Impact of water intake on reticulorumen temperature dynamics and microbial diversity in cattle

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Statement of originality

This is to certify that the content of this thesis is my own work. This thesis has not been submitted for any other degree or purpose.

I certify that the intellectual content of this thesis is the product of my own work, and that all assistance received in preparing this thesis and all sources have been duly acknowledged.

Md Shaheenur Rahman

6 May 2026

“To the paths behind me, and the hearts beside me”

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Abstract

The quest for highly efficient cattle has emerged as a critical priority, given their growing role in animal-sourced food production under the threat of global warming. For improving the efficiency and sustainability of cattle production, a comprehensive understanding of reticulorumen temperature (RT) dynamics and their role in shaping microbial fermentation is crucial. While water intake evokes a sudden decrease in RT, the consequences of these rapid thermal fluctuations for microbial adhesion, fermentation efficiency, and community composition remain relatively unexplored. Driven by the potential association between water consumption and RT dynamics, this thesis aims to develop a deeper understanding of reticulorumen thermoregulation through the application of thermodynamic principles and explore its impacts on enteric microbial fermentation. This thesis begins with a comprehensive literature review (Chapter 2) examining the physicochemical and microbial environment of the reticulorumen and its susceptibility to external and internal perturbations, with an emphasis on the impact of water consumption on RT variability. Chapter 3 used thermodynamic principles to analyse published data on declines in RT following drenching with water, revealing a proportional relationship between water temperature and the magnitude of the RT decline. The outcomes confirm the predictive utility of a thermodynamic framework for RT dynamics. Chapter 4 extended this framework by incorporating real-time RT records from feedlot cattle, enabling the characterisation of drinking events and revealing individual variability in drinking frequency, magnitude of RT decline, and recovery duration. Building on these insights, Chapter 5 established an *in vitro* model capable of replicating RT fluctuations associated with water intake, which provided a controlled platform for investigating temperature perturbations induced by drinking events. Chapter 6 revealed that short-term RT fluctuations, induced by simulated drinking events, affect fermentation kinetics and microbial composition in a substrate-dependent manner, with frequent drops delaying early fermentation and altering microbial diversity and fermentation profiles. Collectively, this thesis identifies that reticulorumen thermoregulation associated with water consumption is a critical but previously overlooked factor shaping microbial fermentation and community resilience. By linking drinking behaviour, RT dynamics, and microbiome function, this thesis describes focused avenues for microbiome-informed management aimed at improving production efficiency and reducing environmental impact.

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Preface

This thesis adopts a thesis-with-publications format and is written using Australian English conventions. Hyperlinks have been enabled throughout the document to facilitate easy navigation for e-readers. Two chapters have been published (Chapter 3; Chapter 5), and the remaining chapters have been presented in a publication format. Some parts of this thesis were also presented at scientific conferences, and institutional research showcases (Chapter 4; Chapter 6).

List of publications, presentations, and achievements

Publication in peer-reviewed journal

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Authorship attribution statement

This thesis contains four research chapters, which are the result of my own investigations.

Chapter 2 presents a review of published literature. Shaheenur Rahman (SR) conducted the investigation and preparation of the original draft under the supervision of Anna Chlingaryan (AC), Peter Thomson (PT), Angela M. Lees (AML), Rafiq Islam (RI), and Cameron Clark (CC). All authors contributed to the conceptualisation and participated in the review and editing process.

Chapter 3 has been published in the European Conference of Precision Livestock Farming conference proceedings entitled 'Modelling reticulorumen temperature change from drinking events using Richmann's Law'. Shaheenur Rahman conducted the investigation and preparation of the original draft under the supervision of PT, AC, AML, Rafiq Islam (RI), and CC. SR and PT conducted data visualisation. All authors devised the conceptualisation and contributed to the review and editing process.

Chapter 4 has been prepared for submission to an international journal and is titled 'Identifying and characterising drinking events using reticulorumen temperature of feedlot beef cattle'. All authors contributed to the conceptualisation of the methods. Data curation, validation, and formal analysis conducted by SR and PT. The original draft was written by SR and reviewed and edited by PT, AC, AML, RI, and CC.

Chapter 5 has been published in *MethodsX* and is titled '*In vitro* simulation of drinking events in cattle'. Shaheenur Rahman, PT and CC, conceptualised the method. Data curation, validation, and formal analysis was conducted by SR under the supervision of PT and AC. The original draft was written by SR and reviewed and edited by AC, PT, AML, RI, Pablo Gregorini, Fabiellen Pereira, and CC.

Chapter 6 has been prepared for submission to an international journal and is titled 'Fermentation characteristics, gas production kinetics, and microbial diversity in response to drinking: an *in vitro* study'. All authors contributed to the conceptualisation of the methods. Data curation, validation, and formal analysis was conducted by SR under the supervision of PT. The original draft was written by SR and reviewed and edited by PT, AC, AML, and CC.

Artificial intelligence statement

During the preparation of this thesis, the author used Microsoft Copilot to assist in the understanding of data analysis methods, minor sentence restructuring, and clarity enhancement. The author confirms that where text was modified by generative AI, the content was reviewed for possible errors, inaccuracies, and bias. The author takes full responsibility for the submitted thesis and ensures the work is their own and has used generative AI in accordance with the University guidelines and policies (refer to the University of Sydney generative AI guide for researchers).

Md Shaheenur Rahman
6 May 2026

Supervisor statement

As the primary supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Anna Chlingaryan
6 May 2026

Abbreviations

Below is a list of alphabetised abbreviations used throughout this thesis. Abbreviations are also defined at first use within respective chapters.

CBT	Core body temperature
DM	Dry matter
FDR	False discovery rate
GHG	Greenhouse gas
GLMM	Generalised linear mixed model
PCoA	Principal coordinate analysis
PERMANOVA	Permutational Multivariate Analysis of Variance
RA	Relative abundance
RE-RRS	Restriction enzyme reduced representation sequencing
RT	Reticulorumen temperature
SCFA	Short-chain fatty acid
TNZ	Thermoneutral zone
VFA	Volatile fatty acid

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Chapter 2. Literature Review

Chapter 3: Modelling reticulorumen temperature changes from drinking events using Richmann's Law

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CHAPTER 1

General Introduction

Introduction

The global human population is rising, with an increasing demand for animal-based protein, which is driving a substantial growth in overall food requirements. The livestock sector is expected to cover a projected 60% increase in demand for animal-sourced protein by 2050 (Makkar, 2018). Ruminant animals, especially cattle, play an important role in meeting this demand by converting low-quality fibrous forages into high-quality animal proteins, such as milk and meat, through microbial fermentation. However, this fermentation process also produces methane (CH_4) (Appuhamy et al., 2016), a potent greenhouse gas (GHG) for which the cattle system is considered a contributor to global climate change. Therefore, the production of animal protein with a minimum environmental footprint has gained a lot of attention globally. Genetic selection of superior animals has been recognised as a potential tool in this regard; however, selecting and measuring appropriate traits is still challenging (Hess et al., 2023; Richardson et al., 2021). Innovative approaches are therefore required to advance understanding and integrate the physiological, behavioural, and microbiological aspects of cattle into a comprehensive framework for sustainable cattle systems.

Enteric fermentation is a fundamental anaerobic process in the reticulorumen, in which a complex microbial community converts ingested feedstuffs into volatile fatty acids (VFAs), microbial protein, and water-soluble vitamins. The VFAs produced in this process provide approximately 70–80% of the energy needs of ruminants (Cammack et al., 2018; Kebreab et al., 2009). Although this microbial fermentation is crucial for ruminants' nutrition, it also incurs a loss of 2-15% of gross dietary energy intake through methanogenesis, producing enteric methane (Johnson et al., 1990; VanNevel & Demeyer, 1996). Enhancing feed efficiency has become a critical objective for sustainable ruminant production, as more efficient animals consume less feed, emit less GHG, and yield higher levels of milk or meat (Ahola & Hill, 2012; Moraïs & Mizrahi, 2019). Traits such as residual feed intake and methane output being

heritable have been considered for genetic selection (Berry & Crowley, 2013; Hegarty et al., 2007). Moreover, there is growing evidence that the composition and function of the rumen microbiome are governed by host genetics and is closely linked to feed efficiency and methane emissions (Li et al., 2019; Wallace et al., 2019). It suggests that microbiome-informed selection strategies could be employed to modify microbial composition in the next generations. There are numerous technical and economic challenges in assessing the feed efficiency and methane emissions on a large scale. Metagenomic profiling offers a high-throughput, cost-effective proxy for selecting animals with superior environmental and production traits (Hess et al., 2023; Ross et al., 2013; Sasson et al., 2017).

The reticulorumen is a site of mutualistic interaction between the host and its resident microbiome, in which a stable environment is critical for supporting microbial activity, animal health, and productivity (Liu et al., 2020). Reticulorumen temperature (RT) is typically regulated between 38 and 42 °C and is closely associated with providing an optimal environment for microbial survival and function (Hicks et al., 2001; Yokoyama & Johnson, 1988). The symbiotic interaction between the host and microbes depends on this stable environment, which is beneficial for both of them (Russell, 2002). However, several physiological (Weimer et al., 2010), behavioural (Prendiville et al., 2010) and environmental (Jami et al., 2013) factors can disrupt ruminal homeostasis, potentially compromising microbial function and host–microbiome interactions. A fundamental component of ruminant physiology is water intake that aids in thermoregulation, digestion, and metabolic processes (NRC, 2001). In cattle, water consumption is accompanied by a sudden and dramatic drop in RT, especially when cold water is consumed (Bewley et al., 2008; Cunningham et al., 1964; Dale et al., 1954). This transient temperature drop is followed by a gradual recovery to the baseline RT. A range of durations (of recovery) from 20 minutes to over 3 hours have been reported, as a recovery period for different magnitudes of RT drop following the consumption of varying amounts of

water (Bewley et al., 2008; Cantor et al., 2018; Serviento et al., 2024). In contrast, Dracy et al. (1963) reported that reticulorumen temperatures consistently returned to baseline RT within 60 minutes, irrespective of the amount of water consumed or the extent of RT drop. It provides an opportunity to investigate what drives this variation and whether there are any influences other than temperature and the volume of consumed water on the RT recovery period. Moreover, in previous studies, individual variability in RT fluctuations was often ignored when determining baseline RT, which may explain the wide range of recovery periods reported. Besides these, RT has become the most prominent method for estimating core body temperature (CBT) in cattle studies (Lees et al., 2022). Therefore, there is a demand for developing a robust method to explore RT dynamics associated with water consumption. A deeper understanding of RT dynamics, using thermodynamic principles may provide insights for its accurate use in cattle production systems.

Although cattle tightly regulate RT to maintain optimal rumen function, under heat-stressed conditions, the heat dissipation mechanism is impaired, leading to elevated RT and disrupting microbial activity and fermentation (Collier & Gebremedhin, 2015; Lees et al., 2018). Changes in microbial community diversity, reductions in richness and abundance of particular groups of microbes and compositional shifts of specific taxa have been revealed in heat-stressed cattle, through high-throughput metagenomic studies (Romero-Pérez et al., 2011; Zhong et al., 2019). In addition, alterations in ruminal pH and shifting VFA profiles have been reported as a consequence of cattle experiencing heat stress (Chen et al., 2018; Kang et al., 2019; Li et al., 2021). While increased RT due to heat exposure has been studied extensively, limited research has addressed the short-term effects of sudden reticulorumen temperature drops, caused by drinking. A few *in vitro* studies using Rumen Simulation Technique (RUSITEC) have demonstrated that reductions in incubation temperature and maintained it for 24 hours to several days substantially reduced fibre digestibility and methane production, and disrupted

microbial ecology (Duarte et al., 2017; Petersen et al., 2016). Cattle generally consume water between two to eleven times daily, with the heritability of water intake behaviour found to be as high as 0.88 in feedlot cattle (Campbell & Munford, 1959; Cardot et al., 2008; Dressler et al., 2023). Shirley et al. (2025) discovered a greater magnitude of RT drop in pasture-based dairy cows drunk less frequently (indicating more water consumption per event) than those drunk more frequently (less amount of water consumption per event). This variation in drinking behaviour and associated changes in RT may signify variances in heat tolerance, suggesting its usefulness as an indirect indicator of thermal resilience. Examining the impact of drinking events associated with RT fluctuations on fermentation characteristics and microbial diversity could strengthen the selection of drinking events as a potential trait of interest for a resilient cattle system.

In vitro rumen models have long been employed as valuable tools in rumen microbiology and nutrition research, serving as cost-effective alternatives to *in vivo* studies. The success of these systems mainly depends on their ability to sustain a microbial community that closely replicates the *in vivo* rumen environment under defined experimental conditions (Warner, 1956). Recent advancements in *in vitro* fermentation systems, coupled with high-resolution omics and analytical techniques, have significantly enhanced their utility in understanding rumen function and its response to dietary manipulations at both microbial and molecular levels (García-Rodríguez et al., 2020; Wetzels et al., 2018). These systems typically operate at a constant temperature of 39 °C, reflective of average RT; however, the temperature can be adjusted to meet specific experimental goals. Despite these capabilities, currently available *in vitro* methodologies do not replicate the dynamic temperature fluctuations associated with drinking events, characterised by an abrupt decline in RT followed by gradual recovery. Developing an *in vitro* approach that simulates these RT dynamics would provide a novel framework for

investigating the temporal effects of water intake on rumen fermentation characteristics and microbial diversity.

Thesis outline and objectives

Considering the identified gaps in knowledge, the overarching objective of this thesis is to develop a deeper understanding of reticulorumen thermoregulation through the application of thermodynamic principles and explore its implications on enteric microbial fermentation. In agreement with this aim, the objectives of this thesis are to:

1. To assess current knowledge and gaps on cattle reticulorumen thermoregulation, reticulorumen temperature changes following water intake, and their effects on rumen microbial community.
2. Evaluate post-drinking reticulorumen temperature drop and determine recovery period following a mathematical approach using thermodynamic principles.
3. Develop a novel *in vitro* method to simulate the impact of drinking events on the reticulorumen environment with particular emphasis on reticulorumen temperature.
4. Investigate the temporal effect of drinking events on fermentation parameters, gas production kinetics, and microbiome diversity using the developed method.

This thesis is composed of a comprehensive review of existing literature (Chapter 2), four independent experimental studies (Chapter 3, Chapter 4, Chapter 5, and Chapter 6), and a general discussion and conclusion (Chapter 7). Experimental chapters are presented as stand-alone scientific manuscripts, containing an abstract, introduction, materials and methods, results, discussion, and conclusion, with any additional information provided in the appendix as supplementary material.

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CHAPTER 2

Literature Review

Reticulorumen facilitates microbial fermentation in a controlled environment, which is sensitive to any disturbances impacting the fermentation process, resulting in a low-productive ruminant system. **Chapter 2** sequentially explores the factors influencing the reticulorumen environment, enteric fermentation, and ruminal microbial communities, with a particular emphasis on drinking-associated reticulorumen temperature dynamics, as informed by existing literature. *In vitro* fermentation methods and high-throughput metagenomic approaches for studying microbiomes have been reviewed. The limitations of existing methods and gaps in knowledge are identified, and key directions for future research are proposed.

Abstract

As global demand for animal-sourced protein is growing, cattle production systems are increasingly required to enhance efficiency while minimising environmental impacts. Central to improving efficiency is the rumen microbiome, the driver of feed degradation, nutrient release, and enteric fermentation, ultimately determining animal performance. The reticulorumen maintains a tightly regulated physicochemical environment essential for microbial function; disturbances in this environment, particularly variations in reticulorumen temperature (RT), can compromise microbial adhesion, disrupt metabolic activity, and diminish fermentation efficiency. Drinking behaviour, a highly heritable trait, individual variability, and environmental factors play a critical role in regulating RT. High volumes of water intake evoke sudden declines in RT, likely, albeit temporarily, inhibiting microbial activity and potentially compromising fibre digestion. The composition of the microbial community, shaped by host genetics and environmental influences, underlies critical fermentation pathways associated with feed efficiency, methane production, and nitrogen utilisation. Methanogens rely on hydrogen supplied by fibrolytic microbes, whereas most bacterial species utilise ammonia for microbial protein synthesis, thereby linking fermentation processes to nitrogen excretion. Heat load, ambient conditions, and water availability collectively influence drinking patterns and RT fluctuations, thereby influencing microbial diversity and community stability. Recent advances in high-throughput sequencing have enabled comprehensive characterisation of these interactions, underscoring the importance of integrating drinking behaviour, RT regulation, and microbial ecology into strategies aimed at enhancing ruminant productivity, resilience, and sustainability of cattle production systems.

Introduction

Climate change, and the associated changes in extreme weather events, are predicted to intensify climatic conditions in the coming decades, posing significant challenges to livestock systems worldwide. The consequences of climate change have been previously described (Jasrotia et al., 2022; Nardone et al., 2010; Rojas-Downing et al., 2017), and this has highlighted that the number of heat stress events has increased (Carvajal et al., 2021, IPCC, 2021), adversely affecting production (Key et al., 2014), welfare (Poisky & von Keyserlingk, 2017), sustainability (Das et al., 2016), as such impacting the long-term viability of livestock systems globally (Gaughan et al., 2010). Ruminants, particularly cattle, can be susceptible to extreme climate events, which can lead to compromised health, reduced production, and impaired welfare (Hansen, 2019; Thorton et al., 2009). At the same time, cattle contribute to climate change through enteric methane emissions, a potent greenhouse gas produced during enteric fermentation (Chhabra et al., 2009; Ingale et al., 2013). With the global demand for animal-sourced protein steadily increasing, there is growing pressure on cattle systems to improve efficiency, while reducing their environmental footprint.

Improving feed efficiency and resilience represents a key strategy in this context, which is associated with multidimensional factors. Feed efficiency is determined mainly by rumen microbes, which degrade and ferment feed into volatile fatty acids (VFAs), supplying approximately 70% to 80% of the ruminants' energy needs for their maintenance and production (Guan et al., 2008; Myer et al., 2015; Perea et al., 2017). Methane emission and feed efficiency of cattle are closely associated, where efficient animals divert less energy to methanogenesis (Zhou et al., 2009), and consequently produce a lower amount of methane (Hegarty et al., 2007; Nkrumah et al., 2006). Additionally, methane production and residual feed intake (RFI); a measure of feed efficiency (Khiaosa-ard & Zebeli, 2014) are positively correlated, and both are heritable traits, suggesting that breeding for reduced CH_4 emissions can be achieved through direct selection for low CH_4 or indirect selection for low RFI (Crowley et al., 2010; Hegarty et al., 2007; Kirkham et al., 2025; Nkrumah et al., 2006). However, any disturbances, whether external or internal, that impact the reticulorumen environment can influence ruminal microbial ecology and fermentation dynamics, therefore impacting feed efficiency (Cani et al., 2019; Fregulia et al., 2021; Petersen et al., 2016). While dramatic changes in reticulorumen temperature have been observed following water intake (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964b), information regarding its impact on microbial communities is limited. Moreover, no information is available on whether there is an

association between cattle drinking behaviour and microbial community structure. Since drinking is a highly heritable trait of cattle and is influenced by several physiological and environmental factors (Delagarde & Lamberton, 2015; Dressler et al., 2023; Dutta et al., 2015) understanding this nexus is crucial to exploring any association between feed efficiency and cattle's water intake behaviour. *In vitro* fermentation at a lowered incubation temperature for a prolonged period, combined with a metagenomic study, revealed the differences in microbial community diversity and reduced methane production (Duarte et al., 2017). As such, drinking-associated reticulorumen temperature dynamics and their corresponding impacts on rumen microbes might be insightful for cattle studies. Advancements in high-throughput sequencing technologies are facilitating the comprehensive characterisation of rumen microbes and their interactive roles on production efficiency (Myer, 2019; Pitta et al., 2018). Given that both methane emissions and residual feed intake are heritable traits, the interactions between water intake, reticulorumen temperature, microbial diversity, and animal efficiency warrant closer examination. Understanding these linkages is crucial for identifying knowledge gaps and developing a framework for resilient cattle systems. This literature review therefore examines the interplay among water intake, reticulorumen temperature dynamics, and rumen microbiome diversity, highlighting their implications for enteric fermentation, methane emissions, and microbial diversity in cattle.

Reticulorumen Structure and Environment

Ruminants with their unique digestive and metabolic adaptations effectively utilise fibrous feedstuffs, agricultural byproducts, and unconventional feed resources such as those high in lignocellulose and other complex polysaccharides indigestible to humans, to produce meat and milk suitable for human consumption (Forbes, 2007; Robbins et al., 1995; Wanapat et al., 2013). Reticulorumen is the primary site where billions of rumen microbes contribute to this process, and the efficiency and stability of rumen fermentation depends on a tightly regulated rumen environment, including key factors such as reticulorumen temperature (RT), pH, feed composition, and microbial community structure (Castillo-González et al., 2014; Linville et al., 2017; Öztürk & Gur, 2021). A deeper understanding of the reticulorumen environment and the consequences of its alteration on ruminal fermentation is crucial for sustainable animal production, environmental responsibility, and human well-being.

What is Reticulorumen?

Ruminants are characterised by their unique digestive tract, comprised of four compartments: the reticulum, the rumen, the omasum, and the abomasum. The rumen is the largest compartment of the ruminant's forestomach, which is a complex organ that allows ruminant animals to efficiently digest complex carbohydrates, synthesise microbial proteins, and convert fibrous plant materials into valuable nutrients and energy, making ruminants important contributors to food production and agricultural sustainability (Mizrahi et al., 2021; Tishkoff et al., 2007). Anatomically, it is positioned dorsal to the reticulum and ventral to the omasum and abomasum. It is situated between the diaphragm and the abdominal floor, occupying the entire left side of the abdominal cavity. The reticulum is the second compartment of the ruminant stomach, located cranially and slightly to the right of the rumen and collectively referred to as reticulorumen (Parish et al., 2009). It is primarily responsible for assisting in rumination contractions and distributing feed within the rumen system.

At birth, the omaso-abomasum weighs more and occupies a greater volume than the reticulorumen (Becker et al., 1951). By four weeks of age, the reticulorumen constitutes 64% of the total stomach volume in calves receiving milk, forage, and grain, and by 12 weeks, this value is increased to about 75% (Tamate et al., 1962; Warner et al., 1956). This trend continues until the reticulorumen makes up about 87% of the total stomach volume in adult ruminants (Warner, R. 1965). In adult cattle, the rumen accounts for 1/7th to 1/10th of their body weight and has a volume ranging from 50 to 100 litres (Church, 1993; Nagaraja, 2016a; Oehme, 1988; Russell, 2009). However, the relative size of the rumen varies according to the age of the animals and mainly by the type of feed ingested (Millen et al., 2016). The rumen, also known as the "paunch," functions as a fermentation vat, where microbial fermentation happens (Valadares Filho & Pina, 2006; Wu & Papas, 1997). The development of the reticulorumen, digestive physiology, biochemical reactions, and its functions has been studied extensively; however, the incorporation of thermodynamic principles, behavioural attributes, and next-generation microbiome sequencing technologies carries significant importance for sustainable ruminant production.

Reticulorumen environment

The reticulorumen is a microbially-friendly environment that provides an ideal habitat for the proliferation of anaerobic microorganisms and serves as a continuous-flow fermentation vat

(Russell, 2002). This fermentation vat contains a small gas cap in the dorsal sac composed primarily of carbon dioxide (~65%) and methane (~26-35%) along with small amounts of other gases, including 0.2% hydrogen, 0.5% oxygen establishing it as strictly anaerobic with a redox potential of 150 to 350 mV (Cunningham, 2008; Nagaraja, 2016a). It also contains heterogeneous substances consisting of a complex mass of digesta, which may either float (forage) or settle at the bottom (grain), together with a liquid portion containing microbial cells and fine feed particles (Millen et al., 2016; Nagaraja, 2016a). This fermentation chamber is buffered between pH 5.7 and 7.3 by phosphate and bicarbonate from saliva, as well as bicarbonate from rumen fermentation (Rode, 2000). The temperature in the reticulorumen is regulated between 38 and 42 °C, with an average temperature of 39 ± 0.5 ^{0}C, providing a conducive environment to ensure the survival and proper functioning of microbes (Hicks et al., 2001; Yokoyama & Johnson, 1988). Maintaining an optimum reticulorumen environment is crucial for both ruminal microbes and host animals, as they mutually benefit from one another (Russell, 2002). Naturally, the reticulorumen environment is homeostatic in terms of pH, osmolarity, reducing potential, and temperature (Ungerfeld et al., 2023). However, various factors, including but not limited to diet (McCann et al., 2014), feeding programs (Golder et al., 2014), environment (Jami et al., 2013), feeding and drinking (Prendiville et al., 2010), and individual characteristics (Weimer et al., 2010), may affect reticulorumen environments through the disruption of mutualistic relationships between the animals and microbiomes. Exploring the impact of prominent behaviours, such as drinking, may help develop strategies to explore associated economic and environmental benefits and/or losses from the cattle system.

Reticulorumen microbial ecology and functional diversity

Rumen microbial ecology encompasses a complex and dynamic ecosystem of microorganisms within the reticulorumen, notable for its functional and phylogenetic diversity as well as complex interactions among various microbial species (Clemmons et al., 2019; Dias et al., 2018). This ecosystem comprises prokaryotic microbial groups such as bacteria, archaea, bacteriophages, viruses, and eukaryotes such as fungi and protozoa, with their concentration ranging from 10^{3-4} to 10^{10-11} per ml of rumen content (Dias et al., 2018; Dill-McFarland et al., 2017; Nagaraja, 2016; Russell & Rychlik, 2001). These microorganisms live symbiotically with the host animal, collaborating synergistically to transform lignocellulosic feeds as a major energy source for the host and have a direct influence on animal physiological parameters and

productivity (Goodacre, 2007; Jami & Mizrahi, 2012). Although it has been observed that no single microorganism is capable of the complete degradation of complex substrates in the rumen (Bladen et al., 1961), studies have shown that even slight alterations in these populations can have a significant impact on ruminants' nutrition and productivity (Canibe et al., 2005; Hales et al., 2014; Kim et al., 2014). Microbial colonisation of the reticulorumen starts within the first 24-30 hours after birth, begins with facultative anaerobic bacteria such as Enterococcus and Streptococcus, along with archaea (Guzman et al., 2015; Jami et al., 2013). These initial colonisers allow for the proliferation of anaerobic microbes, including members of the *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* phyla (Du et al., 2023; Vlková et al., 2006) by utilising available oxygen in the gut. The taxonomic diversity in both mass and density increases as calves grow and are introduced to various types of feeds and forages (Jami et al., 2013; Li et al., 2012). Generally, the gastrointestinal (GI) tract of cattle predominantly consists of *Firmicutes* (approximately 40%), followed by *Bacteroidota* (around 30%) and *Proteobacteria* (about 10%). However, the composition of ruminal microbiota is not conserved nor does it remain constant throughout life. The composition is influenced by numerous factors, including limited to host genetics, diet, age, geographic locations, and the specific compartments within the GI tract (Benson et al., 2010; Cammack et al., 2018; de Oliveira et al., 2013; Keum et al., 2024; Myer et al., 2015). Cellulose-degrading bacteria (members of the *Firmicutes)* are abundant in the reticulorumen when cattle consume more forage-based diets (Koike & Kobayashi, 2009), with dominant genera including *Prevotella, Ruminococcus, Butyrivibrio*, *Prevotellaceae*, *Rikenellaceae,* RC9 gut group, and *Ruminococcaceae,* NK4A214 group (Chen et al., 2021; Ramos et al., 2021; Wang et al., 2020). *Streptococcus bovis*, a lactate-producing bacterium, is commonly associated with high-grain, rapidly fermentable diets (Clemmons et al., 2019). Similarly, *Lactobacillus* species increase in abundance with high-grain diets, further contributing to lactate production in the rumen (Nagaraja & Titgemeyer, 2007).

Archaea, a separate domain of life characterised by distinctive physiological traits, are only distantly related to bacteria (Krause et al., 2013). In the rumen, archaea typically represent a small fraction of the total microbial richness and abundance, contributing less than 4% to the total ruminal microbial community (Matthews et al., 2019). These archaea are primarily methanogens, the sole methane producers in the rumen ecosystem. All methanogens are classified within the archaeal phylum Euryarchaeota, comprising seven orders. Among them, Methanobacteriales, Methanomicrobiales, Methanomassiliicoccales, and Methanosarcinales

are prevalent in the rumen ecosystem (Mizrahi et al., 2021). The diversity of methanogens in the rumen is limited, with most belonging to the Methanobacteriaceae family. Within the Methanobacteriaceae family, the genus Methanobrevibacter is predominant, comprising up to 70% of the rumen archaeal community (Friedman et al., 2017).

Protozoa are comparatively large, unicellular eukaryotes and less diverse than bacteria and archaea, comprising around 25–50% of the total microbial biomass (Abubakr et al., 2013; Newbold et al., 2015; Solomon & Jami, 2021; Sylvester et al., 2004). This microbial domain accounts for 30–40% of overall fibre digestion, participates in bacterial predation, and contributes to methane production through its association with methanogens (Morgavi et al., 2013). Rumen Fungi also constitute a vital part of the rumen microbiome community, significantly contributing to the degradation of plant fibre by secreting degradative enzymes, including cellulases, hemicellulases, and xylanases, from their rhizoids (Akin & Borneman, 1990; Bauchop, 1979), which is essential for the digestive process in ruminants. Although fungi are less abundant than bacteria in the rumen, they also maintain a close connection with methanogenic archaea (Bauchop & Mountfort, 1981).

The reticulorumen microbial community consists of more than 2,500 known species; however, it is noteworthy that a substantial portion, above 80%, of the ruminal microbial population remains uncharacterised (Cammack et al., 2018; Henderson et al., 2015; Henderson et al., 2019). Since the host, its microbiome, and environment all interact to affect an animal's overall performance, it is crucial to fully understand these intricate relationships in order to maximise rumen function, increase animal productivity, and lessen the environmental impact of ruminant production (Huws et al., 2018; Mizrahi et al., 2021).

Reticulorumen Digestion and Enteric Fermentation

The reticulorumen functions as a controlled fermentation system where diverse microbes break down fibrous plant material and complex feed components into simpler compounds essential for microbial growth and host nutrition. Ruminal fermentation pathways of carbohydrates, proteins, and lipids have been extensively investigated. According to these studies, in the case of plant materials digestion, at the first stage, polysaccharides (cellulose and hemicellulose) are degraded by fibre-digesting microbes into di- and monosaccharides (predominantly hexose sugars). At the second stage, these simple sugars are immediately taken up by rumen microbes, reaching the microbial cells with the help of specific transporters. Subsequently, these sugars undergo metabolism through diverse pathways, including the pentose phosphate pathway and

the Embden-Meyerhoff-Parnas pathway (Glycolysis pathway) with pyruvate as the major intermediate product (Russell & Wallace, 1997). In the third stage, certain metabolites, including hydrogen, carbon dioxide, lactate, and succinate, are produced from pyruvate, and finally, pyruvate and these metabolites undergo additional transformations to produce short-chain fatty acids (SCFAs) such as acetic (40-70%), propionic (15-40%), butyric (10-20%), methane, and various other byproducts (Bergman, 1990; Demeyer, 1991; Krause et al., 2003; Li et al., 2016; Makanya et al., 2020). The genus Fibrobacter is widely recognised as a representative bacteria producing acetate (Russell & Rychlik, 2001), whereas *F. succinogenes* is considered a key cellulolytic species that converts cellulose to soluble sugars and succinate to fuel the metabolism of other microbiomes (Arntzen et al., 2017; Suen et al., 2011). Prevotella takes part in starch, protein and hemicellulose degradation, leading to the production of more propionate. A high relative abundance of Prevotella with a high concentration of propionic acid in yak has been reported by Zhang et al. (2016). These SCFAs are volatile fatty acids (VFAs) resulting from ruminal fermentation, providing approximately 70% to 80% of the net energy requirements of the animal (Kebreab et al., 2009; Mizrahi, 2011; Seymour et al., 2005; Wu & Papas, 1997). However, the composition and balance of the rumen microbiome, along with the type of diet, primarily determine the production and proportion of SCFAs during ruminal fermentation and thus significantly influence the energy that is available for the animal to metabolise (Ben Shabat et al., 2016; Carberry, Kenny, et al., 2014; De La Torre et al., 2019; Guan et al., 2008).

During the fermentation of plant fibre by bacteria and protozoa, substantial amounts of H_2 and CO_2 are accumulated in the reticulorumen along with some organic acids and VFAs (Gomez et al., 2019; Louis & Flint, 2017). Methanogenic microbes use this H_2 to reduce CO_2 by forming CH_4 in a process called methanogenesis (Berghuis et al., 2019; Carberry, Waters, et al., 2014; de Mesquita et al., 2023). Furthermore, by utilising H_2, methanogens may influence other microbial species that are sensitive to H_2 partial pressure and uphold fermentation equilibrium through microbial growth kinetics and fermentation thermodynamics (Janssen, 2010). This interspecies H_2 transfer is crucial for optimal microbial fermentation and VFA production (Morgavi et al., 2010). Short-chain fatty acids and CH_4, produced in the rumen as fermentation end-products, are intricately yet distinctively linked to the efficiency of cattle in feed utilisation and production (Khiaosa-ard & Zebeli, 2014).

Recent evidence indicates that host genetics help shape an individual's rumen microbiome profile, and that efficient fermentation depends on the proper functioning of these microbes (Li

et al., 2019; Wallace et al., 2019). Ruminant performance, therefore, reflects the interplay of host genotype, environmental factors, and the rumen microbial community (Brito et al., 2020; Mizrahi et al., 2021). Thus, traits such as water consumption behaviour, influenced by various environmental factors, subsequently affect the reticulorumen environment, which is closely associated with microbial functionality. Therefore, a comprehensive understanding of ruminal fermentation is essential for developing strategies to enhance cattle systems. The multidimensional role of ruminal digestion and microbial fermentation is presented in Figure 2. 1**.**

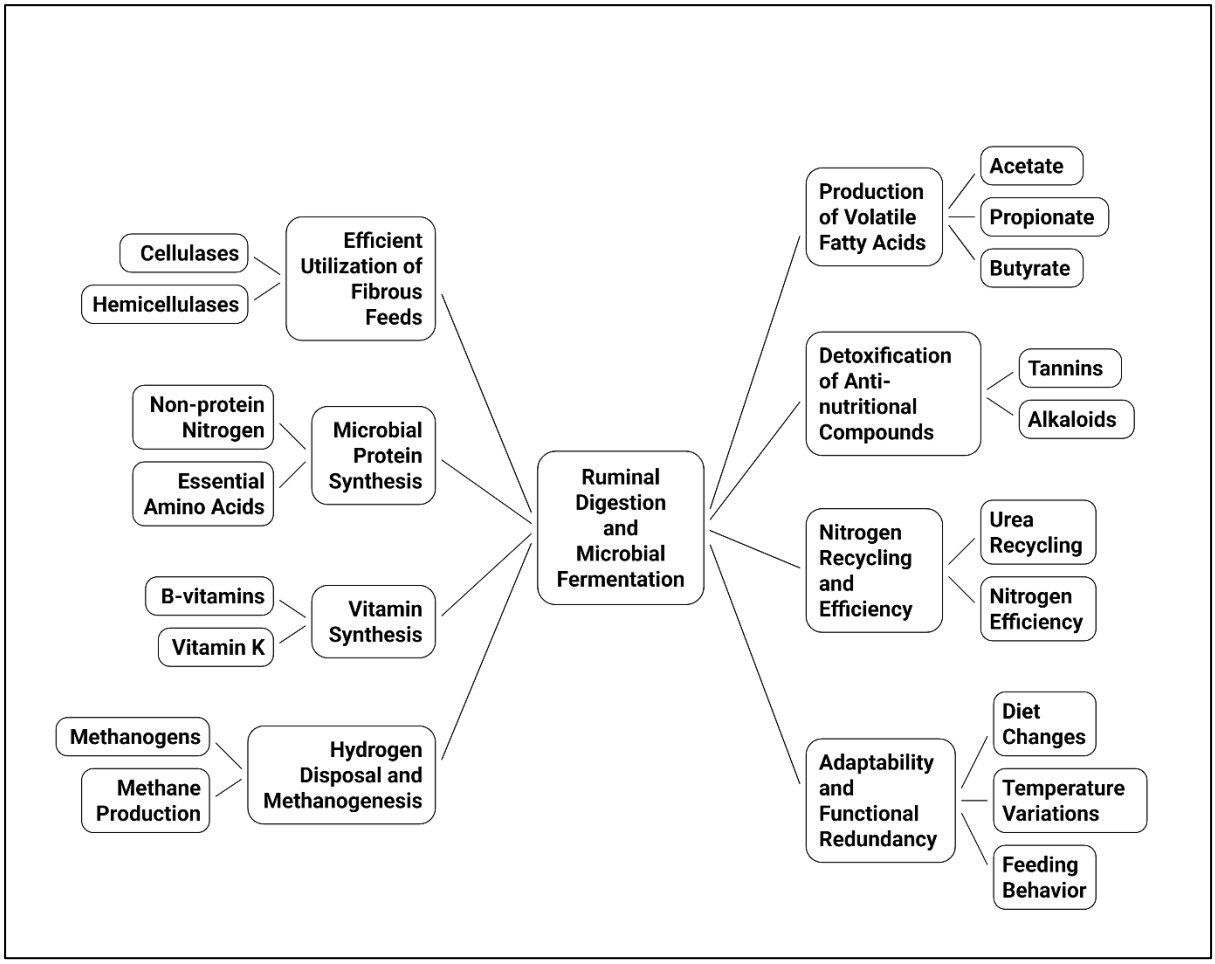

Figure 2. 1. Schematic diagram of the benefits of ruminal digestion and microbial fermentation

Emissions and excretions from reticulorumen fermentation

Microbial fermentation in the reticulorumen not only supports ruminants with the required nutrition but is also associated with the release of greenhouse gases, mainly CH_4, excessive nitrogen in manure, and a potential decrease in the nutritional quality of ruminant products (Scollan et al., 2011). Annual CH_4 emissions from ruminants are approximately 2.1 $GtCO_2e$

(gigatons of carbon dioxide equivalent), representing 20.2% of global CH_4 emissions (Shukla et al., 2019), exclusively generated by methanogenic archaea during microbial fermentation in the reticulorumen (Boone et al., 1993), the process known as methanogenesis (McAllister et al., 2015). Enteric CH_4 emission through methanogenesis not only impacts global warming but also represents a loss of energy from 2 to 12% of gross energy intake, which in principle could otherwise be available for animal growth and milk production (Johnson & Johnson, 1995; Moss et al., 2000; Shabat et al., 2016). The principal substrates for methanogenesis in the rumen are H_2, CO_2, formate (HCOOH), and methyl-containing compounds such as the methylamines and methanol, generated through microbial fermentation of fibrous feedstuffs (Janssen & Kirs, 2008). Methanogens utilise these H_2 and carbon substrates to produce CH_4 following hydrogenotrophic (utilisation of H_2 to reduce CO_2, or formate to CH_4), methylotrophic (utilisation of methanol or methyl amines), and acetoclastic (utilisation of acetate) pathways (Coleman, 1986; Guyader et al., 2014). Alternatively, during the fermentation of starch-rich feedstuffs, H_2 is metabolised in a different pathway, contributing to propionate production, which is a desirable pathway of H_2 removal. The efficient H_2 removal from the rumen favours VFA formation and increases feed fermentation rates (McAllister & Newbold, 2008).

A dynamic interaction occurs in the rumen between hydrogen-producing and hydrogen-utilising microbes, known as interspecies hydrogen transfer. This mechanism is considered central to maintaining ecosystem homeostasis and plays a crucial role in shaping the composition of the microbial community (Wolf et al., 2016). Methanogens are occasionally related to hydrogen-producing microbes in the rumen. Protozoa are one of the major hydrogen providers, interacting significantly with methanogens (Newbold et al., 2015; Ushida et al., 1997). The abundance of methanogens has been found positively associated with recognised H_2 producers such as Fibrolytic bacteria, including Cellulolytic Ruminococcus and several Firmicutes in different animals (Morvan et al., 1996). Anaerobic fungi also produce significant amounts of H_2, along with CO_2, formate, and acetate, contributing to methanogenesis. Syntrophic interactions related to H_2 production and utilisation have been documented among methanogens, the rumen fungus Neocallimastix frontalis (Bauchop & Mountfort, 1981), the bacterium F. succinogenes (Rychlik & May, 2000), hemicellulolytic bacteria, and other carbohydrate fermenters (Leahy et al., 2010).

Despite methanogenic archaea comprising less than 4% of the entire ruminal microbiota, both their abundance and diversity, as well as their interactions with other species, significantly influence the quantity of CH_4 produced (Pitta et al., 2016; Tapio et al., 2017). Sequencing of

the rumen microbiome demonstrated that microbial genes are directly associated with CH_4 emissions. Furthermore, metatranscriptomic technologies have demonstrated that less prevalent, and previously overlooked methanogenic lineages may play a more substantial role in CH_4 production than the predominant methanogens in the rumen (Söllinger et al., 2018). Methane emission has been linked to the feed efficiency of cattle (Hegarty, 2004), and it has been suggested that efficient cattle produce 24–28% less methane (L/kg of BW) compared to inefficient ones (Hegarty et al., 2007; Jones et al., 2011; Nkrumah et al., 2006). From a metabolic point of view, the methanogenic metabolic pathway is enriched in low-efficiency cattle, while in the high-efficiency groups, the lactic acid-propionate conversion pathway is enriched (Shabat et al., 2016). Finally, a reduction in methane production is associated with an increase in propionate availability for host energy utilisation, indicating that reduced methanogenesis can enhance meat and milk production (Shabat et al., 2016). The metabolic pathways of CH_4 formation during enteric fermentation are shown in Figure 2. 2.

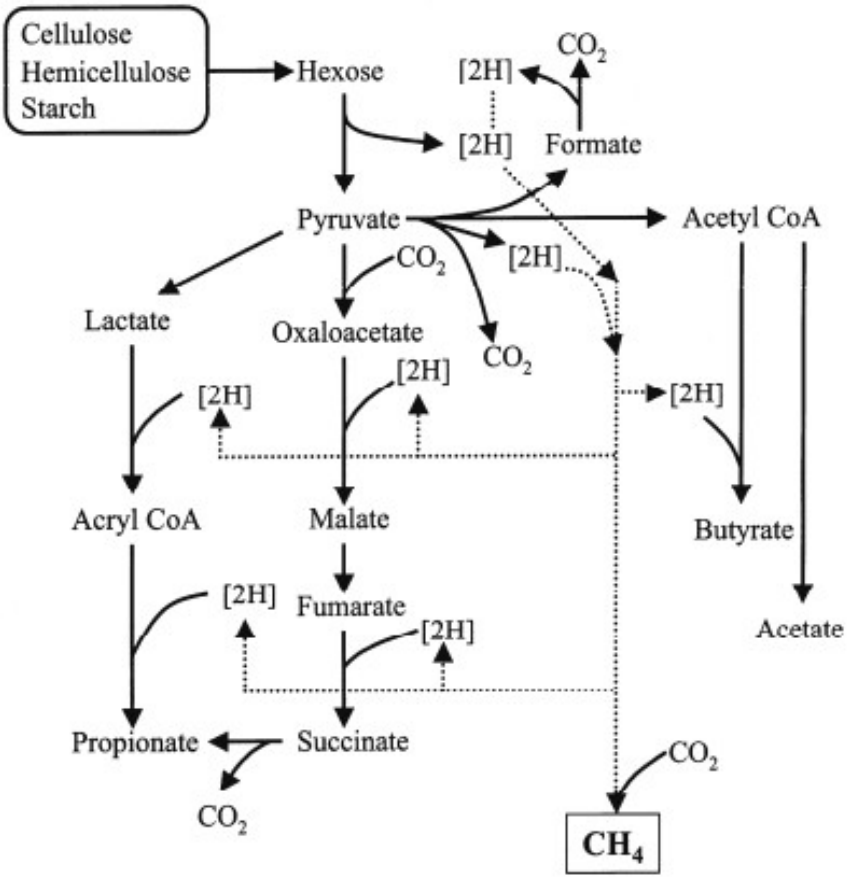

Figure 2. 2. Possible fermentation pathways of methane production in the rumen, adopted from Mitsumori and Sun (2008).

Ammonia (NH_3) is the central intermediary in the degradation and assimilation of protein and non-protein nitrogenous substances (NPN) in the rumen. The pathway of N assimilation into microbial protein within the reticulorumen is an essential component of protein flow to the small intestine of ruminant animals. Ammonia is generated in the rumen mainly from microbial degradation of nitrogenous compounds from the protein component (rumen degradable protein) of the diet within the rumen, and microbial hydrolysis of urea passing across the gut wall from the blood and intestinal fluids (Parker et al., 1995). It has been reported that about 90% of bacterial species utilise NH_3 as the primary source of N for microbial protein synthesis (Bryant & Robinson, 1962), and a maximum of 40 – 68% of microbial N was derived from the rumen NH_3 pool (Hristov & Broderick, 1994). The resultant microbial protein is essential for animal products, while excess ammonia is absorbed from the rumen, metabolised, and subsequently excreted in the urine. This represents an inefficient utilisation of dietary proteins, resulting in detrimental environmental impacts through nitrogen pollution (Puniya et al., 2015). A particular group of bacteria whose ammonia production rate is much higher than the capacity of ruminal microbes to utilise it for other functions, including microbial protein synthesis (Rychlik & Russell, 2000; Wang & Tan, 2013), known as the hyper-ammonia-producing bacteria (HAB) (Bach et al., 2005; Van Soest, 1994). There are several strains of hyper-ammonia-producing bacteria (HAB) with varying biological capacities for ammonia production; Selenomonas ruminantium, Peptostreptococcus elsdenii, and Bacteroides ruminicola are such HAB strains. Dietary manipulation with a proper ratio of rumen-degradable protein and rapidly fermentable carbohydrates is a suggested approach to address the low efficiency of nitrogen utilisation in ruminants. Since microbial community structure has been evident as central to enteric fermentation and associated emissions, every possible factor impacting microbial diversity is worth investigating.

Factors impacting reticulorumen fermentation

Reticulorumen digestion is a complex mechanical and biochemical process by which plant polysaccharides are hydrolysed to small saccharides that, in turn, are fermented to simpler substances and utilised by microbes and the host for their survival and growth. While degradation of feedstuffs by rumen microorganisms is systematic, influenced by their preferences for various feed structures and substrates, the optimum rumen environment is a prerequisite for effective fermentation (Firkins, 2010; Welkie et al., 2010).

Reticulorumen pH and fermentation

The optimal rumen pH is crucial for the survival and stability of rumen microbes, as these microbes are susceptible to minor fluctuations in pH levels, which are generally regulated by the type of feed ingested (Penner, 2016). In practice, the interaction of feed, rumen pH, and rumen microbes is interdependent, with each factor influencing the others (Grünberg & Constable, 2009). The effect of pH on fibre digestion in the rumen is well established (Erfle et al., 1982; Hoover, 1986; Hoover et al., 1984; Mould & Orskov, 1983; Mould et al., 1983). From the studies, rumen pH typically ranges from 6.0 to 7.0 on a forage-based diet, but it declines when cattle consume diets rich in highly fermentable starch (Grünberg & Constable, 2009; Russell & Diez-Gonzalez, 1997) from which large amounts of VFA, especially propionic acid, are produced (Fondevila & Dehority, 1996). An increase in VFA production in the reticulorumen greater than absorption will lead to a decrease in rumen pH, thereby disrupting the rumen microbial community (Clarke, 1977), particularly fibrolytic microbes, and thereby reducing fibre digestion (Cheng et al., 1980; Russell & Dombrowski, 1980). The effect of pH, diet, and their interactions explained 79% of the variation in the organic matter digestibility, with an average value at a mean pH (5.95) of 49.4% (Calsamiglia et al., 2008). However, Russell (1999), in a controlled *in vitro* study, demonstrated that the change in the acetate: propionate ratio was attributed mainly to the diet (75%) and to a lesser extent to pH (25%). Furthermore, Weimer (1996) observed that ruminal acid detergent fibre (ADF) digestion, an indicator of fibre digestibility and feed quality, declined by 3.6% for every 0.1 unit drop in pH below 6.3. According to Palmonari et al. (2010), when the pH drops below 6.0, the population and growth of cellulolytic bacteria and the ruminal fungi decline, which impairs fibre digestibility and results in overproduction of lactic acid. An increase in the percentage of rapidly degradable starch in the diet generally favours the development of protozoa as long as the rumen pH does not fall below 5.5 (Leng, 2014). In addition, acute or chronic low rumen pH, referred to as acidosis, can dramatically alter the ruminal microbial ecosystem, leading to a decrease in feed efficiency and reduced growth or milk production (Hernández et al., 2014; Nagaraja & Lechtenberg, 2007).

Reticulorumen temperature and fermentation

Reticulorumen temperature is closely associated with microbial growth and, consequently, ruminal fermentation, metabolic functions, and the overall health of ruminants. It is a crucial component of the reticulorumen environment, maintained in a narrow range between 38 and

40 °C, for the effective digestion and fermentation of complex carbohydrates by rumen microbes (Dehority, 2003; Linville et al., 2017). Reticulorumen temperature typically runs higher than rectal temperatures, and because of the activity of heat-producing rumen microorganisms, as such RT have been described as 1- 2 °C higher than other proxy measures of core body temperatures (Dale et al., 1954). However, RT has been reported to be influenced by a variety of factors, including environmental factors such as ambient temperature, relative humidity, rainfall, physiological conditions like oestrous, pregnancy, excitement, and behavioural activities such as drinking, eating, and lying (Al-Haidary et al., 2002; Lefcourt et al., 1999; Liang et al., 2013; Nakamura et al., 1984; Piccione & Refinetti, 2003; Rutherford et al., 2019). Changes in RT via hypo- and hyperthermia have been shown to influence the gut microbiota of ruminants, with consistent effects on community diversity and stability (Huus & Ley, 2021), and hence it is probable that these factors will also influence enteric fermentation.

Water consumption

Adequate water is an indispensable resource for all livestock, essential for maintaining their physiological processes and production attributes. Utley et al. (1970) reported that the provision of adequate water to livestock yields physiological and productivity benefits that are similar to those associated with the consumption of high-quality forage. Research has shown that both the volume of water consumed by cattle and the frequency of their drinking behaviour intricately associated with feed intake, physiological health, lactation performance, and growth rates (Grout et al., 2006; Lardner et al., 2005; Silanikove, 1992; Williams et al., 2017). Murphy (1992) identified that daily water requirements for cows vary between 24 and 136 litres, depending on their stage of lactation. Furthermore, Burgos et al. (2001) found that a decline in water consumption can lead to a substantial reduction in milk production, with reductions reaching up to 26%. In contrast, Daros et al. (2019) reported that unrestricted access to water can enhance milk yield by as much as 1.7 litres per day. Although cattle drink intermittently and for short periods, this behaviour is biologically vital and significantly impacts their health and productivity (Delagarde & Lamberton, 2015; Dutta et al., 2015).

Drinking behaviour of cattle

The drinking behaviour of cattle is associated with several factors, including but not limited to breed, body size and growth, health, stage of production, feed intake, water quality, water availability, and climatic conditions (Ahlberg et al., 2019; Arias & Mader, 2011; Meyer et al.,

2004; Sowell et al., 1999; Wagner & Engle, 2021). Drinking behaviour in terms of number of sessions per day, intake rate (L/min), session size (kg), time per session (sec), daily water intake (kg), session interval (min) have been investigated in dairy and beef cattle and reported heritability of these traits ranged from 0.56 to 0.88 (Dado & Allen, 1994; Dressler et al., 2023). Drinking behaviour varies between different breeds. Cattle with a higher heat-tolerant capacity have a lower metabolic rate, and they efficiently utilise ingested feed (Montalenti, 1978). This may be one of the reasons for lower water intake in zebu cattle compared to taurus cattle in similar environmental conditions (Payne & Hutchison, 1963). Lactating dairy cows (*Bos taurus*) in temperate regions often drink 2 to 4 times daily, with a maximum of 6 to 11 drinking episodes per day (Campbell & Munford, 1959; Chiy et al., 1993). Similarly, the average drinking frequency for *Bos taurus* beef cattle raised in cool climates, particularly temperate regions, is noted to be 4-7 times daily (Coimbra et al., 2010; Lardner et al., 2013). Availability of water also impacts drinking behaviour. In arid environments, both lactating and non-lactating *Bos taurus* and *Bos taurus* crossbred cows have been observed to drink between 1 and 2.5 times per day, with maximum frequencies ranging from 3 to 4 drinking episodes in large paddocks of 23 to 300 km², equipped with a single water source (Low et al., 1981; Rouda et al., 1994). Recently, Shirley et al. (2025) identified in lactating dairy cows an average of 3.6 drinking events per day with a large variation of individual drinking events per day from one to 20.

