THE UNIVERSITY OF
SYDNEY

Increased Understandings of Ruminal Acidosis in Dairy Cattle

by

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A thesis submitted in fulfillment

of the requirements for the Degree of

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CONTENTS

ABSTRACT

Ruminal acidosis remains an important and prevalent disorder of economic and welfare concern to the dairy industry worldwide. There are inconsistencies in the diagnostic techniques and definitions of ruminal acidosis and a requirement for further information on the pathogenesis of ruminal acidosis, in particular in regard to the role of feed substrates, such as starch, sugar, and protein (Chapter 1). A greater understanding of changes to the microbiome during ruminal acidosis, feed management, and the possible synergistic effects of feed additive control agents is also required (Chapter 1). Consequently, the overall hypothesis of this thesis, which was supported, is that starch-, sugar-, and protein- or amino acid-based feed substrates would produce different ruminal and blood measures and distinct rumen bacterial community composition associated with different risks of ruminal acidosis. Secondly, that partial mixed ration feeding strategies and feed additive control agents would promote favorable ruminal conditions and reduce the risk of ruminal acidosis, which was also supported; however, whether feed additive control agents reduced the risk of ruminal acidosis was equivocal. Heifers exposed to a single feeding of grain and fructose had an increased risk of ruminal acidosis and accumulated ruminal lactate, compared to those fed grain only (Chapter 2). This highlights that diets with high sugar content should be fed with caution and increase the risk of ruminal acidosis when physically effective fiber is inadequate. Different oxidative stress responses were not observed among treatment groups of heifers fed single exposures of different substrates (Chapter 3) or different feed additives over a 20 d period (Chapter 7), but were evident in a heifer with acute clinical ruminal acidosis (Chapter 8). This suggests oxidative stress responses may only occur during acute clinical ruminal acidosis. Distinct ruminal bacterial community composition occurred among heifers fed a single exposure to different substrates (Chapter 4) and also among lactating cows fed different feeding strategies at different supplementary feeding amounts (Chapter 5) and these communities were associated with rumen fermentation characteristics. Cattle appeared to have host specific rumen bacteria and a core microbiome (Chapters 4 and 5). This suggests that host specificity in rumen ecosystems may be associated with the individual susceptibilities of cattle to ruminal acidosis and a need to tailor feed management and control for ruminal acidosis for individual cattle. Supplementary feeding amount and ruminal concentrations of propionate and valerate appeared to have the largest association with ruminal bacterial communities in Chapter 5

and may be good predictors of ruminal acidosis. A partial mixed ration feeding system, compared with component feeding, decreased ruminal acidosis (Chapter 5), suggesting benefits of this feeding system; however, milk production and milk component benefits were not observed for this feeding system. Feed additive control agents perturbed the rumen by different mechanisms but had minimal synergistic effects when combinations of feed additives were fed and ruminal acidosis control was equivocal (Chapters 6 and 7). Feed additives may not be capable of controlling ruminal acidosis in all cattle when large amounts of readily fermentable carbohydrates are fed (Chapter 7). Concentrations of the volatile fatty acids (**VFA**): butyrate, propionate, valerate, isobutyrate, isovalerate, and caproate were below detectable limits in a heifer with acute clinical ruminal acidosis 24 h after she consumed a ration with 19.1% sugar and 54.1% starch on a DM basis and her acetate concentration was <20 m*M*. However, concentrations of these VFA were higher 55 h after she consumed the ration. These findings demonstrate that the rumen is extremely dynamic and can rapidly recover from severe perturbation.

Throughout this thesis it has been evident that classic models of ruminal acidosis may not be sufficient to describe the pathogenesis of ruminal acidosis when diets with a high sugar content are fed and uncharacterized rumen bacteria may be involved in the pathogenesis of ruminal acidosis. Definitions of ruminal acidosis to describe acidosis when cattle are fed different substrates, in particular diets with a high sugar content are required. The rumen appears to be better adapted to respond to changes in starch intakes, compared with sugar intakes and cattle have individual rumen responses and susceptibilities to ruminal acidosis during shifts in feed substrates. In summary, this thesis has increased our understandings of the pathogenesis of ruminal acidosis and control strategies for ruminal acidosis in cattle.

PREFACE

All chapters of this thesis have been written in publication style. Chapters 2, 3, 4. 5, 7, and 8 have been published in peer-reviewed journals, while chapter 6 has been submitted for publication in a peer-reviewed journal, with H. M. Golder as first author and are indicated accordingly on the cover page. Sections of chapter 1 are intended for publication in peer-reviewed journals with modifications. Assistance given by others is indicated in the Confirmation of Co-authorship of Published Work and Acknowledgments of each chapter.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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B.AgSc. (Hons.)

October 2014

ACKNOWLEDGMENT OF CONTRIBUTION TO THE RESEARCH WORK AND/OR AUTHORSHIP

This thesis includes 6 original papers published in peer-reviewed journals and 1 submitted to a peer-review journal.

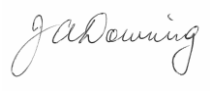
The core theme of the thesis is understanding ruminal acidosis in dairy cattle.

The ideas, development and writing up of all the papers in this thesis were the principal responsibility of the candidate, working within the Faculty of Veterinary Science under the supervision of Dr. Pietro Celi and Adj. Prof. Ian Lean.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

CONFIRMATION OF CO-AUTHORSHIP OF PUBLISHED WORK

Because the PhD candidate Ms Helen Golder collaborated widely with a large number of researchers, the Faculty of Veterinary Science's Board of Postgraduate Studies (BPGS), requested that the student provide good evidence to support her contribution to the research included in the thesis and have this acknowledged by the collaborators. These documents have been included with the thesis under examination. Dr Ahmad Rabiee's unavailability was discussed by the BPGS, and the committee has agreed that the absence of Dr Rabiee's signature should not delay the examination of the thesis and awarding of the degree.



Dr Jeff Downing

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Board of Postgraduate Studies

The following details the contribution of each of the co-authors to one or more peer-reviewed publications within this thesis.

Adj. Prof. Ian Lean and Dr. Ahmad Rabiee contributed to study design, animal experiments, statistical analysis, and finalizing of manuscripts prior to publication.

Dr. Pietro Celi contributed to study design, laboratory analysis methods, animal experiments, and finalizing of manuscripts prior to publication.

Prof. Cord Heuer and Dr. Idris Barchia contributed to statistical analysis and Dr. Murray Hannah contributed to study design and statistical analysis.

Dr. Elizabeth Bramley contributed to statistical analysis, the acidosis model, and finalizing of manuscripts prior to publication.

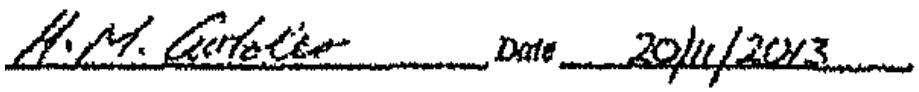
Assoc. Prof. David Miller contributed to laboratory analysis methods and finalizing of a manuscript prior to publication.

Dr. Ray King and Dr. Elliot Block contributed to study design and finalizing of manuscript(s) prior to publication.

Dr. Chris McSweeney and Dr. Stuart Denman contributed to laboratory analysis methods, statistical analysis, and finalizing of manuscripts prior to publication.

Dr. Bill Wales and Dr. Martin Auldist contributed to study design, animal experiments, and finalizing of a manuscript prior to publication.

Ms. Marlie Wright, Dr. Leah M arett, and Ms. Jae Greenwood contributed to animal experiments and data collation.

Candidate  Date 20/11/2013

I, as a co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate.

Adj. Prof. Ian Lean Date 20/11/2013

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CHAPTER 2: Effects of Grain, Fructose and Histidine Challenges on Ruminal pH and Fermentation Measures in Dairy Heifers

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	N for idea of research but Y for framing hypotheses
DESIGN	planning the methods to generate results	N
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Y
RESOURCES	dollars, equipment, space, personnel vital to the project	N
MATERIAL	biological materials, reagents, referred patients	Y
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Y
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	N/A

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CHAPTER 3: Effects of Grain, Fructose and Histidine Challenges on Endotoxin and Oxidative Stress Measures in Dairy Heifers

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Y
DESIGN	planning the methods to generate results	N
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Y
RESOURCES	dollars, equipment, space, personnel vital to the project	N
MATERIAL	biological materials, reagents, referred patients	Y
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Y
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	Y

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CHAPTER 4: Ruminal Bacterial Community Shifts in Grain, Fructose, and Histidine Challenged Dairy Heifers

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Y
DESIGN	planning the methods to generate results	Y
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Y
RESOURCES	dollars, equipment, space, personnel vital to the project	Y
MATERIAL	biological materials, reagents, referred patients	Y
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Y
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	Y

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CHAPTER 5: Effects of Partial Mixed Rations and Supplement Feeding Amounts on Ruminal Function, Bacterial Communities, and Ruminal Acidosis

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Hypotheses 3, 4, and 5 only relating to bacterial work
DESIGN	planning the methods to generate results	Bacterial work only
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Responsibility for bacterial work in the project and responsibility for the manuscript
RESOURCES	dollars, equipment, space, personnel vital to the project	Bacterial work only
MATERIAL	biological materials, reagents, referred patients	N
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Responsibility for bacterial work experiments only and responsibility for reporting data from whole project
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y except materials and methods for non-bacterial work
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
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CHAPTER 6: Effects of Feed Additives on Ruminal pH, Fermentation Products and Oxidative Stress Responses in Dairy Heifers Challenged with Grain

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Y
DESIGN	planning the methods to generate results	N
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	N for responsibility of organization of study 1, Y for responsibility of organization of study 2 and for manuscript
RESOURCES	dollars, equipment, space, personnel vital to the project	N
MATERIAL	biological materials, reagents, referred patients	N for study 1 and Y for study 2
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Responsibility for organising and reporting data
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
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WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	Y

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CHAPTER 7: Effects of Feed Additives on Ruminal and Blood Measures During a Grain and Fructose Challenge

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Y
DESIGN	planning the methods to generate results	Y
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Y
RESOURCES	dollars, equipment, space, personnel vital to the project	Y
MATERIAL	biological materials, reagents, referred patients	Y
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Y
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	Y

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CHAPTER 8: Case Report: Ruminal Acidosis in 21-month-old Holstein Heifer

CATEGORY	DESCRIPTION	INVOLVED Y/N
CONCEPT	the idea for the research or article, framing the hypothesis or key hypotheses	Y
DESIGN	planning the methods to generate results	Y
SUPERVISION	oversight and responsibility for the organization and course of the project and the manuscript	Y
RESOURCES	dollars, equipment, space, personnel vital to the project	Y
MATERIAL	biological materials, reagents, referred patients	Y
DATA COLLECTION/ PROCESSING	responsibility for doing experiments, managing patients, organizing and reporting data	Y
ANALYSIS/ INTERPRETATION	responsibility for making sense of and presenting the results	Y
LITERATURE SEARCH	responsibility for this necessary function	Y
WRITING	responsibility for creating all or a substantive part of the manuscript	Y
CRITICAL REVIEW	reworking the manuscript for intellectual content before submission, not just spelling and grammar checking	Y
OTHER	for novel contributions	Y

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I would also like to thank my supervisor, Pietro Celi who barely missed a sampling day throughout my cattle trials, has supported me throughout my PhD, and encouraged me to be involved in Tuesday night soccer and social Friday drinks when I first arrived at Camden. Fellow staff and students at the Franklin lab also deserve a thank you for supporting me with my trials and always being welcoming and helpful.

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I am grateful for the support of my family throughout my PhD, who always showed interest in my work, and continue to look after my pets since I moved to Camden.

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Bill Inglis and the staff from Craigend deserve a special mention for trusting me with their cattle during trial work, and feeding and weighing the cows before the trial.

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I'd like to thank my housemates during the last 4 years from the many friends I made during my time at Nepean Hall, to my housemates at the Nepean Lodges Tori Scott, Kate Hartcher, Sonia Lui, and Saranika Talukder for putting up with my irregular working hours and for sharing the highs and lows of my PhD. It was also a pleasure to work and socialize with the other Camden postgrads, postdocs, and university employees.

I will never forget the experiences I have had throughout my PhD and the people that shared my PhD journey which has shaped me as a person and given me many new skill sets. I am proud to have contributed work that will be of benefit to the dairy industry.

LIST OF ABBREVIATIONS

The following abbreviated terms have been used throughout the thesis and are defined at first use in each chapter. Abbreviations used exclusively in tables are not listed and are defined below the table.

%	percent
µg	microgram(s)
µL	microliter(s)
µm	micrometer(s)
µmol	micromole(s)
ADF	acid detergent fiber
ADG	average daily gain
ADICP	acid detergent insoluble crude protein
AOPP	advanced oxidation protein products
AR1	first-order autoregressive
ATCC	American Type Culture Collection
BAP	biological antioxidant potential
BCC	bacterial community composition
BRA	Bovine Research Australia
BCS	body condition score
BUF	sodium bicarbonate + magnesium oxide
BW	bodyweight
Carr U	Carratelli units
catalog no.	catalog number
ca.	circa
cDNA	Complementary deoxyribonucleic acid
CFU	coliform forming units
CP	crude protein
CV	coefficient of variation
d	day(s)
DCAD	dietary cation-anion difference
DEPI	Department of Environment and Primary Industries
DFM	Direct-fed microbial(s)
DGGE	denaturing gradient gel electrophoresis
DIM	days in milk
DM	dry matter
DMI	dry matter intake
DNA	deoxyribonucleic acid
dROM	derivatives of reactive oxygen metabolites
ECM	energy corrected milk
ESC	ethanol-soluble carbohydrates
EU	endotoxin units
FCR	feed conversion ratio
FDR	false discovery rate
FE	Fermenten®
FL	flavophospholipol
g	gram(s)

GEE	generalized estimating equations
GSH-Px	glutathione peroxidase
h	hour(s)
ICP-OES	inductively coupled plasma-optical emission spectroscopy
Inc.	Incorporation
kg	kilogram(s)
L	liter(s)
LAL	*Limulus* amebocyte lysate
Ln	natural logarithm
Log_{10}	logarithm base 10
m	meter(s)
M	monensin
ME	metabolizable energy
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
MLY	monensin + live yeast
mm	millimeter(s)
mmol	millimole(s)
m*M*	millimole(s) per liter
mo	month
Mt	Mount
MT	monensin + tylosin
N or no.	number
NDF	neutral detergent fiber
NDICP	neutral detergent insoluble crude protein
NEFA	non-esterified fatty acid
NFC	non-fiber carbohydrates
nm	nanometer
NPN	non-protein nitrogen
NSC	non-structural carbohydrate
NSP	non-starch polysaccharide
NSW	New South Wales
^oC	degree Celsius
OSI	oxidative stress index
OTU	operational taxonomic unit
P	probability
PC	principal component
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
pH	hydrogen ion concentration
pKa	logarithmic acid dissociation constant)
PMR	partial mixed ration
Pty Ltd.	Proprietary limited
qPCR	Real-time polymerase chain reaction
r	correlation coefficient
R^2	R-squared (coefficient of correlation)
rDNA	ribosomal deoxyribonucleic acid
RDP	rumen degradable protein
RISA	ribosomal intergeric spacer region

RNA	ribonucleic acid
ROC	receiver operator characteristic
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RUP	rumen undegradable protein
SARA	subacute ruminal acidosis
s	second(s)
SED	standard error of the difference
SEM	standard error of the mean
SD	standard deviation
SDM	total supplement DM/cow per day
SOLiD	Sequencing by Oligo Detection
SMRT	Single-molecule real-time
spp.	species
SSCP	Single-strand-conformation
SSU	small subunit
T	tylosin
T-FLRP	terminal restriction fragment analysis
TGGE	terminal gradient gel electrophoresis
TMR	total mixed ration
UK	United Kingdom
UV	ultraviolet
VFA	volatile fatty acids
VIC	Victoria
VM	Virginiamycin
vol/vol	volume per volume
wk	week(s)
WSC	water-soluble carbohydrates
yr	year(s)

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CHAPTER 3: Effects of Grain, Fructose, and Histidine Challenges on Endotoxin and Oxidative Stress Measures in Dairy Heifers

CHAPTER 8: Case Report: Ruminal Acidosis in 21-month-old Holsten Heifer

milled wheat).[1]Ruminal and blood samples were collected on d 0, 7, 14, and 21 during their respective experimental periods from both heifer 1250 and her cohort of heifers.[2]Ruminal and blood samples were collected from heifer 1250 on d 22 and 23. [3]Introductory doses were offered for the initial days before the full rate was offered. 353

Figure 2. Ruminal concentrations of total volatile fatty acid (VFA) (A); acetate (B); propionate (C); acetate:propionate (D); butyrate (E); valerate (F); ammonia (G); and total lactate (H); and pH (I); plasma oxidative stress index (OSI) (J); plasma ceruloplasmin concentrations (K); and serum β-hydroxybutyrate concentrations (L) in heifer 1250 that was diagnosed with acute ruminal acidosis and the mean (±SEM) of 7 cohort heifers. Ruminal fluid samples were collected using a stomach tube over the following 5 experimental periods on the study day indicated in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); Chall = challenge (d 21); and PC = postchallenge (d 22 and 23). Refer to Figure 1 for rations offered during each experimental period. On challenge (d 21) ruminal fluid samples were collected approximately 3.6 h after the challenge rations were consumed. *Ruminal fluid was not collected from the 7 cohort heifers during the postchallenge period. 356

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CHAPTER 4: Ruminal Bacterial Community Shifts in Grain, Fructose, and Histidine Challenged Dairy Heifers

CHAPTER 5: Effects of Partial Mixed Rations and Supplement Amounts on Milk Production and Composition, Ruminal Fermentation, Bacterial Communities, and Ruminal Acidosis

LIST OF PUBLICATIONS FROM THIS THESIS

Peer-reviewed Publications

Golder, H. M., P. Celi, A. R. Rabiee, and I. J. Lean. 2014. Effects of feed additives on rumen and blood profiles during a starch and fructose challenge. J. Dairy Sci. 97:985-1004.

Golder, H. M., S. E. Denman, C. McSweeney, P. Celi, and I. J. Lean. 2014. Ruminal bacterial community shifts in grain, sugar, and histidine challenged dairy heifers. J. Dairy Sci. 97:5131-5150.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION

The dairy industry is a vital multi-million dollar industry worldwide producing approximately 378 billion liters of milk annually (Anon, 2013). Milk or milk derived products are not only sold as direct consumer products, but are essential ingredients in an array of other products. Increasing world population, land use pressures, and declining terms of trade for farmers dictate that the dairy industry must further increase efficiency without compromising animal welfare. The major challenges for the dairy industry are rising costs of farm inputs, variable milk price, poor reproductive efficiency, lameness, mastitis, and nutritional disorders such as ruminal acidosis (Wells et al., 1998; Leddin et al., 2011) and some of these challenges are linked.

Ruminal acidosis is the most important nutritional disorder of ruminants and presents economic and welfare concerns to the dairy industry worldwide (Oetzel, 2003; Enemark et al., 2008). Ruminal acidosis is also considered to be a metabolic disorder and has been recognized by a multitude of names since the 17th century. It has a prevalence of between 10.0 to 26.7% in cross-sectional surveys of dairy herds (Bramley et al., 2008; Tajik et al., 2009) and although recent estimates are not available, in 1999, ruminal acidosis was estimated to cost between US$500 million and US$1 billion annually (Stone et al., 1999). Economic losses are mainly due to reduced milk and milk components production, lameness, premature culling, increased death loss (Krause and Oetzel, 2005), and costs of preventive treatments. Ruminal acidosis also has a number of sequalae disorders such as rumenitis, metabolic acidosis, laminitis, and liver abscesses that further contribute to economic and welfare concerns (Enemark et al., 2008; Plaizier et al., 2008).

Ruminants rely on energy generated from feed by microbes in the rumen and rumen bypass products for their maintenance, growth, and production. Overall, rumen function occurs as a result of the complex symbiotic relationship between the rumen and its ecosystem, which is highly responsive to dietary changes (Taijma et al., 2000). Ruminal acidosis occurs when ruminants consume large amounts of readily fermentable carbohydrates with inadequate physically effective dietary fiber (Bramley et al., 2008). The consumption of large amounts of readily fermentable carbohydrates perturb the rumen and impair its function, leading to clinical signs of ruminal acidosis such as

reduced milk yield, diarrhea, low body condition, low milk fat, lameness, cyclic feeding, and death (Enemark et al., 2008; Plaizier et al., 2008).

The complexity of ruminal acidosis and interactions within the ruminal ecosystem means that many questions about the pathogenesis of ruminal acidosis remain unanswered, despite a large number of reviews of ruminal acidosis (Nagaraja and Titgemeyer, 2007; Enemark et al., 2008; Plaizier et al., 2008; Tajik and Nazifi, 2011). This thesis attempts to address some of the pertinent questions surrounding the pathogenesis of ruminal acidosis to improve strategies for ruminal acidosis control; however, some questions remain unanswered and a number of new questions are raised.

First and foremost are questions surrounding how to accurately and consistently diagnose ruminal acidosis, as there are inconsistencies in regard to diagnostic methods and definitions of ruminal acidosis. Lean et al. (2009) note the need for definitions of metabolic disease to be accurate, standardized, repeatable, and based on clinical outcomes including measurable changes in metabolism, morbidity, mortality, or production. The work in this thesis continues on from that of Bramley et al. (2008) who produced a model to categorize ruminal conditions of cattle based on ruminal fermentation measures associated with health and production measures. The model produced by Bramley et al. (2008) was validated in this thesis and used to interpret ruminal conditions within this thesis.

Secondly, there are a number of questions surrounding the role of different feed substrates in ruminal acidosis. It is known that a number of feeds can induce ruminal acidosis such as cereal grains, brassicas, citrus pulp, root crops, forages with a high water soluble carbohydrate content, and fruits (Dunlop and Hammond, 1965). It is also known that several factors can increase the risk that feed substrates will cause ruminal acidosis, such as the type of processing, amount of feed fed, and feeding strategy (Britton et al., 1989); however, detail on responses of the rumen to these different substrates and responses when substrates are subjected to different factors are not fully elucidated.

Thirdly, ruminal acidosis models have been developed in order to describe the pathogenesis of ruminal acidosis (Hungate, 1966; Baldwin and Allison, 1983; Nocek et al., 1997; RAGFAR, 2007; Owens et al., 1998). These models describe pathways for the

generation of organic acids in the rumen and changes in ruminal bacteria but it is unclear where pro-inflammatory molecules such as histamine (Ahrens et al., 1967), and endotoxin (Khafipour et al., 2009), and oxidative stress (Wullepit et al., 2007) and inflammatory responses (Gozho et al., 2007) that have been associated with ruminal acidosis fit within these models.

Fourthly, knowledge of the ruminal ecosystem is rapidly advancing with the integration of use of more recently developed molecular techniques and pioneering culture-based methods. Multivariate analysis allows interpretations of associations between rumen microbiology, ruminal fermentation measures, and other information from the host (Dray et al., 2003). Links between structural analysis and functional gene activity (McSweeney et al., 2009) will provide improved understandings of rumen function in the future. Despite continued evolution in the field of rumen microbiology, only a minute portion of the rumen microbiome is known. Numerous questions remain in regard to the dynamic responses of ruminal bacteria under different ruminal conditions and how we can manipulate these to achieve a healthy rumen and improved control of ruminal acidosis.

Fifth, control of ruminal acidosis is required across many feeding systems and is attempted through a number of simultaneous feed management decisions (RAGFAR, 2007). Potential control strategies for ruminal acidosis and their influence on the rumen need to be evaluated. Inclusion of feed additives such as antibiotics, direct-fed microbials, yeasts, buffers, or neutralizing agents is part of the management strategies implemented in the industry (The et al., 1985; Coe et al., 1999; Krehbiel et al., 2003). Elucidating how feed additives modify the rumen is important for improving control of ruminal acidosis. Evaluation of efficacy between feed additives and the development of prudent use strategies for feed additives in the dairy industry are also important. Some of the questions that remain are: what is the most cost-effective and beneficial control strategy for ruminal acidosis? Is there a feed additive or control strategy capable of controlling ruminal acidosis in all cattle? Are there synergistic or antagonistic effects when feed additives are combined?

The overall aim of this thesis was to increase our understandings of the pathogenesis of ruminal acidosis and control strategies for ruminal acidosis in dairy cattle. This was achieved through the investigation of the role of substrates in rumen perturbation and

evaluation of feeding strategies and potential feed additive control agents for ruminal acidosis in 4 short or longer term randomized challenge studies. The overall hypothesis, which was supported, was that starch-, sugar-, and protein- or amino acid-based feed substrates would produce different ruminal and blood measures and distinct ruminal bacterial community composition associated with different risks of ruminal acidosis. Secondly, that partial mixed ration feeding strategies and feed additive control agents would promote favorable ruminal conditions and reduce the risk of ruminal acidosis, which was also supported; however, whether feed additive control agents reduced the risk of ruminal acidosis was equivocal.

This work has increased our understandings of the pathogenesis of ruminal acidosis and identified feed management practices that produce rumen responses that are favorable to reducing the risk of ruminal acidosis. The findings of this thesis, although focused at the dairy industry, have relevance for other ruminant production industries, such as the beef and sheep industries.

THESIS OUTLINE

This thesis is comprised of a critical review of ruminal acidosis (Chapter 1), a series of 4 randomized animal studies and interpretations (Chapters 2 to 7), a case report on clinical ruminal acidosis in a dairy heifer (Chapter 8), and a general discussion and conclusion. Each chapter is a stand-alone manuscript, each with its own abstract, introduction, materials and methods, results, discussion, and conclusion.

Chapter 1 is a critical review of understanding ruminal acidosis and focuses on defining acidosis and evaluation of diagnostic measures for ruminal acidosis, including an emphasis on comparisons between ruminal fluid collection methods, the role of substrates in ruminal acidosis, key ruminal bacteria associated with ruminal acidosis, and evaluation of feed additives as control agents for ruminal acidosis. As this review is a stand-alone manuscript with sections intended for publication with modifications; it incorporates key findings from the subsequent chapters of this thesis.

Chapter 2 is an experimental study that investigated the effects of a single exposure to combinations of grain, fructose, and histidine on ruminal pH and fermentation products in nonpregnant dairy heifers. It was hypothesized that the fructose would contribute to the onset of ruminal acidosis and alter ruminal pH, volatile fatty acid, and lactate

measures in grain-fed heifers, and further that histidine would increase ruminal histamine and induce ruminal acidosis. It was concluded that fructose increased the risk of ruminal acidosis compared to grain, with lactate accumulation occurring in the fructose-fed cattle. This study formed the basis for the hypotheses presented in Chapters 3 and 4. The addition of histidine did not have significant effects on ruminal fermentation. The study also showed that a single exposure to 0.8% of bodyweight (**BW**) dry matter (**DM**) grain and 0.4% of BW fructose was adequate as an induced ruminal acidosis challenge model capable of creating rumen perturbation. A modification of this challenge model was subsequently used to evaluate the ability of feed additives to reduce the risk of ruminal acidosis in Chapter 7.

Chapter 3 focuses on investigations of ruminal endotoxin concentrations and oxidative stress responses to the single exposure of combinations of grain, fructose, and histidine in Chapter 2, with responses hypothesized to occur in the fructose-fed cattle. Minimal oxidative stress responses were observed and it was hypothesized that responses may occur in cattle exposed to longer term carbohydrate challenges, this hypothesis was tested in Chapter 7.

Chapter 4 examines the ruminal bacterial community composition of cattle fed the single exposure of combinations of grain, fructose, and histidine in Chapter 2, both among treatment groups and at 3 rumen fluid collection points over a 3.6 h period after ration consumption. Associations between ruminal fermentation measures identified in Chapter 2 and ruminal bacterial community composition were investigated and bacterial communities were evaluated in the context of existing understandings of ruminal acidosis models (RAGFAR, 2007; Nocek, 1997). *Lactobacillus* spp. generally associated with ruminal acidosis were not identified as shifting in relative abundance in this initial period of rumen perturbation; however, an operational taxonomic unit closely associated with *Streptococcus bovis* was identified in heifers from the grain + fructose group. Despite distinct bacterial community composition among treatment groups a large variation in ruminal bacterial community composition was observed both among- and within-groups. Cattle appeared to be able to adapt to short term exposures to grain compared to fructose with bacterial community compositions from the fructose-fed cattle associated with increased lactate and butyrate concentrations and decreased

ruminal pH. This chapter led to the investigation of ruminal bacterial community composition in a longer term substrate exposure study (Chapter 5).

Chapter 5 examines milk and ruminal measures, ruminal acidosis, and ruminal bacterial community composition and their associations in lactating dairy cattle fed a partial mixed ration at linearly increasing supplement amounts for 19 d, as part of the Flexible Feeding System Project (Future Farming Systems Research Division, Department of Environment and Primary Industries, Ellinbank, Victoria, Australia). The incorporation of estimated metabolizable protein above NRC (2001) requirements was also investigated. Ruminal acidosis was evaluated in this chapter using the model by Bramley et al. (2008) and validated the use of this acidosis model to evaluate ruminal acidosis and ruminal conditions in Chapters 2 and 7. Similar to Chapter 4, distinct ruminal bacterial community composition was observed among treatment groups, a number of bacteria believed to be associated with ruminal acidosis were dominant, and a large among- and within-group variation in ruminal bacterial community composition occurred. Ruminal acidosis was reduced in cattle offered diets as a partial mixed ration, compared with cattle offered diets as components.

After developing an understanding of the role of the substrates: starch, fructose, and protein in rumen perturbation and ruminal acidosis in Chapters 2 to 5, the ability of feed additives to reduce the risk of ruminal acidosis was evaluated in Chapters 6 and 7. Use of feed additives is common practice in the dairy industry; however, literature on use of feed additive combinations is limited. There is a need to develop prudent use strategies for feed additives and assess non-antibiotic feed additive control strategies for ruminal acidosis. Chapter 6 combined data from 2 single grain challenge randomized block studies focused on the effects of Fermenten®, monensin, tylosin, and flavophospholipol, and synergistic effects of their combinations on ruminal fermentation and plasma measures, and identified Fermenten® and monensin as having possible beneficial effects.

The potential of both antibiotic and non-antibiotic feed additives to reduce the risk of ruminal acidosis was evaluated in heifers fed readily fermentable carbohydrates for a longer period in Chapter 7. Virginiamycin, and the combinations of monensin and tylosin, monensin and live yeast, and sodium bicarbonate and magnesium oxide were fed in a 21 d readily fermentable carbohydrate feeding study. The first 20 d consisted of

an adaptation period to the 62:38 forage:concentrate total mixed ration, followed by a modification of the single grain and fructose ruminal acidosis induction challenge model validated in Chapter 2. A control heifer was diagnosed with clinical ruminal acidosis 10 h after consuming the grain and fructose challenge ration. Her clinical signs, dry matter intake, locomotion scores, and ruminal and blood measures are compared to those in her cohort, in a case study reported as Chapter 8.

The thesis concludes with a general discussion and conclusion that focuses on several interpretations and implications from this thesis. Key findings include firstly, that the ruminal acidosis model by Bramley et al. (2008) adequately defines starch-based ruminal acidosis but is not suitable for diagnosis of sugar-based ruminal acidosis and valerate and propionate are good diagnostic measures of ruminal acidosis. Secondly, that feed substrates have an important influence on the risk of ruminal acidosis and the rumen microbiome, with sugars increasing the risk of ruminal acidosis, compared to grain fed when physically effective fiber is inadequate. Thirdly, that the involvement of ruminal histamine and endotoxin concentrations and plasma oxidative stress responses in the pathogenesis of ruminal acidosis remains unclear and requires further investigation. Fourth, that individual cattle were observed to have host specific bacterial community composition that share a common microbiome. Fifth, that feeding systems and feed additives have an important role in ruminal acidosis control, but require refinement and prudent use, respectively. Genetic by environment interactions, development of ruminal acidosis models for different feed substrates, modes of action of feed additives, and characterization of a greater proportion of microbiota in the rumen are yet a few potential areas for continued research in this field.

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CHAPTER 1

Critical Review: Understanding Ruminal Acidosis in Dairy Cattle

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OVERVIEW OF CHAPTER 1

Ruminal acidosis remains a complex nutritional and metabolic disorder impacting cattle production and welfare worldwide. To increase our understandings of ruminal acidosis and identify required areas of research in order to reduce the impact of ruminal acidosis Chapter 1 critically reviews definitions and use of diagnostic measures for ruminal acidosis, ruminal fluid collection techniques, the involvement of feed substrates and ruminal bacteria, and the use of feed additives as control agents for ruminal acidosis.

ABSTRACT

Ruminal acidosis is an important nutritional and metabolic disorder with a point prevalence of between 10.0 to 26.7% across a range of dairy feeding systems worldwide. It appears to occur along a continuum of severity reflecting the degree of hydrogen sequestration in safe pools. Diagnosis of ruminal acidosis is best achieved through an evaluation of a combination of clinical signs, feed management history, ruminal fermentation measures, and production performance. The volatile fatty acids, propionate and valerate were both sensitive (0.93 and 0.90, respectively) and specific (0.87 and 0.90, respectively) predictors of ruminal acidosis. Ruminal pH collected by rumenocentesis or stomach tube were moderately sensitive (0.68 and 0.74, respectively) and specific (0.84 and 0.79, respectively) predictors of ruminal acidosis. Milk fat to protein ratio was specific (0.81) but not sensitive (0.54).

There are advantages and disadvantages for the use of ruminal fluid collection by fistula, rumenocentesis, or stomach tube. Each ruminal fluid collection method is valid for measuring ruminal pH provided these are carried out correctly and consistently. Continuous ruminal pH measurement systems provide robust data. Feeding sugars as substrates produces different fermentation profiles to starches, and may be more conducive to causing ruminal acidosis than starch. Molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphisms (RFLP) in combination with traditional culture-based techniques are rapidly increasing our understandings of the rumen microbiome. Advances in sequencing and metagenomics are allowing examination of phylogenetic, physical, and functional properties of microbial communities. Future steps are the association between structural analysis and functional gene activity. Individuals appear to have a unique rumen ecosystem comprised of a core microbiome. Feed additives used to control ruminal acidosis could include antibiotics, buffers, neutralizing agents, and direct-fed microbials, and appear to influence the rumen by different mechanisms. The potential for synergistic use of these agents is largely unknown.

Keywords: bacterial microbiome, feed additives, ruminal acidosis, rumen ecology

INTRODUCTION

Ruminal acidosis is an important complex nutritional disorder of ruminants, referred to by many names (Underwood, 1992) that presents economic and welfare concerns for the dairy industry (Oetzel, 2003). It occurs across a range of feeding systems in the dairy industry worldwide and has a prevalence of between 10.0 to 26.7% in cross-sectional surveys of dairy herds (Table 1).

The substantial economic impacts of ruminal acidosis are associated with reduced milk production and components, lameness, premature culling, increased death loss (Krause and Oetzel, 2005), and costs of preventive interventions. There is no recent estimate of economic loss; however, in herds diagnosed with the subacute form of ruminal acidosis it was estimated to cost US$1.12 per dairy cow per day (Stone, 1999). A number of sequalae to ruminal acidosis such as laminitis, liver abscesses, and rumenitis (Enemark, 2008; Plaizier et al., 2008) further contribute to its economic impact.

Despite a number of recent comprehensive reviews of ruminal acidosis (Nagaraja and Titgemeyer, 2007; Enemark, 2008; Plaizier et al., 2008; Tajik and Nazifi, 2011), a quantitative review is warranted that focuses on ruminal acidosis definitions, diagnosis, critical review of diagnostic measures, comparisons among ruminal fluid sampling techniques, responses to different feed substrates, involvement of ruminal bacteria in acidosis, and the use of feed additives as control strategies for ruminal acidosis.

DEFINITIONS

The ruminal acidosis complex can manifest in a variety of forms (Kleen et al., 2003) and is primarily associated with feed management practices. A lack of consistency in both definitions and diagnostic techniques for ruminal acidosis has led to a multitude of names being applied to the different presentations of ruminal acidosis and has created confusion in diagnosis (Kleen et al., 2003). Britton et al. (1989) described ruminal acidosis as ‘not one disease, but rather a continuum of degrees of ruminal acidity’. Perhaps this understanding could be reworded in the context of degrees of safe sequestration of hydrogen. However, since the 1990’s the continuum of ruminal acidosis has been frequently simplified into 2 forms of severity, subacute or acute, whose ruminal, blood, microbial, and sequalae characteristics are summarized by Nagaraja and

Table 1. Summary of cross-sectional surveys of the prevalence of ruminal acidosis in dairy herds

Country	Farming system	Herd selection criteria (or details)	Cow selection	Number of herds sampled (number of cows sampled/herd)	Measures	Diagnosis basis	Prevalence (%)	Reference
Nederlands	grass & maize silage, potatoes, concentrate	typical to the area and serviced by the same veterinary practice mean 8600 kg milk yield	group1: ≤25 DIM group 2: 25 to 182 DIM	18 herds (ca. 12 cows)	*Ruminal*: pH (RC), VFA, protozoa, consistency, fill *Faecal:* consistency, structure *Milk:* yield, components, long chain fatty acids *Urine:* pH, net acid-base excretion *Other:* body condition scoring	SARA if ruminal pH <5.5	13.7	Kleen et al. (2009)
Iran	TMR	willing participation >250 cows	random selection group 1: 3 to 20 DIM group 2: 60 to 150 DIM	10 herds	*Ruminal:* pH (RC), contractions *Milk*: components *Other*: body condition *Urine*: pH *Faecal*: quality	SARA if ruminal pH ≤5.5	26.7	Tajik et al. (2009)
Australia	predominately pasture based	randomly selected with a random numbers table	<100 DIM 3 primiparous 5multiparious	100 herds (8 cows)	*Ruminal:* pH (RC and stomach tube), VFA, ammonia, and lactate *Milk:* yield and components *Feed*: composition, predicted diet composition (CPM)	discriminant analysis including ruminal pH (RC), VFA, ammonia, and lactate	10	Bramley et al. (2008)
Ireland	pasture based	<2kg of supplement/milking 95±38 herd size 8114±734 kg/cow milk yield	random selection 80 to 150 DIM	12 herds (12 cows)	*Ruminal:* pH (RC) and VFA *Milk:* yield and components Faecal: consistency and fiber scores *Feed:* pasture composition *Other*: health status, locomotion & body condition scoring	SARA if ruminal pH <5.5	11	O'Grady et al.(2008)
Italy	TMR	ca. 10,000 kg/yr milk yield >100 cows step up and down lactation diets, TMR	no clinical signs of disease random selection 5 to 60 DIM	10 herds (12 cows)	*Ruminal*: pH (RC), VFA, ammonia, and lactate *Feed:* composition *Other:* health data	SARA if ruminal pH <5.5 critical risk of SARA if ruminal pH <5.8	3 herds 5 herds at critical risk of SARA	Morgante et al. (2007)

RC = rumenocentesis; SARA = subacute ruminal acidosis.

