemnae*]	–	–	–	–	–	–
multispecies: tagaturonate reductase [*Prevotella*]	2.26	0	2.37	0	0.95	0.74
L-arabinose isomerase [*Prevotella* sp. tf2-5]	–	–	–	–	–	–
L-arabinose isomerase [*Lachnospiraceae* bacterium]	0.53	0.21	0.36	0.06	1.47	0.24
hypothetical protein SteCoe-20439 [*Stentor coeruleus*]	2.17	0.15	2.84	0.1	0.76	0.69
unnamed protein product [*Vitrella brassicaformis* CCMP3155]	2.23	0.36	5.37	0.11	0.42	0.1
nucleotide sugar dehydrogenase [*Succinatimonas hippei*]	1.2	0.66	1.19	0.58	1.01	0.97
xylose isomerase [*Bacteroidales* bacterium]	–	–	–	–	–	–
L-ribulose-5-phosphate 4-epimerase [*Prevotella* sp. tc2-28]	2.33	0.05	3.04	0.01	0.77	0.24
multispecies: xylose isomerase [*Prevotella*]	–	–	–	–	1.24	0.34
L-ribulose-5-phosphate 4-epimerase [*Prevotella* sp. AGR2160]	–	–	–	–	0.85	0.83
multispecies: altronate dehydratase [*Prevotella*]	2.82	0.11	3.36	0.07	0.84	0.72
multispecies: mannonate dehydratase [unclassified *Prevotella*]	2.8	0.16	–	–	–	–
mannitol dehydrogenase family protein [*Prevotella* sp. lc2012]	1.33	0.57	2.14	0.18	0.62	0.55
tagaturonate reductase [*Prevotella* sp. CAG:1092]	1.42	0.09	1.01	0.94	1.41	0.01
multispecies: rhamnulokinase [unclassified *Prevotella*]	1.21	0.63	2.75	0.05	0.44	0.2
L-ribulose-5-phosphate 4-epimerase [*Selenomonas ruminantium*]	2.6	0.09	2.81	0.08	0.92	0.62
UDP-glucose 6-dehydrogenase [*Alphaproteobacteria* bacterium]	0.86	0.81	2.2	0.34	0.39	0.26
carbohydrate kinase [*Prevotella* copri]	1.09	0.78	1.07	0.83	1.02	0.93
tagaturonate reductase [*Bacteroides pectinophilus*]	–	–	–	–	–	–
tagaturonate reductase [*Prevotella* sp. tc2-28]	2.96	0.04	3.57	0.02	0.83	0.74
5-dehydro-4-deoxy-D-glucuronate isomerase [*Lachnospiraceae* bacterium NK3A20]	6.83	0	–	–	–	–
L-arabinose isomerase [*Lachnobacterium bovis*]	–	–	–	–	–	–
pectin esterase [*Prevotella* sp. PMUR]	–	–	–	–	–	–
clathrin heavy chain [*Ascobolus immersus* RN42]	–	–	–	–	0.79	0.07
4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase [*Prevotella ruminicola*]	2.1	0	2.69	0	0.78	0.04
lysophospholipase L1 [*Treponema berlinense*]	–	–	–	–	–	–

Table S6.6 (continued).

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
unnamed protein product [*Vitrella brassicaformis* CCMP3155]	0.84	0.55	1.55	0.2	0.54	0.15
L-arabinose isomerase [*Prevotella* sp. P5-92]	2.06	0.09	2.09	0.12	0.99	0.97
arabinose isomerase [*Prevotella* sp. tc2-28]	–	–	–	–	–	–
Galactose metabolism pathway						
multispecies: alpha-D-glucose phosphate-specific phosphoglucomutase [*Ruminobacter*]	0.1	0	0.07	0	1.47	0.12
clathrin heavy chain 1 [*Stylonychia lemnae*]	1.08	0.85	2.57	0.11	0.42	0.2
phosphoglucomutase [*Lachnospiraceae* bacterium]	0.55	0.26	0.47	0.14	1.17	0.73
hypothetical protein FGO68-gene7773 [*Halteria grandinella*]	0.81	0.53	1.68	0.38	0.48	0.23
galactose-1-epimerase [*Prevotellaceae* bacterium LKV-178-WT-2A]	0.37	0.52	0.15	0.06	2.58	0.26
phospho-sugar mutase [*Prevotella ruminicola*]	0.25	0.01	0.19	0	1.35	0.1
UDP-glucose 4-epimerase GalE [*Prevotella* sp. P6B4]	4.68	0	5.04	0	0.93	0.71
galactokinase [*Prevotella* sp. tf2-5]	2.43	0.02	2.67	0.01	0.91	0.57
UDP-glucose 4-epimerase GalE [*Prevotella brevis*]	1.4	0.11	1.27	0.22	1.11	0.72
glucokinase [*Escherichia coli*]	0.19	0.01	0.17	0.01	1.17	0.55
ubiquitin family [*Ruminococcus albus* 8]	1.4	0.2	2.79	0.03	0.5	0.08
phosphoglucomutase [*Prevotella* sp. tf2-5]	–	–	–	–	–	–
phospho-sugar mutase [*Prevotella brevis*]	0.82	0.73	2.6	0.14	0.31	0.34
clathrin heavy chain 1 [*Stylonychia lemnae*]	1	0.99	3.53	0.07	0.28	0.07
UTP-glucose-1-phosphate uridylyltransferase GalU [*Anaerobiospirillum succiniciproducens*]	–	–	–	–	2.98	0.11
6-phosphofructokinase [*Prevotella* bryantii]	0.79	0.71	0.91	0.9	0.87	0.88
galactokinase [*Prevotella brevis*]	2.42	0.07	2.25	0.04	1.08	0.89
UDP-glucose 4-epimerase GalE [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	0.73	0.08
clathrin heavy chain 1 [*Stylonychia lemnae*]	–	–	–	–	–	–
ROK family protein [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	0.9	0.9
UDP-glucose 4-epimerase GalE [*Prevotella lascolaii*]	1.18	0.72	0.6	0.18	1.98	0.12
galactokinase [*Prevotella oris*]	0.74	0.44	1.65	0.25	0.45	0.01
putative glucokinase [*Prevotella* sp. CAG:1185]	0.93	0.8	1.35	0.61	0.69	0.35
hypothetical protein SteCoe-20439 [*Stentor coeruleus*]	2.17	0.15	2.84	0.1	0.76	0.69
unnamed protein product [*Vitrella brassicaformis* CCMP3155]	2.23	0.36	5.37	0.11	0.42	0.1
6-phosphofructokinase [*Bacteroidales* bacterium]	–	–	–	–	–	–
DUF4982 domain-containing protein [*Prevotella* sp. 885]	–	–	–	–	–	–
galactose mutarotase [*Prevotella* sp. tf2-5]	2.13	0.03	1.85	0.09	1.15	0.62
galactose mutarotase [*Prevotella* sp. PMUR]	2.54	0	3.05	0	0.83	0.12

Table S6.6 (continued).

Pathways/enzymes [related microbe]	L vs C		L vs S		S vs C	
	FC	*P*-value	FC	*P*-value	FC	*P*-value
phosphoglucomutase [*Bacteroidales* bacterium]	1.99	0.05	2.97	0	0.67	0.38
galactokinase [*Prevotellaceae* bacterium LKV-178-WT-2A]	–	–	–	–	–	–
RpiB/LacA/LacB family sugar-phosphate isomerase [*Lachnospiraceae* bacterium oral taxon 500]	–	–	–	–	–	–
UDP-glucose 4-epimerase GalE [*Prevotella brevis*]	1.17	0.68	3.8	0.01	0.31	0.16
6-phosphofructokinase [*Treponema saccharophilum*]	–	–	–	–	1.9	0.52
hypothetical protein FGO68-gene11367 [*Halteria grandinella*]	1.35	0.56	–	–	–	–
alpha-galactosidase [*Prevotella* sp. P4-98]	–	–	–	–	–	–
6-phosphofructokinase [*Lachnospiraceae* bacterium]	0.59	0.19	0.41	0.02	1.44	0.03
6-phosphofructokinase [*Oribacterium* sp. FC2011]	–	–	–	–	0.63	0.62
UDP-glucose 4-epimerase GalE [*Bacteroides graminisolvens*]	–	–	–	–	–	–
clathrin heavy chain [*Ascobolus immersus* RN42]	–	–	–	–	0.79	0.07
6-phosphofructokinase [*Bacteroides rodentium*]	0.86	0.8	0.42	0.13	2.03	0.19
ROK family protein [*Prevotella* sp. P6B4]	2.53	0.21	4.62	0.16	0.55	0.64
unnamed protein product [*Vitrella brassicaformis* CCMP3155]	0.84	0.55	1.55	0.2	0.54	0.15
UDP-glucose 4-epimerase GalE [*Lachnospiraceae* bacterium P6A3]	0.47	0.27	0.17	0.07	2.76	0.15

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. FC, fold change. −, no value was detected.

Reference

Aspeborg, H., Coutinho, P. M., Wang, Y., Brumer, R. H., and Henrissat, B. 2012. Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5). *BMC Evol. Biol.* 12(1):186.

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. 2009. The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37:D233-D238.

Deusch, S., Camarinha-Silva, A., Conrad, J., Beifuss, U., Rodehutscord, M., and Seifert, J. 2017. A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments. *Front. Microbiol.* 8.

Deusch, S., Tilocca, B., Camarinha-Silva, A., and Seifert, J. 2015. News in livestock research-use of Omics-technologies to study the microbiota in the gastrointestinal tract of farm animals. *Comput. Struct. Biotechnol. J.* 13:55-63.

Hart, E. H., Creevey, C. J., Hitch, T., and Kingston-Smith, A. H. 2018. Meta-proteomics of rumen microbiota indicates niche compartmentalisation and functional dominance in a limited number of metabolic pathways between abundant bacteria. *Sci. Rep.* 8(1).

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., and Janssen, P. H. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5(1).

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280:309-316.

Jami, E., Mizrahi, I., and López-García, P. 2012. Composition and similarity of bovine rumen microbiota across individual animals. *PLoS One* 7(3):e33306.

Jose, V. L., Appoothy, T., More, R. P., and Arun, A. S. 2017. Metagenomic insights into the rumen microbial fibrolytic enzymes in Indian crossbred cattle fed finger millet straw. *AMB Express* 7(1).

Kevin, L. A. 2000. Degradation of cellulose and starch by anaerobic bacteria, Page: 359-386, Glycomicrobiology. Doyle, ed. Kluwer academic/plenum, New York.

Kotarski, F. S., Waniska, D. R., and Thurn, K. K. 1992. Starch hydrolysis by the ruminal microflora12. *Symposium* 92:22-3166.

Miron, J., Ben-Ghedalia, D., and Morrison, M. 2001. Invited review: adhesion mechanisms of rumen cellulolytic Bacteria. *J. Dairy Sci.* 84(6):1294-1309.

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

NRC, 2001. Nutrient requirements of dairy cattle, seventh ed. National Academy Press, Washington, DC.

Shen, J., Zheng, L., Chen, X., Han, X., Cao, Y., and Yao, J. 2020. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of dairy goats fed different rumen degradable starch. *Front. Microbiol.* 11:1003.

Stam, M. R., Danchin, E. G. J., Rancurel, C., Coutinho, P. M., and Henrissat, B. 2006. Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of amylase-related proteins. *Protein Eng. Des. Sel.* 19(12):555-562.

Stevenson, D. M. and Weimer, P. J. 2007. Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. *Appl. Microbiol. Biotechnol.* 75(1):165-174.

Stewart, R. D., Auffret, M. D., Warr, A., Walker, A. W., Roehe, R., and Watson, M. 2019. Compendium of 4,941 rumen metagenome-assembled genomes for rumen microbiome biology and enzyme discovery. *Nat. Biotechnol.* 37(8):953-961.

Stewart, S. C., Flint, H. J., and Bryant, M. P. 1997. The rumen bacteria, Page: 79, The rumen microbial ecosystem. Hobson, P. N., and Stewart, C. S., ed. Blackie acadamic professional, London.

Sukharnikov, L. O., Alahuhta, M., Brunecky, R., Upadhyay, A., Himmel, M. E., Lunin, V. V., and Zhulin, I. B. 2012. Sequence, structure, and evolution of cellulases in glycoside hydrolase family 48. *J. Biol. Chem.* 287(49):41068-41077.

Viborg, A. H., Terrapon, N., Lombard, V., Michel, G., Czjzek, M., Henrissat, B., and Brumer, H. 2019. A subfamily roadmap of the evolutionarily diverse glycoside hydrolase family 16 (GH16). *J. Biol. Chem.* 294(44):15973-15986.

Vishnu, C., Naveena, B. J., Altaf, M., Venkateshwar, M., and Reddy, G. 2006. Amylopullulanase-a novel enzyme of L. amylophilus GV6 in direct fermentation of starch to L (+) lactic acid. *Enzyme Microb. Technol.* 38(3-4):545-550.

Voget, S., Steele, H. L., and Streit, W. R. 2006. Characterization of a metagenome-derived halotolerant cellulase. *J. Biotechnol.* 126(1):26-36.

Vorgias, C. and Antranikian, G. 2002. Glycosyl hydrolases from extremeophiles, Glycomicrobiology. Doyle, R. J., ed. Kluwer academic publishers, New York.

Wang, L., Zhang, G., Xu, H., Xin, H., and Zhang, Y. 2019. Metagenomic analyses of microbial and carbohydrate-active enzymes in the rumen of Holstein cows fed different forage-to-concentrate ratios. *Front. Microbiol.* 10.

Zhu, N., Yang, J., Ji, L., Liu, J., Yang, Y., and Yuan, H. 2016. Metagenomic and metaproteomic analyses of a corn stover-adapted microbial consortium EMSD5 reveal its taxonomic and enzymatic basis for degrading lignocellulose. *Biotechnol. Biofuels* 9(1).

Chapter 7

General discussion

Carbohydrates are the major energy supplier in the ration for ruminants. Starch and fibre form the main ingredients for ruminant nutritionists to modulate the energy supply of dairy cows. Research to date mainly focused on the types, ratios, processing, or interactions of starchy and fibrous ingredients (Mendoza et al., 1999, Zhou et al., 2015).

The rumen microbiome plays a critical role in the degradation of feedstuffs in ruminants including amylolytic, fibrolytic, proteolytic, etc., activities. Numerous studies have worked on the ruminal microbes and enzymes involved in starch- and fibre-degradation (Huntington, 1997, Krause et al., 2003, **Chapter 2**). There is still a lack of knowledge on the metabolic mechanisms of the functional species in the rumen due to limitations of traditional approaches. 16S rRNA gene-based approaches have been widely used to characterize microbial communities and establish the ribosomal database project (RDP) database (Kim et al., 2011). The development of next-generation sequencing technologies and rapid evolution of computational tools and reference databases have promoted the application of 'omics' techniques in the study of the rumen microbiome by exploring their gene (metagenomics), protein (metaproteomics) and metabolite (metabolomics) expressions (Deusch et al., 2015). With the assistance of these advanced omics approaches, extensive and accurate information can be obtained which can bring new insights of our understanding of the mechanisms and enables improving rumen functioning. Furthermore, the establishments of genomic, proteomic and metabolic data make it easier to manipulate certain functions of rumen performance through diets. In addition, the integration of the main omics technologies aids our understanding of molecular changes in response to internal and external environmental factors (Wallace et al., 2017).