Ambient conditions and water consumption

High ambient temperatures have been found to greatly influence both the frequency and volume of water intake in cattle (Arias & Mader, 2011). Specifically, exposure to ambient temperature of 32°C can lead to a two to fourfold increase in water consumption compared to cooler temperatures ranging from 2°C to 10°C (McDowell, 1967). However, Singh (2002) reported that cows drank marginally more water at 37 °C of water temperature as compared with at 15 °C and 25 °C drinking water temperature in crossbred cattle (Haryana × HF) during the summer months, indicating the influence of water temperature on water consumption. According to West (2003), there is a consistent increase in water intake of dairy cattle by 1.2 kg/°C during periods of heat load. A linear increase of water intake (22.8 L to 67.0 L/day) with a similar increase in environmental temperature (17 to 35°C) was observed by Kamal et al. (1959). A notable increase in water consumption among beef cattle was also observed when exposed to simulated heat waves (Brown-Brandl et al., 2005). Under conditions of heat load,

cattle consume smaller amounts of water more frequently, thereby prolonging the overall duration of water intake (Cook et al., 2007). Pereyra et al. (2010) indicated that Holando-Argentino cows demonstrated the highest average frequency of drinking episodes when the Temperature-Humidity Index (THI) was between 74.91 and 83.95. It has been reported that the proximity of the water source also influences drinking frequency if no shade or sprinklers are available to alleviate the heat load (Wang et al., 2013). A negative association between low ambient temperature and water intake has been reported by Serviento et al. (2024). However, cold conditions can also lead to increased water consumption, as dry matter intake rises substantially at low ambient conditions, thereby influencing overall water requirements (Shalit et al., 1991).

Water consumption and reticulorumen temperature

Maintaining an optimum reticulorumen temperature is essential for facilitating efficient microbial fermentation in the rumen. However, water consumption leads to a dramatic decrease in reticulorumen temperature, distorting the reticulorumen environment for at least a short period of time. Considering the importance of reticulorumen temperature, several studies have investigated the factors that regulate water consumption along with associated temperature drops in the reticulorumen (Arias & Mader, 2011; Golher et al., 2021; Meyer et al., 2004; Mishra, 2021; Serviento et al., 2024). It has been revealed that the volume and temperature of ingested water determine the magnitude of the reticulorumen temperature drop (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964). A range of temperature drops from 0.4 ± 2 °C (Bewley et al., 2008) to 12.84 °C (Cunningham et al., 1964) has been reported in various experimental studies, where cattle were drenched with different volumes of water at varying temperatures. This temperature drop in the reticulorumen after water intake follows the thermodynamic principle of mixing fluids (Rahman et al., 2024; Welsh et al., 2025).

Generally, RT is tightly maintained within a range of 38 to 40°C, with an average temperature of 39 ± 0.5 °C. However, when cattle drink cold water (less than the RT), the RT is immediately depressed. Following the lowest temperature level, reticulorumen temperature exhibits a rapid increase due to ingesta mixing, followed by a gradual rise as the chilled ingesta warms, eventually approaching pre-drinking levels (Cunningham et al., 1964; Dale et al., 1954). From the experimental studies where lactating or non-lactating cows were drenched with chilled or near body temperature water, the reported length of time for RT to return to near baseline (pre-drinking) varies from 15 minutes to 210 minutes (termed as recovery period) (Bewley et al.,

2008; Cantor et al., 2018; Cunningham et al., 1964; Dale et al., 1954). On the other hand, long-term observational studies utilising rumen bolus sensor technology for continuous monitoring of RT have identified characteristic RT fluctuations as water intake events using different types of algorithms. The length of recovery periods of algorithm-driven drinking events ranges from 35 minutes to 160 minutes (Cantor et al., 2018; Serviento et al., 2024; Shirley et al., 2025; Vázquez-Diosdado et al., 2019). Several studies have suggested that the duration of RT recovery is correlated with the extent of the temperature decline, with a greater decline taking longer to recover (Bewley et al., 2008; Dracy et al., 1963; Serviento et al., 2024). Furthermore, Dracy and Kurtenbach (1968) observed a 9.0°C decline in rumen temperature following the administration of 2.5 kg of milk at 8.0°C to calves, with a subsequent recovery period of 52 minutes. In contrast, Bewley et al. (2008) reported a longer recovery duration of approximately 3.5 hours after a 9.2°C temperature drop resulting from drenching cows with 18.9 kg of water at 5.1°C. However, little is known about the consequences of this duration (recovery period) on rumen function. Further investigation of the recovery period, considering animals body weight, physiological states and environmental parameters, might assist in understanding reticulorumen thermodynamics and associated impact on ruminal fermentation.

Ambient conditions, reticulorumen temperature, and the microbiome

The thermoneutral zone (TNZ) represents the range of ambient conditions at which cattle do not need to adjust their metabolic heat production or increase evaporative heat loss to maintain thermal balance (Kingma et al., 2012). Within the TNZ, cattle maintain an equilibrium between heat accumulation and dissipation, facilitating optimal growth, performance and wellbeing (Li et al., 2021). When ambient conditions fall below the TNZ, animals undergo cold stress due to heat loss exceeding heat generation, consequently disrupting thermal balance (Roland et al., 2016). Conversely, heat stress occurs when animals are unable to effectively dissipate excess body heat, triggering physiological and behavioural adjustments to mitigate the effects of internal and external thermal challenges (Bernabucci et al., 2010; Das et al., 2016; Dikmen & Hansen, 2009; Silanikove, 2000). During the period of heat load, heat dissipation can be disrupted, which can result in elevated RT. Elevated relative humidity intensifies the impact of high ambient temperatures by reducing the efficiency of evaporative cooling mechanisms, especially from the respiratory surfaces (Jericho & Magwood, 1977). This can further increase rumen temperature and stress levels in cattle (Brown-Brandl et al., 2006; Hahn, 1999). Reticulorumen temperature is also influenced by breed and shade availability, with Lees et al.

(2018) reported an RT of 43.7 °C in an unshaded Angus steer during a heat wave event. Metabolic heat from microbial fermentation contributes 3–8% of an animal's total heat production (Czerkawski, 1980), and an inadequate dissipation of this heat leads to elevated RT (Beatty et al., 2008). Conversely, cold ambient temperatures can cause a reduction in RT as the animal diverts more energy towards maintaining core body temperature (Giannone et al., 2023; Nkrumah et al., 2006; West, 2003).

Under high heat load conditions, an animal's CBT reflects the net balance of heat accumulation and heat dissipation to the environment, making estimates of CBT a reliable indicator of thermal status (Lees et al., 2018; Yan et al., 2021). Generally, rectal temperature is considered the gold standard of CBT; however, it has been reported reported a strong relationship between rectal temperature and RT during periods of reticulorumen hyperthermia (Beatty et al., 2008; Rose-Dye et al., 2011; Timsit et al., 2011). Further, RT has become the most prominent method for estimating core body temperature in cattle studies (Lees et al., 2022). During excessively hot conditions, an animal's CBT elevates, corresponding to an increase in RT. It has been reported that during the period of high heat load, cattle experience shorter rumination periods, and lower salivary bicarbonate infusion into the rumen impacting ruminal pH, which subsequently results in changing ruminal microbial community structure and metabolism (Kocherginskaya et al., 2001; Tajima et al., 2001; West, 1994; Zhao et al., 2019). Under conditions of elevated heat load, cattle exhibit altered feeding behaviour, characterised by a reduction in voluntary intake of high-fiber forage relative to concentrate to ensure their maintenance energy needs (Beede & Collier, 1986). However, because of higher consumption of concentrate diet (rich in soluble sugars), the activities of saccharolytic bacteria such as Streptococcus, unclassified Enterobacteriaceae, Ruminobacter, Treponema, and unclassified Bacteroidaceae increase (Zhao et al., 2019) and results in a higher concentration of lactic acid, which leads to lower pH in the rumen. This lower pH further inhibits the growth and activities of fibrolytic bacteria such as *Fibrobacter succinogenes*, *Flavonifractor*, *Prevotella ruminicola*, *Ruminococcus flavefaciens*, and *Treponema* decrease (Sales et al., 2021; Yadav et al., 2013). Alteration of this rumen microbiota composition further leads to a marked reduction in SCFAs concentrations and shifts in their proportional distribution (Acetate: Propionate) in the rumen (Tajima et al., 2007). However, Uyeno et al. (2010) observed no changes in the overall bacterial diversity (richness) at the genus and species levels, even under heat stress conditions.

When ruminants are exposed to cold ambient conditions, the reticulorumen environment remains thermally stable since endothermic mammals increase feed consumption and

metabolic heat generation to maintain thermal homeostasis in cold seasons (Crater & Barboza, 2007; Goel et al., 2005). However, the temperature of free water and feed, which are closely linked to ambient temperatures, are crucial in maintaining rumen temperature, especially in open barn systems during colder seasons (Serviento et al., 2024). Consumption of feed and water, and even air inhalation during rumination in extreme cold environments, could result in temperature fluctuations within the reticulorumen by lowering the overall endogenous temperature (Degen & Young, 1984; Nicol & Young, 1990). Crater and Barboza (2007) reported that the RT of muskoxen (*Ovibos moschatus*) decreased to 31.4 °C in early winter, 35.7 °C in mid-winter, and 32.5 °C in late winter. It has been suggested that while there is an inverse relationship between microbial diversity and ambient temperature, there are some microbes that adapt better to adverse environmental conditions, which may potentially impact ruminal functions (Romero-Pérez et al., 2011) and/or greenhouse gas production. In the rumen of sheep under simulated cold conditions, Guo et al. (2021) observed an increased abundance of fibre-digesting bacteria *Lachnospiraceae* and at the family level a lower abundance of Prevotellaceae. Prevotellaceae typically dominate in the rumen, with their primary fermentation products being acetate and propionate (Herrmann et al., 2017; Poeker et al., 2018). Therefore, the decrease of Prevotellaceae can be accompanied by a decrease in the content of acetate and propionate. Studies suggest that bacterial growth at suboptimal temperatures is limited because protein synthesis becomes inhibited under such conditions (Broeze et al., 1978). The causes of alterations in microbial community composition are not yet fully understood; temperature may indirectly affect these changes by modifying host physiology under heat stress or cold stress conditions, which warrants further investigation.

Simulating reticulorumen environment and microbiome modelling

For several decades, ruminant nutrition research has predominantly relied on a range of *in vitro* techniques, which allow for more controlled conditions and require fewer animals than *in vivo* experiments, therefore reducing the economic and management costs (López, 2005). Anaerobic *in vitro* fermentation has been widely utilised to simulate rumen kinetics and to analyse microbial composition and metabolite profiles in a controlled laboratory environment (Dhakal et al., 2024). Both traditional batch fermentation systems and relatively advanced continuous fermentation models, such as the rumen simulation technique (RUSITEC), have played a pivotal role in enhancing our understanding of rumen metabolism (Kour et al., 2025). While batch culture *in vitro* systems allow the investigation of a large number of treatments in

a relatively short period of time, complex continuous culture systems simulate and maintain a stable fermentation for longer periods (Czerkawski & Breckenridge, 1977). It has been reported that batch cultures exhibit significant shifts in microbial abundance and composition, whereas continuous-culture fermenters tend to preserve a more stable microbiota and community structure throughout the incubation period (Soto et al., 2013). Batch cultures operate as closed systems, with no continuous inflow or outflow. In contrast, semi-continuous systems such as the Rumen Simulation Technique (RUSITEC) (Czerkawski & Breckenridge, 1977) and fully continuous rumen simulation models (Hannah et al., 1986; Hoover et al., 1976) incorporate controlled inflow and outflow to more accurately replicate ruminal fermentation dynamics. Although these models significantly reduce the need for live animals and facilitate high-throughput hypothesis testing, they still fall short of fully replicating the complexity of the rumen environment. These systems generally lack key physiological features such as rumen motility, salivary flow, epithelial absorption, immune interactions, and diurnal feeding and drinking patterns, all of which influence pH, redox potential, and microbial turnover *in vivo* (Scicutella et al., 2025). Basically, the efficacy of ruminal fermentation simulation systems depends primarily on their ability to sustain microbes that accurately reflect that of the rumen under specific experimental conditions (Slyter & Putnam, 1967; Warner, 1956), which provides opportunities for developing experiment-specific methods.

Together with improvements in fermentation systems, the introduction of high-throughput sequencing technologies has advanced rumen microbiome research. Recent developments in these technologies have enabled the identification of subtle alterations in microbial community composition, reflected in shifts in abundance and diversity (Henderson et al., 2013). Traditionally, two primary strategies have been employed for sequencing metagenomic samples: targeted sequencing and shotgun metagenomic sequencing. Initial studies primarily utilised 16S rRNA gene amplicon sequencing to characterise bacterial and archaeal communities, offering a cost-effective means of estimating the abundance of microbial communities (Denman et al., 2018). Amplicon-based methodologies appeared promising for *in vitro* investigations due to their cost-effectiveness, scalability, and analytical capabilities. However, this method typically depends on long sequence reads (Franzén et al., 2015), captures phylogenetic variation at a single gene, and is prone to PCR primer bias arising from mismatches in the flanking regions where primers anneal (Sim et al., 2012). Shotgun metagenomic sequencing addresses many of these limitations by analysing total community DNA without locus-specific PCR, enabling the reconstruction of metagenome-assembled

genomes, functional annotation of carbohydrate-active enzymes, and integration with metabolomic and host databases (Stewart et al., 2019). Nevertheless, full-depth shotgun metagenomics remains comparatively costly for the very large sample sizes required to support genome-wide association studies, selective breeding analyses, or extensive factorial *in vitro* screening (Hillmann et al., 2018). Restriction Enzyme-Reduced Representation Sequencing (RE-RRS), also referred to as Genotyping-by-Sequencing (GBS), is a next-generation sequencing approach introduced by Hess et al. (2020). This method reduces genome complexity by digesting genomic DNA with restriction enzymes, followed by sequencing fragments within a defined size range. Unlike 16S rRNA sequencing, RE-RRS is not constrained by the presence of a specific gene, enabling it to profile a broader spectrum of organisms, including the host, viruses, and fungi that may influence the trait of interest, such as methane yield (Hess et al., 2020). RE-RRS represents a promising approach for rapid, high-throughput, and cost-efficient sequencing of metagenomic samples, offering a substantially lower cost compared to conventional metagenomic shotgun sequencing (Hess et al., 2023; Hess et al., 2020).

Conclusion

The reticulorumen environment and its microbial community have always been considered key to ruminant nutrition. The majority of the energy and protein supply for ruminants, as well as greenhouse gases, is synthesised in the reticulorumen through microbial fermentation under a controlled environment. Several factors, including fluctuations in reticulorumen temperature, have been found to alter fermentation parameters. Notably, drastic temperature changes are often associated with water intake events, highlighting the importance of understanding reticulorumen thermodynamics in relation to water intake for advancing cattle studies. Considering that microbial composition is influenced by both host genetics and environmental factors, certain animals may exhibit distinct microbial profiles that make them less vulnerable to external disturbances. This review identified specific gaps in knowledge in this field, which this thesis addresses in the subsequent chapters. These are –

- Insufficient explanation of RT fluctuations following water intake from the thermodynamic point of view.
- Lack of methods to simulate RT fluctuations mimicking cattle drinking events.
- Unexplored interplay among water intake, RT dynamics, enteric fermentation and reticulorumen microbiome diversity.

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CHAPTER 3: PUBLISHED MANUSCRIPT

Modelling reticulorumen temperature changes from drinking using Richmann's Law

Chapter 3 compiles and synthesises published data on reticulorumen temperature drops and interprets these observations within the framework of thermodynamic principles. Relative changes in reticulorumen temperatures correspond to intake water temperatures, along with the relationship between observed and predicted reticulorumen temperature explored in this chapter. Alignment of thermodynamic principles to literature data provides the basis of exploring drinking behaviour using reticulorumen temperature data. This chapter was published as a peer-reviewed conference paper at the 11th European Conference of Precision Livestock Farming 2024 in Bologna, Italy. http://www.eaplf.eu/.

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Modelling reticulorumen temperature change from drinking events using Richmann's Law

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Abstract

A rapid decline and exponential recovery in rumen temperature occur due to drinking, with differences in such behaviour between animals potentially impacting the rumen ecosystem. Previous research has highlighted diverse temperature drops in the reticulorumen following drinking events. To evaluate these drops from the thermodynamic perspective, we compiled a reticulorumen temperature drop dataset from previously published studies in dairy cattle and applied Richmann's law of mixing fluids. Our outcomes revealed a proportionality between the changes in ingested water temperature from the baseline reticulorumen temperature of 39°C and the resulting shift in reticulorumen temperature for a given volume of water. Further, the specific heat capacity of rumen content was determined to be 3595.6 ± 177.3 (SE) J/(kg.K) using a nonlinear model to estimate the temperature drop in the reticulorumen from the actual drop of the compiled dataset. The estimated drops from the model were compared to actual reticulorumen temperature drops reported in studies, showing a strong correlation (R^2 = 0.93) over a range of documented temperature drops from 0.4°C to 12.8°C. Currently, we are incorporating another principle of thermodynamics (Newton's law of cooling) to add a model of temperature recovery to provide an overall reticulorumen temperature model for water consumption. This thermodynamic model will then be employed to determine the potential impact of (the differences in) water intake behaviour on the rumen ecosystem.

Keywords: reticulorumen temperature, Richmann's law, thermodynamics, drinking, temperature drop, specific heat capacity

Introduction

The rumen functions as an anaerobic fermentation chamber, maintaining stringent temperature control within a narrow range of 38 degrees Celsius (°C) to 42 degrees Celsius (°C) and a buffered pH range from 5.7 to 7.3, thus providing a consistent and conducive habitat for microbial communities (Yokoyama & Johnson, 1988). However,

rumen temperature deviates from this optimal range due to various factors including but not limited to physiological conditions, fluctuations in ambient temperature, environmental stressors, and differences in livestock management practices. Deviations from the optimal temperature can potentially disrupt the rumen environment's balance, altering microbial activity that may negatively impact productivity (Bewley et al., 2008; Liang et al., 2013). Drinking has been shown to induce the most dramatic reductions in reticulorumen temperature. Notably, when cattle and sheep are drenched with cold water of varying temperatures, rumen temperature drops ranging between 5°C and 10°C have been reported (Bewley et al., 2008; Cantor et al., 2018; Simmons et al., 1965).

After drinking, water mixes with the rumen contents, which contain 86 to 91% water; the rest is dry matter (DM) of feed particles (Alexander et al., 1969; Dado & Allen, 1995). When multiple bodies with different temperatures are mixed, thermal equilibrium can be explained by Richmann's law of mixture (Tillmann & Bohn, 2015). According to this law, when the temperature of the ingested water differs from that of the rumen content, the resulting mixture will eventually achieve thermal equilibrium. Richmann's law calculates the final equilibrium temperature by considering the distinct initial temperatures and specific heat capacities of these two bodies (Richmann et al., 1750). However, rumen content, having an undefined specific heat capacity, introduces challenges in accurately predicting the temperature dynamics in the reticulorumen following drinking events.

This study aims to compile the published data on reticulorumen temperature drops and evaluate the corresponding findings in light of the thermodynamic principles governing the mixing of fluids at different temperatures. By doing so, we seek to advance the understanding of reticulorumen temperature dynamics. This is crucial for developing a robust model of reticulorumen temperature change associated with drinking events and assessing its broader implications for rumen temperature changes in the rumen ecosystem.

Materials and Methods

Literature search strategy

A comprehensive literature search was performed to identify research investigating the relationship between water consumption and subsequent decline in reticulorumen temperature. The literature search was undertaken using targeted search terms, across three databases: Scopus, CABI's CAB Abstracts (covering 1910 to the present), and Google Scholar. Initial searches utilising inclusive phrases including: 'water and related terms' combined with 'cattle', 'cow', and 'ruminants' resulted in many articles. To refine the results, more specific search terms such as 'water intake', 'water consumption', 'drinking', 'rumen temperature', and 'reticulorumen temperature' were employed in conjunction with 'cattle', 'cow', and 'ruminants' resulted in 15 studies relevant to our research focus.

Study selection

For our study, we exclusively selected experimental studies that examined the effects of treatment variables, specifically the volume and temperature of consumed water. Observational studies, which involved monitoring rumen temperature without the application of any treatment intervention, were systematically excluded. This selection

process resulted in 5 studies on cattle which included nine experiments with 27 treatments involving a total of 39 animals (Table 1). Four of the studies were conducted with lactating and non-lactating dairy cows, while one study involved dairy bull calves aged 6 to 10 weeks. This deliberate focus on experimental designs ensured that the data set comprised only those studies capable of directly correlating the act of water consumption with measurable changes in reticulorumen temperature.

Table 1: Selected publications matching the inclusion criteria for temperature change analysis.

Research papers	Techniques to record reticulorumen temperature	Number of experiments	Number of treatments	Number of cattle
Dale et al. (1954)	Thermocouple and recording potentiometer.	1	1	1
Cunningham et al. (1964)	Radio telemetry system with temperature-modulated transmitters.	2	4	4
Dracy and Kurtenbach (1968)	Radio telemetry system with temperature-modulated transmitters.	2	4	4
Bewley et al. (2008)	Phase IV Cattle Temperature Monitoring System (a passive radio frequency transponder).	2	6	18
Cantor et al. (2018)	Reticulorumen bolus transponder (Smart bolus).	2	12	12

Temperature monitoring methods and experimental protocol

The selected studies employed diverse techniques to observe alterations in reticulorumen temperature as presented in the Table 1. Rumen temperature recordings were taken at intervals ranging from 2 to 15 minutes. The amount of water allotted to the animals was determined based on the drinking bout sizes (Litres/drinking bout) observed across the studies and the temperature of the water provided to the cattle ranged from 1.1°C to 38.9°C. A mean baseline reticulorumen temperature was calculated for each experiment to estimate the decline in reticulorumen temperature following water consumption.

Estimation of ΔT water and ΔT rumen

To evaluate the reticulorumen temperature drop from the published studies, the change in ingested water temperature (ΔT water) and the change in reticulorumen temperature (ΔT rumen) were calculated. ΔT water was defined as the difference in temperature between the baseline rumen temperature (RT_0 = 39 ^{0}C) and the ingested water temperature (T_w) and was calculated as

$$\Delta T \text{ water } (^0C) = RT_0 - T_w \quad \ldots\ldots\ldots(1)$$

ΔT rumen was defined as the difference in temperature between the lowest rumen temperature (RT_f) after consumption of water, and the baseline rumen temperature (RT_0) and was obtained as

$$\Delta T \text{ rumen } (^0C) = RT_0 - RT_f \quad \ldots\ldots\ldots(2)$$

Estimation of reticulorumen temperature drop (ΔRT)

To estimate reticulorumen temperature drop, we applied Richman's law of mixing fluids. According to this law, the overall final temperature of the mixture (T_f) results from achieving thermodynamic equilibrium upon mixing two liquids with different initial temperatures (degrees Celsius) (T_1 and T_2), volumes (Litres) (m_1 and m_2) and distinct specific heat capacities (Jule per kilogram kelvin) (c_1 and c_2) can be determined using the following equation:

$$T_f = \frac{m_1 c_1 T_1 + m_2 c_2 T_2}{m_1 c_1 + m_2 c_2} \quad \ldots\ldots\ldots(3)$$

In the context of our study, T_f is the rumen temperature (RT_f) after drinking water, with subscript 1 indicating the rumen content (r) and 2 indicating the water consumed (w), i.e.

$$RT_f = \frac{m_r c_r RT_0 + m_w c_w T_w}{m_r c_r + m_w c_w} \ldots\ldots\ldots\ldots(4)$$

where RT_0 is the baseline reticulorumen temperature before the drinking event. Then the temperature drop in the reticulorumen from the baseline was estimated as

$$\Delta RT = RT_0 - RT_f = \frac{m_w c_w (RT_0 - T_w)}{m_r c_r + m_w c_w} \ldots\ldots\ldots\ldots(5)$$

Since a known volume of consumed water (m_w) is mixed with an unknown volume of rumen content (m_r) which is a heterogeneous substance with an unknown specific heat capacity (c_r), estimation of m_r and c_r, was thus required. The m_r was estimated using findings in published research studies and c_r has been estimated through a nonlinear model by application of Richmann's law to literature data. The estimated ΔRT was then compared with the actual reticulorumen temperature drop from the selected studies.

Estimation of rumen content volume (m_r)

Several direct and indirect methods to estimate m_r have been developed by using DM (Alexander et al., 1969) and neutral detergent fibre (NDF) (Dado & Allen, 1995) percent of rumen digesta. However, no information regarding DM and NDF content was available in the selected studies. It has been reported that m_r, as determined by different methods, is significantly correlated (r = 0.60 to 0.79) with live body weight of cattle Salem (2005). Moloney et al. (1993) utilised body weight information to estimate the rumen volume suggesting the following equation:

$V = 12.7 + 0.083 \times W$ ……..(6)

Where V is rumen volume in litre (L), and W is body weight in Kilogram (kg).

We utilized equation (6) to estimate m_r based on the fact that all experiments described in the selected studies were conducted in the USA with lactating and non-lactating Holstein cows, with an average body weight between 650 kg and 750 kg.

Estimation of rumen content's specific heat capacity (c_r)

To estimate c_r we applied Richmann's law to the data compiled from the selected studies. A nonlinear least-squares regression analysis was performed using the `nls()` function in R to fit the predefined model formula (5), with an addition of a random error term to the model. Note that ΔRT and T_w are the output and input variables respectively, and c_r is the only parameter to be estimated, the other terms in (3) are taken as known constants. The set constants were fixed with the following values: a baseline rumen temperature (RT_0) of 39°C, a specific heat capacity of water (c_w) of 4184 J/(Kg.K), and a range of rumen content volumes (m_r) estimated by using equation (6) with ranges of body weights mentioned above (650 to 750 kg) and evaluated at 55, 65, and 75 litres (L).

Normalisation of intake volume and associated temperature drop data

Various volumes of intake water at different temperatures and the resulting temperature drops in the reticulorumen have been taken from the selected studies. Cunningham et al. (1964) administered 21.6 L of water at 1.1°C, Bewley et al. (2008) used 25.2 and 18.9 L of water at temperatures from 5.1°C to 38.9°C, and Cantor et al. (2018) provided 22.7, 11.4, and 5.7 L of water at temperatures ranging from 1.7°C to 29.4°C. We normalised these data by calculating the temperature drop (°C) per 10 L of water intake and plotted it against the intake water temperature (Figure 1).

The data analysis and processing were conducted using Microsoft® Excel® (Version 2401, Build 16.0.17231.20290, Microsoft Corporation, Washington, USA) and R (version 4.3.0, Rstudio, Boston, USA).

Results and Discussion

Evaluation of reticulorumen temperature drop: Figure 1 depicts the relative changes in reticulorumen temperature from a baseline of 39°C following the administration of various volumes of water to cows at differing temperatures. The data indicate that the relative change in ΔT rumen (°C/10 L of water intake) increases as the intake water temperature decreases. Additionally, the magnitude of ΔT rumen is correlated with the volume of water consumed, with larger volumes of colder water leading to a greater decrease in ΔT rumen. This finding is consistent with Cunningham et al. (1964), who observed a 12.84°C drop in the lower rumen temperature of Holstein cows given 21.06 L of water at 1.1°C. Similarly, Cantor et al. (2018) reported that the intake of 22.7 L of water at 1.7°C resulted in a 9.2°C change in ΔT rumen. Furthermore, the study found that the magnitude of ΔT rumen decreased with smaller volumes of ingested water at temperatures closer to that of the reticulorumen, suggesting a proportional relationship between intake water temperature and ΔT rumen. This trend was consistently observed across the reticulorumen temperature records in all other selected studies.

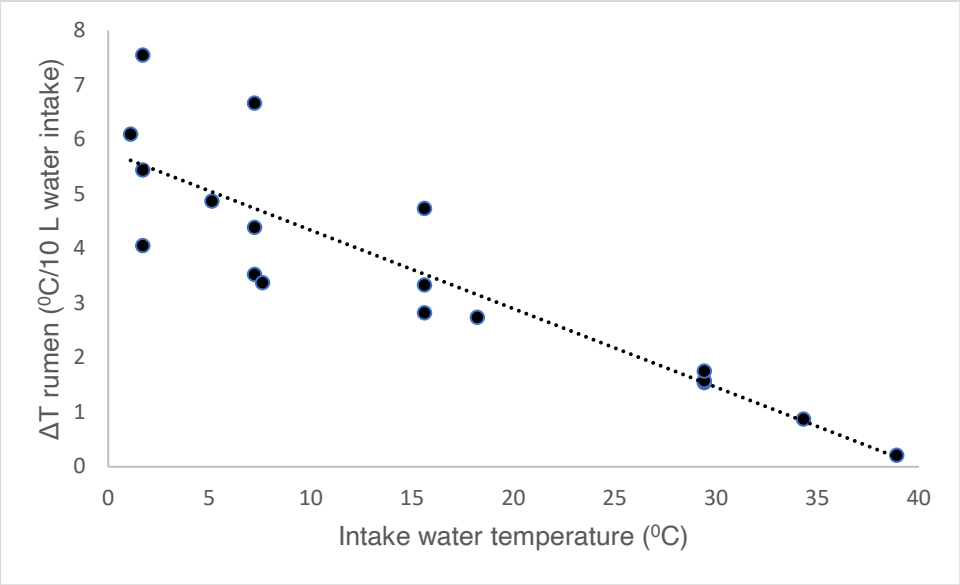

Figure 1: Relative changes in reticulorumen temperatures correspond to intake water temperatures

Estimation and evaluation of specific heat capacity of rumen content (c_r). The estimated values of c_r were 4249.4 ± 209.5, 3595.6 ± 177.3, and 3116.2 ± 153.6 (SE) J/(Kg.K) for m_r of 55, 65, and 75 L respectively (Table 2). However, the model produced the output (ΔRT) with the residual sum of squares (Res SS) as 15.46 in all cases.

Table 2: Estimated specific heat capacity of rumen content for different rumen content volumes

Rumen content volumes (m_r) (L)	Specific heat capacity (c_r) J/(Kg.K)	SE	Res SS
55	4249.4	209.5	15.46
65	3595.6	177.3	15.46
75	3116.2	153.6	15.46

SE = Standard Error; Res SS = Residual Sum of Square

It has been observed that the estimated c_r decreased ($p < 0.001$) with increasing m_r (Table 2), however, the product of m_r and c_r (m_r*c_r) remained almost the same (~234) across the volumes. These results give an indication of plausible values of c_r along with most likely m_r. Bonan (2019) highlighted that a leaf's fresh mass comprises its dry mass and water mass. Water, with a specific heat of 4184 J/(Kg.K), is three times greater than that of dry biomass (Blanken et al., 1997), resulting in leaves having a specific heat capacity of 3350 J/(Kg.K), or 80% of that water. Rumen content also contains water and dry matter, and its estimated specific heat capacity also remains lower than that of water. Therefore, assuming a specific heat capacity of 3595.6 J/(Kg.K) for rumen content (c_r) and a rumen volume (m_r) of 65 L could provide a reasonable approximation for understanding reticulorumen temperature dynamics with Richmann's law. The strong association (R^2 = 0.93) observed between actual and predicted temperature drop (Figure 2) indicates the accuracy of model-based estimates of these parameters (m_r and c_r). However, the

estimated parameters may vary among individuals due to several reasons, including but not limited to the dry matter content of the feed, the amount of fluid portion of rumen content, and the body weight of cattle. Moreover, due to the scarcity of relevant previous studies, data from five published studies were included for analysis in the current study; therefore, we cannot make inferences, only speculations in this regard.

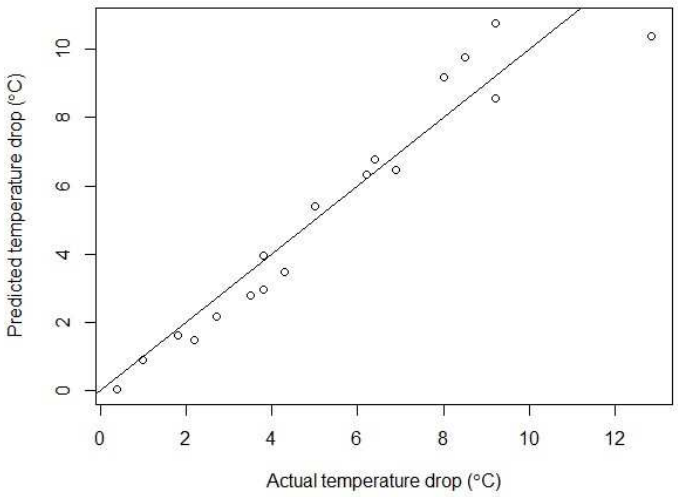

Figure 2: Relationship between actual and predicted temperature drop (^{0}C)
Here, y = 1.0x – 0.21, R^2 = 0.93, Res SS = 15.46

Conclusions

This study applied Richmann's law of mixing fluids to model reticulorumen temperature declines associated with drinking water at varying temperatures. A proportional change in temperature between reticulorumen and consumed water has been revealed. Additionally, the study provided a model-based estimate for the specific heat capacity of rumen content (c_r = 3595.6 ± 177.3 (SE) J/(Kg.K). The observed relationship between predicted and actual rumen temperatures confirms the utility of Richmann's law in modelling temperature dynamics in the reticulorumen. The data provided here also highlights an opportunity to undertake further research to understand the impact of water intake on changes to the rumen ecosystem.

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CHAPTER 4

Identifying and characterising drinking events using reticulorumen temperature of feedlot beef cattle

In Chapter 3, the magnitudes of reticulorumen temperature drop following water consumption have been explored using thermodynamic laws, with literature data providing an opportunity to characterise a full drinking event. **Chapter 4** incorporates reticulorumen temperature data from feedlot steers and employs a drinking algorithm to identify and characterise drinking events. The relationship between reticulorumen temperature drops and the duration of the recovery period is explored, with a continued focus on the impact of water intake on ruminal microbial diversity.

Abstract

Water intake is a significant behaviour in cattle, intricately related to their thermoregulation where abrupt reticulorumen temperature (RT) changes following drinking may impact the reticulorumen environment. Identifying and analysing RT data could provide insights regarding drinking behaviour and physiological reactions to environmental stressors. Previously developed generalised or fixed-threshold-based drinking algorithms provide an opportunity to consider a drinking event detection model featuring dynamic RT fluctuations following water consumption. In this study, we employed such an algorithm to identify drinking events from rumen bolus-driven RT data in 36 feedlot steers (Angus, Charolais, and Brahman; n = 12 per breed) subjected to shaded and unshaded treatments. A total of 13,320 drinking events were identified, with Charolais exhibiting the highest mean daily frequency (4.14 ± 0.40), followed by Brahman (3.27 ± 0.31) and Angus (3.13 ± 0.30). The estimated reductions in RT following drinking ranged from 0.22 °C to 8.6 °C (median: 1.28 °C; mean: 1.56 ± 0.04 °C), with Charolais exhibiting the largest mean decrease. The median recovery duration, defined as the duration required for RT to return to 90% of its initial temperature, was 76.5 minutes (mean: 118.2 minutes), with longer recovery observed in Angus relative to Charolais and Brahman. However, the frequency of drinking, extent of RT drops, and associated recovery duration exhibited considerable variation across months, breeds, and treatments, with a significant three-way interaction (breed × treatment × month; $p < 0.001$). It has also been revealed that, except for minor RT decreases (< 1.5 °C), the duration of the recovery period remains consistent (~120 mins) for greater reticulorumen temperature declines, aligning with Newton's law of cooling/heating. Temporal variability in drinking behaviour across the breeds and the recovery duration provides the basis for investigating its impact on enteric fermentation and microbial diversity.

Introduction

Climate change is anticipated to pose substantial challenges to global ruminant livestock production with the predicted increases in ambient temperatures, altering rainfall patterns, and intensified frequency and severity of extreme weather events (Thornton et al., 2022). Australian beef cattle production systems are predominantly pasture-based, encompassing a wide range of Agro-climatic zones from tropical regions in the northern areas to temperate regions in southern regions, and comprises a variety of tropically-adapted and temperate genotypes and their crossbreeds (Cheruiyot et al., 2020; Greenwood et al., 2018). In line with the global trend, extreme climate events such as frequent heat waves, longer and hotter summers, and their impacts on the cattle system are predicted to increase in Australia over the coming decades (Cowan et al., 2024; Howden et al., 2008). At the same time, cattle systems are becoming a vital part in the broader context of mitigating climate change by reducing enteric emissions of greenhouse gases (GHG) and enhancing carbon sequestration (Sejian et al., 2015; Singh et al., 2017). Therefore, the integration of adaptation and mitigation strategies is crucial for achieving effective climate actions in the livestock sector (Howarth & Robinson, 2024) to ensure maximum production while minimising their environmental footprint. The high-yielding animals are generally under greater metabolic pressure, which subsequently makes them more susceptible to adverse climatic conditions, especially to extremely high ambient temperature, which results in heat stress (Silanikove, 2000; Van Iaer et al., 2014). The primary responses of cattle to hot environmental conditions are to evoke behavioural and physiological responses that are targeted towards maintaining thermal equilibrium, generally at the cost of production such as growth (Brown-Brandl, 2018; Bunning & Wall, 2022; Callegaro et al., 2024). Several methods have been developed to detect animals' responses to extreme weather events to facilitate management decisions (Dunshea et al., 2013; Gaughan et al., 2008); however, their effectiveness is generally limited to the herd level, which warrants the development of a method whereby individual animal variability is able to be considered (Islam et al., 2021).

Drinking is a highly heritable and biologically significant behaviour in cattle (Delagarde & Lamberton, 2015; Dressler et al., 2023; Dutta et al., 2015), which is closely linked to numerous factors, including but not limited to ambient temperature, physiological states, type of breed, season, availability of shade, exposure to heat or cold, availability of water, and water temperature (Arias & Mader, 2011; Brown-Brandl et al., 2005; Cardot et al., 2008; Mishra, 2021; Sexson et al., 2012). Cattle require water for physiological processes associated with maintenance, growth, fattening, pregnancy, and lactation (ARC, 1980), and as such,

deprivation of water for 72 h negatively impacts health, behaviour, and performance (Ahmed & ElHadi, 1996; Cardot et al., 2008; Scharf et al., 2009). Typically, cattle consume between 15 and 172 L of water per day, with over 83% of this requirement fulfilled by drinking, while the remaining is derived from feed and metabolic water (Cardot et al., 2008; Meyer et al., 2004). The daily drinking frequency of cattle ranges from three to 11 events (Coimbra et al., 2010; Lardner et al., 2013), with an average of seven per day (Cardot et al., 2008). However, it has been observed that the frequency of drinking and the volume of water consumed are markedly influenced by high ambient temperature (Arias & Mader, 2011). During periods of heat stress, it is thought that cattle drink more frequently and/or consume more water to support heat dissipation, by providing the water needed to facilitate evaporative cooling and cooling the reticulorumen (Bernabucci et al., 2010; Collier et al., 1982; Valente et al., 2015). Furthermore, cattle drinking behaviour exhibits variability both between and within breeds, with research indicating that *Bos indicus* typically consumes less water than *Bos taurus* (Payne & Hutchison, 1963). This variation is additionally impacted by numerous physiological and environmental factors that influence water consumption in both dairy and beef cattle (Arias & Mader, 2011; Golher et al., 2021). Although water consumption is intermittent and of short duration, drinking activities have been identified as the most influential factor that can drop the RT drastically, with potential impacts on the reticulorumen environment crucial for ruminants' nutrition, health, and production (Ipema et al., 2008; Rutherford et al., 2019). The RT is typically maintained within a range of 38°C to 42°C, with an average temperature of 39 ± 0.5°C, providing an optimal environment for the survival and proper functioning of rumen microbes (Hicks et al., 2001; Yokoyama & Johnson, 1988). Further, RT has become the most prominent method for estimating core body temperature in cattle studies (Lees et al., 2022). However, drinking-associated RT drop impacts negatively on these ruminal attributes, necessitating a deeper understanding of drinking-related RT dynamics. Reticulorumen temperature drops due to drinking in lactating cows have been investigated previously in experimental studies (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964; Dracy & Kurtenbach, 1968), where different magnitudes of RT drops were observed across varying volumes and temperatures of consumed water. Previously, Rahman et al. (2024) applied thermodynamic principles of mixing fluids and investigated these RT drops, describing a proportional relationship between the RT drop and the temperature of intake water. Following a drop in RT due to drinking, a recovery period ensues whereby RT returns to its baseline level. Previous studies have reported different durations of recovery period ranging from 15 to 210 minutes (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964; Serviento et al., 2024),

suggesting a robust method to develop for accurate estimation of recovery period from each drinking event that might facilitate investigating their temporal impact on enteric fermentation and/or thermoregulation of cattle.

Monitoring the reticulorumen environment using a sensor technology (rumen bolus) has gained popularity in precision livestock farming. Commercially available bolus systems can identify and remove drinking events from raw reticulorumen temperature data; however, the underlying algorithms are often not clear, and most of them are developed based on generalised or fixed thresholds (Vázquez-Diosdado et al., 2019). Considering these limitations, Shirley et al. (2025) recently developed a drinking event detection model based on the rapidity of the RT drop within a defined timeframe and identified drinking events across various individuals in the pasture-based dairy herds. In the current study, we applied this model to rumen bolus-derived RT data from feedlot beef steers to identify and characterise drinking events. Specifically, the objective of this study was to investigate the frequency of drinking events, the magnitude of RT drops, and the drinking recovery period across three breeds of cattle, managed under feedlot conditions.

Materials and Methods

A full description of the experimental approach has been reported previously by Lees et al. (2018). Briefly, data for this study were collected at The University of Queensland's research feedlot in Southeast Queensland, Australia (27.54° S, 152.34° E; 100 m above sea level) between October 2012 and April 2013. A total of 36 steers (12 each of Angus, Charolais, and Brahman) with an initial non-fasted body weight of 318.5 ± 6.7 (mean ± SD) kg were enrolled into the study. The study adopted a 2 × 3 factorial design incorporating two treatments: 1) unshaded and 2) shaded, where each pen had six steers, comprising two animals from each breed (2 Angus, 2 Brahman, and 2 Charolais). For the shaded treatment, shade was provided by shade-cloth (black, 90% solar block, Darling Downs Tarpaulins, Toowoomba, Qld, Australia) attached to a 4-m high steel frame, providing a shade footprint of 3.0 m^2/animal at 1200 h. Nutritional management included, twice daily bunk calls (0700 h and 1200 h) using a modified 'clean bunk at midday' methodology (Lawrence, 1998), and fed once daily at 1430 h. Cattle were weighed (non-fasted condition) at 7-day intervals for the duration of the study.

Reticulorumen temperature data

Radio frequency identification (RFID) rumen boluses (Smartstock, Pawnee, OK, USA) were administered orally on day 0 to monitor rumen temperature (RT). Data were transmitted every 10 minutes by a Yagi antenna to a base station and later recorded in a database using proprietary software (TechTrol Inc., Pawnee, OK, USA). Continuous records of RT were collected throughout the study duration.

Data preprocessing

From 36 feedlot beef cattle, 522,239 data points of RT were recorded and utilised for the current study. Initially, raw data were visualised, and it was found that the bolus-driven RT records of the first few days and in some cases several times within the experimental period were physiologically low (< 30 °C), and as such were excluded from this data set. To have a consistent record of RT, initial records of 72 hours (burn-in period) and any records < 30 °C were filtered from the primary dataset. Then, repeated values of RT (duplicate records) that were recorded for a specific period were removed, keeping the first and last observations of that repeated sequence. This cleaned data set was then used to identify drinking events and estimate the duration of the recovery period following each drinking event. The pre-processing steps and their results are summarised in Table 4. 1.

Table 4. 1. Processing of rumen bolus-driven reticulorumen temperature records

Pre-processing steps	Number of initial observations	Observations dropped	Number of final observations
1. Original data length	522,239		
2. Removal of 'burn-in' records and other inconsistent records	522,239	17,655	504,584
3. Removal of consecutive repeated records	504,584	253,948	250,636
4. Final data points	522,239	271,603	250,636

Identifying drinking events

Fluctuations of RT were assumed to occur due to drinking water cooler than the body temperature of cattle. An algorithm, as described by Shirley et al. (2025), was used to detect significant drops in reticulorumen temperature to accommodate variations in water temperature and volume during drinking events. The algorithm identified significant rapid temperature drop

events as drinking events, either a sharp drop within a single 10-minute interval or a more moderate decline sustained over up to three consecutive 10-minute intervals. To detect such drinking events, the algorithm was applied in this study under a different production system to that described by Shirley et al. (2025), i.e. beef vs dairy cattle. Briefly, a drinking event was defined when the rate of temperature decline (RateRT) met any of the following criteria: (i) ≤ – 0.5 °C per 10 minutes over the last time interval or (ii) ≤ – 0.25 °C per 10 minutes over the last time interval and ≤ – 0.5 °C 10 min over the last two time intervals or (iii) ≤ – 0.25 °C per 10 minutes during one and two time intervals and ≤ – 0.5 °C over the last three time intervals. The cumulative temperature decrease (ΔRT) associated with a drinking event was calculated from a maximum of three consecutive 10-minute intervals and transformed into a positive number for analysis. It is important to note that three observations were deemed adequate to encompass the total drop (about 30 minutes), as any effects of ingestion would be apparent within that timeframe, aligning with the empirical rumen temperature plots, Figure 4. 1.

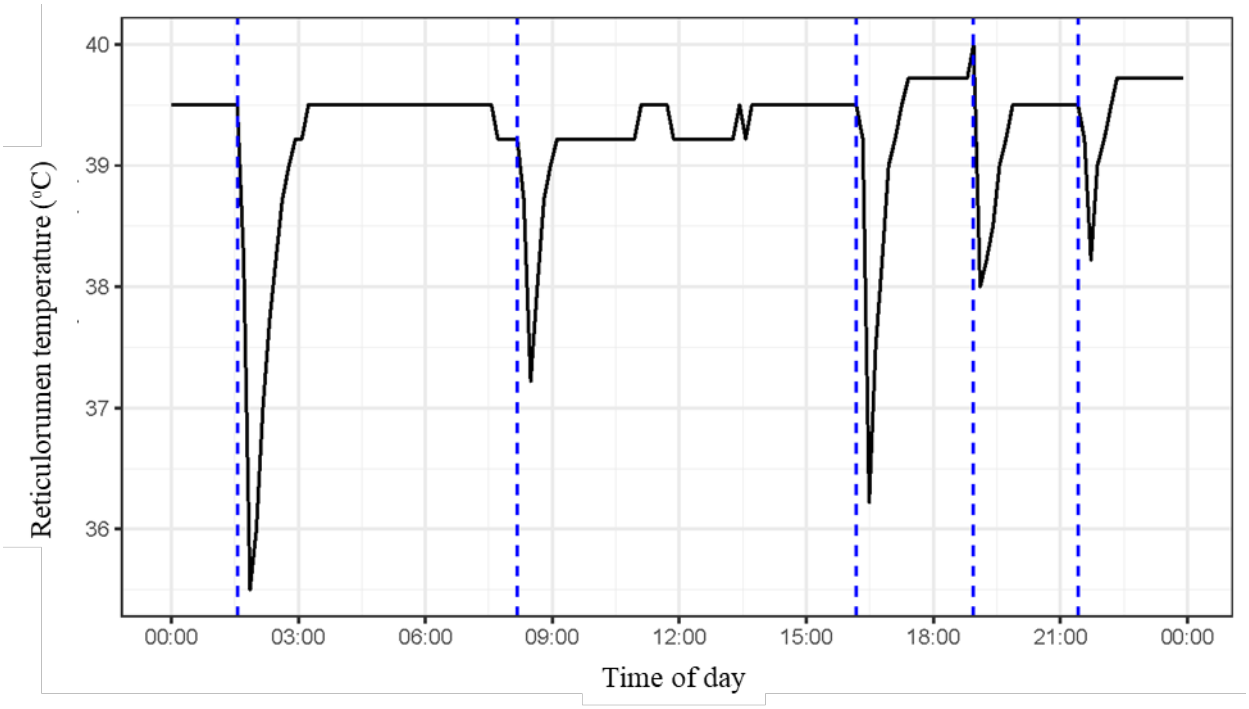

Figure 4. 1. Detection of drinking events based on fluctuation of reticulorumen temperature (ºC), where the blue vertical lines indicate the onset of drinking events, identified based on a sharp decline in RT with a predefined magnitude.

Estimation of the duration of the recovery period

The reticulorumen temperature drop is accompanied by an initial rapid and then gradual return to its pre-drinking level over time. The time required for the recovery of reticulorumen temperature was estimated as the time when the temperature returned to 100*k*% of its pre-

drinking value. For 90% recovery (i.e., $k = 0.9$), the corresponding recovery temperature (T_k) was calculated as:

$$T_k = RT - (1 - k) \times \Delta RT$$

where RT is the initial rumen temperature at the onset of the drop, and ΔRT is the magnitude of the temperature drop. In the analysis, k was set to 0.9, representing 90% return to the initial temperature. The recovery duration $t^{(k)}$ for each drinking event was calculated as the time interval from the onset of the temperature drop to when the rumen temperature returned to 90% of its pre-drinking level, expressed as 'Duration' in minutes. Instances where a new drinking event was detected before recovery were considered as 'censored' and not considered in the analysis.

Statistical analysis

The number of detected drinking events each day by individual animals was analysed to investigate the shade effect as well as any difference between the three breeds of cattle. A generalised linear mixed-effects model (GLMM) was fitted to the identified drinking events data using the *'glmer'* function from the "*lme4*" package (Bates et al., 2015) in R (R, v.4.3.0). The '*anova*' function from the *'car'* package (Fox & Weisberg, 2018) was used to perform Type II Wald chi-square tests to determine the impact of breeds, treatments, time (months), and their interactions on the frequency of drinking events. Fixed effects were breed, treatment, month, and their interaction, and a random effect for the individual animal within the treatments was considered. For the analysis of RT drop, a linear mixed-effects model was fitted to the RT drop data using the '*lmer'* function from the '*lme4*' package in R with the same fixed and random effects considered for the analysis of the number of drinking events. To meet model assumptions, the RT drop data were log-transformed, and the model-based means were then calculated on the back-transformed scale. The time to 90% recovery of the RT drop was analysed using only uncensored individual drinking event data, converting the response variable to log-transformed to meet model assumptions, and the same fixed and random effects were included in the analysis as used in previous mixed models. The relationship between recovery duration and RT drops was visualised in a scatter plot with a log-transformed scale.

Results

Algorithm-driven drinking events

As per the criteria of the RT drop outlined in the drinking algorithm, drinking events are flagged with a blue dashed line as depicted in Figure 1. A total of 13,320 drinking events were identified, comprising 4,957 events from Angus, 4,375 events from Charolais, and 3988 events from Brahman cattle. The highest number of drinking events was identified in unshaded Angus, whereas the lowest number was found in unshaded Brahman. Drinking events of Charolais under shaded and unshaded conditions were similar. A total of 2,143 drinking events were identified from shaded Brahman and 2020 events from shaded Angus cattle.

Frequency of drinking events

The frequency of daily drinking events ranged from 0 to 13, with variations across cattle breeds (Figure 4. 2), and the median number of drinking events per day was 3.00, with a mean value of 3.55. The estimated number of drinking events per day was higher in Charolais (4.14 ± 0.40) compared to Angus (3.13 ± 0.30) and Brahman (3.27 ± 0.31).