Table 1 (continued). Summary of cross-sectional surveys on the prevalence of ruminal acidosis in dairy herds

Country	Farming system	Herd selection criteria (or details)	Cow selection	Number of herds sampled (number of cows sampled/herd)	Measures	Diagnosis basis	Prevalence (%)	Reference
USA (Wisconsin)	-	-	-	57 herds	*Ruminal:* pH	SARA if ruminal pH <5.5	23	Oetzel et al.(2004)
USA (Wisconsin)	TMR	<80 herd size >8200 kg milk yield rolling herd average adequate chemical fiber in diets	6 cows 2 to 30 DIM 6 cows 90 to 120 DIM	14 herds (6 early and 6 peak lactation cows)	*Ruminal:* pH and D-and L-lactate	SARA if ruminal pH <5.5	20.1	Oetzel et al. (1999)
USA (Wisconsin)	TMR	<80 herd size >8200 kg milk yield rolling herd average adequate chemical fiber in diets	6 cows to 30 d pre-calving 6 cows 2 to 30 DIM 6 cows 90 to 120 DIM	15 herds (6 pre-calving, 6 early, and 6 peak lactation cows)	*Ruminal*: pH (RC) *Feed*: composition	SARA if ruminal pH <5.5	>40% of herd in 33% of herds 19% (early lactation cows) 26% (mid lactation cows)	Garrett et al. (1997)
Finland[1]	-	-	treated by a veterinarian in 1983 for acidosis	61,124 cows	-	-	0.3% (lactation incidence risk)	Grohn and Bruss (1990)

[1]Veterinary diagnosis data from 2 d pre-partum until next subsequent calving.
RC = rumenocentesis; SARA = subacute ruminal acidosis.

Titgemeyer (2007). Subacute ruminal acidosis (**SARA**) is also commonly referred to as subclinical ruminal acidosis and was defined by Nordlund and Garrett (1994) as 'a temporarily altered ruminal fermentation rate or pattern which causes an accumulation of fermentation end products in the rumen, a decrease in ruminal pH, and changes in the microbial population distribution; but the aberration is not severe enough or of sufficient duration to cause overt, pathognomonic clinical signs in the animal'. Milder forms of ruminal acidosis such as SARA can be regarded as a herd disease (Nordlund and Garrett, 1994) and acute ruminal acidosis as occurring in the individual cow. The complex nature of ruminal acidosis prevents classification of all cases of ruminal acidosis into the simplified forms of severity, such as subacute and acute.

CATTLE AT RISK

Herds are likely to be at risk of ruminal acidosis if they belong to one or more of the following categories: (1) have inadequate ruminal buffering caused by inadequate dietary fiber and/ or inadequate physical fiber, (2) have excessive intake of rapidly fermentable carbohydrates, or (3) have inadequate ruminal microbial and papillae adaptation to a highly fermentable diet (Krause and Oetzel, 2006). Ruminal buffering results from a combination of both endogenous buffers, such as saliva, and dietary buffering (Krause and Oetzel, 2006). Kleen et al. (2003) described early post-partum and mid-lactation as the 2 most critical periods when cows are at risk of ruminal acidosis for a number of reasons. Cows in the transition period are subject to a large amount of stress due to calving, variable dry matter intake (**DMI**), change of management, and negative energy balance and are often changed from a low energy, high fiber diet in the dry period to a high energy diet with less fiber during lactation without adequate adaptation, increasing their risk of ruminal acidosis (Kleen et al., 2003). Ruminal acidosis in mid-lactation cattle usually results from changes in feed management, such as mistakes in feed ration formulation, poor timing of feed management, and over processing of total mixed rations (Kleen et al., 2003).

DIAGNOSIS

A correct diagnosis of ruminal acidosis and associated secondary disorders is critical for the implementation of strategies to control and optimize rumen conditions that will be cost-effective and beneficial to animal health. Diagnosis of false positives of any

disorder can lead to unnecessary treatment interventions that may be uneconomical, while the diagnosis of false negatives will compromise animal health and production.

In practice, veterinarians and research scientists use different diagnostic measures for rumen disturbance or ruminal acidosis based on their differing reasons for diagnosis, available diagnostic timeframes, and access to critical information for diagnosis. Veterinarians need to provide an individual cow-side diagnosis and implement treatment immediately, in the case of severe ruminal acidosis, or provide a herd diagnosis for herds suspected of milder forms of ruminal acidosis. Therefore, veterinarians are limited to the use of diagnostic measures and on-farm information that returns results that can be immediately interpreted. Research scientists have greater control over the environment and greater access to feeding history, resources, and monitoring over longer periods, compared to veterinarians. Research scientists have the luxury to use diagnostic measures that require a longer processing time for results. Although each research scientist will have individual research aims, in general ruminal conditions need to be monitored in cattle at a single point in time or over a period of time, often in response to interventions, hence different diagnostic measures to those used by veterinarians may be required.

The process of diagnosis of ruminal acidosis by either veterinarians or research scientists begins with an assessment of clinical signs, inspection of feed and diets, and the integration of information on the history of feed and feed management, production, and clinical disorders. Critical information and history to be obtained for the individual and herd includes: the time since last feeding, ration component breakdown, previous disease history, milk yield and composition or weight gain records, feed and feed management history, cull records, and death losses. Problems identified that could be related to ruminal acidosis should be characterized in detail and evaluated using methods such as those described by Oetzel (2000) before ruminal acidosis is considered as the cause. Diagnosis should then be supported by analysis of ruminal, blood, urine, fecal, milk, and feed measures when permitted.

Clinical Signs

The clinical signs of ruminal acidosis usually reflect the severity of the case and are frequently not recognized or are subtle for milder cases of ruminal acidosis as their onset can occur after a time-lag from a predisposing event (Nordlund and Garrett, 1994). The occurrence of milder ruminal acidosis is often a herd problem (Enemark, 2008) and is difficult to diagnosis in individual cows. Many of the clinical signs associated with ruminal acidosis have differential diagnoses (Britton and Stock., 1986); therefore, collective interpretation of all clinical signs observed is important.

Individual Cow Level

In individual cows suspected of ruminal acidosis, a physical examination should be performed that includes measurements of heart rate, respiration rate, rate of rumen contractions, and body temperature. Locomotion, body condition, perineal staining, fecal consistency, and rumen fill should also be scored (Sprecher et al., 1997; Atkinson, 2009; Bramley et al., 2012). Further, demeanor, dehydration, and sites of pain should be assessed.

Cattle with rumen perturbations consistent with ruminal acidosis may present with a range of clinical and subclinical signs that include poor body condition, diarrhoea, a dull and lethargic demeanor, dehydration, a moderately distended rumen, lameness, weak rumen contractions, anoxia, abdominal pain, tachycardia, tachypnea, staggering, recumbency, coma, poor immune function, sporadic nosebleeds, a decline in milk yield, depression in milk fat, and death may occur (Oetzel, 2000; Krause and Oetzel, 2006). The speed of progression of clinical signs may depend on the severity of rumen perturbation, but death can occur within 12 to 24 hours in peracute cases (Dirksen, 1970).

Herd Level

At the herd level the same signs assessed in individual cows can be measured or scored for a random selection of cows in subgroups at risk of ruminal acidosis (Nordlund and Garrett, 1994). However, heart rate, respiration rate, rate of rumen contractions, and body temperature measurements are usually omitted. Garrett et al. (1999) suggested a sample size of 12 cows or more be evaluated. An increase in, or above average

percentage of premature culling for the herd may be an indication of ruminal acidosis (Nordlund and Garrett, 1994). Within a herd, groups of cattle may be diagnosed with different ruminal conditions (Bramley et al., 2012).

Secondary Disorders

Signs of one or more secondary disorders of ruminal acidosis may assist in the diagnosis of ruminal acidosis; however, many links between these disorders and ruminal acidosis have not been completely elucidated. At an individual cow level, clinical signs of secondary disorders of ruminal acidosis may have a differential diagnosis; however, a high prevalence of these signs at the herd level suggests that ruminal acidosis is also prevalent (Nordlund et al., 1995). There are several disorders believed to be secondary to ruminal acidosis including: laminitis (Nilsson, 1963), rumenitis (Enemark, 2008), epistaxis (Dirken, 1985), vena caval syndrome (Nordlund, 1995), cerebro-corticol necrosis (polioencephalomalacia) (Enemark et al., 2002), parakeratosis (Dirken, 1985), metabolic acidosis (Dunlop, 1972), and liver abcesses (Oetzel, 2000).

Feeding Behavior and Assessment

Feeding behavior of the herd including the following should be observed: average rumination time, sorting behavior of a total mixed ration (**TMR**), and dry matter intake. Cows that have a low rumination time, are sorting their feed, have a cyclic feeding pattern, or low DMI may be at risk of ruminal acidosis (Britton et al., 1989; Maekawa et al., 2002). Cows that are low in the social order, which are frequently first lactation cows, often eat last and therefore can be exposed to feed with a different effective fiber content or chemical composition resulting from sorting from the previous cows and may increase their risk of ruminal acidosis (Kleen et al., 2003). All feed sources should be assessed for chop length or particle size if applicable, and quality using relevant characteristics ie stage of maturity of pasture, type of pasture or forage. Chemical analysis can be performed on individual feed components and residual TMR after feeding to obtain their percentage of dry matter (**DM**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), crude protein (**CP**), starch, sugar, and non-structural carbohydrate (**NSC**) content (RAGFAR, 2007). This will allow estimation of the overall chemical composition of the cow's daily diet and for comparison with recommended requirements. This information, combined with the evaluation of the physical

characteristics of the feed will indicate possible sub-optimum rumen function and ruminal acidosis (RAGFAR, 2007).

DIAGNOSTIC MEASURES

A range of ruminal, blood, urine, fecal, milk, and other monitoring measures can be used to support diagnosis and monitoring of ruminal acidosis, but the diagnostic value of each measure has not always been validated. With the exception of the measurement of ruminal pH and calculation of milk fat to protein, the diagnostic measures critically reviewed here are limited to research scientists due to the processing time.

To better define ruminal acidosis, diagnostic measures must be validated and be sensitive, specific, repeatable, and cost-effective. The value of a diagnostic measure can be assessed using a receiver operator characteristic (**ROC**) curve which indicates the sensitivity and specificity of the test and the area under the curve created between these measures. Sensitivity of a measure defines the ability of a test to correctly identify individual cattle with the disorder; whereas, specificity defines the ability of a measure to correctly identify individual cattle that do not have the disorder (Ospina et al., 2013). Thus a measure can be sensitive and not specific or specific and not sensitive. The use of diagnostic measures that are not both sensitive and specific will result in misdiagnosis and likely result in treatment that is not optimal and provide research scientists false indications of research outcomes.

Standardization of methods for measuring indicators of ruminal acidosis should be used; in particular collection site of samples, sample storage, and collection time relative to feeding should be consistent among measurements. Interpretation of diagnostic measures can be based on statistical differences between experimental groups, specific cut-point values, or optimal ranges.

Ruminal Measures

pH

Diagnosis of ruminal conditions in cattle during experimental studies has been based on ruminal pH cut points, mean ruminal pH, nadir pH, or time or area that ruminal pH is less than a defined pH cut point. Defined cut points vary, but in general have been

defined as ranging between ≤5.5 (Garrett et al., 1999) to ≤5.8 for diagnosis of SARA (Beauchemin et al., 2003b) and <5.0 for acute ruminal acidosis (Nagaraja and Titgemeyer, 2007); however, the pH cut-point where acute ruminal acidosis occurs is hard to define and probably not very important (Stock, 2000). Ruminal pH <5.5 for >3 h has recently also been used to diagnosis SARA (Gozho et al., 2007). Plaizier et al. (2008), importantly, noted that there is no agreement on when ruminal pH depressions are detrimental to the health and production of dairy cattle.

The rumen is heterogeneous and ruminal pH fluctuates throughout the day (Keunen et al., 2002). In particular ruminal pH is influenced by the consumption of readily fermentable carbohydrates. Ruminal pH shifts of 0.5 to 1.0 pH units, which reflect 5 to 10 fold changes in hydrogen, have occurred over a 24 h period (Dado and Allen, 1993; Nocek et al., 2002). The changes in ruminal pH after feeding and the dynamic nature of the rumen are a challenge to evaluate (Garrett, 1996; Oetzel, 2003). It has been noted that the rumen is dynamic, as opposed to being in a steady state, to create a favourable environment for digestion of the diet (Kleen, 2004). Feeding different substrates can create different ruminal pH environments, which should not be interpreted using generic rumen pH reference cut-points. Therefore, despite the common use of ruminal pH measures for ruminal acidosis diagnosis, these should be used with caution and in conjunction with other measures or reference values defined for different feeding systems.

Ruminal pH measures also differ between ruminal fluid samples collected using different methods (Table 2). The majority of ruminal pH differences in ruminal fluid collected using a stomach tube, rumenocentesis, or rumen fistula were less than 0.35 pH units, with r^2 values ranging from 0.11 to 0.73 (Table 2). Ruminal pH values from ruminal fluid collected by rumenocentesis were higher than those from fistulated cattle; while, stomach tube ruminal pH values were higher than those collected by rumenocentesis or from fistulated cattle (Table 3). Hence, an adjustment factor may be required for comparison of ruminal pH values measured in ruminal fluid collected using different techniques.

Table 2. Difference and relationship between ruminal pH measurements in ruminal fluid collected using stomach tubing, rumenocentesis, and rumen fistula methods in cattle

Methods compared	No. of cows sampled	Difference in ruminal pH values between methods[1]	Relationship between methods (r^2)	Reference
Stomach tube and rumenocentesis				
	6	+0.04		Shen et al. (2012)
	58	+0.76	0.11	Enemark et al. (2004)
	5	+1.1		Nordlund et al. (1995)
	800	+0.2	0.47	Bramley, unpublished
	16	+0.35	0.25	Duffield et al. (2004)
Rumenocentesis and fistula				
	30	+0.28	0.52	Garrett et al. (1999)
	16	+0.33	0.42	Duffield et al. (2004)
	30	+0.34	0.73	Garrett et al. (1995)
Stomach tube and fistula				
	16	+0.34	0.58	Duffield et al. (2004)
Continuous ruminal pH measurement system and fistula			Correlation coefficient (r)	
Mean over 1 min	14	Mean of 1 and 5 min -0.03	0.98	Penner et al.(2006)
Mean over 5 min	14		0.97	Penner et al.(2006)
	4	-0.04	0.99	Sato et al. (2012)
	4	+0.39	0.93	Phillips et al. (2010)
	12	+0.11	0.85	Dado and Allen (1993)
	6		0.65	Graf et al. (2005)
	1	-0.07	0.88	Al Zahal et al., (2007)
	16	cranial-ventral site	0.68	Duffield et al. (2004)
	16	caudal-ventral site	0.61	Duffield et al. (2004)
	16	central site	0.35	Duffield et al. (2004)
	16	cranial-dorsal site	0.50	Duffield et al. (2004)
Continuous ruminal pH measurement system and stomach tube				
	16	First sample	0.15	Duffield et al. (2004)
	16	Second sample	0.31	Duffield et al. (2004)
Continuous ruminal pH measurement system and rumenocentesis				
	16		0.43	Duffield et al. (2004)
	6		0.56	Marchesini et al. (2013)

[1]Difference in ruminal pH values were calculated by subtracting the mean ruminal pH value for the second named ruminal collection method from the first named collection method ie Mean ruminal pH of stomach tube ruminal sample - Mean ruminal pH of rumenocentesis ruminal sample.

Receiver operator characteristic curves produced from the dataset of Bramley et al. (2008) that assessed ruminal status of 800 cows from 100 dairies using ruminal, performance, feed, and fecal characteristics showed that ruminal pH measured from ruminal fluid obtained using a stomach tube or rumenocentesis were only moderately sensitive (0.68 and 0.74, respectively) and specific (0.84 and 0.79, respectively) indicators of ruminal acidosis (Table 3).

Table 3. Sensitivity (Se), specificity (Sp), area under the curve (AUC), and cut points from receiver operator characteristic curves for the value of ruminal and milk measures for diagnosing ruminal acidosis (Rabiee and Lean, 2012) based on the dataset of Bramley et al. (2008)

Measure	Se	Sp	AUC	Cut point
Acetate (m*M*)	0.94	0.27	0.627	36.7
Butyrate (m*M*)	0.94	0.20	0.530	5.28
Propionate (m*M*)	0.93	0.87	0.955	23.1
Valerate (m*M*)	0.90	0.90	0.954	1.62
pH (stomach tube)	0.68	0.84	0.801	6.45
pH (rumenocentesis)	0.74	0.79	0.822	5.96
Milk fat:milk protein	0.54	0.81	0.716	1.02

There are advantages and disadvantages for the use of each ruminal fluid collection method, with fistulas enabling collection of a mixed, representative ruminal fluid sample from multiple sites in the rumen; however, fistulation surgery is required, the anaerobic integrity of the rumen is breached, and this method is limited to relatively small numbers of experimental cattle. Larger numbers of cattle can be sampled using rumenocentesis and samples can be taken from a relatively consistent site; however, there is a risk of peritonitis and more animal restraint is required (Nordlund and Garrett, 1994). Large numbers of cattle can be sampled multiple times using a stomach tube, as it is less invasive and has less chance of infection, but there is limited control over the position of the tube in the rumen. Saliva contamination has been raised as a concern in ruminal fluid collected using a stomach tube (Duffield et al., 2004); however, is rarely of concern if the correct insertion technique is used (Lodge-Ivey et al., 2009; Shen et al., 2012). Each ruminal fluid collection method is valid provided it is carried out correctly and consistently. The rumen is not homogenous; therefore, perhaps ruminal evacuation, mixing, and sampling is the only method of obtaining an accurate ruminal pH.

Standardization of timing of ruminal fluid collection after feeding allows improved comparison of ruminal measures between samplings (Plaizier et al., 2008) and sampling

should occur within 2 to 4 h after concentrate consumption in cattle fed separate components and within 5 to 8 h for TMR fed cattle (Nordlund and Garrett, 1994; Garrett, 1996). In the field, a single ruminal pH measurement is often the only pH value obtained by clinicians, but is less accurate than continuous ruminal pH measurements (Duffield et al., 2004; AlZahal et al., 2007) and is not suitable for a herd diagnosis (Nordlund and Garrett, 1994).

Continuous ruminal pH measurement systems that are also referred to as rumen in-dwelling pH probes and intra-ruminal boluses or sensors can be used experimentally to provide continuous ruminal pH measurement (Penner et al., 2006). Lampila (1955) made the first attempt at continuous ruminal pH measurement in cattle, but technology has progressed markedly since and continues to evolve. The earlier probes were only suitable for use in fistulated cattle due to their size and requirement for daily removal for cleaning and recalibration (Dado and Allen, 1993). Movement of cattle was also restricted to within limited range of the transceiver units (Dado and Allen, 1993).

Current intra-ruminal pH boluses are considerably smaller and can be orally administered into the rumen of non-fistulated cattle and transmit ruminal pH data using radio frequency to a transceiver unit (Mottram et al., 2008). In general, they consist of a ruminal pH sensor which can be accompanied by a temperature and pressure sensor, a component to transduce and condition the signal and store the data, radio transceiver, aerial, and battery that are enclosed in a container (Mottram et al., 2008).

There are a number of different continuous ruminal pH measurement systems and therefore caution should be used when making comparisons between systems. Ruminal pH values collected by continuous ruminal pH measurement systems were either higher or lower than those obtained by spot sampling in fistulated cattle with no consistency observed (Table 2). Correlation coefficients between ruminal pH values obtained from continuous ruminal pH monitoring systems or fistula were ≥0.88 for studies conducted after 2006, which shows how the technology has progressed over time (Table 2). Correlation coefficients between continuous ruminal pH measurement systems and stomach tube or rumenocentesis obtained ruminal pH values were low (≤0.43; Table 2).

Use of continuous ruminal pH measurement systems has the advantages of allowing easy and non-invasive monitoring of fluctuations in ruminal pH (Penner et al., 2006),

which may aid in detection of undesirable rumen conditions and the occurrence of ruminal acidosis (Mottram et al., 2008). Potential concerns with the use of continuous measurement systems are; upward ruminal pH drift and sensitivity (Penner et al., 2006), lack of control over the location of the probe because probes migrate within the rumen (Penner et al., 2006; Kaur et al., 2010), fibrous material can pack around the probe and affect the pH (Nocek et al., 2002; Kaur et al., 2010), the probes may lodge in one point in the rumen, limited battery life, and cattle must return to within distance of the data logger. Consequently, the probes are currently restricted largely to research use (Sato et al., 2012).

Veterinarians are likely to only collect ruminal fluid by rumenocentesis or using a stomach tube and should measure ruminal pH immediately using a calibrated pH meter. In the case of a herd diagnosis a sample should be collected from a minimum of 12 cows (Garrett et al., 1999). For research scientists, if large numbers of cattle are available and a single ruminal sample is required, collection of ruminal fluid samples by rumenocentesis may be the most suitable; however, if multiple samples are required stomach tubing may be the preferred method. During experiments with relatively small numbers of cattle and access to fistulated cattle, fistula ruminal fluid collections may be preferred. Ruminal in-dwelling probes may be a suitable option if continuous ruminal monitoring is required in a relatively small number of cattle; however, interpretation of ruminal conditions is limited without the use of other monitoring tools.

Volatile Fatty Acids (VFA)

Volatile fatty acids, primarily acetate, butyrate, and propionate, are the end-products generated during microbial fermentation of hexose to generate ATP for microbial maintenance and growth (France and Dijkstra, 2005). They provide the major energy source of ruminants and influence milk production (France and Siddons, 1993). Individual and total VFA concentration or proportion can be measured in ruminal fluid as indicators of rumen perturbation and ruminal acidosis and reflect responses to feed substrates and feed management. Concentrations of ruminal VFA, at any time, reflect rates of production and loss but normal concentrations of ruminal total VFA are between 70 to 130 m*M* with the ratio of acetate to propionate to butyrate usually approximately 70:20:10 for cattle fed forage diets (France and Dijkstra, 2005). Hexose is metabolized to pyruvate via the Embden-Meyerhof glycolytic pathway for all 3 of the primary VFA

(Figure 1). Acetate and butyrate are then generated via the intermediate, acetyl-CoA from pyruvate; while, propionate is generated mainly from the succinate pathway and also via the acrylate pathway (France and Dijkstra, 2005; Figure 1).

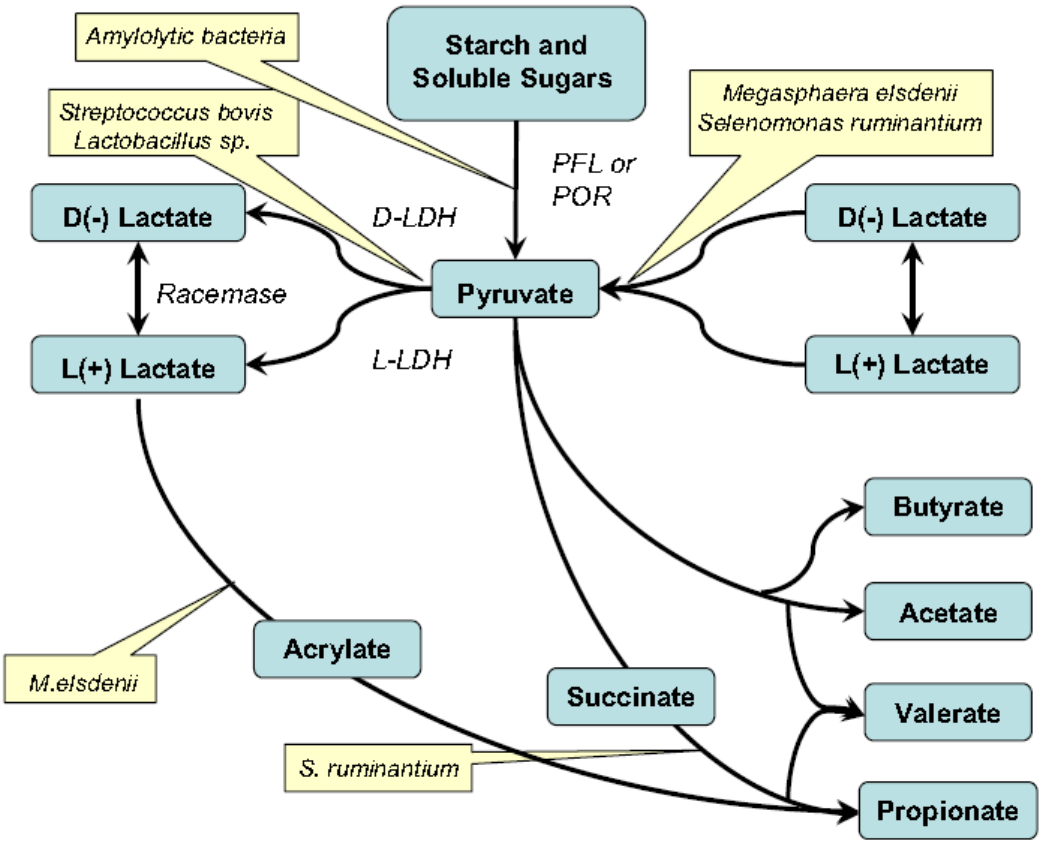

Figure 1. Major metabolic intermediates and groups of ruminal bacteria involved in the fermentation of starch and soluble sugars to lactate and volatile fatty acids (acetate, propionate, butyrate, and valerate). D-LDH = D-lactic dehydrogenase; L-LDH = L-lactic dehydrogenase; PFL = pyruvate formate lyase; POR = pyruvate oxidoreductase (Nagaraja and Lechtenberg, 2007).

In general, during mild ruminal acidosis or increased grain feeding, total VFA concentrations increase, with increases in propionate concentrations to approximately 35 to 45% of the total VFA pool at the expense of acetate (Ørskov, 1986; France and Dijkstra, 2005). Both increases (Khafipour et al., 2009b) and decreases in butyrate concentrations have been reported (Kennelly et al., 1999). High concentrations of valerate have also been associated with ruminal acidosis (Enemark et al., 2004; Morgante et al., 2007; Bramley et al., 2008) and may indicate that valerate is acting as a safe hydrogen sink for the removal of lactate.

In more severe cases of ruminal acidosis, total VFA concentrations decline (Wilson et al., 1975; Nagaraja and Titgemeyer, 2007) and complete absence of propionate, butyrate, and valerate and low concentrations of acetate can occur within 4 to 24 h of exposure to readily fermentable carbohydrates (Ryan, 1964; Kezar and Church, 1979; Golder et al., 2014a). Ruminal VFA concentrations returned to normal within 55 h of diet ingestion when a heifer with clinical ruminal acidosis had recovered (Golder et al., 2014a), supporting the merit of ruminal VFA measurement as an indicator of ruminal acidosis.

Receiver operator characteristic curves produced from the dataset of Bramley et al. (2008) showed valerate and propionate were both sensitive (0.90 and 0.93, respectively) and specific (0.90 and 0.87, respectively) indicators of ruminal acidosis (Rabiee and Lean, 2012; Table 3). Acetate and butyrate concentrations were sensitive, but not specific indicators of ruminal acidosis (Table 3).

Volatile fatty acids have value as indicators of ruminal acidosis because their concentrations do not fluctuate to the extent of those of ruminal pH, are relatively stable, are not influenced by saliva contamination, and can indicate where hydrogen is partitioned within the rumen. Ruminal osmolality may affect VFA concentrations (M. Hall, pers. comm.) but further research is required in this area.

Ammonia

The majority of ruminal ammonia is generated from the fermentation of dietary rumen degradable protein (**RDP**) and non-protein nitrogen (**NPN**) and is probably the most important source of nitrogen for ruminal bacteria (Allison, 1969; Russell and Strobel, 1987). Approximately 60% of true protein is degraded to ammonia and the remainder escapes the rumen (Satter and Roffler, 1975). The ammonia passively diffuses across cell membranes of bacteria and is used for their growth, along with peptides and amino acids; however, some bacteria may use active transport of ammonia (Russell and Strobel, 1987). Utilization of ammonia by bacteria depends on their number, growth rate, and availability of energy obtained from carbohydrates (Satter and Roffler, 1975). If carbohydrates are not available, protein degradation ends with the production of VFA and ammonia and, as a consequence, the availability of microbial nitrogen to the host is limited (Russell et al., 1983).

It is estimated that approximately 90% of ammonia is incorporated into microbial nitrogen and >90% could be incorporated when mean ammonia concentration is low (<20 g ammonia nitrogen/L of ruminal fluid). Satter and Slyter (1974) estimated that maximum microbial production is achieved at a concentration of 50 g of ammonia nitrogen (approximately 2.9 mmol of ammonia)/L of ruminal fluid, a finding supported by Russell and Strobel (1987). Efficiency of microbial production decreases as ammonia accumulates in the rumen (Russell and Strobel, 1987). Excess ammonia is absorbed by the rumen and eventually is excreted as urea in the urine and milk and increases ammonia in the blood (Russell and Strobel, 1987). When ruminal bacteria pass into the abomasum their microbial protein is degraded to peptides which pass into the small intestine and are broken down to amino acids that are absorbed. A deficiency of ruminal available ammonia reduces the flow of microbial protein from the rumen and hence vital amino acid supply to the host (Forero et al., 1980).

Measurement of ruminal ammonia concentrations can give an important indication of rumen function and bacterial activity. Bramley et al. (2008) associated low ruminal ammonia concentrations with ruminal acidosis, but high concentrations occurred in a heifer with clinical ruminal acidosis (Golder et al., 2014a). The merit of using ruminal ammonia as a diagnostic indicator of ruminal acidosis requires further investigation and could be used in conjunction with other indicators of ruminal acidosis, as interpretation of results in isolation may largely reflect dietary protein content.

Lactic Acid

Ruminal acidosis was formerly referred to as D-lactic or lactic acidosis as accumulation of lactic acid in the rumen produced by *Streptococcus bovis* and *Lactobacillus* spp. was considered to be the major contributing factor to acidity in the rumen and the development of ruminal acidosis (Turner and Hodgetts, 1949-1959; Dunlop and Hammond, 1965; Figure 1). It should be noted that much of the pioneering studies in this field were conducted in sheep (Turner and Hodgetts, 1949-1959). A combination of concentrations of VFA and lactic acid contributes to the acidity of the rumen (Britton et al., 1989). It has since been suggested that accumulation of ruminal lactic acid occurs in cattle with acute ruminal acidosis only, as opposed to those with SARA (Garrett, 1996; Oetzel et al., 1999). Normal ruminal total lactate concentrations are defined as up to 5 m*M* and concentrations above 40 m*M* are proposed to indicate severe ruminal acidosis

(Owens et al., 1998). However, Golder et al. (2012) showed D- and L-lactate concentrations were >5 m*M* in grain and fructose challenged dairy heifers that displayed no clinical signs of ruminal acidosis. It appears that feed substrates may influence ruminal lactic acid concentrations as greater lactic acid concentrations were observed when sugars as opposed to starches were fed (Harmon et al., 1985; Heldt et al., 1999; Golder et al., 2012). This suggests ruminal lactic acid reference ranges may need to be distinct for sugar and starch dominant diets. Transient spikes in ruminal lactic acid concentrations have also been reported (Kennelly et al., 1999), which add to the challenge of interpretation of ruminal lactic acid results.

Lactate in the rumen can be absorbed into the bloodstream, accumulate in the rumen, pass out of the rumen with ingesta, or be fermented primarily to ruminal propionate, butyrate, isobutyrate, and valerate (Huntington, 1988; Stewart et al., 1997; Table 4). Accumulation of lactic acid in the rumen lowers ruminal pH to a greater extent than VFA, as it has a pKa of 3.9, compared to 4.8 for ruminal VFA (Oetzel, 2003). However, ruminal pH is not always correlated with ruminal lactate concentrations ($r = -0.14$; Britton et al., 1989).

The role of the D- and L-stereoisomers of lactate in ruminal acidosis remains unclear, with different proportions of the stereoisomers reported in different studies (Hibbard et al., 1995; Golder et al., 2014b). It has been suggested the ratio of stereoisomers may be influenced by ruminal pH (Giesecke and Stangassinger, 1980) and the D-stereoisomer was metabolized at approximately one third of the rate of the L-lactate stereoisomer in ruminal epithelial tissue slices (Prins et al., 1974). The stereoisomers can also be interconverted by racemase (Figure 1); hence, their ratio does not always reflect their production (Asanuma and Hino, 2002).

Ruminal lactate measurements may have value in the diagnosis of ruminal acidosis when cattle are fed diets with a high sugar content or are abruptly exposed to high-grain, rapidly fermentable diets; however, lactate may not be a useful measure for diagnosing milder forms of ruminal acidosis when forage or grain based diets are fed, or when cattle are adapted to high-grain diets (Huntington, 1988). Owing to the inter-conversion between stereoisomers, both lactate stereoisomers should be measured. Further, distinct reference ranges may be required for sugar and starch-based diets.

Histamine

Histamine is generated in the rumen after feeding and can accumulate in the rumen during acidotic conditions (Nilsson, 1963; Ahrens, 1967; Golder et al., 2014a; Table 4); however, its involvement in the pathogenesis of ruminal acidosis and its possible association with laminitis remains unclear. While accumulation of histamine is not directly responsible for epithelial damage in the rumen (Ahrens, 1967), it delays epithelial regeneration (Aschenbach et al., 1998). Absorption of histamine can occur across an intact rumen epithelium; however, a combination of low epithelial permeability and inactivation by catabolism of >90% result in low net absorption of histamine, a result that supported earlier studies (Kay and Sjaastad, 1974). Epithelial damage appears to increase the net absorption of histamine (Aschenbach and Gabel, 2000); however, once absorbed, histamine is rapidly metabolized to inactive forms by either methylation or oxidation (Goth, 1974).

Given that the net absorption of histamine is low, and it is inactivated either during or after absorption, it appears that histamine generated in the rumen may be less likely to cause laminitis associated with ruminal acidosis than endogenous sources of histamine. Therefore, the value of ruminal histamine concentration as a diagnostic measure for ruminal acidosis remains equivocal and reference values need to be established.

Endotoxin

Endotoxins are a lipopolysaccharides released from the cell walls of gram negative bacteria during bacterial multiplication and lysis (Rietschel et al., 1994). A meta-analysis found that increases in ruminal endotoxin were linear when cattle were fed above 44.1% concentrate or below 39.2% NDF and were associated with increased plasma haptoglobin, and serum amyloid A levels (Zebeli et al., 2012). Similar depressions in ruminal pH for cattle challenge fed with alfalfa pellet and ground alfalfa to those observed in cattle challenged with high amounts of concentrate suggest that ruminal pH depressions and increased ruminal endotoxin concentrations alone do not cause an acute phase response (Plaizier et al., 2012). Normally, the rumen epithelium has a low permeability to endotoxin (Andersen et al., 1994) and translocation is reported in only 2 studies (Khafipoor et al., 2007; Khafipour et al., 2009b; Table 4), but epithelial damage may increase epithelial permeability to endotoxin (Owens et al., 1998).

Although ruminal endotoxin concentrations may be linked to ruminal acidosis and nonspecific acute phase responses (Gozho et al., 2007; Khafipour et al., 2009a), it's use as a diagnostic measure for ruminal acidosis should be approached with caution until ruminal endotoxin's proposed involvement in ruminal acidosis is further established, and standard measurement protocols and reference values for cattle are validated.

Table 4. Summary of attributes of ruminal lactic acid, histamine, and endotoxin concentrations when cattle are fed diets rich in rapidly fermentable carbohydrates. Adapted from Lean et al. (2013b)

Attribute	Compound or molecule		
	Lactic acid	Histamine	Endotoxin
Generated in the rumen	√	√	√
Absorbed by healthy rumen	√	√	√[1]
Absorbed by damaged rumen	?[2]	√	√
Induced acidosis when injected	×[3]	×[3]	×[4]
Induced laminitis when injected	√	√	√

[1]Inconsistent evidence.
[2]Appear probable.
[3]Infusions in sheep induced ruminal acidosis.
[4]Induced acute phase responses and endotoxic shock.

Milk Measures

Milk fat depression has been associated with ruminal acidosis and a milk fat to protein ratio of <1.15:1 has been suggested to be an indicator of ruminal acidosis (RAGFAR, 2007). A ROC curve based on the dataset from Bramley et al. (2008) suggests milk fat to protein ratio has some merit as an indicator for ruminal acidosis with a sensitivity and specificity of 0.54 and 0.81, respectively (Table 3). Milk fat percentage and yield can also be affected by a number of other factors apart from the presence of ruminal acidosis including: stage of lactation, breed, ration composition, and body fat mobilization (Grummer, 1991). Further, milk fat content had low correlation coefficients with ruminal pH in cows >30 DIM with r = 0.305 (Allen, 1997) and r = 0.390 (Enemark et al., 2004). Cows <30 DIM had an r = –0.06 (Enemark et al., 2004), suggesting milk fat measures may not be suitable indicators of ruminal acidosis in cows at this stage of lactation. Kleen et al. (2003) concluded that milk fat depression appears to occur in the same situations as SARA, but might not depend on the presence of SARA. Therefore, milk fat measures should be used in combination with other indicators of ruminal

acidosis to diagnose ruminal acidosis. Other milk markers that have also been associated with ruminal acidosis include: lactose, chloride, sodium, potassium, and milk urea nitrogen (Enemark et al., 2002), but these require further validation.