In this thesis, three practical rations were formulated including two glucogenic diets with starch as the main energy source but differing in corn processing (C, ground corn and corn silage diet; S, steam-flaked corn and corn silage diet) and one lipogenic diet where fibre was the main energy source (L, sugar beet pulp and alfalfa silage diet), with the aim to 1) characterize the sensitivities and interactions of the amylolytic and fibrolytic bacterial species, 2) reveal the activities of the associated enzymes including amylase, cellulase and hemicellulase in the rumen of dairy cows, and 3) further evaluate the application of omics techniques by studying the ruminal microbial metabolism. The

technological approaches employed in this thesis included 16S rRNA gene-based sequencing, metagenomics, metaproteomics and metabolomics analysis.

In this chapter, the applications of omics on understanding the ruminal microbiome will be studied through a combination of metagenomics, metaproteomics and metabolomics analysis, in terms of 1) the microbial communities and 2) the synthesis pathways of VFA including propionate, acetate and butyrate.

Application of 'omics' on rumen microorganisms

The rumen microbes hydrolyse dietary plant components like cellulose, hemicellulose, pectin, fructosan, starch and other polysaccharides to monomeric or dimeric sugars, which subsequently are fermented by enzymes, along with any simple sugars in the vegetation, to yield various products, some of which might be subject to further microbial action.

Ruminal microbial communities

The dietary composition of ruminants is one of the major drivers of the taxonomic composition of the rumen microbiome. Understanding how changes in microbial diversity affect rumen functioning or enable sustained feed utilisation facilitated by the above-mentioned analytical technologies is current of great interest in ruminant nutritional science. Systematic cataloguing of microbial diversity and functionality in rumen fluid samples using both 16S rRNA gene-based and metagenomics approaches has been attempted in the present study to evaluate the characterization of certain ruminal communities, including amylolytic, fibrolytic and lactate-producing bacteria, etc.

Bacteria community

Nowadays, 16S rRNA gene sequencing has been widely used to identify bacterial and archaeal species. The 16S rRNA gene is evolutionarily specific and conserved, which makes it possible to quantify the target organisms (Zoetendal et al., 2004, Deng et al., 2008). The 16S rRNA gene clone library of rumen microorganisms has been constructed with molecular tools, which have revealed a several-fold enlargement in the diversity of ruminal microbes compared to when the more traditional cultural

techniques were used. This microbial diversity was based on ruminants receiving different diets and also across different animal species (Tajima et al., 2001, Larue et al., 2005).

In this thesis, the results of 16S rRNA sequencing in **Chapter 5** show that 20 bacterial phyla were collectively detected from the 18 rumen fluid samples of dairy cows, which were dominated by *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (**Figure S5.2a**, **Chapter 5**). The most abundant bacterial genera were *Prevotella_1*, *Ruminococcus_1* and *Succinivibrionaceae_UCG-001* (**Figure S5.2b**, **Chapter 5**). As knowledge of the bacterial population increased, it became apparent that cultivated cellulolytic bacterial genera such as *Ruminococcus* and *Fibrobacter* were not among the most abundant members in the community and the presence of various other fibre-degrading genera (Pitta et al., 2010) were identified. The same insights occurred for the amylolytic bacterial genera including *Streptococcus*, *Ruminobacter* and *Succinimonas*, which were not among the dominant bacterial members.

In addition, the microbial composition data from the metagenomics at the species level shows that the fibrolytic (cellulolytic, hemicellulolytic and pectinolytic) bacteria mentioned in **Table 7.1** account for approximately 4.6% (including *Prevotella ruminocola*, which degrades both starch and fibre, as well as protein) of all ruminal bacteria and the amylolytic bacteria (*Prevotella ruminocola*, *Ruminobacter amylophilus*, *Ruminoccus bromii* and *Succinimonas amylolytica*) account for approximately 3.3% (**Figure 7.1**). Similar results were observed in the study of Stevenson and Weimer (2007) where the main cellulolytic bacteria, including *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* accounted for less than 10% of the total bacteria in the rumen. Overall, from both 16S rRNA sequencing and metagenomics data, the cultivated fibrolytic and amylolytic communities were not among the dominant bacteria in the rumen population which suggested that various other fibre- and starch-degrading microbes exist which still are to be discovered and characterized.

Table 7.1. Rumen microbial classification based on functions

Microbial category	Representative genera/species
Bacteria	
Acetogens	*Acetitomaculum ruminis*, *Eubacterium limosum*
Acid utilizers	*Megasphaera elsdeni*, *Wolinella succinogenes*, *Veillonella gazogene*, *Micrococcus lactolytica*, *Oxalobacter formigenes*, *Desulfovibrio desulfuricans*, *Desulfotomaculum ruminis*, *Succiniclasticum ruminis*
Amylolytic	*Streptococcus bovis*, *Ruminobacter amylophilus*, *Ruminobacter bromii*, *Prevotella ruminicola*, *Clostridium polysaccharolyticum*, *Succnivibrio amylolytica*
Cellulolytic	*Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Clostridium cellobioparum*, *Clostridium longisporum*, *Clostridium lochheadii*, *Clostridium polysaccharolyticum*, *Eubacterium cellulosolvens*
Hemicellulolytic	*Ruminococcus flavefaciens*, *Ruminococcus albus*, *Clostridium polysaccharolyticum*, *Prevotella ruminicola*, *Eubacterium xylanophilum*, *Eubacterium uniformis*, *Butyrivibrio fibrisolvens*
Lipolytic	*Anaerovibrio lipolytica*
Pectinolytic	*Treponema bryantii*, *Treponema saccharophilum*, *Lachnospira multiparus*, *Succinivibrio dextrinosolvens*
Proteolytic	*Prevotella ruminicola*, *Ruminobacter amylophilus*, *Clostridium bifermentans*
Saccharolytic	*Succinivibrio dextrinosolvens*, *Succnivibrio amylolytica*, *Selenomonas ruminantium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Bifidobacterium globosum*, *Bifidobacterium longum*, *Bifidobacterium thermophilum*, *Bifidobacterium ruminale*, *Bifidobacterium ruminantium*
Tanninolytic	*Streptococcus caprinus*, *Eubacterium oxidoreducens*
Ureolytic	*Megasphaera elsdenii*
Archaea	
Methanogens	*Methanobacterium formicicum*, *Methanobacterium bryantii*, *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanomicrobium mobile*, *Methanosarcina barkeri*, *Methanoculleus olentangyi*
Eukaryotes	
Fungi	*Piromyces communis*, *Piromyces mae*, *Piromyces minutus*, *Piromyces dumbonicus*, *Piromyces rhizinfl atus*, *Piromyces spiralis*, *Piromyces citronii*, *Piromyces polycephalus*, *Anaeromyces mucronatus*, *Anaeromyces elegans*, *Caecomyces communis*, *Caecomyces equi*, *Caecomyces sympodialis*, *Cyllamyces aberensis*, *Cyllamyces icaris*, *Neocallimastix frontalis*, *Neocallimastix patriciarum*, *Neocallimastix hurleyensis*, *Neocallimastix variabilis*, *Orpinomyces joynii*, *Orpinomyces intercalaris*
Protozoa	*Entodinium bovis*, *Entodinium bubalum*, *Entodinium bursa*, *Entodinium caudatum*, *Entodinium chatterjeei*, *Entodinium parvum*, *Entodinium longinucleatum*, *Entodinium dubardi*, *Entodinium exiguum*, *Epidinium caudatum*, *Isotricha prostoma*, *Isotricha intestinalis*, *Dasytricha ruminantium*, *Diplodinium dendatum*, *Diplodinium indicum*, *Oligoisotricha bubali*, *Polyplastron multivesiculatum*, *Eremoplastron asiaticus*, *Eremoplastron bubalus*
Bacteriophages	*Methanobacterium phage Ψ M1*, *Methanobacterium phage Ψ M10*, *Methanobacterium phage Ψ M100*, *Methanothermobacter phage Ψ M100*, *Methanobacterium phage ΨM2*

Adapted from previous studies (Pfister et al., 1998, Luo et al., 2001, Kamra, 2005, Janssen and Kirs, 2008, Wright and Klieve, 2011, Choudhury et al., 2012, Sirohi et al., 2012, Kumar et al., 2014).

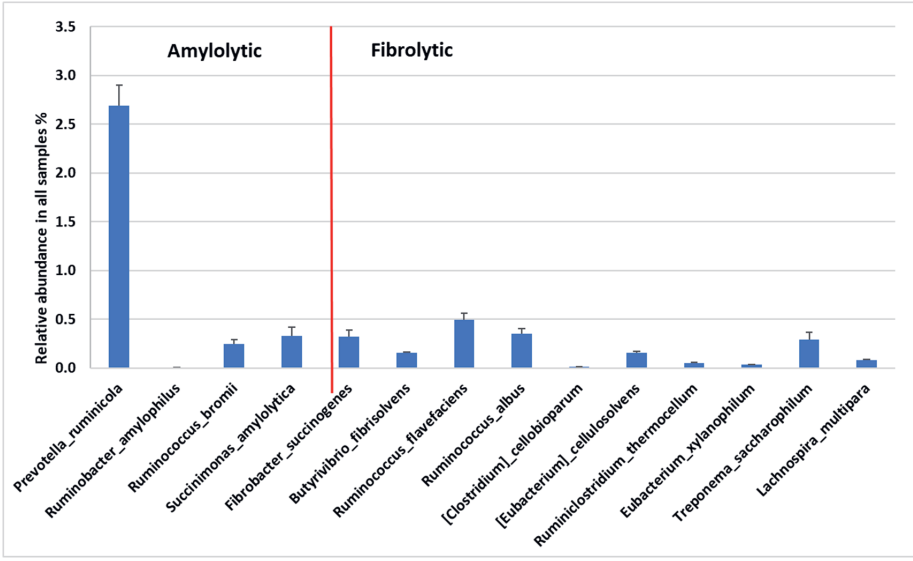


Figure 7.1. The average relative abundance of amylolytic and fibrolytic bacteria relative to total bacteria based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet investigated in the present thesis.

Archaea community

It has been reported that the domain archaea members accounted for 0.3 to 3.3% of the microbial small subunit (16S and 18S) rRNA in the rumen (Lin et al., 1997, Sharp et al., 1998, Ziemer et al., 2000). In the present study, the taxonomical analysis from the ruminal metagenome showed that the archaea contributed 1.1% of the ruminal microbial community detected from rumen fluid samples (**Figure S6.1a**, **Chapter 6**). Within the archaeal community, a total of 12 phyla were annotated with the *Euryarchaeota* being the most dominant constituting 98.4% of the archaeal community. A total of 139 genera including 20 unclassified taxa were annotated which were dominated by *Methanobrevibacter* (73.7%) (**Figure 7.2**). The dominant archaeal genus in the *in vitro* trial was also *Methanobrevibacter* (69.1% on average, **Table 3.5**, **Chapter 3**). These results are in line with the previous studies (Janssen and Kirs, 2008, Zhu et al., 2021). The dominant genus was very stable among published studies, but the proportion was diverse.

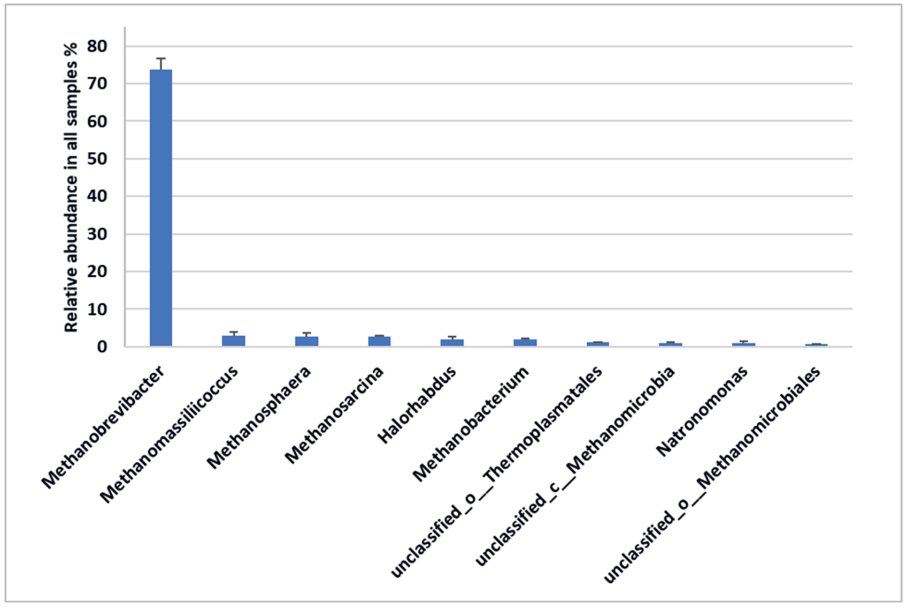


Figure 7.2. The average relative abundance of the top 10 archaea genera based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet investigated in the present thesis.

Fungi community

The presence of fungi in the rumen was identified relatively late compared to other microbiota as they were confused with flagellate protozoa and were categorized with them (Agarwal et al., 2015). The fungi account for a very small part of the microbial population in the rumen (5-8% of total rumen biomass) and colonize the rumen compartment within 8-10 days after birth (Agarwal et al., 2015). Although the abundance of rumen fungi is far less than that of bacteria, their degradation ability is higher than that of bacteria and they have been reported to account for as high as 20% of the total microbial biomass when the roughage was added in the rumen (Rezaeian et al., 2004).

For the fungal taxonomic analysis through the metagenomics approach in previous studies, the community structure and the dominant taxa had a large diversity. Liang et al. (2021) showed that the dominant phyla were *Chytridiomycota*, *Ascomycota* and *Mucoromycota* and the dominant genera consisted of *Piromyces*, *Neocallimastix* and *Anaeromyces*. Fouts et al. (2012) detected 46 fungal genera through Sanger and 454 sequencing in the rumen of cows consuming a forage diet, with the *Nectria*,

Penicilliopsis, *Cystofilobasidium* and *Delphinelaa* being the most abundant comprising over 25% of the community.