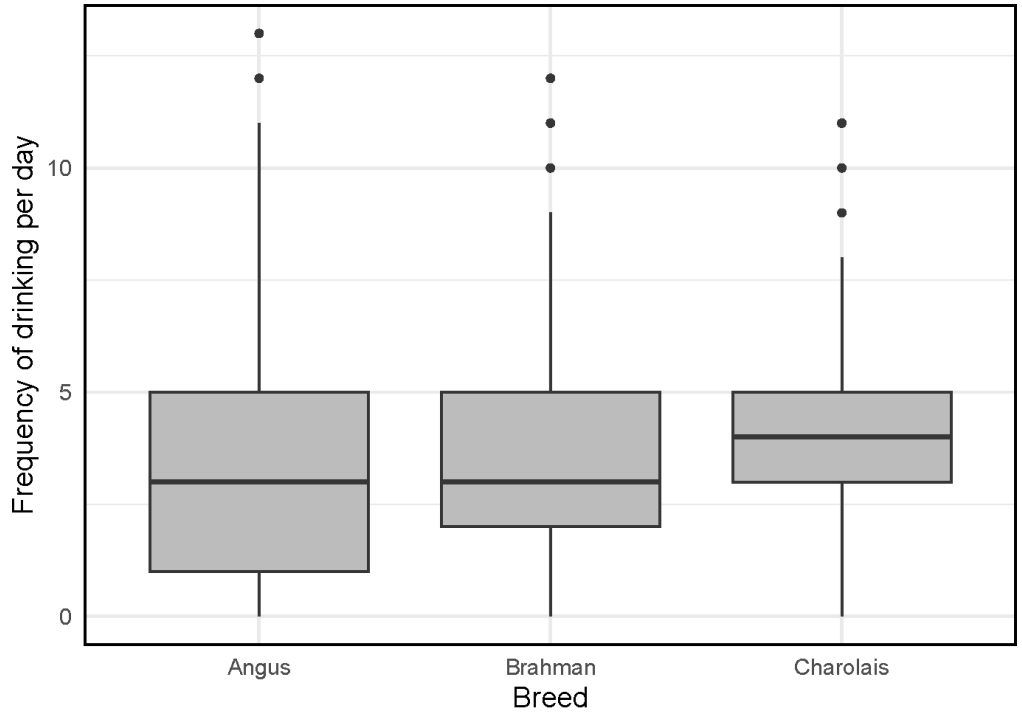

Figure 4. 2. Frequency of daily drinking events across cattle breeds illustrates breed-specific variation in drinking behaviour.

The frequency of drinking events exhibited significant variation across months, breeds, and treatments. The most substantial variations were observed in Charolais during the warmer

months (January-February), as shown in Figure 4. 3. We identified a significant three-way interaction between breed, treatment, and month ($p < 0.001$) for the frequency of daily drinking events; however, treatment alone and breed × treatment interaction were not significant Table 4. 2.

Table 4. 2. Type II Wald chi-square tests of fixed effects for factors affecting the number of drinking events per day.

Factors	χ^2	DF	*p*-value
Month	136.32	5	< 0.001
Breed	7.11	2	0.03
Treatment	0.001	1	0.97
Month × Breed	64.98	10	< 0.001
Month × Treatment	49.59	5	< 0.001
Breed × Treatment	0.44	2	0.80
Month × Breed × Treatment	70.12	10	< 0.001

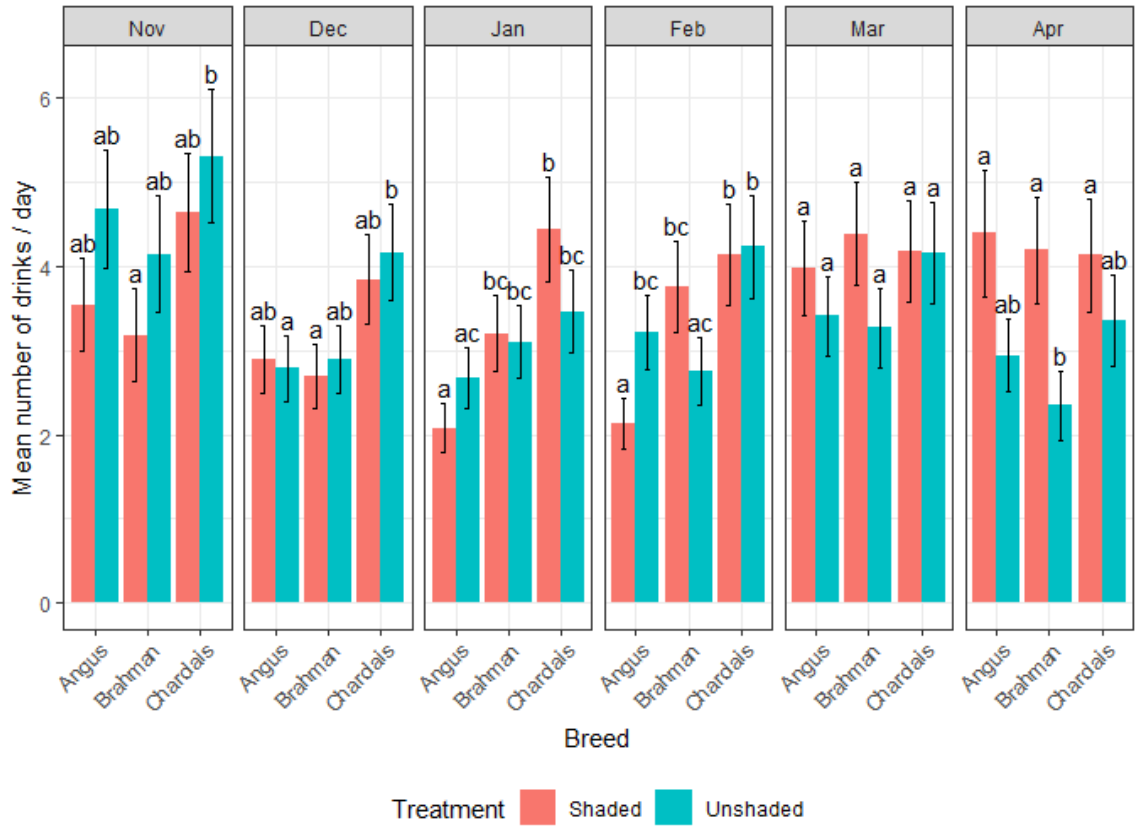

Figure 4. 3. Mean daily number of drinking events over six months for three cattle breeds under two treatment conditions. Bars show mean values with standard error. a, b, c means within each month with a different letter indicate significant differences among breed × treatment combinations ($p < 0.05$).

Reticulorumen temperature drop

The estimated RT drop associated with identified drinking events ranged from 0.22 °C to 8.6 °C, with variations across the breeds shown in Figure 4. 4. The median RT drop with each drinking event was 1.28 °C, with an overall estimated mean of 1.56 ± 0.04°C. The estimated mean RT drop was higher in Charolais (1.46 ± 0.07) compared to Brahman (1.32 ± 0.0.06) and Angus (1.11 ± 0.05). The magnitude of the RT drops following drinking events increased gradually from November to April, with the most significant drop observed in April, particularly in Charolais cattle, as shown in Figure 4. 5.

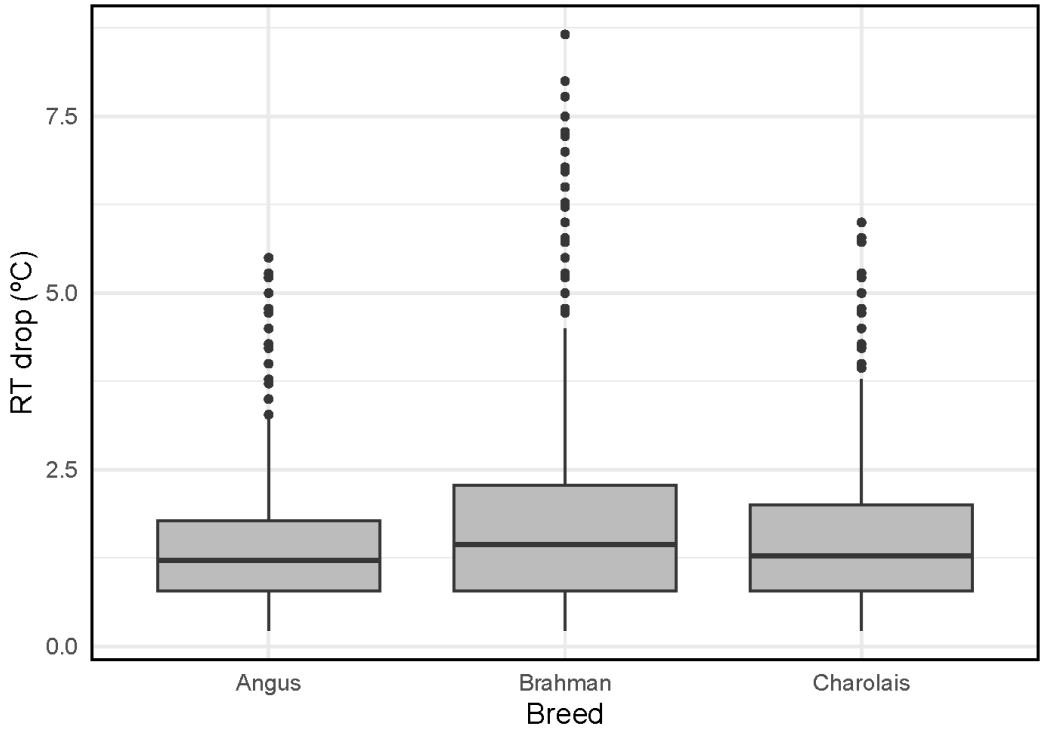

Figure 4. 4. Reticulorumen temperature (RT) drops across three breeds of cattle, illustrating breed-specific variation in RT dynamics.

There was a significant three-way interaction among breed, month, and treatment for RT drop ($p = 0.005$). In addition, significant fixed effects were identified for month and breed and their interaction; however, treatment alone and when accompanied by breed also had no significant impact on RT drop Table 4. 3.

Table 4. 3. Type II Wald F-tests of fixed effects for factors affecting reticulorumen temperature drops

Factors	Num. DF	Den. DF	*F*-value	*p*-value
Month	5	13243.0	261.28	< 0.001
Breed	2	31.3	9.31	0.0007
Treatment	1	31.4	0.0083	0.93
Month × Breed	10	13238.0	17.69	< 0.001
Month × Treatment	5	13243.0	6.75	< 0.001
Breed × Treatment	2	31.3	0.31	0.73
Month × Breed × Treatment	10	13238.0	2.53	0.005

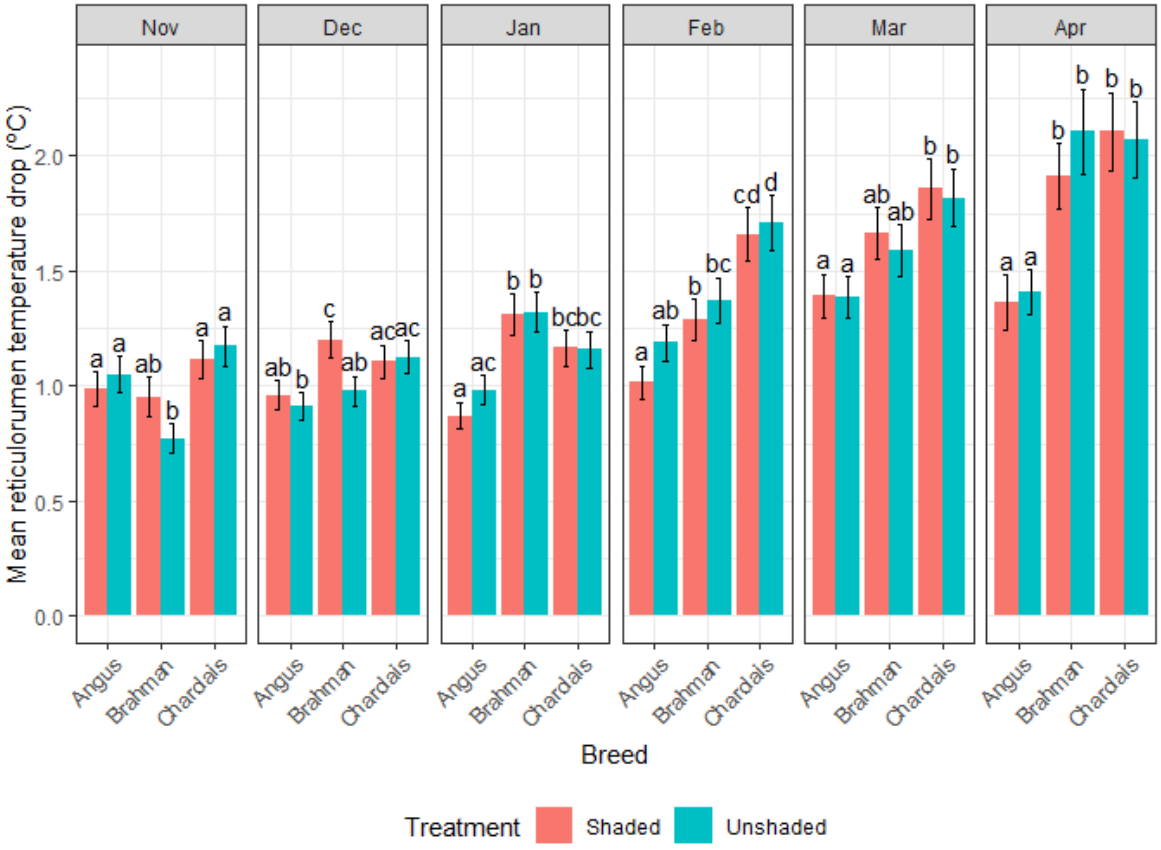

Figure 4. 5. Mean reticulorumen temperature (°C) following drinking events from November to April for three cattle breeds under two treatment conditions. The bar represents the mean ± standard error. Different lowercase letters within each month indicate statistically significant differences among breed treatment combinations (p <0.05).

Recovery period

The cumulative duration of drinking recovery, defined as the time to achieve 90% of the temperature recovery, had a median of 76.5 minutes, with a mean of 118.2 minutes. The

estimated mean of the recovery period was highest in Angus (94 ± 4.24 mins), followed by Charolais (83 ± 3.59 mins) and Brahman (78 ± 3.78 mins). Distribution of recovery period durations (in minutes) following drinking events for Angus, Brahman and Charolais breeds under shaded and unshaded treatments is depicted in Figure 4. 6 and Supplementary Figure 4S. 1, indicating that Brahman remained consistent irrespective of the treatment conditions compared to Angus and Charolais.

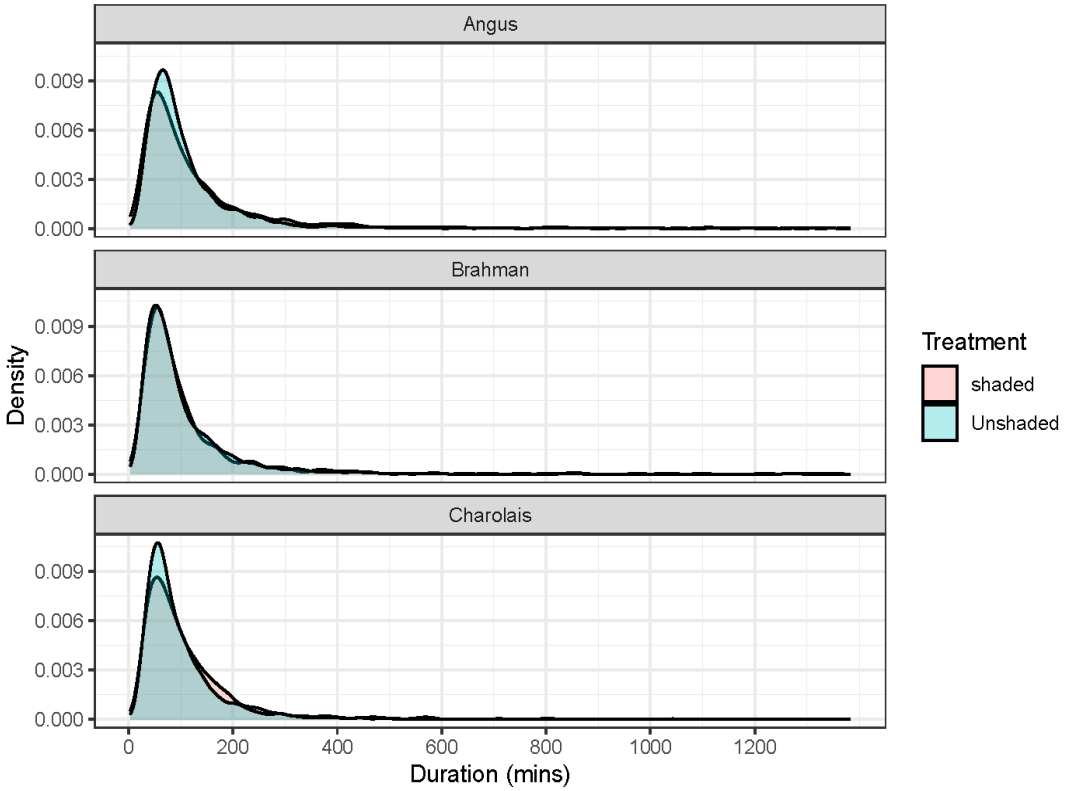

Figure 4. 6. Distribution of recovery period durations (in minutes) following drinking events for Angus, Brahman, and Charolais breeds under shaded and unshaded treatments. Each plot represents an individual breed, along with density curves comparing treatment effects.

The mean recovery period duration following drinking events varied across months, breeds, and treatments. The longest recovery duration period (slower recovery) was observed in February and April, particularly in the shaded Angus breed in February (Figure 4. 7). Type II Wald tests identified a significant three-way interaction among month, breed and treatment for the response variable as shown in Table 4. 4.

Table 4. 4. Type II Wald F-tests of fixed effects for factors affecting the duration of the recovery period.

Factors	Num. DF	Den. DF	*F*-value	*P*-value
Month	5	9418.0	19.82	< 0.001
Breed	2	30.3	4.40	0.02
Treatment	1	30.4	1.13	0.30
Month × Breed	10	9405.6	7.62	< 0.001
Month × Treatment	5	9418.0	5.50	< 0.001
Breed × Treatment	2	30.3	0.008	0.99
Month × Breed × Treatment	10	9405.6	3.41	0.0002

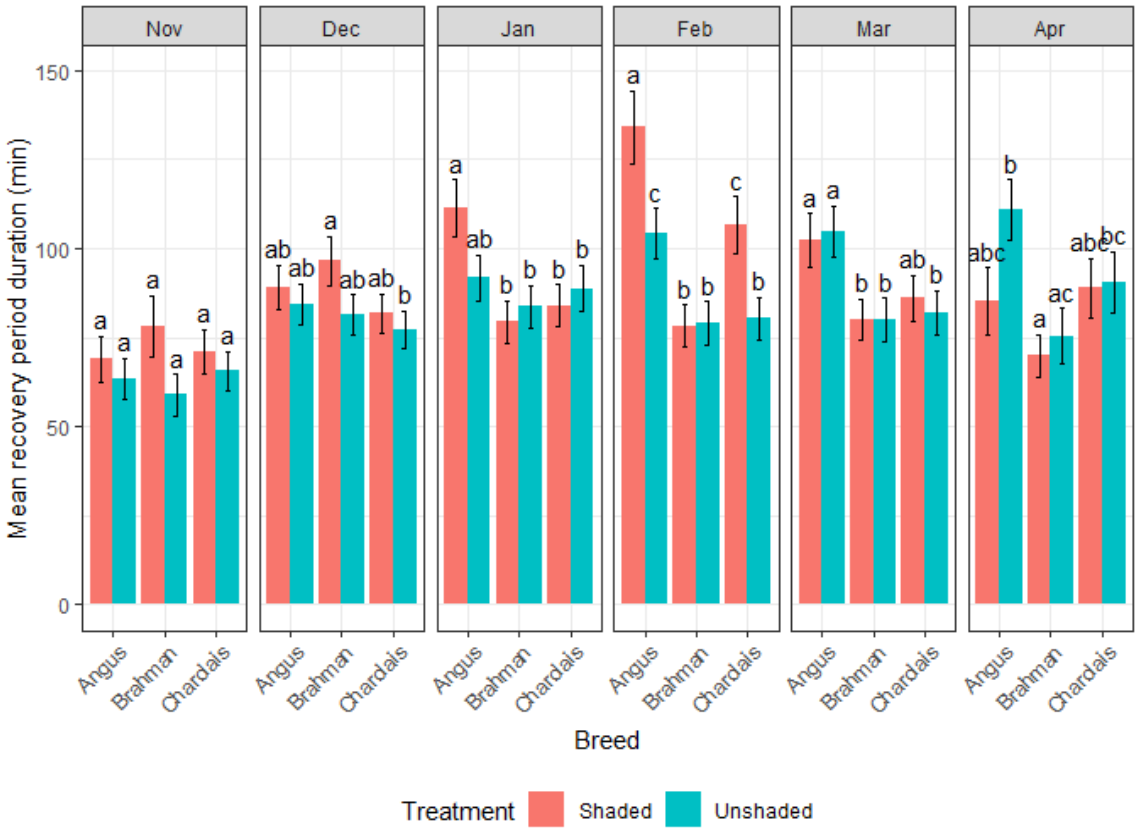

Figure 4. 7. Mean recovery period duration (min) following drinking events, defined as the time required to reach 90% of baseline rumen temperature, across six months for three cattle breeds (Angus, Brahman, and Charolais) under two treatment conditions (Shaded and unshaded). Bars represent mean ± standard error. Different lowercase letters within each month indicate statistically significant differences among breed × treatment combinations ($p < 0.05$) based on post hoc comparisons.

Relationship between reticulorumen temperature drop following drinking and duration of recovery period

The relationship between the magnitude of the reticulorumen temperature drops (ºC) and estimated recovery duration (mins) is depicted in Figure 4. 8. Except for the smaller reticulorumen temperature drops (< 1.5 °C), the duration of the recovery period remains the same for the reticulorumen temperature drops with greater magnitudes.

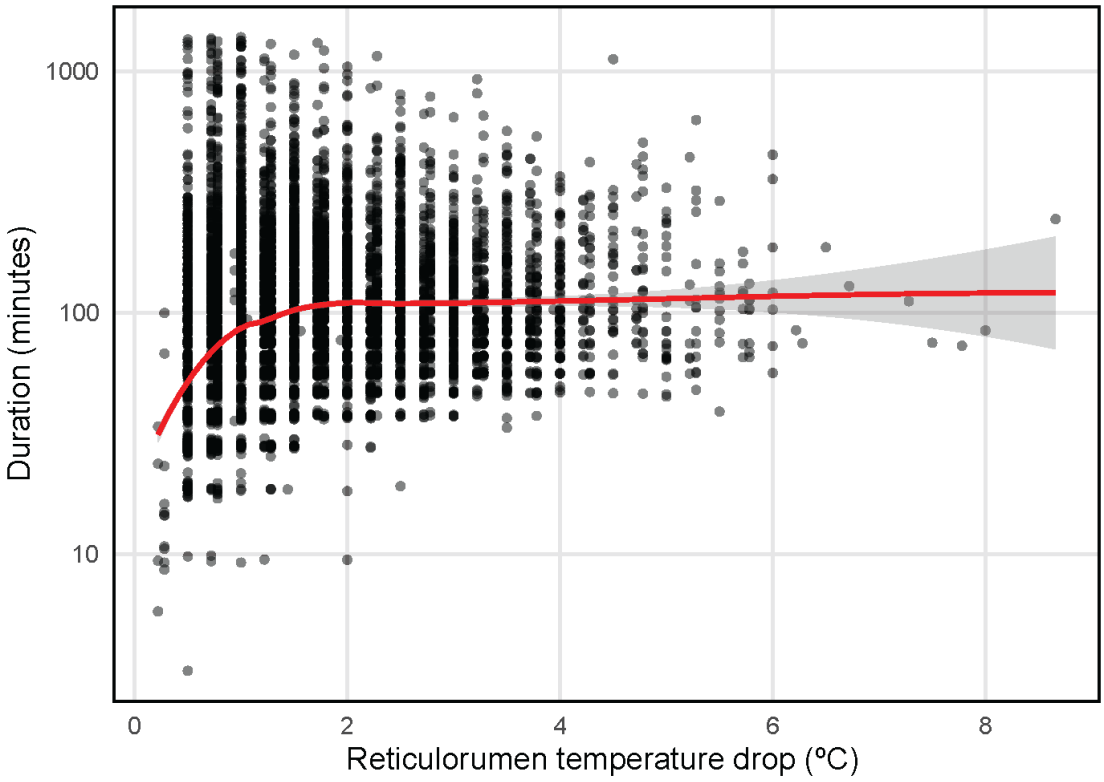

Figure 4. 8. Duration of time to 90% temperature recovery in relation to the reticulorumen temperature drop following drinking events. A logarithmic scale is used on the y-axis to visualise the distribution of recovery duration. The red line represents an empirically estimated smoothing curve for the scatterplot.

Discussion

The drinking event detection algorithm used in this study has previously been validated and applied in lactating dairy cows under pasture-based systems by Shirley et al. (2025). Unlike conventional algorithms that rely on a fixed or generalised baseline reticulorumen temperature threshold, this algorithm emphasises the rate of temperature change over a defined time interval, enabling precise estimation of RT declines following water consumption. Furthermore, by accounting for individual variability in baseline RT, this algorithm facilitates

characterisation of animal-specific thermoregulatory responses based on detected drinking events. Because of this dynamic nature of the algorithm developed by Shirley et al. (2025), it was applied in the current study to identify drinking events using rumen bolus-driven RT of feedlot beef steers and to characterise the frequency of drinking, reticulorumen temperature changes following drinking, and the duration of time needed for reticulorumen temperature to recover post drinking.

The behavioural patterns of water consumption were evaluated for 36 feedlot cattle comprising three breeds: Angus, Charolais, and Brahman, provided with shaded and unshaded pens over six months, using this drinking event detection algorithm. In line with the variation among breeds, the average number of drinking events per day across Charolais, Angus, and Brahman were 4.14, 3.13, and 3.27 events, respectively. Variations in the number of drinking events per day reported in the literature ranged from an average of four to seven for growing *Bos taurus* beef cattle, with a range between three and 11 drinks per day (Coimbra et al., 2010; Lardner et al., 2013), while an average of 2.6 times per day has been reported for *Bos indicus* steers in tropical climate (Lampkin & Quarterman, 1962). Whilst the mean number of drinking events per day in the Taurine and Indicine breeds in our study aligns with the number reported in the literature, between-animal variations in Taurine cattle have also been observed. Although water intake is primarily influenced by dry matter intake (McDowell, 1967), cattle drinking behaviour is also linked to heat tolerance capacity with *Bos indicus* cattle being well-documented as more heat-tolerant than *Bos taurus* cattle (Hansen, 2004). Moreover, cattle with greater heat tolerance possess lower metabolic rates and demonstrate higher feed efficiency (Ingram & Mount, 2012), which might explain why Zebu (indicine) cattle consume less volume of water than taurine cattle under the same environmental conditions (Payne & Hutchison, 1963). In the current study, we didn't have records of water temperature or the volume of water consumed by cattle, which makes it challenging to explain drinking behaviour, specifically the frequency of drinking and the associated RT drops. Comparatively higher frequencies (numerically) of drinking events in unshaded Charolais cattle in November, December, and February indicate their greater heat susceptibility compared to the Brahman and Angus cattle under the same environmental conditions. However, the significant three-way interaction among month, breed, and treatment suggests that the effect of shade on drinking behaviour varied across months and breeds. It has been reported in previous studies that one of the main reasons behind the variation in drinking frequency in cattle is the amount of water consumed in each drinking episode (Cardot et al., 2008), accompanied by factors such as

ambient temperature, water temperature, and dry matter intake (Golher et al., 2014; Horrocks & Phillips, 1961; NRC, 1996). Studies have found that cattle consume water at approximately three times the amount of dry matter intake, indicating adequate water intake is crucial for maintaining feed intake (Ahlberg et al., 2018; Alves et al., 2017). Conversely, water deprivation can lead to sudden changes in weight gain and overall health and well-being of cattle (Scharf et al., 2008; Williams et al., 2017). Monitoring drinking frequency may aid in selecting animals with better adaptive capability and higher feed efficiency. Future studies should focus on accommodating all physiological, environmental (across the seasons), and nutritional aspects of feed that influence the drinking behaviour of cattle to explore their potential for incorporation in an efficient cattle production system. The difference in frequency of drinking between Brahman and Charolais may be due to their inherent response to climatic conditions (Arias & Mader, 2011; Scheffler, 2022). However, the Charolais and Angus data presented here suggest that the between-animal variation within the same breed type needs attention. Within-breed variation may also present a pathway to explore the connection between heat tolerance and the physiological and behavioural drivers around drinking events.

The average drop in RT following each drinking event in Charolais, Angus, and Brahman were 1.46 °C, 1.11 °C, and 1.32 °C, respectively. The highest mean magnitude of RT drop was observed in unshaded Brahman (2.1 ± 0.18 °C) and shaded Charolais (2.1 ± 0.016 °C) in April, while the lowest was observed in shaded Angus (0.87 ± 0.06 °C) in January. The overall average decline in RT of 1.56 ± 0.04 °C was identified in this study. This decline in RT is slightly lower than the median drop of 1.68 °C observed by Serviento et al. (2024); however, it falls within the range of 0.47 °C to 4.11 °C reported by Cantor et al. (2018) under variable volumes and water temperature. The current study was conducted under feedlot conditions where cattle had *ad libitum* access to linear open water troughs and feed bunks, where it is anticipated that water temperature was influenced by ambient environmental temperature. Previous experimental studies have shown that cattle drenched with different volumes of water at different temperatures resulted in different magnitudes of reticulorumen temperature drop, confirming that the extent of the RT drop is regulated by the volume and temperature of water drenched (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964). Specifically, Bewley et al. (2008) observed a maximum drop of 9.2 °C and 0.4 °C when they drenched cows with cold water at 5.1 °C (18.9 kg) and body temperature water at 38.9 °C (18.9 kg), while Welsh et al. (2025) observed a similar trend of RT drop with the volume of water in sheep. The coldest water temperature at the highest drenched quantity resulted in a greater temperature

drop in that experimental study compared to the present study. However, RT drops from drenching body temperature water were lower than that of the current study. The RT drop observed by Serviento et al. (2024) and Cantor et al. (2018) was closer to the current study because water temperature and quantity in their studies were not controlled as in the current study. The variations in ambient temperature between the studies might be the reason behind the slight difference in the magnitude of RT drop. Similar to the highest frequency of drinking events, the greatest RT drop was also observed in Charolais cattle, and a similar trend was observed for Angus and Brahman in our study, which is opposite to the trend observed by Shirley et al. (2025) in pasture-based dairy cattle. However, the highest drinking frequency with the greatest RT drop in Charolais indicates that they might consume a larger volume of water in each drinking episode. An *ad libitum* supply of water with unrestricted access might influence their water consumption. In addition, the magnitude of the RT drops gradually increased from February to April as the season shifted from summer to Autumn, resulting in cooler water. However, significant three-way interactions among breed, month, and treatment on reticulorumen temperature drops indicate the changes in RT due to drinking across breeds and shade treatments depend on the month of the year. These findings demonstrate the significance of considering seasonal and breed-specific aspects when evaluating changes in RT in feedlot cattle. Although drinking events' associated RT drops align with thermodynamic principles (Welsh et al., 2025), the composition of reticulorumen content and its specific heat capacity, volume (size) of rumen, physiological status, ambient conditions, and frequency of water intake need to be considered in future studies.

Every drinking event is associated with a certain degree of RT drop, followed by a gradual recovery to the pre-drinking level. Previous studies with controlled water intake and temperature have reported wide variations in RT recovery periods following drinking, ranging from as short as 20 minutes to over three hours in dairy cows (Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964; Serviento et al., 2024; Vázquez-Diosdado et al., 2019). The variations in these findings may be due to the differences in the methods used to record drinking events and the definition of the recovery period. To the best of our knowledge, the drinking recovery period in feedlot beef cattle of any specific breed has not been reported yet, which made it difficult for us to make an accurate comparison. This study, identified an average drinking-recovery time of 118.2 min, based on the time to 90 % temperature recovery from the start of the drinking events. The 90% recovery threshold was applied based on the thermodynamic characteristics of temperature recovery following drinking. Specifically,

temperature increases rapidly during the initial phase following reaching the lowest mixing temperature, whereas beyond a certain point, the rate of recovery slows, and a longer duration is required to return to baseline levels. Thus, the time required to reach 90% temperature recovery after drinking reflects the period during which the cooling effect is likely to persist within the reticulorumen. This duration closely aligns with the minimum two-hour recovery period reported in studies conducted under controlled conditions (Bewley et al., 2008; Boehmer et al., 2009) but is notably longer than findings from field-based studies in dairy cattle, ranging between 29.98 and 35.55 minutes (Vázquez-Diosdado et al., 2019). Even, it is slightly longer than the average recovery duration of 97.73 minutes estimated by Shirley et al. (2025) in pasture-based dairy cows. This difference might be due to the breed and physiological state of the animals. This study identified the longest drinking recovery period in Angus, followed by Charolais and Brahman, which was opposite to the direction of RT drop, except for Brahman, which took the shortest time for temperature recovery. This suggests that the differences in RT between *Bos taurus* and *Bos indicus* cattle, mediated through differences in patterns of drinking events (behaviour), might be associated with the differences in thermoregulation between the breeds. The significant three-way interaction between month, breed, and treatment also suggests that the impact of shade on recovery duration varied depending on both the time of the year and breed. As the volume and temperature of consumed water were not monitored during the study, we cannot confirm the variability observed between individuals; however, Brahman cattle remained consistent under both shaded and unshaded conditions compared to Angus and Charolais. This suggests a hypothesis to be tested in future studies, as heat-exposed (unshaded) cattle will have more density to the left (Figure 4. 6) with a smaller number of drinking events. We also revealed a relationship between the magnitude of RT drop and duration of the recovery period (Figure 8), which explains that, except for smaller RT drops (<1.5 °C), the duration of the recovery period (time to 90% of temperature recovery) remains relatively constant at around 120 minutes. This is consistent with Newton's law of cooling/heating, which describes that the time of gradual/exponential temperature recovery does not depend on the magnitude of the temperature drop but on the 'decay' rate parameter that determines how fast the temperature will return to its baseline. Dracy et al. (1963) reported that, irrespective of the amount of water consumed or the extent of the temperature drop, reticulorumen temperatures generally returned to normal within 60 minutes. However, the difference between the normal and post-drinking was as much as 1.2°C after 60 minutes in their study. The trend of recovery duration from the current study aligns with this trend, with differences in the length of duration, and it could be worth investigating the factors associated

with the temperature recovery rate parameter, incorporating thermodynamic principles in future studies.

Drinking is a highly heritable and biologically significant behaviour of cattle (Dressler et al., 2023; Dutta et al., 2015), providing cattle with ample water is as beneficial as providing high-quality forages (Utley et al., 1970). As drinking is associated with dramatic changes in RT, and is influenced by ambient temperature, it is important to understand its dynamics across breeds and seasons to identify resilient individuals. Maintaining a stable reticulorumen environment is crucial for efficient microbial fermentation, which might be disrupted by frequent RT drops, and a longer recovery period warrants further investigation. Although the variability of water intake behaviour between Zebu and Taurine cattle is well known, the extent of variation in terms of frequency of drinking, rate of recovery, and duration of recovery in different production settings needs to be investigated by incorporating computer vision technology.

Conclusion

This study develops a foundation for an advanced understanding of beef cattle drinking event identification, characterisation, and behavioural orientation. The findings of this study open a new avenue to explore the nexus between the ambient environment, drinking behaviour, RT fluctuations, and duration of drinking recovery period. Specifically, revealing the relationship between RT drop and recovery duration provides us with an opportunity to explore the impact of drinking-associated RT dynamics on enteric fermentation and microbial diversity in future studies.

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CHAPTER 5: PUBLISHED MANUSCRIPT

In vitro simulation of drinking events in cattle

Based on the thermodynamic features of a drinking event from Chapters 3 and 4, this Chapter 5 develops a novel method to simulate temperature fluctuations that mimic drinking events outside the reticulorumen. This method provides opportunities to investigate the impact of temperature fluctuations in the reticulorumen following drinking on fermentation characteristics and microbial community structure in an *in vitro* setting. This chapter was published as a manuscript in the *MethodsX* journal in August 2025. (https://doi.org/10.1016/j.mex.2025.103593).

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In vitro simulation of drinking events in cattle☆,☆☆

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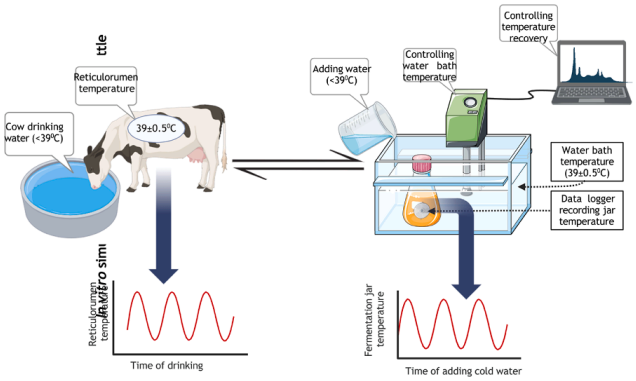
GRAPHICAL ABSTRACT



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ABSTRACT

Drinking causes a rapid decline in reticulorumen temperature (RT) followed by an exponential recovery, which may potentially impact the reticulorumen ecosystem. However, the nexus between drinking events and their effects on ruminal fermentation and microbial diversity has not yet been studied, either *in vitro* or *in vivo*. Although artificial (*in vitro*) rumen systems are widely

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used in ruminant research to simulate the reticulorumen environment, no such simulation has been described to consider the impact of drinking events on the reticulorumen environment. Therefore, we have developed a method for the *in vitro* simulation of drinking events in the fermentation jar where the jar temperature was considered a proxy for RT is reduced by adding a measured amount of cold water to the water bath, and the subsequent recovery period is achieved following a temperature profile regulated by a heating immersion circulator. This method enables the replication of RT fluctuations from drinking events, allowing for the monitoring of their impact on fermentation characteristics and microbial ecology in future research. The features of this method are:

Creation of a hypothetical drinking event
Estimation of volume and temperature of cold water for a drinking event
Establishing a temperature profile to regulate the recovery period

Specifications table

Subject area	Agricultural and Biological Sciences
More specific subject area	*In vitro* study of the reticulorumen environment of cattle
Name of your method	*In vitro* simulation of drinking events in cattle
Name and reference of original method	*None*
Resource availability	Water bath (food quality stainless steel), Heating immersion circulator, Cold water, Temperature data logger, Computer, Fermentation jar (Glass jar, 250 ml)

Background

The reticulorumen is a critical and complex microbial habitat that enables ruminant animals to efficiently digest complex carbohydrates, synthesise proteins, and convert fibrous plant materials into valuable nutrients and energy [1,2]. The temperature within the reticulorumen environment is tightly regulated between 38 °C and 42 °C, with an average temperature of 39 ± 0.5 °C, providing an optimal environment for the billions of microbes for their survival and proper functioning [3–6]. Temperature deviations outside these normal ranges, either lower or higher, have been observed to decrease the adhesion of microbes to the rumen solids (fibrous substances) [7]. It has been reported in several studies that drinking can cause significant reticulorumen temperature (RT) fluctuations, ranging from 0.4 °C to 12.8 °C, with subsequent recovery periods of 15 min to 120 min [8–13]. However, information regarding the impact of RT fluctuations due to drinking on feed digestibility, fermentation characteristics, and microbial diversity is limited. *In vitro* models of rumen fermentation have been developed to simulate the reticulorumen environment and have been extensively used in ruminant research, particularly in the fields of ruminant microbiology and nutrition. These models include continuous systems [14, 15], which maintain a constant inflow of buffer and substrate along with a continuous outflow to mimic the steady-state conditions of the rumen; semi-continuous systems [16], which feature a continuous inflow of buffer, intermittent substrate feeding, and continuous outflow to simulate the periodic feeding patterns of ruminants; and batch systems [17,18], which have no inflow or outflow, with substrate and buffer added at the beginning and the system sealed for the duration of the experiment, making them suitable for short-term studies. These models have been instrumental in advancing our understanding of rumen fermentation dynamics over several decades. Among them, the Rumen Simulation Technique from a semi-continuous system and the ANKOM system from a batch fermentation system have been recommended to simulate rumen conditions and reticulorumen microbiome modelling [19]. However, simulating a reticulorumen environment, considering the effect of drinking events and their impact on microbial ecology, has not yet been studied in either of the *in vitro* systems. Compared to the semi-continuous system, the batch system is simple, easy to operate, relatively fast, and produces accurate results [19,20]. This system utilises fermentation jars that require minimal substrate and inoculum for fermentation and are placed in a water bath or equipped with a heating jacket to maintain a consistent temperature, *i.e.*, incubation temperature, of 39 ± 0.5 °C, simulating reticulorumen conditions [21,22]. The objective of this study was to develop an *in vitro* method to simulate drinking events in the fermentation jar and determine a protocol to establish the impact of RT declines and recovery periods. The establishment of such a protocol would allow for the impact of RT fluctuations due to drinking on feed digestibility, fermentation characteristics, and microbial diversity to be evaluated.

Method details

An experiment was conducted using a water bath, cold water, and a fermentation jar at The University of Sydney's Dairy Science laboratory (J.L. Shute Building, Camden campus, New South Wales, Australia), to create a drinking event and its associated temperature dynamics. The method of simulating drinking events with associated temperature drops and recovery periods was based on two basic thermodynamic principles: Richmann's Law of Mixtures [23] and Newton's Law of Cooling/ Heating [24]. According to Richmann's law, when two volumes of fluids at different initial temperatures are combined, they exchange heat until a thermal

equilibrium is established [25]. On the other hand, Newton's Law of Cooling/Heating states that the rate of change of temperature of an object is directly proportional to the temperature difference between the object and its surroundings [26]. In cattle, RT is maintained approximately at 39.0 ± 0.5 °C. When cattle consume water below 39.0 °C, RT decreases as heat is exchanged between the water consumed and rumen content, then gradually returns to pre-drinking temperature over a certain time. In this study, RT dropping and its recovery were achieved in the fermentation jar by reducing the water bath temperature by adding cold water. These changes in temperature over time in the water bath and fermentation jar can be explained by Newton's Law of Cooling/Heating. Simulating drinking events and their associated RT dynamics in an *in vitro* set-up was done as described below.

Creation of a hypothetical drinking event

Based on the concept of drinking and the thermodynamics of water, a hypothetical graph of a drinking event was created. This hypothetical drinking event considered a temperature drop of 9 °C from the baseline temperature of 39 °C and a recovery period (considered 99 % recovery of the dropped temperature) of 120 min, which aligned with the published literature mentioned earlier (Fig. 1).

Equipment assembly

List of necessary materials and equipment for simulating drinking events:

- Food-grade stainless steel water bath (20 L)
- Heating immersion circulator
- Temperature data logger
- Data logger reader
- Cold water (≤4 °C)
- Plastic bucket
- Plastic tray
- Over sink rack
- Plastic mug/beaker
- Fermentation jar (250 mL)

Assemble the heating immersion circulator with a water bath and run the device

The heating immersion circulator is designed for temperature control applications with liquid media in a water bath. This facilitates temperature control within the water bath using a predefined temperature regime. A commercially available CORIO CD heating immersion circulator (JULABO Technology, Germany https://www.julabo.com) with a working temperature range from +20 °C to +150 °C, temperature stability ± 0.03 °C, and temperature resolution 0.01 °C was used. A 20 L food-grade deep stainless-steel tray with dimensions of 53 cm × 32.5 cm × 15 cm (length × width × depth) was used as a water bath in this study. The heating circulator was fitted on the top edge of the water bath with a universal clamp, which was then connected to a socket and the computer with a

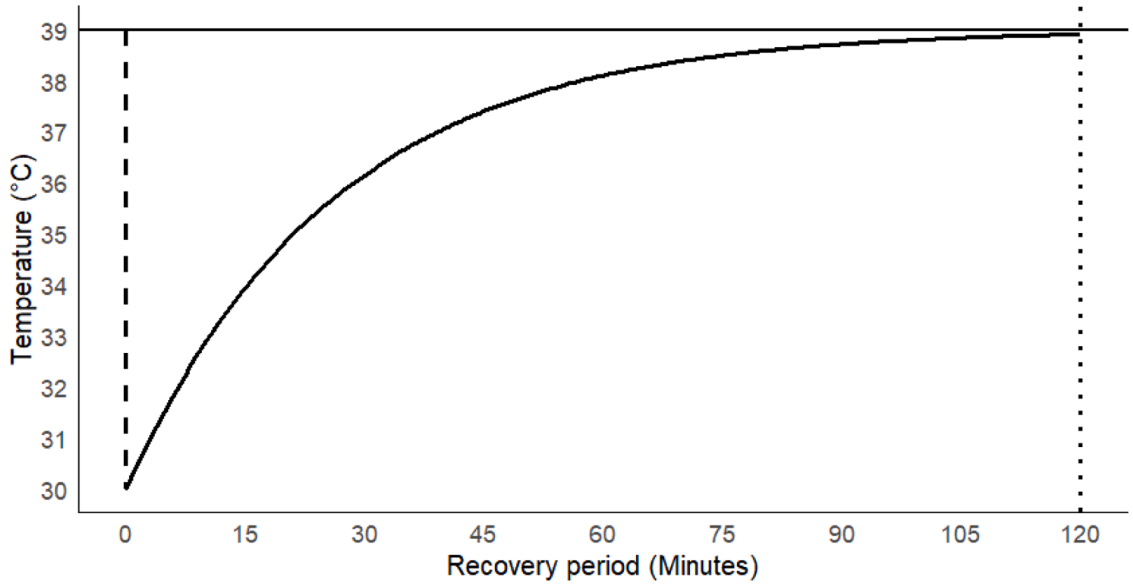

Fig. 1. Hypothetical graph of a drinking event with the initial temperature drops of 9 °C (dashed vertical line) from the baseline temperature of 39 °C (solid horizontal line) and recovery period of 120 min (vertical dotted line).

provided cord and USB cable, respectively. According to the manufacturer's specifications and operating instructions, the pump and heater of the heating immersion circulator must always be covered entirely with bath fluid/water to operate this heating device. The current assembly requires 12.5 L of water to cover them. This assembly was used as media to simulate the drinking events with associated temperature drops and recovery periods in 250 mL fermentation jars (considered reticulorumen), generally used in the *in vitro* batch fermentation system.

Placement of the fermentation jar and the temperature data logger

A temperature data logger was used to record the temperature inside the fermentation jar (iButton temperature logger, Thermochron eXtreme, range: −30 °C to 85 °C, resolution: 0.5 °C to 0.0625 °C, https://thermochron.com.au/). The activation time and the resolution of the data logger for temperature data logging were programmed using eTemperature software (https://etemperature.com.au) before placing it in the fermentation jar. After programming the data logger, it was placed into the fingertip of a disposable nitrile glove (Nitrile Gloves Large LC N338PF-L-LC MicroAnalytix Pty Ltd., Taren Point, NSW 2229, Australia) and tightened by making a knot for waterproofing. Then, it was placed inside the fermentation jar containing 100 mL of 39 °C water. The jars were then sealed with an ANKOM RF module and positioned in a water bath, held in place by an over-sink rack to prevent them from moving during simulating a drinking event by adding cold water to the water bath (Fig. 2).

Estimating the volume and temperature of water required for creating a drinking event with a temperature drop of 9 °C

A drinking event is characterised by its associated temperature drop in RT and subsequent recovery period. For creating an *in vitro* drinking event to achieve a desired temperature drop of 9 °C, an estimate of water volume across temperatures is required. Richmann's law of mixtures was applied to estimate the desired temperature drops in the water bath and in the fermentation jars from the baseline temperature of 39 °C. Richmann's law calculates the final temperature of a mixture (T_f) when two liquids of masses m_1 and m_2 at temperatures T_1 and T_2 are mixed, taking into account their respective heat capacity coefficients c_1 and c_2 represented as

$$T_f = \frac{m_1 c_1 T_1 + m_2 c_2 T_2}{m_1 c_1 + m_2 c_2} \quad (1)$$

For example, mixing 1.0 L of water (m_1) at 39.5 °C (T_1) with 2.0 L of water (m_2) at 4 °C (T_2) results in the final temperature of the mixture (T_f) at 15.8 °C. This is because of the mixture of two volumes of water whose specific heat capacity is 4.18 J/g °C.

This method aimed to reduce the fermentation jar temperature by around 9 °C using cold water; however, the batch fermentation system does not allow any inflow or outflow within the jar. For this reason, cold water was introduced into the water bath to force a lower temperature in the fermentation jar. Despite the fermentation jar being situated within the water bath, the temperature decrease in the water bath from the baseline temperature (39 °C) does not equal the reduction observed in the fermentation jar (less than the drops in the water bath) (Fig. 3). This could be attributable to the density difference between water (1.0 g/cm^3) and the fermentation jar (silica glass jar, 2.20 g/cm^3) [27], as well as the heat transfer mechanism between two media. To resolve this issue, multiple simulation attempts have been conducted to determine the minimum temperature reduction in the water bath that will simultaneously generate around 9 °C decreases inside the fermentation jar. Considering the capacity of the water bath (20 L) used in our experiments,

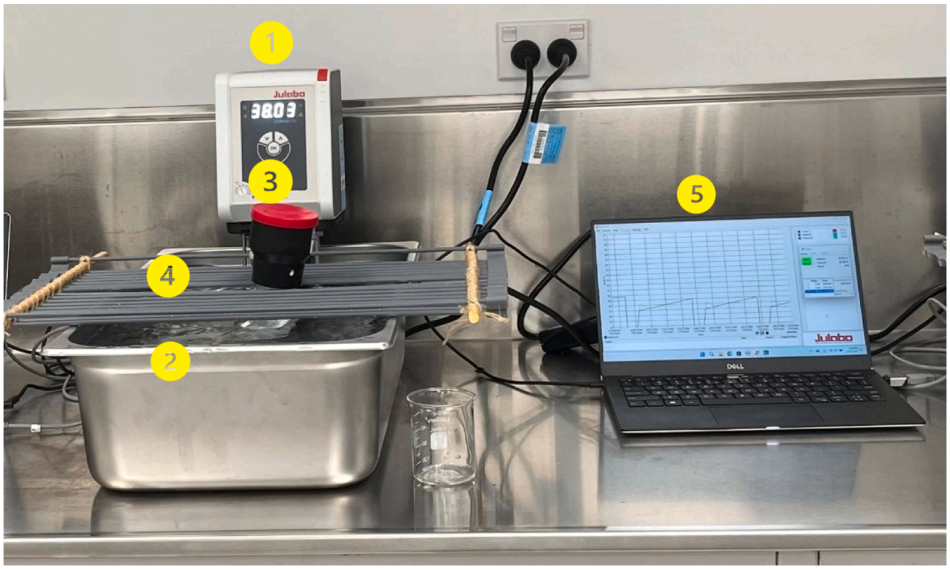

Fig. 2. The assembly of a drinking event simulating unit consists of a heating immersion circulator[1] mounted on a stainless-steel water bath[2], a fermentation jar sealed with an ANKOM module[3] holding a data logger inside, an over-sink rack[4] positioned across the water bath to hold the jar in place securely, and a data acquisition system comprising a laptop computer[5] connected to the circulator.

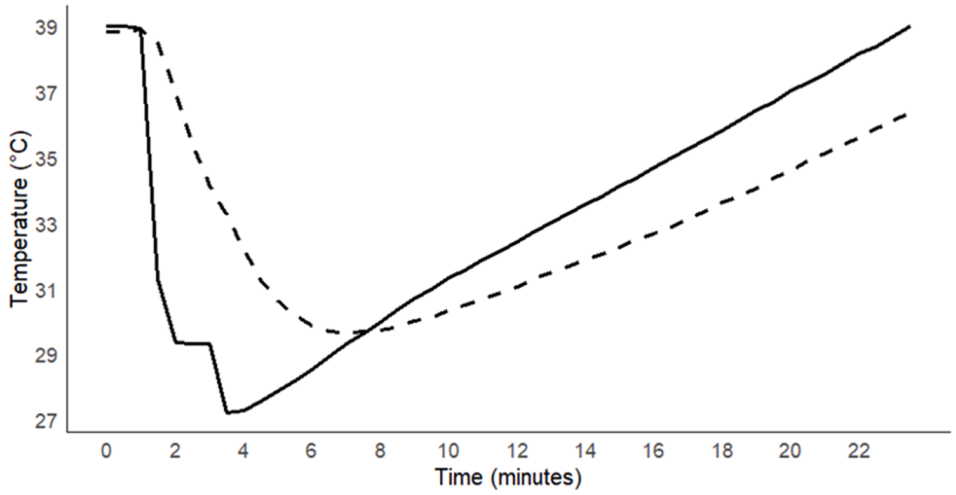

Fig. 3. The observed temperature drops in the water bath (solid line) and the fermentation jar (dashed line) after adding cold water.