Other Measures

Urine (Furll, 1994), faecal (Oetzel, 2000), ruminal gases (Dewhurst et al., 2001), blood and blood gas measures, electrolytes (Enemark and Jorgensen, 2002), and metabolomic profiles (Saleem et al., 2012) can be used as indirect indications of ruminal acidosis. Oxidative stress responses have been influenced by concentrate diets (Gabai et al., 2004; Wullepit et al., 2009); however, responses were not observed when cattle were fed a single challenge feed of carbohydrates (Golder et al., 2013) or in a long-term carbohydrate challenge study (Golder et al., 2014b). Further research is required to investigate the potential of oxidative stress responses as indicators of ruminal acidosis. Acute phase proteins have been used as inflammatory biomarkers for ruminal acidosis (Gozho et al., 2007; Khafipour et al., 2009ab); however, these are non-specific indicators of inflammation and should be interpreted in combination with other indicators of ruminal acidosis.

There appears to be currently no sufficient single diagnostic indicator or monitoring tool for ruminal acidosis; however, there is merit in the use of multiple measures. Bramley et al. (2008), Morgante et al. (2007), and O'Grady et al. (2008) sampled 800, 120, and 144 head of dairy cattle, respectively, and investigated associations between diets, ruminal fermentation measures, and milk production. All three studies provided similar findings of associations between low ruminal pH and a ruminal environment in which total VFA concentrations were increased, but propionate and valerate concentrations were particularly increased and ammonia concentrations were low. Bramley et al. (2008) developed a model which predicts ruminal acidosis from ruminal samples by calculating eigenvalues which represent the statistical distance of each ruminal sample from the centroid of known ruminal acidosis cases based on standardized variates of concentrations of ruminal acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, caproate, ammonia, and total lactate, and pH. The model related health, production, and feed data to the categories of ruminal acidosis created by the model (Bramley et al., 2008). The Bramley et al. (2008) model has been used for other datasets (O'Grady et al.,

2008; Golder et al., 2012; Golder et al., 2014b) and validated by data provided by Golder et al. (unpublished).

Simultaneous observations and assessment or monitoring of multiple measures associated with clinical observations, feed assessment, and feeding behavior supported by ruminal, milk, urine, blood, and other measures should be used for assessment of rumen conditions and ruminal acidosis. Refinement or adoption of ruminal acidosis models such as that of Bramley et al. (2008) that use multiple indicators will aid in interpretation of results and ruminal acidosis diagnosis. Implementation of validated reference values for measures used will also be required to aid diagnosis.

FEED SUBSTRATES

A number of the following factors related to feed substrates influence ruminal fermentation measures and may be substantial risk factors for ruminal acidosis. The substrate type, cultivar within plant species, substrate amount, substrate processing, substrate composition, combination of substrates, duration of exposure to substrate, duration and rate of adaptation to substrate, and delivery method and feeding time of substrates all influence fermentation (Britton et al., 1989).

Feeds contain various proportions of constituents that influence their relative capacity to induce ruminal acidosis (Dunlop and Hammond, 1965) through various pathways and microbial interactions. A variety of feeds have been implicated as risk factors for ruminal acidosis (Underwood, 1992) with the major feed risks considered to be readily fermentable carbohydrates, including starches, sugars, and pectin. The biochemical pathways involved in carbohydrate metabolism have been extensively reviewed (Hungate, 1966; Baldwin and Allison, 1983). The involvement of dietary proteins and amino acids is less defined; while, the involvement of lipids is likely to be negligible as they comprise only a small portion of the diet and only contribute small amounts of VFA to the total VFA pool (France and Dijkstra, 2005). Other feed constituents such as hydroxyl acids, amines, L-tartaric acid, lactic acid, phytate, phosphate, and potassium have also been suggested to be involved in the development of ruminal acidosis (Dunlop and Hammond, 1965). Lethal doses of various feeds or feed constituents have not been established.

The recommended requirements of feed components for cattle and amounts of each readily fermentable carbohydrate or the sum of these required to induce ruminal acidosis may vary between cattle and differ depending on factors including other dietary components, feed management, or stage of lactation. Bramley et al. (2008) showed that cows in herds fed low NDF (30.4 ± 4.3%) and high NFC diets (40.3 ± 4.4%) had an increased ruminal acidosis risk. Recommended chemical composition of diet components are provided by the NRC (2001). The recommended non-fiber carbohydrate content (**NFC**) is 30 to 45% of the diet on a DM basis (Hall et al., 2010); however, it has been suggested to restrict NFC to 350 to 400 g/kg of diet DM if the NFC is largely sugar or starch, or 400 to 500 g/kg when other carbohydrates predominate (Hoover and Miller, 1995). Target levels of 5 g/kg DM sugars, 100 g/kg DM soluble fiber, and 200 g/kg DM starch have also been suggested (Hall, 1999).

Starches

Starch is a major storage polymer of grains which are commonly fed as an energy source to dairy cattle. The ratios of the 2 polymers of starch, amylose and amylopectin vary between cereal grains and are believed to be genetically controlled (Theurer, 1986). The quality of the starch is dependent on the type of starch (Van Soest, 1994). Alpha and beta amylase released from amylolytic and also some cellulolytic bacteria are the 2 enzymes that hydrolyze starch (Van Soest, 1994). The alpha amylase randomly cleaves starch chains of either amylose or amylopectin; whereas, beta amylase only cleaves units from the ends of amylose or amylopectin (Van Soest, 1994). The rumen remains the main site for starch digestion, regardless of the amount of starch fed, except for when large amounts of ground maize are fed (Beever, 1993).

The rate of starch fermentation by amylolytic and also some cellulolytic bacteria is highly variable and effects the risk of ruminal acidosis, with rate of starch fermentation being influenced by grain type, grain cultivar, and grain processing among other factors (Opatpatanakit et al., 1994; Lean et al., 2013a). The ranking of acidotic risk of cereal grains based on *in vivo*, *in sacco*, and *in vitro* measures in cattle and grain chemical composition in descending order was: wheat > triticale > barley > oats > sorghum (Lean et al., 2013a). *In vitro* gas estimates ranked grains for risk in the following orders wheat > barley > corn > sorghum (Lanzas et al., 2007) and wheat > triticale > oats > barley > maize > rice > sorghum (Opatpatanakit et al., 1994). Steam flaking, rolling, popping, or

other grain processes that increase the surface area or gelatinize starch influence the rate and site of digestion and can change a grain's ruminal acidosis index ranking (Huntington, 1988; Lanzas et al., 2007).

The immediate end-products of microbial degradation are primarily hexoses and pentoses, which are then used for microbial maintenance or growth (Beever, 1993). Hexose is also used to generate ATP which is required by bacteria for maintenance and growth reactions, with approximately 0.662 mol of hexose and 3.914 mol of ATP required for 100 g of microbial biomass (Baldwin et al., 1970). The fermentation process to generate ATP produces primarily the VFA: acetate, propionate, and butyrate (Beever, 1993). Starches favor propionate production during microbial fermentation in the rumen and a decrease in acetate (France and Dijkstra, 2005) and induce accumulation of lactic acid in the rumen when fed in high doses until the rumen is adapted to the diet (Huber, 1976).

It appears that extremely high intra-ruminal doses of grain at 3 to 7% of bodyweight (DM-basis) provided in very fermentable form induce acute ruminal acidosis; while, moderate concentrations of grain such as 1.5% of bodyweight (DM-basis) cause rumen perturbation consistent with SARA (Dougherty et al., 1975; Hibbard et al., 1995; Brown et al., 2000).

To reduce rumen perturbation and the risk of ruminal acidosis and increase production, adaptation to diets with high starch content is advised to allow the rumen microbes and rumen papillae to adapt to the new diet and adequate physically effective fiber should also be provided (Garrett, 1996).

Sugars

Cattle can be exposed to a range of sugar contents from different sources in their diet including molasses, crystalline sugars, fruits, whey, brassicas, root crops, citrus pulps, and forages. The sugar content of pastures is often measured by their water soluble carbohydrate (**WSC**) content, which is the sum of fructans and simple sugars (Longland and Byrd, 2006). Fructans, β-D-fructose polymers with terminal glucose monomers, are important carbohydrate reserves synthesized in the vegetative tissues of over 36,000 plant species (Carpita et al., 1989) and can comprise up to 50% of the total DM content of temperate grasses (Cairns and Longland, 1998). The WSC content of pasture plants is

lowest in the morning after the plant has used fructan for respiration overnight and is higher in the afternoon after photosynthesis has accumulated fructan (Fulkerson and Donaghy, 2001). Only trace amounts of WSC are found in silages due to previous fermentation of these carbohydrate fractions (Beever, 1993).

Perennial ryegrass cultivars with increased expression of fructan could shift ruminal fermentation towards a higher proportion of propionate production and lower ruminal pH (Taweel et al., 2005), and therefore increase the risk of ruminal acidosis. Higher ruminal propionate and butyrate and lower acetate concentrations occurred in cows fed pastures with higher WSC (Lee et al., 2002), but no difference in ruminal fermentation measures were observed in cows fed pasture with a higher WSC content in another study (Taweel et al., 2005). However, the WSC content of pasture fed in this study was lower than that fed by Lee et al. (2002). Further research is required into the possible association between pastures with increased WSC content and ruminal acidosis.

Sugars are broken down at a faster rate than starch and other carbohydrate fractions (Sniffen et al., 1992). Sugars produce different fermentation profiles to starches as dietary sugar additions increased the concentrations of ruminal butyrate and valerate compared to those of cattle fed starch-based diets (Heldt et al., 1999; DeFrain et al., 2004). Golder et al. (2012) observed a decrease in ruminal valerate concentration in fructose-fed cattle compared to those fed grain. In contrast, no differences in ruminal VFA were reported between sugar supplemented and unsupplemented cattle in other studies (DeFrain et al., 2006; Oelker et al., 2009). Reductions in ruminal ammonia in nearly all sugar studies examined by Hoover and Webster (2001) lead them to suggest sugars allowed more efficient utilization of rapidly available nitrogen.

Dietary sugar addition produced more lactic acid than starch-based diets (Harmon et al., 1985; Heldt et al., 1999; Golder et al., 2012), hence sugars are likely to increase the risk of ruminal acidosis. Giesecke et al. (1976) reported generation of ruminal D- and L-lactate within the first 15 to 20 min of sugar consumption. The amount of sugar required to produce ruminal acidosis is relatively unknown, but from ruminal acidosis challenge models reviewed by Nagaraja and Titgemeyer (2007), it was suggested that approximately half the amount of glucose (0.3 to 0.6% of BW) compared to grain was required to induce SARA. Lactic acid concentrations higher than those specified for cattle with normal ruminal conditions (5 m*M*; Owens et al., 1998) occurred in cattle fed

grain and fructose, despite supplementation with antibiotic feed additives. These cattle displayed no signs of clinical ruminal acidosis (Golder et al., 2014b). Ruminal fluid measures from these cattle did not fit the model of ruminal acidosis developed by Bramley et al. (2008), which suggests models of ruminal acidosis are different when sugars are fed to those for starch-based diets. The rumen appears to be better able to adapt to starch-based diets compared to those that contain sugar.

Proteins

Crude protein requirements for cattle in early lactation are suggested to be between 16.5 to 17.5% of the DM supply (NRC, 2001). Feeds supplemented to dairy cattle to increase dietary crude protein content include: legume grains and legume forages, canola and soybean meals, brewers and distillers, byproducts including gluten feeds and meals, grains and byproducts of lysine production such as Fermenten® and Biochlor®. Feedstuffs vary widely in their relative proportions of RDP and NPN, extent of rumen degradation, and intestinal digestibility of undegraded feed protein (NRC, 2001). The ultimate purpose of supplementing feeds high in protein is to meet the metabolizable protein requirements of the cattle (NRC 2001). Rumen degradable protein from the diet is broken down by proteolytic bacteria in the rumen to peptides and amino acids and is later further digested to ruminal VFA and ammonia; while, NPN provides ruminal ammonia (France and Dijkstra, 2005). The rumen undegradable portion of protein from the diet bypasses the rumen and is absorbed as amino acids in the small intestine, supplying approximately 30% more energy than starch (Klopfenstein, 2001) without generation of hydrogen in the rumen.

Energy for the proteolytic bacteria to ferment RDP is required from readily fermentable carbohydrates; therefore, increased synchrony of ruminally available protein and carbohydrate has been proposed as a method to increase efficiency of microbial nitrogen production and animal productivity (Johnson, 1976) and could influence the risk of ruminal acidosis. Increases in ruminally available protein have increased organic acid concentrations regardless of ruminally available carbohydrate (Herrera-Saldana and Huber, 1989).

The potential for dietary protein content to be involved in the pathogenesis of ruminal acidosis has been largely unexplored; however, the supply of RDP has influenced

organic acid and lactate pool sizes (Hall, 2013). However, 8% of the dry weight of bacteria is hydrogen (Todar, 2012), therefore providing a considerable sink for hydrogen generated in ruminal catabolism of carbohydrates. Consequently, it appears possible that protein can be either beneficial or detrimental for risk of ruminal acidosis, depending on substrate availability, concentration, and dietary management.

Hall (2013) proposed that the effects of protein on organic acids and lactate may be related to indirect effects of RDP on the energy demand of cells and be controlled through microbial storage of glycogen. Increasing the supply of amino acids and ammonia increases the efficiency of microbial growth and possibly leads to an increase in ATP demands, which can lead to signaling of decreased glycogen storage and increased glycolysis, which will also increase lactate production (Counette and Prins, 1981; Hall, 2013). Therefore, an excess in available peptides and ammonia may have detrimental effects on the rumen and increase the risk of ruminal acidosis through increased accumulation of VFA and lactate. However, the movement of hydrogen into VFA and ammonia and microbial protein, which are relatively safe sinks, during the degradation of protein may also be beneficial and reduce the risk of ruminal acidosis. Ammonia produced may 'buffer' changes in ruminal pH by neutralizing up to 10 to 15% of ruminal VFA concentrations produced (Owens et al., 1998), which could reduce the risk of ruminal acidosis.

In vivo studies that have assessed the effects of dietary protein on ruminal acidosis are limited; however, increasing estimated metabolizable protein content of rations above NRC (2001) requirements created more favorable ruminal conditions, although not significantly, as indicated by lowered acidosis eigenvalues compared to cows on iso-energetic diets (Golder et al., unpublished). 'Protein sparing' effects demonstrated by feed additives used to control ruminal acidosis (Plaizier et al., 2000) suggest the importance of protein metabolism in ruminal acidosis. Fermenten® and Biochlor® have increased *in vitro* ruminal ammonia nitrogen concentrations and stimulated microbial protein nitrogen production by approximately 24.6 and 13.5%, respectively (Lean et al., 2005) and Fermenten® showed potential to reduce ruminal acidosis *in vivo* (Golder et al., unpublished).

Starch, sugar, and protein content of feed appear to affect ruminal fermentation characteristics and the risk of ruminal acidosis; therefore, perhaps additional definitions

for ruminal acidosis are required to define ruminal acidosis during specific feeding conditions.

RUMEN ECOLOGY

The rumen microbial ecosystem is a complex community that works in a dynamic, symbiotic relationship with the host to convert feed into energy (Kamra, 2005) and is highly responsive to dietary changes (Tajima, 2001). The vast diversity of ruminal anaerobic bacteria (10^{10} cells/mL), protozoa (10^5 to 10^6 cells/mL), archaea (10^7 to 10^9 cells/mL), bacteriophages (10^8 to 10^9 cells/mL), and fungi (10^3 to 10^5 zoospores/mL) and their diverse synergies and antagonism allow ruminants to efficiently utilize a range of feeds (Bergen, 2004; Ferrer et al., 2005; Kamra, 2005). Microbial interactions have been reviewed by Wolin and Miller (1988). The composition of the rumen microbiome results from effects of several factors including: diet, feed additives, health, age, condition, and species of the host, season, and geographical conditions (Stewart et al., 1997; Pers-Kamczyc et al., 2011).

Techniques Used to Study Rumen Microbial Ecology

Limited capacity, culture-based methods provided the foundations of characterization of microbial shifts in general and during ruminal acidosis (Hungate, 1966; Slyter, 1976; Goad et al., 1998). More recently, advances in molecular technologies that are sensitive, high-throughput, and culture-independent allow rapid determination of ruminal bacterial community structures, diversity, and richness (McSweeney et al., 2007). There are many recent reviews available on the complete range of traditional and molecular techniques used in the study of microbial ecology in animals (Zoetendal et al., 2004; McSweeney et al., 2007; Deng et al., 2008; Pers-Kamczyc et al., 2011). The newer molecular technologies are more effective and efficient than culture-based, hybridization or fingerprinting techniques, owing to their high sensitivity, reproducibility and dynamic ranges (Table 5). They also allow characterization, quantification, and prediction of phylogenetic relationships of the microbiota (Zoetendal et al., 2004).

The choice of molecular technique used in a rumen ecological study is determined by the question being addressed (Zoetendal et al., 2004), as each method has its own advantages and disadvantages (Table 5). The foundation of a number of molecular techniques is the small subunit (**SSU**) 16S rRNA/rDNA gene from prokaryotes or SSU

18S rRNA/rDNA gene from eukaryotes, because these are highly conserved and provide a species-specific signature aiding in identification (Meyer et al., 2010). The molecular techniques with the exception of florescence in situ hybridization (FISH) involve extraction of DNA or RNA from a ruminal sample and the DNA is subsequently subjected to a polymerase chain reaction (**PCR**; Pers-Kamczyc et al., 2011; Figure 2), a technique developed by Kary Mullis in the 1980's that revolutionized science (Valasek and Repa, 2005). The PCR copies and amplifies DNA in steps known as denaturation, annealing and elongation (Freeman et al., 1999). The amplicons, which are pieces of DNA or RNA that have been amplified, can then be examined by denaturing or terminal gradient gel electrophoresis (DGGE and TGGE, Muyzer and Smalla, 1998), ribosomal intergenic spacer analysis (RISA, Fisher and Triplett, 1999), single-strand-conformation polymorphism (SSCP, Lee et al., 1996) or terminal restriction fragment analysis (T-RFLP, Osborn et al., 2000) to provide information on community composition. The amplicons can also be cloned or sequenced to determine phylogenetic diversity.

There are many variants of PCR such as reverse transcription PCR (**RT-PCR**; Freeman et al., 1999), Real-time polymerase PCR (**qPCR**; Mackay, 2007), which can also be referred to as quantitative PCR, quantitative real-time PCR, or real-time quantitative PCR, and a combination of RT-PCR and qPCR can be referred to as qRT-PCR (Varkonyi-Gasic and Hellens, 2010), or RT-qPCR (Taylor et al., 2010). These PCR methods can also be competitive and can be referred to as cPCR, or with 'competitive' as a prefix. The variants of PCR perform different functions (Table 5).

The majority of previous studies have used DNA based methods to identify and classify microbial diversity because degradation of RNA commonly occurs during extraction and co-extraction of phenolic compounds is common (McSweeney et al., 2009). A new RNA extraction protocol has been developed, allowing the use of RNA to examine microbial diversity (Kang et al., 2009). RNA-based approaches more accurately represent bacterial growth activity than DNA approaches (Wagner, 1994).

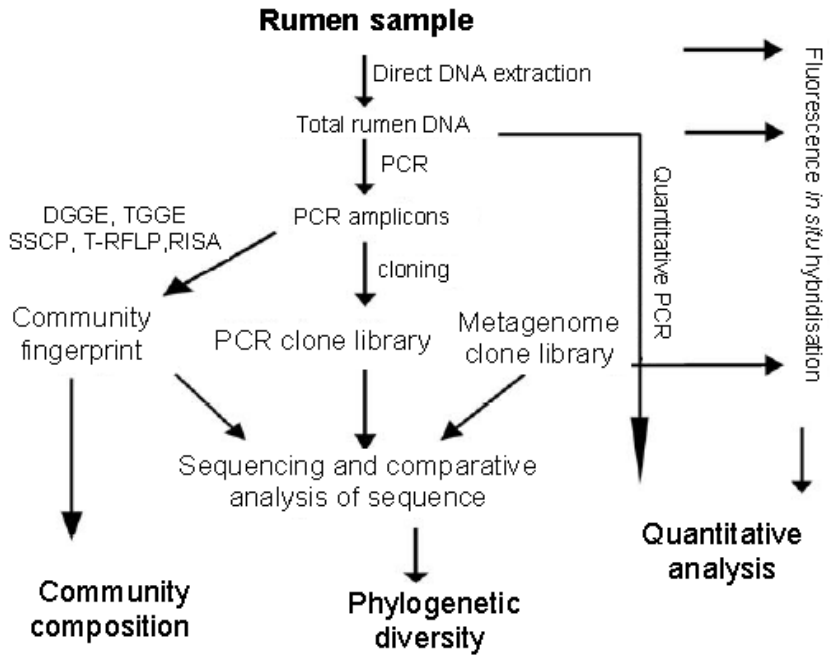

Figure 2. Analysis of microbial communities in ruminal samples by culture-independent molecular methods (Pers-Kamczyc et al., 2011). PCR = polymerase chain reaction; DGGE = denaturant gradient gel electrophoresis; TGGE = temperature gradient gel electrophoresis; SSCP = single strand conformation polymorphism; T-RFLP = terminal restriction fragment length polymorphism; RISA = ribosomal intergenic spacer.

The design and subsequent publishing of new primer sets for amplifying specific ruminal bacteria is facilitating rapid investigation of these bacteria. Primer sets are now available for the following bacteria: *Anaerovibrio lipolytica, Butyrivibrio fibrisolvens, Eubacterium ruminantium, Prevotella albensis, P. brevis, P. bryantii, P. ruminicola, Ruminobacter amylophilus, Selenomonas ruminantium, Streptococcus bovis, Succinivibrio dextrinisolvens, Treponema bryantii*, and *Prevotella* (Tajima, 2001; Ouwerkerk et al., 2002; Stevenson and Weimer, 2007).

Many molecular technologies involve and can be limited by DNA sequencing, which can be a labor intensive and time consuming procedure. The first generation of DNA sequencing was developed by Frederick Sanger in 1977 and was based on a chain-termination method, referred to as Sanger sequencing (Liu et al., 2012). It was labor intensive and required radioactive materials. Sequencing became faster and more accurate when automatic sequencing was introduced in 1987 (Liu et al., 2012). Beginning in 2005 second generation sequencing, also known as next generation

sequencing, was launched with 3 main sequencing systems available that have since been upgraded and are currently known as the 454 GS FLX Titanium System, Sequencing by Oligo Ligation Detection (SOLiD), and HiSeq 2000, each with their advantages and disadvantages (Table 5) (Liu et al., 2012). New-generation sequencing is more cost-effective, has high throughputs, and improved accuracy over first generation sequencing (McSweeney et al., 2007). Third generation sequencing, including single-molecule real-time (SMRT) sequencing and nanopore sequencing are the latest sequencing systems but are yet to be used for rumen microbiology (Liu et al., 2012). In third generation sequencing fluorescent or electric current signals are monitored in real-time and PCR is not required (Liu et al., 2012).

At present, a *metagenomic* approach toward investigation of rumen microbiology is being adopted. Metagenomic analysis is used to examine the phylogenetic, physical, and functional properties of microbial communities (Handelsman, 2004). This approach involves studying the genomes of all organisms within entire rumen microbial communities collectively (Singh et al., 2008). The main steps involve DNA extraction and cloning of the DNA fragments in a host to produce a clone library (McSweeney et al., 2009). This library can be screened by PCR or hybridization methods to identify genes encoding for specific steps in known metabolic pathways (McSweeney et al., 2009). The metagenomic approach has the advantage of producing a catalogue of genetic information on the entire ecosystem and can identify novel gene sequences (McSweeney et al., 2009). The functional activity of rumen micro-organisms can also be described using gene expression analysis (Yu and Forster, 2005). Problems in extracting RNA and the priming of complementary DNA (**cDNA**) synthesis have slowed the development of gene expression analysis (McSweeney et al., 2009). The next step is to link structural analysis to functional gene activity (McSweeney et al., 2009). McSweeney et al. (2009) noted that the challenge is to understand the biology at the molecular level to allow the adjustment of feeding systems for maximum efficiency in the rumen.

Table 5: Summary of techniques used to study rumen microbial ecosystems. Adapted from Zoetendal et al. (2004) and Deng et al. (2008) with additions from, Lee et al. (1996), Fisher and Triplett (1999), Bustin and Nolan (Bustin and Nolan, 2004), Liu et al. (2012), and Siqueira et al. (2012)

Method	Acronym	Uses	Description	Advantages	Limitations
Culture					
Roll-tube		Isolation Enumeration	Based on cultivation	Isolates populations	Labour intensive Not representative Requires knowledge of growth requirements Only a small portion of microbes are culturable 16S/18S rRNA/rDNA-based analysis required for identification Low sensitivity
Most-probable-number	MPN	Enumeration	Based on several dilutions and incubations of cultures	Only estimates live micro-organisms	Low sensitivity Labour intensive Time consuming Not representative Only a small portion of microbes are culturable
Hybridization					
Dot blot hybridization eg Southern and Northern		Hybridization Detection Relative abundance	PCR products separated by gel electrophoresis and hydridized with probes on a filter membrane	More accurate than micro arrays	Sequence information required Labour intensive at the species level Large amounts of RNA required
DNA micro arrays		Detection Enumeration	cDNA mixed with 2 fluorescent dyes applied to thousands of spots of DNA oligosaccharides	Thousands of genes studied simultaneously High specificity	Low sensitivity

Table 5 (continued): Summary of techniques used to study rumen microbial ecosystems. Adapted from Zoetendal et al. (2004) and Deng et al. (2008) with additions from, Lee et al. (1996), Fisher and Triplett (1999), Bustin and Nolan (Bustin and Nolan, 2004), Liu et al. (2012), and Siqueira et al. (2012)

Method	Acronym	Uses	Description	Advantages	Limitations
Reverse transcription PCR	RT-PCR	Qualitatively detects RNA expression levels	RNA template is converted to cDNA and used as a template for PCR	Ability to identify amplified fragments during the PCR process Amplification to analysis in one tube; hence less contamination	Complex Issues with sensitivity, specificity and reproducibility
Combination of RT-PCR and qPCR	qRT-PCR or RT-qPCR	Quantification of RNA	Detects and measures PCR products by cleavage of an oligonucleotide probe	Powerful Sensitive	Indiscriminate binding to any double-stranded DNA Signal generated following irradiation is dependent on the mass of doublestranded DNA produced in the reaction.
Fingerprinting					
Restriction fragment length polymorphisms	RFLP	Monitors community shifts Comparative analysis	Discriminates by variation in restriction enzyme sites	Very sensitive High throughput	Subject to PCR biases Clone library required for identification Semi-quantitative
Denaturing gradient gel electrophoresis and Temperature gradient gel electrophoresis	DGGE TGGE	Monitors community shifts Comparative analysis	Based on DNA melting points	Doesn't require radioactivity Efficient and accurate at identifying mutations Less labour intensive than blot methods Inexpensive	Subject to PCR biases Clone library required for identification Semi-quantitative

Table 5 (continued): Summary of techniques used to study rumen microbial ecosystems. Adapted from Zoetendal et al. (2004) and Deng et al. (2008) with additions from, Lee et al. (1996), Fisher and Triplett (1999), Bustin and Nolan (Bustin and Nolan, 2004), Liu et al. (2012), and Siqueira et al. (2012).

Method	Acronym	Uses	Description	Advantages	Limitations
Florescence in situ hybridization	FISH	Detection Enumeration Comparative analysis	Fluorescent labelled probes hybridize to target sequences	Many probes can be used at once High sensitivity	Sequence information required Labour intensive at species level Lack of probes
Suppressive subtractive hybridization	SSH	Isolation of DNA fragments Comparative analysis	Suppressive PCR Common DNA sequences eliminated	Can differentiate between 2 genetically similar organisms cDNA can be used as probes to screen libraries High efficiency	Subtraction fragment redundancy Labour intensive
Intergenic spacer					
Ribosomal intergenic spacer analysis	RISA	Microbial diversity Community composition	PCR of DNA from the intergenic region between the small (16S) and large (23S) subunit and RNA genes in the rRNA operon	Rapid Sensitive Reproducible No gel electrophoresis required	Possible preferential amplification of shorter templates Underestimates of diversity when intergenic spacer size classes overlap among unrelated organisms
PCR					
Competitive polymerase chain reaction	cPCR	Detection Quantifies absolute abundance	Compares known copies of internal standards to target sequence	High sensitivity	Labour intensive Requires preparation of internal standards
Real-time PCR quantitative PCR, quantitative real-time PCR, or real-time quantitative PCR	qPCR	Detection Quantifies absolute abundance	Monitoring DNA amplification by fluorescence	Quantifies wide dynamic ranges High sensitivity No post PCR steps Minimal contamination risk High throughput Easy, reliable and reproducible	Expensive equipment False negatives Relies on accuracy of standards and quality of PCR products

Table 5 (continued): Summary of techniques used to study rumen microbial ecosystems. Adapted from Zoetendal et al. (2004) and Deng et al. (2008) with additions from, Lee et al. (1996), Fisher and Triplett (1999), Bustin and Nolan (Bustin and Nolan, 2004), Liu et al. (2012), and Siqueira et al. (2012)

Method	Acronym	Uses	Description	Advantages	Limitations
Single-strand-conformation polymorphisms	SSCP	Detects polymorphisms and mutations	DNA fragments with the same size but different sequences are separated by gel electrophoresis	Simple and effective Radioactive substances not required Long primers not required Inexpensive	Relatively insensitive Accuracy is dependent on optimized physical factors
Phylogenetic					
Clone libraries		Phylogenic identification New microbe discovery	Databank of known DNA sequences	Enables phylogenetic classification and discovery of new organisms Easy access	Labour intensive Subject to PCR bias Expensive
Sequencing Systems					
454 GS FLX Titanium	454 Pyrosequencing	Sequencing	Detects light emitted as a nucleotide is added (Pyrosequencing)	Fast High accuracy No need for gel-electrophoresis or labelled primers Wide variety of applications Large sequencing depth (no of sequences per sample) and breath (number of samples analyzed)	Detects homopolymers (repeated nucleotides) resulting in sequencing errors Short length of reads High cost Low throughput
HiSeq 2000		Sequencing	Sequencing by synthesis	High throughput Inexpensive	Short read assembly
Sequencing by Oligo Ligation Detection	SOLiD	Whole genome resequencing, targeted resequencing, trnacriptiome research, small RNA analysis, epigenome	Ligation and 2-base coding	High accuracy	Short read assembly

Integration of knowledge obtained from classical culture-based microbiology and molecular techniques is rapidly increasing our understandings of the rumen microbiome and its functions in general (Pers-Kamczyc et al., 2011) and during ruminal acidosis. Despite constant evolution of methods for studying the rumen ecosystem, only approximately 10% of the rumen microbiome is known (Pers-Kamczyc et al., 2011), which limits knowledge of shifts in rumen microbes during rumen perturbation and ruminal acidosis.

There are now opportunities to examine associations between the host genome, rumen microbiomes, ruminal fermentation measures, and production measures, hence further expanding our knowledge of changes at the whole animal level. Multi-variate analysis such as co-inertia analysis that measures the concordance of 2 datasets (Dray et al., 2003) is a method that can be used to examine the strength and direction of the relationship between rumen microbial communities and other known factors of the host animal.

Ruminal Bacteria

Reviews on the role of protozoa, archaea, bacteriophages, and fungi in the rumen are available elsewhere (Orpin and Joblin, 1988; Williams and Coleman, 1988; Janssen and Kirs, 2008) and will not be the focus of this review, as knowledge of the involvement of these rumen microbes in ruminal acidosis is limited. However, protozoa engulf starch particles, storing them as glycogen, hence act as ruminal buffers by delaying bacterial fermentation and potentially stabilizing the rumen, reducing the risk of ruminal acidosis (Slyter, 1976). The remainder of this section of the review will focus on ruminal bacteria and their changes associated with ruminal acidosis.

The rumen contains approximately 200 species of bacteria (Pers-Kamczyc et al., 2011) that can be classified into 4 groups based on their location of colonization in the rumen: (1) free-living bacteria associated with the rumen liquid phase, (2) bacteria associated with feed particles, (3) bacteria associated with rumen epithelium, or (4) bacteria attached to the surface of protozoa (Czerkawski and Cheng, 1988; McAllister et al., 1994). The taxonomy of bacteria in the rumen is assigned to the species level when possible to provide information on the characteristics of individual bacteria. Traditionally, phenotypic properties of bacteria formed the basis for classification;

however, SSU rDNA sequences are now the standard phylogenetic classification tool (Stackebrandt and Goebel, 1994; Zoetendal et al., 2004). Bacteria are also commonly classified into groups based on their substrate utilization (Table 6).

Table 6. Grouping of bacterial species based on type of substrate fermented (Church, 1988)

Cellulolytic	Proteolytic	Methane-producing
Fibrobacter (Bacteroides) succinogenes	*Ruminobacter (Bacteroides) amylophilus*	*Methanobrevibacter ruminantium*
Ruminococcus flavefaciens	*Prevotella (Bacteroides) ruminicola*	*Methanobacterium formicium*
Ruminococcoccus albus	*Butyrivibrio fibrisolvens*	*Methanomicrobium mobile*
Butyrivibrio fibrisolvens	*Streptococcus bovis*	
Pectinolytic	Lipid-utilizing	Ureolytic
Butyrivibrio fibrisolvens	*Anaerovibrio lipolytica*	*Succinivibrio dextrinosolvens*
Prevotella (Bacteroides) ruminicola	*Butyrivibrio fibrisolvens*	*Selenomonas sp.*
Lachnospira multiparus	*Treponema bryantii*	*Prevotella (Bacteroides ruminicola*
Succinivibrio dextrinosolvens	*Eubacterium sp.*	*Ruminococcus bromii*
Treponema bryantii	*Fusocillus sp.*	*Butyrivibrio sp.*
Streptococcus bovis	*Micrococcus sp.*	*Treponema sp.*
Sugar-utilizing	Hemicellulolytic	Ammonia-producing
Treponema bryantii	*Butyrivibrio fibrisolvens*	*Prevotella (Bacteroides) ruminicola*
Lactobacillus vitulinus	*Prevotella (Bacteroides) ruminicola*	*Megasphaera elsdenii*
Lactobacillus ruminus	*Ruminococcus sp.*	*Selenomonas ruminantium*
Amylolytic	Acid-utilizing	
Ruminobacter (Bacteroides) amylophilus	*Megasphaera elsdenii*	
Streptococcus bovis	*Selenomonas ruminantium*	
Succinimonas amylolytica		
Prevotella (Bacteroides) ruminicola		

Core Bacterial Microbiome

Individuals appear to have a unique rumen ecosystem comprised of a core rumen microbiome (Jami and Mizrahi, 2012) that has a unique ability to adapt to different feed substrates (Dougherty et al., 1975; Brown et al., 2000) and may be associated with an individual animals' susceptibilities to disorders such as ruminal acidosis. Host genetics may play a vital role in bacterial profiles, as bacterial profiles are related to breed (Guan et al., 2008; Brulc et al., 2009) and breed of cattle had a greater influence than diet on bacterial profiles (Lee et al., 2012). Weimer et al., (2010) showed that when >95% of

ruminal fluid with differing pH, total VFA concentration, and bacterial community composition from 2 cows fed the same diet was exchanged the ruminal pH, total VFA, and bacterial profiles of the 2 cows returned to their original profiles within 24 h, further suggesting that host genetics may be responsible for individual rumen microbiomes. Host specificity of ruminal bacteria may pose a challenge when examining interventions or management changes that effect the rumen and emphasizes the need for large sample sizes for *in vivo* studies. In particular, such variation in ruminal bacterial communities between cattle will pose challenges for strategies designed to control ruminal acidosis.

The core microbiome appears to be dominated by bacteria from the Bacteroidetes (predominately gram negative) and Firmicutes phyla (predominately gram positive) (Tajima et al., 2000; Kong et al., 2010). Proteobacteria are commonly the third dominant phylum of the rumen (Khafipour et al., 2009c; Jami and Mizrahi, 2012), but in some studies the candidate phylum, TM7 are more dominant (Golder et al., unpublished). The most well studied members of the Bacteroidetes are those from the Bacteroidia class and include the *Prevotella* genus, which are the dominant genus in most cattle (Tajima et al., 2000; Fernando et al., 2010; Jami and Mizrahi, 2012). *Prevotella* appear to be the dominant bacterial genus in the rumen (Tajima et al., 2000; Stevenson and Weimer, 2007; Jami and Mizrahi, 2012). The large genetic diversity of the *Prevotella* and their capacity to selectively utilize a large variety of sugars, amino acids, and peptides allows them to dominate over a range of diets (Matsui et al., 2000). Only 2 to 4% of total bacterial 16S rRNA gene copies were accounted for by the 4 characterized *Prevotella* spp. in ruminal samples from lactating cows 3 h after consuming a 27.5% NDF TMR (Stevenson and Weimer, 2007), suggesting a need for further identification and characterization of *Prevotella* spp. Many of the bacteria associated with ruminal acidosis such as *Streptococcus bovis, Lactobacillus* spp., and *Megasphaera elsdenii* belong to the Firmicutes phylum (Hungate, 1966; Bergey et al., 2011).