In this study, a total of 10 fungal phyla were annotated with the *Ascomycota* being the most dominant (43.7%) in the rumen fluid samples (**Figure 7.3**). A total of 276 genera were annotated with the *Rhizophagus* constituting 18.8% of the fungal community being the most prevalent (**Figure 7.4**). The present fungal community was not in agreement with previous studies (Fouts et al., 2012, Liang et al., 2021). These results showed that the current landscape of the fungal diversity in the rumen is largely incomplete. Specifically, there is a greater diversity of *Pleosporales*, *Neocallimastix*, *Sordariomyceteideae*, *Udeniomyces* and others, than previously appreciated.

Besides the identification of bacteria, archaea and fungi, metagenomics has also allowed the identification of the rumen microbiome of viruses and unclassified taxes. Although the present work did not focus on viruses and other unclassified taxes, their interactions with bacteria could also be a factor affecting rumen fermentation, which may warrant further studies in the future.

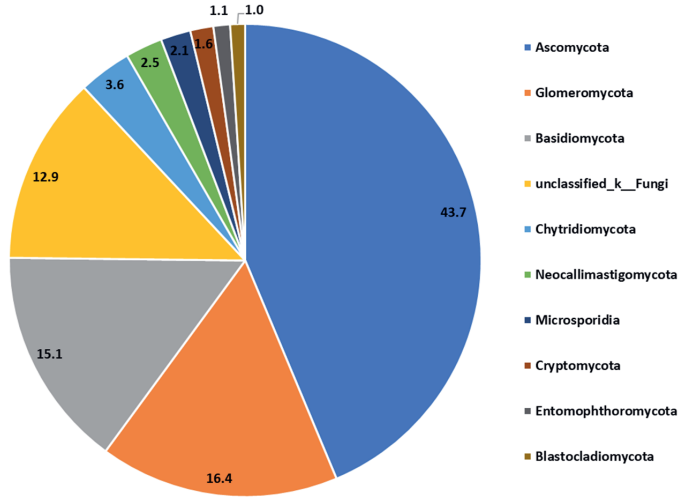


Figure 7.3. The average relative abundance of the top 10 fungi phyla based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet investigated in the present thesis.

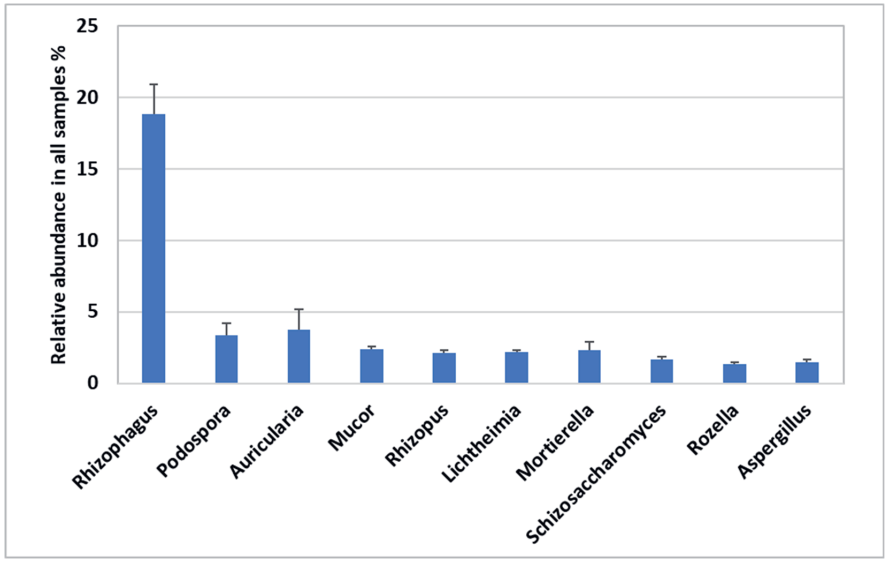


Figure 7.4. The average relative abundance of the top 10 fungi genera based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic and a lipogenic diet investigated in the present thesis.

Application of omics on rumen metabolism

To evaluate the application of omics technologies in studying the rumen microbial functioning, the volatile fatty acid (VFA) profiles in the rumen were chosen as the phenotypic parameters, their synthetic pathways were analysed through metagenomics and metaproteomics approaches. The total VFA concentration and molar proportions of individual VFA were tested both in the *in vitro* and *in vivo* trials. The total VFA concentration was not influenced by dietary treatments (C, L and S), irrespective whether diets were incubated under *in vitro* conditions (**Chapter 3**) or degraded in the rumen (**Chapter 5**). Similar results were also observed in **Chapter 4** when the diets with different ratios of starch to fibre were incubated under *in vitro* conditions. However, the individual VFA proportions both in **Chapter 3** and **5** showed a slight difference (**Figure 7.5**). In an attempt to determine the mechanism underneath the alterations of individual VFA proportions, their metabolic pathways and enzymes involved were further studied. The productions of propionate, acetate and butyrate belonged to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of

propanoate, pyruvate and butanoate metabolism, separately, as shown in **Figure**s **S7.1**, **S7.2** and **S7.3**.

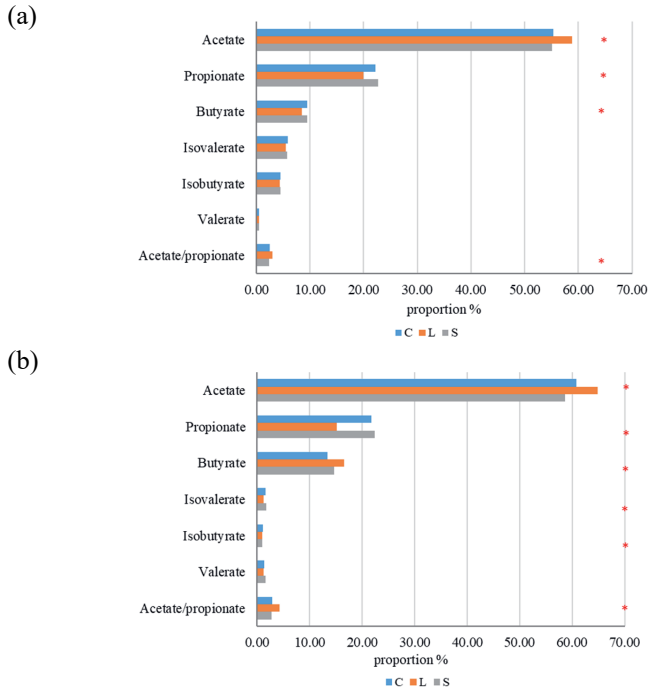


Figure 7.5. Comparison of the ruminal volatile fatty acid profile in *in vitro* (a) and *in vivo* (b) conditions in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. Proposition, the percentage of individuals in the total volatile fatty acids. * $P \leq 0.05$

Data analysis

The enzymes involved in the abovementioned KEGG pathways were analysed from both the metagenomics and metaproteomics data. For the latter, the enzyme comparison was performed with the Kruskal-Wallis H test with a Welch's *post-hoc* test using the stats package in R software (version 3.3.1) and SciPy package in Python (version1.0.0). The $P \leq 0.05$ was considered a significant level and $0.05 < P \leq 0.1$ as a trend. As for the metaproteomics data, the differentially expressed proteins in each group set (L vs C, L vs S and S vs C) within these pathways were analysed by Student's t-test in R (version 3.3.1).

Enzymes involved in the synthesis of propionate, acetate and butyrate

Propionate is produced in the rumen through two main pathways: the succinate pathway and the acrylate pathway (**Figure 7.6a**) (Fouts et al., 2012, Liang et al., 2021), which mostly fall within the KEGG pathway of propanoate metabolism (Supplementary **Figure S7.1**). The acetate synthesis includes two main pathways (**Figure 7.7a**), originating from acetyl-CoA and are mainly associated with the KEGG pathway of pyruvate metabolism (Supplementary **Figure S7.2**). The butyrate synthesis consists of four main pathways as shown in **Figure 7.8a** (Vital et al., 2014), which mostly belonged to the butanoate metabolism KEGG pathway (Supplementary **Figure S7.3**).

KEGG pathways associated with the synthesis of propionate, acetate, butyrate

For the propanoate metabolism, after comparative metagenomics data analyses, eight enzymes were detected with a *P*-value ≤ 0.1 (**Figure 7.6b**). Thereinto, the enzymes of lactaldehyde reductase (Enzyme commission number: EC, EC1.1.1.77, $P = 0.012$) and propionaldehyde dehydrogenase (EC1.2.1.87, $P = 0.019$) were significantly higher, while the 4-aminobutyrate aminotransferase (EC2.6.1.19, $P = 0.008$) and 1,3-propanediol dehydrogenase (EC1.1.1.202, $P = 0.041$) were significantly lower in the samples of diet L compared to diets C and S. As for the metaproteomics analysis (**Table 7.2**), the two detected enzymes (both classified into EC6.2.1.5) of ADP-forming succinate-CoA ligase subunit beta (originated from *Thalassolituus* sp. C2-1) and succinate-CoA (originated from *Glaciecola pallidula*) and the enzyme of fumarate reductase (quinol) flavoprotein subunit (EC1.3.5.4) originated from *Ruminobacter* sp. kh1p2 ($P = 0.015$ and 0.002) and *Aeromonas australiensis* ($P = 0.002$ and 0.001, respectively) were up-regulated by diets C and S relative to diet L. In addition, the EC2.8.3.18 (succinyl-CoA:acetate CoA-transferase, originating from *Succiniclasticum ruminis*) was observed to be down-regulated by diet L compared to diets C and S both in the metagenomics ($P = 0.036$, **Figure 7.7b**) and metaproteomics analysis ($P = 0.004$ and 0.011) (**Table 7.2**). This enzyme specifically catalyses the conversion of succinyl-CoA together with acetate to succinate and acetyl-CoA (Mullins et al., 2008, Mullins and Kappock, 2012), which is involved in the KEGG pathways of the pyruvate metabolism (**Figure S7.2**) and butanoate metabolism (**Figure S7.3**). The higher-yielding succinate from acetate in diets C and S might lead to a higher propionate production through the succinate pathway and meanwhile resulted in their lower acetate proportion. *Succiniclasticum ruminis* was reported to be a propionate-producing

bacteria through the succinate pathway (van Gylswyk, 1995). In summary, based on metagenomic and metaproteomic data analysis, it is safe to conclude that the succinyl-CoA:acetate CoA-transferase (EC2.8.3.18) produced by *Succiniclasticum ruminis* might lead to the higher propionate productions by promoting the succinate pathway in diets C and S.

For the pyruvate metabolism (**Figure 7.7b**), from the metagenomics data, lactaldehyde dehydrogenase (EC1.2.1.22, $P = 0.030$), glycolaldehyde dehydrogenase (EC1.2.1.21, $P = 0.030$), acetyl-CoA synthetase (EC6.2.1.1, $P = 0.100$) and malate dehydrogenase (EC1.1.1.37, $P = 0.100$) were observed to have higher proportions in diet L than in diets C and S, while propionyl-CoA carboxylase (EC6.4.1.3, $P = 0.040$), phosphoenolpyruvate carboxykinase (EC4.1.1.49, $P = 0.100$) and homocitrate synthase (EC2.3.3.14, $P = 0.100$) were potentially higher in diet L. As for the metaproteomics analysis, only the malate dehydrogenases (EC1.1.1.37) were detected that were up-regulated by diet L compared to diets C and S, which were derived separately from *Prevotella* sp. bacterium ($P = 0.007$ and 0.002), *Prevotella* sp. ($P = 0.008$ and 0.002) and *Prevotella* sp. tc2-28 ($P = 0.005$ and 0.002). From metagenomic and metaproteomic data, even though it was not observed that more enzymes which were equally influenced by diets in the acetate synthesis pathways except for the succinyl-CoA:acetate CoA-transferase (EC2.8.3.18), the malate dehydrogenases which might play an important role in the higher acetate proportion in diet L through the pyruvate metabolism.

As for the butanoate metabolism, according to the metagenomics analysis (**Figure 7.8b**), the samples in diet L had significantly lower levels of EC1.1.1.157 (3-hydroxybutyryl-CoA dehydrogenase, $P = 0.039$) and EC2.6.1.19 (4-aminobutyrate aminotransferase/(S)-3-amino-2-methylpropionate transaminase, $P = 0.010$), but higher level of EC1.1.1.35 (3-hydroxyacyl-CoA dehydrogenase, $P = 0.005$) compared to diets C and S. As for the metaproteomics analysis, the enzymes of EC1.2.7.1 (pyruvate:ferredoxin (flavodoxin) oxidoreductase), which originated from *Prevotella* sp. bacterium ($P = 0.001$ and 0.006, separately), *Prevotella bryantii* ($P = 0.027$ and 0.013), *Prevotella* sp. tc2-28 ($P = 0.012$ and 0.008), *Prevotella* sp. tf2-5 ($P = 0.022$ and 0.024) and bacterium F083 ($P = 0.075$ and 0.020), and the enzymes of EC1.2.7.11 (3-methyl-2-oxobutanoate dehydrogenase subunit VorB), which originated from *Prevotella* sp. bacterium ($P = 0.039$ and 0.034) and *Prevotella* sp. kh1p2 ($P = 0.010$

and 0.014) were up-regulated when diet L was fed than diets C and S, which both promote the reaction from pyruvate to acetyl-CoA (**Figure S7.3**). Even though the same enzymes that were influenced by diets both in metagenomic and metaproteomic data were not observed, the abovementioned enzymes provided us with the candidate enzymes which might determine the differences in butyrate production. Besides, the succinyl-CoA:acetate CoA-transferase (EC2.8.3.18) catalysed the production of succinate and acetyl-CoA, which are substrates for butyrate production. The succinyl-CoA:acetate CoA-transferase might be the reason resulting in the higher butyrate proportion in diets C and S.

In total, through the combination of metagenomics and metaproteomics analysis, the succinyl-CoA:acetate CoA-transferase originated from *Succiniclasticum ruminis* was observed to be up-regulated in diets C and S relative to diet L, which catalysed the conversion reaction from acetate to succinate, and resulted in the higher proportions of propionate and butyrate but a lower acetate proportion in diets C and S.