4.0 °C water was used to achieve the desired temperature reduction. To determine the estimated temperature reduction in the water bath, Richmann's Law of Mixtures was applied. As outlined previously, 12.5 L (m_1) of normal water must be added first to start the heating device, which regulates an increase in water bath temperature and maintains it at 39 °C (T_1). The estimated temperature drops in the water bath from the estimated final temperature (T_f) of the mixture (39 °C and 4 °C water) following the addition of different volumes of cold water (m_2) at 4 °C (T_2) are detailed in Table 1:

Following these estimated temperature drops in the water bath, several trials were undertaken with different volumes of water from 1.0 L to 7.0 L of 4 °C water to ensure that the desired temperature decline in the fermentation jar could be achieved. During these trials, data loggers were maintained inside the fermentation jar, per the description provided previously. It was found that the addition of 7.0 L of 4 °C water reduced the water bath temperature by approximately 12 °C while simultaneously reducing the fermentation jar temperature by around 9 °C (Fig. 3).

The water bath, having a capacity of 20 L, was initially filled with 12.5 L of water at 39 °C, to which 7.0 L of water at 4 °C was added in two steps to reduce the temperature of the water bath. Around 4.5 L of cold water was added to the water bath at the first step and waited until the water bath temperature reached equilibrium. At this point, the fermentation jar was temporarily transferred to a rectangular plastic tray, and subsequently, around 3–4 L of water from the water bath was shifted to this plastic tray to make room for adding the remaining cold water and maintaining the fermentation jar temperature during this transition time. Immediately after the water removal, the fermentation jar was returned to the water bath, and the remaining cold water was introduced. This stepwise addition of cold water slightly interrupted the decline in the water bath temperature (Fig. 3).

Creating a recovery period for a drinking event

After reaching the RT to its lowest level, the time required to achieve within ± 0.10 °C of the pre-event level or 90 % of the dropped temperature is termed the recovery period [10]. After drinking, upon reaching the minima, RT increases exponentially toward the pre-drinking temperature. In this method, we used a software-programmed heating immersion circulator to regulate the recovery period from the minima to the initial baseline temperature in the water bath and fermentation jar (39 °C). Using this software, a temperature profile considering the initial rapid and gradual return of the water bath and fermentation jar temperature was created.

Table 1
Temperature drop in the water bath estimated by Richmann's Law.

The volume of water m_1 (L)	Water bath temp T_1 (°C)	Sp. heat capacity of water c_1 (J/g °C)	The volume of cold water m_2 (L)	Temp of cold water T_2 (°C)	Sp. heat capacity of water c_2 (J/g °C)	Final temperature in the water bath (T_f) (°C)	Temperature drops in the water bath (T_1-T_f) (°C)
12.5	39.0	4.2	1	4	4.2	36.41	2.59
12.5	39.0	4.2	2	4	4.2	34.17	4.83
12.5	39.0	4.2	3	4	4.2	32.22	6.78
12.5	39.0	4.2	4	4	4.2	30.52	8.48
12.5	39.0	4.2	4.5	4	4.2	29.73	9.27
12.5	39.0	4.2	5	4	4.2	29.00	10.00
12.5	39.0	4.2	6	4	4.2	27.65	11.35
12.5	39.0	4.2	7	4	4.2	26.43	12.57

*m_1 = The volume of water in the water bath, T_1 = Baseline temperature in the water bath, c_1 = Specific heat capacity of water, m_2 = The volume of added cold water, T_2 = Temp of the added cold water, c_2 = Specific heat capacity of cold water, T_f = Estimated final temperature in the water bath, T_1-T_f = Estimated temperature drops in the water bath.

This profile was set to achieve the recovery period in 2.0 h following the 9 °C drop in the fermentation jar (Table 2).

This temperature profile became activated when the water bath temperature reached 27 ± 0.01 °C due to the addition of 7.0 L of 4.0 °C water. Once the profile is activated, the water bath temperature is set to be reached at 36 °C from 27 °C in 30 min, then from 36.0 °C to 38.0 °C in another 30 min, and finally, it returned to the initial 39.0 °C from 38.0 °C in 1.0 hour. This completes a single drinking event episode with a 9.0 °C drop in the fermentation jar and a 120-minute recovery period (Fig. 4).

Method validation

Following the criteria of the hypothetical drinking event, an event with a temperature drop of around 9 °C and a subsequent recovery period of 120 mins was simulated in the fermentation jar. However, it was observed that it took several minutes (6–7 mins) for the jar temperature to be minimal (lagged response of the jar) compared to the time required for the hypothetical event and the event created in the water bath following the addition of cold water (Fig. 1; Fig. 3). Further, the temperature change in the jar across the recovery period was consistent with the temperature change in the water bath until reaching the pre-event level temperature at 39 °C (Fig. 5). To explore the association between changes in the bath temperature and changes in the jar temperature, Newton's Law of Cooling/Heating was used. The water bath temperature was considered as input, and the fermentation jar temperature as output. Let $b(t)$ be a function describing the temperature of the bath at time t, and let $j(t)$ be the corresponding temperature of the jar within the bath at time t. Then according to Newton's Law of Cooling/Heating, the rate of change of the jar temperature is proportional to the difference in temperature between the jar and the surrounding bath water, *i.e.*

$$\frac{dj(t)}{dt} = -k_j[j(t) - b(t)] \tag{2}$$

where k_j is a (positive) rate constant. Assuming the initial jar temperature, $j(0)$, is j_0, for an arbitrary input bath temperature function, $b(t)$, the solution to this differential equation using the 'integrating factor' method is

$$j(t) = \exp\left(-k_j t\right)\left[j_0 + k_j \int_0^t b(u)\exp\left(k_j u\right)du\right] \tag{3}$$

While the form of $b(t)$ may be known in some situations, in the present study $b(t)$ was under experimental control, so the above solution requires numerical methods. The bath temperature data set consisted of observations every 3 s, and to create a continuous function, linear interpolation was applied between consecutive time points using the 'approxfun' function in R, and numerical integration was undertaken using the integrate function in R.

The single parameter in the current model, k_j, was estimated by least squares by choosing the value of k_j to minimise

$$\mathrm{RSS} = \sum_{i=1}^{n} (j_i - j(t_i))^2 \tag{4}$$

where j_i is the recorded (observed) jar temperature at time t_i, $j(t_i)$ is the model-based predicted jar temperature, and n is the number of observations. The observed *versus* predicted temperature of the fermentation jar was assessed using the Root Mean Squared Error (RMSE), where

$$\mathrm{RMSE} = \sqrt{\mathrm{RSS}/\mathrm{n}} \tag{5}$$

For the data set displayed in Fig. 5, k_j was estimated as 0.33, resulting in an RMSE of 0.22 °C.

It is anticipated that the magnitude of this temperature drop will be influenced by the volume and temperature of the cold water added to the water bath. Based on the maximum reticulorumen temperature drops following the drenching of cold water reported in published *in vivo* studies, we replicated this temperature drop of around 9 °C within the fermentation jars. This relatively small RMSE indicates the temperature time course in the jar follows what is predicted. This method effectively simulated a full drinking episode (Fig. 4). By adjusting the iteration number in the heating device software, multiple drinking events can be simulated using the protocol established here. Although our *in vitro* method is developed based on *in vivo* observations of reticulorumen temperature fluctuations following drinking, further validation under controlled *in vivo* experiments could help to address external and internal factors influencing RT drop and recovery and improve its applicability on a broader scale.

Table 2
Target water bath temperatures and the corresponding time intervals for achieving each temperature step.

Water bath Temperature to be reached at (deg C)	Time set for reaching the next step (hh:mm:ss)
27.00	00:00:00
36.00	00:30:00
38.00	00:30:00
39.00	01:00:00

*This table was created following the template outlined in JULABO EasyTemp software.

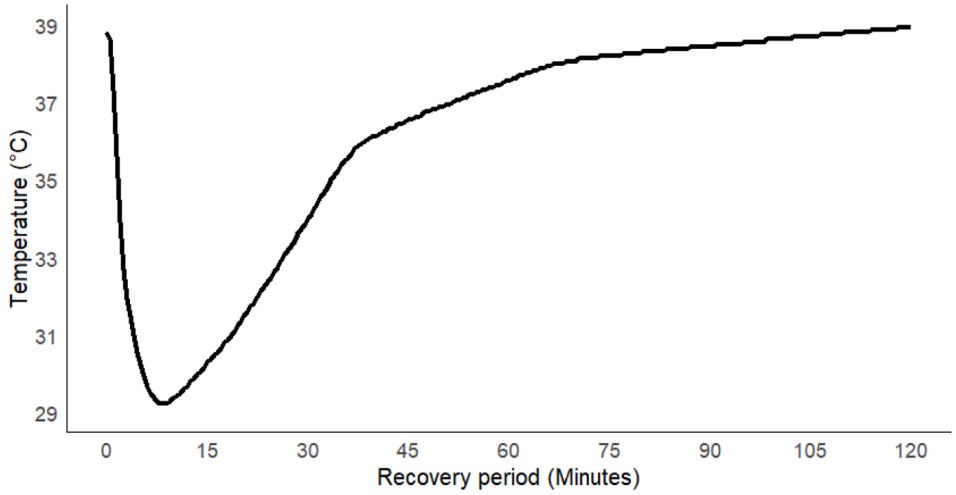

Fig. 4. A single drinking event with a temperature drop of around 9 °C and a recovery period of about 120 min.

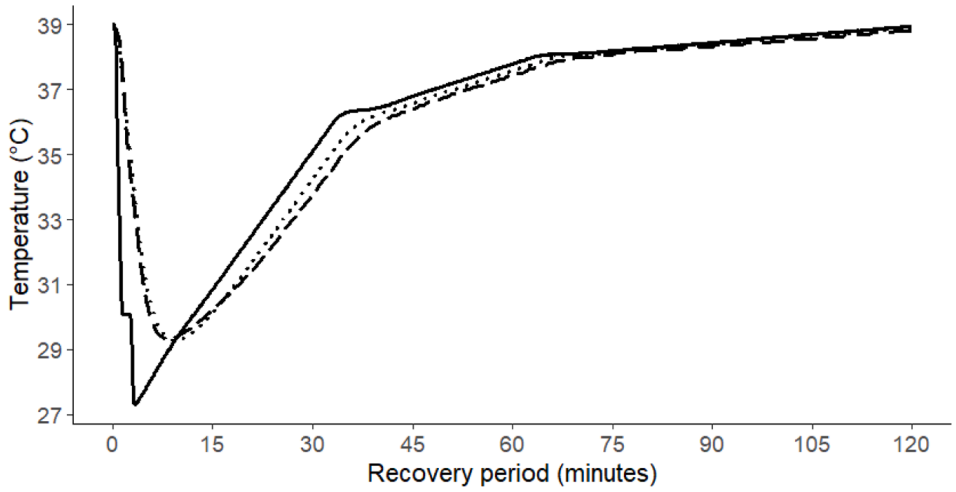

Fig. 5. Comparison of observed (dashed line) and predicted (dotted line) jar temperatures based on water bath temperatures (solid line), as predicted by the heat transfer model.

Limitations

- Water baths <14 cm in depth are unsuitable for this method.
- If water is not sufficiently cold (>4 °C), the volume of water needs to be readjusted to achieve the desired temperature drop
- This is an indirect way of mimicking temperature fluctuations inside the fermentation jar, as no cold water is added to the jar
- This method is suitable for *in vitro* batch-type fermentation, so it incurs the limitation of batch fermentation

Ethics statements

Not applicable

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the author(s) did not use any AI and AI-assisted technologies.

CRediT authorship contribution statement

Md Shaheenur Rahman: Methodology, Investigation, Validation, Writing – original draft, Data curation, Visualization. **Anna Chlingaryan:** Methodology, Supervision, Writing – review & editing. **Peter C. Thomson:** Software, Data curation, Visualization, Formal analysis, Writing – review & editing. **Mohammed Rafiq Islam:** Writing – review & editing, Methodology, Supervision. **Angela M. Lees:** Methodology, Writing – review & editing. **Pablo Gregorini:** Methodology, Writing – review & editing. **Fabiellen Cristina**

Pereira: Methodology, Writing – review & editing. **Cameron E.F. Clark:** Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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Temperature control in the water bath and fermentation jar across multiple trials is provided in the appendix of this thesis, in Supplementary Table 5.1 on page 177.

CHAPTER 6

Fermentation characteristics, gas production kinetics, and microbial diversity in response to drinking: an *in vitro* study

Chapter 6 builds upon the *in vitro* method developed in Chapter 5 to simulate drinking events in cattle. This chapter explores the potential impact of drinking frequency and associated drops in reticulorumen temperature on fermentation characteristics and microbial diversity in cattle. The results of this *in vitro* study highlight the relevance of drinking behaviour as a trait of interest for future *in vivo* research.

Abstract

While prolonged exposure to low incubation temperatures compromises rumen fermentation, the short-term effects of RT variability induced by drinking remain unexplored. This experiment determines the impact of controlled, transient temperature fluctuations representing simulated drinking events on fermentation parameters, gas production kinetics, and microbial community diversity across two *in vitro* experiments. The studies were undertaken using different substrates: a kale and silage mixture in Experiment 1 and ryegrass in Experiment 2. The treatments included a control (T1: constant 39 °C), 12 drinking-induced RT fluctuation events (T3) in both experiments, and intermediate frequencies (T2: two events in Experiment 1; six in Experiment 2), with each event lowering temperature by ~ 9.5 °C, followed by a 2-hour recovery period during the 26-hour fermentation. In Experiment 1, frequent drinking events (T3) significantly increased total gas production ($p < 0.001$), while dry matter degradability and major VFAs were unaffected except for iso-butyrate ($p = 0.01$). Whereas in Experiment 2, total gas and CH_4 production remained unchanged, but NH_4 and N_2O concentrations were significantly higher in T1 and lowest in T3 ($p < 0.05$), with propionic and hexanoic acids increasing under T3 ($p < 0.05$). Across both experiments, gas-kinetic analysis consistently showed that frequent RT drop events (T3) reduced the fractional rate constant (c) while increasing the theoretical asymptote ($a + b$), indicating slower initial fermentation but greater cumulative substrate degradation. Bacterial and archaeal α-diversity (Shannon and Simpson index) remained largely stable across treatments, except for significant shifts in archaeal genus-level diversity ($p < 0.05$). However, in Experiment 2, microbial communities exhibited stronger substrate-dependent responses: bacterial phylum-level diversity declined under T3 ($p < 0.01$), genus-level diversity remained stable, and species-level diversity increased in T2 and T3 compared to T1 ($p < 0.001$). β-diversity analyses (Bray–Curtis and Aitchison) showed no treatment-related shifts in community composition, with only one genus (Streptococcus) was found differentially abundant in Experiment 1. In contrast, Experiment 2 exhibited clear treatment-based clustering of bacterial and archaeal communities, and differential abundance analysis revealed extensive restructuring, with 38 bacterial genera and 59 bacterial species differing between T3 and T1. Correlation analysis revealed treatment-specific shifts in associations between microbial phyla and fermentation parameters, with frequent cooling strengthening positive correlations between certain phyla and VFAs or methane in both experiments. Overall, RT fluctuations on mixed substrate exhibited minimal disruption, whereas ryegrass substrate showed pronounced microbial and functional shifts. These findings suggest that short-term RT fluctuation events, analogous to natural drinking, alter fermentation kinetics and microbial community structure in a substrate-dependent manner, particularly affecting taxa within the Bacillota lineage and *Methanobrevibacter species.* Maintaining thermal stability within the rumen may promote microbial resilience, optimise fermentation efficiency, and enhance overall ruminant performance. As such, this study highlights the potential of incorporating drinking behaviour as a trait in *in vivo* research aimed at selecting animals with greater resilience.

Introduction

Ruminants host a diverse community of anaerobic microorganisms, which form a symbiotic relationship with the host and extract nutrients from fibrous plant materials through enteric fermentation (Ben Shabat et al., 2016; Gruninger et al., 2019; Van Soest, 1994). This enteric fermentation is a complex process in which rumen microbes (bacteria, protozoa and fungi) degrade and convert ingested lignocellulosic components into volatile fatty acids (VFAs), ammonia (NH_3), carbon dioxide (CO_2), and hydrogen (H_2), supplying the majority of ruminants' energy, via VFAs, and between 50 and 90% of protein requirements, through microbial protein synthesis (Kebreab et al., 2009; Li et al., 2016; Makanya et al., 2020). At the same time, a group of archaea known as methanogens utilise H_2 and carbon substrates, mainly CO_2, acetate, or methanol, to produce methane (CH_4), reducing the hydrogen pressure in the rumen in a process known as methanogenesis (Carberry et al., 2014; Hedderich & Whitman, 2013). This fermentative process causes a significant energy loss (2–15%) relative to the dietary gross energy intake (Johnson et al., 1990; VanNevel & Demeyer, 1996), that could be saved and redirected for the performance and growth of the animal, instead of being released to the atmosphere in the form of CH_4 (Carberry et al., 2014).

The efficiency and stability of rumen fermentation depend on a tightly regulated rumen environment, including factors such as temperature, pH, feed composition, and microbial community (Castillo-González et al., 2014; Linville et al., 2017; Öztürk & Gur, 2021) while any changes in the physicochemical properties of the rumen environment due to intrinsic or extrinsic factors can induce changes in the rumen fermentation patterns (Russell & Hespell, 1981). In fact, the integrated interactions between the host, rumen microbiome, and the external environment mutually contribute to animal performance. Recent studies in ruminant nutrition have established that the rumen microbial community composition is intricately associated with economically and environmentally important traits such as feed efficiency (Ben Shabat et al., 2016; Sasson et al., 2017) and methane emission (Difford et al., 2018; Kittelmann et al., 2014), with evidence indicating that efficient cattle produce 24 to 28% less methane (L/kg of BW) compared to inefficient ones (Hegarty et al., 2007; Jones et al., 2011; Shabat et al., 2016). Efficiency has been further associated with a less diverse rumen microbiome, which produces large amounts of fermentation products utilised for energy and protein synthesis (Shabat et al., 2016). Given that the microbial profile of an individual is under host genetic control (Li et al., 2019; Wallace et al., 2019), this could be used as a potential tool for selecting cattle with high efficiency, along with a favourable carbon footprint. Consequently, there is growing interest in

exploring factors that influence microbial community structure and resulting fermentation characteristics.

Feed composition impacts the ruminal microbial community structure and fermentation characteristics, with shifts from roughage-based to concentrate-based diets modifying bacterial diversity and proportions of VFAs (Carberry et al., 2014; De La Torre et al., 2019; Stanton et al., 2020). Heat stress has been shown to alter rumen fermentation by reducing fibrolytic species such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* and increasing lactate accumulation (Kang et al., 2019; Sales et al., 2021; Yadav et al., 2013; Zhao et al., 2019). Conversely, low ambient temperatures are associated with decreased microbial diversity and these shifts favouring cold-adapted species (Cani et al., 2019; Serviento et al., 2024). Very low ambient temperature can also impact RT. In muskoxen (*Ovibos moschatus*), RT can decrease to as low as 31.4 °C during winter (Crater & Barboza, 2007), whereas in sheep subjected to simulated cold conditions, Lachnospiraceae increased and Prevotellaceae decreased (Guo et al., 2021), indicating temperature-dependent modulation of rumen microbiota and function.

Effective fibre degradation in the rumen depends on cellulolytic microbes attaching firmly to plant particles, enabling their enzymes to hydrolyse structural carbohydrates (McAllister et al., 1994; McAllister & Cheng, 1996). This attachment is sensitive to temperature, with maximum adhesion observed at 38 °C (Roger et al., 1990). Deviations from this optimal temperature sharply reduce adhesion of fibre digesting microbial (especially *Fibrobacter succinogenes)* cells, reflecting its reliance on outer membrane and enzyme-associated proteins for cellulose binding, which are susceptible to thermal disruption (Petersen et al., 2016; Qi et al., 2007; Wang & McAllister, 2002). The consumption of water by cattle and sheep through drinking have been shown to induce dramatic reductions in RT of between 5 and 10 °C, depending on the volume and temperature of water consumed (Arias & Mader, 2011; Bewley et al., 2008; Cantor et al., 2018; Cunningham et al., 1964). It has also been evident that drinking behaviour varies between breeds and between individuals (Ahlberg et al., 2019; Arias & Mader, 2011; Wagner & Engle, 2021); however, information regarding the impact of drinking associated RT drops on microbial community structure and fermentation is scarce.

Providing heated drinking water during cold seasons has been shown to increase water intake in both dairy cows and beef cattle compared to ambient-temperature water, and this rise in water consumption is strongly associated with greater feed intake (Osborne et al., 2002; Petersen et al., 2016). Beyond increasing feed and water consumption, heated water helps

maintain optimal rumen temperature, which stabilises microbial attachment and activity and this thermal stability supports fibrolytic enzyme function, enhances VFA production, and promotes efficient fermentation pathways (He et al., 2023; Roger et al., 1990). While providing beef cattle with heated water, closer to rumen temperature, has been shown to improve performance compared to room-temperature water during the cold season (Grossi et al., 2021; Khonkhaeng & Cherdthong, 2019), the impact of short-term temperature changes due to drinking remains unknown. Evidence from *in vitro* systems, such as RUSITEC, has shown sustained reductions in incubation temperature to negatively affect microbial ecology, fibre digestibility, and methane production (Duarte et al., 2017). According to Petersen et al. (2016), the *in vitro* neutral detergent fibre (NDF) disappearance decreased from 41% to 14% when the incubation temperature was reduced from 39 °C to 31°C for 48 hours. However, these studies have primarily focused on prolonged exposure to low incubation temperatures rather than the acute, transient temperature fluctuations experienced by cattle during drinking. To the best of the authors' knowledge, no research has specifically investigated how such short-term drops in reticulorumen temperature influence fermentation dynamics and the associated ruminal microbial community. Therefore, the objective of this study is to evaluate the effects of controlled, short-term incubation temperature reductions through simulated drinking events on fermentation characteristics, methane production, gas production kinetics, and microbial diversity in an *in vitro* setting. In this study, RT fluctuations were simulated in an *in vitro* setting following the method developed by Rahman et al. (2025). This method enabled the simulation of RT fluctuations associated with drinking events, providing an opportunity to investigate the objectives of this study. Since the current study applied the concept of RT fluctuations following drinking, the term “drinking events” is used interchangeably with RT fluctuation events in this chapter. We considered an extreme level of RT drop (~9.5 °C) and the frequency of drinking events (2 – 12/day) aligned previously published studies (Bewley et al., 2008; Cantor et al., 2018).

Materials and Methods

All animal handling protocols were approved by the Lincoln University Animal Ethics Committee (AEC2024-11 and AEC2024-52). Two *in vitro* experiments were conducted at the Agriculture and Life Sciences (AGLS) laboratory, Lincoln University, New Zealand, to achieve the objectives of this study.

Experimental Design and Treatments

Experiment 1.

For experiment 1, *in vitro* fermentation was conducted using the ANKOM RF gas production system (ANKOM Technology Corp., Fairport, NY) with the RF1 gas production module and the method for *in vitro* simulation of drinking events in cattle developed by Rahman et al. (2025). A 3×3 Latin square design was employed to investigate the effects of frequency of incubation temperature fluctuations through simulated drinking events on fermentation characteristics, gas production kinetics, and rumen microbial diversity. The experiment consisted of three treatments applied across three water baths in three separate runs. Treatment 1 (T1) was the control, with no drinking events, and maintained a constant temperature of 39°C (incubation temperature) over a 26-hour incubation period. Treatment 2 (T2) applied two drinking events 12 hours apart, causing ~9.5 °C drops in each event, followed by a 120-minute recovery period for each event. Treatment 3 (T3) applied 12 drinking events, two hours apart, causing ~9.5 °C drops in each event, with the same recovery period for each drinking event over a 26-hour incubation period. The water baths served as the experimental units for treatment application, with each water bath containing four 250 mL glass fermentation jars; thus, there were two fermentation jars per cow, representing two replications per cow per water bath. As such, a total of 12 jars (2 × 2 per water bath × 3 water baths) were used in each *in vitro* run.

Experiment 2.

In Experiment 2, the impact of incubation temperature fluctuations on fermentation characteristics, gas production kinetics, and microbial diversity was investigated using an *in vitro* assembly similar to that in Experiment 1, with the RF1 gas production modules replaced by RF1X modules. This time, a randomised complete block design (RCBD) was implemented, in which treatment 2 (T2) consisted of six drinking events spaced at four-hour intervals. Each event imposed ~ 9.5 °C temperature reduction, followed by a 120-minute recovery period, across two separate *in vitro* fermentation runs. However, treatment (T1) and treatment 3 (T3) applied in Experiment 2 were the same as T1 and T3 used in Experiment 1. Furthermore, within each water bath, two fermentation jars containing substrate, buffer, and inoculum (described below) were used for fermentation, while two fermentation jars served as blanks (without

substrate). Jar allocation to treatments was randomised within each run, and fermentation was carried out for 26 hours.

Preparation of substrate and inoculum

In Experiment 1, the substrate was composed of a 70:30 (w/w) mixture of kale forage (*Brassica oleracea*) and corn silage (*Zea mays*), as the donor cows were grazing kale forage at Ashley Dene Research and Development Station (Lincoln University, Springston,New Zealand) and were supplemented with corn silage during the period of the experiment. The composition of the substrate for Experiment 1 was estimated as the weighted average of its content. During the period of Experiment 2, as cows were grazed solely on ryegrass, the substrate was composed of 100% ryegrass. Fresh kale forage, corn silage and ryegrass were collected from the farm, freeze-dried, and then ground separately by a centrifugal mill (ZM200; Retsch) to pass through a 1-mm screen. The prepared substrates were weighed into ANKOM Filter Bags (F57, porosity 25 μm) to a total mass of 0.5 g of substrate (0.35 g of kale & 0.15 g of corn silage, on a dry matter basis) in each bag. In the case of Experiment 2, 0.5 g of ryegrass was weighed into F57 bags. Two bags together comprising 1.0 g of substrate (DM basis) were used in each fermentation jar for fermentation. The chemical composition of kale, silage and ryegrass was determined using near-infrared spectroscopy (Model: FOSS NIRS system 5000, FOSS). The chemical composition of substrates used in both experiments is presented in Table 6. 1.

Table 6. 1. Chemical attributes of substrates used in 26-h fermentation with ANKOM RF Gas Production Systems

	Experiment 1	Experiment 2
Content (% Dry Matter)	Substrate (Kale & Silage)	Substrate (Ryegrass)
Dry Matter (DM)	88.96	91.42
Organic Matter (OM)	90.38	89.68
Carbohydrate (CHO)	26.92	19.32
Acid Detergent Fibre (ADF)	20.09	23.68
Neutral Detergent Fibre (NDF)	33.54	39.77
Protein	17.77	18.04

In Experiment 1, around 200 mL of rumen fluid (inoculum) was collected early in the morning (around 8:00 am) from non-lactating, rumen-canulated Holstein Friesian cows (n = 2) grazing

in the paddock at Lincoln University's Ashley Dene Farm. Rumen fluid was collected by taking rumen digesta from different locations within the rumen of cows and gently squeezed into a warmed (39.5 °C) thermos flask, and purged with CO_2 to maintain anaerobic conditions (Garrett et al., 2021). Considering the influence of host genetics on the rumen microbiome community (Difford et al., 2018), rumen fluids from two cows were kept separately in two flasks and used accordingly while preparing the fermentation jar for incubation. In Experiment 2, rumen fluid was collected from two lactating Holstein Friesian cows immediately after morning milking (around 8:00 am) using the same procedure as in Experiment 1. However, rumen fluid from two cows was mixed and used as the inoculum in the fermentation jar in Experiment 2 to minimise cow-level variation.

In Vitro Fermentation

Rumen fluid was strained through cheesecloth and subsampled (20 mL per jar) into fermentation jars containing 80 mL of buffer solution. The buffer solution was prepared according to the operating instructions of ANKOM (2018). It consisted of combining two warmed (39.5°C) solutions: buffer A (KH_2PO_4 at 10 g/L, $MgSO_4 \cdot 7H_2O$ at 0.5 g/L, NaCl at 0.5 g/L, $CaCl_2 \cdot 2H_2O$ at 0.1 g/L, and reagent grade urea at 0.5 g/L) and buffer B (Na_2CO_3 at 15.0 g/L and $Na_2S \cdot 9H_2O$ at 1.0 g/L) at a 5:1 ratio, adjusted to a pH of 6.8. Each fermentation jar contained 1.00 g of DM of substrate (corrected for residual DM), as per the randomised treatment to jar allocation for each run. Throughout the loading of fermentation jars, the mixture of rumen fluid and buffer medium was maintained at 39.5°C within a water bath and purged with CO_2. Temperature data loggers (Thermochron eXtreme-iButton, Code: DI 0000081A21941, Resolution: 0.0625 °C, Accuracy: 0.5 °C) were placed in one fermentation jar in each water bath to record incubation temperature inside the fermentation jar over the incubation period. The loaded ANKOM jars fitted with the ANKOM RF Gas Production System (ANKOM) were placed in water baths fitted with heating immersion circulators to regulate the incubation temperature and recovery period. According to treatment allocation, two and twelve drinking events (12 hours apart, and two hours apart) were created within 24 hours in Experiment 1, and six and 12 events (four and two hours apart) were created in Experiment 2. Since each drinking event had a recovery period of 2 hours, the 12th event, which occurred at the 24th hour, resulted in fermentation continuing for a total of 26 hours. While the ecological niches of microbial fermentation shift over time in response to substrate availability, leading to significant changes in microbiome diversity and community structure,

it has been suggested that the optimal time to end in vitro fermentation is after 18–24 hours of incubation (Dhakal et al., 2024; Pinnell et al., 2022). Considering the objectives of the current work to investigate microbiome diversity, an incubation period of 26 hours was maintained in both experiments. In each drinking event, the temperature in the fermentation jar was dropped by approximately 9.5 °C by adding 8.5 L of 4°C water to the water baths, which contained 12.5 L of 39°C water (Rahman et al., 2025). Due to the addition of the cooled water to the water bath, there were two instances where the temperature drop was slightly greater than 9.5°C (Figure 6. 1). The ANKOM RF Gas Production System automatically recorded the gas pressure every 5 minutes over a 26-hour period. At the termination of the 26-hour gas production period, the pH of the fluid was measured using a benchtop pH meter (Orion 2-star, Thermo Scientific, Waltham, MA, USA). The temperature data loggers were then recovered and downloaded to determine the temperature profile inside the jars.

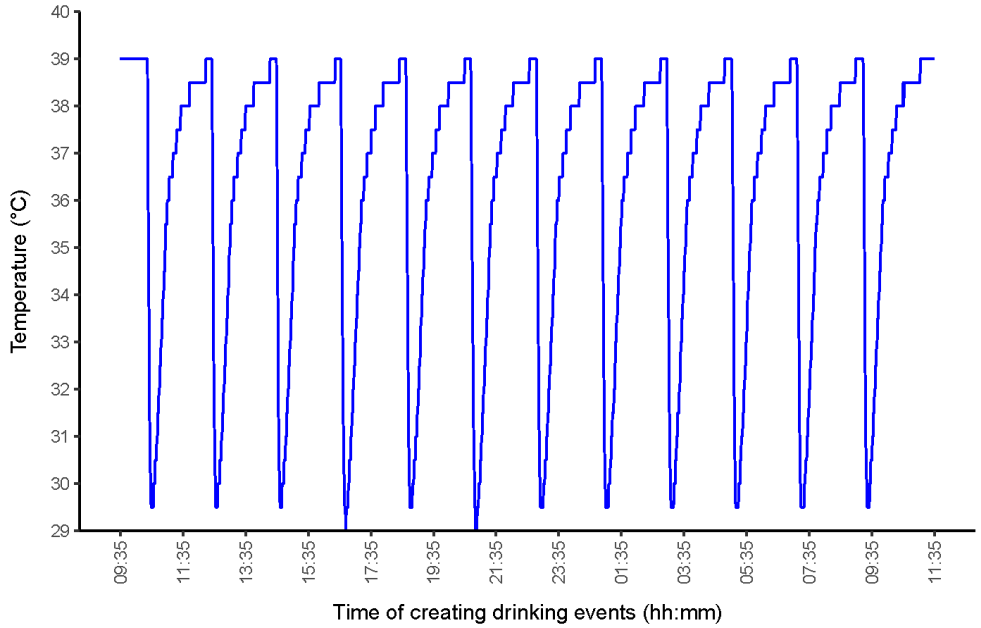

Figure 6. 1. Simulated drinking events with associated temperature drops by around 9.5 °C, followed by a 120-minute recovery period in the fermentation jar during *in vitro* fermentation for 26 hours.

Sample collection and laboratory analysis

Headspace gas was collected in gas vials from each fermenter jar at the end of fermentation, using a gas-tight syringe to determine the concentrations of CH_4, CO_2 and N_2O. After the collection of gas, fermenters were opened and subsamples of the rumen fluid–buffer solution were collected into 2-mL Eppendorf tubes to determine VFA concentration and microbial community diversity, and another subsample was acidified (99% H_2SO_4) to

determine NH_3 and Urea. These subsamples were stored at −20°C until they were analysed for VFA, NH_3, and Urea, and at -80 ºC for microbiome. The ANKOM bags (F57) with the nondegraded substrate were collected from each of the fermentation jars, rinsed with cold water (tap water) until the water ran clear, overnight dried in open air (room temperature) for 24 hours then dried at 105 ºC for at 4.0 hours and kept in a desiccator for at least 30 minutes to take the final weight to estimate *in vitro* dry matter degradability of substrate. Volatile fatty acid concentrations were measured using gas chromatography, following the method described by Chen and Lifschitz (1989). The analysis was performed with a gas chromatograph (Shimadzu GC-2010, with AOC-20i autosampler, Kyoto, Japan), equipped with a SGE BP21 30 m × 530 µm × 1 µm wide-bore capillary column. Methane and nitrous oxide concentrations were determined using gas chromatography (Model 8610C, SRI Instruments, Torrance, CA, USA) with an automated Gilson GX-271 auto sampler (Gilson Inc., Madison, MI, USA). The NH_3 concentration of the acidified rumen samples was measured using a clinical analyser (RX Daytona+, Randox Laboratories Ltd., London, UK, https://www.randox.com/rx-daytona-plus-overview/) and a commercial test kit (Cat. No. AM3979; Randox) based on the enzymatic UV method described by Neeley and Phillipson (1988). Methane production (mL/g of DM) was calculated by multiplying total gas volume (mL/ g of DM) by the percentage of CH_4 in the gas sample.

Microbiome DNA Extraction, Fragment Sequencing and Bioinformatics Analysis

Subsamples of rumen fluid and buffer solution were stored at -80 °C until analysed for DNA extraction and the subsequent sequencing in AgResearch, Mosgiel, New Zealand. Microbial samples were processed and sequenced using the methodology outlined in Hess et al. (2020). Briefly, DNA was extracted using a combined bead-beating, phenol, and column purification protocol and sequenced using restriction enzyme-reduced representation sequencing (RE-RRS) using the restriction enzyme *Pst*I (CTGCA|G). Samples were split across 1 (384-well) plate for DNA extraction and were run on 1 library of sequencing on an Illumina NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA). In total, 42 samples from Experiment 1 were sequenced along with 2 positive controls and 6 negative controls, whereas 31 samples from Experiment 2 were sequenced along with 2 positive controls and 1 negative control.

The Genotyping-By-Sequencing Taxonomic and Functional Feature Extraction (GBS-TaFFE) pipeline (https://github.com/BenjaminJPerry/GBS-TaFFE.git) was used to profile rumen GBS data against the Genome Taxonomy Database (GTDB; Parks et al., 2022). Sequenced reads

were demultiplexed and trimmed for adapters using Cutadapt (Martin, 2011). Samples with fewer than 25,000 raw reads were excluded. BBDuk and PRINSEQ++ were applied for low-entropy and homopolymer trimming and filtering, while KneadData removed tandem repeats and rRNA (SILVA138) sequences. Taxonomic profiling was performed using Kraken2 with the GTDB v2.20 (https://gtdb.ecogenomic.org/), supplemented with additional protozoa and fungi genomes. Assignments from domain to genus were included for Experiment 1, with species-level resolution added in Experiment 2.

Analysis of bacterial and archaeal diversity across the treatments was undertaken through alpha (α) and beta (β) diversity metrics to understand the impact that drinking events may have on the rumen microbial community structure. The bacterial and archaeal community richness and abundance (diversity) in each sample were calculated using the Shannon index (Shannon, 1948) and Simpson index (Simpson, 1949). Between-treatment differences in community structure (beta diversity) were quantified using the Bray–Curtis dissimilarity index (Bray & Curtis, 1957) on relative abundance data, and Aitchison distance (Aitchison et al., 2000) on Centered Log-Ratio (CLR) transformed data, and community composition was visualised by principal coordinate analysis (PCoA). Differentially abundant taxa were identified to investigate which taxa change in abundance across the treatments applied in *in vitro* fermentation experiment.

Statistical analysis

Analysis of fermentation parameters and gas production data

All fermentation data were analysed in R (v4.3.0). Gas production data were converted from pounds per square inch to millilitres using the ideal gas law and Avogadro's number (Ankom operations manual). To evaluate treatment effects on total gas production (mL/g of DM), concentrations of CH_4, VFA, NH_3, pH, and dry matter digestibility, a one-way analysis of variance (ANOVA) was conducted using the *aov* function in R, with Treatment as a fixed effect and 'Water bath', 'cow' and 'run' as random blocking factors. Model residuals were confirmed to be normally distributed with homogeneous variance, satisfying the assumptions of the model. Estimated marginal means for treatment, water bath, and run effects were computed using the *emmeans* package, and 95% confidence intervals were derived. Gas production kinetics were characterised by fitting the Ørskov and McDonald (1979) nonlinear model to the cumulative gas production data:

$$G(t) = a + b(1 - e^{-ct})$$

where *G(t)* is the cumulative gas volume (mL) at time *t* (*h*), *a* is the initial gas volume, a + *b* is the theoretical asymptote of the gas curve (hence *b* is the change from the initial value), and *c* is the fractional rate constant of gas production (% h^{-1}). Nonlinear mixed models were fitted using the *nlme* package in R, with *Treatment* included as a fixed effect on all parameters (*a*, *b*, and *c*), and random effects for *Run* and nested *Jar within Run* on the *b* parameter. Since the *b* parameter most likely captures between-jar variation in gas production, this parameter was allowed to vary between jars. Model predictions were generated using the *emmeans* package, and all figures showing the fitted response curves were created using the *ggplot2* package of R (Wickham, 2016). Model fitting used maximum likelihood estimation with starting values informed by preliminary data. The model's fit was assessed using Akaike's Information Criterion (AIC), residual diagnostics, and parameter significance testing using Wald *t*-tests and ANOVA on the mixed-effects model. Significance was declared at $p < 0.05$, and trends were discussed where $p < 0.05$.

Analysis of microbiome composition and diversity metrics

Rarefaction curves were generated at the phylum and genus level using the *vegan* package in R to assess sequencing depth sufficiency and sampling completeness across the treatments (Supplementary Figure 6S. 3 - *6S. 6.*) Differences in microbial counts between treatments were assessed using a generalised linear mixed-effects model (GLMM) with a Poisson distribution. The fixed effects included treatment and its interaction with taxonomic group (taxa), while run and cow were included as random intercepts to account for repeated measures and individual variability. Model fitting was performed using the *glmer* function from the *lme4* package in R. Diversity metrics were also analysed in R. Alpha diversity indices (Shannon and Simpson indices) were estimated and analysed with the *vegan* package in R from the RE-RRS-generated count matrix table. The resulting Shannon (H) and Simpson (1-D) diversity indices were then compared across treatments using a linear mixed-effects model with the *lmer* function from the lmerTest package (Kuznetsova et al., 2017). For beta diversity, the Bray-Curtis dissimilarity and Aitchison distance were calculated through the vegan package. The effects of treatments on the beta diversity index were tested using PERMANOVA with the *adonis* function (Anderson, 2001) in the *vegan* package. Homogeneity of dispersion was tested with the '*betadisper'* function and ANOVA to verify PERMANOVA assumptions. Differential abundance of microbial taxa at the phylum and genus level (Experiment 1) and species level

(Experiment 2) across treatments was assessed using the DESeq2 package (Love et al., 2014) with the default parameters in R. Any taxa where the mean relative abundance of a cohort is less than 0.01% were filtered before analysis to minimise noise. The DESeq2 pipeline was then used to estimate dispersion parameters and fit a negative binomial generalised linear model for each taxon. Pairwise contrasts between treatments (T2 vs T1, T3 vs T1, and T3 vs T2) were computed, and $\log_2$ fold changes ($\log_2$FC) were shrink-estimated using the adaptive prior method implemented in *apeglm* (Zhu et al., 2019) to improve stability and interpretability of fold-change estimates for low-abundance taxa. Differentially abundant phyla, genera and species were identified based on an adjusted *p*-value (Benjamini–Hochberg false discovery rate, FDR < 0.05). Significant results were summarised and visualised using volcano plots and heat-tile maps of log two fold change (log2FC) values across contrasts to highlight treatment-responsive taxa. Pairwise Pearson correlations were calculated between the log-transformed bacterial abundances and fermentation variables (volatile fatty acids, ammonia, pH, gas, and methane) for each treatment group using the *cor()* function in R. Correlation matrices were visualised as clustered heatmaps with hierarchical clustering applied to both taxa and fermentation traits using heatmap.2 function from the *gplots* package.

Results

Results of Experiment 1

Fermentation characteristics

The impact of drinking events on pH was significant ($p = 0.04$); however, it did not impact the dry matter degradability (%) of substrate (kale & silage mixture). More frequent drinking events (12 events in 26 hours) resulted in significantly ($p < 0.001$) higher total gas production (mL/g DM) compared to control and two events, with a similar trend observed in total methane production. However, concentrations of CH_4 in total gas, N_2O, NH_3, and urea were not affected by the frequency of drinking events during *in vitro* fermentation. In the case of VFA production, no effects on VFA production were detected, except for Iso-butyric acid, which was highest for 12 events compared to control and two events (Table 6. 2).

Gas production kinetics

The gas production trends, derived from empirical values recorded over 26 hours of *in vitro* fermentation with varying numbers of simulated drinking events, are presented in Figure 6. 2.

The initial gas production was negative (due to negative gas pressure), and the gas production curves from different treatments did not reach their asymptotes. However, the theoretical asymptote $a + b$ derived from a nonlinear mixed-effects model (Table 6. 3) was significantly affected by the treatments (p<0.0001), with the highest value observed in the 12-event treatment (253 mL) and the lowest was in the control (112 mL). The fractional rate constant of gas production c (h^{-1}) was substantially reduced in the 12-event treatment (T3) (0.0265 h^{-1}) relative to T1 (0.0521 h^{-1}) and T2 (0.0502 h^{-1}), with strong statistical support ($p < 0.0001$). In addition, the initial gas production parameter (a) varied significantly across the treatments (Table 6. 3).

Table 6. 2. *In vitro* gas production and fermentation output information on the impact of drinking associated RT fluctuation events after 26 hours of incubation within the ANKOM RF gas production system.

Variables	Treatments			SE	*p*-Value
	Control (T1)	2 events (T2)	12 events (T3)		
pH	6.08	6.11	6.13	0.01	0.04
DM degradability (%)	60.2	59.9	60.9	0.67	0.62
Total gas (mL/g of DM)	68.1	63.6	112.5	4.46	< 0.001
CH4/total gas (%)	9.42	9.26	9.50	0.67	0.97
Total methane (mL/g of DM)	6.44	6.49	10.73	0.68	< 0.001
N_2O (ppm)	2.05	4.00	3.36	0.70	0.15
NH_3 (mmol/L)	17.9	17.4	17.1	0.43	0.40
Urea (mmol/L)	5.03	4.53	5.11	0.37	0.48
VFA (mmol/L)					
Acetic acid	29.3	29.3	29.8	0.71	0.84
Propionic acid	10.9	10.8	11.0	0.21	0.67
Butyric acid	7.37	6.93	7.42	0.19	0.15
Iso-butyric acid	0.49	0.48	0.44	0.009	0.01
Valeric acid	1.06	1.03	1.07	0.03	0.52
Iso-valeric acid	0.52	0.49	0.46	0.02	0.23
Hexanoic acid	0.53	0.49	0.49	0.03	0.61
Total	50.2	49.5	50.7	1.06	0.71

*SE = Standard error

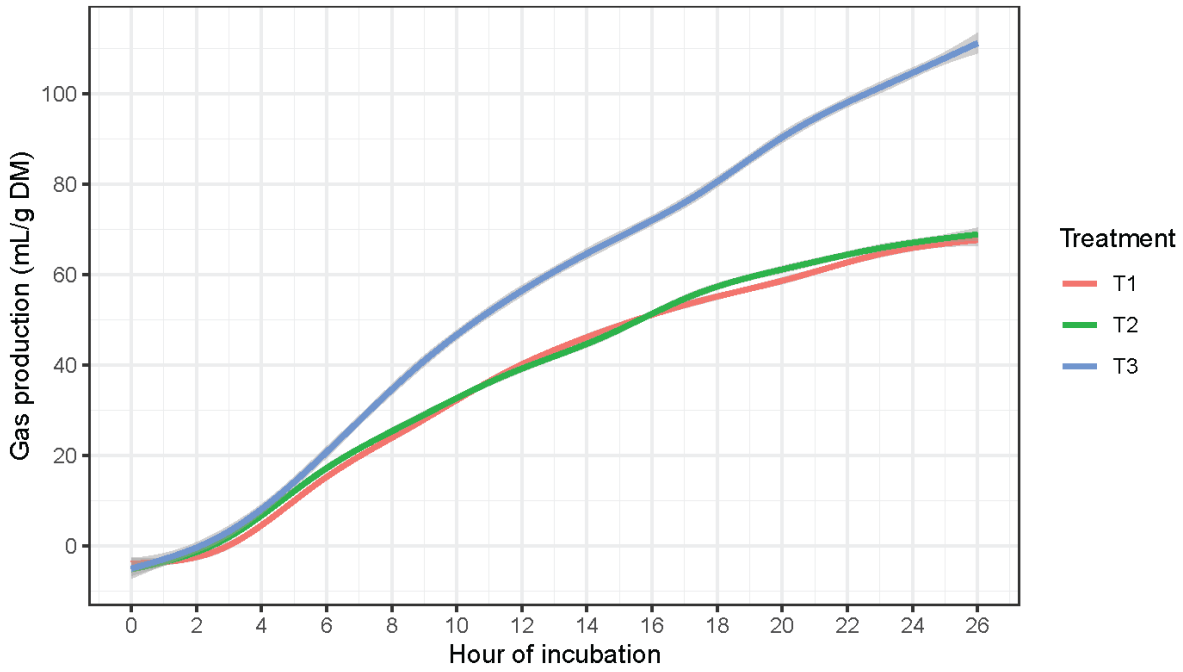

Figure 6. 2. *In vitro* gas production from kale & silage mixture with drinking associated RT fluctuation events (T1 = control (no event in 26 h), T2 = 2 events in 26 h, and T3 = 12 events in 26 h) in a 26-h incubation period. Grey bands represent ± 1 SE.

Table 6. 3. Estimated nonlinear gas production parameters (estimate ± SE) under different drinking event treatments and their pairwise comparisons.

Parameters	Treatment	Estimated mean	SE	Contrast	*p*-value
a	T1	-12.9	0.27	T1 vs T2	0.0047
	T2	-11.8	0.28	T1 vs T3	0.0948
	T3	-13.5	0.25	T2 vs T3	<0.0001
a+*b*	T1	112	6.76	T1 vs T3	<0.0001
	T2	114	7.08	T2 vs T3	<0.0001
	T3	253	7.88	T1 vs T2	0.8009
c	T1	0.052	0.001	T1 vs T3	<0.0001
	T2	0.050	0.001	T2 vs T3	<0.0001
	T3	0.027	0.0006	T1 vs T2	0.1869

*Model parameters: *a* = the initial gas volume, a + *b* = the theoretical asymptote of the gas curve, and *c* = the fractional rate constant of gas production (% h^{-1}) under different drinking event treatments (T1 = control, T2 = two events, T3 = 12 events). Pairwise comparisons (Tukey-adjusted) indicate significant differences where $p < 0.05$.

Rumen microbial composition in the *in vitro* system

Microbial abundance

The count matrix generated through the RE-RRS pipeline represented the distribution of sequencing reads assigned to each identified taxon, from domain to genus, across the treatments (T1, T2 & T3) and the original rumen sample (H0). The analysis of sequencing reads at the domain level revealed that the microbial community of the rumen sample subjected to treatments was predominantly composed of bacteria, with a relative abundance (RA) of 97.6%, whereas archaea represented only 2.4%. However, in the zero-hour (H0) sample (original rumen sample before incubation), bacterial abundance was slightly higher than in the fermented sample (Figure 6. 3).

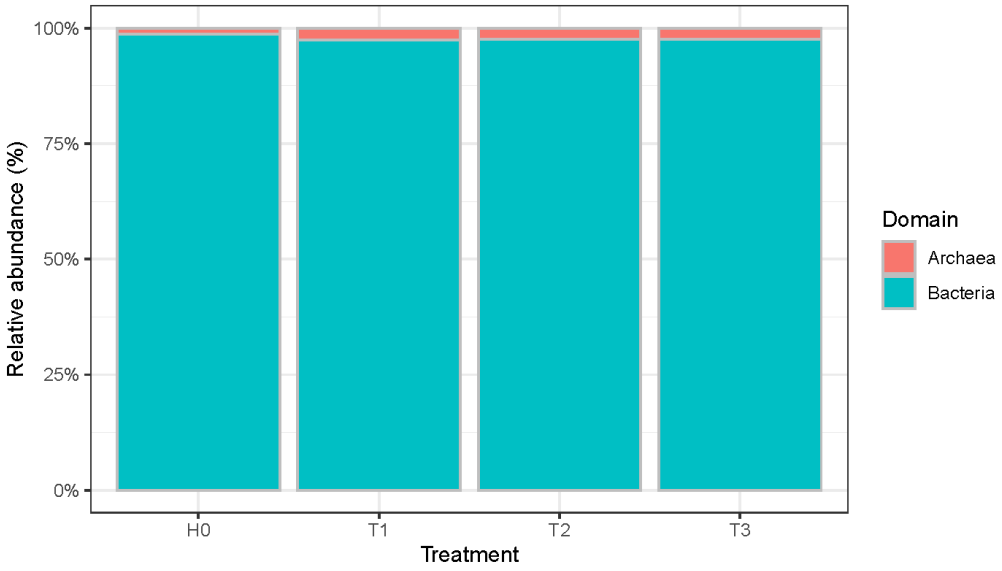

Figure 6. 3. Community composition of microbial domains across the treatments (T1 = Control, T2 = two drinking events, T3 = 12 drinking events), and the original rumen sample (H0).

Based on the QC-passed sequence reads, a total of 173 different microbial phyla, including 17 archaeal phyla and 13,203 distinct genera, including 471 archaeal genera, were classified. Within the bacterial domain, the most abundant (RA > 0.01%) phyla were Bacillota_A (Firmicutes) 63.3%, Bacteroidota (Bacteroidetes) 23.2%, Bacillota (4.17%) Actinomycetota (Actinobacteria) 2.76%, and Pseudomonadota (Proteobacteria) 1.34% (Figure 6. 4). At the same time, the archaeal domain is dominated by Methanobacteriota (58.7%) and Thermoplasmatota (40.3%). The most relatively abundant (RA > 0.01%) genera in the bacterial community were unclassified CAG-791 (27.8%), followed by Prevotella (12.3%), Limivicinus

(5.39%), UBA1066 (4.67%), and RUG740 (3.15%) (Figure 6. 5). In the archaeal domain, around 80% belonged to methanogens, including the most abundant genus, Methanobrevibacter (35.5%), followed by Methanobrevibacter_A (21.7%), Methanomethylophilus (18.4%), unclassified UBA71 (17.5%), SIGS (4.36%), and Methanosphaera (1.22%) across the treatments (Figure 6. 6).