The Proteobacteria can be highly variable in abundance (Jami and Mizrahi, 2012) and have highly diverse metabolic functions (Garrity et al., 2005). *Escherichia coli*, a member of the Proteobacteria, which can be responsible for endotoxin release, has been associated with SARA induced by grain or alfalfa hay (Khafipour et al., 2009c). Knowledge of the characteristics, substrate utilization, and end products of bacteria belonging to the candidate phylum, TM7, are limited. The phylum, Fibrobacteres, is

considered to be critical for fiber degradation (Ransom-Jones et al., 2012) and may be a part of the core microbiome in forage-fed cattle. There is a need to define the core microbiome of dairy cattle to aid in the development of control strategies for ruminal acidosis.

Changes to the Microbiome

Ruminal bacteria can be influenced by diet and a number of host and environmental factors including: age, antibiotic consumption, geographic location, season, and feeding management (Stewart et al., 1997). The most important factor influencing bacterial community structure may be the feeding cycle, during which there are intermittent supplies of fermentable energy for bacterial growth and catabolism (Welkie et al., 2010). Bacterial populations normally increase after feeding (Bryant and Robinson, 1968) and decline as substrate availability declines as the subsequent feeding approaches (Mullins et al., 2013). Initial changes in ruminal bacterial population after feed consumption appear to be an increase in bacteria that ferment sugars and RDP, as these substrates are easier to degrade, while increased activity from cellulolytic bacteria, starch utilizers, and lactate producers are likely to follow (Huntington, 1988). As cattle become adapted to their change in diet, the population of the lactate producer *Streptococcus bovis* decreases and numbers of lactate utilizers increase. Populations of lactate producers in general may not necessary decrease depending on the supplementation of feed additives, but the rumen microbial community will become more stable and lactate may no-longer accumulate beyond concentrations that impair the function of the rumen. A low ruminal pH may persist but the rumen is adapted to function at this new pH. Sampling site, method, and time have had little impact on bacterial diversity assessment (Li et al., 2009; Lodge-Ivey et al., 2009).

In general, gram negative bacteria predominate in forage- or roughage-fed cattle with increases in the number of gram positive bacteria observed as concentrate feeding increases (Hungate, 1966; Latham et al., 1971) and the rumen environment may adapt to feed changes over a period of time. Gram negative bacteria appear to be replaced with gram positive bacteria during ruminal acidosis (Dirksen, 1970; Nagaraja and Titgemeyer, 2007).

Ruminal acidosis models (Hungate, 1966; Baldwin and Allison, 1983; Nocek, 1997; Owens et al., 1998; RAGFAR, 2007) describe an initial increase in growth of all ruminal bacteria when large amounts of readily fermentable carbohydrates are fed, followed by an increase in population of *S. bovis* (Figure 3). *Streptococcus bovis* produces lactate as an end product and drops the ruminal pH to favorable conditions for *Lactobacillus* spp. to proliferate and further decrease the pH of the rumen. *Lactobacillus* spp. outcompete *S. bovis* and other ruminal bacteria that are acid intolerant due to the release of bacteriocins, as the decline in ruminal pH alone is not adequate to prevent their growth (Wells et al., 1997). Lactate utilizing bacteria such as *Megasphaera elsdenii*, *Selenomonas ruminantium,* and *Veillonella parvula* are proposed to proliferate under these conditions and convert ruminal lactate to primarily ruminal propionate, butyrate, isobutyrate, and valerate (Stewart et al., 1997; Figure 1). However, relative to *S. bovis* these lactate utilizing bacteria are relatively slow growing and increase with adaptation to readily fermentable carbohydrate diets (Huber, 1976). They were reported to increase by 6 to 8 fold over a 4 wk exposure to readily fermentable carbohydrates (Huntington et al., 1981). Hence, bacterial profiles of cattle initially exposed to high amounts of readily fermentable carbohydrates will be different to those adapted to the same diets.

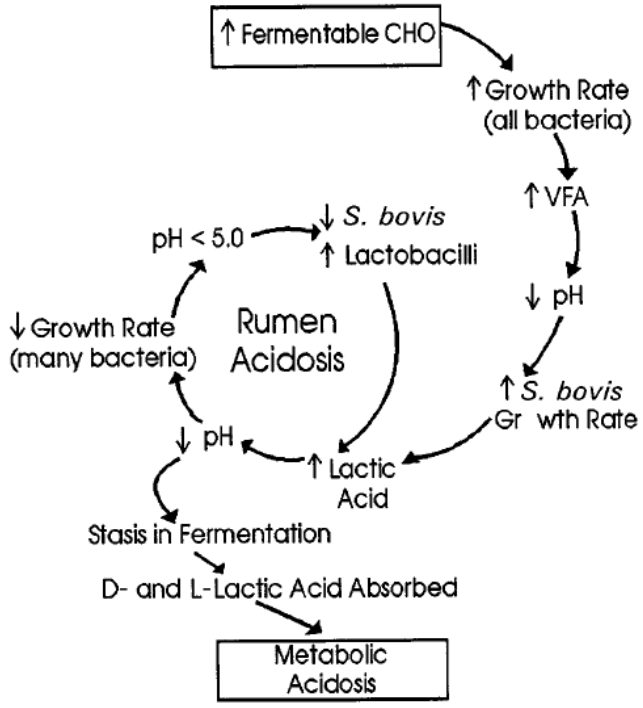

Figure 3. Sequence of events associated with inducement of acute ruminal acidosis. CHO = carbohydrate; VFA = volatile fatty acids; S. bovis = *Streptococcus bovis* (Nocek, 1997).

S. bovis, that was first associated with high grain diets in sheep (Hungate et al., 1952), is often considered to be the causative agent of ruminal acidosis and reside in low populations when roughage-based diets are fed (<1% of the total bacterial population; Wells et al., 1997) and proliferate during shifts to rapidly fermentable carbohydrate diets (Russell and Robinson, 1984). *S. bovis's* ability to tolerate an acidic environment (Russell, 1991), rapid growth (Russell and Robinson, 1984), production of lactate as the principal fermentation product if fermentation rates are rapid (Russell and Baldwin, 1979), and very high amylase activity (Cotta, 1988) support its proposed role in ruminal acidosis during rapidly fermentable carbohydrate feeding. However, it has not always increased or been identified in grain-fed cattle (Tajima et al., 2000; Klieve et al., 2003; Golder et al., unpublished) and is not always the main cause of high ruminal acidity (Hungate, 1966). *S. bovis* numbers declined by 10,000 fold in cattle adapted to a grain diet (Wells et al., 1997). *S. bovis* appears to increase in the short term under certain feeding conditions, namely rapid changes to readily fermentable carbohydrates, and may contribute to the pathogenesis of ruminal acidosis. However, as its proliferation is not essential for the aetiology of ruminal acidosis; it is outcompeted by other microbes, and no-longer persists in adapted cattle. Therefore, it may not be the most significant causal agent in ruminal acidosis. There are a number of other bacteria that produce lactate and undergo population shifts during feeding changes (Owens et al., 1998). It is possible that the interactions of the many microbes that are not currently identified and characterized are more responsible for triggering ruminal acidosis than bacteria currently identified and characterized. The specific bacteria or other microbes involved in the pathogenesis of ruminal acidosis may vary between individuals and diets fed.

Lactobacillus spp. have been implicated in the pathogenesis of ruminal acidosis in a number of ruminal acidosis models. These can produce at least half of their end product carbon as lactate (Schleifer, 2009). A number of *Lactobaccilli* spp. are acid tolerant and predominate at low pH with growth generally occurring when ruminal pH is <5.0 (Schleifer, 2009). The Lactobacillceae family did not show an increased relative abundance in cattle supplemented with fructose that had increased ruminal lactate concentrations compared to those fed grain (Golder et al., unpublished). *Escherichia coli* are often prominent in the rumen when high-corn or readily fermentable carbohydrate diets are fed and have a large reservoir of virulence factors for inflammation, but require epithelial damage to cause pathological change (Plaizier et al., 2008). *E. coli* have not

always been identified in the rumen (Golder et al., unpublished), raising questions in regard to the significance of these in the pathogenesis of ruminal acidosis.

Ruminal acidosis models (Hungate et al., 1966; Baldwin and Allison, 1983; Nocek, 1997; Owens et al. 1998; RAGFAR, 2007) may have merit for certain cases of ruminal acidosis. However, owing to the complexity of interactions within the rumen microbiome and host responses it is likely other members of the rumen microbiome have vital roles in the pathogenesis of ruminal acidosis in different physiological situations. A number of these microbes are most likely yet to be identified or cultured, given the low percentage of organisms in the rumen that have been identified and cultured, and microbes involved may also vary between individual cattle. There is a need to further understand ruminal microbial shifts during dietary changes to give insights into associations between biology, health, and performance (Brown et al., 2006) and to improve animal health and production. New models to explain the pathogenesis of ruminal acidosis are needed.

BUFFERING AND SALIVA

The rumen is capable of buffering changes in rumen pH by a number of methods of hydrogen removal and therefore can mitigate the risk of ruminal acidosis. The use of various routes of ruminal hydrogen removal from the rumen is dynamic and is influenced by the amount of fermentation acids produced and ruminal pH with a balance between the production of fermentation acids and the secretion of buffers a large factor determining rumen pH (Allen, 1997). The largest source of hydrogen buffering is from VFA and ammonia formation and their removal from the rumen when they are absorbed across the rumen wall or flow out of the rumen (Allen, 1997).

The majority of hydrogen ions not removed by VFA absorption are removed from solution in the rumen by saliva from a combination of alkalization and buffering; however, the rate of acid production in the rumen is more than double the rate of saliva secretion (Allen, 1997). Saliva contains both bicarbonate and hydrogen phosphate that are capable of removing hydrogen, is relatively constant in composition, and is not affected by feed substrates or intake (Erdman, 1988). Thus the supply of endogenous buffers from saliva is determined by the amount of saliva sceretion (Cassida and Stokes, 1986). Maekawa et al. (2002) reported both particle size and moisture content of feed

affect the amount of saliva added to feed during mastication and attributed differences in ensalivation rate between feeds to differences in eating rate between feeds.

Bicarbonate and hydrogen phosphate remove hydrogen from the rumen by different mechanisms. The bicarbonate ion binds with a hydrogen ion creating carbonic acid which is then dehydrated to form water and carbon dioxide (Allen, 1997). The dehydration of carbonic acid is favored by its equilibrium constant. The carbon dioxide is removed by belching (Allen, 1997). In the hydrogen phosphate system a hydrogen ion combines with hydrogen phosphate to form dihydrogen phosphate which flows out of the rumen through the omasal orifice (Allen, 1997). Saliva secretion is stimulated by physically effective fiber and saliva flow rates vary with chewing, with a 1.8 times higher flow rate occurring during rumination compared to resting (Cassida and Stokes, 1986).

$$H^+ + HCO^-_3 \longleftrightarrow H_2CO_3 \longleftrightarrow H_2O + CO_2$$

bicarbonate ion carbonic acid carbon dioxide gas removed by belching

Direct buffering from the diet is less than one fifth of buffering from saliva and is largest when rumen pH <5 (Allen, 1997). Cereal grains have the lowest buffering capacity: while, high protein feeds have the highest (Jasaitis et al., 1987). Hydrogen ions can also be removed by the flow of particulate matter and as free hydrogen from the rumen (Allen, 1997), as well as by methane.

FEED ADDITIVES

As highlighted throughout this review, ruminal acidosis is a complex disorder and there remain a number of questions in regard to its pathogenesis. It is likely individual cattle have unique susceptibilities to ruminal acidosis and ruminal responses differ for a number of reasons, but are largely based on the type of substrate fed and feeding strategy. This poses challenges for developing effective control strategies for ruminal acidosis which are likely to vary between herds.

Current control strategies for ruminal acidosis involve feeding management (RAGFAR, 2007), previously addressed in this review. This can include the provision of adequate access to physically effective fiber, a balanced ration composition, sufficient chop length and grain particle size, forage access close to concentrate feeding, avoidance of

rapid feed changes, and consistent pasture access (RAGFAR, 2007). Feed additives, have been defined by a number of definitions and broadly cover any products that are used in animal nutrition for health or production benefits. These can be used in combination with other aspects of feed management to modify rumen function.

Feed additives that can influence ruminal acidosis include: antibiotics, buffers, neutralizing agents, and direct-fed microbes (Teh et al., 1985; Coe et al., 1999; Krehbiel et al., 2003). However, the registration for use of these products in dairy cattle differs throughout the world and *in vivo* information on these feed additives is limited or inconsistent in some cases with *in vitro* findings, particularly when different feed substrates are involved.

Antibiotics

A number of antibiotics are used in the dairy industry; however, use of antibiotics as feed additives in animal nutrition has been banned in the European Union (AnadÓN, 2006). Virginiamycin, monensin, flavophospholipol, and tylosin are antibiotic feed additives that may have potential to reduce the risk of ruminal acidosis (Coe et al., 1999; Lean et al., 2000; Golder et al., 2014b; Golder et al., unpublished data).

Virginiamycin

The streptogramin antibiotic, virginiamycin (**VM**) is used for ruminal acidosis control in Australia; however, is banned in the European Union, and not used in the New Zealand or The United States' dairy industries. Cocito (1979) proposed that VM stabilized ruminal fermentation and reduced feed intake variation by inhibition of protein synthesis of gram positive and select gram negative bacteria and Nagaraja et al. (1987) found that VM lowers lactate production *in vitro*. Further, VM may promote propionate producing bacteria (Dennis et al., 1981) and has a protein-sparing effect *in vitro* (Van Nevel et al., 1984).

Virginiamycin reduced counts of *S. bovis*, *Lactobacillus* spp., and *Fusobacterium necrophorum* in supplemented cattle compared to controls (Coe et al., 1999), supporting proposed models of action. However, VM had no effects of total VFA (Salinas-Chavira et al., 2009; Golder et al., 2014b) or propionate (Clayton et al., 1999; Valentine et al., 2000; Golder et al., 2014b) in cattle studies compared to controls, a finding which

contrasts the proposal that VM selects for propionate producing bacteria (Dennis et al., 1981). An absence of effects of VM on ruminal ammonia concentrations in cattle studies (Coe et al., 1999; Ives et al., 2002; Golder et al., 2014b) also does not support the protein-sparing effect of VM identified *in vitro* (Van Nevel et al., 1984).

Coe et al. (1999) concluded that VM can moderate ruminal fermentation in situations that could lead to rapid production of lactic acid and reduced ruminal L-lactate concentration in cattle with ruminal acidosis induced by an intra-ruminal slurry of powdered corn and corn starch at 12.5 g/kg of BW (Coe et al., 1999). However, VM had no effect on ruminal lactate concentration in other cattle studies (Salinas-Chavira et al., 2009; Golder et al., 2014b). Golder et al. (2014b) showed that VM did not prevent total lactate concentrations accumulating >10 m*M* and large amounts of histamine also accumulated in the rumen during a starch and fructose challenge. The ruminal lactate concentration in a heifer in the study by these authors exceeded 60 m*M*, suggesting that VM is not capable of lactate control in all cattle.

Overall, VM showed limited effects on ruminal fermentation characteristics and discrepancies between *in vivo* and *in vitro* results. Similarly, VM supplementation had no effects on milk production or milk composition compared to controls (Clayton et al., 1999; Valentine et al., 2000). Therefore, VM's mode of action under different feeding conditions and potential to reduce the risk of ruminal acidosis through changes in the ruminal environment needs to be further elucidated in *in vivo* studies. It may be of interest to investigate the efficacy of VM in combination or in rotation with other feed additives.

Monensin

Monensin is a carboxylic polyether ionophore produced by a naturally occurring strain of *Streptomyces cinnamonensis* (Haney and Hoehn, 1967). It is approved for use in lactating cattle in several countries including: Australia, Argentina, Brazil, New Zealand, South Africa, and the United States (Gallardo et al., 2005). It is proposed to favor increased ruminal propionate, and decreased acetate, butyrate, and methane percentages (Richardson et al., 1976), and have a 'protein sparing' effect (Russell and Houlihan, 2003). A proposed mode of action of monensin is the selective inhibition of gram positive bacteria, in particular inhibition of lactate producing bacteria without

affecting most lactate utilizing bacteria (Dennis et al., 1981; Weimer et al., 2008); however, lactate concentrations have been rarely reported in *in vivo* cattle studies with monensin.

There is a large disparity in ruminal fermentation responses between monensin studies (Table 7) that may result from differences in monensin dose rates, cattle management, physiological state of the cattle, and diet. Despite these discrepancies a meta-analysis showed monensin supplemented dairy cattle had improved body condition score, bodyweight, milk and milk protein yields by 0.03, 0.06 kg/d, 2.5%, and 0.016 kg/d, respectively (Duffield et al., 2008). Milk fat and protein percentages were decreased by 0.13% and 0.03%, respectively, and DMI was reduced by 0.7 kg (Duffield et al., 2008).

Weimer et al. (2008) note the lack of systematic studies on the effect of monensin on populations of ruminal bacteria and the challenge in linking changes in specific ruminal bacterial groups to identified production benefits of monensin. Monensin does not always suppress gram positive bacteria and its effects on ruminal bacteria are more complex (Weimer et al., 2008) with bacterial species varying a lot in resistance to monensin (Russell and Houlihan, 2003). This could explain some of the heterogeneity in ruminal fermentation results and emphasizes the need to further elucidate mode of action in different feed management systems.

Monensin may have beneficial additive effects when fed in combination with other feed additives as the combination of monensin and tylosin has increased total VFA, milk yield, and tended to increase weight gain in lactating cows during a starch challenge (Lean et al., 2000). This combination also increased ruminal pH, and decreased total VFA and propionate concentrations in feedlot steers (Ives et al., 2002). The interaction of monensin and tylosin decreased butyrate concentrations in a 1.2% of BW grain single challenge feeding (Golder et al., unpublished data). Monensin can also be fed in combination with Flavophospholipol; however, additive effects of this combination were inconclusive (Golder et al., unpublished data).

Although the effects of monensin on ruminal bacteria and ruminal fermentation measures were variable and its mode of action is not fully elucidated, monensin had beneficial production effects suggesting its effects of rumen perturbation and ruminal acidosis were positive. Use of monensin in combination with other feed additives also appeared to be beneficial to ruminal conditions.

Table 7. Summary of effects of monensin on ruminal total and individual volatile fatty acid, ammonia, and lactate concentrations and pH from *in vivo* cattle studies

Cattle type	No.	Additional treatments	Rate	Effects on ruminal measures		Reference
				Total and individual VFA (m*M*)	pH, ammonia & lactate (m*M*)	
Steers	6 (cross over)	Low, medium and high roughage + or - monensin	200 mg/steer	+ prop low & high monensin diets (molar proportion) - acet low & high monensin diets	Not measured	Thorton and Owens (1981)
Feedlot cattle	48		0, 100, 500 mg/hd/d	- acet with inc dosage - but with inc dosage + prop with inc dosage - val NS total VFA	Not measured	Richardson et al. (1976)
Fistulated Holstein steers	4		0, 11, 22, 33 ppm	- acet with increased dosage + prop with increased dosage	NS pH NS ammonia	Dinius et al. (1976)
Non-lactating Holstein cows	2	+ /- 1 & 2 kg/d soybean meal	350 mg/hd/d	- acet + prop NS but, val, total VFA	NS pH - ammonia	Yang and Russell (1993)
Fistulated Holstein heifers	2		33 ppm	- acet:prop	+ lactate	Bartley et al. (1979)
Crossbred yearling heifers	150		33 ppm	NS VFA	Not measured	Dyer et al. (1980)
Crossbred steers	4	4×4 latin square	29 mg monensin + 11 mg tylosin/kg daily diet	- but proportions - val proportions NS acet & prop proportions	NS pH NS ammonia	Morris et al. (1990)
Lactating fistulated Holsteins	3	3×3 latin square	33 ppm	+ prop proportion (trend) - but proportion (trend) NS total VFA	NS pH - ammonia	Haimoud et al. (1995)

(+) = increase; (-) = decrease; acet = acetate; but = butyrate; prop = propionate; val = valerate; isobut = isobutyrate; isoval = isovalerate; VFA = volatile fatty acid; BW = bodyweight; DM = dry matter.

Table 7 (continued). Summary of effects of monensin on rumen total and individual volatile fatty acid, ammonia, and lactate concentrations and pH from *in vivo* cattle studies

Cattle type	No.	Additional treatments	Rate	Effects on ruminal measures		Reference
				Total and individual VFA (m*M*)	pH, ammonia & lactate (m*M*)	
Lactating Holsteins	16	CRC pre and post-calving	CRC 32 g 335 ± 33 mg/d	Not measured	- (numerical) ammonia pre- & post calving	Plaizier et al. (2000)
Fistulated Lactating Holsteins	4	4×4 latin square	350 mg/d	NS VFA	+ pH (trend P = 0.08) - ammonia	Benchaar et al. (2006)
Fistulated Lactating Holsteins	6	Double 3×3 latin square	24 mg/kg DM	NS total VFA	Square × treatment interaction for pH - ammonia	Martineau et al. (2007)
Lactating Holsteins	24		350 mg/d	Not measured	NS pH - ammonia	Ghorbani et al. (2011)
Fistulated Lactating Holsteins	4	4×4 latin square	16 mg/kg DM	NS VFA	NS pH NS ammonia	da Silva-Kazama et al. (2011)
Fistulated Holstein steers	4	70% lucerne hay 30% corn	33 ppm	- acet molar proportion + prop molar proportion + but (trend) molar proportion NS total VFA)	Not measured	Armentano and Young (1983)
Lactating Holsteins	4	High and low forage: concentrate 70:30 and 50:50	300 mg/d	- acet proportion + prop proportion - but proportion NS total VFA More profound effects on low forage	NS pH NS ammonia N	Ramanzin et al. (1997)
Fistulated Lactating Holstein	8	Corn meal followed by fresh forage	350 mg/cow/d	- acet:prop NS acet, prop, but, total VFA	NS ammonia NS pH	Ruiz et al. (2001)

(+) = increase; (-) = decrease; acet = acetate; but = butyrate; prop = propionate; val = valerate; isobut = isobutyrate; isoval = isovalerate; VFA = volatile fatty acid; BW = bodyweight; DM = dry matter.

Table 7 (continued). Summary of effects of monensin on ruminal total and individual volatile fatty acid, ammonia, and lactate concentrations and pH from *in vivo* cattle studies

Cattle type	No.	Additional treatments	Rate	Effects on ruminal measures		Reference
				Total and individual VFA (m*M*)	pH, ammonia & lactate (m*M*)	
Fistulated Lactating Holsteins	8	-	248 mg/d	- acet - but + prop NS val & total VFA	NS pH NS ammonia N	Broderick (2004)
Lactating Holsteins	41	2 wk prepartum 6 wk postpartum	CRC 335 ± 33 mg/d	- acet:prop - but NS acet, prop , & total 6 wk postpartum	+ pH at 6 wk postpartum NS ammonia N	Green et al. (1999)
Cows per group	10	1. Sampled before and after 2 wks of monensin 2. Sampled before and after 2 wks of monensin on cows that had never received monensin and then on cows that had previously received monensin	24 ppm	1. + prop molar percentage + isobut molar percentage + isoval molar percentage - but molar percentage - acet:prop 2. Treated once + prop, + isobut & + isoval - but & - acet:prop molar percentage Twice treated = + isobut	Not measured	Sauer et al. (1998)
Fistulated steers	4	High roughage High grain High roughage + monensin High grain + monensin	150 mg/d	+ prop (both diets) - but NS acet	NS pH	Van Maanen et al. (1978)
Fistulated steers	4	4×4 latin square	33ppm	+ acet (- proportion) + prop (+ proportion) NS val - total VFA	NS pH	Rogers and Davis (1982)
Yearling steers		Corn base Sorghum grain based	33 ppm	- acet corn based diet + prop corn based diet NS total VFA & but NS sorghum diet	- ammonia corn based diet	Muntifering et al. (1980)

(+) = increase; (-) = decrease; acet = acetate; but = butyrate; prop = propionate; val = valerate; isobut = isobutyrate; isoval = isovalerate; VFA = volatile fatty acid; BW = bodyweight; DM = dry matter; CRC = control release capsule.

Table 7 (continued). Summary of effects of monensin on ruminal total and individual volatile fatty acid, ammonia, and lactate concentrations and pH from *in vivo* ruminal acidosis induction cattle studies

Cattle type	No.	Additional treatments	Rate	Effects on ruminal measures		Reference
				Total and individual VFA (m*M*)	pH, ammonia & lactate (m*M*)	
Fistulated steers (Epx # 1)	2	1 day before and day of intraruminal glucose (12.5 g/kg BW)	1.3 mg/kg BW intraruminal	Not measured	+ pH - lactate Prevented acidosis	Nagaraja et al. (1981)
Fistulated cows (Epx # 2)	3	Same as Exp # 1 above except ground corn instead of glucose. 2×3 latin square	1.3 mg/kg BW intraruminal	Not measured	- pH + lactate Acidosis not prevented	Nagaraja et al. (1981)
Fistulated cows (Epx # 3)	4	Same as Exp # 2 except treatments given for 7 d prior to acidosis induction	1.3 mg/kg BW intraruminal	-acet + prop -but + VFA	+ pH - lactate	Nagaraja et al. (1981)
Fistulated Lactating Holstein cows	6	Grain induced SARA (Cross over design) Control vs monensin premix	22 mg/kg	No VFA measured	NS pH	Osbourne et al. (2004)
Fistulated cows	4	12.5g/kg BW glucose (intraruminal) 4×4 latin square	0.33, 0.65 and 1.3 mg/kg BW	- acet + prop + total VFA	+ pH (minor) - lactate	Nagaraja et al. (1982)
Lactating Holsteins	6	Cross-over design Exp #1 CRC 10 d substitution 15% grain pellets (wheat:barley) of TMR ad lib intake 6x/d for 10 d	335 mg/d	NS VFA - acet:prop	NS pH	Mutsvangwa et al. (2002)
		Exp # 2 monensin premix instead of CRC in Exp # 1	22 mg/kg DM	NS VFA - acet:prop	NS pH	

(+) = increase; (-) = decrease; acet = acetate; but = butyrate; prop = propionate; val = valerate; isobut = isobutyrate; isoval = isovalerate; VFA = volatile fatty acid; BW = bodyweight; DM = dry matter; CRC = control release capsule.

Flavophospholipol

Flavophospholipol is a phosphoglycolipid antimicrobial produced by strains of *Streptomyces* spp. (Gallo et al., 2010) which inhibits cell wall synthesis in gram positive bacteria and has some activity against *Salmonella* spp. and *Escherichia coli* (Butaye et al., 2003). No effects of flavophospholipol on total and individual VFA concentrations in cattle have been demonstrated (Albert et al., 1991; Mogentale et al., 2010; Golder et al., unpublished data), except for a decrease in total VFA in bulls (Alert et al., 1993). This raises questions around the mechanisms responsible for flavophospholipol's improvement of average daily gain (**ADG**) in a number of feedlot studies (Galbraith et al., 1983; Scott et al., 1984; Rowland et al., 1999) and increased milk yield (Arana et al., 1992; Blaziak et al., 1992), and milk protein and fat yields in dairy cattle (Bahrecke et al., 1984). However, VFA profiles were not always measured in studies that reported production measures so these may still be influenced. Perhaps there is potential for synergistic effects when flavophospholipol is combined with other feed additives as the interaction of Fermenten® and flavophospholipol reduced total VFA, acetate, and isobutyrate concentrations in a 1.2% of BW grain single challenge feeding (Golder et al., unpublished data). Flavophospholipol may have potential to reduce ruminal acidosis based on its ability to improve production but further research is required to support this and further ascertain flavophospholipol's mode of action and influence on ruminal fermentation.

Tylosin

Tylosin is a macrolide antibiotic produced by *Streptomycetes fradiae* that inhibits protein biosynthesis in gram positive bacteria (Liu and Douthwaite, 2002). Its main use is to reduce the incidence of liver abscess in feedlot cattle by controlling the etiological agent of liver abscess, the gram negative opportunistic bacteria, *Fusobacterium necrophorum* (Nagaraja et al., 1999). Wileman et al. (2009) concluded that tylosin reduced the risk of liver abscess from 30 to 8% in beef cattle, but had no consistent benefits on ADG, gain to feed ratio, or DMI by using a general linear mixed model incorporating results from 6 studies.

Studies on the effects of tylosin on ruminal fermentation are somewhat limited but tylosin increased total VFA and butyrate concentrations and tended to decrease plasma lactate concentrations in lactating dairy cattle during a ruminal acidosis challenge (Lean et al., 2000). In steers, tylosin had no effect on VFA or ammonia proportions (Horton and Nicholson, 1980) or their concentrations and ruminal lactate concentration (Nagaraja et al., 1999). Tylosin appears to have synergistic effects on ruminal fermentation profiles when administered in combination with monensin (Lean et al., 2000; Ives et al., 2002), a common practice in the beef industry. Owing to its positive effects on liver abscess reduction and ruminal fermentation measures in combination with monensin, it is probable that tylosin administration will reduce the risk of ruminal acidosis in feedlot cattle and have beneficial effects on rumen perturbation in dairy cattle when combined with monensin.

Buffers and Neutralizing Agents

A buffer, by definition, reduces the decrease in pH without causing an increase in pH (Staples and Lough, 1989). Sodium bicarbonate, derived from natural deposits of trona (Staples and Lough, 1989), is a weak base that buffers hydrogen ions of organic acids (Ha et al., 1983) and is the most common buffer used in the dairy industry (Hu and Murphy, 2005). Others include potassium carbonate, potassium bicarbonate, and sodium sesquicarbonate (Erdman, 1988). Evidence has suggested that sodium bicarbonate's primary mode of action is not as a buffer, but through indirect increases in DM and water intakes caused by sodium, facilitated though a higher ruminal fluid dilution rate and lower starch digestion rate (Russell and Chow, 1993; Valentine et al., 2000). It has additive effects with magnesium oxide (Thomas and Emery, 1969; Erdman et al., 1980), hence this combination is often incorporated in dairy rations. Magnesium oxide is an alkalizing agent; however, it is not established if the mode of action is through the alkalizing ability (Herod et al., 1978), alleviation of magnesium deficiency (Staples and Lough, 1989), or improved digestibility (Erdman et al., 1980).

Quantitative reviews have demonstrated the effects of buffers and neutralizing agents on ruminal fermentation and milk production measures in dairy cattle varies among substrates, with benefits being primarily observed in maize silage-fed cattle (Erdman, 1988; Staples and Lough, 1989; Hu and Murphy, 2005). Ruminal pH differences to controls were not observed in quantitative reviews when buffers, neutralizing agents, or

their combinations were fed to dairy cattle (Erdman, 1988; Hu and Murphy, 2005); however, ruminal pH was numerically increased in the majority of studies reviewed by Staples and Lough (1989) and was lower for those fed maize diets (Hu and Murphy, 2005). Propionate concentration and the ratio of acetate to propionate were not affected, unless maize or >30% forage diets were fed, in which case propionate concentrations decreased and the ratio of acetate to propionate was increased (Erdman, 1988; Hu and Murphy, 2005). No effects on butyrate and valerate concentrations were observed in dairy cattle fed a combination of sodium bicarbonate and magnesium oxide (Stokes et al., 1986; Arambel et al., 1988). Lactate (Kilmer et al., 1981; Kennelly et al., 1999) and ammonia concentrations were not affected in sodium bicarbonate-fed dairy cattle (Solorzano et al., 1989; González et al., 2008).

When corn silage was offered as the main forage type, supplementation with buffers or neutralizing agents increased milk yield by 0.8 kg/d, and increased milk fat by 0.22%, which equated to an increase in 1.6 kg/d more 4% fat corrected milk (Staples and Lough, 1989). Production effects of buffer or neutralizing agent supplementation were inconsistent when other feed types were fed, with no production benefits reported when a combination of sodium bicarbonate and magnesium oxide were supplemented in diets with common forage to grain ratios (Staples and Lough, 1989). In the review of Hu and Murphy (2005), sodium bicarbonate had no effects on milk production, milk protein yield or proportion, regardless of forage type, but cows fed maize silage diets produced 2.7 g/kg more milk fat. The combination of sodium bicarbonate and magnesium oxide was effective at increasing DMI above that of unsupplemented controls (Golder et al., 2014b) and sodium bicarbonate increased DMI in maize silage-fed dairy cattle (Hu and Murphy, 2005).

Supplementation of buffers and neutralizing agents in maize silage diets appear to have production benefits and increase the ratio of acetate to propionate, which might suggest a healthy rumen, but they may not be effective in other diets.

Direct-fed Microbials

A number of different terms have been used for supplementing microbes in the rumen (Yoon and Stern, 1995). The term 'probiotic' was first used by Parker (1974) and was more clearly defined by Fuller (1989) as 'a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance'. In The United States probiotics are considered to include viable microbial cultures, culture extracts, enzyme preparations, or their combinations (Yoon and Stern, 1995). To avoid confusion regarding a number of terminologies for probiotics in 1989 The United States Food and Drug Administration redefined probiotics as 'Direct-fed microbials' (**DFM**) with the definition 'a source of live (viable) naturally-occurring micro-organisms' (Yoon and Stern, 1995).

Animal responses to DFM have been inconsistent owing to the supplementation of many different organisms, strains of organisms, and combinations of multiple organisms, and differences in micro-organism inclusion level, diet, feeding management, and animal factors (Raeth-Knight et al., 2007). Hence evaluation of DFM performances is a challenge. Ruminal fermentation and production responses to individual DFM have been recently reviewed (Yoon and Stern, 1995; Beauchemin et al., 2003a; Krehbiel et al., 2003; Beauchemin et al., 2006; Seo et al., 2010). In general, DFM have increased milk production in dairy cattle, improved health and performance in calves, and ruminal responses indicated that they could reduce the risk of ruminal acidosis (Krehbiel et al., 2003). Responses to DFM in the rumen include a decrease in the area below SARA ruminal pH, an increase in propionate concentrations, increased protozoa counts, and altered counts of bacteria such as those of lactate producing and utilizing bacteria (Krehbiel et al., 2003).

With concerns around the use of antibiotics in the animal industries there is interest in the use of DFM to reduce or replace the use of antibiotics (Seo et al., 2010). A number of products of single or mixed bacterial cultures are used in the ruminant industries, largely strains from the following bacterial genera: *Bifidobacterium, Enterococcus, Streptococcus, Prevotella, Bacillus, Lactobacillus, Megasphaera,* and *Propionibacteria* (Seo et al., 2010). *Lactobacillus acidophilus* and *Propionibacterium freudenreichii* are the primary bacterial DFM used in the dairy industry (Raeth-Knight et al., 2007). The primary yeast and fungal products used contain *Saccharomyces cerevisiae* and

Aspergillus oryzae strains, respectively (Yoon and Stern, 1995; Seo et al., 2010). The majority of exogenous enzyme products incorporated in ruminant feeds are fiber-degrading enzymes that are products of microbial fermentation from bacterial (mostly *Bacillus* spp.) or fungal (mostly *Trichoderma longibrachiatum, Aspergillus niger, A. oryzae*) origin (Pendleton, 2000) and many do not contain live micro-organisms (Beauchemin et al., 2003a), so may not be classified as DFM. Practical matters related to the effects of DFM include a need to optimize dosage, timing, strains of DFM, and animal conditions (Seo et al., 2010). DFM that target the rumen must be active in the rumen and remain viable during delivery (Seo et al., 2010).

Feed additives have potential as control agents for ruminal acidosis; however, they need to overcome individual animal variation in rumen microbiomes and responses to any changes in diet. They appear to influence the rumen by different mechanisms, but our understanding of these mechanisms is largely based on *in vitro* ruminal responses and may not reflect *in vivo* responses. Further work is required to elucidate these mechanisms, particularly during different feeding situations. Prudent use strategies for feed additives need to be implemented; however, animal variation suggests that no single feed additive will be capable of controlling ruminal acidosis in all cattle (Golder et al., 2014b). Different feed additives may be need to be supplemented depending on feed substrates; however, a degree of ruminal acidosis may be evitable (Enemark, 2008). Feeding combinations of feed additives may have synergistic effects, but literature is limited and further research is required in this field.

CONCLUSION

Ruminal acidosis remains an important economic and welfare issue for the dairy industry worldwide. It appears to occur along a continuum of severity based on degrees of hydrogen sequestration. There is a range of indicators that can be used to diagnose ruminal acidosis; however, many require further validation and the establishment of reference values. Ruminal fluid collection techniques may influence ruminal pH values; however, each collection method is valid, provided it is carried out correctly and consistently. Caution is advised for clinicians when diagnosing ruminal acidosis based on a single ruminal pH measurement. We suggest diagnosis of ruminal acidosis is best achieved through an evaluation of a combination of clinical signs, feed management history, ruminal fermentation characteristics, and production performance. Feed sources

with high sugar contents can increase the risk of ruminal acidosis when physically effective fiber is inadequate and result in ruminal conditions that do not fit models of ruminal acidosis previously described (Hungate, 1966; Baldwin and Allison, 1983; Nocek, 1997; Owens et al., 1998; RAGFAR, 2007). Development of specific definitions for ruminal acidosis during different feeding conditions is warranted. The rumen appears to consist of a core microbiome that is influenced by feed management. Feed additives have potential to control ruminal acidosis by different mechanisms; however, prudent use strategies need to be implemented.

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CHAPTER 2

Effects of Grain, Fructose, and Histidine Challenges on Ruminal pH and Fermentation Measures in Dairy Heifers

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OVERVIEW OF CHAPTER 2

The importance of the influence of substrates and need to further investigate their role in the risk of ruminal acidosis was one of the conclusions of the critical review in Chapter 1. The involvement of ruminal histamine also remained unclear in Chapter 1. The majority of studies on ruminal acidosis have examined effects in cattle fed readily fermentable carbohydrates for several days or weeks or administered substrates into the rumen via fistulas. Consequently, the effects of grain, fructose, and the amino acid histidine, which is a precursor for histamine, on ruminal pH and fermentation measures was examined in unadapted cattle fed a single challenge feed of combinations of these substrates.