Table 7.2. Pairwise comparison of protein information involved in KEGG pathways of propanoate, pyruvate and butanoate metabolism based on metaproteomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet

Enzymes [originated microbe]	L vs C		L vs S		S vs C	
	Regulation	*P*-value	Regulation	*P*-value	Regulation	*P*-value
Enzymes in propanoate metabolism						
formate C-acetyltransferase [*Succinivibrio* sp.]	down	0.020	down	0.001	NS	0.735
formate C-acetyltransferase [*Clostridiales* bacterium]	down	0.003	down	0.014	NS	0.923
formate C-acetyltransferase [*Clostridiales* bacterium NK3B98]	up	0.002	up	0.002	NS	0.442
phosphate acetyltransferase [*Prevotella ruminicola*]	up	0.005	up	0.006	NS	0.310
multispecies: phosphate butyryltransferase [*Bacteroidales*]	down	0.063	down	0.033	NS	0.565
acetyl-CoA C-acetyltransferase [*Candidatus* Weimeria bifida]	down	0.043	down	0.027	up	0.167
acetate kinase [*Prevotella* sp. bacterium]	up	0.027	up	0.025	NS	0.555
bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase [*Succinimonas amylolytica*]	up	0.063	up	0.095	NS	0.450
methylmalonyl-CoA epimerase [*Prevotella pectinovora*]	up	0.013	up	0.005	NS	0.133
methylmalonyl-CoA mutase family protein [*Succiniclasticum ruminis*]	down	0.019	down	0.031	up	0.036
ADP-forming succinate-CoA ligase subunit beta [*Thalassolituus* sp. C2-1]	down	0.030	down	0.020	up	0.106
succinate-CoA ligase subunit alpha [*Glaciecola pallidula*]	down	0.014	down	0.014	NS	0.272
multispecies: acetyl-CoA carboxylase carboxyl transferase subunit alpha [*Pseudoalteromonas*]	down	0.039	down	0.003	NS	0.539
Enzymes in pyruvate metabolism						
malate dehydrogenase [*Succinimonas amylolytica*]	down	0.010	down	0.007	up	0.176
malate dehydrogenase [*Selenomonas bovis*]	down	0.021	down	0.003	up	0.028
malate dehydrogenase [*Prevotella* sp. bacterium]	up	0.007	up	0.002	NS	0.213
malate dehydrogenase [*Prevotella* sp.]	up	0.008	up	0.002	up	0.062
malate dehydrogenase [*Prevotella* sp. tc2-28]	up	0.005	up	0.002	NS	0.406
malate dehydrogenase [*Succinimonas amylolytica*]	down	0.001	down	0.001	NS	0.108

Table 7.2 (continued).

Enzymes [originated microbe]	L vs C		L vs S		S vs C	
	Regulation	*P*-value	Regulation	*P*-value	Regulation	*P*-value
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. bacterium]	up	0.001	up	0.006	NS	0.895
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella bryantii*]	up	0.027	up	0.013	NS	0.523
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. tc2-28]	up	0.012	up	0.008	NS	0.656
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. tf2-5]	up	0.022	up	0.024	NS	0.710
pyruvate:ferredoxin (flavodoxin) oxidoreductase, homodimeric [Bacterium F083]	up	0.075	up	0.020	NS	0.100
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Candidatus* Weimeria bifida]	down	0.069	down	0.008	up	0.085
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Eubacterium* sp. *OM08-24*]	down	0.100	down	0.094	NS	0.606
3-methyl-2-oxobutanoate dehydrogenase subunit VorB [*Prevotella* sp. bacterium]	up	0.039	up	0.034	NS	0.607
2-oxoacid:ferredoxin oxidoreductase subunit beta [*Prevotella* sp. kh1p2]	up	0.010	up	0.014	NS	0.932
fumarate reductase (quinol) flavoprotein subunit [*Ruminobacter* sp. kh1p2]	down	0.015	down	0.002	NS	0.912
fumarate reductase (quinol) flavoprotein subunit [*Aeromonas australiensis*]	down	0.002	down	0.001	NS	0.303
formate C-acetyltransferase [*Succinivibrio* sp.]	down	0.009	down	0.003	NS	0.775
formate C-acetyltransferase [*Clostridiales* bacterium]	down	0.003	down	0.014	NS	0.923
formate C-acetyltransferase [*Succinivibrio* sp.]	down	0.020	down	0.001	NS	0.735
formate C-acetyltransferase [*Clostridiales* bacterium NK3B98]	up	0.002	up	0.002	NS	0.442
phosphate acetyltransferase [*Prevotella ruminicola*]	up	0.005	up	0.006	NS	0.310
multispecies: phosphate butyryltransferase [*Bacteroidales*]	down	0.063	down	0.033	NS	0.565
acetyl-CoA C-acetyltransferase [*Candidatus* Weimeria bifida]	down	0.043	down	0.027	up	0.167
pyruvate kinase PykF [*Aeromonas jandaei*]	down	0.001	down	0.007	up	0.136
acetate kinase [*Prevotella* sp. bacterium]	up	0.027	up	0.025	NS	0.555
pyruvate phosphate dikinase [*Alphaproteobacteria* bacterium CG11_big_fil_rev_8_21_14_0_20_39_49]	up	0.058	up	0.057	NS	0.693
pyruvate phosphate dikinase [*Prevotella* sp. bacterium]	up	0.076	up	0.083	NS	0.962
pyruvate phosphate dikinase [*Rhodospirillaceae* bacterium LM-1]	up	0.073	up	0.032	NS	0.154

Table 7.2 (continued).

Enzymes [originated microbe]	L vs C		L vs S		S vs C	
	Regulation	*P*-value	Regulation	*P*-value	Regulation	*P*-value
multispecies: pyruvate phosphate dikinase [unclassified *Roseburia*]	up	0.037	up	0.010	NS	0.500
pyruvate phosphate dikinase [*Prevotella ruminicola*]	up	0.006	up	0.008	NS	0.256
pyruvate phosphate dikinase [*Prevotella* sp. bacterium]	up	0.037	up	0.035	NS	0.860
pyruvate phosphate dikinase [*Firmicutes* bacterium CAG:555]	NS	0.082	up	0.035	NS	0.332
pyruvate phosphate dikinase [*Ruminococcaceae* bacterium P7]	down	0.031	down	0.038	NS	0.982
Succinyl-CoA:acetate CoA-transferase [*Succiniclasticum ruminis*]	down	0.004	down	0.011	NS	0.195
sodium-extruding oxaloacetate decarboxylase subunit alpha [*Treponema pectinovorum*]	up	0.001	up	0.001	NS	0.347
oxaloacetate decarboxylase subunit alpha [*Clostridiales* bacterium]	up	0.054	up	0.048	NS	0.486
phosphoenolpyruvate carboxykinase (GTP) [*Treponema* sp. bacterium]	up	0.001	up	0.001	NS	0.552
phosphoenolpyruvate carboxykinase (GTP) [*Treponema berlinense*]	up	0.004	up	0.003	NS	0.111
phosphoenolpyruvate carboxykinase [ATP] [*Firmicutes* bacterium CAG:475]	up	0.096	up	0.093	NS	0.815
phosphoenolpyruvate carboxykinase (ATP) [*Prevotella brevis*]	up	0.008	up	0.023	NS	0.482
phosphoenolpyruvate carboxykinase (ATP) [*Lachnospiraceae* bacterium NE2001]	up	0.080	up	0.023	NS	0.445
phosphoenolpyruvate carboxykinase (ATP) [*Prevotella* sp. P5-92]	up	0.010	up	0.005	NS	0.049
multispecies: phosphoenolpyruvate carboxykinase (ATP) [*Alistipes*]	down	0.001	down	0.006	NS	0.230
phosphoenolpyruvate carboxykinase (ATP) [*Lachnospiraceae* bacterium]	down	0.001	down	0.003	up	0.100
multispecies: acetyl-CoA carboxylase carboxyl transferase subunit alpha [*Pseudoalteromonas*]	down	0.039	down	0.003	NS	0.539
Enzymes in butanoate metabolism						
beta hydroxybutyryl-CoA dehydrogenase [*Butyrivibrio fibrisolvens*]	up	0.008	up	0.024	up	0.335
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. bacterium]	up	0.001	up	0.006	NS	0.895
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella bryantii*]	up	0.027	up	0.013	NS	0.523
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. tc2-28]	up	0.012	up	0.008	NS	0.656
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Prevotella* sp. tf2-5]	up	0.022	up	0.024	NS	0.710

Table 7.2 (continued).

Enzymes [originated microbe]	L vs C		L vs S		S vs C	
	Regulation	*P*-value	Regulation	*P*-value	Regulation	*P*-value
pyruvate:ferredoxin (flavodoxin) oxidoreductase, homodimeric [Bacterium F083]	up	0.075	up	0.020	NS	0.100
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Eu*bacterium sp. *OM08-24*]	down	0.100	down	0.094	NS	0.606
pyruvate:ferredoxin (flavodoxin) oxidoreductase [*Candidatus* Weimeria bifida]	down	0.069	down	0.008	up	0.085
3-methyl-2-oxobutanoate dehydrogenase subunit VorB [*Prevotella* sp. bacterium]	up	0.039	up	0.034	NS	0.607
2-oxoacid:ferredoxin oxidoreductase subunit beta [*Prevotella* sp. kh1p2]	up	0.010	up	0.014	NS	0.932
bifunctional NADH-specific enoyl-ACP reductase/trans-2-enoyl-CoA reductase [*Succinivibrionaceae* bacterium]	down	0.008	down	0.022	NS	0.592
succinate dehydrogenase/fumarate reductase iron-sulfur subunit [*Prevotella* sp. tc2-28]	up	0.081	up	0.057	NS	0.339
fumarate reductase/succinate dehydrogenase flavoprotein subunit [*Prevotella ruminicola*]	up	0.022	up	0.025	NS	0.734
fumarate reductase (quinol) flavoprotein subunit [*Ruminobacter* sp. kh1p2]	down	0.015	down	0.002	NS	0.912
fumarate reductase (quinol) flavoprotein subunit [*Aeromonas australiensis*]	down	0.002	down	0.001	NS	0.303
acetolactate synthase 3 large subunits [*Gammaproteobacteria* bacterium]	down	0.001	down	0.002	NS	0.753
acetolactate synthase 3 large subunits [*Agarivorans* sp. Alg241-V36]	down	0.016	down	0.024	NS	0.368
formate C-acetyltransferase [*Succinivibrio* sp.]	down	0.020	down	0.001	NS	0.735
formate C-acetyltransferase [*Clostridiales* bacterium NK3B98]	up	0.002	up	0.002	NS	0.442
formate C-acetyltransferase [*Succinivibrio* sp.]	down	0.009	down	0.003	NS	0.775
formate C-acetyltransferase [*Clostridiales* bacterium]	down	0.003	down	0.014	NS	0.923
multispecies: phosphate butyryltransferase [*Bacteroidales*]	down	0.063	down	0.033	NS	0.565
acetyl-CoA C-acetyltransferase [*Candidatus* Weimeria bifida]	down	0.043	down	0.027	up	0.167
Succinyl-CoA:acetate CoA-transferase [*Succiniclasticum ruminis*]	down	0.004	down	0.011	NS	0.195

Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. KEGG, Kyoto Encyclopedia of Genes and Genomes. up/down (A vs B), this protein is up/down regulated in group A than in group B.

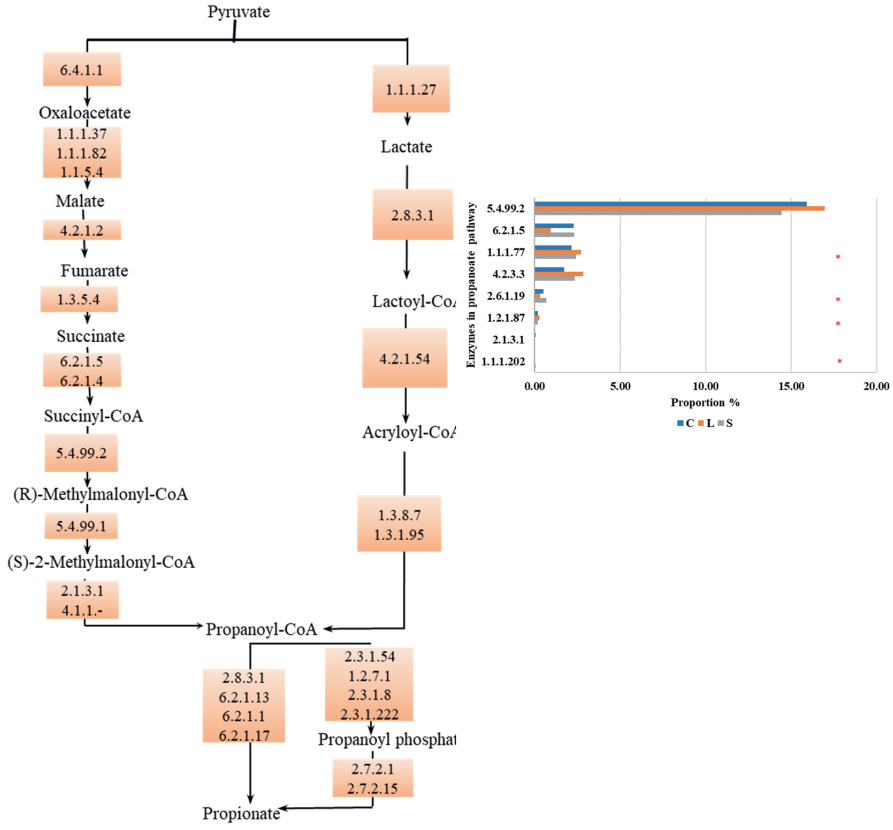


Figure 7.6. (a) Propionate synthesis pathways and (b) the proportion of enzymes in the propanoate metabolism pathway with $P \leq 0.1$ based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. 5.4.99.2, methylmalonyl-CoA mutase; 6.2.1.5, succinyl-CoA synthetase alpha subunit; 1.1.1.77, lactaldehyde reductase; 4.2.3.3, methylglyoxal synthase; 2.6.1.19, 4-aminobutyrate aminotransferase; 1.2.1.87, propionaldehyde dehydrogenase; 2.1.3.1, methylmalonyl-CoA carboxyltransferase 5S subunit; 1.1.1.202, 1,3-propanediol dehydrogenase. * $P \leq 0.05$. Figure (a) was created in BioRender.com.

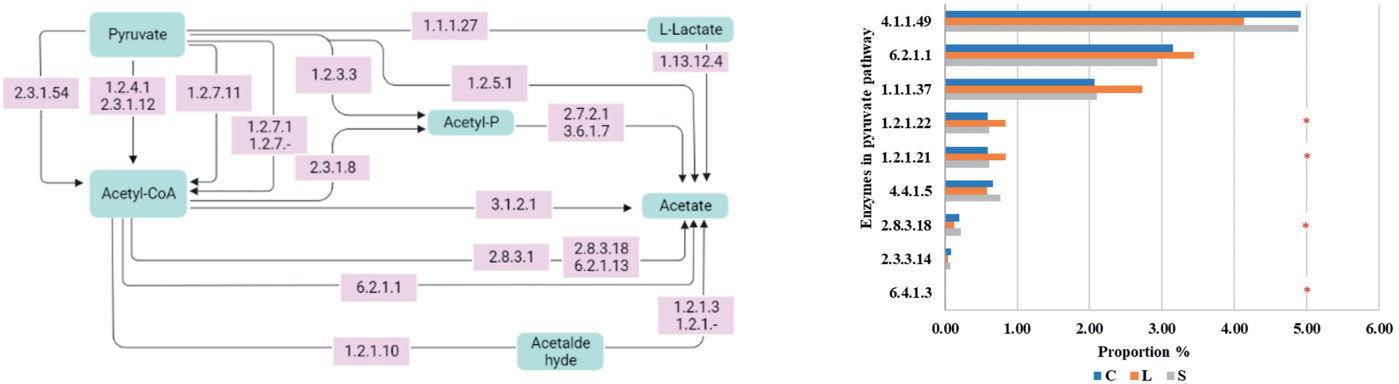


Figure 7.7. (a) Acetate synthesis pathways and (b) the proportion of enzymes in the pyruvate metabolism pathway with $P \leq 0.1$ based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. 4.1.1.49, phosphoenolpyruvate carboxykinase (ATP); 6.2.1.1, acetate-CoA ligase; 1.1.1.37, malate dehydrogenase; 1.2.1.22, lactaldehyde dehydrogenase; 1.2.1.21, glycolaldehyde dehydrogenase; 4.4.1.5, lactoylglutathione lyase; 2.8.3.18, succinyl-CoA:acetate CoA-transferase; 2.3.3.14, homocitrate synthase; 6.4.1.3, propionyl-CoA carboxylase. * $P \leq 0.05$. Figure (a) was created in BioRender.com.