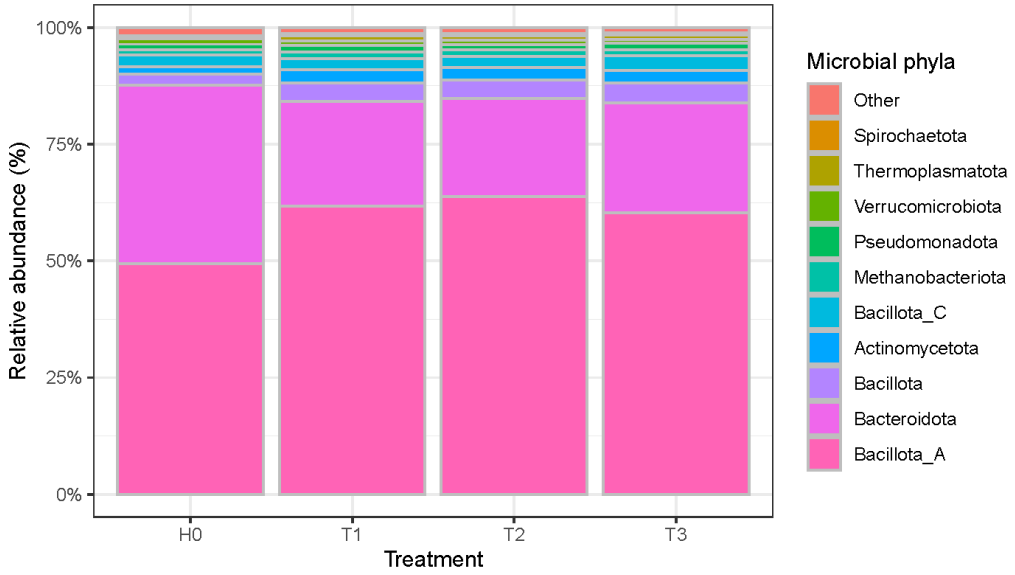

Figure 6. 4. Ruminal microbiome community composition at the phylum level across the treatments (T1 = control, T2 = two drinking events, and T3 = 12 drinking events) and original rumen sample (H0) with a relative abundance greater than 0.01%. Only the ten most relatively abundant phyla are displayed.

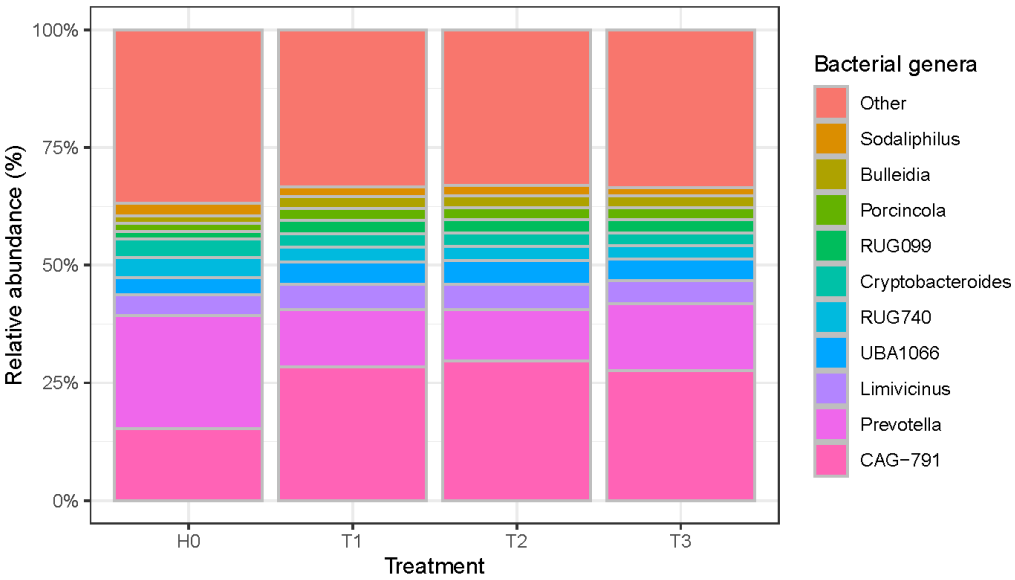

Figure 6. 5. Relative abundance of bacterial genera across the treatments (T1 = control, T2 = two events, and T3 = 12 events) and original rumen sample (H0) with a relative abundance greater than 0.01%.

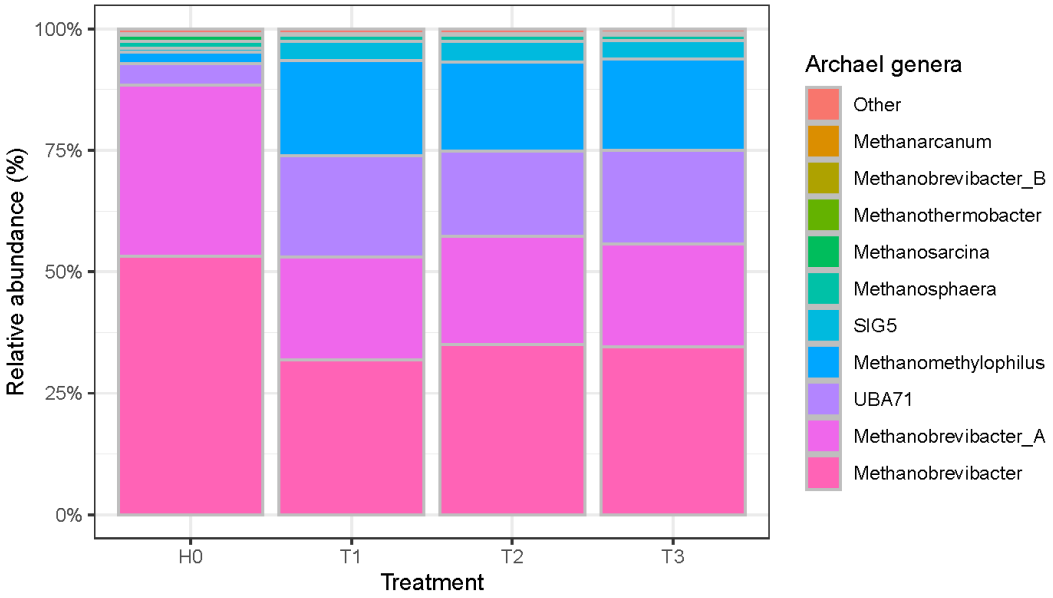

Figure 6. 6. Ruminal archaeal community composition at the genus level across the treatments (T1 = control, T2 = two events, and T3 = 12 events) and original rumen sample (H0) with a relative abundance greater than 0.01%. Only the ten most relatively abundant genera are displayed.

Bacterial and archaeal diversity and richness

The microbial α-diversity at the phylum and genus levels was assessed using the Shannon (H) and Simpson (1 – D) indices and compared among treatments by linear mixed-effects models, revealing no significant differences among treatments for either index (H: $p = 0.88$; 1 – D: $p = 0.84$). Mean Shannon diversity at the phylum level was 1.25 ± 0.05, and mean Simpson diversity was 0.56 ± 0.03 across all samples. At the genus level, treatment had a marginal effect on the bacterial Shannon diversity index ($p = 0.067$). Conversely, Simpson diversity remained unaffected ($p = 0.14$), suggesting overall bacterial community evenness was largely stable across treatments. Mean Shannon diversity of bacterial community at the genus level was 3.43 ± 0.14, and mean Simpson diversity was 0.89 ± 0.02 across the samples. However, in the archaeal community, both diversity metrics were significantly influenced by treatment. The Shannon index differed significantly among treatments ($p = 0.003$), and a similar pattern was observed for the Simpson index ($p = 0.002$), indicating apparent treatment-dependent shifts in archaeal genus-level diversity. The mean Shannon diversity of the archaeal genus was 1.58 ± 0.02, and the mean Simpson diversity was 0.75 ± 0.01 across all samples. The effects of

treatments on Shannon and Simpson diversity metrics are depicted in Figure 6. 7 & Figure 6. 8**,** and in the Supplementary Figure 6S. 3 and Supplementary Figure 6S. 4, respectively.

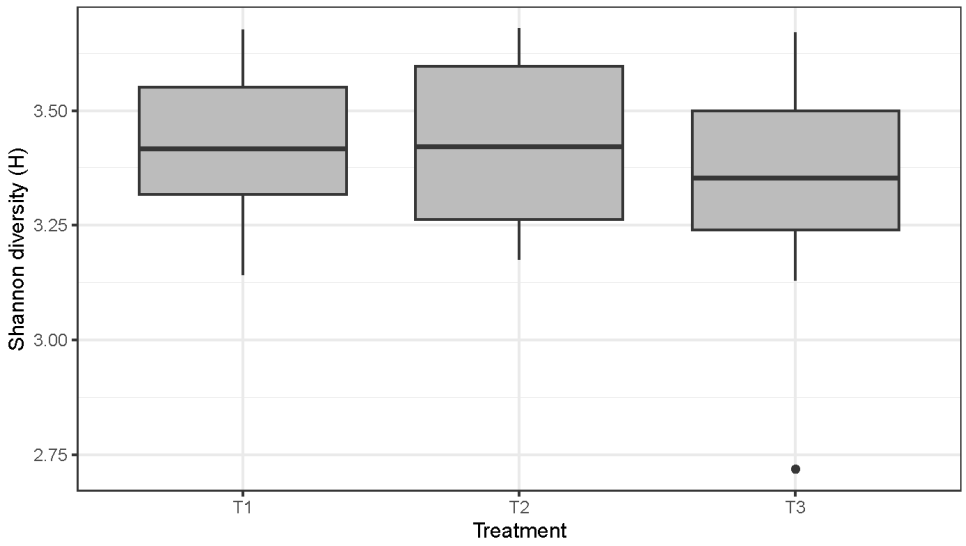

Figure 6. 7. Impact of RT fluctuations on Shannon diversity (H) of bacterial genera. Boxplots represent the distribution of Shannon diversity across treatments (T1 = control, T2 = two events, and T3 = 12 events).

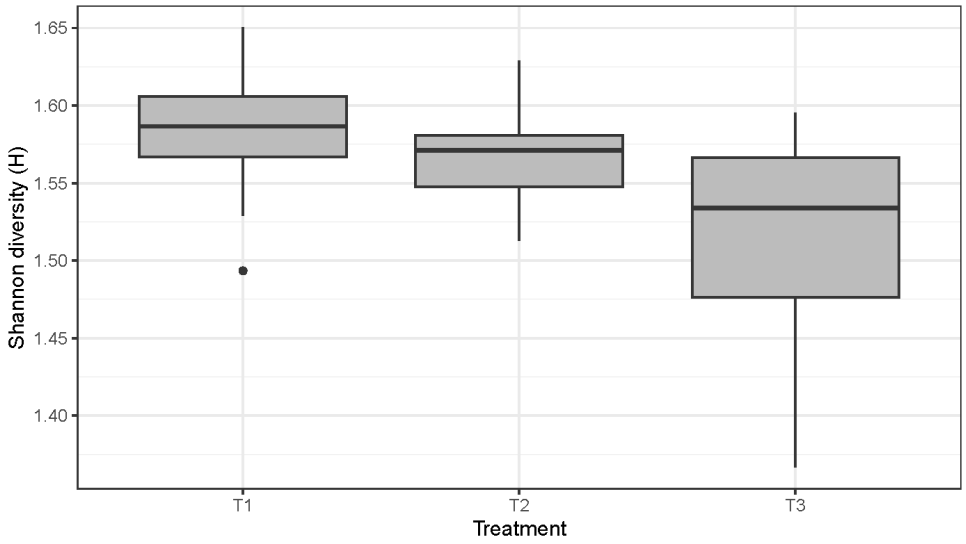

Figure 6. 8. Impact of RT fluctuations on Shannon diversity (H) of archaeal genera. Boxplots represent the distribution of Shannon diversity across treatments (T1 = control, T2 = two events, and T3 = 12 events).

Beta diversity

To investigate whether alterations in α-diversity are associated with changes in community composition, β-diversity analyses were performed using Bray–Curtis dissimilarity and the

Aitchison distance method. Principal Coordinate Analysis based on Bray–Curtis dissimilarity revealed no distinct clustering of bacterial and archaeal communities at the genus level among treatments (Figure 6. 9 & Figure 6. 10). In the case of bacterial genera, PCoA1 explained 68%, and PCoA2 explained 20% of the total variation among the samples across the three treatment groups. PERMANOVA analysis confirmed that community composition did not differ significantly across treatments ($R^2 = 0.023$, $p = 0.89$). For archaeal genera, PCoA1 and PCoA2 explained 70% and 19.4% of the total variation of archaeal genera across the treatments, respectively, with insignificant differences ($R^2 = 0.01$, $p = 0.98$) across treatment groups. The Aitchison-based PCoA indicated no clear separation in the composition of bacterial or archaeal genera among the treatment groups depicted in Supplementary Figure 6S. 5 & Supplementary Figure 6S. 6. PERMANOVA results consistently demonstrated no significant differences in β-diversity among treatments (Bacteria: $R^2 = 0.30$, $p = 0.99$; Archaea: $R^2 = 0.03$, $p = 0.97$).

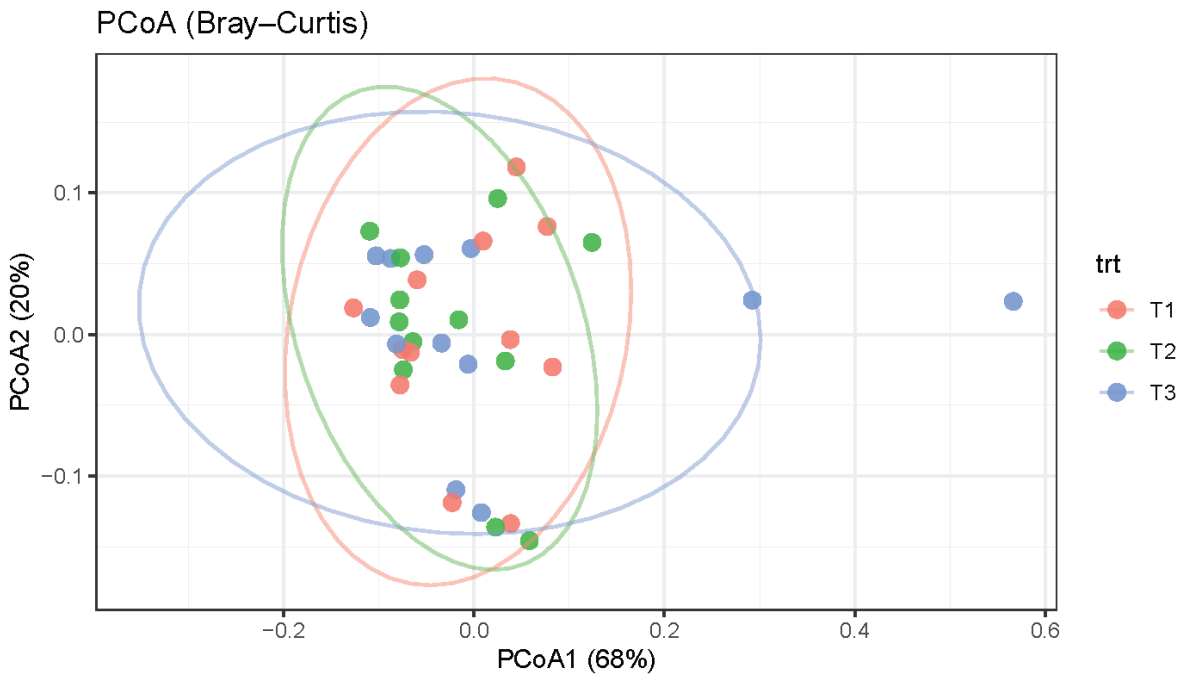

Figure 6. 9. Principal Co-ordinate Analysis (PCoA) utilising Bray–Curtis dissimilarity showing the β-diversity of bacterial communities at the genus level across treatments (T1 = control, T2 = two events, and T3 = 12 events). Each point denotes the bacterial community of an individual sample, while ellipses indicate 95% confidence intervals for each treatment group.

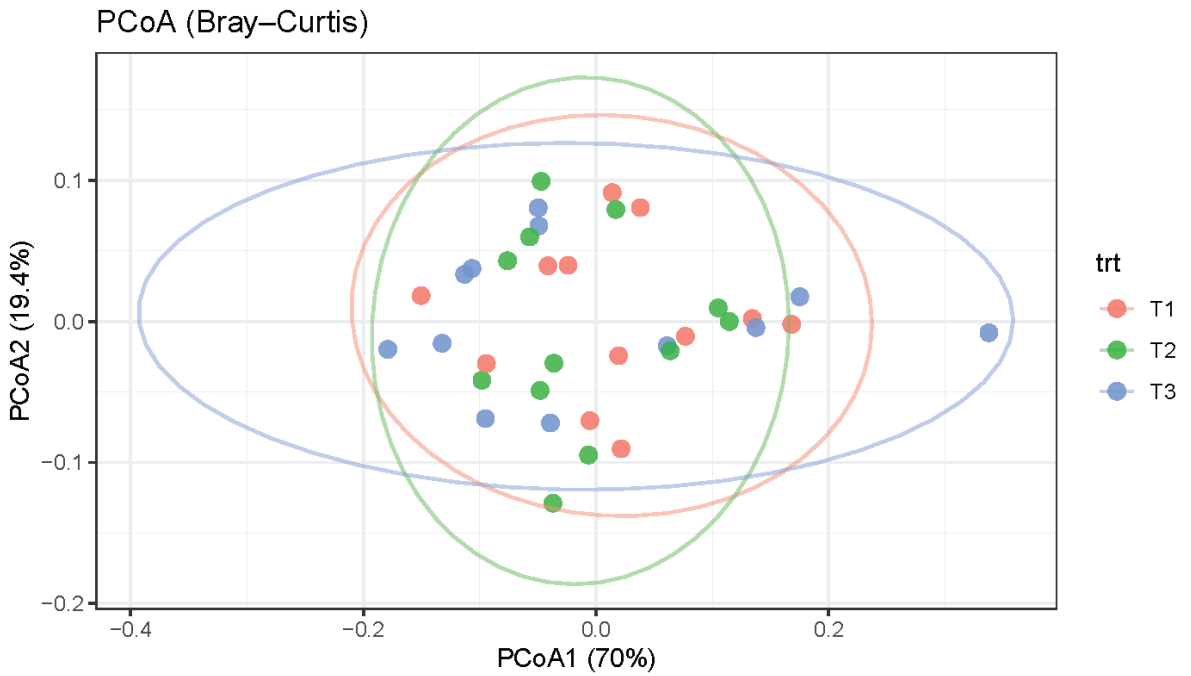

Figure 6. 10. Principal Co-ordinate Analysis (PCoA) utilising Bray–Curtis dissimilarity showing the β-diversity of archaeal communities at the genus level across treatments (T1 = control, T2 = two events, and T3 = 12 events). Each point denotes the archaeal community of an individual sample, while ellipses indicate 95% confidence intervals for each treatment group.

Differences in microbial abundances

Following the β-diversity studies, DESeq2 was used to identify differentially abundant microbial taxa among treatment groups. With a false discovery rate (FDR) threshold of <0.05, only one genus, Streptococcus, exhibited significant differences in T3 contrast to T2. No differentially abundant genera were detected in the T2 vs. T1 or T3 vs. T1 contrasts (FDR < 0.05), indicating minimal compositional change relative to the control. Moreover, no phyla of the microbiome showed differential abundance among treatments contrast (FDR < 0.05).

Correlation analysis

Correlations between fermentation parameters and the relative abundance (RA) of microbial phyla (RA > 0.01%) were explored across the treatments using pairwise Pearson correlations and visualised in a correlation heatmap (Figure 6. 11 - 6. 13). Methane concentration, NH_3, urea, iso-valeric, and iso-butyric acid were moderately to strongly negatively correlated with Bacteroidota, Bacillota lineages, Methanobacteriota, Thermoplasmatota, Desulfobacterota_F, Actinomcetota, and Planctomycetota. These fermentation parameters were also negatively

(moderate to weak) correlated with all other phyla, except Fibrobacterota and the candidate phylum UBP6. Hexanoic acid showed strong positive correlations across several phyla, such as Bacillota lineages, Actinomycetota, Cyanobacteriota, and Methanoges. Spirochaetota showed a strong positive correlation with propionic acid, acetic acid, and Total VFA. Whereas all other fermentation parameters showed weak or mixed correlations with all the relatively abundant phyla under control treatments (T1) (Figure 6. 11).

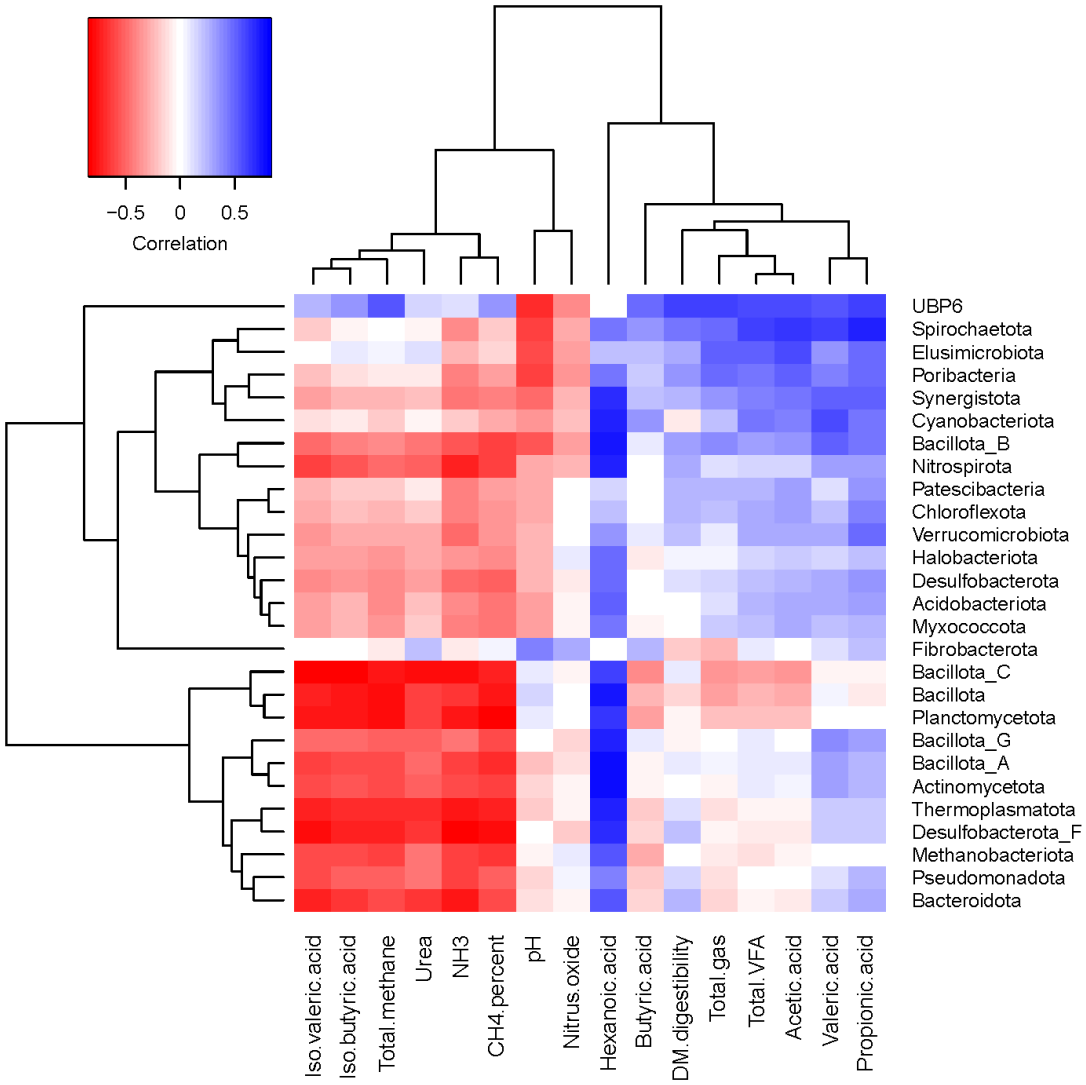

Figure 6. 11. Correlation heatmap showing relationships between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T1** (control). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

Under treatment T2 (two events), most of the phyla showed weak to moderate correlations with fermentation parameters, except for a few that exhibited strong positive correlations, mainly with VFA-related parameters. Total VFA and acetic acid are strongly correlated with the phylum Elusimicrobiota, whereas hexanoic acid showed positive correlation with Actinomycetota, Cyanobacteriota, and some Bacillota lineages. Methane parameters, NH_3, and branched-chain VFAs showed moderate to strong correlations with some Bacillota lineages and Thermoplasmatota (Figure 6. 12).

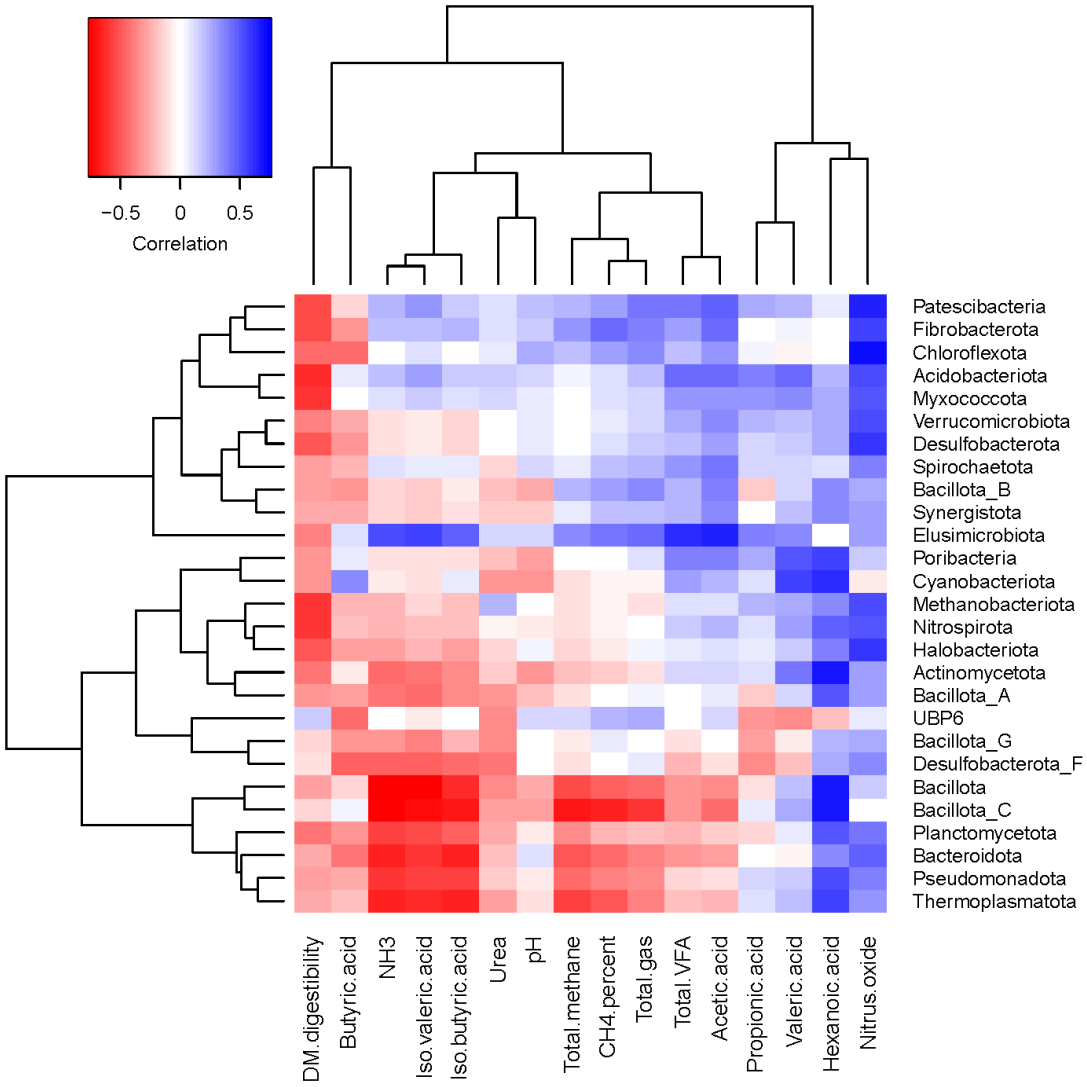

Figure 6. 12. Correlation heatmap showing relationships between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T2** (two events). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

Correlations between fermentation parameters and the abundance of microbial phyla showed more positive associations under treatment T3. Total VFA and acetic acid were strongly positively correlated with Elusimicrobiota and Poribacteria phyla whereas Hexanoic acid was positively correlated with all microbiome phyla except unclassified UBP6. Methane attributes also showed a strong positive correlation with Bacillota_G, Bacillota_B, and Bacillota_A. Conversely, a strong negative correlation was observed between fermentation products NH_3, Urea, and Bacteroidota, Bacillota_C, and Pseudomonadota. Branched chain VFAs were negatively correlated with Bacteroidota and Bacillota_C (Figure 6. 13).

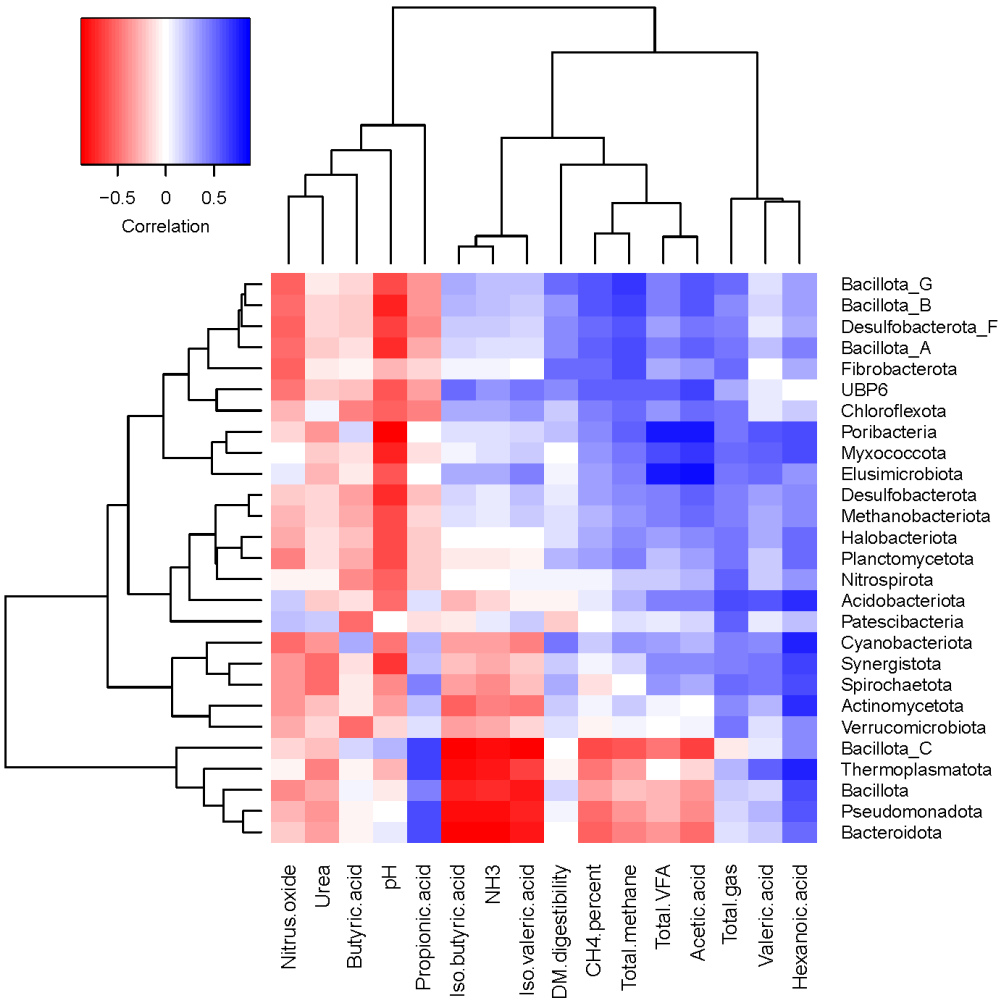

Figure 6. 13. Correlation heatmap showing relationships between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T3** (12 events). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

Results of Experiment 2

Fermentation characteristics

Reticulorumen temperature fluctuation through simulated drinking events did not significantly affect ($p > 0.05$) pH and substrate degradability during the 26-hour in vitro fermentation in Experiment 2. Gas production, in terms of total gas (mL/g of DM), total CH_4, methane concentration (% in total gas), and CO_2 (%), was also not significantly affected by the drinking-associated RT fluctuation events during incubation. However, significant variations were observed in the concentration of N_2O and NH_3, with the highest concentration found in T1 and the lowest in T3 in both cases. In the case of VFA, RT fluctuations significantly impacted the concentrations of propionic acid ($p = 0.02$) and hexanoic acid ($p = 0.001$), with the highest concentrations observed in T3 compared to T1 and T2. In contrast, no impact was observed on the total amount of VFAs and concentration of other VFAs (Table 6. 4).

Table 6. 4. In vitro gas production and fermentation output information on the effect of drinking associated RT fluctuation events after 26 hours of incubation within the ANKOM RF Gas production system. Values are means ± SE.

Variables	Treatments			*p*-Value
	Control (T1)	6 events (T2)	12 events (T3)	
pH	6.20 ± 0.02	6.22 ± 0.02	6.22 ± 0.02	0.73
DM Digestibility	55.6 ± 1.17	54.3 ± 1.17	54.2 ± 1.17	0.89
Total gas (ml/g of DM)	57.1 ± 4.38	54.9 ± 3.54	59.7 ± 3.77	0.15
CH_4/total gas (%)	6.79 ± 0.71	5.93 ± 0.57	5.77 ± 0.61	0.93
Total CH_4 (ml/g of DM)	3.4 ± 0.55	2.90 ± 0.45	2.85 ± 0.48	0.93
N_2O ppm	96.9 ± 16.7	60.1 ± 15.6	49.4 ± 16.7	0.03
CO_2 (%)	108.3 ± 12.3	100.5 ± 8.97	86.6 ± 10.6	0.38
NH_3 (mL/L)	28.1 ± 0.50	27.0 ± 0.46	26.70 ± 0.45	0.02
VFA (mL/L)				
Acetic acid	34.10 ± 1.29	33.8 ± 1.18	33.34 ± 1.18	0.18
Propionic acid	14.64 ± 0.58	14.85 ± 0.53	15.33 ± 0.53	0.02
Butyric acid	7.43 ± 0.28	7.29 ± 0.26	7.22 ± 0.26	0.40
Iso-butyric acid	0.80 ± 0.05	0.77 ± 0.04	0.69 ± 0.04	0.68
Valeric acid	1.27 ± 0.04	1.24 ± 0.04	1.22 ± 0.04	0.45
Iso-valeric acid	1.39 ± 0.06	1.37 ± 0.06	1.26 ± 0.05	0.85
Hexanoic acid	0.16 ± 0.01	0.15 ± 0.01	0.20 ± 0.01	0.001
Total	59.78 ± 2.20	59.44 ± 2.01	59.27 ± 2.01	0.13

Gas production kinetics

Compared to Experiment 1, gas production patterns in Experiment 2 showed a distinct trend, with the control treatment (T1) generating higher gas volumes than T2 and T3 (Figure 6. 14). However, similar to Experiment 1, initial gas pressure was negative, and none of the gas production curves reached their asymptotes under any treatment. The theoretical asymptote ($a + b$), estimated using a nonlinear mixed-effects model, was significantly affected by treatment ($p < 0.0001$), with the highest value observed in the 12-event treatment (118.4 mL) compared to T1 and T2, reflecting a trend similar to that reported in the previous experiment (Table 6. 5). The fractional rate constant of gas production, $c(h^{-1})$, was significantly lower in the 12-event treatment (T3) (0.027 h^{-1}) compared to T1 (0.051 h^{-1}) and T2 (0.049 h^{-1}). This difference was highly significant ($p < 0.0001$) and aligned with the pattern observed in Experiment 1. However, the fractional rate constant of gas production did not differ significantly between control and six events. In addition, the initial gas production parameter (a) tended to be lower with increasing number of drinking events (Table 6. 5).

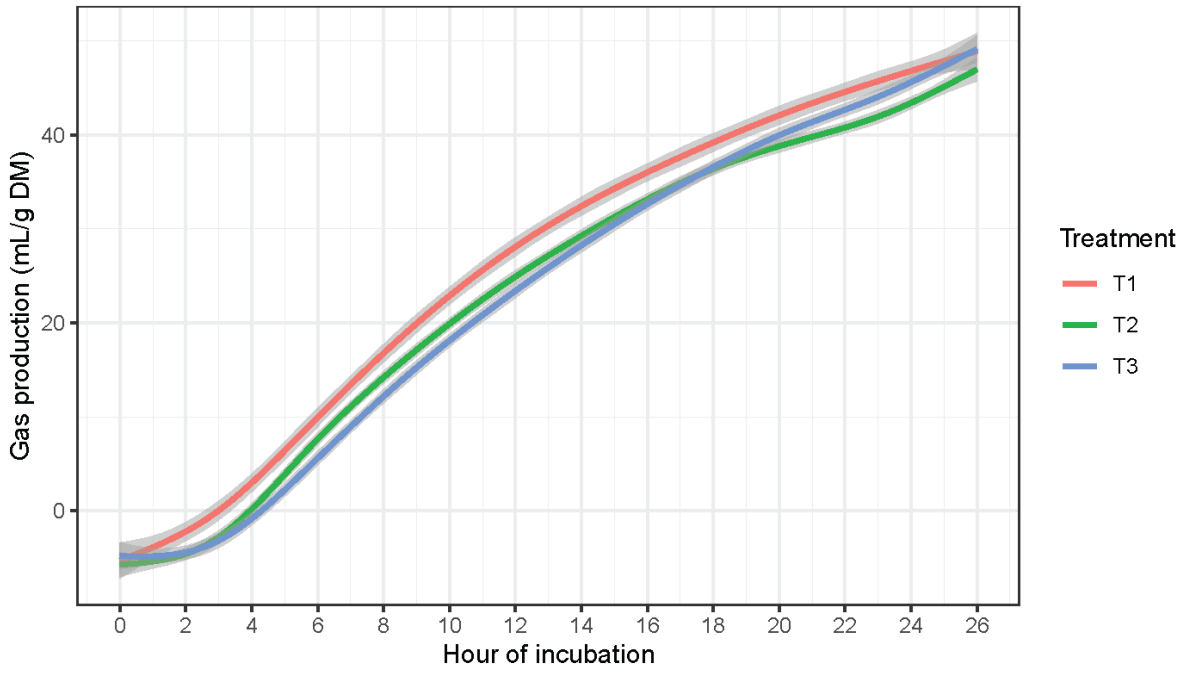

Figure 6. 14. In vitro gas production from ryegrass with simulated drinking events (T1 = control, T2 = 6 events, and T3 = 12 events) in a 26-h incubation period. Grey bands represent ± 1 SE.

Table 6. 5. Estimated nonlinear gas production parameters (mean ± SE) under different RT fluctuation events and their pairwise comparisons.

Parameters	Treatment	Estimated mean	SE (±)	Contrast	*p*-value
a	T1	–9.93	0.354	T1 vs T2	0.0001
	T2	–11.66	0.275	T1 vs T3	0.1152
	T3	–10.65	0.290	T2 vs T3	0.0116
a+*b*	T1	82.3	5.94	T1 vs T2	0.7026
	T2	79.8	5.00	T1 vs T3	<0.0001
	T3	118.4	6.88	T2 vs T3	<0.0001
c	T1	0.051	0.0019	T1 vs T2	0.4656
	T2	0.049	0.0015	T1 vs T3	<0.0001
	T3	0.027	0.0015	T2 vs T3	<0.0001

*Model parameters: a = the initial gas volume, $a + b$ (hence b) = the theoretical asymptote of the gas curve, and c = the fractional rate constant of gas production (h^{-1}) under different drinking event treatments (T1 = control, T2 = six events, T3 = 12 events). Pairwise comparisons (Tukey-adjusted) indicate significant differences where $p < 0.05$.

Rumen microbial composition in the *in vitro* system

Microbial abundance

The count matrix for Experiment 2 was generated using the same procedure as in Experiment 1; however, sequencing reads were classified across taxonomic levels from domain to species for all treatments (T1, T2, and T3) as well as the original rumen sample (H0). Similar to Experiment 1, the microbial community in both treated samples (T1, T2 and T3) and the original rumen sample (H0) was dominated by bacteria (relative abundance: 98.5%), while archaea accounted for only 1.46% (Figure 6. 15). Based on QC-passed sequence reads, a total of 199 microbial phyla—including 18 archaeal phyla—and 16,620 distinct genera, of which 588 were archaeal, were identified. Additionally, 67,216 sequence reads were classified at the species level, with 1,367 assigned to the archaeal domain and the remainder corresponding to bacterial species. This represents a higher taxonomic richness compared to the previous experiment. Consistent with Experiment 1, the dominant bacterial phyla (RA > 0.01%) in Experiment 2 with ryegrass substrate were Bacteroidota (41.9%), Bacillota_A (38.1%), Pseudomonadota (6.83%), Bacillota_C (4.52%), and Actinomycetota (2.90%) (Figure 6. 16).

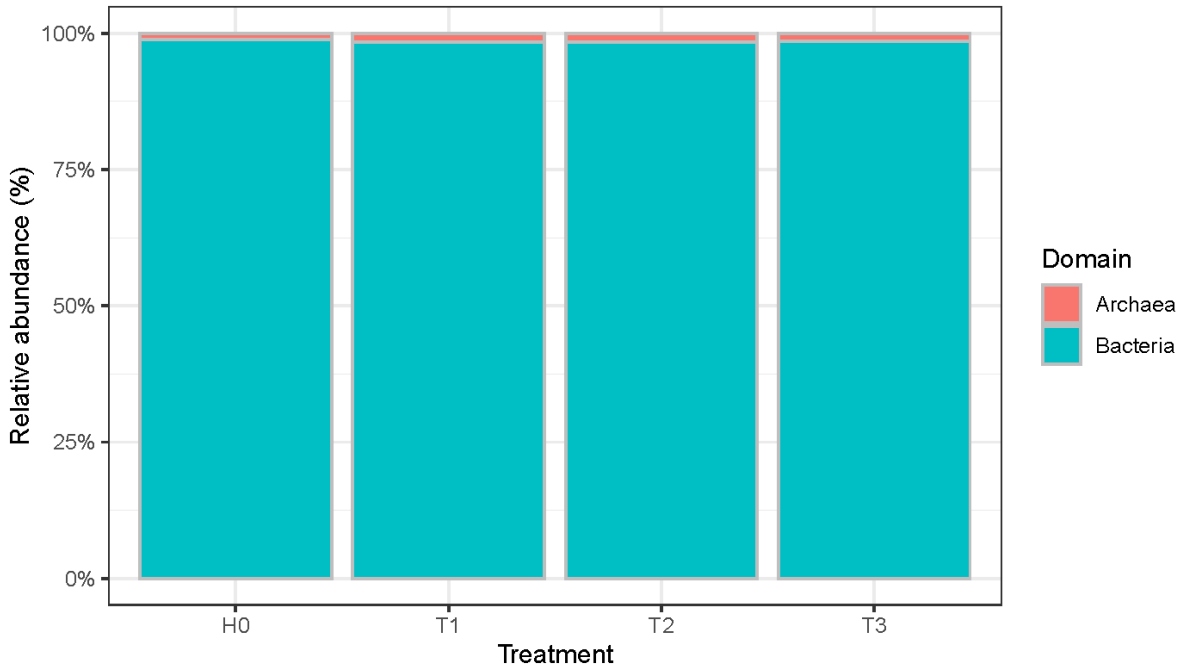

Figure 6. 15. Community composition of microbial domains across the treatments (T1 = control, T2 = six events, T3 = 12 events) after 26 h of incubation and the original rumen sample (H0).

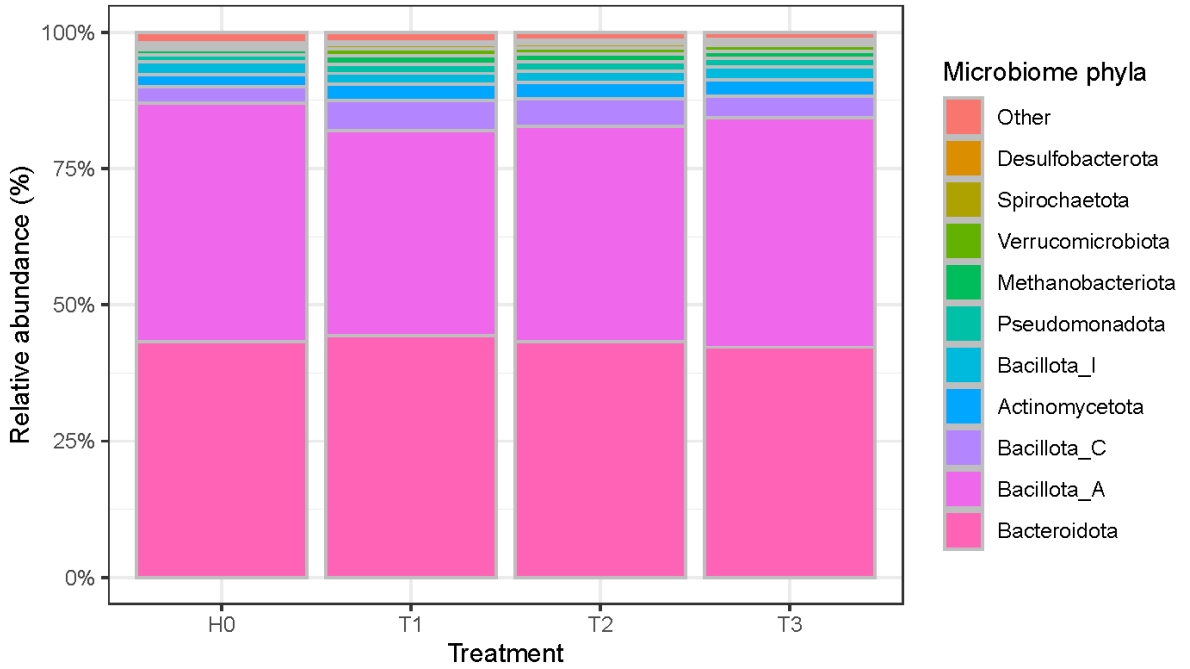

Figure 6. 16. Ruminal microbiome community composition at the phylum level across the treatments (T1 = control, T2 = six events, T3 = 12 events) and original rumen sample (H0) with a relative abundance greater than 0.01%. Only the ten most relatively abundant phyla are displayed.

However, in the archaeal domain, the relative abundance of the Methanobacteriota phylum was 92.4%, markedly higher than in the previous experiment, whereas Thermoplasmatota accounted for only 5.18%, representing a substantial decrease compared to its earlier abundance with kale and silage substrates. At the genus level, the most abundant bacterial genus (RA > 0.01%) were Prevotella (25.2%), followed by Limivicinus (6.71%), Sodaliphilus (6.09%), Comamonas (5.32%), and candidate genus CAG-791 (5.19%), while the remaining genera among the top ten exhibited relative abundances below 5% across treatments (Figure 6. 17). Although the relative abundance of Archaeal genera followed a similar trend to Experiment 1, Methanobrevibacter accounted for 88.5% in Experiment 2, more than twice its proportion in the previous experiment. The RA of the top ten archaeal genera depicted in Figure 6. 18. In Experiment 2, a total of 177 bacterial species and 100 archaeal species, most of which were candidate taxa and were identified from the *in vitro* fermented rumen sample, each with a relative abundance greater than 0.1%.

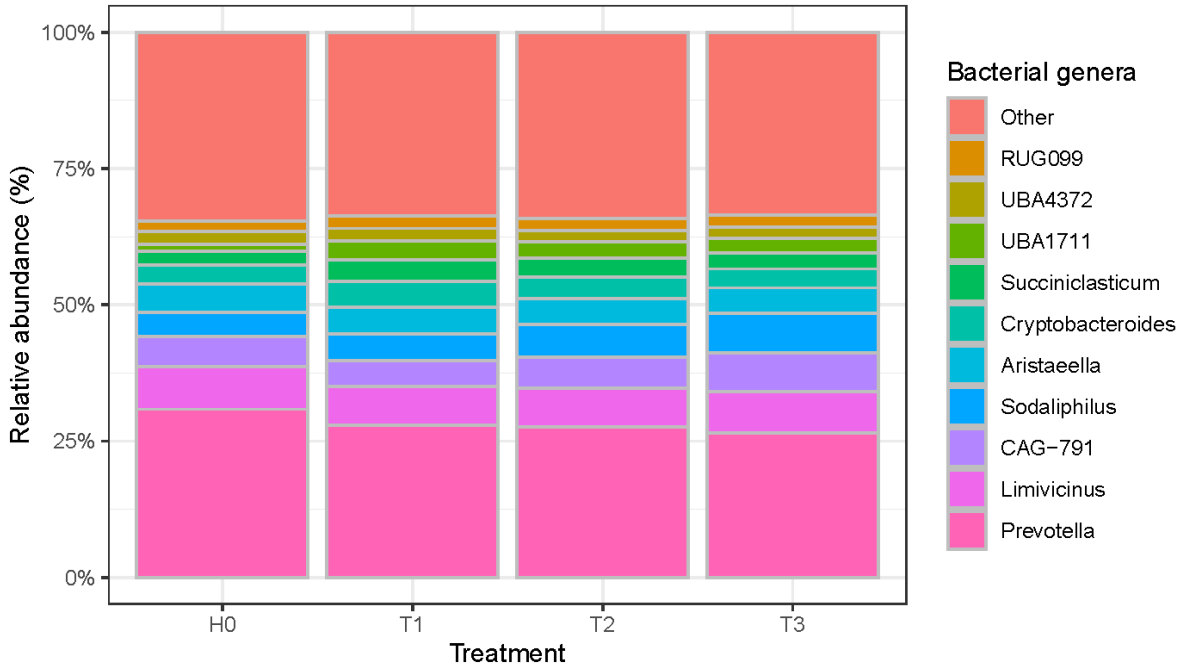

Figure 6. 17. Relative abundance of bacterial genera across the treatments (T1 = control, T2 = six events, T3 = 12 events) and original rumen sample (H0) with a relative abundance greater than 0.01%.

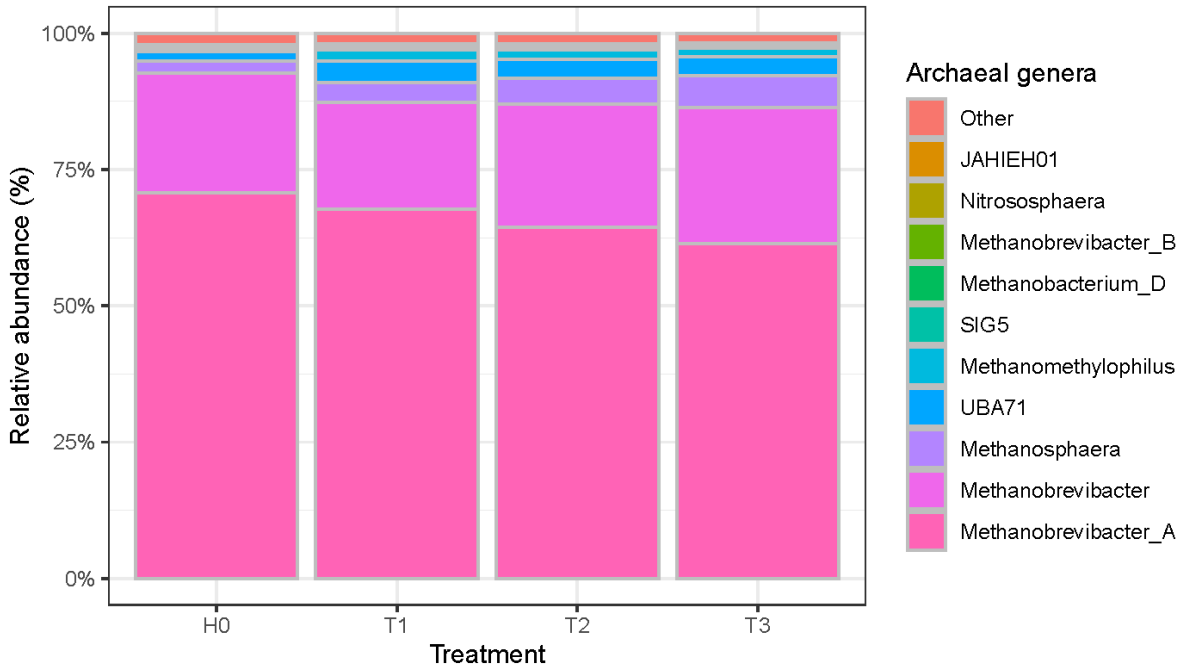

Figure 6. 18. Ruminal archaeal community composition at the genus level across the treatments (T1 = control, T2 = six events, T3 = 12 events) and original rumen sample (H0) with a relative abundance greater than 0.01%. Only the ten most relatively abundant genera are displayed.