ABSTRACT

The effects of grain, fructose, and histidine on ruminal pH and fermentation measures were studied in dairy cattle in a partial factorial study. Holstein-Friesian heifers (n = 30) were randomly allocated to 5 treatment groups: (1) control (no grain); (2) grain [fed at a crushed triticale dry matter intake (**DMI**) of 1.2% of bodyweight (**BW**)]; (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); (4) grain (1.2% of BW DMI) + histidine (6 g/head); and (5) Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). Heifers were fed 1 kg of grain daily with *ad libitum* access to ryegrass silage and alfalfa hay for 10 d. Feed was withheld for 14 h before challenge day, on which heifers were fed 200 g of alfalfa hay and then the treatment rations immediately thereafter. Ruminal samples were collected 5 min after ration consumption, 60 min later, and at 3 subsequent 50-min intervals. Grain decreased ruminal pH and increased ammonia, total volatile fatty acid (**VFA**), acetate, butyrate, propionate and valerate concentrations compared to controls. The addition of grain had no effect on ruminal D- and L-lactate concentrations. Fructose markedly decreased ruminal pH and markedly increased D- and L-lactate concentrations. Fructose increased total VFA and butyrate and decreased valerate concentrations. Although histidine did not have a marked effect on ruminal fermentation, increased concentrations of histamine were observed following feeding. This study demonstrates that the substitution of some grain for fructose can lower ruminal pH and increase VFA and lactate concentrations, warranting further investigation into the role of sugars on the risk of ruminal acidosis in dairy cattle.

Key words: fructose, histidine, lactate, ruminal acidosis

INTRODUCTION

Ruminal acidosis is a complex and diverse nutritional disorder that affects cattle. It is associated with an accumulation of organic acids, including volatile fatty acids (**VFA**) and lactate, and subsequent decrease in ruminal pH (Nagaraja and Titgemeyer, 2007). These changes reflect the feeding of diets that contain large amounts of readily fermentable carbohydrates and are low in neutral detergent fiber (**NDF**) or that are high in preformed organic acids and are fed to cattle adapted to forage diets (Bramley et al., 2008). Bramley et al. (2008) found that dairy cows with higher ruminal concentrations of acetate, propionate, butyrate, valerate and D-lactate, lower concentrations of ammonia, and lower pH had lower milk fat percentage, and were more prevalent in herds with higher ratios of non-fiber carbohydrate (**NFC**) to NDF in diets fed.

The acute form of ruminal acidosis can result in incoordination, rumenitis, metabolic acidosis, lameness, hepatic abscesses, pneumonia, and death (Nagaraja and Lechtenberg, 2007). Greater economic losses result from subacute ruminal acidosis (**SARA**) associated with reduced milk yield, lower fat and protein yields, decreased body condition, laminitis, diarrhoea and increased cull rate (Enemark, 2008; Plaizier et al., 2008).

The specific dietary precursors that influence the risk for ruminal acidosis have not been well studied. The aim of this study was to examine the effects of grain, fructose, and histidine, fed to dairy heifers unadapted to these in a single challenge, on ruminal VFA, ammonia, and lactate concentrations.

The polymer of fructose, fructan, is the primary form of excess carbohydrate storage in cool-season forages (Pollock and Cairns, 1991). Interest has increased in the potential benefits of *Lolium perenne* varieties with greater water-soluble carbohydrate (**WSC**) content in pasture-based dairying (Miller et al., 2001; Tas et al., 2006). Fructan administered as an oligofructose drench at 13, 17, or 21 g/kg of bodyweight (**BW**) induced metabolic acidosis (Thoefner et al., 2004) and ruminal and systemic acidosis when 17 g/kg of BW of oligofructose was administered to dairy heifers (Danscher et al., 2009, 2010). Chemical analysis results (I. J. Lean, unpublished data) from more than 100 ryegrasses collected under experimental protocols involving immediate icing, freezing, and freeze-drying of samples found that WSC averaged 18% of dry matter

(**DM**) and ranged between 3 to 31% of DM. It was our intention to pulse feed heifers with fructose at a dry matter intake (**DMI**) of 0.4% of BW (33% of DM) and examine the effects on ruminal fermentation products. The concentration of fructose used is similar to amounts of WSC ingested by cattle over a day. We hypothesized that this soluble carbohydrate might contribute to the onset of ruminal acidosis and alter ruminal pH, VFA and lactate measures in dairy heifers fed a single grain-based challenge developed by Lean and Rabiee (2009a).

Release of histamine has been hypothesized to have an important role in ruminal acidosis (Dain et al., 1955; Ahrens, 1967), as has endotoxin release (Gozho et al., 2005; Khafipour et al., 2009). The amino acid histidine is decarboxylated at low ruminal pH by the bacteria *Allisonella histaminiformans* to produce the inflammatory molecule histamine (Garner et al., 2002). Histidine is considered the first limiting amino acid in grass silage- and cereal-based diets (Vanhatalo et al., 1999; Korhonen et al., 2000); however, histidine is present in relatively high concentrations in white clovers (*Trifolium repens*, 4.7 to 5.1 g/kg of DM; Penkov et al., 2003), ryegrass (*L. perenne*, 2.8 g/kg of DM), and kikuyu, (*Pennisetum clandestinum*, 2.9 g/kg of DM; Reeves et al., 1996). There is a lack of clearly defined pathways with regard to the absorption of histamine from the rumen and entry into the circulatory system (Brent, 1976; Motoi et al., 1984) and a need to investigate the involvement of ruminal histamine in ruminal acidosis and its sequelae, including laminitis. We hypothesized that histidine orally administered at a rate representative of 160% of a dairy cow's histidine requirement would increase ruminal histamine concentrations and could induce ruminal acidosis.

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted on 30 nonpregnant Holstein-Friesian heifers <18 mo of age with a mean BW of 359.3 ± 47.3 kg of BW at Camden, New South Wales (**NSW**), Australia. The heifers were from a commercial dairy herd and all experimental procedures were approved by the Bovine Research Australasia Animal Ethics Committee (BRA 0609-0610).

All heifers were housed on a dry lot and were locked in individual head stanchions in a feed pad twice a day for approximately a total of 3 h/d. In the stanchions, heifers were

individually offered 1 kg (as-fed basis) of grain daily and had *ad libitum* access to ryegrass silage and alfalfa hay twice daily for a 10-day adaptation period before challenge day. The target feed intake during this period was 2 kg/d of alfalfa hay, 7.2 kg/d of ryegrass silage, and 1 kg/d of triticale (as-fed basis). The estimated chemical composition of the diet was calculated using CPM Dairy Ration Analyzer (version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 1) from forage samples analyzed by near-infrared spectroscopy (AOAC 2000) and wet chemistry by George Weston Technologies (Sydney, NSW, Australia) and wet chemistry by Dairy One Inc, Forage Testing Laboratory (Ithaca, NY; Table 2). Wet chemistry techniques were as follows: DM (AOAC 2000; method 930.15), NDF (Van Soest et al., 1991), crude protein (**CP**)(AOAC 2000; method 990.03), soluble protein (Cornell sodium borate-sodium phosphate buffer procedure), crude fat (AOAC 2000; method 2003.05), ash (AOAC 2000; method 942.05), lignin (AOAC 2000; 973.18), acid detergent fiber (**ADF**) (AOAC 2000; method 973.18), acid and neutral detergent insoluble crude protein (**ADICP** and **NDICP**) (Leco TruMac N Macro Determinator; Leco Corp., St. Joseph, MI), starch (YSI 2700 SELECT Biochemistry Analyzer; YSI Inc., Yellow Springs, OH), WSC (Hoover and Miller-Webster, 1998), ethanol-soluble carbohydrates (Hall et al., 1999). The NFC was calculated as NFC = 100 – [(NDF – neutral detergent soluble CP) + CP + crude fat + ash]. The minerals were analyzed by inductively coupled plasma-optical emission spectroscopy (**ICP-OES**; George Weston Technologies).

Table 1. Estimated chemical composition of the diet during the adaptation period

Item	Chemical composition (% of DM)
DM	73.0
NDF	42.3
Forage NDF (% of NDF)	97.3
Forage NDF (% of DM)	41.1
Physically effective NDF	39.2
Lignin	5.6
NFC[2]	30.2
Silage acids	6.5
Sugar	7.0
Starch	7.7
Soluble fiber	8.9

DM = dry matter; NDF = neutral detergent fiber; NFC = non-fiber carbohydrates.
[1]Estimations were performed using CPM Dairy Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) based on *ad libitum* feeding with a target intake of 2 kg/d of alfalfa hay, 7.2 kg/d of ryegrass silage, and 1 kg/d of triticale cultivar 'Berkshire' (as-fed basis). Estimates were based on 400 kg of BW heifers with a body condition score of 3.25 and growth rate of 0.73 kg/d.
[2]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash]. NDICP = neutral detergent insoluble crude protein.

Table 2. Chemical composition of alfalfa hay and ryegrass silage fed during the adaptation period, and triticale cultivar 'Berkshire' fed during the adaptation and challenge periods[1]

Item (% of DM)	Feed		
	Alfalfa hay	Ryegrass silage	Triticale
DM	87.7	76.2	88.8
CP	20.7	17.7	16.7
Soluble protein (% of CP)	43	40.5	26.5
Crude fat	2.5	2.6	1.5
Ash	9.0	10.4	2.4
Lignin	6.8	6.5	2.3
ADF	33.6	35.6	5.3
NDF	45.9	52.3	22.1
ADICP	1.2	1.5	0.3
NDICP	3.5	5.6	2.9
NFC[2]	25.5	22.8	60.0
Available protein	19.5	16.2	16.5
Degradable protein (% CP)	69	64	70
Starch	2.5	1.7	51.7
WSC	7.2	7.5	-
ESC (simple sugars)	4.9	7.3	3.8
DCAD (mEq/100 g)	20	5	1
Minerals (mg/kg)			
Chloride	9,388	16,367	1,358
Calcium	10,002	9,828	357
Cobalt	<0.5	1.60	<0.5
Copper	7.7	9.6	6.1
Iron	228	1,393	57.6
Phosphorus	3,400	2,700	3,000
Potassium	24,170	19,730	6,625
Magnesium	2,906	3,608	1,300
Manganese	56.2	139	57.3
Molybdenum	0.8	0.7	0.6
Sodium	1,440	4,510	100
Sulfur	3,000	3,400	1,900
Zinc	29	27	42

DM = dry matter; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; ADICP = acid detergent insoluble protein; NDICP = neutral detergent insoluble crude protein; NFC = non-fiber carbohydrate; WSC = water-soluble carbohydrate; ESC = ethanol-soluble carbohydrate; DCAD = dietary cation-anion difference.

[1]Values are means obtained from near-infrared spectroscopy and wet chemistry.

[2]NFC = 100 – [(NDF-NDICP) + CP + crude fat + ash].

Treatment Groups

The heifers were randomly allocated using Stata v.11 (StataCorp. LP, College Station, TX) to 5 treatment groups (n = 6 heifers/group) in a partial factorial arrangement: (1) control (no grain); (2) grain (crushed triticale at 1.2% of BW DMI); (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); (4) grain (1.2% of BW DMI) + histidine (6 g/head); and (5) Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). The statistical power of the study was based on previous experiments conducted by our group that demonstrated significant differences in VFA using a smaller number of cattle per group than used in this study. The chemical composition of triticale cultivar 'Berkshire' was analyzed by wet chemistry (Table 2; George Weston Technologies and Dairy One Inc.). The fructose [(Melbourne Food Depot, East Brunswick, Victoria (**VIC**), Australia] was a 99.5% pure crystalline powder and was mixed through the grain ration on the morning of the challenge.

The histidine (Merck KGaA, Darmstadt, Germany) was an L-histidine powder dissolved in 50 mL of tap water, and it was administered via a stomach tube immediately after consumption of 200 g of alfalfa hay. The dose rate of 6 g of histidine per head corresponds to approximately 160% of the average daily histidine requirement of 400 kg heifers as calculated in CPM Dairy. This percentage of histidine in respect to requirement equates to a similar percentage expected for lactating cattle when fed ryegrass (16 kg of DM/d) and 6 kg of DM of grain. Daily DMI was estimated based on maintenance and 0.7 kg of growth for heifers and approximated 2.75% of BW. Heifers that were not enrolled in 1 of the 2 histidine-containing treatment groups received approximately 100% of their average daily histidine requirement as calculated in CPM Dairy.

Challenge Procedure

The challenges were conducted over 4 consecutive days, with 7 or 8 heifers randomly allocated to 1 of 4 d with at least 1 heifer from each treatment group challenged on each day. Feed was withheld for 14 h before challenge. On the day of challenge, each heifer was offered and ate 200 g of alfalfa hay to reduce saliva contamination of the ruminal samples. Immediately after consumption of the hay, heifers were fed their allocated treatment rations with the exception of the control group, which received no further

ration. From previous work, we found that feeding a small proportion of hay or silage immediately before feeding the challenge rations prevents cattle from salivating excessively before sampling. All heifers were fed individually and were locked in head stanchions for the 215-min duration of the trial without access to water. The time for each heifer to consume their allocated treatment ration was recorded, and orts were weighed using an electronic scale to calculate the percentage of allocated ration consumed. Orts that contained fructose were sieved to separate the grain from the fructose and were weighed individually.

Sampling Procedure

Ruminal fluid (250 mL) was collected 5 min after consumption of the challenge ration, 60 min later, and at 3 subsequent 50-min intervals via a stomach tube and custom-designed stomach pump. The 3 m stomach tube was inserted to a length of >2 m. Ruminal fluid was scored for saliva contamination as described by Bramley et al. (2008) using a 3-point scoring system (3 being the highest level of contamination). No ruminal fluid retained for analysis had a saliva score >1. Ruminal fluid was then centrifuged at 1,512 × *g* for 15 min at 5°C and stored at –20°C for VFA, ammonia, lactate, and histamine analysis.

Blood samples were taken via jugular venipuncture using blood collection tubes containing lithium heparin (BD Vacutainer, Plymouth, Devon, UK), immediately after the first and last ruminal fluid sample collections from each heifer. Blood samples were centrifuged at 1,512 × *g* for 15 min at 5°C and plasma was decanted and stored at –20°C for L-lactate, D-lactate, and histamine analysis.

Laboratory Analysis

Ruminal fluid samples were analyzed for pH immediately after collection using a pH meter (Merck Pty Ltd., Kilsyth, VIC, Australia) and fermentation products, following storage at –20°C within 4 wk of collection. Ammonia (catalog no. 11 112 732 035; Arrow Scientific, Lane Cove, NSW, Australia) and D- and L-lactate concentrations in ruminal fluid and L-lactate concentrations in plasma were analyzed using a Boehringer Mannheim kit (catalog no. 11 112 821 035; Arrow Scientific) and spectroscopy. Volatile fatty acid concentrations were analyzed by an Agilent series gas chromatograph with HP6890 injection, 30 mm × 0.53 mm × 1.0 μm capillary column (Agilent Technologies,

Inc., Wilmington, DE) and Chemstation software (Agilent Technologies, Inc.) based on methodology from Supelco Inc. (1975).

Ruminal and plasma histamine concentrations were analyzed using a human histamine ELISA kit (IBL International, Hamburg, Germany) according to the manufacturer's instructions for human plasma samples. Ruminal fluid was passed through a 0.22-μm filter before analysis. The kit was validated for bovine ruminal and plasma histamine by Rabiee et al. (2009). The validation process included examination of the parallelism and fitted regression between a human plasma histamine standard reference curve and a bovine ruminal histamine curve [parallelism: $y = -0.2983\text{Ln}(x) + 1.2106$; $R^2 = 0.959$; fitted line: $y = -0.2628\text{Ln}(x) + 1.7003$; $R^2 = 0.99$] and a serially diluted bovine plasma curve [parallelism: $y = -0.3387\text{Ln}(x) + 1.9724$; $R^2 = 0.964$; fitted line: $y = -0.274\text{Ln}(x) + 1.4775$; $R^2 = 0.99$]. The results of both bovine ruminal and plasma histamine curves were in agreement with the human plasma histamine standard curve (Rabiee et al., 2009).

Locomotion Scoring

Heifers were locomotion scored during the adaptation period, 2 d postchallenge, and 1wk after the final day of challenge using the 5-point scoring system developed by Sprecher et al. (1997). The locomotion scoring was conducted by 2 of the study investigators while heifers were individually walked on a concrete surface.

Statistical Analysis

The raw means and standard deviations for the ruminal and plasma variables of the 5 treatment groups are presented in Table 3. To obtain the least squares means, standard error of the means, main effects of grain, fructose nested within grain (fructose), histidine nested within grain (histidine), and time and the interactions with time, data from the 5 treatment groups were merged into a factorial arrangement and analyzed using a repeated-measures generalized estimating equations PROC MIXED model in SAS (version 9.2, SAS Institute Inc., Cary, NC). Grain was used as a base substrate for all treatment groups with the exception of the control group.

The model used was:

$$Y_{ijk} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + R\varepsilon_{ijk,}$$

where Y_{ijk} = response of treatment group i (i = 1 to 6) at time j (j = 0 to 4) by heifer k (k = 1 to 30); μ = mean effect of treatment group; β_i = effect of treatment group; γ_j = effect of time j; $(\beta\gamma)_{ij}$ = effect of treatment group by time interaction; $R\varepsilon_{ijk}$ = random residual error adjusted for repeated measurements within heifer k at time j at treatment i using a first-order autoregressive correlation pattern (**AR1**) in PROC GENMOD (SAS Institute Inc.). This procedure uses the sandwich estimator in a marginal model (generalized estimating equations; Diggle et al., 2002).

The variables D- and L-lactate, butyrate, caproate, plasma histamine, and plasma L-lactate were transformed using a natural logarithm in SAS to achieve a normal distribution of residuals. A residual analysis was performed for each response variable, testing for the distributional assumption, homogeneity of the variance, and influential observations using residual and deviance plots. The random effect of day was included in the original model but did not approach significance for any variable and was consequently eliminated from the model.

A correlation was performed using PROC MIXED in SAS (Roy, 2006) to determine the relationship between ruminal and plasma L-lactate and histamine concentrations, regardless of treatment groups.

The acidosis category of heifers was defined according to the methods of Bramley et al. (2008). Briefly, a discriminate analysis was conducted on standardized variates of the following variables: ruminal pH, acetate, propionate, butyrate, isobutyrate, isovalerate, caproate, D-lactate, and ammonia based on the 3 K-Means Cluster acidosis categories defined by Bramley et al. (2008; PASW Statistics 18, SPSS Inc., Chicago, IL).

RESULTS

Heifers exhibited no visible signs of clinical ruminal acidosis during or after the experimental periods. No signs of lameness or laminitis were observed during the course of locomotion scoring. Only 2 out of 150 ruminal samples contained saliva contamination and scored above zero. Heifers in the 2 nonfructose groups consumed 99.5% of the allocated grain in a mean time of 28 ± 5 min. Fructose-fed heifers consumed 75.7% ± 5.3 of allocated grain and 74% ± 8.5 of fructose in a mean time of 65 ± 4.4 min.

The least squares means and standard error of the means, main effects of grain, fructose nested within grain (fructose), histidine nested within grain (histidine), and time, and the interactions with time for the ruminal and plasma measures are presented in Table 4. The main effects and interactions are displayed in Table 5.

Ruminal Results

The grain and fructose consumed by the heifers increased ruminal total VFA concentrations. The concentrations of VFA increased over the sampling period in grain-fed groups (Table 5; Figure 1A).

Ruminal acetate concentrations were higher in grain-fed heifers compared to control heifers. Ruminal acetate concentrations increased in fructose groups; however, the inclusion of histidine had no effect on the ruminal concentration of acetate (Tables 4 and 5). The effect of time alone on acetate concentrations was not significant; however, ruminal concentrations of acetate increased over the sampling period in the grain-fed groups (Table 4 and 5; Figure 1B).

Table 3. Raw mean concentrations (±SD) of ruminal and plasma measures for each treatment group

Item	Group[1]					SD
	Control	GR	GR + FR	GR + FR	GR + FR + HIS	
No. of heifers	6	6	6	6	6	
Ruminal (m*M*)						
Total VFA	63.74	91.72	101.72	87.63	102.36	25.07
Acetate	44.07	59.63	63.31	56.97	64.82	14.60
Butyrate	6.58	10.15	17.44	10.23	15.58	5.75
Isobutyrate	1.21	1.39	1.08	1.46	1.18	0.30
Propionate	8.73	15.49	15.78	13.77	16.05	4.70
Caproate	0.21	0.46	0.51	0.38	0.59	0.25
Valerate	0.98	2.18	1.73	2.19	2.01	0.94
Isovalerate	1.95	2.42	1.87	2.64	2.13	0.58
D-lactate	0.18	0.16	11.03	0.19	7.28	3.77
L-lactate	0.07	0.09	5.78	0.09	4.05	3.87
Ammonia	8.28	12.87	11.03	15.19	10.13	5.42
Histamine (ng/mL)	61.33	103.19	107.73	132.55	114.60	58.54
pH	7.14	6.89	6.44	6.94	6.54	0.41
Plasma						
L-lactate (m*M*)	1.44	1.23	1.28	1.42	1.34	0.63
Histamine (ng/mL)	0.25	0.29	0.26	0.46	0.35	0.20

[1]Control (no grain); GR = (crushed triticale 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = Grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head).

Table 4. Least squares means concentrations (±SEM) of ruminal and plasma measures obtained from a merged factorial generalized linear model

Item	Group			
	Control	Grain	Fructose[1]	Histidine[2]
No. of heifers	6	24	12	12
Ruminal (m*M*)				
Total VFA	63.74 ± 5.10	95.86 ± 2.55	102.04 ± 3.60	95.00 ± 3.60
Acetate	44.07 ± 3.03	61.18 ± 1.52	64.07 ± 2.15	60.90 ± 2.15
Ln Butyrate[3]	1.83 ± 0.11	2.50 ± 0.05	2.75 ± 0.08	2.50 ± 0.08
Isobutyrate	1.21 ± 0.07	1.28 ± 0.04	1.13 ± 0.05	1.32 ± 0.05
Propionate	8.73 ± 0.89	15.27 ± 0.45	15.91 ± 0.63	14.91 ± 0.63
Ln Caproate[3]	-1.69 ± 0.30	-0.90 ± 0.15	-0.65 ± 0.21	-0.79 ± 0.21
Valerate	0.98 ± 0.15	2.03 ± 0.07	1.87 ± 0.10	2.10 ± 0.10
Isovalerate	1.95 ± 0.13	2.26 ± 0.07	2.00 ± 0.94	2.38 ± 0.09
Ln D-lactate[3]	-2.13 ± 0.49	-0.59 ± 0.25	0.97 ± 0.35	-0.74 ± 0.35
Ln L-lactate[3]	-2.87 ± 0.43	-1.32 ± 0.30	0.23 ± 0.43	-1.47 ± 0.43
Ammonia	8.28 ± 1.04	12.30 ± 0.52	10.58 ± 0.74	12.66 ± 0.74
Histamine (ng/mL)	61.33 ± 17.92	114.52 ± 8.96	111.16 ± 12.67	123.57 ± 12.67
pH	7.14 ± 0.87	6.70 ± 0.04	6.49 ± 0.06	6.74 ± 0.06
Plasma				
Ln L-lactate (m*M*)[3]	0.16 ± 0.11	0.22 ± 0.06	0.21 ± 0.08	0.26 ± 0.08
Ln Histamine (ng/mL)[3]	-1.55 ± 0.16	-1.25 ± 0.08	-1.30 ± 0.11	-1.07 ± 0.11

VFA = volatile fatty acids; Ln = natural logarithm.

[1]Fructose is nested within grain.

[2]Histidine is nested within grain.

[3]Exponentiated least squares means for the 4 groups, respectively: butyrate: 6.23, 12.18, 15.64, 12.18; caproate: 0.18, 0.41, 0.52, 0.45; D-lactate: 0.12, 0.55, 2.64, 0.48; L-lactate: 0.06, 0.27, 1.24, 0.23; plasma L-lactate: 1.17, 1.25, 1.23, 1.30; plasma histamine: 0.21, 0.29, 0.27, 0.34.

Table 5. The significance of main effects and interactions (*P*-values) with time for ruminal and plasma measures obtained from a merged factorial generalized linear model

Item	Main effects				Interactions		
	Grain (G)	Fructose (F)[1]	Histidine (H)[2]	Time (T)	G × T	F × T	H × T
Ruminal							
Total VFA	0.001	0.021	0.734	0.218	0.006	0.236	0.145
Acetate	0.001	0.065	0.849	0.192	0.013	0.129	0.187
Ln Butyrate	0.007	<0.001	0.972	0.333	0.014	0.354	0.303
Iso-butyrate	0.082	<0.001	0.251	0.402	0.035	0.160	0.074
Propionate	<0.001	0.162	0.428	0.079	<0.001	0.208	0.051
Ln Caproate	0.267	0.111	0.494	0.018	0.317	0.623	0.659
Valerate	<0.001	0.042	0.339	<0.001	<0.001	0.078	0.113
Iso-valerate	0.013	<0.001	0.083	0.512	0.011	0.132	0.004
Ln D-lactate	0.835	<0.001	0.548	<0.001	0.869	0.090	0.442
Ln L-lactate	0.768	<0.001	0.622	0.032	0.891	0.273	0.419
Ammonia	0.001	0.003	0.510	<0.001	0.233	0.062	0.918
Histamine	0.054	0.709	0.318	<0.001	0.061	0.868	0.128
pH	0.030	<0.001	0.389	0.298	0.220	0.686	0.109
Plasma							
Ln L-lactate	0.856	0.880	0.541	0.001	0.030	0.582	0.665
Ln Histamine	0.550	0.649	0.104	0.003	0.125	0.193	0.009

VFA = volatile fatty acids; Ln = natural logarithm.
[1]Frutose is nested within grain.
[2]Histidine is nested within grain.

Ruminal butyrate concentrations were higher in all grain-fed heifers compared with controls, with the highest concentrations being observed in the fructose groups. Histidine had no effect on butyrate concentrations. Butyrate concentrations increased over the sampling period in the grain-fed groups (Table 4; Figure 1C).

Ruminal concentrations of isobutyrate were lower in the fructose-fed heifers compared with those receiving no fructose. Isobutyrate concentrations increased over the sampling period in the grain-fed groups (Table 5).

Ruminal concentrations of propionate were markedly higher in the grain-fed heifers compared to the control heifers (Tables 4 and 5). Fructose and histidine supplementation did not affect concentrations of propionate. Ruminal concentrations of propionate increased over the sampling period in the grain- and histidine-fed groups (Table 5; Figure 1D).

The main effects of grain, fructose, and histidine did not affect ruminal caproate concentrations. Ruminal caproate concentrations increased over the sampling period (Tables 4 and 5); however, there were no significant interactions observed among grain, fructose, or histidine treatments by time (Table 5).

Ruminal concentrations of valerate increased approximately 2-fold in the grain compared to the control group. Fructose decreased ruminal valerate concentrations. Ruminal concentrations of valerate increased over the sampling period in the grain groups, but declined for the control (Table 5; Figure 1E).

Ruminal concentrations of isovalerate were higher in the grain and fructose groups. Isovalerate concentrations in the rumen increased over the sampling period in the grain and histidine groups (Table 4 and 5).

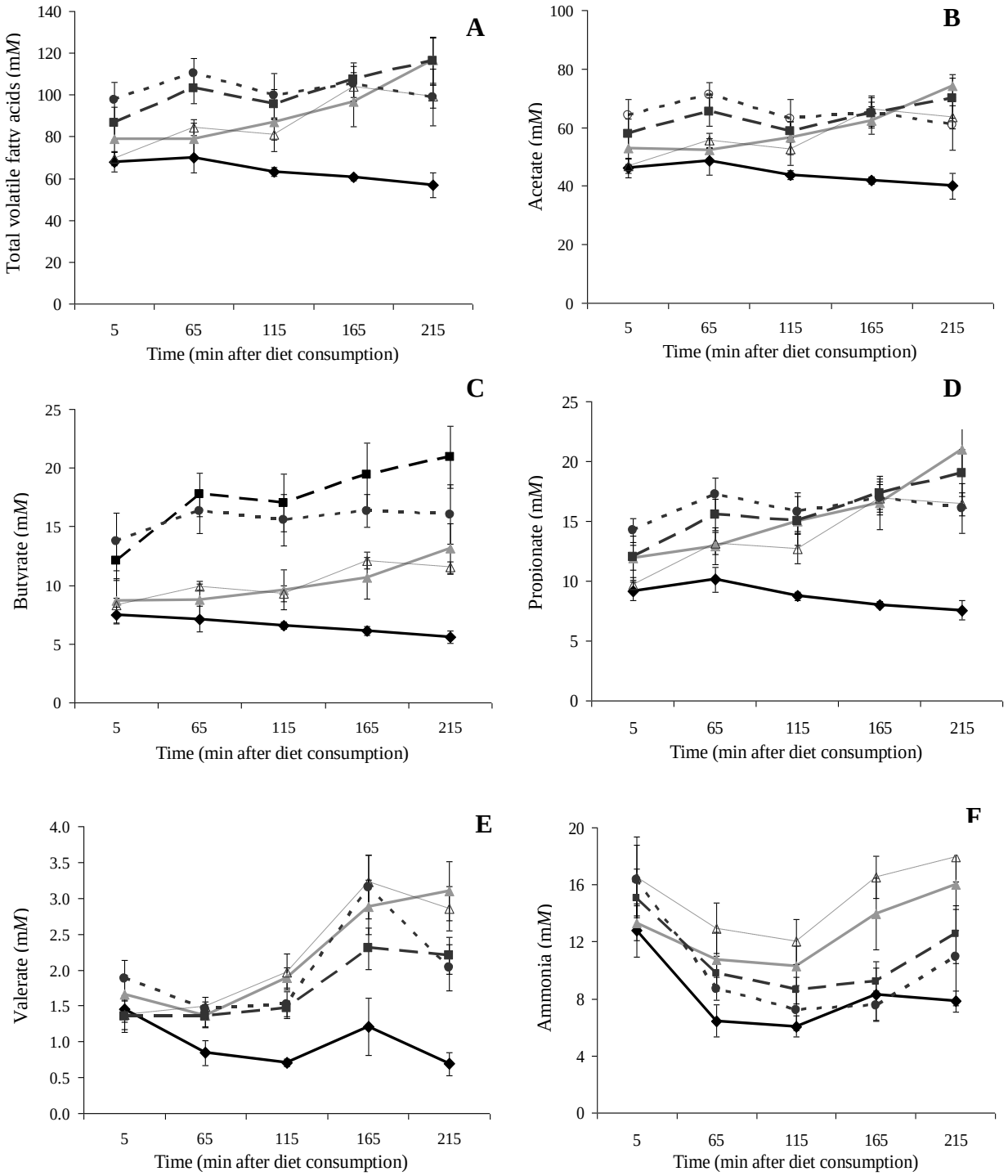

Figure 1. Concentrations of (A) total VFA, (B) acetate, (C) butyrate, (D) propionate, (E) valerate, (F) ammonia, (G) D-lactate, (H) L-lactate, and (I) histamine, and (J) pH in the 5 treatment groups. All values are means ± SEM from ruminal fluid taken at 5, 65, 115, 165, and 215 min after completion of treatment consumption. GR = (crushed triticale at 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head); (n = 6 heifers/group).

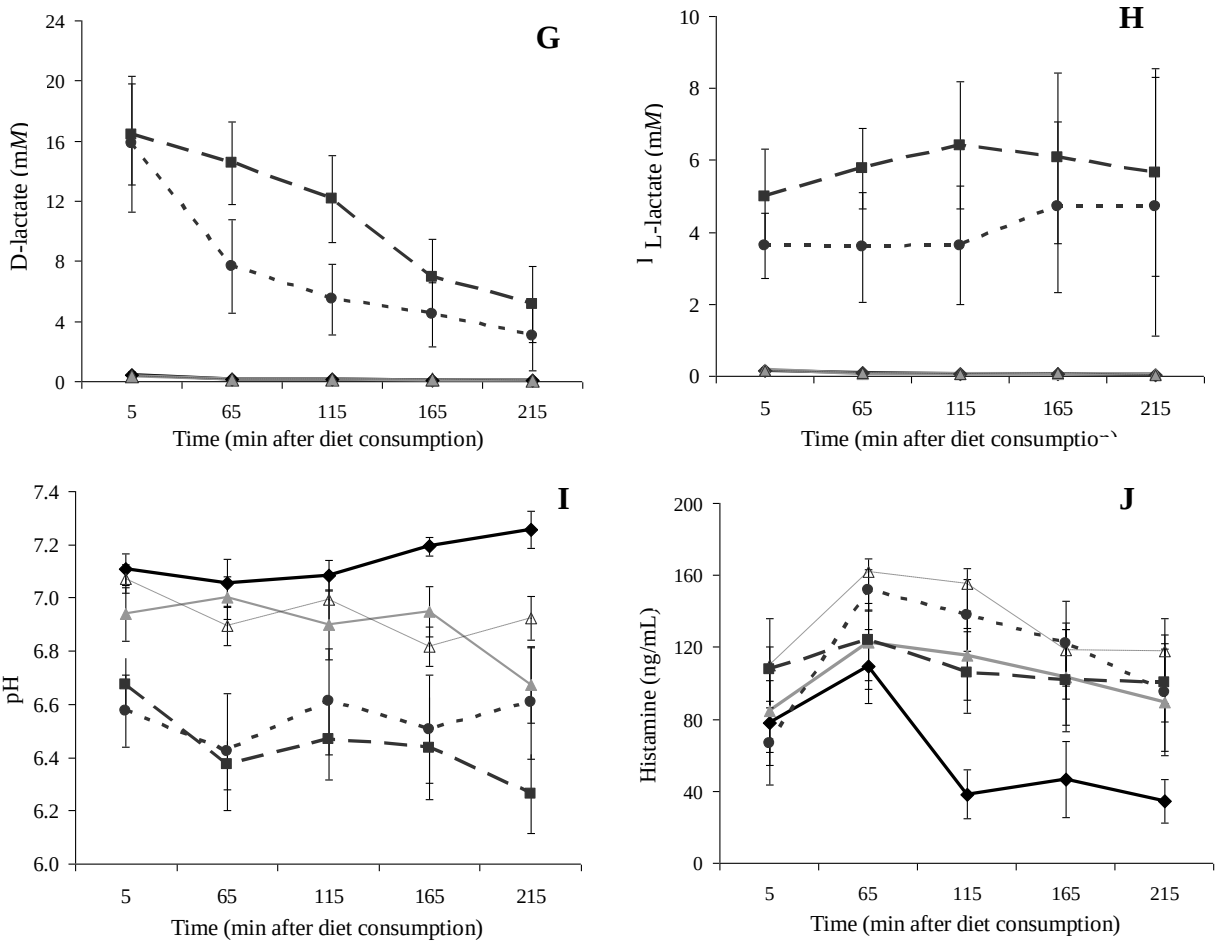

Figure 1 (continued). Concentrations of (A) total VFA, (B) acetate, (C) butyrate, (D) propionate, (E) valerate, (F) ammonia, (G) D-lactate, (H) L-lactate, and (I) histamine, and (J) pH in the 5 treatment groups. All values are means ± SEM from ruminal fluid taken at 5, 65, 115, 165, and 215 min after completion of treatment consumption. GR = (crushed triticale at 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head); (n = 6 heifers/group).

Feeding grain had no significant effect on ruminal D- and L-lactate concentrations compared with control heifers (Tables 4 and 5). Ruminal concentrations of D- and L-lactate were markedly increased in the fructose groups. The average concentrations of D- and L-lactate in the fructose groups increased by 22- and 21-fold, respectively, compared with the mean concentrations for the non-fructose-fed groups (data not shown). In the grain + fructose + histidine group, ruminal concentrations of D-lactate peaked at the 5-min sampling at 16.42 m*M* (Figure 1G), whereas ruminal concentrations of L-lactate peaked at the 115-min sampling at 6.41 m*M* (Figure 1H). A significant decline occurred in ruminal concentrations of D-lactate over the sampling period (Figure 1G), whereas ruminal L-lactate concentrations increased over the sampling period (Table 5; Figure 1H).

Ruminal ammonia concentrations were increased in grain-fed heifers and decreased in the fructose-fed heifers (Tables 4). Ruminal concentrations of ammonia gradually declined until the 115-min sampling. This was followed by a gradual increase throughout the remaining sampling period (Table 5; Figure 1F).

Ruminal histamine concentrations were not significantly affected by treatment groups; however, the ruminal concentrations of histamine were higher in all grain-fed heifers (Table 4 and 5; Figure 1I). Ruminal concentrations of histamine increased up to the 65-min sampling in all treatment groups and then subsequently declined.

Ruminal pH was lower in the grain- and fructose-fed groups; however, the decline in pH was more pronounced in the fructose-fed heifers. The effects of time and time by treatment group interactions on ruminal pH were not significant (Table 5; Figure 1J).

Plasma Results

Plasma concentrations of D-lactate were below the minimum detection limit during preliminary analysis; consequently, we were unable to measure and analyze the D-lactate data. Plasma concentrations of L-lactate were not affected by treatment group (Table 5). Plasma L-lactate concentrations decreased over the sampling period with a higher concentration in the control group at the 5-min sampling period (Table 5; Figure 2A).

Plasma histamine concentrations were not affected by treatment groups (Table 5). Plasma histamine concentrations decreased over the sampling period, and plasma histamine declined over the sampling period in the histidine groups (Table 5; Figure 2B).

No significant correlation was observed between ruminal and plasma concentrations of L-lactate ($r = 0.009$) or histamine ($r = –0.141$), regardless of treatment group.

Results of cluster and discriminate analyses, based on Bramley et al. (2008), showed that all heifers enrolled in this study could be classified as cattle with normal rumen function, except one, which was classified with suboptimal rumen function.