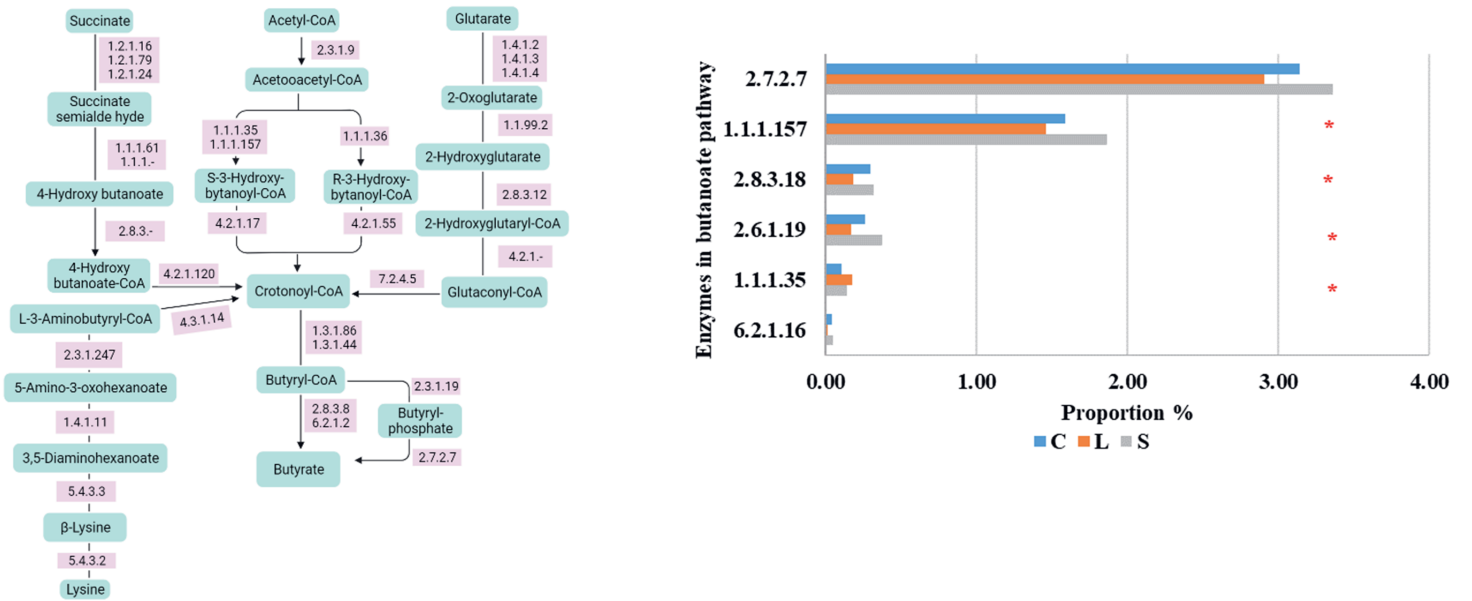


Figure 7.8. (a) Butyrate synthesis pathways and (b) the proportion of enzymes in the butanoate metabolism pathway with $P \leq 0.1$ based on metagenomics analysis in rumen fluid of dairy cows fed two glucogenic (C, S) and a lipogenic (L) diet. Diets: C, corn and corn silage diet; L, sugar beet pulp and alfalfa silage diet; S, steam-flaked corn and corn silage diet. 2.7.2.7, butyrate kinase; 1.1.1.157, 3-hydroxybutyryl-CoA dehydrogenase; 2.8.3.18, succinyl-CoA:acetate CoA-transferase; 2.6.1.19, 4-aminobutyrate aminotransferase/(S)-3-amino-2-methylpropionate transaminase; 1.1.1.35, 3-hydroxyacyl-CoA dehydrogenase. * $P \leq 0.05$. Figure (a) was created in BioRender.com.

Integration of omics techniques

The rumen microbiome combined with metabolomics has been used to link changes in the rumen microbiome to changes in the ruminal fermentation and metabolite formation, which allows evaluation of the rumen functioning in response to dietary strategies including new rations, feed ingredients, or additives (Zhang et al., 2017, Abecia et al., 2018, Xue et al., 2020, Wang et al., 2021). Correlations between the abundance of microbial phyla or genera and specific rumen metabolites have been commonly conducted (Belanche et al., 2019), in an attempt to provide a functional context to changes in the rumen microbial population. In the present thesis, the 16S rRNA sequencing and metabolomics approaches were used in **Chapters 3** and **5** in order to detect the alterations in the bacterial communities, metabolites and metabolic pathways, and then to further correlate the affected bacteria to affected metabolites. Here the correlations between the relative abundance of microbial genera and specific rumen metabolites are provided (**Chapters 3**, **4** and **5**). However, it should be noted that the analyses were limited to contain a small range of well-defined rumen metabolites. The Bovine Metabolome Database (BMDB) which only lists the bovine-derived metabolites is still under development (Foroutan et al., 2020). The Human Metabolome Database (HMDB) is the most widely used database for annotating rumen metabolome (Wishart et al., 2009). In the future, the addition of more samples and the inclusion of more studies are required to improve the quality and reliability of the data in the BMDB (Foroutan et al., 2020).

Another study revealed that even though the rumen microbiomes showed differences in the taxonomic compositions, their metabolic functions remain the same (Taxis et al., 2015). This indicates that the microbial diversity at the composition and taxonomic level may not be directly associated with metabolic functions that affect the host. It suggests that differences might occur at the transcription or protein level. Recent studies in which amplicon sequencing has been combined with metaproteomics analysis have established that integrated omics approach allows for a greater insight into the complex network of microbial adaptation in the rumen (Deusch et al., 2017). The combination of metagenomics and metaproteomics was already applied in ruminant animal studies (Zhu et al., 2016, Zhang et al., 2020), but was barely related to rumen functions. In **Chapter 6**, the metabolic pathways influenced by diets both at the gene and protein

level through a combination of metagenomics and metaproteomics approaches, in which alterations of the starch and sucrose metabolism, the differential influenced enzymes and their originated microbes were revealed. In **Chapter 7**, the pathways and enzymes involved in VFA production through a combination of metagenomics and metaproteomics approaches were detected. The result showed that the succinyl-CoA:acetate CoA-transferase produced by the *Succiniclasticum ruminis* might be the main reason resulting in the higher propionate and butyrate proportions but a lower acetate proportion in the starch-containing diets. The current findings provide insight into the exploration and exploitation of the metabolic pathways of certain phenotypes.

Future directions

Characterization of the rumen microbiome

The omics technologies have provided a picture of which species are present and their potential function in the rumen. As more uncultured ruminal microbes are detected, it will require an increased focus and revival of culture-based techniques through new high throughput culturing methods and media to isolate and characterize novel microbes (Kenters et al., 2011, Lagier et al., 2016). For example, this thesis observed multiple unclassified species from the rumen which need to be characterized.

In addition, as many of the current studies focus only on the bacterial and archaeal communities in the rumen, the other microbial communities with small proportions for instance, the fungi, protozoa and virus populations are easily ignored. The rumen microbial functions will be better understood and utilized only when all aspects of the microbiome are considered (Newbold and Ramos-Morales, 2020).

Monitoring of rumen microbial functions

Due to our limited ability to rationally design and drive rumen microbial composition and function in this highly complex and dynamically changing environment, Ark et al. (2017) came up with the construction of genome-scale metabolic models (GEM) for specific isolates which contain accurate annotation of their functional gene products. Most microbes have the capacity to utilise a wide array of nutrients using varied metabolic pathways. Then, the minimal nutrient requirements of the un-cultivated microbes can be predicted based on their GEM (Henry et al., 2010). For instance, 1) a desired rumen phenotype of interest can be predicted by modelling the co-cultures and

interactions of multispecies, and 2) modelling the competitive and cooperative metabolism draw the conclusion that competition is generally dominated by versatile fast-growing species (Freilich et al., 2011).

Since a large number of microbial metabolites and microbial-host co-metabolites are also present in plasma and other body fluids, the discovery of biomarkers which can be linked to microbiota function can be applied in research or farms to monitor changes in rumen functioning. For example, 1) saliva and buccal swab samples have already been proved to reflect the rumen microbiome, 2) changes in plasma fatty acid profiles were suggested as biomarkers for weight gain, and 3) levels of trimethylamine N-oxide as a marker for methylamine utilising methanogens (Kittelmann et al., 2015, Morgavi et al., 2015, Tapio et al., 2016, Artegoitia et al., 2017).

Host-microbiome interactions

Future research should not only focus on interactions among microbes but also on how microbial metabolites alter host gene expression in various tissues. Fouhse et al. (2017) examined the connection between the immune system in ruminants and rumen microbes which found the host-specific interaction between salivary immunoglobulin lgA and some Toll-like receptors in the rumen epithelia. Similarly, Liu et al. (2015) reported that several ruminal epithelial Toll-like receptors which were involved in the recruitment of immune cells and the production of inflammatory cytokines were up-regulated by high-grain diets. The alterations in the rumen environment may cause systemic changes in the host and *vice versa*.

The omics techniques lead to targeted gene or enzyme interventions that direct rumen composition and activity towards desired performance. For example, Kumar et al. (2014) detected the key enzymes and proteins that can be targeted for methane inhibition by comparing two strains of methanogenic archaea, *Methanobrevibacter boviskoreani* strain JH1 and strain AbM4.

Conclusion

The ruminal microbial community structure, amylolytic and fibrolytic communities from all rumen fluid samples were detected through the 16S r RNA sequencing and metagenomics approaches. The results show that the metagenomics analysis detected the microbial community with high relative abundance like bacteria but was less able to detect lower abundant microbiota such as archaea, fungi and protozoa, for which targeted metagenomics would be a better approach. The amylolytic and fibrolytic bacteria were not among the dominant population in the rumen. The succinyl-CoA:acetate CoA-transferase which originated from *Succiniclasticum ruminis* was observed to be up-regulated in diets C and S relative to diet L from the metagenomics and metaproteomics analysis. This enzyme catalyses the conversion reaction from acetate to succinate and resulted in the higher proportions of propionate and butyrate but a lower acetate proportion in diets C and S. The interaction of various omics technologies was used in exploring the rumen functions, but interpretation is limited by the lack of an accurate database.

In the future, the applications of omics technologies will promote the development of characterizing rumen microbiome including the relatively small communities. Monitoring the rumen functions will become easier through the use of a modelling approach where the metabolic activities of certain microbes or detecting certain biomarkers from other tissues is employed. Finally, rumen functioning can be manipulated towards desired performance through targeted gene or protein intervention of rumen microbiota.

Supplementary material

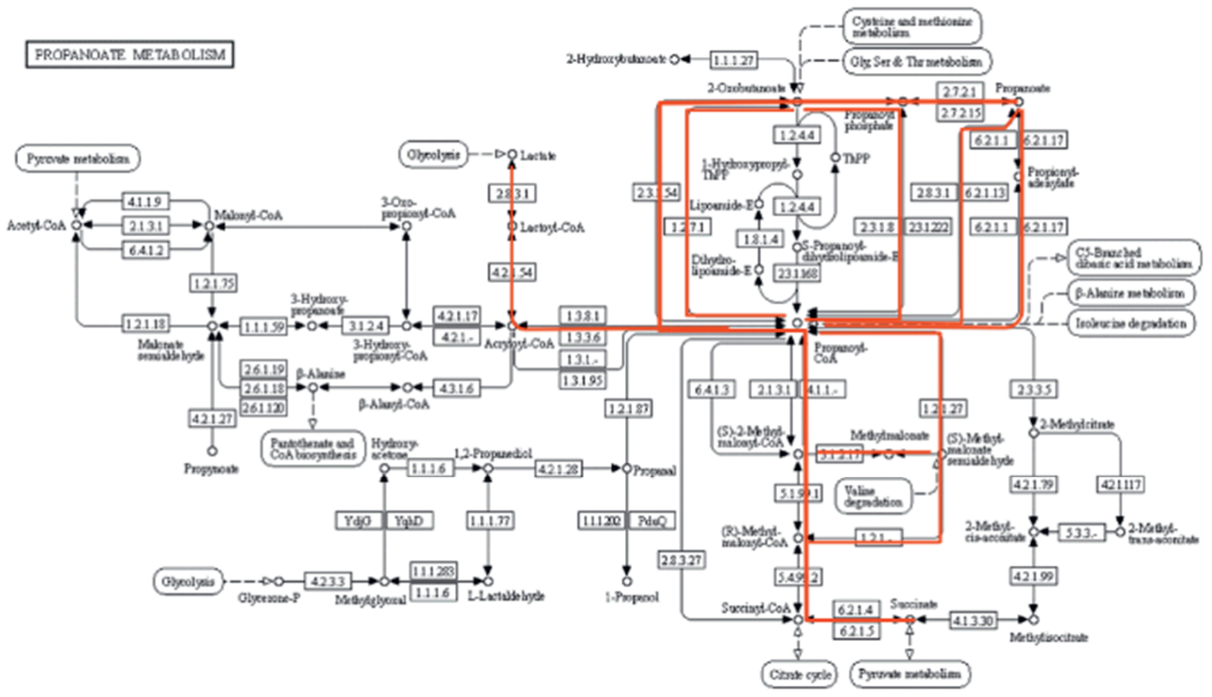


Figure S7.1. KEGG pathway map of propanoate metabolism. The red lines are associated with the propionate synthesis pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes. The figure was adapted by BioRender.com.