Bacterial and archaeal diversity and richness

Alpha diversity

In Experiment 2, microbial diversity was evaluated using the same metrics applied in Experiment 1, with the addition of species-level taxa. Unlike Experiment 1, Experiment 2 demonstrated a significant treatment impact on microbial α-diversity, with pronounced differences at the phylum level for both Shannon (H) ($p = 0.0002$) and Simpson (1 - D) indices ($p = 0.001$). Pairwise comparison revealed that the microbial community diversity in terms of H and 1 - D was significantly lower in T3 than in T1 (H: $p = 0.0004$, 1 - D: $p = 0.001$) and T2 (H: $p = 0.008$,1- D: $p = 0.01$), whereas no difference was observed between T1 and T2 (H: $p = 0.14$, 1- D: $p = 0.30$). Estimated marginal means (± SE) of Shannon index were 1.44 ± 0.009, 1.41 ± 0.009, and 1.36 ± 0.009, and Simpson index were 0.66 ± 0.002, 0.65 ± 0.002, and 0.64 ± 0.002, for T1, T2, and T3, respectively, which were slightly higher than the values observed in the previous experiment. Diversity of archaeal phyla did not differ significantly in either of the indices (H: $p = 0.69$; 1- D: $p = 0.71$). At the genus level, treatments did not significantly affect bacterial community diversity, as indicated by both the Shannon and Simpson indices (p

> 0.05), a pattern that differed slightly from the trend observed in Experiment 1. For archaeal genera, the Shannon diversity index did not differ significantly among treatments ($p = 0.53$), whereas the Simpson index indicated a significant treatment effect ($p = 0.03$). According to the Simpson metric, archaeal diversity in T1 was significantly lower than in T3 ($p = 0.03$). The impacts of the treatments on diversity metrics at both the phylum and genus levels are presented in Figure 6. 19 - 6. 21, and Supplementary Figure 6S. 7. Both Shannon and Simpson diversity indices of bacterial community showed a significant treatment effect at the species level (H: $p = 0.0006$, 1- D: $p = 0.0005$). A consistent pattern across both measures showed that T2 and T3 exhibited significantly greater microbial diversity than T1, with no difference between T2 and T3 (Tukey, $p > 0.05$). Estimated Shannon diversity means (± SE) were 4.78 ± 0.02, 4.82 ± 0.02, and 4.85 ± 0.02 for T1, T2, and T3, respectively, showing the same direction of change as the Simpson index (Supplementary Figure 6S. 8). In contrast, archaeal species diversity remained stable across treatments, with no significant impact on either the Shannon ($p = 0.74$) or Simpson indices ($p = 0.76$).

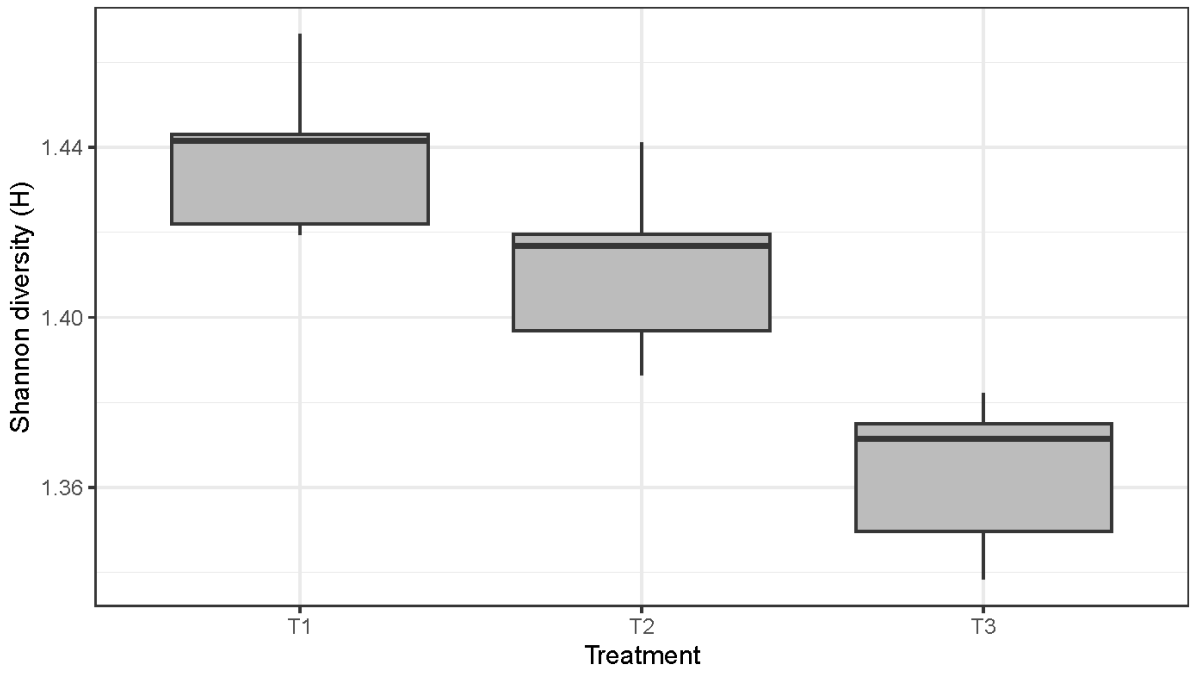

Figure 6. 19. Impact of RT fluctuation events on Shannon diversity (H) of microbial phyla. Boxplots represent the distribution of Shannon diversity across treatments (T1 = control, T2 = six events, T3 = 12 events).

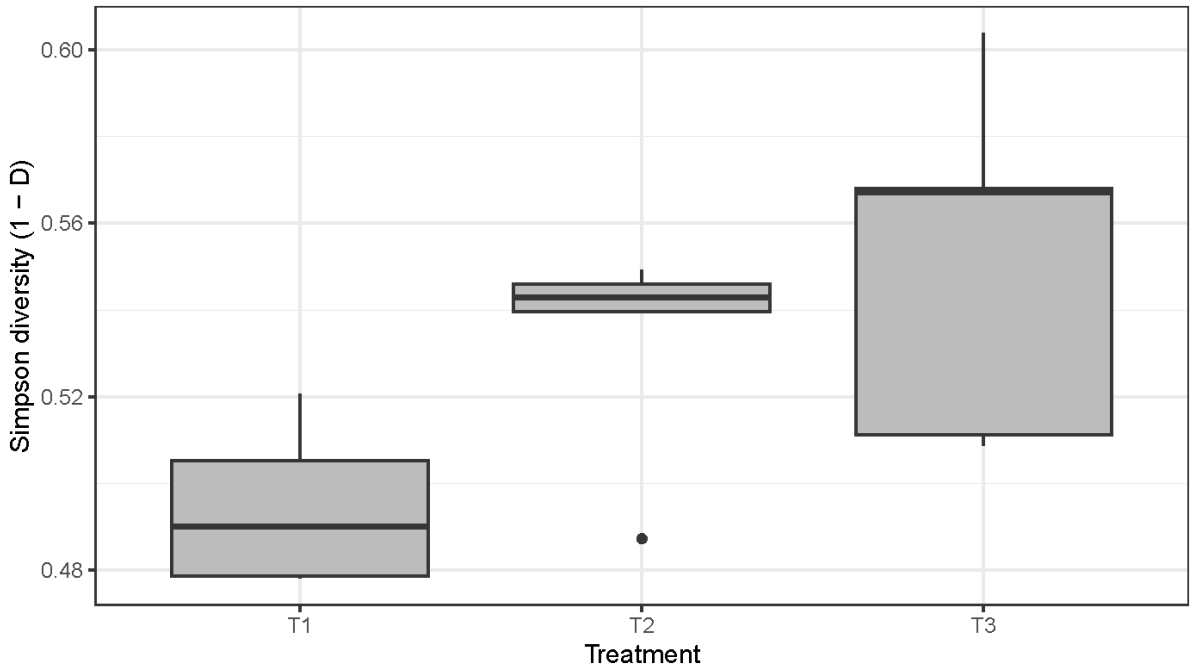

Figure 6. 20. Impact of RT fluctuation events on Simpson diversity (D) of archaeal genera. Boxplots represent the distribution of Simpson diversity across treatments (T1 = control, T2 = six events, T3 = 12 events).

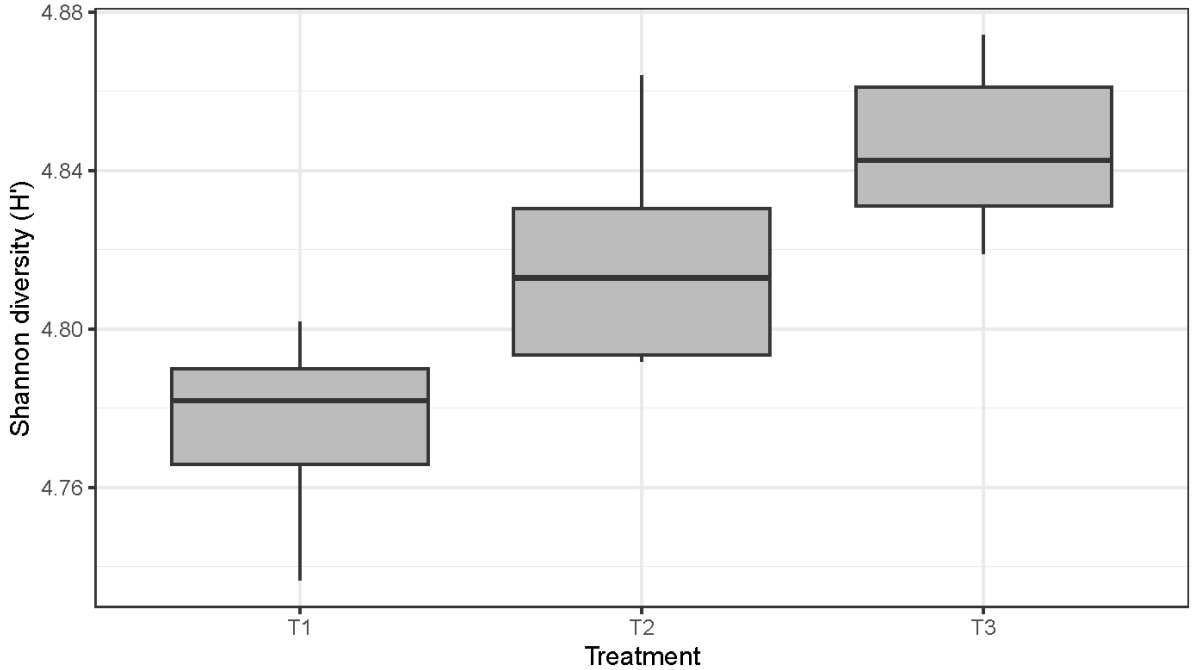

Figure 6. 21. Impact of RT fluctuation events on Shannon diversity of bacterial species. Boxplots represent the distribution of Shannon diversity across treatments (T1 = control, T2 = six events, T3 = 12 events).

Beta diversity

Unlike Experiment 1, PCoA based on Bray–Curtis dissimilarity and Aitchison distance illustrated distinct clustering of microbial communities at the phyla level among treatments in Experiment 2 (Figure 6. 22)**.** Bray–Curtis–based PERMANOVA indicated significant differences in microbial community composition (R^2 = 0.39, p = *0.04*), explaining approximately 39% of the variation among the three treatment groups. At the genus level, β-diversity also varied significantly among treatments, as indicated by both Bray–Curtis (R^2 = 0.35, p = 0.009) and Aitchison (R^2 = 0.15, p = 0.001) distance metrics (Figure 6. 23, and Supplementary Figure 6S. 9). Similarly, species-level β-diversity of bacterial and archaeal communities also differed significantly among the treatment groups, as assessed by both Bray–Curtis (Bacterial species: R^2 = 0.29, p = 0.04, Archaeal species: R^2 = 0.28, p = 0.03) and Aitchison (Bacterial species; R^2 = 0.18, p = 0.002, Archaeal species: R^2 = 0.10, p = 0.002) methods (Figure 6. 24, Figure 6. 25, and Supplementary Figure 6S. 10). In all cases, multivariate dispersion did not differ significantly (Supplementary Figure 6S. 11 - 6S. 13) and Supplementary Table 6. 1 - 6. *6*).

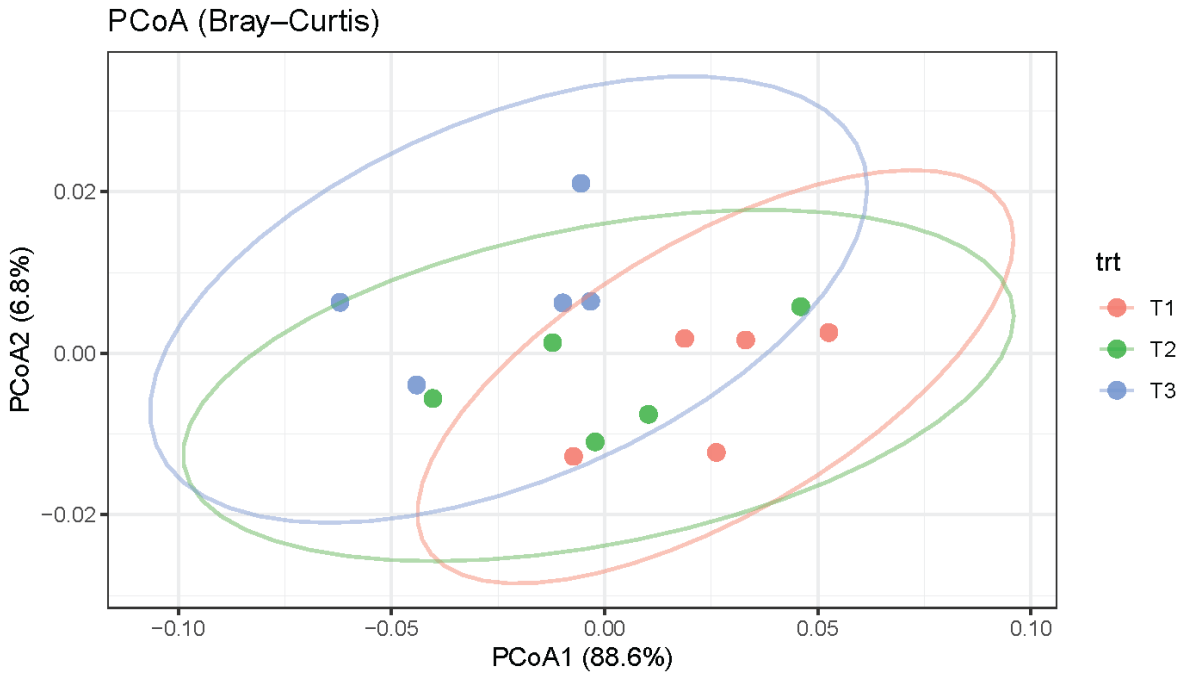

Figure 6. 22. PCoA of microbial phyla composition based on Bray–Curtis dissimilarity, where each point represents a sample under three different treatments (T1 = control, T2 = six events, T3 = 12 events). The first two principal coordinates explain 88.6% and 6.8% of the variance, respectively. PERMANOVA indicated a significant effect of treatment on community composition, while betadisper analysis confirmed homogeneous dispersion (P > 0.05).

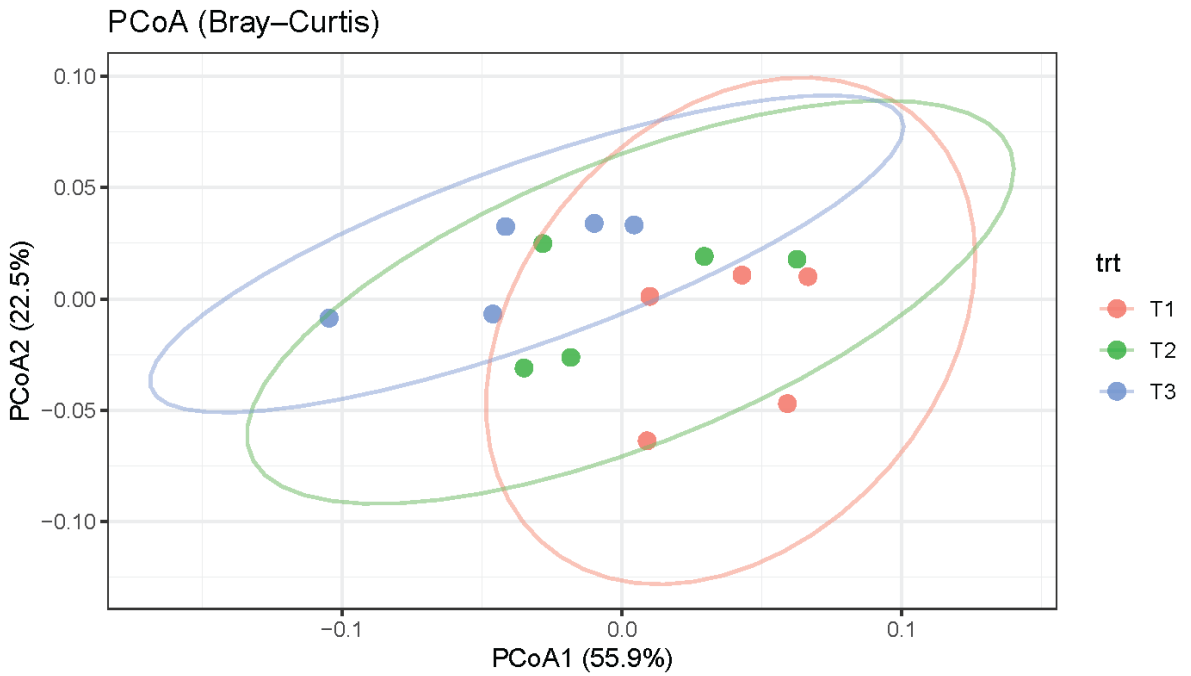

Figure 6. 23. PCoA of microbial community composition based on Bray–Curtis dissimilarity at the genus level. Ellipses represent 95% confidence intervals for the treatments (T1 = control, T2 = six events, T3 = 12 events). PERMANOVA showed a significant treatment effect while betadisper analysis confirmed homogeneous dispersion ($P > 0.05$).

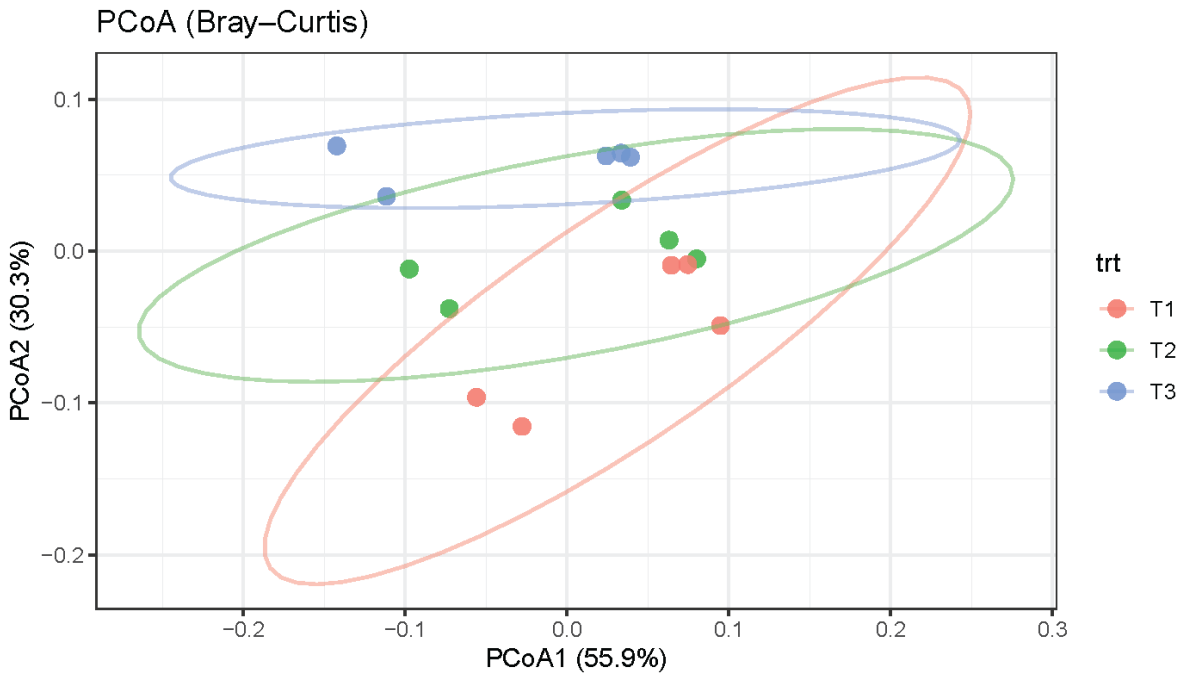

Figure 6. 24. PCoA of bacterial community composition based on Bray–Curtis dissimilarity at the species level. Ellipses represent 95% confidence intervals for the treatments (T1 = control, T2 = six events, T3 = 12 events). PERMANOVA showed a significant treatment effect while betadisper analysis confirmed homogeneous dispersion ($P > 0.05$).

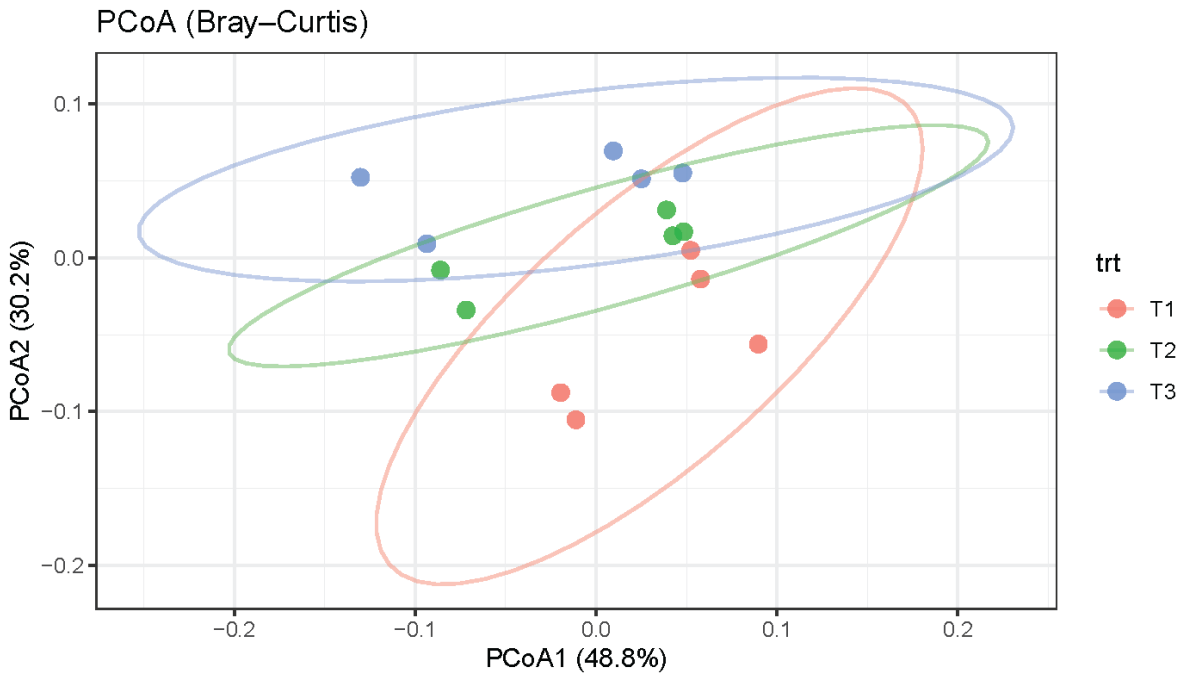

Figure 6. 25. PCoA of archaeal community composition based on Bray–Curtis dissimilarity at the species level. Ellipses represent 95% confidence intervals for the treatments (T1 = control, T2 = six events, T3 = 12 events). PERMANOVA showed a significant treatment effect while betadisper analysis confirmed homogeneous dispersion ($P > 0.05$).

Differentially abundant microbes

While Experiment 1 did not identify any differentially abundant phyla, several phyla were detected as differentially abundant in Experiment 2. Differential abundance analysis (DESeq2, FDR < 0.05) identified five differentially abundant phyla between T3 and T1 (Figure 6. 26), all of which belonged to the Bacillota lineage. In addition, two phyla (Bacillota_A and Bacillota_I) were differentially abundant in T3 vs T2, in contrast to no phyla being detected as differentially abundant in T2 vs T1.

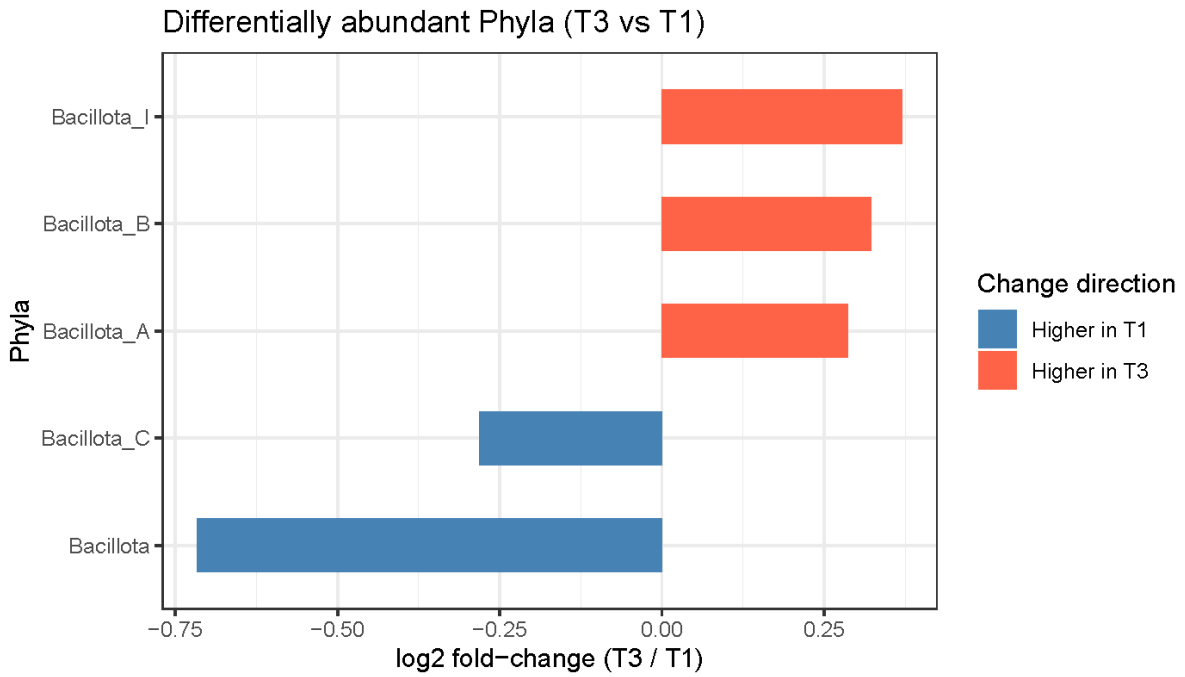

Figure 6. 26. Differentially abundant phyla between T3 (12 events) and T1(control) identified by DESeq2 (FDR < 0.05). Bars represent log2 fold changes in relative abundance (T3/T1), with positive values indicating higher abundance in T3 and negative values indicating higher abundance in T1.

Differential abundance analysis revealed treatment-specific shifts in several microbial genera in Experiment 2 compared to Experiment 1. A total of 38 microbial genera were differentially abundant between T3 and T1, and six genera between T3 and T2 (UBA2943, Limosilactobacillus, RUG11272, UBA3792, UBA4246, Vescimonas), again with no differences between T2 and T1 (Figure 6. 27 & Figure 6. 28). The pattern was even more pronounced at the species level, where 59 microbial species differed significantly between T3 and T1 (Figure 6. 29), and six species between T3 and T2. A similar trend was observed in Archaeal species, with eight species found to be differentially abundant in the T3 vs T1 contrast (Figure 6. 30).

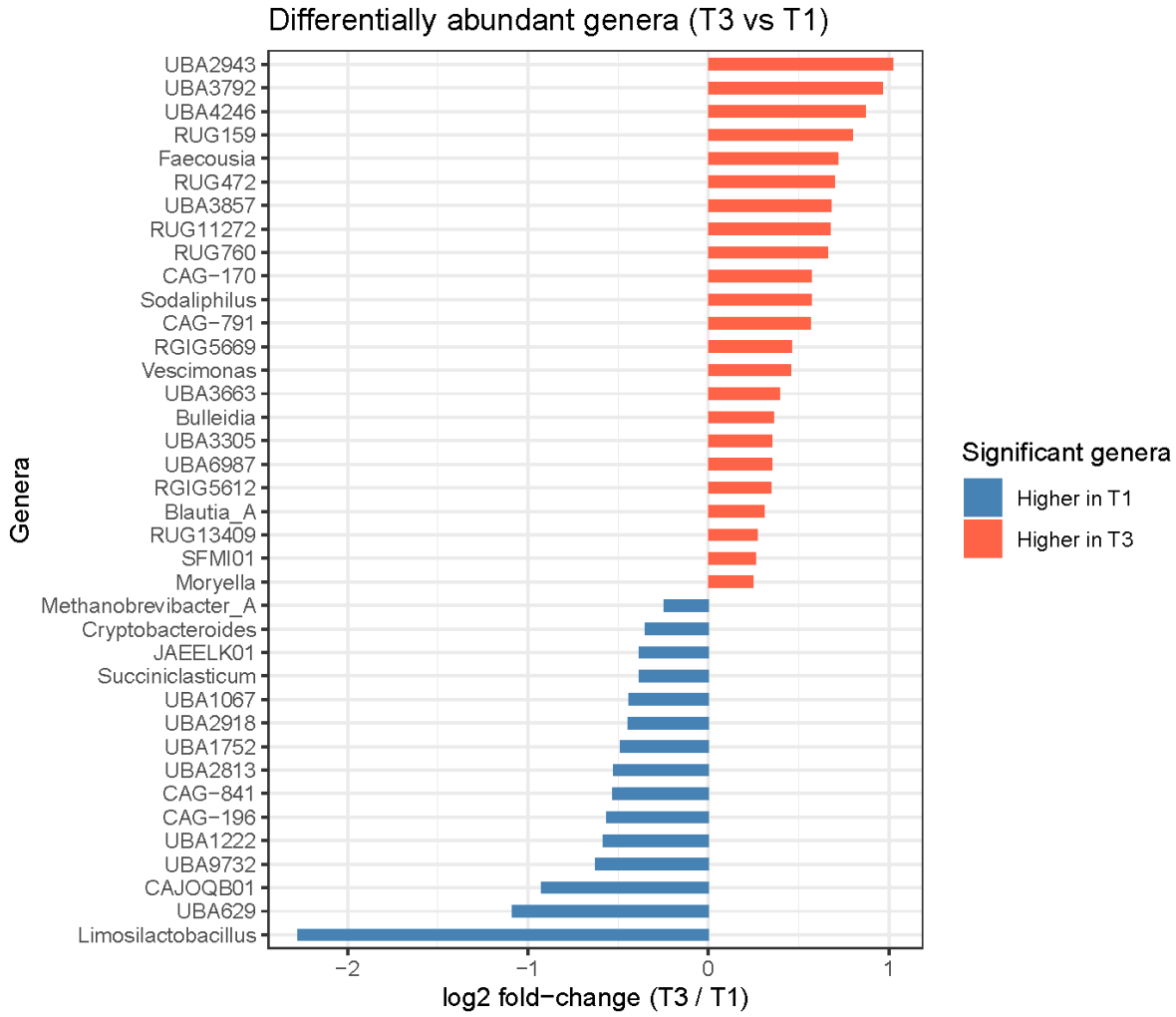

Figure 6. 27. Differentially abundant microbial genera between T3 and T1 identified by DESeq2 (FDR < 0.05). Bars represent log2 fold changes in relative abundance (T3/T1), with positive values indicating higher abundance in T3 (12 events) and negative values indicating higher abundance in T1 (control).

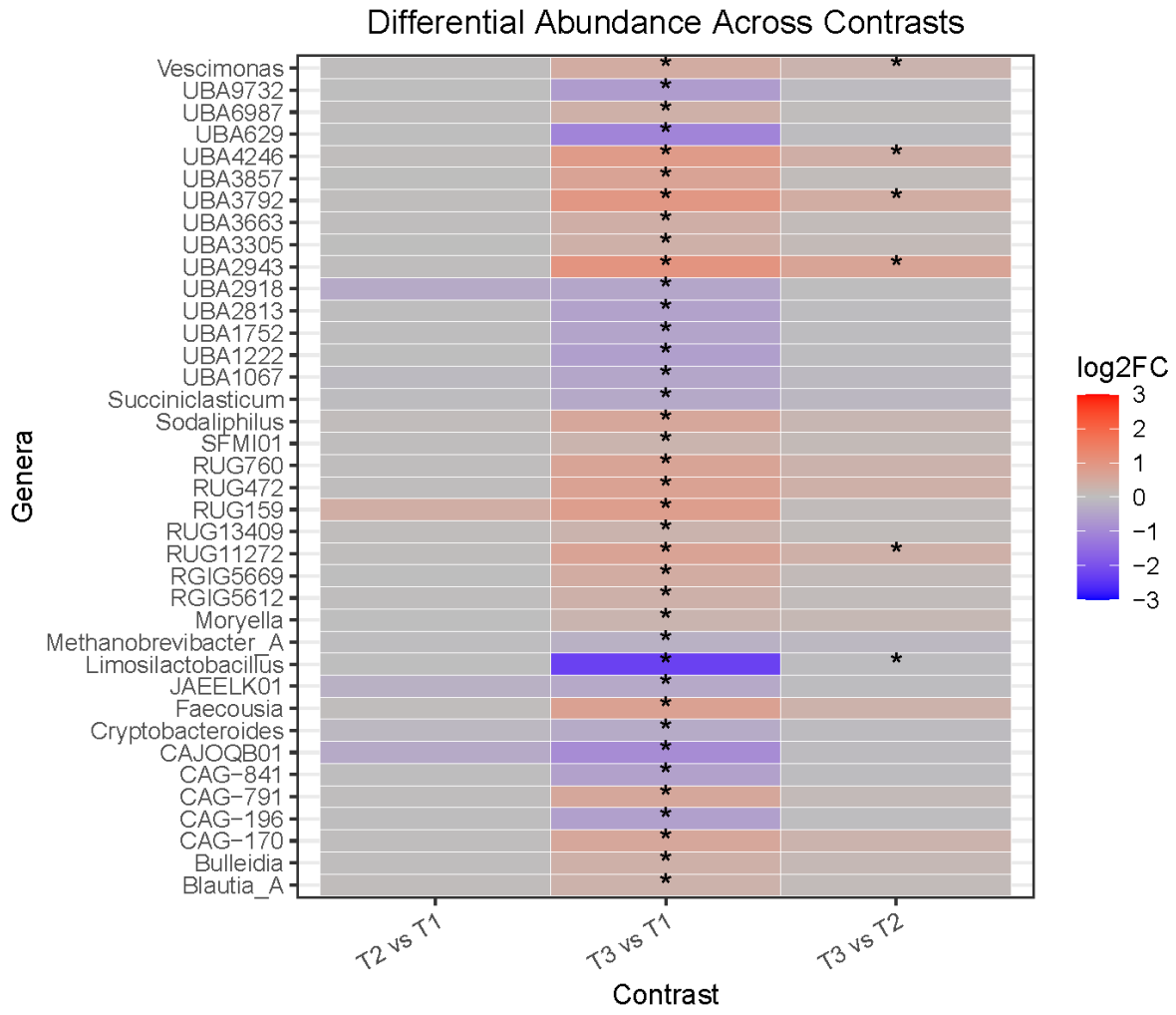

Figure 6. 28. The heatmap displays log2 fold-changes in genus-level abundance across treatment contrasts, with colour intensity indicating the magnitude of change (red = increased, blue = decreased). Asterisks (*) denote genera that were significantly differentially abundant at FDR < 0.05 (Benjamini-Hochberg adjusted p-value).

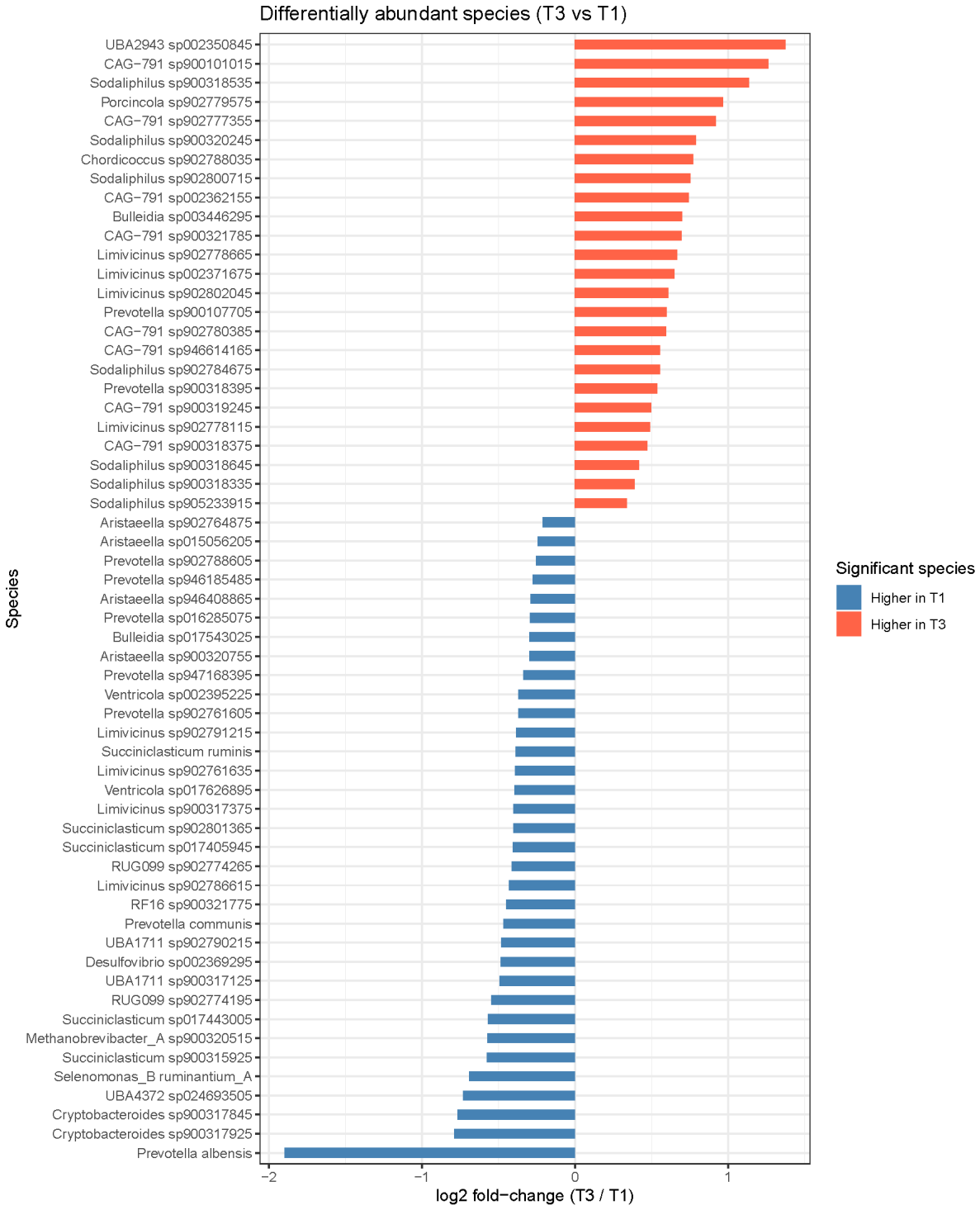

Figure 6. 29. Differentially abundant microbial species between T3 (12 events) and T1 (control) identified by DESeq2 (FDR < 0.05). Bars represent log2 fold changes in relative abundance (T3/T1), with positive values indicating higher abundance in T3 and negative values indicating higher abundance in T1.

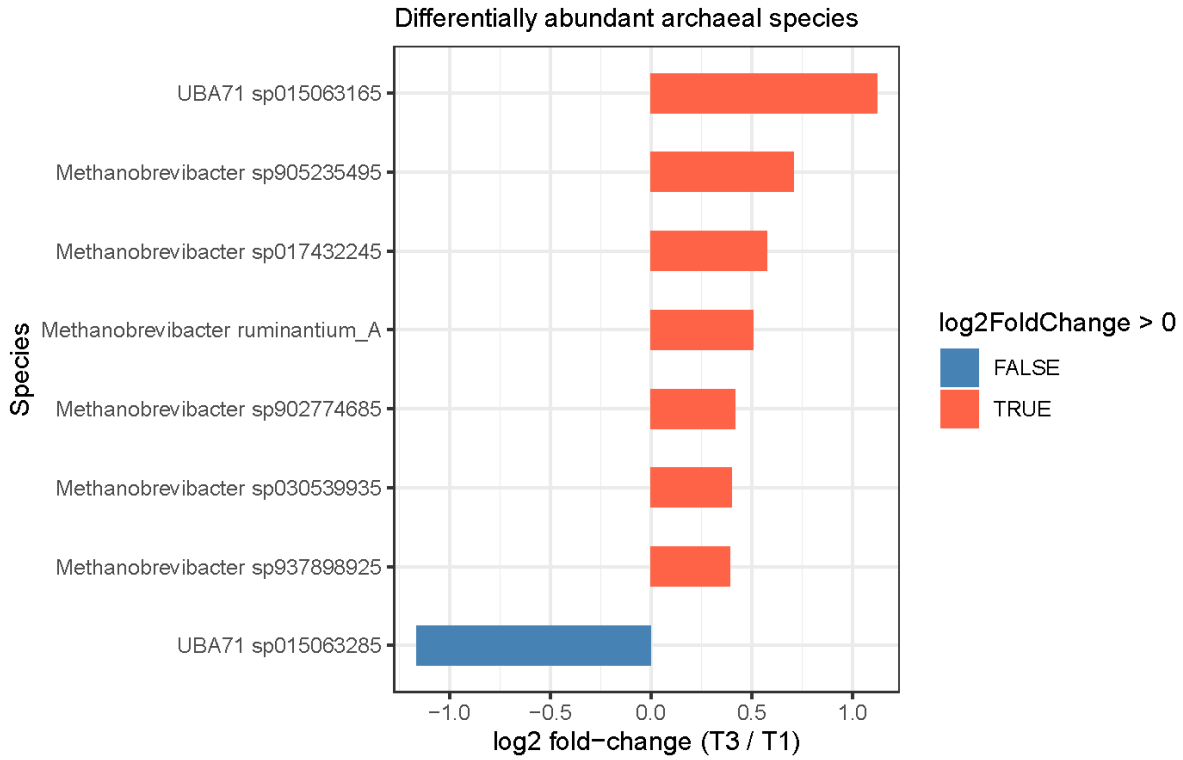

Figure 6. 30. Differentially abundant archaeal species between T3 (12 events) and T1(control) identified by DESeq2 (FDR < 0.05). Bars represent log2 fold changes in relative abundance (T3/T1), with positive values indicating higher abundance in T3 and negative values indicating higher abundance in T1.

Correlation analysis

The strength and direction of correlations between fermentation parameters and microbial phyla abundance varied considerably between Experiment 1 and Experiment 2. The correlations between fermentation parameters and the relative abundance of microbial phyla (RA > 0.01%) are visualised in heatmaps (Figure 6. 31 - Figure 6. 33). In the control treatment (T1), almost all phyla showed a strong negative correlation with iso-valeric, iso-butyric, valeric, hexanoic acid, and NH_3, and a moderate to weak correlation with acetic, propionic, butyric, and total VFAs. In contrast, gas production and CO_2 were positively correlated with all phyla except Bacillota. Bacillota showed a strong negative correlation with methane traits; however, several other phyla, including methanogens, were positively associated with methane concentration and total methane (Figure 6. 31).

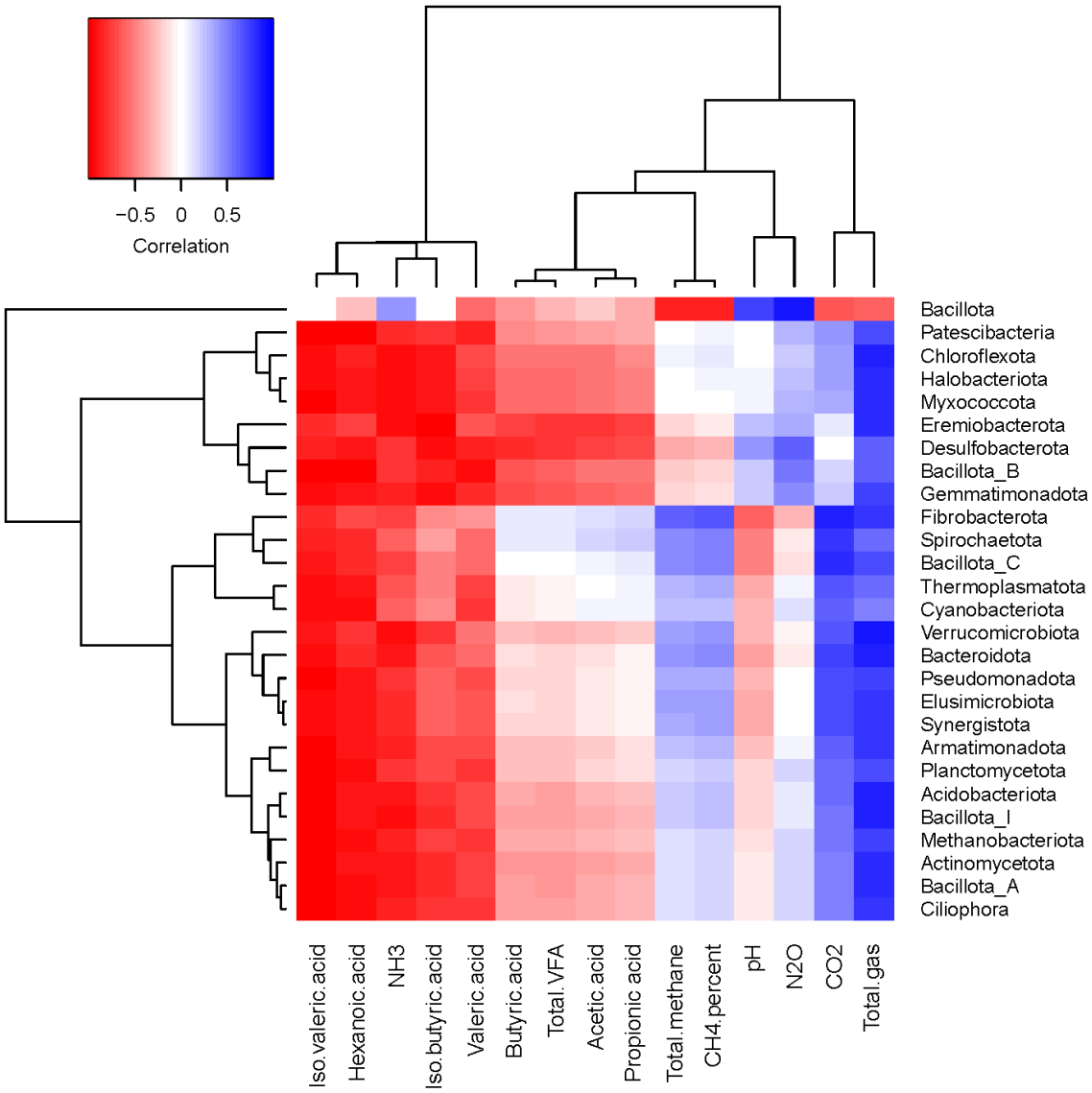

Figure 6. 31. Correlation heatmap showing relationships between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T1** (control). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

In Treatment 2 (six events), gas production, including CO_2, methane variables, and some VFAs (propionic and hexanoic acid) showed moderate to strong positive correlation with almost all phyla. In contrast, valeric, iso-valeric, iso-butyric, and NH_3 exhibited a moderate negative association with all phyla. In line with this trend, pH showed a strong negative correlation with a few phyla, including Fibrobacterota, Thermaplasmatota, and Cyanobacteriota. All other fermentation parameters had weak or mixed correlations with the phyla listed in Figure 6. 32.

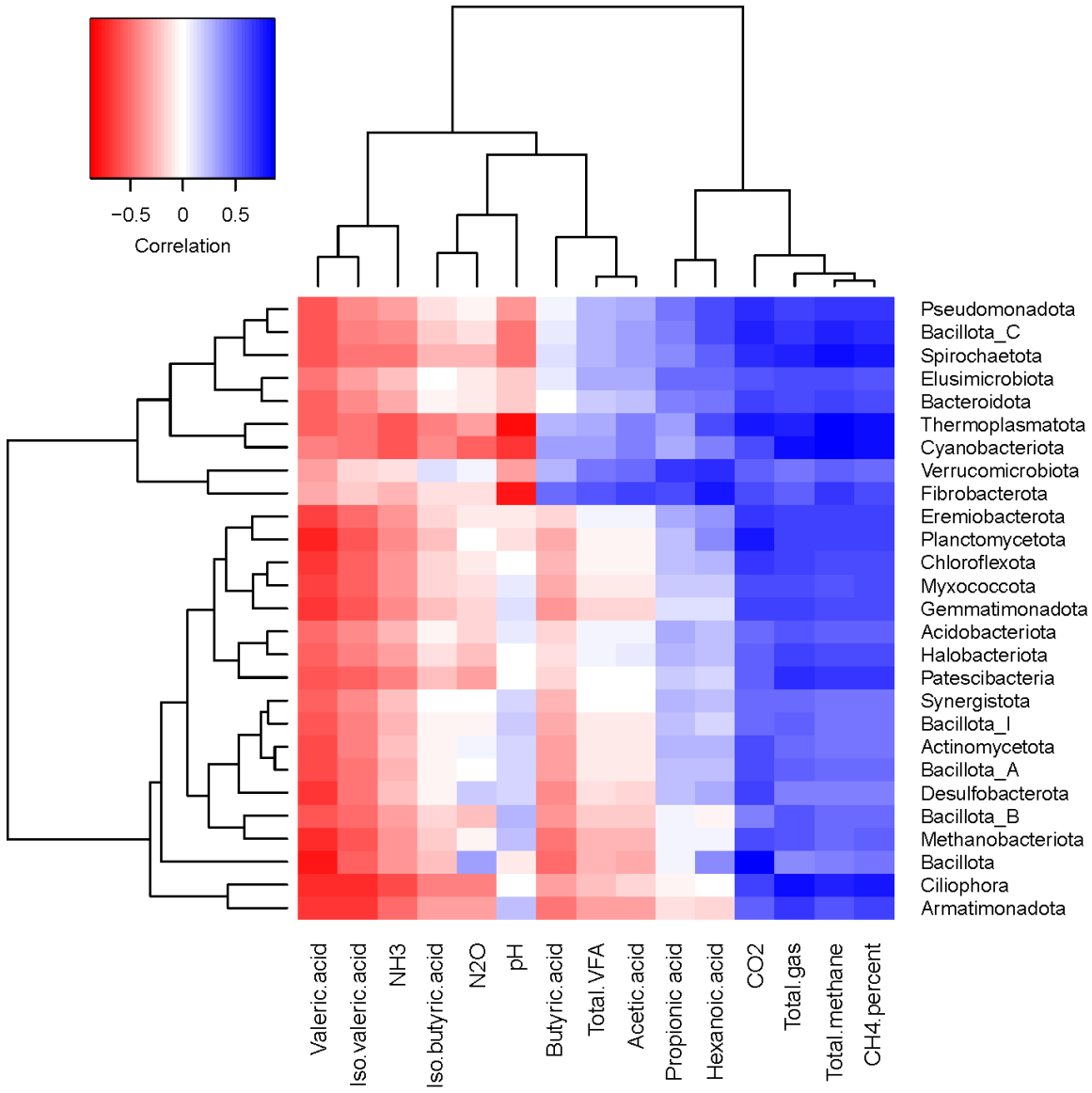

Figure 6. 32. Correlation heatmap showing associations between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T2** (six events). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

A comparable pattern was observed in Experiment 2, with fermentation parameters showing a greater positive association with microbial phyla abundance, similar to the trend in Experiment 1. In treatment T3 (12 events), Cyanobacteriota showed the highest positive correlations with most VFAs, such as propionic acid, acetic acid , hexanoic acid, and total VFA. Fibrobacterota was strongly correlated with hexanoic acid and propionic acid. In addition, Gemmatimonadota and Thermoplasmatota showed consistent positive correlations with iso-

valeric acid. Halobacteriota and Gemmatimonadota strongly correlated with total methane, and Actinomycetota and Bacillota_B also showed positive associations with methane. All other fermentation parameters, such as iso-butyric acid, gas production, pH, N_2O, and CO_2, showed weak or mixed correlation with almost all listed phyla in Figure 6. 33.