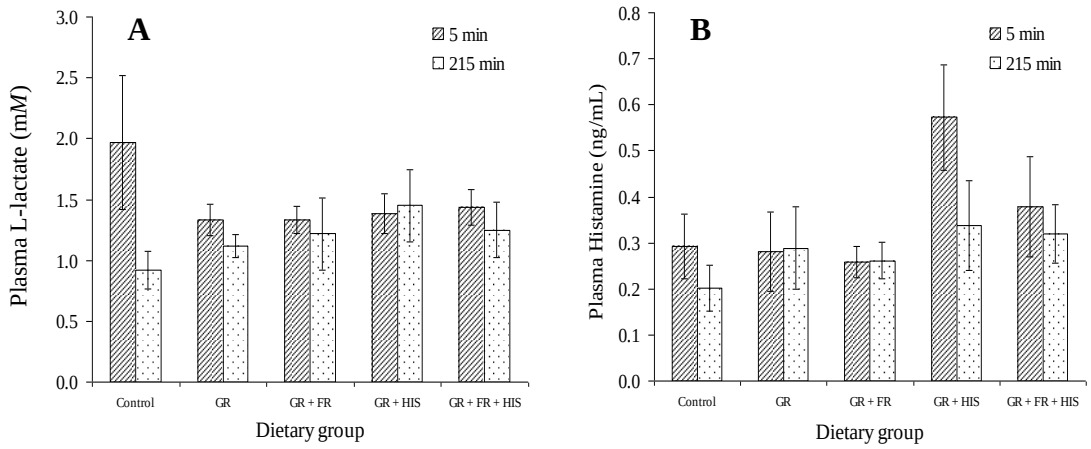

Figure 2. Concentrations of plasma (A) L-lactate and (B) histamine in the 5 treatment groups. All values are means ± SEM from plasma taken at 5 and 215 min after completion of treatment consumption. GR = (crushed triticale at 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head); (n = 6 heifers/group).

DISCUSSION

We hypothesized that increasing the grain and fructose contents of diets and oral administrations of histidine may contribute to the onset of ruminal acidosis, altering ruminal pH, and ruminal measures of histamine, VFA, and lactate in dairy heifers fed a single grain-based challenge. The ruminal acidosis challenge model in this study was capable of decreasing ruminal pH and modifying ruminal fermentation measures in all treatment heifers compared with control (no grain) heifers; however, the level of rumen modification may not have been enough to induce ruminal acidosis and did not distinguish between control and treatment heifers according to the Bramley et al. (2008) model. The starch content of the triticale cultivar 'Berkshire' fed in this study was not as high as predicted or as high as that of triticale cultivars used in previous studies (Lean and Rabiee 2009b). Consequently, the effect of grain on ruminal fermentation may have been less than predicted from previous studies (Lean and Rabiee, 2009b).

Ruminal and metabolic acidosis was induced by Thoefner et al. (2004) and Danscher et al. (2009, 2010) in dairy heifers of comparable age and weight to those of the current study by orally drenching with oligofructose at 13, 17, or 21 g/kg of BW (~ 0.13, 0.17 and 0.21% of BW). The marked difference in acidosis induction between these studies, in which only approximately half the concentration of sugar and no grain were administered compared with the current study, could be the result of differences in oligofructose and free fructose fermentation, or from exposure to sugar before challenge. Oligofructose consists of fructose units linked by β (2 to 1) bonds, and additional degradation is required compared with the fructose used in our study. In theory, this difference in chemical structure should have induced more severe fermentation changes in the current study than in those of Thoefner et al. (2004) and Danscher et al. (2009, 2010). Those authors drenched their cattle with 5% of the challenge dose of oligofructose twice daily for 3 d before the main challenge. Consequently, the ruminal microflora had an opportunity to adapt to the presence of oligofructose. This may have increased the risk of acidosis compared to our study, in which the cattle were not adapted to large amounts of fructose before challenge. Interestingly, these cattle did not consume all the fructose on offer and consumed this ration less rapidly than groups without fructose. This observation suggests a hypothesis

that cattle may control the risk of acidosis by controlling rates of consumption of certain feeds.

Ruminal pH in this trial was relatively high throughout the experimental period. Although pH is often used as a defining measure for ruminal acidosis (Plaizier et al., 2008), no consistently defined pH range exists for ruminal acidosis (Khafipour et al., 2009). The development of ruminal acidosis reflects the movement and concentration of hydrogen ions within the ruminal ecosystem as they are released from precursor pools in feed to produce VFA, lactate, microbial proteins, and waste gases. Volatile fatty acids are one of the major ruminal fermentation products and hydrogen sinks. The general increase in total VFA and individual VFA in all treatment groups compared with the control indicates that microbial fermentation of the diets high in starch and fructose was occurring as expected and is consistent with that of Heldt et al. (1999).

The observed increase in ruminal acetate, butyrate, and propionate concentrations associated with starch feeding in our study were as expected. Ruminal valerate concentrations were increased in all treatment heifers compared with control heifers. Elevated ruminal valerate concentrations, possibly produced from lactate by *M. elsdenii* (Hungate, 1966; Stewart et al., 1997), have been associated with ruminal acidosis (Bramley et al., 2008; Lean and Rabiee, 2009a).

Dietary sugar additions can increase the ruminal concentrations of butyrate and valerate (Heldt et al., 1999; DeFrain et al., 2004). However, other studies reported no significant differences in these VFA when sugars were fed (Oelker et al., 2009). In our study, butyrate concentrations were increased in the fructose-fed heifers, whereas valerate concentrations were lower in the control heifers compared with heifers in the remaining treatment groups. The decrease in valerate concentrations with fructose addition to grain appears anomalous given the higher lactate concentrations, suggesting that the removal of lactate through valerate production needs further examination. The lack of effect of fructose on propionate suggests propionate production may be a more dominant fermentation pathway in grain-fed cattle. Differences in the microbial species responsible for the fermentation of starches and sugars are a likely cause of the difference in these fermentation end products and are the focus of future work by our group.

Fructose feeding increased ruminal lactate concentrations; however, these concentrations were not reflected in clinical signs of ruminal acidosis. Marked increases in ruminal lactate concentrations above 40 m*M* are generally only associated with acute ruminal acidosis (Owens et al., 1998), whereas ruminal lactate concentrations during induction protocols for SARA, or those identified in field studies, do not generally exceed 5 m*M* (Nagaraja and Titgemeyer, 2007; Bramley et al., 2008; Lean and Rabiee, 2009a,b). The high ruminal lactate concentrations in the fructose-fed heifers may result from fermentation by *Streptococcus bovis* (Hungate, 1966) and other microbes. The low plasma L-lactate concentrations suggest ruminal L-lactate was not readily absorbed into the bloodstream, which is expected at the observed ruminal pH values and the relatively low pK_a (logarithmic acid dissociation constant) of L-lactate. Interestingly, the ruminal lactate degradation products, propionate and valerate (Stewart et al., 1997), did not increase in the fructose groups. Other studies also found that sugars produce greater concentrations of lactic acid than starch (Harmon et al., 1985; Heldt et al., 1999), and Giesecke and Stangassinger (1976) reported generation of ruminal D- and L-lactate within the first 15 to 20 min of sugar consumption. In our study, ruminal D-lactate was the dominant isomer. This finding may be a consequence of the slower metabolism of D-lactate in the rumen (Harmon et al., 1985).

The lack of presentation of clinical signs of ruminal acidosis in the fructose-fed heifers despite the large increase in ruminal lactate concentrations and the study of Bramley et al. (2008), in which pH and lactate were not the most critical determinants of ruminal acidosis, suggest that lactate concentrations are not a major determinant of the clinical expression of ruminal acidosis.

The increase in ruminal ammonia as observed in the grain-fed heifers in this study was expected and reflects an increase in protein consumed. The fructose effect may have resulted from the lower nitrogen intake and increased incorporation of ammonia into microbial protein. Alternatively, the fructose effect may result, in part, from an increased rate of metabolism of fructose compared with starch (Firkins, 2011), resulting in more energy being immediately available for microbial proteolysis and subsequent microbial protein synthesis. Decreased ruminal concentrations of ammonia have been reported by Broderick et al. (2008) in dairy cows fed 7.5% sucrose compared with those fed 7.5% starch.

Declines in ruminal pH resulting from high starch diets such as those in this study are well documented (Emmanuel et al., 2008). Studies examining the role of sugars in ruminal fermentation have reported declines in ruminal pH (Thoefner et al., 2004; Danscher et al., 2010) or no effect on ruminal pH (Broderick et al., 2008; Oelker et al., 2009). The decline in pH in the fructose-fed heifers in this study was expected due to the relatively high amount of fructose fed (0.4% BW and approximately 33% of DMI) compared with other sugar studies.

Ruminal pH responses to soluble carbohydrate supplementation have been highly variable. Marked decreases in ruminal pH were observed in dairy cattle administered 13 to 21 g/kg of BW of oligofructose (Thoefner et al., 2004; Danscher et al., 2010) and for cattle fed purified glucose at 20.6% of DM compared with cattle fed fiber and starch at 20.6% of DM (Hristov et al., 2005). Heldt et al. (1999) found that fructose, glucose, and sucrose fed at 0.3% BW of DMI produced rapid declines in pH 3 h after supplementation compared with declines after 9 h in starch-fed steers. No significant decreases in ruminal pH were observed when sucrose (Broderick et al., 2008), molasses (Oelker et al., 2009), or whey were administered (DeFrain et al., 2004). In contrast, Penner et al. (2009) observed increases in mean ruminal pH in cattle fed 5.7% compared with 2.8% DMI sucrose.

Notwithstanding differences in consumption times between heifers in fructose and nonfructose groups, Figure 1 suggests that fructose was more rapidly fermented than the grain. We hypothesize that larger differences in fermentation products and fermentation patterns would have occurred if heifers had consumed the entire fructose dose offered. Sugars are more rapidly metabolized in the rumen than are starches (Firkins, 2011). The fructose was anticipated to produce earlier peaks in fermentation product concentrations and have different fermentation measures over time compared with the other groups.

No studies have fed or infused histidine into the rumen; Vanhatalo et al. (1999), Korhonen et al. (2000), and Huhtanen et al. (2002) investigated histidine infusion of 0 to 6.5 g/d into the abomasum or duodenum. Histidine addition in the current study did not have a significant main effect on any of the measures analyzed, including histamine concentration (Table 5). It can be hypothesized that although histidine was drenched at 160% of daily requirement, the 6 g drenched does not equate to the challenge represented by concentrations in pasture. Reeves et al. (1996) found the histidine

concentration in ryegrass was 2.8 g/kg of DM; hence 44.8 g/d would be consumed by a cow eating 16 kg/d of DM. Drenching with more histidine might have a greater influence on ruminal fermentation. Despite the lack of significant effect, histidine may still be utilized for microbial growth and is the sole substrate for *A. histaminiformans* (Garner et al., 2002). Although the clearance rates of histamine were not measured in this study, the grain effect may support findings that histamine concentrations might be associated with ruminal acidosis (Dain et al., 1955; Ahrens, 1967). The low correlation between ruminal and plasma histamine for all groups supports findings that ruminal histamine is not absorbed across the rumen epithelial wall (Fuquay et al., 1969). Histamine is a basic compound and at low pH the majority of histamine is in the dissociated form; hence, epithelial absorption is impaired (Brent, 1976). However, epithelial damage resulting from ruminal acidosis can increase the permeability of rumen epithelia, thus increasing histamine absorption (Aschenbach and Gabel, 2000). Motoi et al. (1984) reported a positive relationship between ruminal and plasma histamine concentrations in concentrate-fed cattle. The association between increased urinary excretion of histamine and increased dietary histamine concentrations demonstrated by Wrenn et al. (1964) suggests that histamine is absorbed from the gastrointestinal tract. Studies using labeled histidine may clarify the question of absorption of histamine from the rumen and gastrointestinal tract.

We hypothesize that the moderate ruminal acidosis challenge and the relatively high pH observed in this study may have limited absorption of histamine. An assessment of the extent of epithelial damage may increase our understanding of histamine absorption during challenge protocols and help clarify the role of histamine in ruminal acidosis. Elevated endotoxin concentrations reported in dairy cattle with grain-induced SARA are thought to be involved in SARA (Gozho et al., 2005; Khafipour et al., 2009). An evaluation of endotoxin concentrations in ruminal samples from our study is in progress to provide a more complete evaluation of the effects of ruminal acidosis on cattle and the rumen. Our results, showing increased ruminal concentrations of histamine in feeding, suggest a need to continue to examine the role of histamine and histidine in the pathogenesis of ruminal acidosis and associated inflammatory conditions.

Ruminal acidosis provides a major challenge for ruminant production. This study is the first to differentiate responses in ruminal VFA, ammonia, lactate, histamine, and pH

between fructose and grain and the first to examine the effects of added histidine on rumen function.

CONCLUSIONS

The substitution of fructose at 0.4% of BW for grain had marked effects on ruminal fermentation products, particularly lactate concentrations that were increased, in this ruminal acidosis challenge study. Heifers exposed to grain had increased production of VFA, including acetate, butyrate, propionate, and valerate. The substitution of 0.4% BW fructose for grain resulted in marked increases in ruminal D- and L-lactate concentrations and a lower pH than when fructose was not substituted. The addition of histidine to rations did not have significant effects on ruminal fermentation, but ruminal histamine concentrations increased over time irrespective of histidine addition. The results suggest that absorption of ruminal L-lactate and histamine into blood might be limited. Implications of this study include a need to further consider the role that sugar sources, including those in forages, play in increasing ruminal lactate concentrations and the risk of ruminal acidosis. Further studies are warranted to explore interactions among dietary precursors that may influence the risk of ruminal acidosis.

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CHAPTER 3

Effects of Grain, Fructose, and Histidine Challenges on Endotoxin and Oxidative Stress Measures in Dairy Heifers

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OVERVIEW OF CHAPTER 3

Chapter 3 presents findings from a companion study to Chapters 2 and 4. The findings of Chapter 2 showed a single challenge feed of combinations of grain, fructose, and the amino acid, histidine produced different ruminal fermentation measures and a 21- and 22-fold increase in D- and L-lactate occurred in the fructose-fed cattle. Thus, these findings led to the hypothesis that grain would increase endotoxin concentrations and induce oxidative stress responses as a result of increased volatile fatty acids (**VFA**) and decreased ruminal pH compared with heifers fed no grain. Fructose was hypothesized to induce greater concentrations of endotoxin and more pronounced oxidative stress responses than grain because of more rapid fermentation rates and observed higher ruminal total VFA, butyrate, and lactate concentrations than induced by grain alone. Histidine was hypothesized to have no effect on endotoxin and oxidative stress responses because no main effects on ruminal fermentation measures were observed from histidine addition in Chapter 2.

ABSTRACT

Ruminal endotoxin and plasma oxidative stress biomarker concentrations were studied in dairy heifers challenged with combinations of grain, fructose, and histidine in a partial factorial study. Holstein-Friesian heifers (n = 30) were randomly allocated to 5 treatment groups: (1) control (no grain); (2) grain [crushed triticale at 1.2% of bodyweight (**BW**) dry matter intake (**DMI**)]; (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); (4) grain (1.2% of BW DMI) + histidine (6 g/head); and (5) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). Ruminal samples were collected by stomach tube 5, 65, 115, 165, and 215 min after ration consumption and blood samples at 5 and 215 min after consumption. Ruminal fluid was analyzed for endotoxin concentrations. Plasma was analyzed for concentrations of the following oxidative stress biomarkers: reactive oxygen metabolites (**dROM**), biological antioxidant potential (**BAP**), advanced oxidation protein products, and ceruloplasmin, and activity of glutathione peroxidase. Treatment group had no effect on concentrations of endotoxin and oxidative stress biomarkers. We observed no interactions of treatment × time. Ruminal concentrations of endotoxin decreased during the sampling period from $1.12 \times 10^5 \pm 0.06$ to 0.92×10^5 endotoxin units/mL ± 0.05 (5 and 215 min after ration consumption, respectively). Concentrations of dROM and the oxidative stress index [(dROM/BAP) × 100] increased over the sampling period, from 108.7 to 123.5 Carratelli units (**Carr U**), and from 4.1 to 4.8, respectively. Ceruloplasmin concentrations markedly declined 5 min after the consumption of rations, from 190 to 90 mg/L over the 215-min sampling period. Overall, a single feeding challenge for dairy cattle with combinations of grain, fructose, and histidine may not be sufficient to induce marked changes in endotoxin or oxidative stress biomarker concentrations.

Key words: endotoxin, fructose, histidine, oxidative stress

INTRODUCTION

Cattle are at an increased risk of ruminal acidosis during the transition period because they are often exposed to abrupt increases in rapidly fermentable carbohydrates (Penner et al., 2007) following reduced dry matter intake (**DMI**) before parturition (Hayirli et al., 2003). Similarly, abrupt exposures can occur in beef cattle fed feedlot diets. Without a gradual introduction, and sufficient effective fiber, carbohydrates cause organic acids to accumulate in the rumen and exceed its buffering capacity, resulting in ruminal acidosis (Nagaraja and Titgemeyer, 2007; RAGFAR, 2007; Plaizier et al., 2008). Ruminal acidosis is a complex nutritional disorder ranging in severity from the peracute form, resulting in death, to relatively mild forms where symptoms can be subclinical. Measures of ruminal pH are often used for diagnosis, but inconsistencies in cut-off thresholds that define ruminal acidosis severity have created confusion regarding the accurate diagnosis of ruminal acidosis (Kleen et al., 2003; Nagaraja and Titgemeyer, 2007; Plaizier et al., 2008). Bramley et al. (2008) showed that ruminal valerate and propionate were the most effective ruminal fermentation measures for diagnosing ruminal acidosis, and ruminal pH and lactate were the least effective. The model of acidosis developed by Bramley et al. (2008) is the only method that defines ruminal acidosis on the basis of changes in ruminal measures and relates these to diet, health, and production. We hypothesize that ruminal acidosis occurs along a continuum of ruminal conditions ranging from clinical ruminal acidosis to normal and that testing responses of cattle to feeding challenges in this range will increase our understanding of the pathogenesis and means to control ruminal acidosis.

Despite extensive research and many detailed reviews (Owens, 1998; Krause and Oetzel., 2006; RAGFAR 2007; Enemark et al., 2008; Plaizier et al., 2008), the pathogenesis of ruminal acidosis is still not completely understood, particularly in regard to the proposed involvement of specific molecules such as endotoxins (Khafipour et al., 2009a) and histamine (Ahrens, 1967), and involvement of specific microbial species. Most research has focused on changes resulting from several days or weeks of repeated exposure to rapidly fermentable carbohydrates. Changes resulting from single exposure to readily fermentable carbohydrate in the first hours after feeding in cattle not adapted to these diets have received less attention but could provide important insights

into ruminal acidosis development, especially around parturition or introduction to feedlots.

In our previous study, we were able to show that dairy cattle fed a single grain challenge [0.8 or 1.2% of bodyweight (**BW**) DMI] after 14 h off feed had increased concentrations of ammonia, total volatile fatty acids (**VFA**), acetate, butyrate, propionate, and valerate, and decreased pH compared to cattle fed no grain (Golder et al., 2012). We also observed that dairy cattle fed 0.8% of BW DM grain and 0.4% of BW dry matter (**DM**) fructose in a single challenge after feed withholding had markedly increased ruminal lactate, total VFA, butyrate concentrations and decreased ruminal valerate concentration and pH (Golder et al., 2012). We conclude that these carbohydrate challenges, which reflect those occurring in dairy cattle during the transition period and that induced significant changes in ruminal fermentation measures, are worthy of further investigation.

Endotoxins are lipopolysaccharides released from the cell walls of gram negative bacteria during bacterial multiplication and lysis (Rietschel et al., 1994). High concentrations of endotoxin in the rumen have been implicated in contributing to the nonspecific, acute phase response during subacute ruminal acidosis (**SARA**) induced by feeding high levels of concentrate over several days (Gozho et al., 2007; Khafipour et al., 2009a). A meta-analysis has shown increased dietary concentrate content and reduced dietary neutral detergent fiber (**NDF**) content are associated with an increase in ruminal endotoxin, plasma haptoglobin, and serum amyloid A levels (Zebeli et al., 2012). Responses became linear when cattle were fed >44.1% concentrate or <39.2% NDF (Zebeli et al., 2012). Cattle in these studies were exposed to high fermentation carbohydrate diets over a series of days. High concentrations of endotoxin may be responsible for some of the observed clinical signs of ruminal acidosis, including inflammation, laminitis, and liver abscesses (Plaizier et al., 2012). Similar depressions in ruminal pH for cattle challenge-fed with alfalfa pellet and ground alfalfa to those observed in high concentrate challenges suggest that ruminal pH depressions and increased endotoxin alone do not cause an acute phase response (Plaizier et al., 2012). It is unclear whether endotoxin concentrations change during the first few hours after single exposures to rapidly fermentable carbohydrates. Increases by 6 h and peaks at 12 h have been reported in dairy cattle fed grain challenges over several days (Gozho et

al., 2006; Khafipour et al., 2009a). Translocation of free endotoxin into the bloodstream can result in the production of pro-inflammatory cytokines, reactive oxygen, and bioactive lipids (Rietschel et al., 1994). Endotoxin can cause inflammation and increased oxidative reactions. Oxidative stress occurs when the presence of pro-oxidative metabolites exceeds the capacity of antioxidants to neutralize these (Miller et al., 1993). It is a relatively new field of research in ruminants but has been implicated in numerous disease processes in cattle (Celi, 2011a). Prolonged concentrate feeding in dairy cattle increased plasma glutathione peroxidase activity and lipid peroxidation, and decreased α-tocopherol and ferric reducing ability of plasma (Wullepit et al. 2009). An increase in oxidative stress was also reported by Gabai et al. (2004) when high levels of starch were fed to dairy cows at 80 days in milk (**DIM**). Further research is needed to explore links between abruptly feeding rapidly fermentable carbohydrates, endotoxin, and oxidative stress in cattle and to determine thresholds for these changes in metabolism.

The objective of this study was to investigate the effects of grain, fructose, and histidine, and their combinations, fed to dairy heifers unadapted to these in a single challenge on ruminal endotoxin and plasma oxidative stress biomarker concentrations. We hypothesized that grain would increase endotoxin concentrations and induce oxidative stress responses as a result of increased VFA and decreased ruminal pH compared with heifers fed no grain. Fructose was hypothesized to induce greater concentrations of endotoxin and more pronounced oxidative stress responses than grain because of more rapid fermentation rates and observed higher ruminal total VFA, butyrate, and lactate concentrations than induced by grain alone. We hypothesized that histidine would have no effect on endotoxin and oxidative stress responses because no main effects on ruminal fermentation measures were observed from histidine addition (Golder et al., 2012).

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted on 30 nonpregnant Holstein-Friesian heifers <18 mo of age with a mean BW of 359.3 ± 47.3 kg at Camden, New South Wales (**NSW**), Australia. The heifers were from a commercial dairy herd, and all experimental procedures were approved by the Bovine Research Australasia Animal Ethics Committee (BRA 0609-0610). The experimental procedures of this study have previously been detailed by Golder et al. (2012).

Briefly, heifers were housed on a dry lot and were fed 1 kg (as-fed basis) of grain daily, with *ad libitum* access to ryegrass silage and alfalfa hay twice daily in individual head stanchions in a feed pad for a 10-d adaptation period before challenge day. The target feed intake during this period was 2 kg/d of an alfalfa hay, 7.2 kg/d of ryegrass silage, and 1 kg/d of triticale (as-fed basis). The estimated chemical composition of the diet (CPM Dairy Ration Analyzer; version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY) during the adaptation period was NDF = 42.3% of DM, lignin = 5.6% of DM, non-fiber carbohydrates (**NFC**) = 30.2% of DM, silage acids = 6.5% of DM, sugar = 7.0% of DM, starch = 7.7% of DM, and soluble fiber = 8.9% of DM. The chemical composition of the alfalfa hay, ryegrass silage, and triticale cultivar 'Berkshire' were analyzed by near-infrared spectroscopy (AOAC 2000) and wet chemistry by George Weston Technologies (Sydney, NSW, Australia) and Dairy One Inc. Forage Testing Laboratory (Ithaca, NY; Table 1). Wet chemistry techniques were described by Golder et al. (2012).

Treatment Groups

The heifers were randomly allocated to the following 5 treatment groups (n = 6 heifers/group) in a partial factorial arrangement: (1) control (no grain); (2) grain (1.2% of BW DMI) of crushed triticale cultivar 'Berkshire'; (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); 4. grain (1.2% of BW DMI) + histidine (6 g/head); and (5) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). The statistical power of the study was based on previous experiments conducted by our group that demonstrated significant differences in VFA using a smaller number of cattle per group than used in this study. The fructose [Melbourne Food Depot, East

Brunswick, Victoria (**VIC**), Australia] was a 99.5% pure crystalline powder and was mixed through the grain ration. The mean weight of fructose administered at a rate of 0.4% of BW (33% of DM) was 1.44 kg. This is equivalent to the amount of water-soluble carbohydrate (**WSC**) a heifer may consume over an entire day. The histidine (Merck KGaA, Darmstadt, Germany) was a L-histidine powder dissolved in 50 mL of tap water and was administered via a stomach tube immediately after consumption of 200 g of alfalfa hay. The dose rate of 6 g of histidine per head corresponds to approximately 160% of the average daily histidine requirement of 400-kg heifers as calculated in CPM Dairy V3.10.

Challenge Procedure

The challenges were conducted over 4 consecutive days, with 7 or 8 heifers randomly allocated to 1 of the 4 d with at least 1 heifer from each treatment group challenged on each day. Feed was withheld for 14 h before challenge. On the challenge day, each heifer was offered and ate 200 g of alfalfa hay to reduce saliva contamination of the ruminal samples. Immediately after the hay was consumed, their allocated treatment rations were offered, except for the control group, which received no further ration. Heifers in the 2 nonfructose groups consumed 99.5% of the allocated grain, in a mean time of 28 min ± 5. Fructose-fed heifers consumed 75.7% ± 5.3 of allocated grain and 74% ± 8.5 of fructose in a mean time of 65 min ± 4.4 (Golder et al., 2012). Heifers exhibited no visible signs of clinical ruminal acidosis or lameness during and after the experimental periods.

Sampling Procedure

Ruminal fluid samples were collected 5, 65, 115, 165, and 215 min after consumption of the challenge ration via a stomach tube and custom-designed stomach pump. Ruminal fluid was scored for saliva contamination as described by Bramley et al. (2008) using a 1- to 3-point scoring system (3 being highest level of contamination). No ruminal samples retained for analysis had a saliva score >1. Ruminal fluid was then centrifuged at 1,512 × *g* for 15 min at 5^{o}C, and stored at –20^{o}C for endotoxin analysis.

Blood samples were taken via jugular venipuncture immediately after the 5 and 215 min ruminal fluid collections in heparinized blood collection tubes (BD Vacutainer, Plymouth, Devon, UK). Blood samples were centrifuged at 1,512 × *g* for 15 min at 5^{o}C,

and plasma was stored at –20°C for the laboratory analysis of derivatives of reactive oxygen metabolites (**dROM**), biological antioxidant potential (**BAP**), advanced oxidation protein products (**AOPP**), glutathione peroxidase (**GSH-Px**), and ceruloplasmin.

Table 1. Chemical composition of alfalfa hay and ryegrass silage fed during the adaptation period, and triticale cultivar 'Berkshire' fed during the adaptation and challenge periods[1]

Item (% of DM)	Feed		
	Alfalfa hay	Ryegrass silage	Triticale
DM	87.7	76.2	88.8
CP	20.7	17.7	16.7
Soluble protein (% of CP)	43	40.5	26.5
Crude fat	2.5	2.6	1.5
Ash	9.0	10.4	2.4
Lignin	6.8	6.5	2.3
ADF	33.6	35.6	5.3
NDF	45.9	52.3	22.1
ADICP	1.2	1.5	0.3
NDICP	3.5	5.6	2.9
NFC[2]	25.5	22.8	60.0
Available protein	19.5	16.2	16.5
Degradable protein (% CP)	69	64	70
Starch	2.5	1.7	51.7
WSC	7.2	7.5	-
ESC (simple sugars)	4.9	7.3	3.8
DCAD (mEq/100 g)	20	5	1
Minerals (mg/kg)			
Chloride	9,388	16,367	1,358
Calcium	10,002	9,828	357
Cobalt	<0.5	1.60	<0.5
Copper	7.7	9.6	6.1
Iron	228	1,393	57.6
Phosphorus	3,400	2,700	3,000
Potassium	24,170	19,730	6,625
Magnesium	2,906	3,608	1,300
Manganese	56.2	139	57.3
Molybdenum	0.8	0.7	0.6
Sodium	1,440	4,510	100
Sulfur	3,000	3,400	1,900
Zinc	29	27	42

DM = dry matter; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; ADICP = acid detergent insoluble protein; NDICP = neutral detergent insoluble crude protein; NFC = non-fiber carbohydrate; WSC = water-soluble carbohydrate; ESC = ethanol-soluble carbohydrate; DCAD = dietary cation-anion difference.

[1]Values are means obtained from near-infrared spectroscopy and wet chemistry.

[2]NFC = 100 – [(NDF-NDICP) + CP + crude fat + ash].

Laboratory Analysis

Endotoxin concentrations were determined in the 5-, 115-, and 215-min ruminal fluid samples by a chromogenic endpoint *Limulus* amebocyte lysate (**LAL**) assay with a sensitivity of 0.1 to 1 endotoxin units (**EU**)/mL (QCL-1000; Lonza Australia Pty. Ltd., Mount Waverley VIC, Australia). Certified pyrogen free (endotoxin content <0.001 EU/mL) microplates, pipette tips, and glassware were used throughout the experiment. Ruminal fluid samples were diluted 62,500 fold with pyrogen-free water (endotoxin content <0.005 EU/mL) and incubated in a 70^{o}C water bath for 20 min to remove most of the other LAL-gelating compounds (Baek et al., 1985). Microplates were pre-equilibrated to 37^{o}C on a heating block (DBH 20D; Ratek, Boronia, VIC, Australia) and remained on the heating block for the duration of the assay. A total of 50 μL of sample or standard was dispensed into the appropriate microplate well. *Limulus* amebocyte lysate (50 μL) was then added to each well and incubated for 12 min. Thereafter, 100 μL of chromogenic substrate was added and the microplate was incubated for a further 15 min before the reaction was stopped by adding 25% vol/vol acetic acid (Sigma-Aldrich, Sydney, NSW, Australia). The absorbance was then read at 405 nm (POLARstar Optima; BMG Labtech, Melbourne, VIC, Australia). Endotoxin concentrations were calculated from the standard curve and multiplied by the dilution factor.

The amount of free oxygen radicals in plasma samples were determined by measuring the concentrations of dROM, using a colorimetric assay according to kit instructions (d-ROMS Test, Diacron International, Grosseto, Italy; Cesarone et al., 1999). The d-ROMs test works on the principal that the plasma is diluted in an acidic buffer solution, allowing iron ions to be released from the plasma proteins to catalyze the breakdown of hydroperoxide to alkoxyl and peroxyl radicals. A chromogen (*N*,*N*-dietylparaphenylendiamine) is then added and changes color as it is oxidized by hydroperoxyl and alkoxyl radicals. The concentration of the colored complex is directly related to the level of hydroperoxide in the sample and can be photometrically quantified at 505 nm (Alberti et al., 2000). The results are expressed in Carratelli units (**Carr U**), where 1 Carr U corresponds to 0.08 mg/100 mL of hydrogen peroxide.

The concentrations of antioxidants were measured using the BAP test according to kit instructions (Diacron International). This test provides a combined measurement of several antioxidants including uric acid, ascorbic acid, proteins, α-tocopherol, and bilirubin by measuring the BAP of the plasma sample to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron (Benzie and Strain, 1996). The results of BAP are expressed in micromoles per liter of reduced iron. The extent of oxidative stress was expressed as an oxidative stress index (**OSI**), which was estimated by [(dROM/BAP) × 100], as the combination of dROM and BAP results provides a more accurate representation of oxidative stress status (Celi, 2011b).

Advanced oxidation protein products were measured according to the methods of Witko-Sarsat et al. (1998). In summary, 200 μL of plasma was diluted 1/5 in phosphate buffered saline (5 m*M*) and placed into a 96-well plate, and then 20 μL of glacial acetic acid was added. A chloramine-T solution (Sigma-Aldrich) was used to produce a standard curve ranging from 0 to 200 μmol/L. In standard wells, 10 μL of 1.16 *M* potassium iodide (ReagentPlus, Sigma-Aldrich) was added to 200 μL of chloramines-T solution followed by 20 μL of acetic acid. Within 5 min the absorbance was read at 340 nm (POLARstar Optima, BMG Labtech). Concentrations of AOPP were expressed as micromoles per litre of chloramine-T equivalents.

The concentrations of plasma **GSH-Px** were measured based on a spectrometric method according to kit instructions (catalog no. 703102; Cayman, Ann Arbor, MI). The absorbance of the samples was recorded for a 4-min period at 60-s intervals at 340 nm (POLARstar Optima, BMG Labtech).

Plasma ceruloplasmin concentrations were determined according to the methods described by Sunderman and Nomoto (1970) except that absorbance was read at 510 nm (POLARstar Optima, BMG Labtech). Briefly, 2 mL of acetate buffer solution and 100 μL of plasma was added to 2 sets of glass tubes for each sample, a set for the reaction (**R**) and a set for the blank (**B**). All tubes were brought to thermal equilibrium in a 37°C waterbath. To all tubes, 1 mL of 37°C *p*-phenylendiamine solution (Sigma-Aldrich) was added and mixed, and all tubes were incubated in a 37°C waterbath for 5 min unstoppered. To the B tubes only, 50 μL of sodium azide solution (Sigma-Aldrich) was added and mixed. After a 30-min incubation of all tubes, 50 μL of sodium azide solution (Sigma-Aldrich) was added to the R tubes. A total of 300 μL of solution from

each tube was pipetted on a micro-plate and absorbance was read at 510 nm (POLARstar Optima, BMG Labtech). The ceruloplasmin concentration was calculated as follows:

$$\text{Ceruloplasmin (g/L)} = 0.752\ (A_R - A_B),$$

where A_R is the absorbance of sample R, and A_B is the absorbance of sample B.

Statistical Analysis

A general linear model with repeated measures was used to estimate the effect of time, treatment group, and time × treatment interaction (PASW Statistics 18, SPSS Inc., Chicago, IL). The blocking effect of day was not significant and removed from the final model. The covariance structure was independent and a Tukey test was performed for means separation over time. The model was as follows:

$$Y_{ijk} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + R\varepsilon_{ijk,}$$

where Y_{ijk} = response of treatment group i (i = 1 to 5) at time j (j = 1 or 2) by heifer k (k = 1 to 30); μ = overall mean; β_i = effect of treatment group; γ_j = effect of time j; $(\beta\gamma)_{ij}$ = effect of treatment group by time interaction; and $R\varepsilon_{ijk}$ = random residual error adjusted for repeated measurements within heifer k at time j.

To estimate the main effects of grain, fructose, and histidine, and their interactions by time, the 5 treatment groups were merged into a factorial arrangement and analyzed using generalized estimating equations (**GEE**) with repeated measures using the PROC MIXED methods in SAS (version 9.2, SAS Institute Inc., Cary, NC; Golder et al., 2012). A Pearson correlation (PASW Statistics 18, SPSS Inc.) was performed to determine the correlations between all measures of ruminal pH, ruminal fermentation products, and ruminal endotoxin, and plasma oxidative stress biomarkers at 5 and also at 215 min after consumption of the challenge ration.

RESULTS

No time × treatment group interaction or effect of treatment group was observed for endotoxin concentration or oxidative stress biomarkers (Table 2); however, we observed a trend toward a decrease in AOPP concentration over time in the control group (P = 0.076; Table 2; Figure 2D). Concentrations of ruminal endotoxin decreased over the sampling period from 1.12 × 10^5 ± 0.06 EU/mL at the 5-min sampling to 0.92 × 10^5 EU/mL ± 0.05 EU/mL at the 215-min sampling (P = 0.021; Figure 1). Concentrations of dROM increased over the sampling period (P = 0.002) from 108.7 ± 4.1 to 123.5 ± 3.3 Carr U at the 5- and 215-min samplings, respectively (Figure 2A). The OSI also increased over the sampling period, from 4.1 ± 0.02 at the 5-min (P = 0.009) to 4.8 ± 0.03 at the 215-min sampling (Figure 2C). Ceruloplasmin concentrations decreased by half (P < 0.001) over the sampling period from 190 ± 70 at 5-min sampling to 90 ± 10 mg/L at the 215-min sampling (Figure 2F). No effect of time was observed for the concentrations of BAP, AOPP, or GSH-Px (Table 2; Figure 2B, D, E).

Table 2. Least square means (± SEM) of treatment groups and main effects (P-values) of treatment groups, time, and their interaction for ruminal endotoxin and plasma oxidative stress biomarker concentrations (n = 6 heifers/group)

Item	Group[1]					SEM	*P*-value		
	Control	GR	GR + FR	GR + HIS	GR + FR + HIS		Time (T)	Group (G)	T × G
Ruminal endotoxin (× 10^5 EU/mL)	1.08	1.10	0.90	1.00	0.97	0.09	0.021	0.482	0.394
dROM (Carr U)	119	114	114	126	123	8.1	0.002	0.809	0.619
BAP (µmol/L)	2,523	2,725	2,646	2,847	2,919	142	0.117	0.107	0.234
OSI (arbitrary units)	5.3	4.4	4.4	4.5	4.2	0.6	0.009	0.605	0.389
AOPP (µmol/L)	49.4	48.0	43.9	45.7	48.2	2.94	0.060	0.723	0.076
GSH-Px (nmol/min per mL)	32.7	32.7	27.6	30.2	30.5	3.24	0.240	0.840	0.757
Ceruloplasmin (mg/L)	120	122	160	162	121	18.0	<0.001	0.266	0.830

EU = endotoxin units; dROM = reactive oxygen metabolites (Carratelli units; 1 Carr U = 0.08 mg/100 mL of hydrogen peroxide); BAP = biological antioxidant potential; OSI = oxidative stress index [(dROM/BAP) × 100]; AOPP = advanced oxidative protein products; GSH-Px = glutathione peroxidase.
[1]Control (no grain); GR = (crushed triticale 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = Grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head).