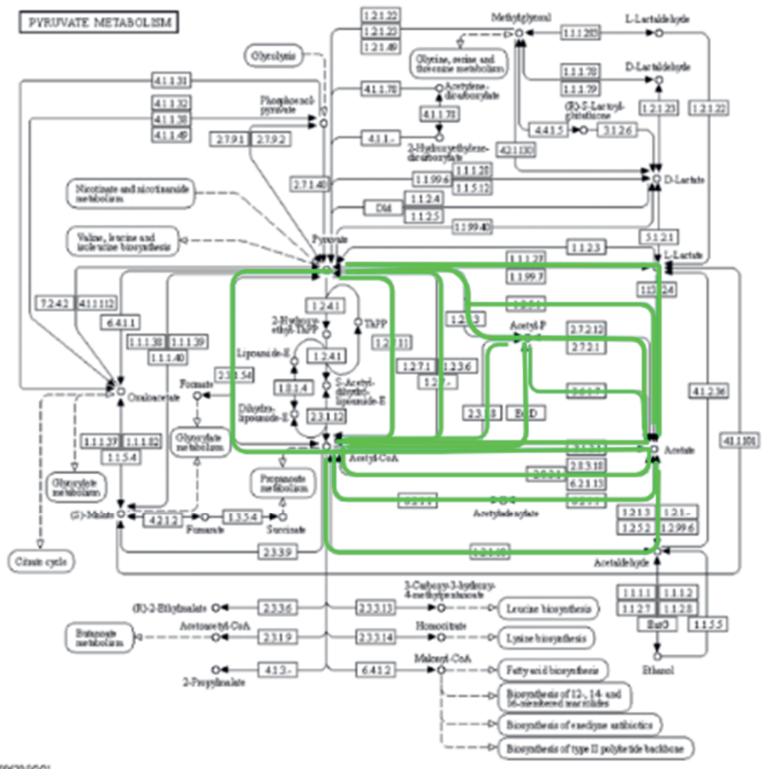


Figure S7.2. KEGG pathway map of pyruvate metabolism. The green lines are associated with acetate synthesis pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes. The figure was adapted by BioRender.com

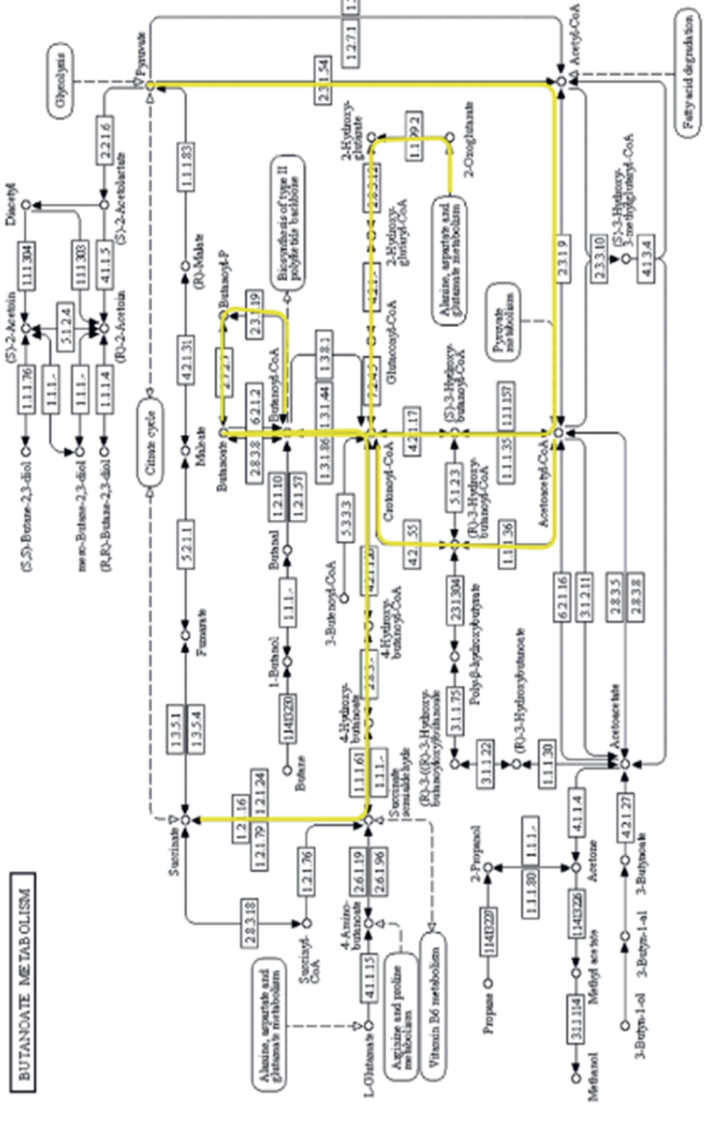


Figure S7.3. KEGG pathway map of butanoate metabolism. The yellow lines are associated with butyrate synthesis pathways. KEGG, Kyoto Encyclopedia of Genes and Genomes. The figure was adapted by BioRender.com

Reference

Abecia, L., Martínez-Fernandez, G., Waddams, K., Martín-García, A. I., Pinloche, E., Creevey, C. J., Denman, S. E., Newbold, C. J., and Yáñez-Ruiz, D. R. 2018. Analysis of the rumen microbiome and metabolome to study the effect of an antimethanogenic treatment applied in early life of kid goats. *Front. Microbiol.* 9:2227.

Agarwal, N., Kamra, D. N., and Chaudhary, L. C. 2015. Rumen microbial ecosystem of domesticated ruminants, in Chapter 2, Page: 17, Rumen microbiology: from evolution to revolution. Puniya, A. K., Singh, R., and Kamra, D. N., ed. Springer, London.

Artegoitia, V. M., Foote, A. P., Lewis, R. M., and Freetly, H. C. 2017. Rumen fluid metabolomics analysis associated with feed efficiency on crossbred steers. *Sci. Rep.* 7:2864.

Choudhury, P. K., Sirohi, S. K., and Puniya, A. K. 2012. Harnessing the diversity of rumen microbes using molecular approaches, in Chapter 6, Page: 65-82, Livestock greenhouse gases: emission and options for mitigation. Sirohi, S. K., Walli, T. K., Singh, B., and Singh, N., ed. Satish Serial Publishing House, Delhi.

Deng, W., Xi, D., Mao, H., and Wanapat, M. 2008. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: a review. *Mol. Biol. Rep.* 35(2):265-274.

Deusch, S., Tilocca, B., Camarinha-Silva, A., and Seifert, J. 2015. News in livestock research-use of omics technologies to study the microbiota in the gastrointestinal tract of farm animals. *Comput. Struct. Biotechnol. J.* 13:55-63.

Foroutan, A., Fitzsimmons, C., Mandal, R., Piri-Moghadam, H., Zheng, J., Guo, A., Li, C., Guan, L. L., and Wishart, D. S. 2020. The bovine metabolome. *Metabolites* 10(6):233.

Fouhse, J. M., Smiegielski, L., Tuplin, M., Guan, L. L., and Willing, B. P. 2017. Host immune selection of rumen bacteria through salivary secretory IgA. *Front. Microbiol.* 8:848.

Fouts, D. E., Szpakowski, S., Purushe, J., Torralba, M., Waterman, R. C., MacNeil, M. D., Alexander, L. J., and Nelson, K. E. 2012. Next generation sequencing to define prokaryotic and fungal deversity in the bovine rumen. *PLoS One* 7(11):e48289.

Freilich, S., Zarecki, R., Eilam, O., Segal, E. S., Henry, C. S., Kupiec, M., Gophna, U., Sharan, R., and Ruppin, E. 2011. Competitive and cooperative metabolic interactions in bacterial communities. *Nat. Commun.* 2:589.

Henry, C. S., DeJongh, M., Best, A. A., Frybarger, P. M., Linsay, B., and Stevens, R. L. 2010. High-throughput generation, optimization and analysis of genome-scale metabolic models. *Nat. Biotechnol.* 28(9):977-982.

Huntington, B. G. 1997. Starch utilization by ruminants: from basics to the bunk. *J. Dairy Sci.* 75:852-867.

Janssen, P. H., and Kirs, M. 2008. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74(12):3619-3625.

Jeyanathan, J., Martin, C., and Morgavi, D. P. 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8(2):250-261.

Kamra, D. N. 2005. Rumen microbial ecosystem. *Curr. Sci. India* 89(1):124-135.

Kenters, N., Henderson, G., Jeyanathan, J., Kittelmann, S., and Janssen, P. H. 2011. Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *J. Microbiol. Meth.* 84(1):52-60.

Kim, M., Morrison, M., and Yu, Z. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. *FEMS Microbiol. Ecol.* 76(2011):49-63.

Kittelmann, S., Kirk, M. R., Jonker, A., McCulloch, A., Janssen, P. H., and Schloss, P. D. 2015. Buccal swabbing as a non-invasive method to determine bacterial, archaeal, and eukaryotic microbial community structures in the rumen. *Appl. Environ. Microbiol.* 81(21):7470-7483.

Krause, D. O., Denman, S. E., Mackie, R. I., Morrison, M., Rae, A. L., Attwood, G. T., and McSweeney, C. S. 2003. Opportunities to improve fibre degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* 27(5):663-693.

Kumar, S., Choudhury, P. K., Carro, M. D., Griffith, G. W., Dagar, S. S., Puniya, M., Calabro, S., Ravella, S. R., Dhewa, T., Upadhyay, R. C., Sirohi, S. K., Kundu, S. S., Wanapat, M., and Puniya, A. K. 2014. New aspects and strategies for methane mitigation from ruminants. *Appl. Microbiol. Biotechnol.* 98(1):31-44.

Lagier, J., Khelaifia, S., Alou, M. T., Ndongo, S., Dione, N., Hugon, P., Caputo, A., Cadoret, F., Traore, S. I., Seck, E. H., Dubourg, G., Durand, G., Mourembou, G., Guilhot, E., Togo, A., Bellali, S., Bachar, D., Cassir, N., Bittar, F., Delerce, J., Mailhe, M., Ricaboni, D., Bilen, M., Dangui Nieko, N. P. M., Dia Badiane, N. M., Valles, C., Mouelhi, D., Diop, K., Million, M., Musso, D., Abrahão, J., Azhar, E. I., Bibi, F., Yasir, M., Diallo, A., Sokhna, C., Djossou, F., Vitton, V., Robert, C., Rolain, J. M., La Scola, B., Fournier, P., Levasseur, A., and Raoult, D. 2016. Culture of previously uncultured members of the human gut microbiota by culturomics. *Nat. Microbiol.* 1:16203.

Larue, R., Yu, Z., Parisi, V. A., Egan, A. R., and Morrison, M. 2005. Novel microbial diversity adherent to plant biomass in the herbivore gastrointestinal tract, as revealed by ribosomal intergenic spacer analysis and rrs gene sequencing. *Environ. Microbiol.* 7(4):530-543.

Liang, J., Fang, W., Wang, Q., Zubair, M., Zhang, G., Ma, W., Cai, Y., and Zhang, P. 2021. Metagenomic analysis of community, enzymes and metabolic pathways during corn straw fermentation with rumen microorganisms for volatile fatty acid production. *Bioresour. Technol.* 342:126004.

Lin, C., Raskin, L., and Stahl, D. A. 1997. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes. *FEMS Microbiol. Ecol.* 22:281-294.

Liu, J., Bian, G., Zhu, W., and Mao, S. 2015. High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of Toll-like receptor genes in goats. *Front. Microbiol.* 6:167.

Luo, Y., Pfister, P., Leisinger, T., and Wasserfallen, A. 2001. The genome of archaeal prophage M100 encodes the lytic enzyme responsible for autolysis of *Methanothermobacter wolfeii*. *J. Bacteriol.* 183:5788-5792.

Mendoza, G. D., Britton, R. A., and Stock, R. A. 1999. Effect of feeding mixtures of high moisture corn and dry-rolled grain sorghum on ruminal fermentation and starch digestion. *Small Ruminant Res.* 32(2):113-118.

Morgavi, D. P., Rathahao-Paris, E., Popova, M., Boccard, J., Nielsen, K. F., and Boudra, H. 2015. Rumen microbial communities influence metabolic phenotypes in lambs. *Front. Microbiol.* 6:1060.

Mullins, E. A., Francois, J. A., and Kappock, T. J. 2008. A specialized citric acid cycle requiring succinyl-Coenzyme A (CoA):acetate CoA-transferase (AarC) confers acetic acid resistance on the acidophile Acetobacter aceti. *J. Bacteriol*. 190(14):4933-4940.

Mullins, E. A., and Kappock, T. J. 2012. Crystal structures of *Acetobacter aceti* succinyl-Coenzyme A (CoA):acetate CoA-transferase reveal specificity determinants and illustrate the mechanism used by class I CoA-transferases. *Biochemistry-US* 51(42):8422-8434.

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

Pfister, P., Wasserfallen, A., Stettler, R., and Leisinger, T. 1998. Molecular analysis of *Methano*bacterium phage M2. *Mol. Microbiol*. 30(2):233-244.

Pitta, D. W., Pinchak, W. E., Dowd, S. E., Osterstock, J., Gontcharova, V., Youn, E., Dorton, K., Yoon, I., Min, B. R., Fulford, J. D., Wickersham, T. A., and Malinowski, D. P. 2010. Rumen bacterial diversity dynamics associated with changing from bermudagrass hay to grazed winter wheat diets. *Microb. Ecol*. 59(3):511-522.

Rezaeian, M., Beakes, G. W., and Parker, D. S. 2004. Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep. *Mycol. Res*. 108(10):1227-1233.

Sharp, R., Ziemer, C. J., Stern, M. D., and A., S. D. 1998. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. *FEMS Microbiol. Ecol*. 26:71-78.

Sirohi, S. K., Singh, N., Dagar, S. S., and Puniya, A. K. 2012. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. *Appl. Microbiol. Biotechnol.* 95(5):1135-1154.

Tajima, K., Nagamine, T., Matsui, H., Nakamura, M., and Aminov, R. I. 2001. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiol. Lett*. 200(2001):67-72.

Tapio, I., Shingfield, K. J., McKain, N., Bonin, A., Fischer, D., Bayat, A. R., Vilkki, J., Taberlet, P., Snelling, T. J., and Wallace, R. J. 2016. Oral samples as non-invasive proxies for assessing the composition of the rumen microbial community. *PLoS One* 11(3):e151220.

Taxis, T. M., Wolff, S., Gregg, S. J., Minton, N. O., Zhang, C., Dai, J., Schnabel, R. D., Taylor, J. F., Kerley, M. S., Pires, J. C., Lamberson, W. R., and Conant, G. C. 2015. The players may change but the game remains: network analyses of ruminal microbiomes suggest taxonomic differences mask functional similarity. *Nucleic Acids Res*. 43:20.

van der Ark, K. C. H., van Heck, R. G. A., Martins Dos Santos, V. A. P., Belzer, C., and de Vos, W. M. 2017. More than just a gut feeling: constraint-based genome-scale metabolic models for predicting functions of human intestinal microbes. *Microbiome* 5:78.

van Gylswyk, N. O. 1995. *Succiniclasticum ruminis* gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int. J. Syst. Bacteriol*. 45(2):297-300.