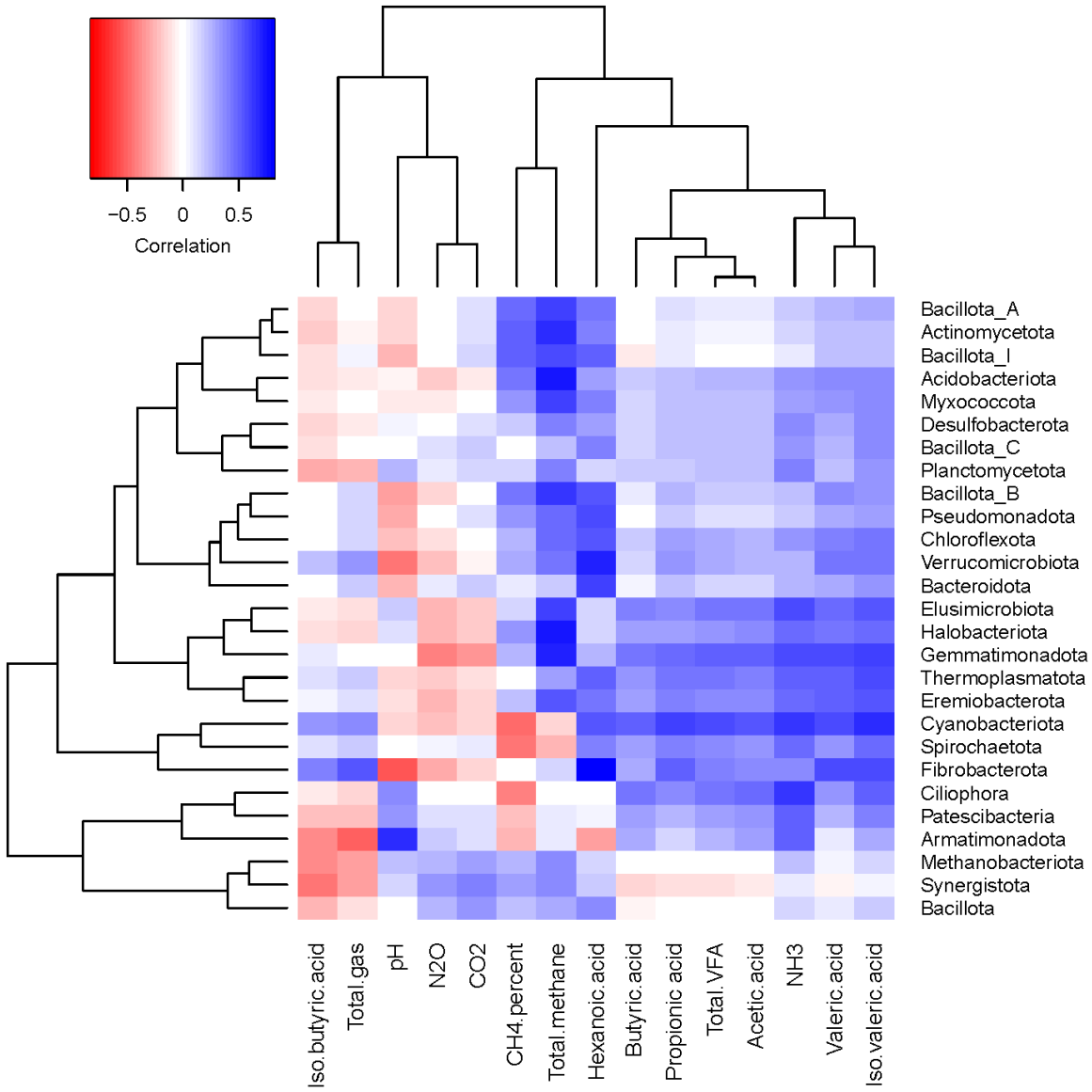

Figure 6. 33. Correlation heatmap showing associations between dominant rumen bacterial phyla (relative abundance > 0.01%) and fermentation parameters under treatment **T3** (12 events). The colour scale represents Pearson correlation coefficients, with blue indicating positive and red indicating negative correlations. Dendrograms show hierarchical clustering of fermentation traits (x-axis) and microbial phyla (y-axis) based on similarity in correlation profiles.

Discussion

Given that the fermentation conditions were generally uniform, any variations in fermentation end products or microbial community diversity among treatments presumably reflect the microbial response to recurrent RT fluctuations in the fermentation jars. Furthermore, because the cows' ration changed from a kale-silage mixture to ryegrass between experiments, substrate types were adjusted to ensure alignment between the inoculum and the substrate during the *in vitro* fermentation. Consequently, results between treatments within experiments are compared quantitatively, while results between experiments are compared qualitatively rather than quantitatively.

We hypothesised that frequent fluctuations in RT would impair fermentation efficiency and alter microbial community structure. Based on the results of the current study, we cautiously accept the general hypothesis and interpret our results within that premise. In the current study, we observed that frequent RT fluctuations (T3) through simulated drinking events significantly influenced certain fermentation traits, such as production of total gas, total CH_4, iso-valeric acid in Experiment 1, and concentration of N_2O, NH_3, propionic acid, and hexanoic acid in Experiment 2, without impacting overall substrate degradability in both experiments. Since this study is the first to investigate RT fluctuations induced by simulated drinking events and their effects on fermentation characteristics, direct comparison with existing literature is challenging. However, the total gas (mL/g DM) in Experiment 2 of the current study aligns with previous observations of 48-hour gas production in an *in vitro* fermentation by de Jong et al. (2025), whereas the total gas in Experiment 1 was less than half of the 24-hour gas production reported by Daza et al. (2019). The markedly higher total gas production observed under treatment T3 in Experiment 1 (112.5 ± 4.46 mL/g DM) relative to Experiment 2 (59.7 ± 3.77 mL/g DM) is likely due to differences in substrate composition, with Experiment 1 utilising a kale–silage mixture and Experiment 2 employing solely ryegrass. Furthermore, the consistently higher dry matter (DM) degradability across treatments in Experiment 1 than in Experiment 2 aligns with the elevated gas production, given the positive correlation between apparent degradability and gas volume (Blummel et al., 1997). Since total CH_4 (mL/g DM) was derived from total gas (mL/g DM), the observed differences in CH_4 output might be attributable to this calculation. Methane concentration (CH_4/ total gas %) remained consistent across treatments in both experiments, suggesting that frequent temperature fluctuations did not affect methanogenesis. However, Duarte et al. (2017) reported that reducing fermentation temperature from 39 °C to 35 °C in the RUSITEC system for 7 days decreased CH_4 production

by 43%, with altered microbial diversity, only in rumen liquid-associated microbes. The fact that the outcomes of gas production and fermentation output from various *in vitro* studies are often difficult to compare due to several confounding factors such as the protocol of rumen fluid collection and treatment (Cornou et al., 2013), the specific buffer used (Patra & Yu, 2013), proportion of feed sample size, fermentation fluid, and headspace volume (Ramin & Huhtanen, 2012). It is important to note that background gas correction could not be applied in Experiment 1 because no blank fermentation jars were available, whereas such blanks were included in Experiment 2 to account for background gas production. Moreover, since the gas production modules RF1 used in Experiment 1 were no longer available during Experiment 2, an updated version, RF1X, was introduced in the laboratory and used in Experiment 2. For these reasons, rather than quantifying and comparing absolute gas volumes and fermentation parameters, we examined directional trends in fermentation responses across different frequencies of RT fluctuations. As such, total gas and CH_4 production changed in a substrate-dependent manner with increased frequency of RT fluctuations. These findings suggest that variations in RT due to drinking may be negatively associated with fermentation efficiency in forage-based diets rich in structural carbohydrates than in diets containing more rapidly fermentable carbohydrates. This highlights the need to account for dietary composition when assessing the physiological and microbial implications of drinking behaviour in cattle.

In Experiment 2 (with ryegrass), NH_3 and N_2O concentrations were significantly higher in T1 (control treatment) compared to treatments with frequent RT drops (T3), whereas no differences were observed in Experiment 1 (with kale–silage). These contrasting nitrogen results between experiments might be attributable to the differences in substrate composition. In Experiment 1, NH_3, N_2O, and urea did not differ significantly among treatments with varying RT fluctuation frequencies. This outcome likely reflects the ample baseline availability of rapidly degradable nitrogen and mixed carbohydrate sources, which masked the relatively transient impacts of RT fluctuations over the 26-hour incubation period. In Experiment 2, elevated levels of NH_3 and N_2O in the T1 (control), compared to repeated RT fluctuation treatments (T2 & T3), suggest that frequent fluctuations in incubation temperature may improve microbial nitrogen utilisation and redox balance, as demonstrated by elevated propionate levels and reduced CO_2 at 6 and 12 RT fluctuation events. It indicates that under frequent RT fluctuations, microbes directed more energy towards propionate production than towards N_2O formation. Overall, these findings suggest that temperature fluctuations do not enhance proteolysis or deamination; rather, they might lead to substrate-dependent adjustments

in fermentation pathways. Concentrations of minor VFAs (Iso-butyric acid and Hexanoic acid) differed between treatments and across experiments, indicating interactions between substrate composition and the frequency of RT fluctuations. Iso-butyric acid, which originates from valine (a Branched-chain amino acid, BCAA) degradation (Allison, 1969) showed the highest concentration under the control treatment in Experiment 1, suggesting uninterrupted BCAA fermentation in the absence of RT fluctuations. Frequent temperature fluctuations might temporarily suppress protease and deaminase activities, reducing BCAA catabolism during recovery phases, in accordance with findings that sub-optimal temperatures can depress enzymatic activity and slow amino acid turnover (Jo et al., 2024; Russell & Cook, 1995). In contrast, in Experiment 2, hexanoic acid concentration was the highest under the frequent RT treatment (T3). This pattern aligned with higher propionate and lower CO_2, NH_3, and N_2O, indicating a more reduced fermentation environment and improved nitrogen assimilation. Under such electron-rich conditions, some rumen bacteria (e.g., *Clostridium* spp.) carry out reverse β-oxidation, extending short-chain volatile fatty acids into longer-chain compounds such as n-hexanoic acid (Angenent et al., 2016). This metabolic flexibility highlights the rumen microbiome's dynamic ability to redirect intermediary metabolism and maintain overall fermentation efficiency during transient thermal stress (Janssen, 2010; Morgavi et al., 2010; Newbold & Ramos-Morales, 2020). In the current study, we applied the method of simulating drinking events developed by Rahman et al. (2025) for the first time, which was essentially a batch-type fermentation system with fermentation jars fitted with an ANKOM RF gas production system (closed system). Although batch fermentation is simple, easy to operate, and widely used in fermentation studies, this system does not accurately reproduce the continuous flow of digesta, dilution rate, and microbial washout dynamics of the rumen, resulting in fermentation outcomes that differ from those in RUSITEC (Czerkawski & Breckenridge, 1977; Shaw et al., 2023). Consequently, to more accurately assess the impact of drinking-induced RT fluctuations on fermentation kinetics and metabolites, future research should integrate controlled RT-fluctuation simulations within a RUSITEC platform.

In this study, the Ørskov and McDonald (1979) model parameter representing initial gas volume (parameter *a*) was negative for all treatments. This is expected in closed, pressure-based systems like the ANKOM RF gas production unit, where the initial headspace pressure is negative and gradually becomes positive as fermentation progresses (Figure 6. 2 & Figure 6. 14) with continued degradation of structural and non-structural carbohydrates producing VFAs and gases (Dijkstra, 1994). Other than the control treatment (T1), the initial RT fluctuation

events of T2 (two/six events) and T3 (12 events) were created during the early phase of incubation; therefore, the parameter *a* is not a critical issue in the current study context. However, the lag phase of the gas production curve, which indicates the time required for hydration, stabilisation, and microbial colonisation of insoluble substrates (Beuvink & Kogut, 1993), was shorter in Experiment 1 than in Experiment 2 (Figure 6. 2 & Figure 6. 14) 2. This variation may be attributed to the chemical composition of the substrates and the rumen inoculum used, as these factors may affect colonisation duration by influencing microbial activity (Nagadi, 2000). Brassica forages contain higher levels of readily fermentable carbohydrates and exhibit greater digestibility (Barry, 2013) compared to perennial ryegrass, which might have triggered the start of fermentation, which aligns with this observed trend (Figure 6. 2 & Figure 6. 14).

Theoretical asymptotic gas production (*b*) was significantly higher under the most frequent RT fluctuation treatments (T3) in both experiments (Supplementary Figure 6S. 1 & 6S. 2), despite a lower fractional rate constant (*c*) of gas production. In a separate study, conducted within the same laboratory using the ANKOM RF gas production system, but with incubation carried out in an oscillating incubator over 48 hours, de Jong et al. (2025) reported *b* and *c* parameters for the ryegrass control treatment that closely matched those observed in T1 (control) and T2 (six event treatment) of Experiment 2 in the present study. However, in T3 (12 event treatment), the current study recorded a substantially higher theoretical asymptote (*b*: 118.4 vs 71.7) and a markedly lower fractional rate constant (*c*: 0.0275 vs 0.0443). Daza et al. (2019) conducted an *in vitro* fermentation using various brassica crops and reported an asymptotic gas production value of 261 mL/g DM for kale forage. This is comparable to the value observed in T3 (12 event treatment) of Experiment 1 in the present study, where the kale and silage mixture produced (asymptotic gas production) 253 ml/g DM (Table 6. 3). Petersen et al. (2016) reported a lower rate of gas production at 31°C than at 39°C during a 48-hour *in vitro* fermentation. However, it is important to note that the *in vitro* methodology, gas collection techniques, and analytical procedures employed by Daza et al. (2019) and Petersen et al. (2016) differed from those used in the current study. Additionally, the *b* and *c* parameters of T1 (control) and T2 (two events treatment) obtained in Experiment 1 were consistent with those observed in Experiment 2. This pattern indicates that repeated temperature drops delayed early gas production but ultimately promoted greater cumulative fermentation. The lower *c* values suggest that microbial activity was initially suppressed or slowed during the early phase due to sudden reductions in incubation temperature, which can temporarily decrease enzyme activity

and substrate hydrolysis rates (Russell & Cook, 1995). The fact that the observed gas production curves did not reach their asymptotes supports the notion of a delayed but extended fermentation phase across the treatments. This trend likely reflects a metabolic adjustment in which microbial communities adapt to environmental stress by maintaining activity over a longer period (Guan et al., 2017). However, to confirm these gas-kinetics trends in response to drinking-associated RT fluctuations, further investigation with different substrates under similar temperature-fluctuation treatments, and with similar substrates under multiple RT-fluctuation treatments over an extended period of 36 or 48 hours, is suggested in both batch-type, continuous and semi-continuous fermentation systems.

Given that a few fermentation parameters differed across treatments, we next investigated whether temperature fluctuations triggered shifts in microbial community structure. Microbial diversity and composition were evaluated using α-diversity (Shannon and Simpson indices), β-diversity (Bray–Curtis dissimilarity and Aitchison distance), and differential abundance analyses, supplemented by correlations between microbial phyla abundance and fermentation parameters. This multi-level approach enabled us to determine whether the observed stability in fermentation results indicated true functional resilience or compensatory alterations within bacterial and archaeal communities. Although the relative abundance of microbial taxa was estimated in both the original rumen sample (H0) and samples subjected to RT fluctuation treatments, we excluded H0 from subsequent diversity analysis to visualise the treatment-driven impacts.

Evidence suggests that dietary composition can alter taxon abundance by up to 50% in cattle (de Melo et al., 2022; Henderson et al., 2016). Therefore, the differences in taxon abundance and community diversity observed between the two experiments in this study may be attributed to the substrates used. At the domain level, the mean RA of Bacteria was slightly higher in Experiment 2 (98.5%) than in Experiment 1 (97.6%), and a similar trend was observed in the Archaeal domain. This difference and proportion were expected given that the archaeal domain is much less diverse than the bacterial domain (Henderson et al., 2015). At the phylum level, Bacillota_A accounted for more than 60% of the total microbial community across all treatments in Experiment 1 (kale–silage substrate) (Figure 6. 4), consistent with the dominance of fibre and protein-fermenting Firmicutes under diets rich in structural carbohydrates and soluble nitrogen (Patel et al., 2014; Zhang et al., 2023). In Experiment 2 (with ryegrass substrate), Bacteroidota (Bacteroidetes) alone represented around 40% of the total microbial genera (Figure 6. 16), indicating a transition towards carbohydrate-specific taxa such as

Prevotella, which are effective in degrading hemicellulose and soluble carbohydrates (Dao et al., 2021). At the genus level, CAG-791 was predominant in Experiment 1 (Figure 6. 5). This unclassified genus, commonly associated with peptide and amino acid fermentation (Zeng et al., 2022) within Bacillota (Firmicutes) lineages, aligns with the elevated iso-butyrate production observed under the control treatment in Experiment 1. On the other hand, Prevotella was the most abundant genus across treatments in Experiment 2 (Figure 6. 17), consistent with its central role in carbohydrate breakdown and propionate formation under readily fermentable substrates (Dao et al., 2021). The dominance of Prevotella coincided with higher propionate and hexanoate concentrations in the treatment with frequent temperature fluctuations (T3), suggesting a link between microbial community structure and shifts in fermentation pathways. Within the archaeal community, Methanobrevibacter and its related lineage, Methanobrevibacter_A, were dominant in Experiments 1 and 2, respectively, suggesting functional consistency in hydrogenotrophic methanogenesis (using H_2 as a substrate for reducing CO_2) despite taxonomic variation. Turnover within the Methanobrevibacter complex has been attributed to slight variations in hydrogen availability and shifts in the composition of associated archaeal partners (Wang et al., 2024). Overall, substrate type predominantly influenced the dominant microbial taxa, but temperature fluctuations induced slight modifications, enabling core fermentative and methanogenic groups to maintain functional stability. Employing time-resolved (repeated sampling at multiple defined time points) multi-omics approaches might be crucial for elucidating mechanistic links between RT fluctuations and shifts in microbial community composition, metabolic pathways, and fermentation efficiency in future studies.

The frequency of RT fluctuations affected microbial diversity and community structure to varying degrees across the treatments between experiments. In Experiment 1, Shannon and Simpson indices did not differ at the phylum level, with a marginal decline at the bacterial genus level ($p = 0.06$) under T3 (12 event treatment), suggesting that overall microbial diversity remained relatively stable. The higher Shannon and Simpson diversity in T1, when compared with T3 for archaeal genera, indicates that methanogenic communities were more sensitive to short-term RT fluctuations than bacterial populations. Similar domain-specific thermal sensitivity has been reported in RUSITEC systems, with lower diversity and richness observed in liquid-associated microbial samples incubated at 35 °C (Duarte et al., 2017). However, β-diversity analyses based on Bray–Curtis dissimilarity and Aitchison distance (PCoA ordination) showed no apparent clustering among treatments (Figure 6. 9 & Figure 6. 10),

suggesting that while minor compositional adjustments occurred, the overall microbial community structure was conserved. This stability supports previous findings that rumen microbiota maintains a core functional structure under environmental stress, likely due to high functional redundancy among bacterial and archaeal groups (Weimer, 2015; Zhao et al., 2019). The identification of *Streptococcus* as the only differentially abundant genus between the T2 (2-event treatment) and T3 (12-event treatments) further supports a resilient yet adaptable community capable of maintaining fermentation activity despite transient thermal stress.

Whereas, in the second Experiment, Shannon and Simpson diversity indices of microbes at the phylum level decreased significantly with increasing frequency of RT fluctuations, indicating reduced richness and evenness under frequent cooling (Figure 6. 19). Such diversity loss is commonly associated with selective enrichment of thermotolerant taxa within dominant rumen phyla, particularly Bacillota (Firmicutes) (Henderson et al., 2015). However, Archaeal phyla remained unaffected, suggesting functional stability in the methanogenic domain. Bacterial diversity at the genus level remained statistically similar across treatments. In contrast, Simpson diversity in archaeal genera under the six (T2) and 12 RT fluctuation events indicated a more even distribution of methanogens, suggesting the usual dynamic nature of microbial adaptation (Henderson et al., 2015). At the species level, significant differences in Shannon and Simpson indices indicated fine-scale taxonomic change within an otherwise stable microbial community. Consistent with these α-diversity shifts, β-diversity analysis (Bray–Curtis and Aitchison PCoA) revealed distinct clustering of bacterial and archaeal taxa across treatments, confirming that repeated temperature fluctuations triggered compositional restructuring. Differential abundance analysis revealed five responsive phyla (Figure 6. 26), all within the Bacillota lineage, indicating diversification within the lineage rather than broad taxonomic shifts. At the genus level, 38 genera, and at the species level, 59 species were identified as differentially abundant taxa between the T3 (frequent-drop treatments) and T1 (control treatment). However, most were unclassified or candidate taxa, which was not unexpected given that more than 80% of rumen microbial lineages lack cultured representatives (Cammack et al., 2018; Henderson et al., 2015). Although these unclassified taxa cannot be linked to specific functions, their consistent response to temperature fluctuations suggests they may represent functionally important yet taxonomically unclassified groups, potentially involved in intermediate fermentation steps or syntrophic interactions. Overall, these findings indicate that short-term RT fluctuations reduced diversity at higher taxonomic levels but enhanced evenness and functional compensation among adaptive taxa, allowing the rumen

microbiome to maintain fermentation efficiency under variable RT conditions. In future studies, revealing the molecular mechanisms by which differentially abundant taxa, including unclassified groups, respond to varying levels of thermal stress may enable the identification of functionally relevant microbial biomarkers to predict and improve rumen fermentation efficiency under dynamic conditions.

The correlation analysis revealed the relationships between gas production, rumen fermentation characteristics, and rumen microbial composition. To avoid unnecessary complexity, we focused on major biological patterns and directional shifts across the treatments. In Experiment 1, under control treatment (T1), methane, NH_3, urea, and branched-chain VFAs showed negative correlations with major bacterial and archaeal groups, indicating a stable, efficient fermentation system where fibre degraders (Bacteroidota, Bacillota lineages) and methanogens function under balanced conditions (Morgavi et al., 2010). Positive correlations of Spirochaetota and Fibrobacterota with acetate, propionate, and total VFA confirm their role in carbohydrate fermentation and acetogenesis (Henderson et al., 2015; Qiu et al., 2020). Under T2 (two-event treatment), most phyla exhibited weak to moderate correlations with fermentation parameters, suggesting a partially stable community structure undergoing adaptive shifts. Frequent RT fluctuations (T3) altered several associations, producing broader positive correlations between fermentation metabolites and microbial phyla (Figure 6. 13). Notably, Elusimicrobiota and Poribacteria showed strong links with acetate and total VFAs, suggesting enhanced syntrophic or hydrogen-scavenging activity (Williams et al., 2019). Hexanoic acid showed positive correlations with most phyla, suggesting extended-chain fatty acid synthesis and broader cross-feeding under thermal fluctuation conditions. Methane also correlated positively with Bacillota_A–C, indicating stronger fermenter–methanogen coupling (Dirks et al., 2025). Overall, these changes signify a reorganisation of metabolic linkages rather than community disruption. Temperature fluctuations appear to promote functional compensation among stress-tolerant taxa, maintaining fermentation performance through cooperative and redundant microbial interactions (Allison & Martiny, 2008; Shade & Handelsman, 2012).

With the ryegrass substrate, temperature fluctuations progressively reorganised microbial–metabolite associations in Experiment 2 (Figure 6. 31 - Figure 6. ***33***). Under control conditions (T1), most phyla showed negative correlations with branched-chain VFAs, hexanoate, and NH_3, indicating efficient nitrogen and branched-chain carbon utilisation under stable incubation. Gas production and CO_2 correlated positively with most phyla, whereas Bacillota

showed a strong negative association with methane traits, contrasting the positive coupling between methanogens and CH_4 production (Hook et al., 2010). Under T2 (6-event treatment), gas production, CO_2, methane, and key VFAs (propionate, hexanoate) correlated positively with most phyla, while NH_3 and branched-chain VFAs remained negatively associated. Under frequent temperature fluctuations (T3), several microbial phyla exhibited significant positive associations with key metabolites: Cyanobacteriota correlated with total volatile fatty acids (VFAs) and propionate; Fibrobacterota correlated with hexanoate; and Halobacteriota and Gemmatimonadota correlated with overall methane production. This pattern indicates an intensified metabolic interplay, suggesting a transition toward syntrophic fermentation and methanogenic pathways during the recurrent temperature drop condition (Day et al., 2022). In addition, an increased abundance of Prevotella is associated with more energy-efficient fermentation pathways toward propionate production via the succinate pathway, which may reduce hydrogen availability and thereby lower the thermodynamic drive for methane formation (Jeong et al., 2024; Yang et al., 2024). On the other hand, an increased abundance of Bacillota is associated with enhanced acetate production, which is linked to milk fat synthesis, while concurrently promoting greater hydrogen generation that can support methanogenesis (Mao et al., 2024; Yang et al., 2024). Overall, the transition from widespread negative to largely positive correlations with increasing temperature fluctuations indicates a collaborative reorganisation of microbial functions. This aligns with microbial ecological models of functional redundancy and compensatory adaptation that maintain fermentation efficacy under stress (He et al., 2024). Future investigations should therefore employ time-resolved, multi-omics strategies such as metatranscriptomics, metaproteomics, and metabolomics to elucidate the metabolic pathways and interspecies interactions that underpin functional redundancy, with particular emphasis on processes governing hydrogen flow and methane production.

Investigating the influence of simulated drinking-induced temperature fluctuations on rumen fermentation characteristics and microbial community diversity *in vitro* is a potentially novel approach. In justification for this experiment, the reduction of RT *in vivo* could be similarly achieved by drenching cattle with cold water and monitoring temperature with an inserted reticulorumen bolus. Alternatively, selecting cattle based on their drinking behaviour and collecting rumen samples would allow further *in vivo* evaluation of drinking-associated temperature dynamics on reticulorumen fermentation and microbial diversity. It has been established that several factors, such as the donor animal's diet, the inoculum source, the substrate composition, and the anaerobic environment, influence the fermentation process in

any *in vitro* fermentation technique. In the current study, we used a batch-type fermentation method (closed method) where there was no direct contact of cold water with the rumen inoculum. It was an indirect way to cool the temperature in the fermentation jar (a proxy for the reticulorumen). Therefore, the impact of temperature drops (through simulated drinking events) on microbial fermentation may not fully reflect what usually occurs in the rumen of a live animal, indicating a limitation of this study.

Conclusion

This study revealed that short-term incubation temperature fluctuations, through simulated drinking events, reorganised the structural configuration of rumen microbiome community without substantially altering their functional capacity. Across both kale–silage and ryegrass substrates, recurrent temperature fluctuations did not compromise substrate degradability or major VFA yields; instead, they reorganised the microbial community toward greater metabolic interdependence. Core bacterial and archaeal phyla such as Bacteroidota, Bacillota, and Methanobacteriota remained dominant throughout. However, repeated RT fluctuation events stimulated stress-resilient and hydrogen-scavenging taxa (e.g., Elusimicrobiota, Poribacteria, Fibrobacterota), thereby strengthening positive associations with key fermentation metabolites. The persistent efficiency of fermentation under fluctuating thermal conditions highlights the rumen microbiome's functional redundancy and compensatory capacity. Future investigations should incorporate metagenomic and metabolomic approaches with continuous culture systems (e.g., RUSITEC) and validate results *in vivo* during natural drinking occurrences to enhance practical relevance. In parallel, investigating the genetic basis of drinking behaviour, RT dynamics, and microbiome responsiveness could enable microbiome-informed selection of cattle with improved fermentation stability and reduced emissions.

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CHAPTER 7

General Discussion and Conclusion

This chapter synthesises and critically discusses the key findings from Chapters 2 to 6. It discusses the nexus among reticulorumen temperature dynamics following water consumption, ruminal microbial ecology, and fermentation characteristics, integrating the physiological, behavioural, and microbiological aspects of cattle into a comprehensive framework for an efficient cattle production system. Finally, it discusses limitations of current research, existing research gaps, and recommends future directions to enhance the production efficiency of dairy and beef systems both economically and environmentally.

Introduction

Improving feed efficiency and reducing environmental footprint have emerged as primary objectives in modern ruminant research. Since feed utilisation is governed mainly by rumen microbial activity under controlled conditions, understanding microbial responses to reticulorumen environmental perturbations has become a crucial part of this effort. The effects of dietary changes and varying ambient conditions on microbial diversity and fermentation profiles in cattle suggest that even minor shifts in microbial community composition can influence reticulorumen function, feed efficiency, and animal performance (Canibe et al., 2005; Hales et al., 2014; Kim et al., 2014). Advances in high-throughput sequencing technologies now enable detailed characterisation of these microbial communities, facilitating links between microbiome community structure and key phenotypic traits such as feed efficiency and methane emissions (Cantalaiedra-Hijar et al., 2018; Chen et al., 2021; Zhao et al., 2024). Reticulorumen temperature (RT), one of the key factors of the reticulorumen environment, fluctuates considerably, particularly following water ingestion (Bewley et al., 2008; Cantor et al., 2018); however, the dynamics of these RT changes and their impact on microbial diversity remain largely unexplored.

Intending to explore this potential issue, the overarching aim of this thesis was to develop a deeper understanding of reticulorumen thermoregulation through the application of thermodynamic principles and to explore its implications for enteric microbial fermentation. The existing literature on RT fluctuations and their impact on microbial diversity was explored in Chapter 2, highlighting the interaction between the reticulorumen environment and the functional diversity of the microbial community, cattle's water consumption behaviour and associated RT changes following drinking, and the potential impacts of RT changes on microbial community structure and fermentation characteristics. The magnitude of the RT drops following water consumption was explored in Chapter 3 using the thermodynamic principle of mixing fluids, based on literature data. Chapter 4 incorporated RT data from feedlot beef steers and employed a drinking detection algorithm to identify and characterise drinking events with a particular emphasis on estimating the duration of the temperature recovery period following RT drop. Building on the characteristics of a drinking event, specifically a rapid decline followed by an exponential recovery, Chapter 5 developed an in vitro method to simulate the reticulorumen environment, representing RT fluctuations following water consumption. Chapter 6 explored the impact of RT fluctuations through simulated drinking events on fermentation characteristics and microbial diversity in an *in vitro* setting. This

concluding chapter synthesises the key findings, discusses their broader implications, and outlines future research directions.

Key findings

Understanding the interactions among water consumption, RT dynamics, and microbial community diversity is crucial for establishing a resilient cattle production framework. Since microbial fermentation is fundamental to ruminant nutrition and depends on a stable reticulorumen environment, factors influencing reticulorumen stability warrant thorough investigation. Variations in RT, including hypo- and hyperthermic conditions, have been shown to affect ruminant gut microbiota, consistently altering microbial functional stability and community diversity (Huus & Ley, 2021). While elevated ambient temperature and relative humidity indirectly influence reticulorumen fermentation through physiological adaptations in the host, water intake is directly associated with a pronounced decline in RT, potentially impacting microbial adhesion to feed substrates and disrupting enteric fermentation (Roger et al., 1990). Reticulorumen microbial profile, residual feed intake, and enteric methane production have been found to be under host genetic control (Berry & Crowley, 2013; Hegarty et al., 2007). The drinking behaviour of cattle has also been identified as a highly heritable trait (Dressler et al., 2023), suggesting a potential link between drinking, RT fluctuations, rumen microbial profiles, and their functionalities. The effects of dietary manipulation and environmental stressors, such as heat and cold, on fermentation characteristics and microbial profiles have been comprehensively reviewed in Chapter 2, providing insights that inform adaptive management strategies. These insights also support selection and breeding programs aimed at improving cattle efficiency and resilience. The current literature review (Chapter 2) also identified that, despite having a pronounced influence of water intake on RT dynamics, its impact on microbial fermentation remains relatively unexplored. As such, a comprehensive understanding of the current state and gaps in knowledge regarding RT dynamics and their potential effects on microbial communities was achieved (Objective 1).

While reviewing the association between drinking water temperature and RT, different magnitudes of RT drop were observed across different volumes of water at varying temperatures. To evaluate these RT drops from a thermodynamic perspective, we compiled a dataset of RT drops from previously published studies and applied Richmann's law of mixing fluids in Chapter 3. In the current study context, the difference between the baseline RT (assumed to be 39 °C) and the lowest RT (achieved after mixing intake water with rumen

content) was defined as the magnitude of the RT drop. This reduction in RT was directly associated with the intake water temperature; specifically, lower intake water temperatures, when accompanied by higher volumes, resulted in a more pronounced decrease in RT (Supplementary Figure 3S. 1). Furthermore, as heat exchange between two substances depends on their specific heat capacities (J/(kg · K), it is challenging to estimate the specific heat capacity of a heterogeneous substance such as reticulorumen content and, hence, the RT drop. Estimation of the specific heat capacity of reticulorumen content by application of Richmann's law to literature data and the relationship between actual (observed) and predicted RT drop (Supplementary Figure 3S. 2) achieved in Chapter 3, and confirmed the application of thermodynamic principles in explaining RT drop dynamics (Objective 2).

This RT drop after drinking is accompanied by a gradual recovery to the pre-drinking level, with recovery times reported to vary widely from about 20 minutes to over three hours in dairy cows. This provides an opportunity to explore the temperature dynamics of a full drinking episode across three beef cattle breeds in Chapter 4, with the intention of estimating the duration of the recovery period based on dynamic fluctuations in RT rather than a generalised or fixed threshold of baseline RT (Objective 2). For the first time, we applied drinking event detection algorithms developed by Shirley et al. (2025) to RT data from feedlot steers and explored variability in drinking frequency, associated RT drops, and recovery duration across breeds, suggesting that breed-specific thermoregulation provides an opportunity to consider drinking as a trait of interest for selecting efficient cattle, along with a proxy for heat-resilient cattle. We identified a relationship between the magnitude of the RT drop and the recovery duration, indicating that, except for minor drops (<1.5 °C), the time to achieve 90% temperature recovery remains relatively stable at approximately 120 minutes. This observation aligned with some published literature, and most importantly, this estimation incorporated a mathematical approach based on thermodynamic principles (Newton's Law of Cooling/Heating). The recovery period suggests the duration of the cooling effect of drinking that persists in the reticulorumen. However, differences in recovery period across breeds suggest that the rate of recovery (decay rate) may be associated with the individuals' heat tolerance capacity, which warrants further investigation. This work showcased RT dynamics in response to shaded and unshaded conditions, thereby improving our understanding of the association between RT fluctuations and cattle's drinking behaviour in varying environmental conditions.

Extending this research, Chapter 5 maintained a specific focus on developing an *in vitro* method to simulate RT fluctuations associated with drinking events in cattle, with the aim of

investigating the impact of short-term RT fluctuations on microbial fermentation (Objective 3). We have developed a method for *in vitro* simulation of drinking events in the fermentation jar, in which the fermentation jar temperature served as a proxy for the RT of cattle. The fermentation jar temperature was reduced by adding a measured amount of cold water to the water bath, and the subsequent recovery period, as estimated in Chapter 4, was achieved using a temperature profile regulated by a heating immersion circulator. While it was an indirect method of altering the internal jar temperature, it corresponded with the thermal dynamics of the water bath, as validated by Newton's Law of Cooling/Heating.

Chapter 6 builds on the *in vitro* method developed in Chapter 5 to simulate drinking events and investigate the impact of temperature fluctuations on fermentation characteristics, gas production kinetics, and microbial diversity (Objective 4). Impact of two, six, and 12 drinking events (reticulorumen temperature fluctuation events) with ~ 9.50 °C drop, followed by a 120-minute recovery period, was compared to the control, where the incubation temperature was maintained at 39 °C for 26 hours of fermentation. In gas kinetics results, frequent temperature fluctuations (12 events over 26 hours) reduced the fractional rate constant (*c*), which might reflect early microbial suppression due to cooling-induced reductions in enzyme activity (Russell & Cook, 1995). Nonetheless, the higher asymptotic gas production observed under these conditions indicates that microbial communities compensated over time, sustaining fermentation and achieving greater cumulative gas production through adaptive metabolic responses (Guan et al., 2017). Most of the fermentation parameters showed no significant difference across the treatments, except total gas production and iso-butyric acid concentration from the kale & silage mixture (Experiment 1), which suggests that such short-term temperature fluctuations did not impact microbial functionality greatly, but rather they adjusted to these temporal perturbations with their inherent dynamic ability (Janssen, 2010; Morgavi et al., 2010; Newbold & Ramos-Morales, 2020). However, several fermentation parameters, including N_2O, NH_3, propionic acid, and hexanoic acid concentrations from the ryegrass (Experiment 2) substrate, showed significant differences across treatments, suggesting that diet type largely determines fermentation patterns. Given that ruminal microbes can influence ruminant phenotypes and are themselves affected by reticulorumen temperature, thereby altering metabolite profiles (Chen et al., 2021; Khonkhaeng & Cherdthong, 2019), we examined whether temperature fluctuations induced shifts in microbial community structure. Results of different diversity metrics indicate that short-term temperature fluctuations during simulated drinking events reorganised the rumen microbial community structure without

substantially affecting its functional capacity. The sustained fermentation efficiency under these conditions highlights the microbiome's functional redundancy and ability to compensate for transient environmental stress.

Practical Implications

Contextually, this research contributes to knowledge development regarding drinking-associated RT dynamics and subsequent impact on microbial community diversity. This research also demonstrates that sudden drops in RT (*in vitro* study) can temporarily suppress microbial activity, alter fermentation dynamics, and shift rumen microbial community structure. The findings of this thesis carry several practical implications for an efficient cattle system, as listed below:

- Providing warmed water during cold seasons or cold water following heat stress may help stabilise RT, thereby supporting consistent microbial adhesion to feed substances, enhancing fibre digestion, and improving fermentation efficiency.

- Identifying microbial taxa or functional pathways resilient to temperature fluctuations could guide the development of probiotics, inoculants, or feed additives that stabilise microbial fermentation under diverse rumen environments.

- Stabilising RT may enhance fermentation efficiency and reduce energy losses through methane and nitrogen excretion, offering an indirect strategy to improve productivity while mitigating emissions.

- Real-time RT monitoring using smart technologies (boluses) can detect significant cooling events, enabling timely management adjustments such as modifying water source, feeding sequence, or environmental conditions.

- Exploring the nexus between drinking behaviour, stability of RT, and microbial community structure and functionalities may pave the way for precision breeding or microbiome-informed management decisions.

Limitations of current research and future directions

While this thesis advances understanding of the dynamic interplay between the reticulorumen environment and microbial fermentation, it also reveals new questions that warrant further investigation. Advances in methods and emerging technologies continually create opportunities to refine and expand existing knowledge. In Chapter 3 , while evaluating RT changes following drinking events using thermodynamic principles, we found only 18 RT drop observations across three published studies (Supplementary Figure 3S. 1). These observations were derived from experimental studies, with RT drops recorded using relatively old technologies. Incorporating smart rumen bolus-driven RT data from observational studies across multiple cattle breeds over longer periods may provide RT dynamics with greater accuracy. In addition, validating model-based estimates of the specific heat capacity of reticulorumen content would strengthen predictions of RT drop from intake water temperature. In Chapter 4, we used RT data recorded between October and April (warmer seasons in Australia) from feedlot steers with no records of water temperature and amount of water consumed. To better understand RT dynamics following drinking, RT data across the seasons and across diverse production systems, along with the volume and temperature of consumed water, should be considered in future studies. The method developed in Chapter 5 used cold water to lower the fermentation jar temperature and a heating circulator to raise it, simulating RT fluctuations following drinking. Although it is a cost-effective method, it requires a lot of cold water during the incubation period, and for each drinking event, it must drain a certain amount of water from the water bath, which is difficult and labour-intensive. Inclusion of a customised refrigerated circulator (heating and cooling) might improve temperature fluctuations in the fermentation jar in an automatic manner. In Chapter 6, two different sets of gas production modules (RF1 in Exp. 1 and RF1X in Exp. 2) were used in two experiments for measuring *in vitro* gas production. Although the same calibration (live interval – 1 Second, recording interval – 5 minutes, Global pressure – 6 psi) was maintained each time, using the same modules across the experiments might improve the accuracy of the gas results. While more replication is advised for *in vitro* fermentation with the ANKOM RF Gas production system, we used only two replications per treatment due to the limited availability of fermentation jars. The DM degradability was measured only after 26 hours of fermentation; however, employing more replicates may facilitate time–resolved DM estimation, providing a better understanding of gas kinetics. Measuring redox potential is crucial for understanding the environment of fermentation media; however, no such facilities were available while conducting *in vitro*

experiments for this study. Furthermore, only bacterial and archaeal community diversity and their correlation with fermentation parameters are investigated. But protozoal and fungal interactions were not explored due to the unavailability of RE-RRS reads from the Eukaryote domain. To obtain comprehensive results, a larger number of replications, along with sequence reads of Eukaryotes, should be considered in future studies. Future research should clarify how rapid reticulorumen temperature drops affect microbial adhesion, key cellulolytic and proteolytic enzyme activity, and early fermentation using mechanistic and multi-omics approaches.

Reticulorumen temperature (RT) monitoring shows considerable potential as a real-time, non-invasive indicator of rumen health and functional stability, while also offering valuable inputs for whole-animal energy balance modelling. Continuous RT monitoring captures dynamic events such as drinking, feeding, fermentation, and thermal stress that reflect short-term changes in ruminal microbial activity, heat exchange, and metabolic processes. Abnormal RT patterns, including dramatic fluctuations or delayed recovery, may signal rumen dysfunction and altered energy dynamics. Incorporating RT-derived metrics such as temperature decline magnitude, recovery rate, and drinking frequency into energy balance frameworks may enhance estimates of energy expenditure and feeding behaviour, particularly under variable environmental conditions. However, a reliable application requires careful interpretation and calibration against intake, metabolic heat production, ambient conditions, and complementary indicators such as reticulorumen pH, feeding patterns, or production traits, given the multifactorial influences on RT signals.

From a breeding perspective, this work highlights drinking behaviour as a potential proxy trait that could be incorporated into selection strategies to improve reticulorumen efficiency and resilience. Given the established role of host genetics in shaping the rumen microbiome, selection for animals exhibiting more stable thermal profiles characterised by reduced temperature fluctuations or faster recovery may favour microbial communities associated with more efficient and stable fermentation. Integrating RT dynamics, drinking behaviour, and microbiome functional traits with genomic data should be prioritised to identify heritable markers of rumen resilience. Such integration could facilitate genomic selection of cattle capable of maintaining efficient microbial fermentation irrespective of RT perturbations. Importantly, integrating drinking behaviour into selection indices would require careful consideration of its genetic correlations with production, thermoregulation, and behavioural traits, as well as its repeatability across varying environmental conditions. Advances in

precision livestock technologies, such as continuous reticulorumen temperature monitoring, provide an opportunity to capture high-resolution phenotypes that can support the development of such indices. Furthermore, *In vivo* validation of drinking-induced cooling effects, alongside studies on host–microbiome–environment interactions, could help translate these findings into production systems.

Conclusion

This thesis explores RT dynamics in relation to water consumption, offering insights into the interactions among water intake, RT, enteric fermentation, and microbial community diversity. A novel method was developed to simulate RT fluctuations representing drinking-associated temperature changes in the reticulorumen and was employed to investigate the impact of these fluctuations on fermentation characteristics and microbial diversity. Collectively, this thesis demonstrates that abrupt RT fluctuations can transiently suppress microbial activity, shift community composition, and subtly influence fermentation profiles, even when overall dry matter degradation remains unchanged. Across two *in vitro* experiments, frequent temperature fluctuations consistently slowed early fermentation kinetics, altered the abundance of several temperature-sensitive taxa, and influenced correlations between microbial groups and some key fermentation metabolites. These findings show that maintaining RT is a crucial factor for microbial function and could help improve fermentation resilience, nutrient utilisation, and manage emissions. More broadly, this work highlights the interaction between environmental changes, host physiology, and microbial ecology, emphasising that stable reticulorumen function depends on conditions that support microbial adhesion, enzyme activity, and the balance of microbial community diversity. Understanding microbial dynamics under transient thermal stress provides a foundation for future *in vivo* validation and for guiding the formulation of management strategies, such as water and feed management, microbial modulation to enhance an efficient rumen ecosystem, and functional efficiency. Ultimately, this thesis advances efforts to develop resilient cattle production systems that maintain performance while mitigating environmental impacts.

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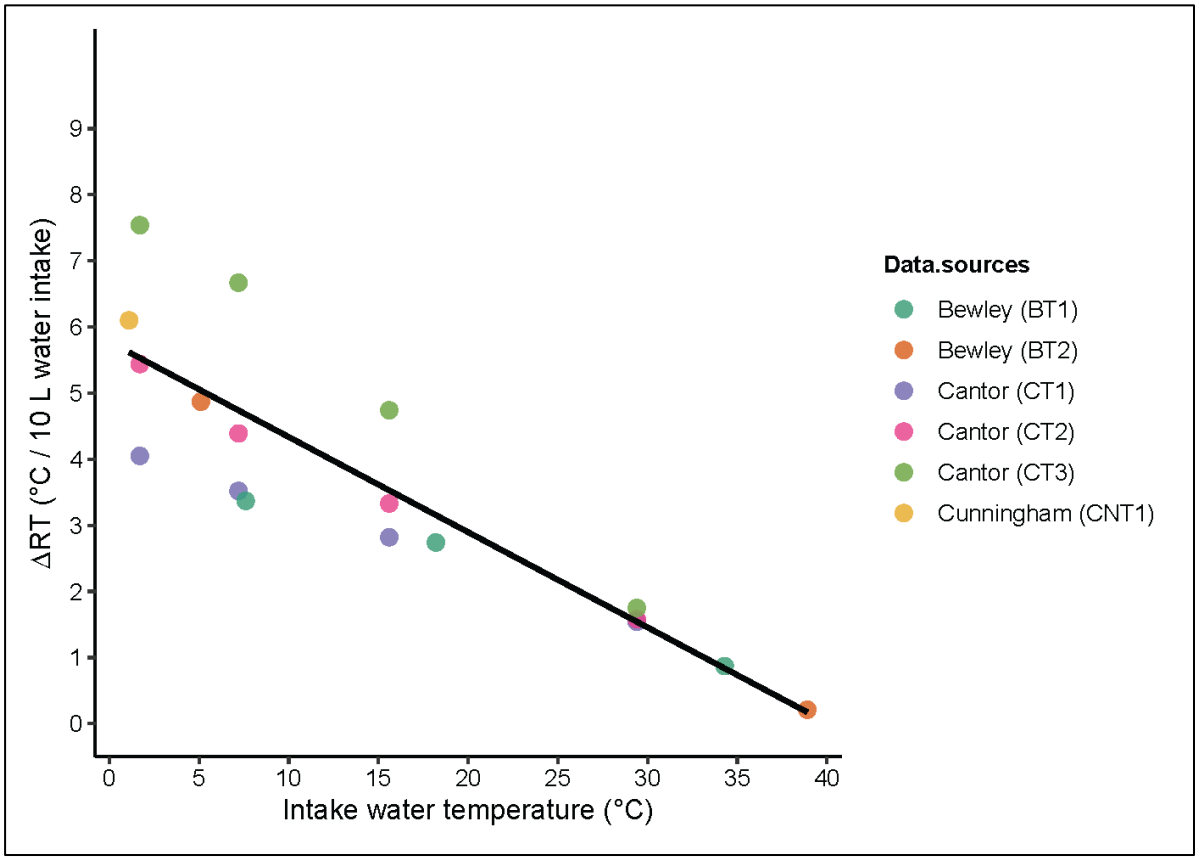
APPENDIX

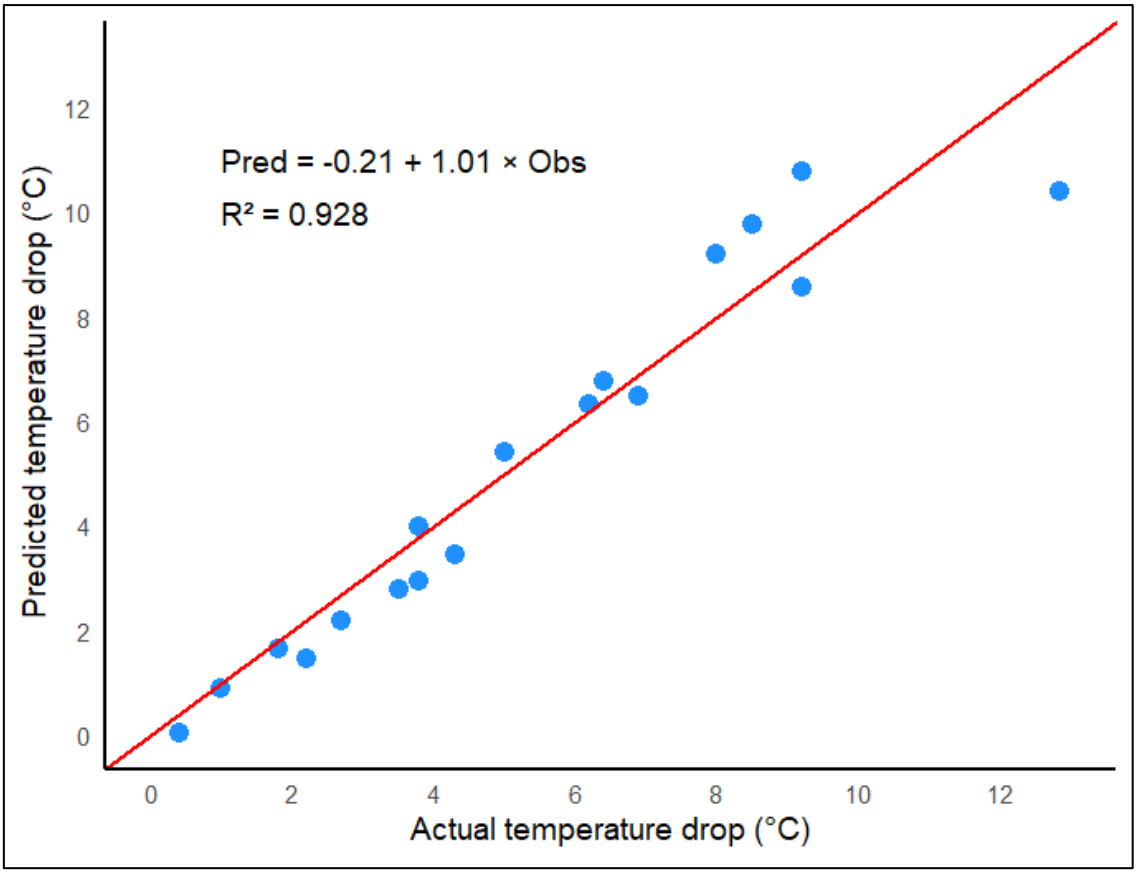
Supplementary Materials

Supplementary Material: Chapter 3

Supplementary Figures



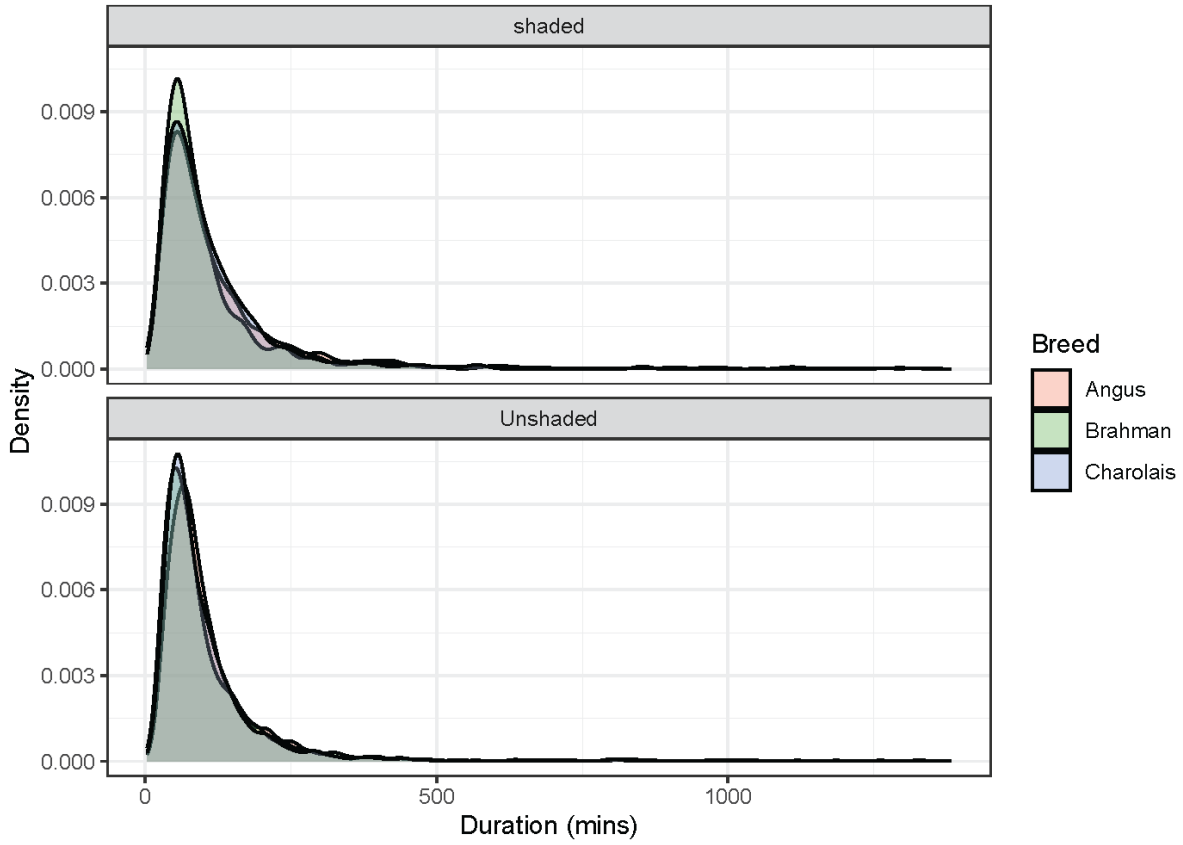
Supplementary Figure 3S. 1. Relative changes in reticulorumen temperature (ΔRT; °C per 10 L water intake) correspond to intake water temperature across multiple experiments. Points represent individual observations from Bewley's experiment (BT1 = 3 observations from Exp. 1 and BT2 = 2 observations from Exp. 2), Cantor's experiment (CT1, CT2, CT3 = 3 * 4 = 12 observations), and Cunningham's experiment (CNT1 = single observation).