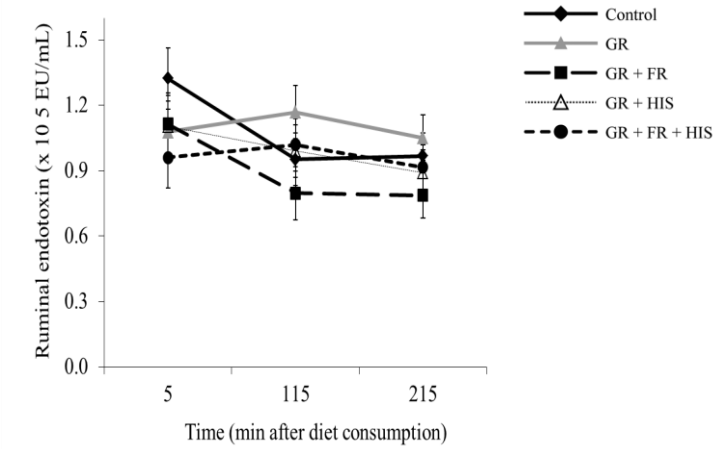

Figure 1. Ruminal concentrations of endotoxin (mean ± SEM) 5, 115, and 215 min after feed consumption in dairy heifers fed 5 different challenge rations (n = 6 heifers/group): control (no grain); GR = (1.2% of BW DMI crushed triticale); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head); (n = 6 heifers/group). EU = endotoxin units.

We observed no effects of grain, fructose, and histidine, or their interactions by time for concentrations of ruminal endotoxin or any plasma oxidative stress biomarkers in the GEE with repeated-measures merged factorial analysis (data not shown).

Ruminal histamine and propionate were negatively correlated with the OSI at the 215-min sampling (r = –0.42; P = 0.021 and r = –0.39; P = 0.087, respectively). Acetate was negatively correlated with the OSI at the 215-min sampling (r = –0.41; P = 0.024). No other correlations were found between the ruminal measures analyzed by Golder et al. (2012) and concentrations of ruminal endotoxin and plasma oxidative stress biomarkers at either the 5- or 215-min samplings.

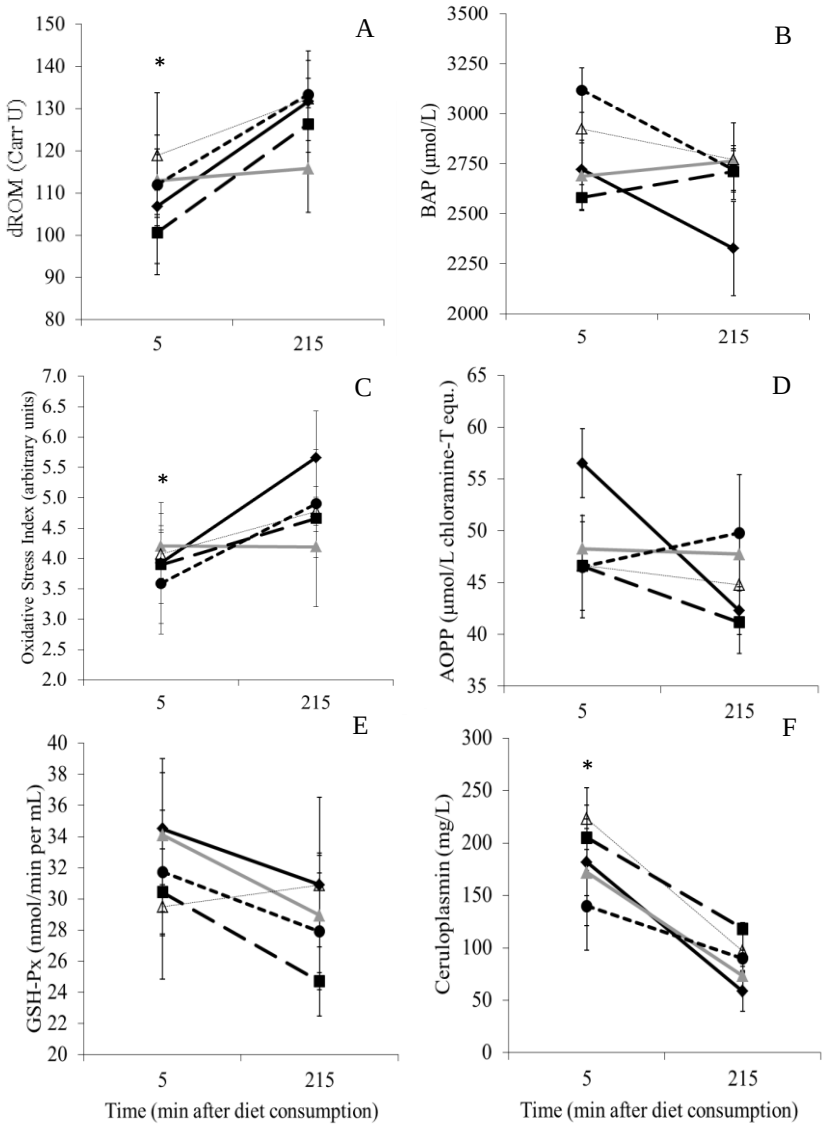

Figure 2. Plasma concentrations (means ± SEM) of oxidative stress biomarkers 5 and 215 min after feed consumption in dairy heifers fed 5 different challenge rations (n = 6 heifers/group); control (no grain); GR = (crushed triticale, 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). (A) reactive oxygen metabolites (dROM), (B) biological antioxidant potential (BAP), (C) oxidative stress index (dROM/BAP), (D) advanced oxidation protein products (AOPP), (E) glutathione peroxidase (GSH-Px) activity and (F) ceruloplasmin concentration. * P <0.05.

DISCUSSION

Although no clinical signs of ruminal acidosis were observed, changes in ruminal fermentation measures, particularly valerate, propionate, and lactate concentrations, suggested these heifers were exposed to a significant challenge ration (Golder et al., 2012). Lactate concentrations were >20 fold higher in the fructose groups than the controls (Golder et al., 2012), indicating the strength of the challenge and potential for endotoxin generation (Zebeli et al., 2011). The ruminal fermentation changes observed by Golder et al (2012) identified group effects of treatment on fermentation. From the results of this study, we rejected the hypothesis that a single grain and fructose challenge in dairy heifers would increase ruminal endotoxin concentrations and induce plasma oxidative stress responses. Histidine had no effect on concentrations of either ruminal endotoxin or plasma oxidative stress biomarkers, as hypothesized. The previous findings (Golder et al., 2012) demonstrate that the lack of significant endotoxin and oxidative stress responses were not primarily attributable to type II error but reflect the efficacy of mechanisms that control ruminal metabolism during periods of significant dietary challenge, such as that encountered around parturition or on feedlot entry diets.

The absence of treatment group effect for both endotoxin and oxidative stress measures provides a useful contribution to the limited field of research into links between nutrition, inflammation, and oxidative stress. It highlights the difference between ruminal environments of carbohydrate-challenged cattle in this study and acute carbohydrate challenge models used in other cattle studies (Andersen and Jarlov, 1990; Andersen et al., 1994). Similarly, it identifies changes occurring during disease progression from initial carbohydrate insults in this study and repeated carbohydrate exposures (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a).

The endotoxin concentrations in our study are consistent with the values reported in concentrate- or alfalfa pellet-fed cattle (Khafipour et al. 2009a,b) and cattle fed concentrate (Gozho et al. 2007). These concentrations are higher than those reported by Plaizier et al. (2012) and Khafipour et al. (2009a) for control and challenge group concentrations. The difference in range of endotoxin values may be accounted for by variation between animals, diets, and assays (Khafipour et al., 2009a).

Ruminal acidosis develops from a progression of changes in bacterial population, fermentation products, and physiological functions, suggesting that a substrate × time interaction for ruminal endotoxin could occur; this effect was not observed. We hypothesized that grain would increase endotoxin concentrations in response to the increased availability of carbohydrate substrates and changes in bacterial populations reflected in decreased ruminal pH, and markedly increased VFA concentrations (Golder et al., 2012). Endotoxin is continuously present in the rumen as the result of release from gram-negative bacteria during multiplication and lysis (Rietschel et al., 1994). The bacterial ecological balance, effects of ruminal pH on metabolism, changes in the cell membrane of bacteria, and other physiological functions of the rumen should modulate the release and accumulation of ruminal endotoxin (Russell and Rychlik, 2001; Ametaj et al., 2010). Substitution of fructose for grain increased mean D- and L-lactate concentrations by 22- and 21- fold, respectively, over other treatment groups, increased total VFA and butyrate concentrations, and decreased valerate concentrations and ruminal pH (Golder et al., 2012). It was therefore hypothesized that fructose would increase endotoxin release compared with that in grain-fed heifers in response to increased bacterial multiplication and lysis reflecting the faster fermentation rate of sugars than starches (Firkins, 2011). Despite the substantial differences in fermentation, feeding a challenge ration of grain and fructose had no effect on ruminal endotoxin concentrations over the first 3.6 h after feeding. Others have reported increased ruminal endotoxin concentrations in grain-based challenge studies in dairy cows (Motoi et al., 1993; Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a). A meta-analysis has shown that increased dietary concentrate content and reduced dietary NDF content are associated with an increase in ruminal endotoxin, plasma haptoglobin, and serum amyloid A levels (Zebeli et al., 2012). Responses became linear when cattle were fed >44.1% concentrate or <39.2% NDF (Zebeli et al., 2012). In these studies, the challenge rations were fed over several days, in contrast to the single challenge in this study. This observation may account for the difference in outcomes observed, as bacterial populations could undergo greater population shifts, possibly resulting in greater bacterial lysis.

It is necessary to establish the thresholds at which endotoxin release is increased after abrupt increases in carbohydrate feeding. Abrupt acute concentrate challenges in dairy cattle have not resulted in consistent endotoxin responses, with no difference reported

by Andersen and Jarlov (1990), an increase reported by Nagaraja et al. (1978), and increases in cattle fed 4 kg of concentrate rather than hay for the previous month reported by Andersen et al. (1994). Differences in findings among these studies may also reflect differences in age, physiological state of the dairy cattle, challenge ration composition, challenge management, and difference in assay methods. The dairy cattle in this study were unmated heifers, and metabolic changes may not be as pronounced in these cattle as in lactating cows under greater metabolic stress (Li et al., 2012). No other studies have examined the effects of sugar or histidine on endotoxin concentrations.

Endotoxin concentrations decreased over the 215-min period of the study, in contrast to the observed increase in VFA production (Golder et al., 2012), whereas increases in ruminal endotoxin concentrations were observed by 6 h after carbohydrate-induced challenge in dairy cattle and peaked 12 h after (Gozho et al., 2006; Khafipour et al., 2009a). Endotoxin concentrations increased over consecutive days during carbohydrate feeding (Gozho et al., 2005; 2007); however, endotoxin concentrations decreased by 25% over 4 d after acute carbohydrate challenge in cattle (Andersen and Jarlov, 1990). The involvement of ruminal endotoxin in the pathogenesis of grain-related disorders remains unclear, given the varied responses to abrupt challenges with carbohydrate including that in this study and requires further investigation.

We hypothesised that the proinflammatory molecule, histamine, which increased with grain feeding in this study (P = 0.054; Golder et al., 2012), would be positively correlated with endotoxin and oxidative stress measures; however, this was not the case.

The measurement of oxidative stress measures in unmated dairy heifers is unique and contributes to the currently small amount of data on oxidative stress in dairy cattle. At present, the normal ranges for oxidative stress measures in dairy cattle in different physiological states are not defined.

Biomarkers of oxidative stress were hypothesized to increase in grain and fructose fed heifers, reflecting the observed decreases in ruminal pH, increases in total VFA, and marked increases in lactic acid in fructose-fed heifers. Changes were proposed to be more pronounced in the fructose-fed heifers compared with the heifers fed grain only or control heifers. However, oxidative stress biomarkers were not affected by treatments, indicating that oxidative stress may not result from a single, albeit significant,

carbohydrate challenge. Oxidative stress can result from more-acute grain challenges, as high levels of starch fed to dairy cows at 80 days in milk (**DIM**) increased oxidative stress (Gabai et al., 2004) and feeding early lactation cattle rations to achieve restricted or high milk production was associated with oxidative stress (Pedernera et al., 2010). Prolonged concentrate feeding in dairy cattle showed increased plasma glutathione peroxidase activity and lipid peroxidation and decreased α-tocopherol and ferric reducing ability of plasma (Wullepit et al. 2009).

The lack of oxidative stress responses to treatment is consistent with the absence of treatment effects on ruminal endotoxin. Endotoxin translocation into the bloodstream may result in the production of oxidative molecules (Rietschel et al., 1994). The absence of the effect of treatment group on the concentrations of ruminal endotoxin suggests that the concentrations of endotoxin in the blood were also unlikely to have been increased.

The increase in OSI and dROM over time may have been a response to feeding after the withholding period or stress, independent of treatments. The increase in OSI and dROM in the controls, supports this hypothesis. Withholding cattle from feed before offering the challenge rations may have increased glycogenolysis and gluconeogenesis and decreased oxidative phosphorylation, a contributor to dROM production. Therefore, the overall oxidative status (OSI) of the cattle could have been low before feeding and increased after. The increase in dROM and OSI may reflect stress from restraint and sampling over the 3.6-h period. Stress of any origin can deplete the body's antioxidant resources (Sconberg et al., 1993) and result in lipid oxidation in muscle (McClelland, 2004).

The observed range of dROM in this study (Figure 2A) was consistent with the ranges reported in dairy cattle (Bernabucci et al., 2005; Celi and Raadsma, 2010; Pedernera et al., 2010) but higher than those reported by Piccione et al. (2007) and Bernabucci et al. (2002). The BAP values reported in this study are slightly higher than those reported in dairy cattle (Celi and Raadsma, 2010; Pedernera et al., 2010). The observed increase in OSI over the experimental period can largely be attributed to the increase in dROM 215 min after feeding. The OSI concentrations were lower than those reported by Pedernera et al. (2010), but within the range reported for lactating dairy cattle by Celi and Raadsma (2010), despite the differing physiological states of heifers and lactating cows.

Advanced oxidation protein products are markers of protein oxidation generated by the reaction between plasma proteins and myeloperoxidase-derived chlorinated oxidants produced by activated neutrophils (Witko-Sarsat et al., 1999). Concentrations of AOPP can be associated with embryonic losses and are considered as an acute indicator of inflammation and oxidative stress in dairy cows (Celi et al., 2011). The lack of effects on AOPP may reflect the abrupt, as opposed to chronic, challenge in the study; however, we observed a trend toward a decrease in AOPP concentration in the control heifers over time.

Glutathione peroxidase is regarded as the most important hydrogen peroxide scavenging enzyme in mammalian cells (Halliwell, 1994) and is therefore considered a good indicator of oxidative stress (Gabai et al., 2004). The activity of GSH-Px was not affected by treatment group, in contrast to alterations observed by Wullepit et al. (2009) in dairy cattle fed a grain carbohydrate challenge protocol. Celi et al. (2010) suggests that GSH-Px activity in the blood may not be very sensitive to nutritional changes. The activity of GSH-Px represents only one aspect of the antioxidant defence system; other enzymes such as catalase and superoxide dismutase may be better acute measures.

Ceruloplasmin (ferroxidase, EC 1.16.3.1) has both anti- and pro- oxidant roles (Healy and Tipton, 2007) and concentrations are within the proposed reference values for lactating Holsteins (Hussein et al., 2012). Ceruloplasmin concentrations decreased consistently across all treatment groups contrary to the expected parallel increase with dROM, again suggesting that metabolic responses coped with this level of carbohydrate challenge.

CONCLUSIONS

Marked changes in ruminal metabolism during a single abrupt challenge with combinations of grain, fructose, and histidine had no effect on ruminal endotoxin concentration or plasma oxidative stress biomarkers. Variable responses in ruminal endotoxin to carbohydrate challenge among studies suggest that further studies are needed to determine the thresholds required to induce increases in endotoxin during abrupt carbohydrate challenges, or on other factors that influence concentrations of endotoxin. The study shows that oxidative stress responses were not involved in responses to abrupt exposure to readily fermentable carbohydrates, both fructose and

starch. This study contributes additional observations on the physiological ranges of oxidative stress biomarkers in ruminants.

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CHAPTER 4

Ruminal Bacterial Community Shifts in Grain, Fructose, and Histidine Challenged Dairy Heifers

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OVERVIEW OF CHAPTER 4

Chapter 4 presents findings from a companion study to Chapters 2 and 3. The findings of Chapter 2 showed a single challenge feed of combinations of grain, fructose, and the amino acid, histidine produced different ruminal fermentation measures and a 21- and 22-fold increase in D- and L-lactate occurred in the fructose-fed heifers. However, treatment groups had no effects on endotoxin and oxidative stress biomarker concentrations in Chapter 3. Thus, it was hypothesized that distinct ruminal bacterial communities would begin to develop over 3.6 h after the single challenge feed among heifers fed combinations of grain, fructose, and histidine and reflect ruminal fermentation measures. We wished to evaluate responses in the context of existing understandings of ruminal acidosis models commonly presented.

ABSTRACT

Ruminal bacterial community composition (**BCC**) and its associations with ruminal fermentation measures were studied in dairy heifers challenged with combinations of grain, fructose, and histidine in a partial factorial study. Holstein-Friesian heifers (n = 30) were randomly allocated to 5 treatment groups: (1) control (no grain); (2) grain [fed at a dry matter intake (**DMI**) of 1.2% of bodyweight (**BW**)]; (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); (4) grain (1.2% of BW DMI) + histidine (6 g/head); and (5) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). Ruminal fluid was collected using a stomach tube 5, 115, and 215 min after consumption of the rations and bacterial 16S rDNA sequence data was analyzed to characterize bacteria. Large variation among heifers and distinct BCC was evident in a between group constrained principal coordinates analysis. Bacterial composition in the fructose-fed heifers was positively related to total lactate and butyrate concentrations. Bacterial composition was positively associated with ruminal ammonia, valerate, and histamine concentrations in the grain-fed heifers. The predominant phyla were the Firmicutes (57.6% of total recovered sequences), Bacteroidetes (32.0%), and candidate phylum, TM7 (4.0%). *Prevotella* was the dominant genus. In general, grain or histidine, or their interactions with time had minimal effects on the relative abundance of bacterial phyla and families. Fructose increased and decreased the relative abundance of the Firmicutes and Proteobacteria phyla over time, respectively, and decreased the abundance of the Prevotellaceae family over time. The relative abundance of the Streptococcaeae and Veillonellaceae families was increased in the fructose-fed heifers and the fructose-fed heifers over time. A total of 31 operational taxonomic units differed among groups in the 3.6 h sampling period, *Streptococcus bovis* was observed in the fructose-fed heifers; while, bacteria similar to Lactobacillus spp. or *Megasphaera elsdenii*, that are commonly associated with ruminal acidosis, were not associated with these operational taxonomic units. The TM7 candidate phylum had an increased abundance of sequence reads by over 2.5 fold due to the introduction of histidine into the diet. Rapid changes in BCC can occur in a short period after a single substrate challenge and the nature of these changes may influence the risk of ruminal acidosis and differ from those in cattle exposed to substrate challenges over a longer time period.

Key words: bacterial community composition, fructose, histidine, ruminal acidosis

INTRODUCTION

The rumen ecosystem is highly responsive to dietary changes, most notably those that occur during weaning and shifts from forage- to concentrate-based diets (Tajima et al., 2000). Examples of marked dietary change occur during the transition period in dairy systems or induction of beef cattle to feedlot diets. These abrupt changes in diet can be associated with ruminal acidosis, a complex of ruminal conditions with a significant economic impact (Stone, 1999). The complex occurs along a continuum of severity reflected in increased disease and losses in production performance. The prevalence of ruminal acidosis has been reported as approximately 20% in Wisconsin dairy herds (Oetzel et al., 1999), 10% in Australian herds (Bramley et al., 2008), and 11% in Irish herds (O'Grady et al., 2008). Bramley et al. (2008) found diets with high non-fiber carbohydrate (**NFC**) and low neutral detergent fiber (**NDF**) increased the risk of ruminal acidosis, a condition associated with increased ruminal propionate, valerate, butyrate, and acetate and decreased ammonia concentrations and a lower milk fat to protein ratio.

Feeding different substrates alters ruminal fermentation responses (Heldt et al., 1999; Golder et al., 2012), presumable partly resulting from different responses in bacterial community composition (**BCC**) in the rumen. Researchers have examined ruminal bacterial composition changes among cattle fed forage- and concentrate-based diets (Tajima et al., 2000; Petri et al., 2012), cattle fed increasing dietary percentages of concentrate (Callaway et al., 2010; Fernando et al., 2010), or in cattle with induced subacute acidosis (Khafipour et al., 2009; Hook et al., 2011). However, these studies have used relatively small numbers of cattle and focused on changes that occurred after adaptation to feed changes. Others have suggested that the rumen microbiome is reasonably resistant to dietary changes (Weimer et al., 2010) and a change in the microbiome is not always related to the severity of ruminal acidosis (Mohammed et al., 2012).

Golder et al. (2012) hypothesized that the addition of 0.4% of bodyweight (**BW**) fructose or histidine (6 g per head), or both, to grain fed as a single challenge would increase the onset of subacute ruminal acidosis. The rationale for this hypothesis is that fructose is rapidly metabolized compared to starch (Firkins, 2011) and metabolic acidosis was induced in heifers drenched with 13, 17, or 21 g/kg (~ 0.13, 0.17, and 0.21% of BW) of oligofructose, a polymer of fructose (Thoefner et al., 2004). Ruminal

and systemic acidosis were also induced when 17 g/kg (~0.17% of BW) of oligofructose was administered to heifers (Danscher et al., 2009; 2010). With increased interests in potential benefits of *Lolium perenne* varieties with greater water-soluble carbohydrate (**WSC**) content in pasture-based dairying (Miller et al., 2001; Tas et al., 2006), it is important to assess the role of sugars in the development of acidosis. The concentration of fructose fed by Golder et al. (2012; 0.4% of BW) is similar to amounts of WSC ingested by cattle over a day.

Release of histamine has been hypothesized to have an important role in acidosis (Dain et al., 1955; Ahrens, 1967), as has endotoxin release (Gozho et al., 2005; Khafipour et al., 2009). The amino acid histidine is decarboxylated at low rumen pH by the bacteria, *Allisonella histaminiformans*, to produce the inflammatory molecule histamine (Garner et al., 2002). The rationale for use of histidine in the study was based on the relatively high concentrations in white clovers, ryegrass, and kikuyu (Reeves et al., 1996; Penkov et al., 2003). Providing fructose and histidine in combination may have a different influence on BBC and subsequent rumen fermentation measures, compared to when they are fed in isolation.

Golder et al. (2012) found that a grain challenge decreased ruminal pH and increased ammonia, total volatile fatty acids (**VFA**), acetate, butyrate, propionate, and valerate concentrations, compared with unfed controls. Fructose fed at 0.4% of bodyweight (**BW**) decreased ruminal pH and valerate concentrations, increased total VFA and butyrate concentrations, and markedly increased D- and L-lactate concentrations, compared with nonfructose-fed heifers. Histidine had limited effects on ruminal fermentation. Combinations of grain, fructose, and histidine had no effects on ruminal endotoxin and plasma oxidative stress biomarker concentrations (Golder et al., 2013). It was concluded that the fructose-fed heifers were at the highest risk of ruminal acidosis.

The aim of this study was to examine the effects of combinations of grain, fructose, and histidine, fed to dairy heifers unadapted to these in a single challenge, on ruminal BCC and its associations with ruminal fermentation measures. We hypothesized that distinct ruminal bacterial communities would begin to develop over 3.6 h after the single challenge feeding among heifers fed combinations of grain, fructose, and histidine and reflect ruminal fermentation measures. We wished to evaluate responses in the context

of existing understandings of ruminal acidosis models commonly presented (Owens et al., 1998; Nagaraja and Titgemeyer, 2007; RAGFAR, 2007).

MATERIALS AND METHODS

Animals and Experimental Design

The experiment was conducted on 30 nonpregnant Holstein heifers <18 mo of age with a mean BW of 359.3 ± 47.3 kg at Camden, New South Wales (**NSW**), Australia. All experimental procedures were approved by the Bovine Research Australasia Animal Ethics Committee (BRA 0609-0610). The experiment consisted of a 10-d adaptation period followed by a single pulse feeding challenge on d 11. All heifers were housed on a dry lot and fed twice daily on a feed pad with individual head stanchions. During the adaptation period, the heifers were fed 1 kg (as-fed) of triticale daily and a target feed intake of 7.2 kg/d of alfalfa hay and 2 kg/d of ryegrass silage (as-fed basis). Rumen and blood samples were collected over approximately a 3.6-h period after consumption of the challenge rations. Dietary information, experimental detail, and ruminal fermentation measures, ruminal pH, endotoxin, and oxidative stress results have been reported previously (Golder et al., 2012; Golder et al., 2013). This article provides data on BCC and interprets other findings in this context.

Treatment Groups

As described by Golder et al. (2012), 30 Holstein heifers (n = 6 heifers/group) were randomly allocated to 5 treatment groups in a partial factorial arrangement: (1) control (no grain); (2) grain [fed at a crushed grain dry matter intake (**DMI**) of 1.2% of BW]; (3) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); (4) grain (1.2% of BW DMI) + histidine (6 g/head); and (5) grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head). The chemical composition of the grain, triticale cultivar 'Berkshire', was analyzed by wet chemistry (Golder et al., 2012). The fructose (Melbourne Food Depot, East Brunswick, Victoria, Australia) was a 99.5% pure crystalline powder and was mixed through the grain ration on the morning of the challenge. The histidine was an L-histidine powder (Merck KGaA, Darmstadt, Germany) dissolved in 50 mL of tap water and was administered by a stomach tube. The challenge-day rations had an estimated NFC of 25.4, 56.5, 56.4, 70.0, and 69.8% of DM for control, grain, grain + histidine, grain + fructose, and grain + fructose + histidine groups, respectively (Table 1).

Challenge and Ruminal Fluid Sampling Procedures

Briefly, as described by Golder et al. (2012), all heifers were withheld from feed for 14 h before being individually offered 200 g of alfalfa hay to reduce salivation and immediately after its consumption were offered their respective challenge rations. Ruminal fluid (250 mL) was collected by a stomach tube 5, 65, 115, 165, and 215 min after ration consumption and immediately scored for saliva contamination using the methodology of Bramley et al. (2008). Ruminal pH was immediately measured and samples of unfiltered rumen fluid that was primarily liquid but contained particulate matter, thus representing a mixture of the liquid and particulate phases of the rumen, were put on ice and later stored in 5-mL tubes at –20^{o}C for bacterial analysis. Ruminal fluid was also processed and analyzed as described by Golder et al. (2012) for VFA, ammonia, D- and L-lactate, histamine, and endotoxin concentrations (Table 2).

Table 1. Estimated chemical composition (CPM Dairy Ration Analyzer version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY) of the challenge rations[1]

Item (% of DM)	Treatment group[2]				
	C	GR	GH	GF	GFH
DM	87.7	88.8	88.8	91.5	91.5
CP	20.7	16.9	17.1	11.5	11.7
RUP (% of CP)	23.5	12.9	14.2	12.8	14.8
RDP (% of CP)	76.5	87.2	85.8	87.2	85.2
RDP	15.8	14.7	14.6	10.0	10.0
Soluble protein (% of CP)	43.0	27.3	28.4	27.7	29.3
ADF	33.6	6.43	6.41	4.74	4.72
NDF	45.9	23.1	23.0	16.0	15.9
Forage NDF (% of NDF)	100.0	7.96	7.96	11.5	11.5
Forage NDF (% of DM)	45.9	1.84	1.83	1.84	1.83
Physically effective NDF	41.3	10.1	10.1	7.31	7.29
Lignin	6.80	2.48	2.47	1.74	1.74
NFC[3]	25.4	56.5	56.4	70.0	69.8
Silage acids	0.00	0.00	0.00	0.00	0.00
Sugar	4.90	3.84	3.83	34.6	34.5
Starch	2.50	49.7	49.6	33.2	33.2
Soluble fiber	18.0	2.91	2.90	2.21	2.21
Total ether extract	2.50	1.54	1.53	1.06	1.06
Total LCFA	1.38	1.36	1.35	0.92	0.92
Ash	9.00	2.66	2.66	1.90	1.89
DCAD (mEq/100g)	35.8	3.03	-0.42	2.50	-0.95
Minerals (mg/kg)					
Chloride	9,400	1,700	1,700	1,300	1,300
Calcium	10,000	3,800	3,800	2,700	2,700
Copper	8	6	6	4	4
Iron	228	64	64	46	46
Phosphorus	3,400	3,000	3,000	2,100	2,000
Potassium	24,200	7,300	7,300	5,200	5,200
Magnesium	2,900	1,400	1,400	900	900
Manganese	56	57	57	39	39
Sodium	4,400	300	300	200	200
Sulfur	3,000	1,900	2,500	1,300	1,900
Zinc	21	41	41	28	28

C = control (no grain); GR = grain [1.2% of bodyweight (BW) dry matter (DM)]; GH = grain (1.2% of BW DM) + histidine (6 g/head); GF = grain (0.8% of BW DM) + fructose (0.4% of BW DM); GFH = grain (0.8% of BW DM) + fructose (0.4% of BW DM) + histidine (6 g/head); CP = crude protein; RUP = rumen undegradable protein; RDP = rumen degradable protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC =non-fiber carbohydrates; LCFA = long-chain fatty acids; DCAD = dietary cation-anion difference.

[1]Estimations were performed using CPM Dairy Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) using the chemical composition components of triticale cultivar 'Berkshire' and alfalfa hay from Golder et al. (2012) and were based on the mean BW of the 30 heifers (360 kg) with a body condition score of 3.25 and growth rate of 0.91 kg/d.

[2]All challenge rations included 200 g (as-fed) of alfalfa hay and were based on

[3]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash. NDICP = neutral detergent insoluble crude protein.

DNA Extraction

Bacterial community composition was only determined on ruminal fluid collected 5, 115, and 215 min after consumption of the challenge rations. Ruminal fluid samples (n = 90) were thawed at room temperature and a 1-mL aliquot was centrifuged at 10,000 × *g* for 1-min and the supernatant discarded. The pellet was resuspended by vigorous vortexing in 200 μL ATL buffer (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany) together with 200-mg silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK). The mixture was homogenized in a FastPrep-24 (MP Biomedicals, Seven Hills, NSW, Australia) at maximum speed for 1-min, twice; heated at 70^{o}C for 15 min and spun at 10,000 × *g* for 5-min. Supernatant (180 μL) was removed for digestion with proteinase K at 50^{o}C for at least 3 h and DNA was extracted according to the QIAmp DNA mini kit protocol (Qiagen GmbH); DNA was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA).

PCR Amplification of 16S Ribosomal DNA Gene Sequences

Genomic DNA from each sample was diluted 1:30 with water and the 16S rRNA gene spanning V1 to V3 was PCR amplified using Platinum taq polymerase (Invitrogen, Carlsbad, CA) as follows: 1 cycle at 94^{o}C for 2 min; followed by 30 cycles of 94^{o}C for 10 s, 55^{o}C for 45 s, 72^{o}C for 45 s; with a final extension of 72^{o}C for 10-min. Primers used in the reaction were modified universal 8F (Snell-Castro et al., 2005) and 515R (Lane, 1991) primers that included 454 sequencing adapters B and A, respectively. In addition, a unique 8 base pair barcode was included in the reverse primer of each amplicon, so that DNA sequence reads can be assigned accurately to each originating sample. The PCR products were visualized on agarose gels and equal amounts of PCR product were pooled and gel extracted (Qiaex gel extraction kit, Qiagen). Approximately 3 μg of pooled amplicon (~40 ng/μl) was sent to Macrogen (Seoul, Korea) for 454 DNA sequencing using a 454 GS FLX Sequencer with titanium chemistry (Roche, Branford, CT).

Table 2. Least square means (±SEM) and effects and interactions of grain, fructose nested within grain, histidine nested within grain, and time for ruminal and plasma measures obtained from a merged factorial generalized linear model (adapted from Golder et al., 2012)

Item	Group mean ± SEM and *P*-value[1]						*P*-value			
	Grain (G)		Fructose (F)		Histidine (H)			Interaction		
	–	+	–	+	–	+	Time (T)	G × T	F × T	H × T
No. of animals	6	24	12	12	12	12				
Ruminal (m*M*)										
Total VFA	63.7 ± 5.1	95.9 ± 2.6	89.7 ± 3.6	102.0 ± 3.6	96.7± 3.6	95.0 ± 3.6				
		0.001		0.021		0.734	0.218	0.006	0.236	0.145
Acetate	44.1 ± 3.0	61.2 ± 1.5	58.3 ± 2.2	64.1 ± 2.2	61.5 ± 2.2	60.9 ± 2.2				
		0.001		0.065		0.849	0.192	0.013	0.129	0.187
ln butyrate[2]	1.83 ± 0.1	2.50 ± 0.1	2.26 ± 0.1	2.75 ± 0.1	2.50 ± 0.1	2.50 ± 0.1				
		0.007		<0.001		0.972	0.333	0.014	0.354	0.303
Iso-butyrate	1.21 ± 0.1	1.21 ± 0.1	1.43 ± 0.1	1.28 ± 0.0	1.24 ± 0.1	1.13 ± 0.1				
		0.082		<0.001		0.251	0.402	0.035	0.160	0.074
Propionate	8.73 ± 0.9	15.27 ± 0.5	14.63 ± 0.6	15.91 ± 0.6	15.63 ± 0.6	14.91 ± 0.6				
		<0.001		0.162		0.428	0.079	<0.001	0.208	0.051
ln caproate[2]	-1.69 ± 0.3	-0.90 ± 0.2	-1.13 ± 0.2	-0.65 ± 0.2	-0.10 ± 0.2	-0.79 ± 0.2				
		0.267		0.111		0.494	0.018	0.317	0.623	0.659
Valerate	0.98 ± 0.2	2.03 ± 0.1	2.18 ± 0.1	1.87 ± 0.1	1.95 ± 0.1	2.10 ± 0.1				
		<0.001		0.042		0.339	<0.001	<0.001	0.078	0.113
Iso-valerate	1.95 ± 0.1	2.26 ± 0.1	2.53 ± 0.1	2.00 ± 0.9	2.15 ± 0.1	2.38 ± 0.1				
		0.013		<0.001		0.083	0.512	0.011	0.132	0.004

VFA = volatile fatty acids; ln = natural logarithm.

[1]P-values are for the comparison of grain (–/+), the comparison of fructose (–/+) nested within grain, and the comparison of histidine (–/+) nested within grain.

[2]Exponentiated least squares means for the 6 groups, respectively: butyrate: 6.23, 12.18, 9.97, 15.64, 12.18, 12.18; caproate: 0.18, 0.41, 0.32, 0.52, 0.90, and 0.45.

Table 2 (continued). Least square means (±SEM) and effects and interactions of grain, fructose nested within grain, histidine nested within grain, and time for ruminal and plasma measures obtained from a merged factorial generalized linear model (adapted from Golder et al., 2012)

Item	Group mean ± SEM and *P*-value[1]						*P*-value			
	Grain (G)		Fructose (F)		Histidine (H)			Interaction		
	–	+	–	+	–	+	Time (T)	G × T	F × T	H × T
ln D-lactate[2]	-2.13 ± 0.5	0.59 ± 0.3	-2.14 ± 0.4	0.97 ± 0.4	-0.44 ± 0.4	-0.74 ± 0.4				
		0.835		<0.001		0.548	<0.001	0.869	0.090	0.442
ln L-lactate[2]	-2.87 ± 0.4	-1.32 ± 0.3	-2.87 ± 0.4	0.23 ± 0.4	-1.17 ± 0.4	1.47 ± 0.4				
		0.768		<0.001		0.622	0.032	0.891	0.273	0.419
Ammonia	8.28 ± 1.0	12.30 ± 0.5	14.03 ± 0.7	10.58 ± 0.7	11.95 ± 0.7	12.66 ± 0.7				
		0.001		0.003		0.510	<0.001	0.233	0.062	0.918
Histamine (ng/mL)	61.3 ± 18	114.5 ± 9	117.9 ± 13	111.2 ± 13	105.5 ± 13	123.6 ± 13				
		0.054		0.709		0.318	<0.001	0.061	0.868	0.128
pH[3]	7.14 ± 0.9	6.70 ± 0.0	6.92 ± 0.1	6.49 ± 0.1	6.67 ± 0.1	6.74 ± 0.1				
		0.030		<0.001		0.389	0.298	0.220	0.686	0.109
Plasma										
ln L-lactate (m*M*)[2]	0.16 ± 0.1	0.22 ± 0.1	0.23 ± 0.1	0.21 ± 0.1	0.18 ± 0.1	0.26 ± 0.1				
		0.856		0.880		0.541	0.001	0.030	0.582	0.665
ln histamine (ng/mL)[2]	-1.55 ± 0.2	-1.25 ± 0.1	-1.20 ± 0.1	-1.30 ± 0.1	-1.43 ± 0.1	-1.07 ± 0.1				
		0.550		0.649		0.104	0.003	0.125	0.193	0.009

ln = natural logarithm.

[1]P-values are for the comparison of grain (–/+), the comparison of fructose (–/+) nested within grain, and the comparison of histidine (–/+) nested within grain.