Vital, M., Howe, A. C., Tiedje, J. M., and Moran, M. A. 2014. Revealing the bacterial butyrate synthesis pathways by analysing (meta)genomic data. *MBio* 5(2):e889.

Wallace, R. J., Snelling, T. J., McCartney, C. A., Tapio, I., and Strozzi, F. 2017. Application of meta-omics techniques to understand greenhouse gas emissions originating from ruminal metabolism. *Genet. Sel. Evol.* 49:9.

Wang, Y., Nan, X., Zhao, Y., Wang, Y., Jiang, L., Xiong, B., and Björkroth, J. 2021. Ruminal degradation of rumen-protected glucose influences the ruminal microbiota and metabolites in early-lactation dairy cows. *Appl. Environ. Microbiol.* 87(2):1.

Wishart, D. S., Knox, C., Guo, A. C., Eisner, R., Young, N., Gautam, B., Hau, D. D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J. A., Lim, E., Sobsey, C. A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li, L., Vogel, H. J., and Forsythe, I. 2009. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res.* 37:D603-D610.

Wright, A. G., and Klieve, A. V. 2011. Does the complexity of the rumen microbial ecology preclude methane mitigation? *Anim. Feed Sci. Technol.* 166-167:248-253.

Xue, M., Sun, H., Wu, X., Liu, J., and Guan, L. L. 2020. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome* 8:64.

Zhang, J., Shi, H., Wang, Y., Li, S., Cao, Z., Ji, S., He, Y., and Zhang, H. 2017. Effect of dietary forage to concentrate ratios on dynamic profile changes and interactions of ruminal microbiota and metabolites in Holstein heifers. *Front. Microbiol.* 8:2206.

Zhang, L., Li, L., Sha, G., Liu, C., Wang, Z., and Wang, L. 2020. Aerobic composting as an effective cow manure management strategy for reducing the dissemination of antibiotic resistance genes: an integrated meta-omics study. *J. Hazard. Mater.* 386:121895.

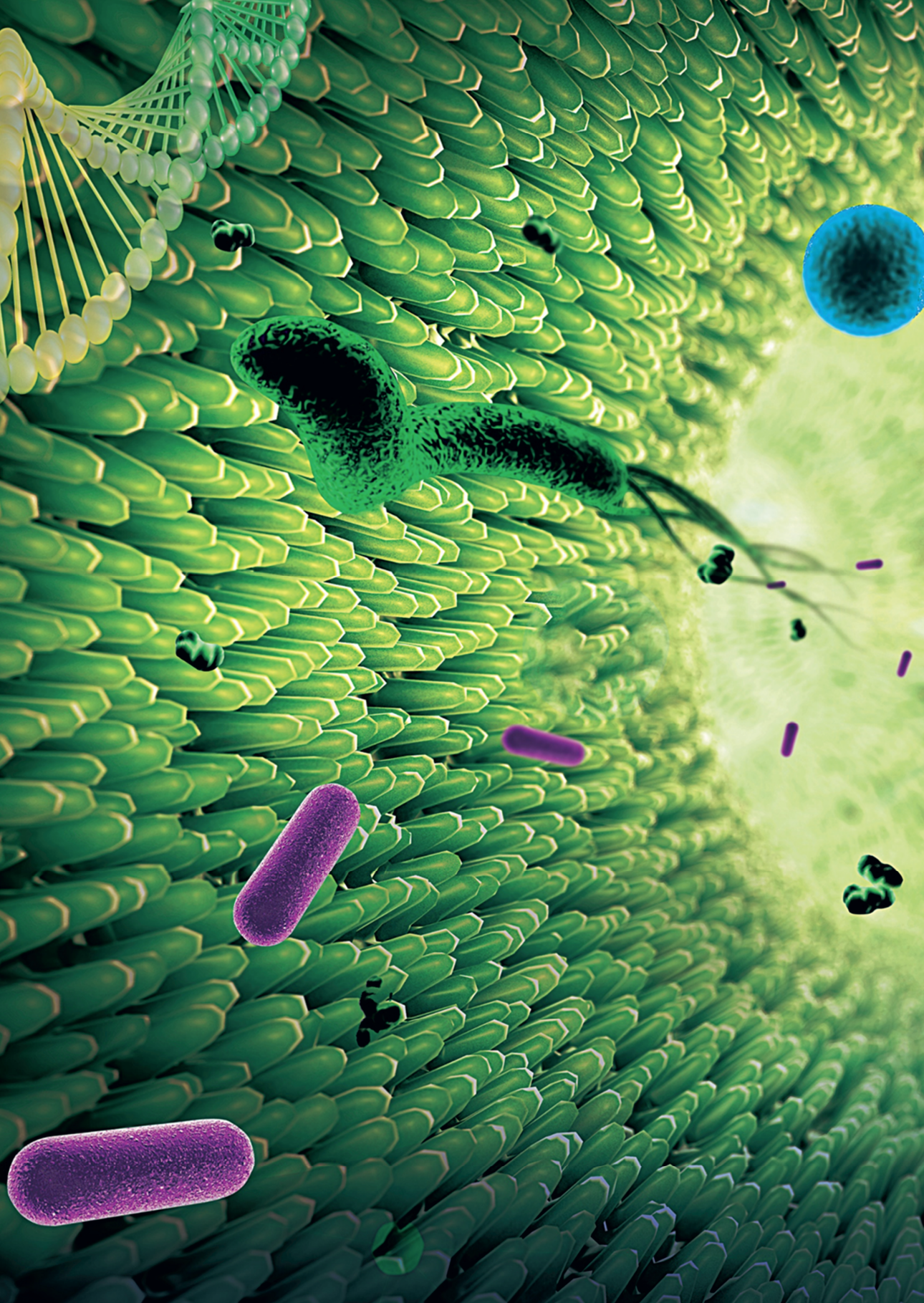
Zhou, X. Q., Zhang, Y. D., Zhao, M., Zhang, T., Zhu, D., Bu, D. P., and Wang, J. Q. 2015. Effect of dietary energy source and level on nutrient digestibility, rumen microbial protein synthesis, and milk performance in lactating dairy cows. *J. Dairy Sci.* 98(10):7209-7217.

Zhu, N., Yang, J., Ji, L., Liu, J., Yang, Y., and Yuan, H. 2016. Metagenomic and metaproteomic analyses of a corn stover-adapted microbial consortium EMSD5 reveal its taxonomic and enzymatic basis for degrading lignocellulose. *Biotechnol. Biofuels* 9:243.

Zhu, Z., Difford, G. F., Noel, S. J., Lassen, J., Løvendahl, P., and 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. *Front. Microbiol.* 12:636223.

Ziemer, C. J., Sharp, R., Stern, M. D., Cotta, M. A., Whitehead, T. R., and Stahl, D. A. 2000. Comparison of microbial populations in model and natural rumens using 16S ribosomal RNA-targeted probes. *Environ. Microbiol.* 2(6):632-643.

Zoetendal, E. G., Cheng, B., Koike, S., and Mackie, R. I. 2004. Molecular microbial ecology of the gastrointestinal tract: from phylogeny to function. *Curr. Iss. Intest. Microbiol.* 5:31-48.



Summary

Adjusting the glucogenic-to-lipogenic nutrient ratios in diets has been an important strategy to improve the energy status of dairy cows. The compositional and functional alterations of the ruminal microbiome in response to diets with different glucogenic and lipogenic nutrients have been investigated. However, as advanced omics techniques including metagenomics, metatranscriptomics, metaproteomics and metabolomics have developed at a rapid pace in recent years, detailed studies on rumen functions in response to different diets can now be more informative and comprehensive regarding rumen functioning.

In this thesis, three iso-nutritious diets were chosen as experimental treatments to yield a moderate (ground corn vs steam-flaked corn, C vs S) and a maximum (glucogenic vs lipogenic, C and S vs L) effects on rumen microbiota functioning. For diets C and S, starch was the main energy sources, while diet L contained mainly fibre as energy source. Multiple omic techniques, including metagenomics, metaproteomics and metabolomics were used to study the changes in the ruminal microbial compositions and functions in response to these dietary treatments.

Chapter 2 reviewed 1) the well-characterized amylolytic and fibrolytic microbes and their associated enzymes reported in the literature and 2) the application of metagenomics approach on the ruminal carbohydrate-active enzymes to date. Amylolytic and fibrolytic microbes mainly consist of bacteria and partly of protozoa, fungi and archaea. Enzymes reported in the rumen were mostly carbohydrate-active enzymes detected through a metagenomics approach. This chapter presents the candidate microbes and enzymes which were focussed on in the following chapters.

In order to explore the microbial composition in rumen fluid and their responses to the above-mentioned dietary treatments, two *in vitro* trials were firstly conducted which are reported in **Chapters 3** and **4**.

In **Chapter 3**, the effects of the three diets on the ruminal bacterial and archaeal structures, the metabolomic products, rumen fermentation and gas production through a combination of 16S rRNA sequencing and metabolomics are evaluated. The two glucogenic diets had greater effects than the lipogenic diet in terms of improving the dry matter digestibility, increasing propionate concentration and promoting amino acid metabolism based on the metabolomics data. The improvement in propionate

production may be attributed to the increased number of bacterial spp. functioning in the succinate pathway. Compared to ground corn, steam-flaked corn did not show more differences in fermentation end-products except for an increase in gas production and down-regulation of the production of some fatty acids and amino acids. Several amylolytic and cellulolytic bacteria were sensitive to the dietary changes, while most highly abundant bacteria were stable or minorly affected. For instance, the cellulolytic bacteria, including the genera of *Ruminococcus*, *Butyrivibrio*, *Eu*bacterium, *Lachnospira*, unclassified *Lachnospiraceae* and unclassified *Ruminococcaceae* had higher relative abundances in diet L, while amylolytic bacteria genera including *Selenomonas_1*, *Ruminobacter* and *Succinivibrionaceae_UCG-002* had higher relative abundances in diet G and S. Correlative analysis between microbes and fermentation metabolites proved to be an effective method to explore the functions of certain microbes.

In **Chapter 4**, the effect of different ratios of glucogenic (G) to lipogenic (L) nutrients (G:L= 3:0, 2:1, 1:2, 0:3) on rumen fermentation end-products and the corresponding bacterial communities was further explored. In experimental diets, where the glucogenic to lipogenic nutrient ratio was above one-third of the dietary energy, the *in vitro* incubation had a higher feed digestibility and lower acetate to propionate ratio. Bacterial genera including *Selenomonas*, *Succinivibrio*, *Ruminobacter*, certain genera in *Ruminococcaceae*, *Christensenellaceae_R-7_group*, *Eu*bacterium and some unclassified taxa were more sensitive to the glucogenic to lipogenic nutrient ratios.

According to the results from the *in vitro* fermentation, it was shown that the microbial compositions and activities were influenced by the dietary treatments, and the difference between the maximum constrasting diets (C and S vs L) were more pronounced than between the two moderately contrasting diets (C vs S). Next, an animal trial was conducted to confirm these influences and to obtain information regarding the alterations in metabolic mechanisms *in vivo* (**Chapters 5** and **6**).

In **Chapter 5**, the changes and interactions of ruminal bacteria and metabolites in response to the dietary treatments were studied through 16S rRNA sequencing and metabolomics analysis. The glucogenic diets resulted in higher ruminal lactic acid, ammonia-nitrogen (NH_3-N) and propionate production, whereas acetate production was lower compared to the lipogenic diet. The two glucogenic diets improved protein

digestion, resulting in an increased availability of amino acids and NH_3-N in the rumen, which might be attributed to species within the genera *Prevotella_7* and *Selenomonas*. The typical amylolytic or cellulolytic bacteria were not observed to be highly abundant in the rumen fluid of cows fed diets high in starch or fibre, respectively. Bacteria belonging to the genera of *Ruminococcus_2* and *Prevotella_7* might contribute to the ruminal amylolytic activities when glucogenic diets are fed, while *Ruminococcus_1* and *Prevotella_1* might contribute to the fibrolytic activities enhanced by the lipogenic diet. The results reported in this chapter provide new insights for the exploration of alternative species of amylolytic and fibrolytic bacteria.

In **Chapter 6**, metagenomics and metaproteomics approaches were performed to explore the changes in the metabolic functioning of ruminal microbes. According to the gene predictions by metagenomics analysis and taxonomic analysis of the secreted proteins by metaproteomics, it was confirmed again that most amylolytic and fibrolytic bacterial communities were unaffected by changes in the glucogenic to lipogenic ratio in the concentrate diets. The amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* were more sensitive to starch-type energy source compared to fibrous energy sources in the diet. The higher number of *Succinimonas amylolytica* in diets C and S led to increased production of pullulanase, thereby, contributing to the upregulation of the pathway of starch and sucrose metabolism. Diet S resulted in a higher proportion of the *Selenomonas ruminantium* than diet C both at the metagenomics and metaproteomics levels, which indicates a higher production of amylopullulanase. The combination of metagenomics and metaproteomics analysis showed to be a powerful approach for future research with an aim of investigating the activities of certain microbial communities in response to diet changes in the rumen of ruminants. These techniques perform well to detect changes in microbial metabolism in response to both the more contrasting treatments (C and S vs L), as well as in the case of more similar treatments (C vs S).

In **Chapter 7**, the integration of multi-omics technologies in this thesis were discussed. Within the ruminal microbial community structure, amylolytic and fibrolytic communities from all rumen fluid samples were detected through the 16S rRNA sequencing and metagenomics approaches. The results show that the metagenomics analysis was capable of detecteing the microbial community with high relative

abundance like bacteria but was less able to detect lower abundant microbiota such as archaea, fungi and protozoa, for which targeted metagenomics would be a better alternative approach. Besides, the results also indicate that the amylolytic and fibrolytic bacteria were not among the dominant population in the rumen. In addition, the enzymes involved in the propionate, acetate and butyrate synthesis were determined through a combination of metagenomics and metaproteomics analysis. The succinyl-CoA:acetate CoA-transferase, which originated from *Succiniclasticum ruminis* was observed to be up-regulated in diets C and S relative to diet L. This enzyme catalyzes the conversion reaction from acetate to succinate and resulted in the higher proportions of propionate and butyrate, but a lower acetate proportion in diets C and S. The interaction of various omics technologies was used in exploring the rumen functions, but interpretation is limited by the lack of an accurate database. In the end, the future directions on the applications of omics technologies were predicted, such as the exploring of high throughput culturing methods to characterize the currently uncultivated microbes, development of techniques to monitor rumen functions and manipulation of the host-microbe interactions.