Supplementary Figure 3S. 2. Association between observed (actual) and model-predicted reticulorumen temperature drops (ΔRT, °C). Points represent individual observations, and the red line denotes the fitted linear relationship between predicted and observed values. The regression equation (Pred = −0.21 + 1.01 × Obs) and high coefficient of determination (R^2 = 0.928) indicate strong predictive accuracy and minimal bias across the range of temperature drops.

Supplementary Materials: Chapter 4

Supplementary Figure



Supplementary Figure 4S. 1. Density distributions of recovery period duration (minutes) for three cattle breeds (Angus, Brahman, and Charolais) under shaded and unshaded conditions. The x-axis represents the duration of the recovery period, while the y-axis shows the probability density. Panels compare recovery dynamics between shaded and unshaded environments, highlighting breed-specific differences in the distribution and spread of recovery times.

Supplementary Material: Chapter 5

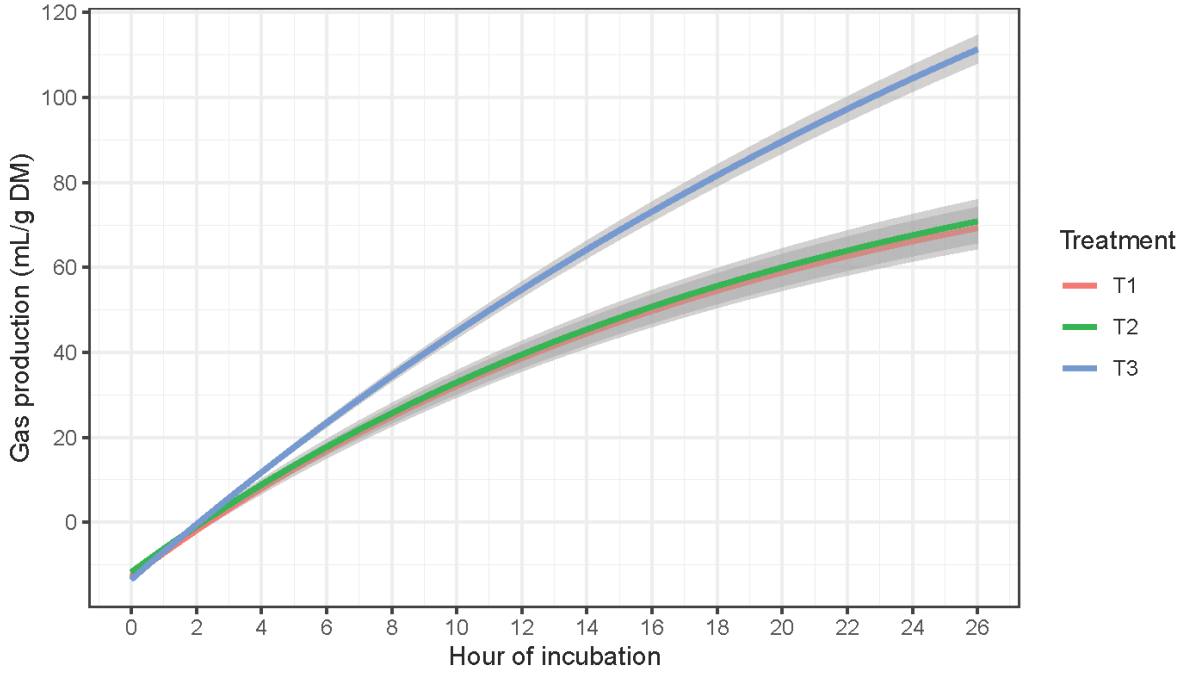
Supplementary Table 5. 1. Temperature drops in the water bath estimated by Richmann's law and subsequent observed fermentation jar temperature

Experiments	m_1 (L)	T_1 (°C)	c_1 (J/g°C)	m_2 (L)	T_2 (°C)	c_2 (J/g°C)	T_f (°C)	(T_1-T_f) (°C)	J_t
Exp 1	12.5	39.0	4.2	1	4	4.2	36.41	2.59	1.8
Exp 2	12.5	39.0	4.2	2	4	4.2	34.17	4.83	3.2
Exp 3	12.5	39.0	4.2	3	4	4.2	32.22	6.78	4.5
Exp 4	12.5	39.0	4.2	4	4	4.2	30.52	8.48	5.8
Exp 5	12.5	39.0	4.2	4.5	4	4.2	29.73	9.27	6.2
Exp 6	12.5	39.0	4.2	5	4	4.2	29.00	10.00	6.9
Exp 7	12.5	39.0	4.2	6	4	4.2	27.65	11.35	7.8
Exp 8	12.5	39.0	4.2	7	4	4.2	26.43	12.57	9.1

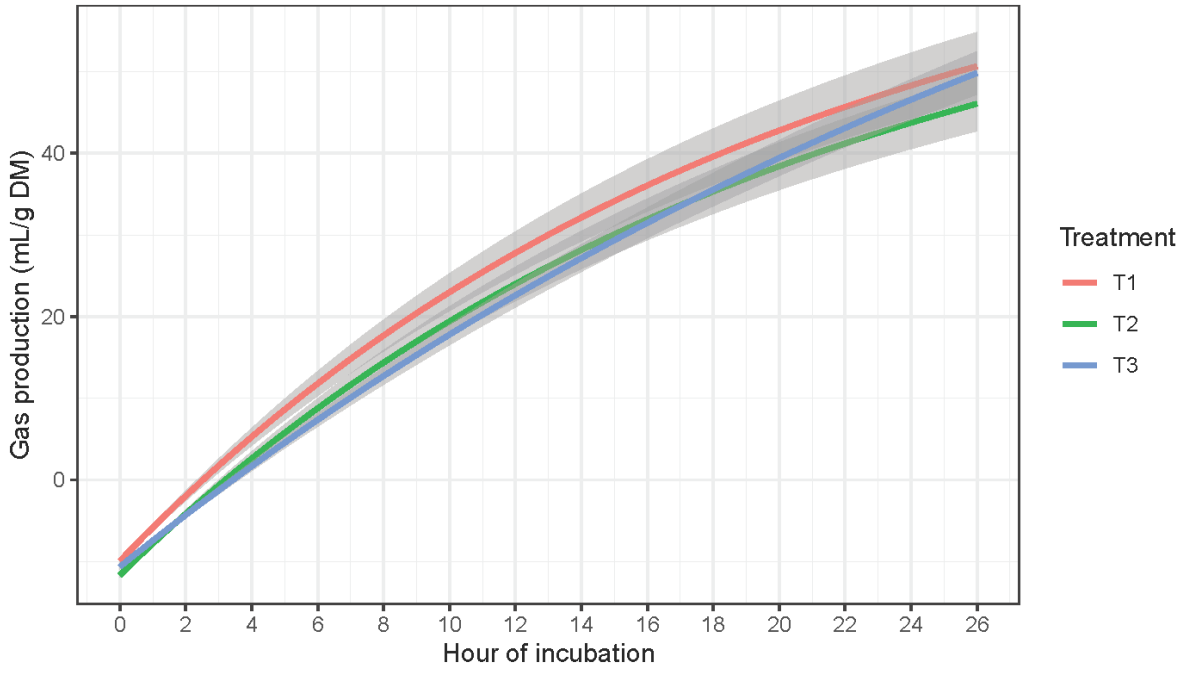
*The volume of water in the water bath (m_1), Baseline temperature in the water bath (T_1), Specific heat capacity of water (c_1), The volume of added cold water (m_2), Temp of added cold water (T_2), Specific heat capacity of cold water (c_2), Estimated final temperature in the water bath (T_f), Estimated temperature drops in the water bath (T_1-T_f), Observed changes in jar temperature (J_t).

Supplementary Material: Chapter 6

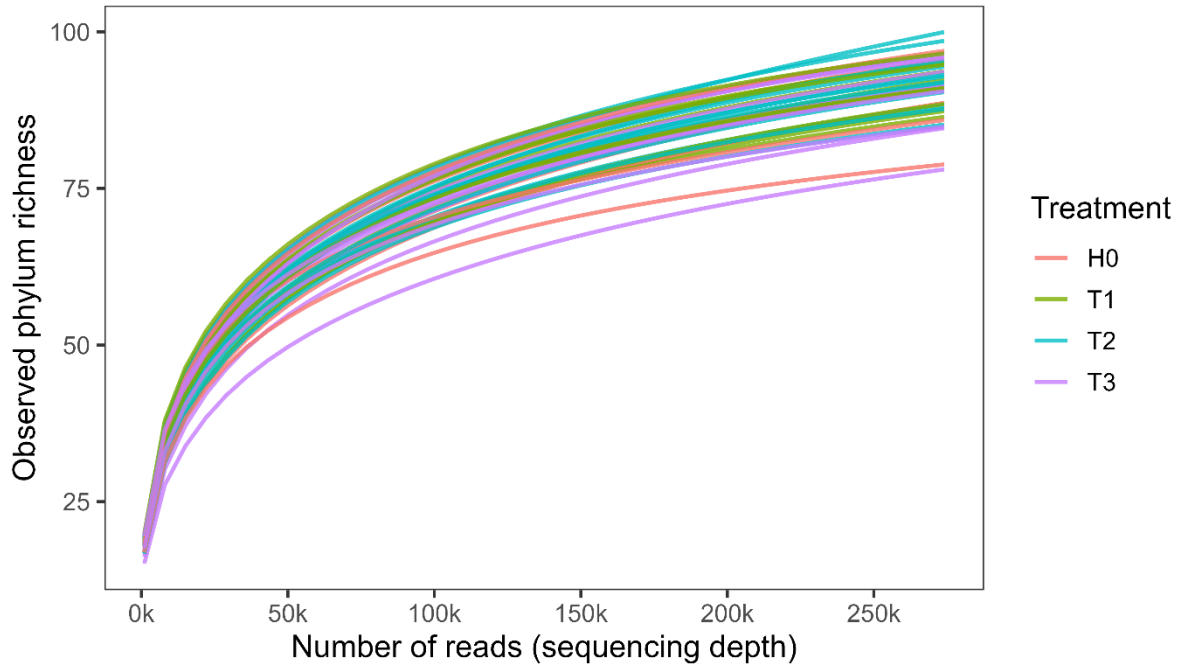
Supplementary Figures



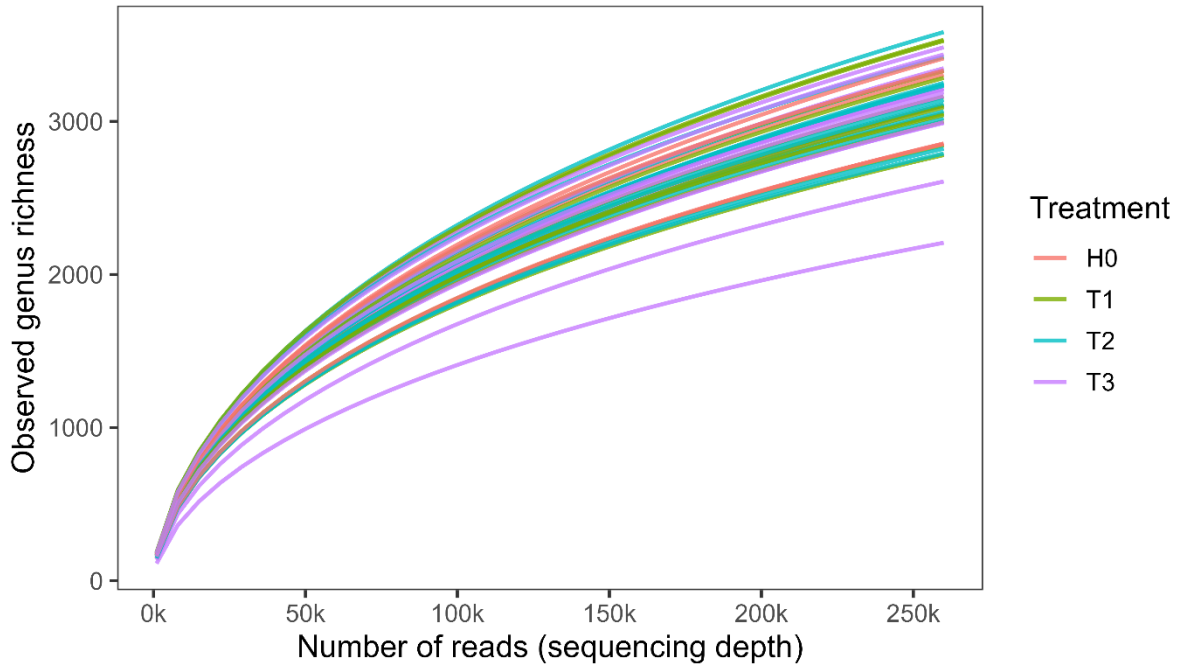
Supplementary Figure 6S. 1. Model-based gas production trends from kale-silage mixture in a 26-h incubation period, where T1 represents control with constant 39 ºC, T2 = two RT fluctuation events and T3 = 12 RT fluctuation events.



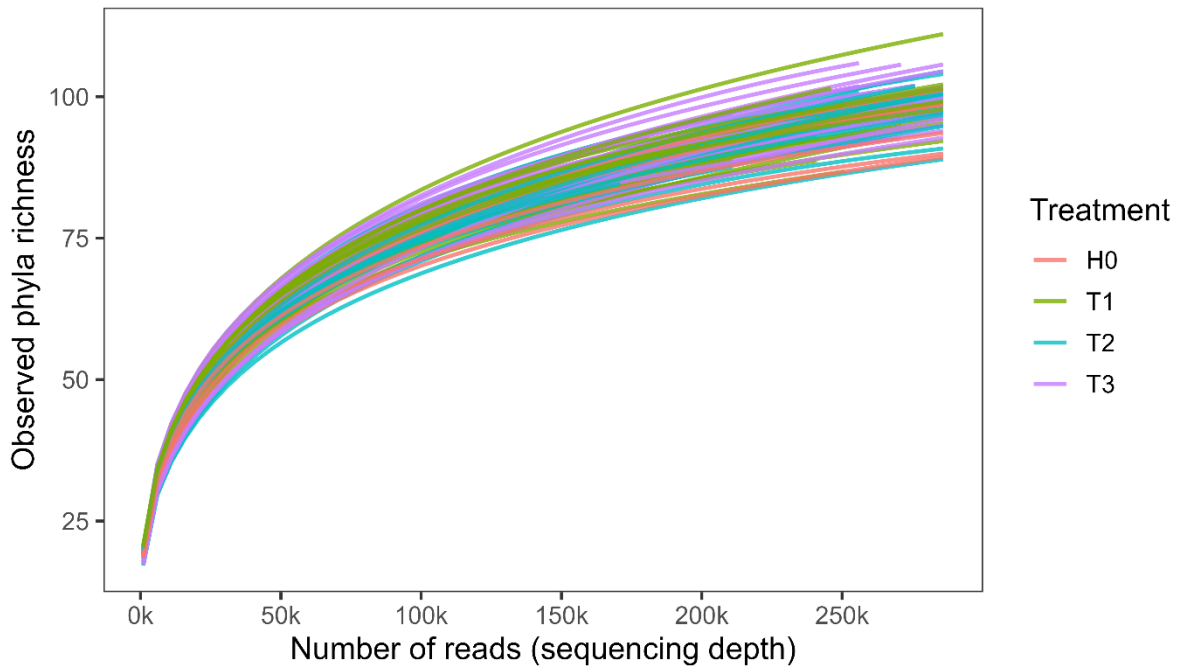
Supplementary Figure 6S. 2. Model-based gas production trends from ryegrass substrate in a 26-h incubation period, where T1 = control, T2 = 6 and T3 = 12 RT fluctuation events.



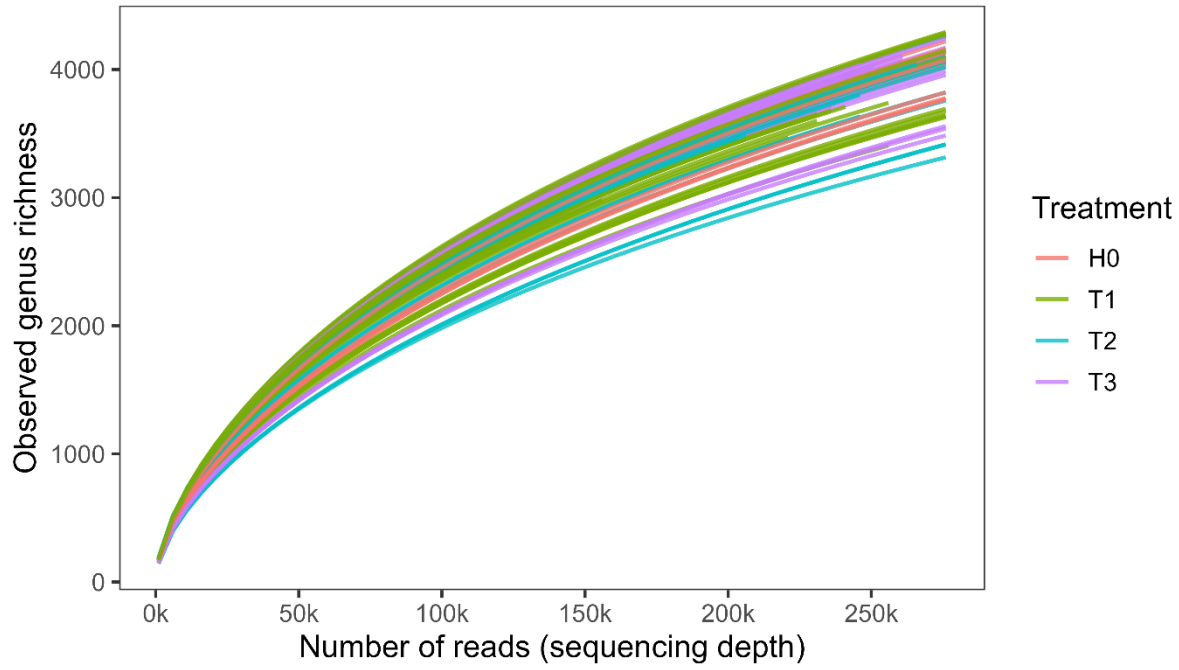
Supplementary Figure 6S. 3. Rarefaction curves at the phylum level in **Experiment 1**, showing observed phylum richness as a function of sequencing depth across treatments (H0 = Samples before fermentation, T1 = control, T2 = 2, and T3 = 12 RT fluctuation events). Curves approached an asymptote with increasing sequencing depth, indicating that most dominant phyla were captured, although complete saturation was not achieved in all samples.



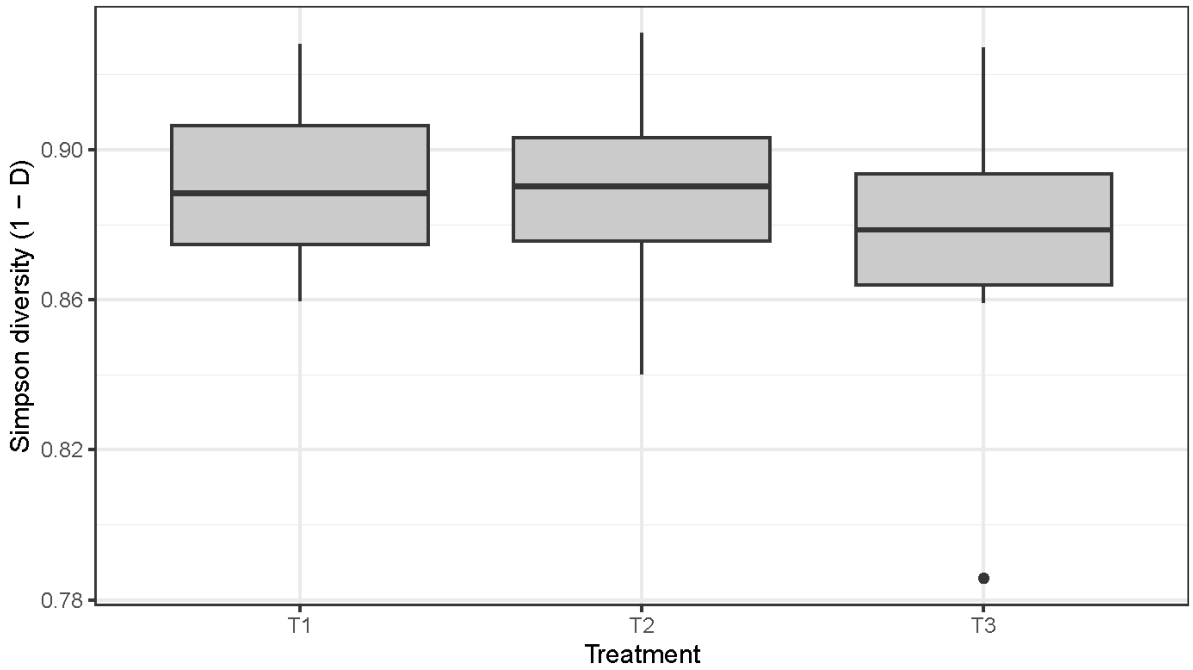
Supplementary Figure 6S. 4. Rarefaction curves at the genus level in **Experiment 1**, showing observed genus richness as a function of sequencing depth across treatments (H0 = Samples before fermentation, T1 = control, T2 = 2, and T3 = 12 RT fluctuation events).



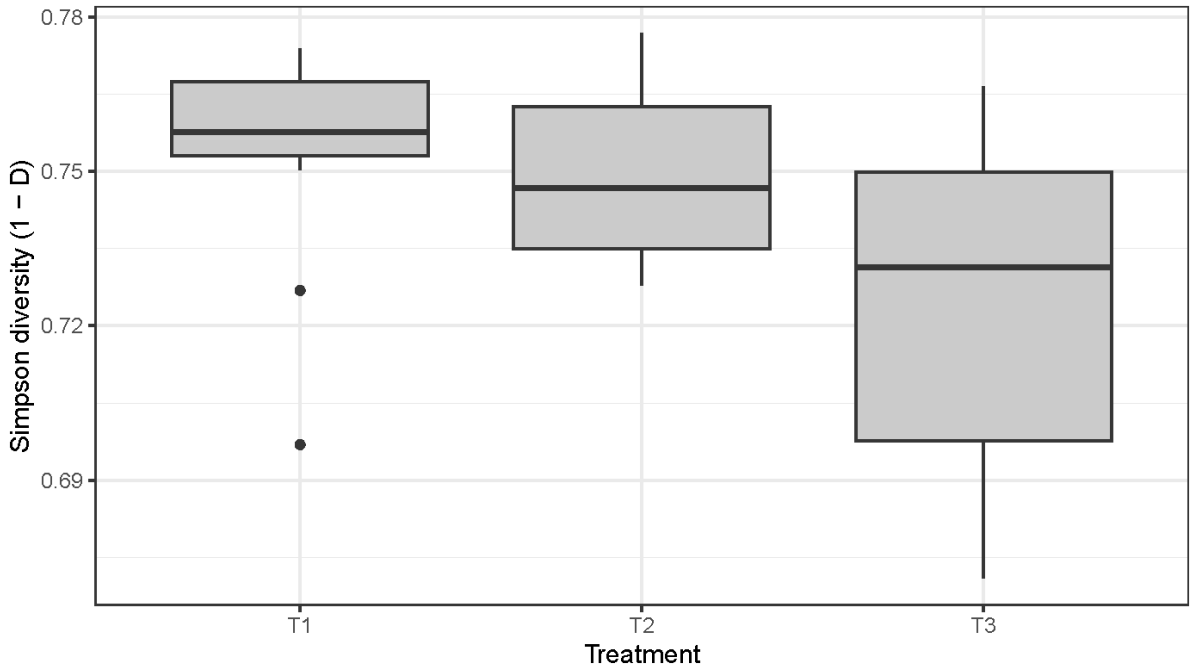
Supplementary Figure 6S. 5. Rarefaction curves at the phylum level in **Experiment 2**, showing observed phylum richness as a function of sequencing depth across treatments (H0 = Samples before fermentation, T1 = control, T2 = 2, and T3 = 12 RT fluctuation events). Curves approached an asymptote with increasing sequencing depth, indicating that most dominant phyla were captured, although complete saturation was not achieved in all samples.



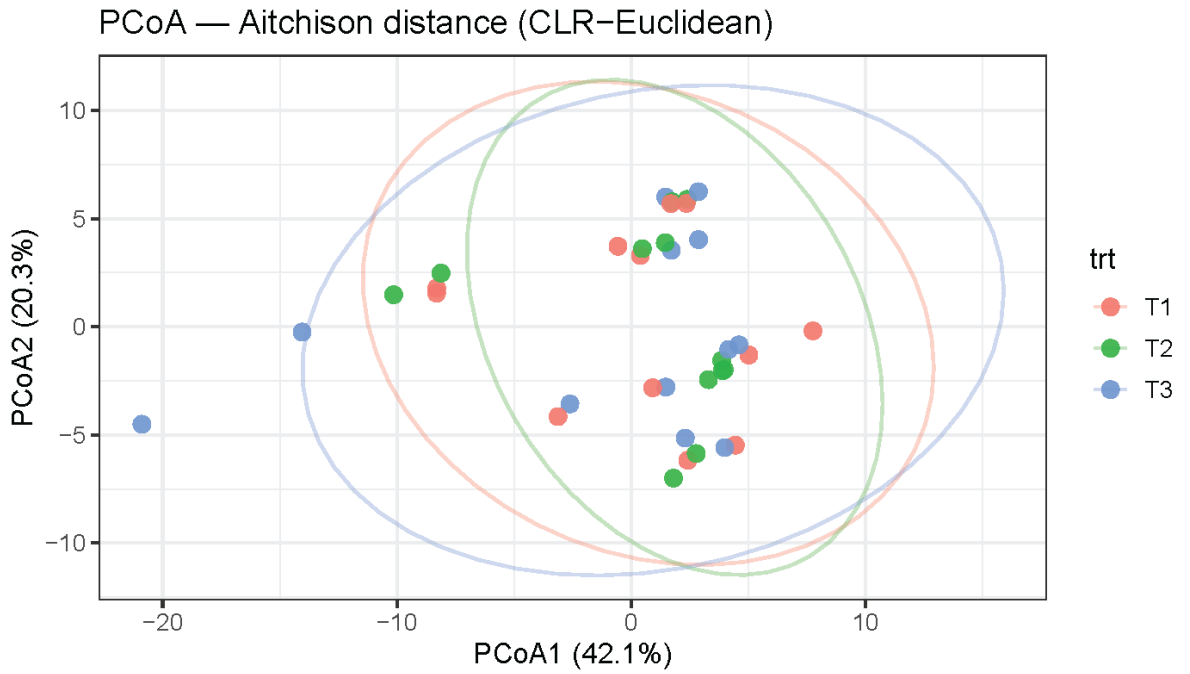
Supplementary Figure 6S. 6. Rarefaction curves at the genus level in **Experiment 2**, showing observed genus richness as a function of sequencing depth across treatments (H0 = Samples before fermentation, T1 = control, T2 = 2, and T3 = 12 RT fluctuation events).



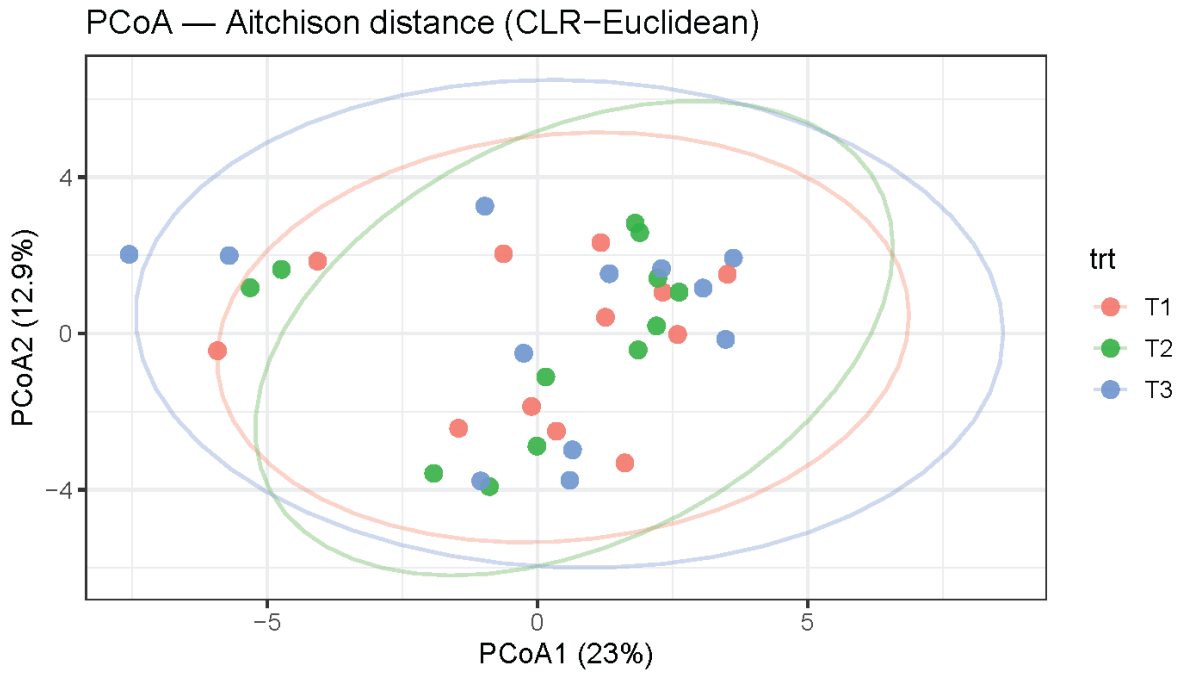
Supplementary Figure 6S. 7. Simpson's diversity index (1 − D) of the bacterial genera across experimental treatments (T1 = control, T2 = two, and T3 = 12 RT drop events) with no significant differences in diversity among treatments (p = 0.14).



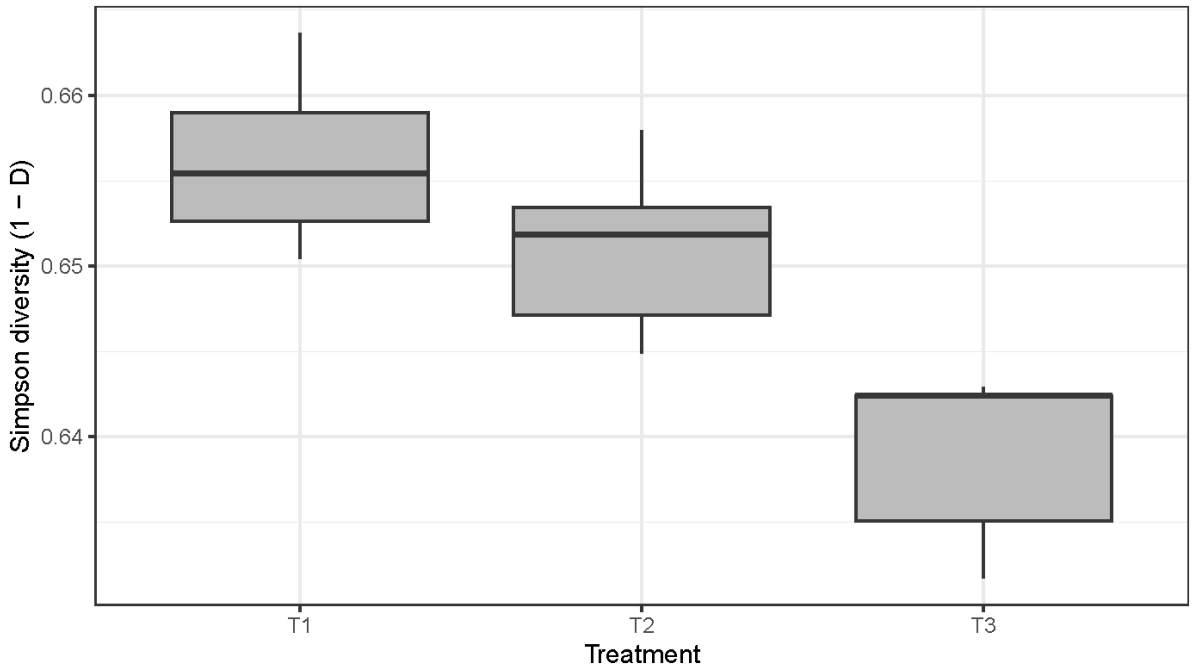
Supplementary Figure 6S. 8. Simpson's diversity index (1 − D) of the archaeal genera across experimental treatments. The diversity index differed significantly between treatments (p = 0.002).



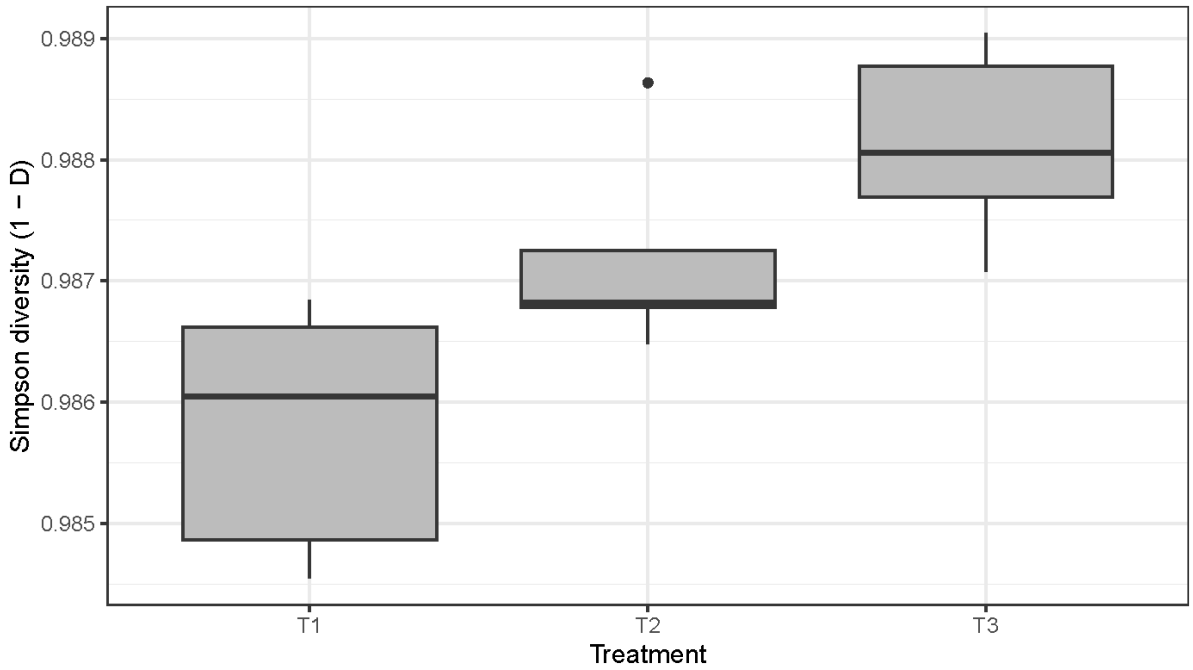
Supplementary Figure 6S. 9. PCoA based on Aitchison distance (CLR–Euclidean) showing genus-level differences in bacterial communities among treatments (T1–T3). Points represent samples, ellipses indicate within-treatment dispersion, and axes show variance explained.



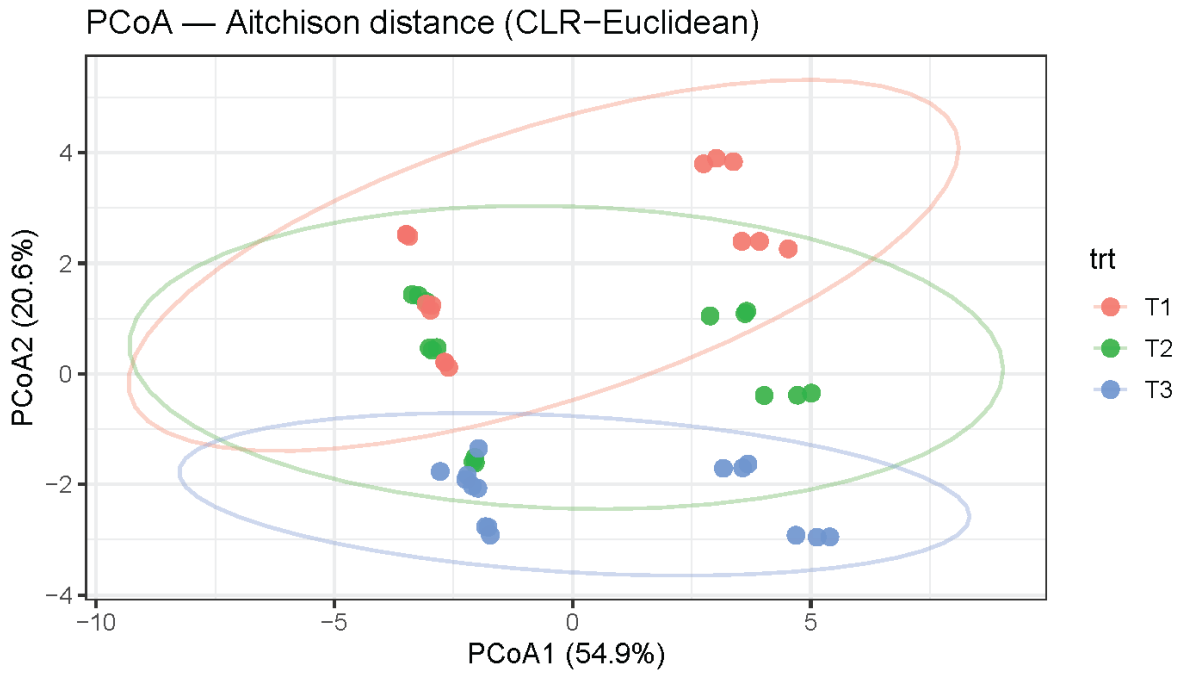
Supplementary Figure 6S. 10. PCoA based on Aitchison distance (CLR–Euclidean) showing genus-level differences in Archaeal communities among treatments (T1–T3). Points represent samples, ellipses indicate within-treatment dispersion, and axes show variance explained.



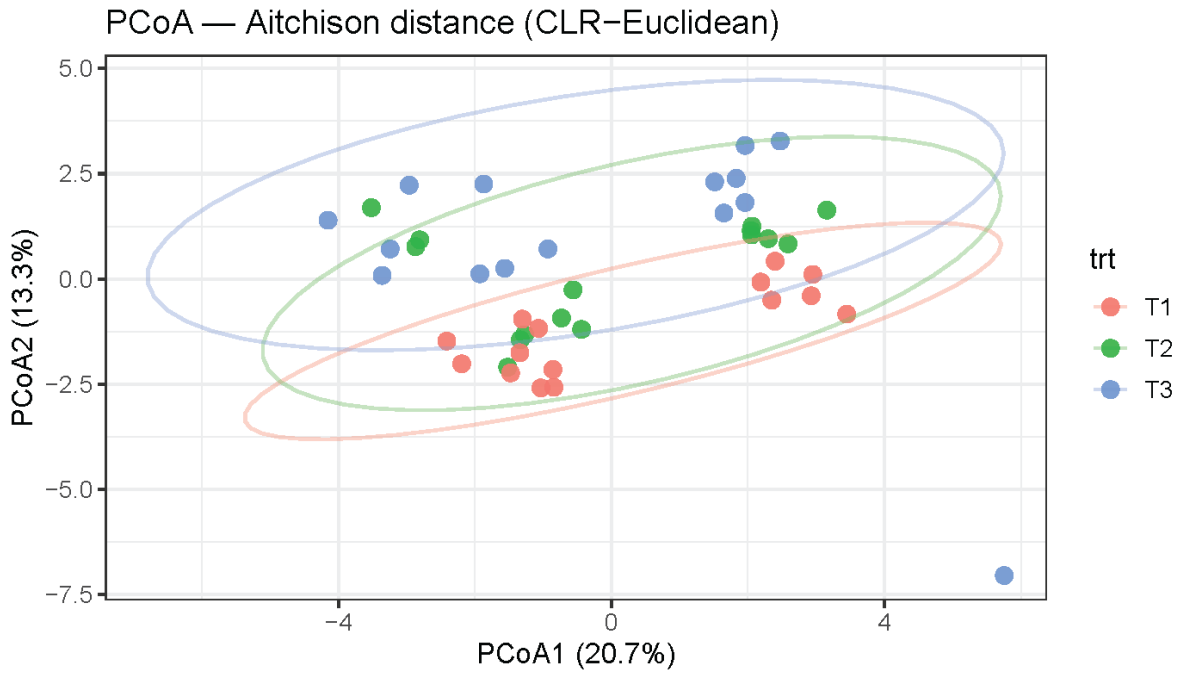
Supplementary Figure 6S. 11. Simpson's diversity index (1 − D) of microbial phyla across treatments: control (T1), six (T2), and twelve RT fluctuation events (T3) within 26 h, with microbial phyla diversity differing significantly among treatments ($p = 0.001$).



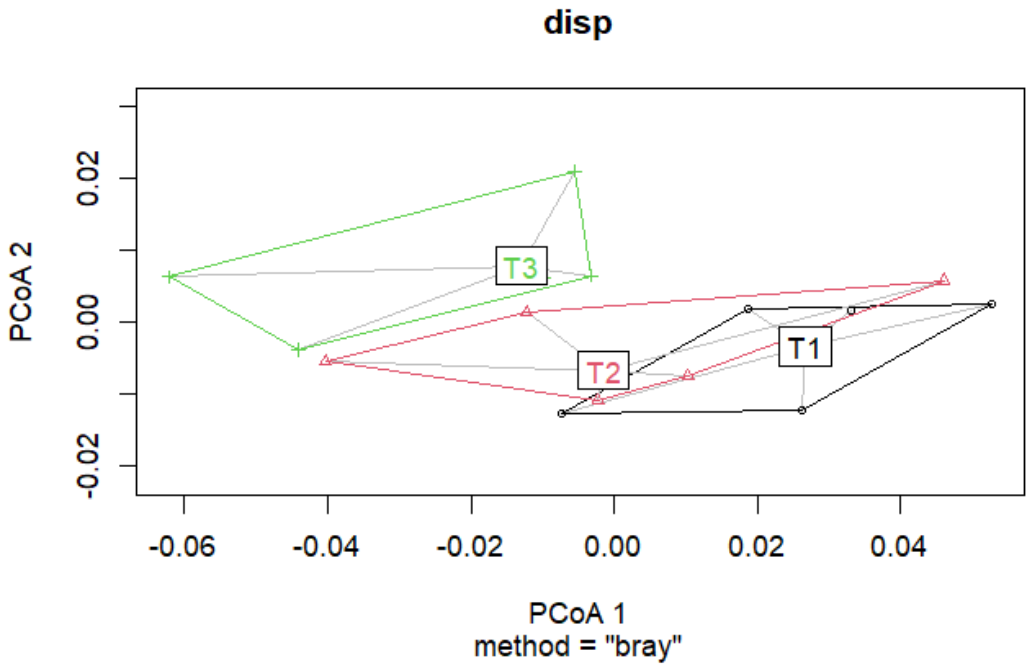
Supplementary Figure 6S. 12. Simpson's diversity index (1 − D) across treatments: control (T1), six (T2), and 12 RT fluctuation events (T3) within 26 h. Boxplots display medians, interquartile ranges, and overall data distribution, with bacterial species diversity differing significantly among treatments ($p = 0.0005$).



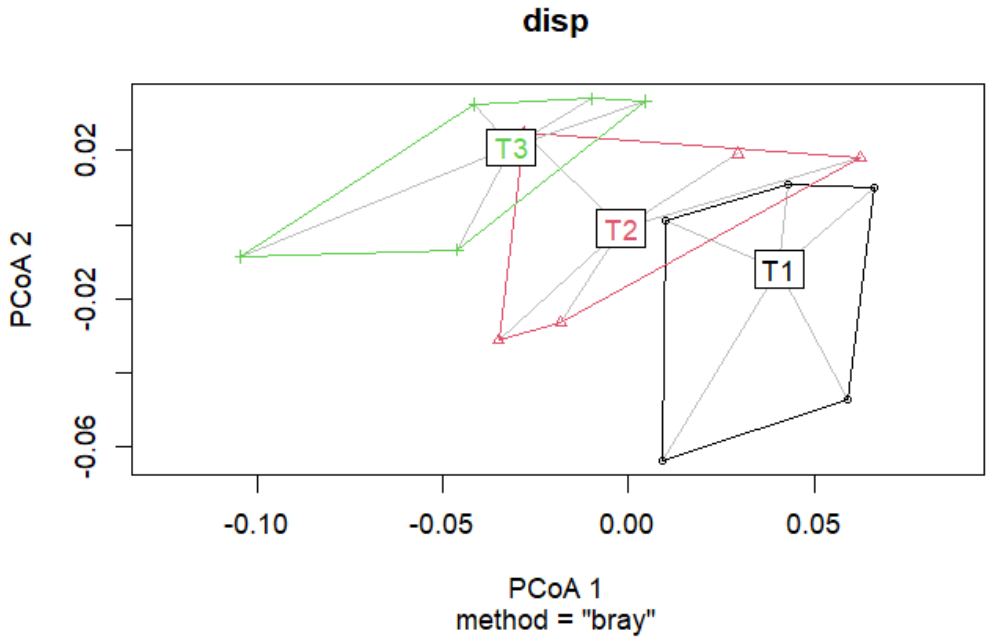
Supplementary Figure 6S. 13. PCoA based on Aitchison distance (CLR–Euclidean) showing species-level differences in bacterial communities among treatments (T1–T3). Points represent samples, ellipses indicate within-treatment dispersion, axes show variance explained, and PERMANOVA confirmed differences ($p = 0.002$).



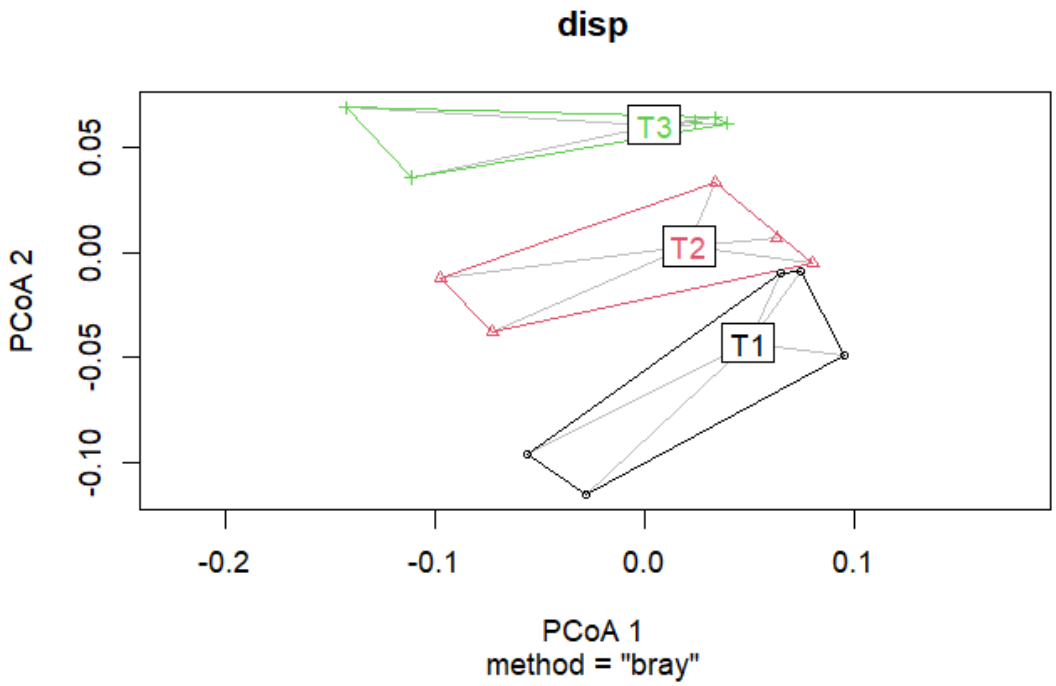
Supplementary Figure 6S. 14. PCoA based on Aitchison distance (CLR–Euclidean) showing species-level differences in Archaeal communities among treatments (T1–T3). Points represent samples, ellipses indicate within-treatment dispersion, axes show variance explained, and PERMANOVA confirmed differences ($p = 0.002$).



Supplementary Figure 6S. 15. Ordination of sample dispersion relative to treatment centroids based on Bray–Curtis distances (betadisper analysis). Points represent samples, polygons group treatments (T1–T3), and a similar spread around centroids indicates comparable multivariate dispersion, supporting PERMANOVA results for microbial phyla.



Supplementary Figure 6S. 16. Ordination of sample dispersion relative to treatment centroids based on Bray–Curtis distances (betadisper analysis). Points represent samples, polygons group treatments (T1–T3), and a similar spread around centroids indicates comparable multivariate dispersion, supporting PERMANOVA results for microbial genera.



Supplementary Figure 6S. 17. Ordination of sample dispersion relative to treatment centroids based on Bray–Curtis distances (betadisper analysis). Points represent samples, polygons group treatments (T1–T3), and similar spread around centroids indicates comparable multivariate dispersion, supporting PERMANOVA results for bacterial species.

Supplementary Tables

Supplementary Table 6. 1. Test of homogeneity of multivariate dispersion (betadisper) based on Bray-Curtis distance at the microbiome phyla level.

Source of variation	Df	Sum of Squares	Mean Square	F	*p*-value
Treatment	2	0.0000300	0.0000150	0.047	0.95
Residuals	12	0.0037907	0.0003159		

Supplementary Table 6. 2. Pairwise Tukey HSD tests of multivariate dispersion for microbial phyla

Comparison	Difference	Lower CI	Upper CI	Adjusted *p*
T2 – T1	0.00337	−0.0266	0.0334	0.95
T3 – T1	0.00237	−0.0276	0.0324	0.98
T3 – T2	−0.00101	−0.0310	0.0290	0.99

Supplementary Table 6. 3. Test of homogeneity of multivariate dispersion (betadisper) based on Bray-Curtis distance at the microbiome genus level.

Source of variation	Df	Sum of Squares	Mean Square	F	*p*-value
Treatment	2	0.0000274	0.0000137	0.052	0.95
Residuals	12	0.0031990	0.000267		

Supplementary Table 6. 4. Pairwise Tukey HSD tests of multivariate dispersion for microbial genera

Comparison	Difference	Lower CI	Upper CI	Adjusted *p*
T2 – T1	0.00322	−0.02433	0.03077	0.95
T3 – T1	0.00095	−0.02660	0.02850	0.99
T3 – T2	−0.00228	−0.02983	0.02527	0.97

Supplementary Table 6. 5. Test of homogeneity of multivariate dispersion (betadisper) based on Bray-Curtis distance at the bacterial species level.

Source of variation	Df	Sum of Squares	Mean Square	F	*p*-value
Treatment	2	0.0000078	0.00000392	0.0023	0.99
Residuals	12	0.0201813	0.00168177		

Supplementary Table 6. 6. Pairwise Tukey HSD tests of multivariate dispersion for bacterial species

Comparison	Mean difference	Lower CI	Upper CI	Adjusted *p*
T2 – T1	0.00173	−0.06747	0.07093	0.99
T3 – T1	0.00120	−0.06800	0.07039	0.99
T3 – T2	−0.00053	−0.06973	0.06866	0.99