In total, the reported experiments further enlarge our knowledge on the applications of omics techniques to understand compositional and functional alterations of the ruminal microbes in response to diets. Most of the amylolytic and fibrolytic microbes reported in the literature were shown to be stable to dietary starch and fibre changes, although some exceptions, for example, the amylolytic bacteria *Succinimonas amylolytica* and *Ruminococcus bromii* were detected. The studies also showed that the microbial activities in the rumen are far more complicated than hitherto known. The microbes sensitive to the dietary treatments detected in the experiments will promote the isolation and characterization of new amylolytic and fibrolytic microbes from the rumen. The metabolic pathways analysis through metagenomics and metaproteomics confirmed that the starch and sucrose metabolism was promoted in the two glucogenic diets, which is associated with a higher level of pullulanase secreted by the *Succinimonas amylolytica*.

About the Author

Curriculum vitae

Scientific output

Training and supervision plan

Curriculum vitae

Dengke Hua was born on January 1, 1989 and grew up in Dezhou, Shandong, China. He received his primary and middle school education in his hometown.

In 2007, Dengke started his bachelor's education in Biotechnology at the Qingdao Agricultural University (Qingdao, China) and obtained his BSc diploma in 2011, after which he continued his MSc study at Yangzhou University (Yangzhou, China) and the Chinese Academy of Agriculture Sciences (CAAS, Beijing, China). He obtained his MSc diploma in Animal Science in 2014. His master thesis was conducted at the Institute of Animal Science in CAAS, focusing on the "Effect of the light program and dietary energy on the performance of yellow-feather broilers", which brought three scientific papers to Chinese journals. From 2014 to 2017, he was employed by Cargill Animal Protein Company (Chuzhou, China) as the manager trainee. During this period, he received systematic training regarding whole chain production of poultry meat. He was promoted to the farm manager of a broiler breeder farm in 2016. In 2017, he won the opportunity to do the PhD program between CAAS and Wageningen University & Research (WUR, Wageningen, the Netherlands). In 2018, he started his PhD journey at the Animal Nutrition Group of WUR working on ruminant nutrition and rumen microbiology which resulted in this thesis. As of November 2022, Dengke will continue his career in ruminant nutrition and production.

Scientific output

This thesis

Hua, D., Zhao, Y., Nan, X., Xue, F., Wang, Y., Jiang, L., and Xiong, B. 2021. Effect of different glucogenic to lipogenic nutrient ratios on rumen fermentation and bacterial community *in vitro*. Journal of Applied Microbiology, 130(6):1868-1882.

Hua, D., Hendriks, W. H., Xiong, B., and Pellikaan, W. F. 2022. Starch and cellulose degradation in the rumen and applications of metagenomics on ruminal microorganisms. Submitted to *Animals*.

Hua, D., Hendriks, W. H., Zhao, Y., Xue, F., Wang, Y., Jiang, L., Xiong, B., and Pellikaan, W. F. 2022. Glucogenic and lipogenic diets affect *in vitro* ruminal microbiota and metabolites differently. Submitted to *Frontiers in Microbiology*.

Hua, D., Hendriks, W. H., Liu, Z., Wang, Y., Wang, Y., Jiang, L., Xiong, B., and Pellikaan, W. F. 2022. Alterations in rumen bacteria community and metabolome characteristics of dairy cows in response to different dietary energy sources. Submitted to *Frontiers in Nutrition*.

Hua, D., Hendriks, W. H., Liu, Z., Wang, Y., Wang, Y., Jiang, L., Xiong, B., and Pellikaan, W. F. 2022. Metagenomic and metaproteomic analysis of microbial carbohydrate digestion in the rumen of dairy cows receiving different dietary energy sources. To be submitted to *Microbiology Spectrum*.

Hua, D.*, Xue, F.*, Hendriks, W. H., Liu, Z., Wang, Y., Wang, Y., Xiong, B., and Pellikaan, W. F. Comparative analysis of different dietary energy sources on physiological nitrogen metabolism of lactating dairy cows. Manuscript in preparation.

* Shared first author.

Others

Hua, D., Xue, F., Xin, H., Zhao, Y., Wang, Y., and Xiong, B. 2021. Effects of monochromatic lights on the growth performance, carcass characteristics, eyeball development, oxidation resistance, and cecal bacteria of Pekin ducks. *Animal Bioscience* 5(34):931-940.

Ma, S.*, Hua, D.*, Guo, Y., Sun, Y., Li, D., Liu, N., Li, Y., Xue, F., Bai, H., Chen, C., Li, F., and Chen, J. 2016. Effect of nutrition levels on the production performance, meat quality, and sexual development of Chinese yellow-feather broilers. *Chinese Journal of Animal Nutrition* 1(28):217-223.

Hua, D., Li, D., Sun, Y., Tang, S., Bai, H., Liu, N., and Liu, R. 2014. Effects of light intensity level on the hormone level, production and carcass performance of Chinese yellow-feather broilers. *Chinese Journal of Animal Husbandry and Veterinary* (5):775-780.

Hua, D., He, H., Jia, Y., Li, D., Tang, S., Bai, H., Liu, N., Qin, N., Sun, Y., Chen, Y., and Chen, J. 2014. Effects of light intensity on the hormone secretion of Chinese yellow-feather broilers. *Chinese Poultry Science* (35):238.

* Shared first author.

Training and supervision plan (TSP)

Completed in fulfilment of the requirements for the education certificate of the Wageningen Institute of Animal Science (WIAS)

A. The Basic Package (1.8 ECTS*)	**year**
WIAS Introduction Day, Wageningen, the Netherlands	2018
Scientific Integrity & Ethics and Animals Science, Wageningen, the Netherlands	2018
B. Disciplinary Competences (15.6 ECTS)	
Applied Multivariate Analysis: Data Mining and Chemometrics, Beijing, China	2017
WIAS Course Statistics for the Life Sciences, Wageningen, the Netherlands	2018
16S rRNA Sequencing, Metagenomics Analysis, Beijing, China	2020
Proteomics and Metabolomics Analysis, Beijing, China	2020
Rmarkdown, Wageningen, the Netherlands	2021
Writing a Literature Survey, Wageningen, the Netherlands	2022
C. Professional Competences (10.2 ECTS)	
Effective Academic Development: Academic Writing and Presenting in English, Beijing, China	2017
Guide to Writing Scientific Papers, Beijing, China	2017
Reviewing a Scientific Paper, Wageningen, the Netherlands	2018
Scientific Writing, Wageningen, the Netherlands	2018
Stress Identification & Management, Wageningen, the Netherlands	2018
Project and Time Management, Wageningen, the Netherlands	2018
Career Orientation, Wageningen, the Netherlands	2021
PhD Workshop Carousel, Wageningen, the Netherlands	2022
D. Societal Relevance (1.5 ECTS)	
Societal Impact of Your Research, Wageningen, the Netherlands	2022
E. Presentation Skills (2 ECTS)	
"Alterations in Rumen Bacteria Community and Metabolome Characteristics of Dairy Cow in Response to Different Dietary Energy Sources", Animal Nutrition and Nutrition Sciences 21NL12ICANNS, Amsterdam, 12/2/2021, Poster	2021
"Alterations in Rumen Bacteria Community and Metabolome Characteristics of Dairy Cow in Response to Different Dietary Energy Sources", WIAS Annual Conference, Wageningen, 02/11/2022, Oral Presentation	2022
F. Teaching Competences (1.5 ECTS)	
Supervise two MSc students for major thesis	2019
Practical Supervision: Animal Nutrition and Physiology	2022
Total 32.6 ECTS	

*one ECTS credit equals 28 hours' study load.

Acknowledgement

Time traces back to 2017. At that time, I decided to resign from my job to pursue a PhD degree, which was one of the best decisions I made in my life. Time flies rather quickly, and the completion of my PhD program is, with these last sentences, coming to an end. I still remember how excited I was at the start of this journey, the prospect of meeting people from all over the world, taking courses and doing research, and having the opportunity to travel to different places. All these and more are now part of my memories and will be part of me during my future career. This journey was full of challenges, stress and uncertainty, but brought me excitement, achievements, and growth at the same time. I am grateful for all the institutions and the individuals, named below or unnamed, who offered assistance, provided guidance, and supported my research and life.

Firstly, I sincerely thank the Chinese Academy of Agricultural Sciences (CAAS) and Wageningen University & Research (WUR) for running this Joint PhD program, which served as the foundation of my research in China and the Netherlands. I am eternally grateful to the China Scholarship Council and the WUR for providing financial support for my PhD program when I studied in the Netherlands. I also thank the CAAS for offering the living allowance when I did my research at the Institute of Animal Nutrition in China.

Secondly, I would like to give my deepest thanks to my promoter, Wouter Hendriks, and my daily supervisor, Wilbert Pellikaan. Thank you for your patience and encouragement, and all the professional advice, comment, and inspiration for my project, especially the positive feedback on my thesis. The conversations I had with the both of you stimulated my critical thinking and I will carry this with me throughout my career.

Next, I would like to show my sincere appreciation to all staff and colleagues of the Animal Nutrition Group (ANU). Great thanks are extended to our respected secretaries, Yvonne van Holland and Betty Looijen. A special thanks is given to Yvonne for the wonderful talks and support during my thesis writing. I appreciate Jan Dijkstra for correcting my abstracts and supervising the modelling practice during the Nutrient Dynamics course. I thank Walter Gerrits and Myrthe Gilbert for their support when I assisted with the fermentation practical. Xuan Huong van der Schans-Le, you are always so kind and willing to share your experiences with me. I would also like to

acknowledge all members of ANU. Thank you for your friendship and kindness which made my PhD life so warm.

I give my great thanks to my helpful paranymphs Corentin Lannuzel and Lei Deng. Thank you for contributing your time and efforts to my defence.

To my lovely colleagues, Hu Yixin, Lve Fang, He Yuan, Mao Lei, Wang Fang, Sholeha Binti Abd Rahim, Nazri Nayan, Kim Jannink, Miranda Elling-Staats, Huyen Nguyen Thi Duong Huyen, Rainer Rauch, Marco Tretola, Sebastian Dorado Montenegro, Evelien Bos, Ilaria Minussi, Tom Bastiaansen, Margreet Edens, Fahmi Mochammad Habibi, Samuel Jansseune, Gabriel Menegazzi Da Conceicao, Tang Tianyue, Wang Zhenyu, Zhang Hanlu, Zhang Yaowen, Zhang Shiyi, Zheng Chen, and other members who I forgot to mention, it is my great honour to have met you all and worked with you.

To the nice friends I met in Wageningen Claudius vande Vijver, Alam Iftakharul Md., Wisse de Beer, Alexander Dachev, Bernard Prasetyo, Amber van der Velden, and Stefan Vroom, I really enjoyed the time we got to know each other. To my dear friend Xai Lor (Shengli Luo), you are a very helpful friend who will always be there when I need help. Thank you for your friendship!

This thesis is dedicated to my late father who always encouraged me to pursue my goals and chase my dreams, to my doting mother who I look up for bravery and motivations to never give up in life, and to all other family members for their consistent supports and encouragements.

时间过得真快，博士旅程即将结束。回想起 2017 年辞去工作，回到北京备考雅思的场景，依然清晰如昨。在荷兰的生活与学习经历，充满了惊喜和挑战。感谢当年的自己，如此坚定地做出读博的决定，感谢自己这些年的坚持和努力，感谢这段经历，不负青春，得偿所愿。此刻落笔，这几年的经历在脑海中快速地闪现，有太多的感动可以铭记，有太多的人需要感谢。

感谢北京畜牧兽医研究所智慧畜牧业团队，我的中方导师熊本海研究员，感谢您对我博士课题的支持和指导；感谢团队赵一广、王辉、南雪梅、郑珊珊、杨亮、罗清尧等老师对我的实验以及生活提供的帮助；特别感谢王亚品、刘子豪同学以及熊本江师傅对我动物饲养实验的帮助；感谢潘晓花、薛夫光、唐志文、孙福昱、汪悦、张帆、蔡萌等同学的陪伴和支持。谢谢你们温暖并欢乐了我在北京的生活。

感谢北京奶牛营养重点实验室提供的实验平台，特别感谢北京农学院的蒋林树教授、方洛云等老师对我动物实验的支持，感谢栗明月、李义、牛慧等同学对我实验的帮助。

感谢北京奶牛中心延庆基地对我的课题提供的实验动物和场地，特别感谢楚康、王欣、王爽、陈立文、段龙飞等对我在牛场实验期间提供的帮助。

在我读博期间，一群老朋友仍然在关心和支持着我。张玉龙和郑先瑞，感谢你们在我备考雅思期间提供的住宿和精神支持以及对我博士生活的帮助，我们是一辈子的好朋友。宋政和王海，我们一起经历了第一份工作带我们的成长，一起为理想奋斗，谢谢跟我的交流和支持。我的高中同学们邓志飞、叶春杨、王欣、任康、钟冠男、李凤娟等，从 2004 算起，18 年的友谊了，感谢你们，希望我们友谊能一直延续下去。

无论是在北京还是瓦村，都有一群好朋友们陪伴着我。感谢在北京学习期间遇到的好友贺小云、刘正群、李硕、刘真、陶林、陈玉霞，感谢老陈簇的李云雷、石雷、许红、葛平壮、倪爱心等好友。感谢在瓦村的好兄弟夏文杰、曾哲、孙秀路、周锡龙、蒋彬、马虚船、刘大志、张陈、王桃军、於嘉闻、程慧、吕志宏、孙培新、万泉兴等，同时特别感谢好朋友马俊南、范盼盼、渠清博、刘迎夏、王艳、胡丽娜、刘宇思、王然、齐烟舟、王越、程哲、谈美秀、蔡慧芳、李巧凤等好友。感谢你们的陪伴和支持，我们曾经一起奋斗、聚餐、跑步、旅游、打球，是你们让我的生活丰富多彩。另外，感谢马伟大哥在我困难的时候给予的安慰和提供的支持。感谢徐名刘、曹瑞、刘钊、张天齐等朋友的陪伴。漫漫人生路，一切安好。

亲爱的魏峥同学，我的好兄弟，很幸运在我最艰难的时候认识你，感谢你耐心地陪伴、倾听、支持和帮助，我们都热爱生活，健身、跑步、考证，让自己变得更健康，让生活更有意思，咱们回国后再聚。

最后，感谢我的家人，感谢妈妈对我无私的关心、支持和唠叨，感谢哥哥嫂子在我出国期间对父母的照顾。感谢所有亲人们对我的关心和问候。

特以此书献给我的父亲！

Colophon

The research described in this thesis was conducted at the Institute of Animal Science, Chinese Academy of Agricultural Sciences (Beijing, China) and supported by the Animal Nutrition Group, Wageningen University & Research (Wageningen, the Netherlands). Dengke Hua was supported financially by the China Scholarship Council. Wageningen University & Research provided additional funding and financially supported the printing of the thesis.

Thesis design & layout	Dengke Hua
Cover design	Guoxiong Nie
Thesis printing	ProefschriftMaken