[2]Exponentiated least squares means for the 6 groups, respectively: D-lactate: 0.12, 0.55, 0.12, 2.64, 0.64, 0.48; L-lactate: 0.06, 0.27, 0.06, 1.24, 0.31, 0.23; plasma L-lactate: 1.17, 1.25, 1.26, 1.23, 1.20, 1.30; plasma histamine: 0.21, 0.29, 0.30, 0.27, 0.24, 0.34.

Sequence Analyses of Gene Amplicons

Sequence data was processed using the quantitative insights into microbial ecology (QIIME) software package (Caporaso et al., 2010). Recovered sequences were assigned to their originating sample based on the attached barcode and filtered based on quality and length parameters. Error correction of 454 was performed using R software (package Acacia; Bragg et al., 2012). Clustering of recovered sequences to an operational taxonomic unit (**OTU**) at a 0.97 distance threshold was used. Taxonomic identification was based on similarity to the Greengenes Database (http://greengenes.lbl.gov). The OTU table was subjected to alpha and beta diversity measures using QIIME and passed through R (package Ade4; Dray and Dufour, 2007) for principal coordinates between group analysis and co-inertia analysis.

Statistical Analysis

The raw means ± SD for the relative abundance of bacterial phyla and families from the 5 individual treatment groups are displayed in Table 3. To obtain the predicted means ± SEM, main effects, and interactions for the relative abundance of bacteria belonging to each phylum and family (Tables 4 and 5, respectively), data from the 5 treatment groups were merged and analyzed in a partial factorial arrangement using a linear mixed model in Genstat (14th edition, VSN International Ltd., Hemel, Hempstead, UK). The mean relative abundances of bacterial phyla or families that were >0.3% for at least 1 group only were analyzed. The model used was:

$$Y_{ijklmn} = \mu + \alpha_i + \beta_{(i)j} + \gamma_{(i)k} + \delta_l + \delta_{(i)jl} + \delta_{(i)kl} + X_m + (XZ)_{mn} + \varepsilon_{ijklmn,}$$

where Y_{ijkl} = response to grain i (i = 1 or 2), fructose j (j = 1 or 2), and histidine k (k = 1 or 2) at l time (l = 1 to 3) from block m (m = A to D) by heifer n (n = 1 to 30); μ = overall mean; α_i = fixed effect of grain; $\beta_{(i)j}$ = fixed effect of fructose nested within grain; $\gamma_{(i)k}$ = fixed effect of histidine nested within grain; δ_l = fixed effect of time; $\delta_{(i)jl}$ = effect of fructose nested within grain by time interaction; $\delta_{(i)kl}$ = effect of histidine nested within grain by time interaction; X_m = random effect of block; $(XZ)_{mn}$ = random effect of heifer nested within block; and ε_{ijklmn} = random residual error within heifer n at time l from block m. The covariance structure of the model was independent. The model was chosen following examination of other covariance structures, including AR1 structures. Minor differences only were observed between the independent and AR1 structures.

A false discovery rate (**FDR**) analysis was performed in R (package ade4) to identify OTU's significantly influenced among the 5 individual treatment groups and these OTU's were matched to the Greengenes Database and fitted into a phylogenetic tree using R software (ARB package; Ludwig et al., 2004).

Table 3. Relative abundance (raw mean ± SD) of bacterial phyla and families

Item	Treatment group relative abundance (%)[1]					SD
	Control	GR	GR + FR	GR + HIS	GR + FR + HIS	
No. of heifers	6	6	6	6	6	
Bacterial phylum						
Firmicutes	52.3	58.5	58.0	60.8	58.2	12.8
Bacteroidetes	40.0	31.9	31.1	25.0	32.0	14.4
TM7	2.73	3.58	3.36	7.30	3.14	5.58
Firmicutes:Bacteroidetes	1.67	2.78	2.49	3.32	2.32	2.02
Tenericutes	1.79	2.43	2.71	2.84	2.51	1.19
Chloroflexi	0.85	0.82	1.50	1.59	1.23	0.98
Actinobacteria	1.07	1.27	0.82	1.15	1.02	0.81
Proteobacteria	0.48	0.50	0.77	0.32	0.74	0.50
Spirochaetes	0.26	0.43	0.60	0.44	0.47	0.47
Lentisphaerae	0.07	0.21	0.54	0.13	0.19	0.36
Bacterial family						
Unclassified Clostridiales	13.3	16.1	15.4	21.2	18.8	6.75
Prevotellaceae	19.9	17.4	12.3	14.7	16.1	11.8
Ruminococcaceae	20.3	15.2	13.5	14.0	17.2	7.04
Unclassified Bacteroidales	19.4	13.4	18.2	9.73	15.1	9.59
Lachnospiraceae	13.7	12.7	11.7	14.8	8.57	5.92
ClostridialesFamilyXIII Incertae	2.08	5.22	4.73	4.36	3.54	3.16
F16	2.73	3.58	3.36	7.30	3.14	5.58
Streptococcaceae	0.44	1.83	7.97	1.76	3.97	6.30
Catabacteriaceae	1.81	4.50	3.12	3.14	3.52	2.58
Erysipelotrichaceae	0.82	1.76	1.83	1.89	1.25	1.12
Anaerolinaceae	0.85	0.82	1.50	1.59	1.23	0.98
Coriobacteriaceae	1.03	1.24	0.78	0.98	1.00	0.77
Unclassified Rickettsiales	0.09	0.06	0.15	0.06	0.08	0.10
Carnobacteriaceae	0.02	1.58	0.02	0.06	1.45	4.00
Porphyromonadaceae	0.74	1.02	0.59	0.52	0.74	0.81
Clostridiaceae	0.20	0.45	0.39	1.01	0.37	0.66
Unclassified RF39	0.54	0.47	0.47	0.72	0.44	0.34
Spirochaetaceae	0.19	0.41	0.55	0.29	0.45	0.44
Eubacteriaceae	0.21	0.47	0.47	0.32	0.31	0.33
Veillonellaceae	0.28	0.19	0.59	0.10	0.36	0.42
Unclassified Alphaproteobacteria	0.28	0.32	0.44	0.10	0.46	0.48
Victivallaceae	0.07	0.21	0.54	0.13	0.19	0.36

[1]Control (no grain); GR = (crushed triticale 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = Grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head).

RESULTS

Bacterial Diversity Analysis

Rarefaction analysis (Hughes et al., 2001), which is used to estimate the depth of coverage of diversity of ruminal bacteria within ruminal fluid samples (Kim et al., 2011), indicated coverage of bacterial diversity appeared to be sufficient to evaluate BCC. Treatment group did not affect the general level of microbial diversity, with all samples possessing similar microbial diversity (Figure 1). The overall microbiomes of the heifers were not distinctly different in their composition either (data not shown). However, principal coordinates analysis (**PCoA**) revealed distinct variation ($P < 0.001$) in the 16S rDNA estimated BCC among groups in a between-group constrained PCoA that accounted for a total of 53.8% of the variation (Figure 2).

Heifers from the control and grain + histidine treatment groups had the most distinct bacterial communities among the treatment groups (Figure 2). Heifers from the grain, grain + fructose, and grain + fructose + histidine treatment groups were closely clustered in the PCoA (Figure 2). A minor overlap in BCC occurred between the grain- and grain + fructose + histidine-fed heifers. Spatial heterogeneity among heifers was relatively large and greatest in the grain + histidine-fed heifers. Clustering of bacterial communities was similar for the 3 sample times within each treatment group and only a total of 22.7% of variation of the community was accounted for ($P = 0.385$, data not shown).

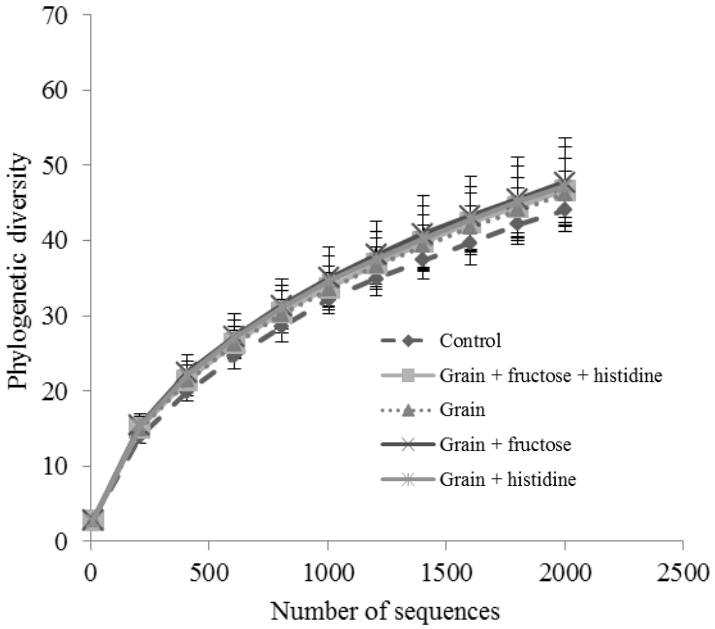

Figure 1. Rarefaction curves for each treatment group shown as the mean ± 95% confidence intervals. Phylogenetic diversity is shown in branch lengths on the y axis and the number of sequences sampled on the x axis.

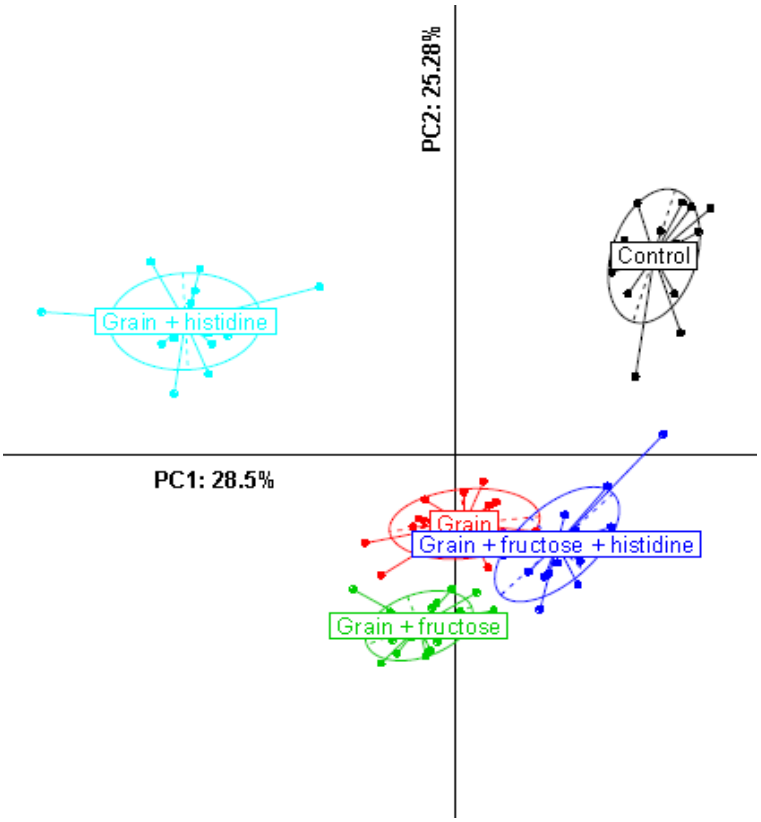

Figure 2. Constrained between group principal coordinates analysis of bacterial 16S rDNA gene sequences at the species level from ruminal fluid collected over approximately a 3.6-h period after heifers consumed the following challenge rations: (1) control (no grain); (2) grain (1.2% of BW DM); (3) grain (1.2% of BW DM) + histidine (6 g/head); (4) grain (0.8% of BW DM) + fructose (0.4% of BW DM) or; (5) grain (0.8% of BW DM) + fructose (0.4% of BW DM) + histidine (6 g/head) (n of heifers = 6/group; n of samples = 18/group). Each point on the plot represents the bacterial community composition of a single sample (heifer by group by sample time combination) with a greater distance between points indicating a greater difference in bacterial community composition.

Co-inertia Analysis

Co-inertial analysis explained associations among BCC, ruminal fermentation measures, and dietary inputs (Figure 3). Bacterial composition in the grain-fed heifers was associated with the amount of grain consumed and ruminal ammonia, valerate, and histamine concentrations. The amount of grain or fructose consumed had the largest influence on bacterial composition, and time of sampling the least. There was a strong positive relationship existed between bacterial composition in the 2 groups that consumed fructose and total lactate and butyrate concentrations (Figure 3).

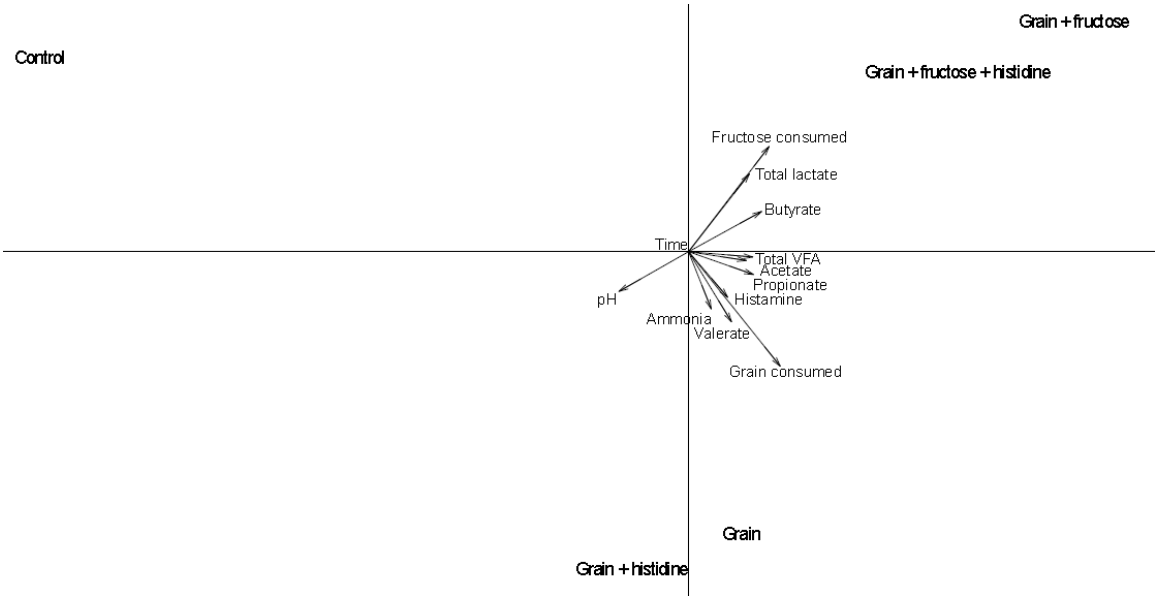

Figure 3. Duality diagram of co-inertia analysis of ruminal bacterial communities from 16S rDNA 454 pyrosequences, measures of ruminal fermentation, and percentages of offered grain and fructose from heifers that consumed the following single challenge rations: (1) control (no grain); (2) grain (1.2% of BW DM); (3) grain (1.2% of BW DM) + histidine (6 g/head); (4) grain (0.8% of BW DM) + fructose (0.4% of BW DM) or; (5) grain (0.8% of BW DM) + fructose (0.4% of BW DM) + histidine (6 g/head) (n of heifers = 6/group). Ruminal fluid was collected over approximately a 3.6-h period after (n of samples = 18/group). On the bi-plot the ruminal fermentation measures are represented as arrows. The direction of the arrow of each ruminal fermentation measure indicates an increasing concentration of that measure. The angle between the arrows indicates their degree of correlation. The magnitude of the arrows indicates the importance of the measure on the bacterial community composition. Measures with long arrows are more strongly correlated with the ordination axes than short arrows and have a greater influence on the pattern of variation (Carberry et al., 2012).

Bacteria Prevalence

A total of 16 phyla and 1 candidate phylum were identified within the ruminal bacterial population from 16S rDNA gene pyrosequences. The majority of recovered sequences were represented by the Firmicutes or Bacteroidetes phyla, accounting for a combined total of 90% of sequences: 58 and 32% of total recovered sequences, on average, respectively (data not shown). The candidate phylum, TM7, represented 4.0% and the phylum, Tenericutes, represented 2.5% of the total recovered sequences, on average. The remaining microbiota were composed of phyla with low relative abundances (<1.3% of total recovered sequences on average) and were not present in heifers from all groups (data not shown). A total of 36 bacterial families were identified and the Prevotellaceae, Ruminococcaceae, and Lachnospiraceae families had the largest relative abundances: 16, 16, and 12%, respectively (data not shown). However, the Unclassified Clostridiales accounted for 17% of the total sequences, on average, when the relative abundance of bacterial families were examined. The relative abundance of the Lactobacillaceae family was below our reported threshold. A total of 55 genera were identified. The *Prevotella* were the predominant genus and represented approximately 16% of the relative abundance of bacterial sequences, ranging from an average of 13 to 21% among groups (data not shown). *Escherichia*, *Megasphaera*, and *Allisonella* were not identified during taxonomic assignment but may have been present and not assigned taxonomy.

The relative abundance results of ruminal bacterial phyla and families are displayed in Tables 4 and 5, respectively. These results include (1) the significance of the model interactions: grain, fructose nested within grain (fructose), and histidine nested within grain (histidine) × time; (2) the main effects of grain, fructose, and histidine; and (3) the predicted means ± standard error of the mean for the main effects. Results are only displayed for ruminal bacterial phyla or families that have relative abundances of >0.3% in at least 1 group. Predicted means of relative abundances ± standard error of the mean for all ruminal bacterial phyla or families that have significant ($P < 0.001$) interactions × time are graphed in Figures 4 and 5, respectively. Within- and among-group variation in relative abundance was high for a number of bacterial phyla and families, which reduced the number of significant effects (Tables 4 and 5).

Table 4. Predicted means ± SEM, and main effects and their interactions of grain, fructose nested within grain, histidine nested within grain, and time for bacterial phyla identified from 16S rDNA recovered sequences with mean relative abundances of >0.3% in at least one treatment group

Item	Group mean relative abundance (%) ± SEM and *P*-values[1]							Interactions (*P*-values)		
	Grain (G)		Fructose (F)		Histidine (H)					
	–	+	–	+	–	+	Time (T)	G × T	F × T	H × T
No. of animals	6	24	12	12	12	12				
Bacterial phylum										
Firmicutes	52.3 ± 3.6	58.9 ± 1.9 0.132	59.7 ± 2.7	58.1 ± 2.7 0.683	58.2 ± 2.7	59.5 ± 2.7 0.738	0.122	0.999	0.008	0.914
Bacteroidetes	40.0 ± 4.3	30.0 ± 2.2 0.052	28.5 ± 3.1	31.5 ± 3.1 0.486	31.5 ± 3.1	28.5 ± 3.1 0.495	0.244	0.882	<0.001	0.672
TM7	2.7 ± 1.6	4.3 ± 0.8 0.350	5.4 ± 1.1	3.2 ± 1.1 0.162	3.5 ± 1.1	5.2 ± 1.1 0.261	0.586	0.588	0.204	0.211
Firmicutes:Bacteroidetes	1.67 ± 0.6	2.7 ± 0.3 0.100	3.0 ± 0.4	2.4 ± 0.4 0.253	2.6 ± 0.4	2.8 ± 0.4 0.748	0.281	0.832	0.003	0.249
Tenericutes	1.79 ± 0.4	2.6 ± 0.2 0.067	2.6 ± 0.3	2.6 ± 0.3 0.949	2.6 ± 0.3	2.7 ± 0.3 0.802	0.067	0.763	0.704	0.156
Chloroflexi	0.89 ± 0.3	1.3 ± 0.2 0.233	1.2 ± 0.3	1.4 ± 0.3 0.573	1.1 ± 0.3	1.5 ± 0.3 0.208	0.151	0.617	0.457	0.631
Actinobacteria	1.04 ± 0.2	1.2 ± 0.1 0.936	1.2 ± 0.2	0.9 ± 0.2 0.197	1.1 ± 0.2	1.1 ± 0.2 0.966	0.003	0.169	0.016	0.636
Proteobacteria	0.48 ± 0.2	0.6 ± 0.1 0.602	0.4 ± 0.1	0.8 ± 0.1 0.037	0.6 ± 0.1	0.5 ± 0.1 0.517	0.484	0.071	0.008	0.044
Spirochaetes	0.27 ± 0.2	0.5 ± 0.1 0.234	0.4 ± 0.1	0.5 ± 0.1 0.528	0.5 ± 0.1	0.5 ± 0.1 0.745	0.987	1.000	0.093	0.435
Lentisphaerae	0.09 ± 0.3	0.3 ± 0.1 0.072	0.2 ± 0.1	0.4 ± 0.1 0.058	0.4 ± 0.1	0.2 ± 0.1 0.030	0.149	0.907	0.288	0.188

[1]*P*-values are for the comparison of grain (–/+), the comparison of fructose (–/+) nested within grain, and the comparison of histidine (–/+) nested within grain.

The relative abundance of bacterial phyla was not influenced in grain-fed heifers over time, compared to control heifers (Table 4). Grain also had no main effect on the relative abundance of bacterial phyla, compared to controls. However, the relative abundance of the Bacteroidetes (P = 0.052) and Tenericutes (P = 0.067) approached a significant decrease and increase, respectively, in grain-fed heifers compared to control heifers (Table 4). Time did not influence the relative abundance of bacterial phyla, with the exception of a trend toward an increase in the relative abundance of the Tenericutes (P = 0.067) at the 115-, compared to the 5- and 215-min samplings (Table 4).

The relative abundance of the Firmicutes, Bacteroidetes, ratio of Firmicutes to Bacteroidetes, Actinobacteria, and Proteobacteria changed over time in the fructose- compared to non-fructose-fed heifers (Table 4; Figure 4A to E, respectively). Fructose tended to increase the relative abundance of the Lentisphaerae, compared to the relative abundance in the non-fructose-fed heifers (P = 0.058; Table 4).

The Proteobacteria were the only bacterial phylum that were influenced in relative abundance in the histidine- compared to non-histidine-fed heifers over time (Table 4; Figure 4F). The relative abundance of the Lentisphaerae was decreased in the histidine-, compared to the non-histidine-fed heifers; whereas, the relative abundance of other bacterial phyla was not influenced among these groups (Table 4).

The relative abundance of the Ruminococcaceae and the Unclassified Rickettsiales differed over time in the grain-fed compared to control heifers (Table 5; Figures 5A and E). Grain-fed heifers had an increased relative abundance of the bacterial families, Unclassified Clostridiales, Clostridiales FamilyXII Incertae, and Erysipelotrichaceae, and tended to increase (P = 0.067) the relative abundance of the Streptococcacaeae, compared to the control heifers (Table 5).

The relative abundance of the Clostridales FamilyXII Incertae, Streptococcacaeae, and Veillonellaceae families increased over time, whereas that of the Unclassified RF39 decreased. The relative abundance of the Porhyromonadaceae families increased in relative abundance at the 115-, compared to the 5- and 215-min samplings (Table 5).

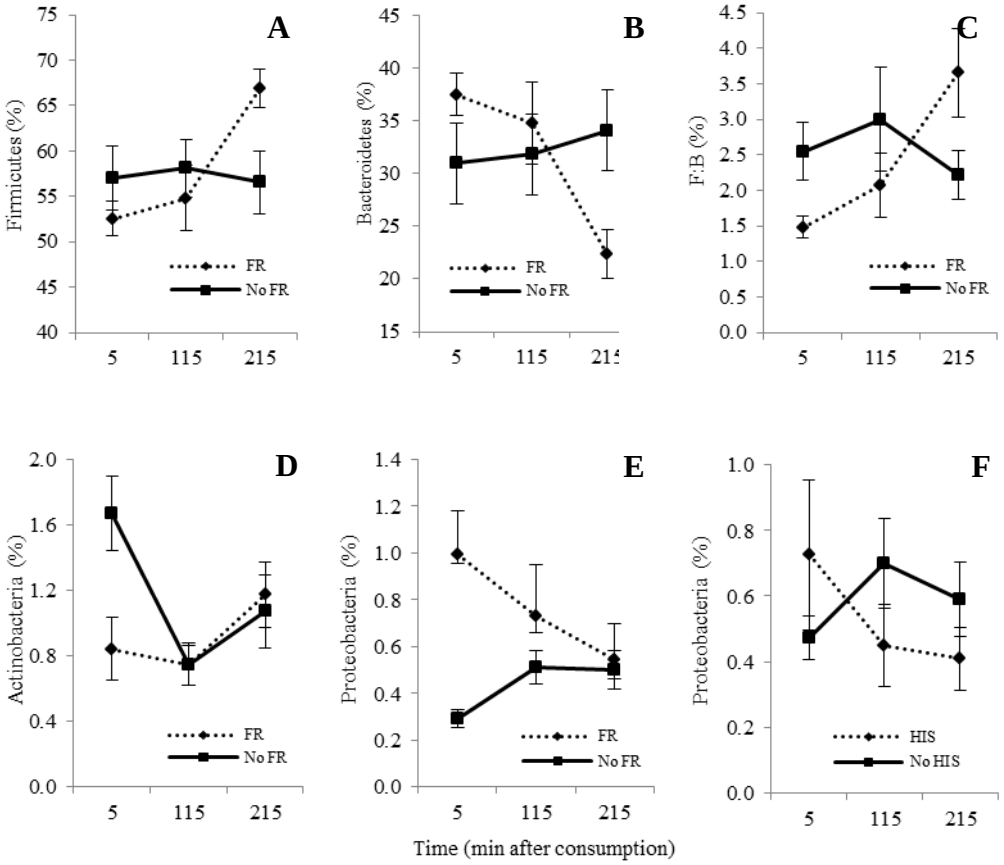

Figure 4. Predicted mean ± SEM percentage of relative abundance of the following ruminal bacterial phyla for fructose- and non-fructose-fed heifers, 5, 115, and 215 min after consumption of their challenge rations: Firmicutes (A); Bacteroidetes (B); Firmicutes:Bacteroidetes (F:B)(C); Actinobacteria (D); Proteobacteria (E); and Proteobacteria in histidine- and nonhistidine-fed heifers. FR = fructose; HIS = histidine.

The relative abundance of the Prevotellaceae and Coriobacteriaceae families and the Unclassified Rickettsiales was influenced over time in the fructose-fed heifers, compared to the non-fructose-fed heifers (Table 5; Figures 5B to D). The relative abundance of the Streptococcacaeae, Veillonellaceae, and Victivallaceae families increased in the fructose- compared to non-fructose-fed heifers and tended to decrease in the Lachnospiraceae family (P = 0.064; Table 5).

The Unclassified Alphaproteobacteria was the only bacterial family that was influenced in relative abundance over time in the histidine-fed heifers, compared to the non-histidine-fed heifers (Table 5; Figure 5F). The relative abundance of the Unclassified Clostridiales and Anaerolinaceae was increased in the histidine- compared to the non-histidine-fed heifers, and decreased in the Victivallaceae family, compared to the non-histidine-fed heifers (Table 5).

A total of 31 OTU's were identified based on their relative abundance as being significantly associated with a treatment group using ANOVA and corrected with the FDR method. The similarity percentage of these OTU's to known bacteria in the Greengenes database from the phylum to the genus level are reported in Table 6.

Of the 31 OTU's identified in the FDR analysis, 11 were identified from the controls and 3, 5, 9, and 3 from the grain, grain + fructose, grain + histidine, and grain + fructose + histidine groups, respectively. The majority of OTU's influenced from the control group belonged to the Lachnospiraceae family and Bacteroidales order. The majority of OTU's for the grain group belonged to the Bacteroidales order, whereas those identified from the treatment groups that received combinations of grain, fructose, and histidine were more diverse and from the Lactobacillales, Bacteroidales, and Clostridiales orders and CW040 from the TM7 candidate phylum. An OTU closely related to *Streptococcus bovis* (OTU No. 4102) had the highest mean value of the OTU's for the heifers from the grain + fructose group and was also increased in the heifers from the grain + fructose + histidine group (Table 6).

OTU No. 1075, identified from the heifers from the grain + fructose group, also had one of the highest mean relative abundances of the identified OTU's and was most closely related to *Levilinea saccharolytica* strain KIBI-1^{T} (Yamada et al., 2006). Two OTU's that differed in the grain + histidine heifers were members of the TM7 phylum and were related to the oral I025 TM7 candidate phylum clone (Table 6).

Table 5. Predicted means ± SEM, and main effects and their interactions of grain, fructose nested within grain, histidine nested within grain, and time for bacterial families identified from 16S rDNA recovered sequences with mean relative abundances of >0.3% in at least one treatment group

Item	Group mean relative abundance (%) ± SEM and *P*-values[1]						Interactions (*P*-values)			
	Grain (G)		Fructose (F)		Histidine (H)					
	–	+	–	+	–	+	Time (T)	G × T	F × T	H × T
No. of animals	6	24	12	12	12	12				
Bacterial family										
Unclassified Clostridiales	13.3 ± 1.6	17.9 ± 1.0 0.049	18.6 ± 1.4	17.1 ± 1.4 0.460	15.8 ± 1.4	20.0 ± 1.4 0.044	0.052	0.559	0.490	0.104
Prevotellaceae	20.6 ± 3.6	15.1 ± 2.0 0.291	16.1 ± 2.8	14.2 ± 2.8 0.632	14.9 ± 2.8	15.4 ± 2.8 0.891	0.709	0.110	<0.001	0.950
Ruminococcaceae	20.3 ± 2.3	15.0 ± 1.2 0.055	14.6 ± 1.7	15.4 ± 1.7 0.746	14.4 ± 1.7	15.6 ± 1.7 0.599	0.120	0.034	0.661	0.646
Unclassified Bacteroidales	19.6 ± 2.8	14.4 ± 1.8 0.175	11.6 ± 2.5	16.7 ± 2.5 0.147	15.8 ± 2.5	12.5 ± 2.5 0.353	0.072	0.065	0.422	0.300
Lachnospiraceae	13.5 ± 2.2	11.9 ± 1.5 0.469	13.7 ± 1.7	10.1 ± 1.7 0.064	12.4 ± 1.8	11.4 ± 1.8 0.599	0.532	0.431	0.175	0.424
Clostridiales FamilyXIII Incertae	2.2 ± 1.1	4.5 ± 0.8 0.039	4.8 ± 0.9	4.2 ± 0.9 0.489	4.9 ± 0.9	4.0 ± 0.9 0.370	0.047	0.682	0.207	0.701
F16	2.8 ± 1.6	4.3 ± 0.8 0.350	5.4 ± 1.1	3.3 ± 1.1 0.162	3.5 ± 1.1	5.2 ± 1.1 0.260	0.584	0.588	0.207	0.211
Streptococcaceae	0.6 ± 1.7	3.9 ± 0.8 0.067	1.8 ± 1.1	6.0 ± 1.1 0.016	4.9 ± 1.1	2.9 ± 1.1 0.217	0.001	0.152	0.003	0.272
Catabacteriaceae	1.7 ± 0.8	3.6 ± 0.4 0.088	3.8 ± 0.6	3.3 ± 0.6 0.576	3.8 ± 0.6	3.3 ± 0.6 0.593	0.854	0.449	0.816	0.467
Erysipelotrichaceae	0.8 ± 0.4	1.7 ± 0.2 0.046	1.8 ± 0.3	1.5 ± 0.3 0.443	3.8 ± 0.3	1.6 ± 0.3 0.546	0.938	0.956	0.810	0.395

[1]*P*-values are for the comparison of grain (–/+), the comparison of fructose (–/+) nested within grain, and the comparison of histidine (–/+) nested within grain.

Table 5 (continued). Predicted means ± SEM, and main effects and their interactions of grain, fructose nested within grain, histidine nested within grain, and time for bacterial families identified from 16S rDNA recovered sequences with mean relative abundances of >0.3% in at least one group

Item	Group mean relative abundance (%) ± SEM and *P*-values[1]							Interactions (*P*-values)		
	Grain (G)		Fructose (F)		Histidine (H)					
	–	+	–	+	–	+	Time (T)	G × T	F × T	H × T
No. of animals	6	24	12	12	12	12				
Bacterial family										
Anaerolinaceae	0.9 ± 0.3	1.3 ± 0.2 0.523	1.2 ± 0.3	1.4 ± 0.3 0.762	1.1 ± 0.3	1.5 ± 0.3 0.045	0.232	0.451	0.565	0.109
Coriobacteriaceae	1.0 ± 0.2	1.0 ± 0.1 0.991	1.1 ± 0.2	0.9 ± 0.2 0.310	1.0 ± 0.2	1.0 ± 0.2 0.714	0.004	0.107	0.036	0.836
Unclassified Rickettsiales	0.1 ± 0.0	0.9 ± 0.0 0.919	0.1 ± 0.0	0.1 ± 0.0 0.025	0.1 ± 0.0	0.1 ± 0.0 0.174	0.199	0.028	<0.001	0.675
Carnobacteriaceae	0.1 ± 1.0	0.8 ± 0.7 0.543	0.8 ± 0.8	0.7 ± 0.8 0.928	0.7 ± 0.9	0.9 ± 0.9 0.858	0.132	0.597	0.991	0.999
Porphyromonadaceae	0.8 ± 0.3	0.7 ± 0.2 0.933	0.8 ± 0.2	0.7 ± 0.2 0.729	0.8 ± 0.2	0.6 ± 0.2 0.574	<0.001	0.892	0.626	0.432
Clostridiaceae	0.2 ± 0.2	0.6 ± 0.1 0.191	1.1 ± 0.2	0.4 ± 0.2 0.158	1.0 ± 0.2	0.7 ± 0.2 0.270	0.131	0.859	0.696	0.437
Unclassified RF39	0.5 ± 0.1	0.5 ± 0.1 0.908	0.6 ± 0.1	0.5 ± 0.1 0.127	0.5 ± 0.1	0.6 ± 0.1 0.307	0.003	0.064	0.587	0.187
Spirochaetaceae	0.2 ± 0.2	0.4 ± 0.1 0.150	0.3 ± 0.1	0.5 ± 0.1 0.315	0.5 ± 0.1	0.4 ± 0.1 0.363	0.806	0.976	0.223	0.673
Eubacteriaceae	0.2 ± 0.1	0.4 ± 0.0 0.071	0.4 ± 0.1	0.4 ± 0.1 0.922	0.5 ± 0.1	0.3 ± 0.1 0.086	0.235	0.350	0.105	0.106
Veillonellaceae	0.3 ± 0.1	0.3 ± 0.1 0.907	0.1 ± 0.1	0.5 ± 0.1 0.005	0.4 ± 0.1	0.2 ± 0.1 0.205	0.021	0.856	0.071	0.535
Unclassified Alphaproteobacteria	0.3 ± 0.1	0.3 ± 0.1 0.810	0.2 ± 0.1	0.5 ± 0.1 0.164	0.4 ± 0.1	0.3 ± 0.1 0.570	0.136	0.388	0.198	0.012
Victivallaceae	0.1 ± 0.1	0.3 ± 0.1 0.073	0.2 ± 0.1	0.4 ± 0.1 0.046	0.4 ± 0.1	0.2 ± 0.1 0.031	0.144	0.918	0.241	0.200

[1]*P*-values are for the comparison of grain (–/+), the comparison of fructose (–/+) nested within grain, and the comparison of histidine (–/+) nested within grain.

Table 6. Operational taxonomic units (OTU) that differ by mean relative abundance among treatment groups identified by false discovery rate analysis

OTU No.	Treatment group relative mean abundance (x 10^{-3}; %)[1]					Group[2]	Taxonomic assignment				
	Control	GR	GR + FR	GR + HIS	GR + FR + HIS		Phylum	Class	Order	Family	Genus
27	6.5×10^{-3}	9.7×10^{-4}	3.1×10^{-4}	2.8×10^{-4}	1.1×10^{-3}	control	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
55	4.8×10^{-3}	5.7×10^{-4}	8.2×10^{-4}	9.8×10^{-4}	1.8×10^{-3}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
115	2.4×10^{-3}	2.2×10^{-4}	6.3×10^{-4}	1.2×10^{-3}	5.1×10^{-4}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
125	1.4×10^{-3}	6.9×10^{-4}	2.6×10^{-4}	3.4×10^{-4}	6.4×10^{-4}	control	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unassigned
188	1.5×10^{-3}	3.4×10^{-5}	1.4×10^{-4}	5.9×10^{-4}	3.6×10^{-4}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
683	1.0×10^{-3}	1.2×10^{-4}	1.7×10^{-4}	2.2×10^{-4}	1.8×10^{-4}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
694	3.7×10^{-3}	7.7×10^{-4}	6.5×10^{-5}	2.5×10^{-4}	8.6×10^{-4}	control	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
771	6.9×10^{-3}	1.6×10^{-5}	1.0×10^{-4}	1.8×10^{-4}	2.2×10^{-4}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
1047	6.0×10^{-4}	4.0×10^{-5}	9.9×10^{-5}	1.8×10^{-4}	8.3×10^{-5}	control	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
2010	4.2×10^{-3}	9.5×10^{-4}	4.4×10^{-4}	4.5×10^{-4}	1.3×10^{-3}	control	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
6567	8.9×10^{-4}	2.0×10^{-4}	3.8×10^{-5}	7.5×10^{-5}	1.9×10^{-4}	control	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
39	2.2×10^{-4}	7.4×10^{-3}	1.1×10^{-4}	4.3×10^{-5}	7.0×10^{-4}	grain	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
262	5.2×10^{-4}	1.8×10^{-3}	1.1×10^{-4}	0.0[3]	4.0×10^{-5}	grain	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
430	1.3×10^{-4}	8.3×10^{-4}	3.9×10^{-4}	7.6×10^{-5}	0.0[3]	grain	Firmicutes	Clostridia	Clostridiales	ClostridialesFamily XIII.IncertaeSedis	Unassigned

[1]Control (no grain); GR = (crushed triticale 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = Grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head).
[2]Treatment group to which the OTU is associated.
[3]Zero or not detected.

Table 6 (continued). Operational taxonomic units (OTU) that differ by mean relative abundance among treatment groups identified by false discovery rate analysis

OTU No.	Treatment group relative mean abundance (x 10^{-3}; %)[1]					Group[2]	Taxonomic assignment				
	Control	GR	GR + FR	GR + HIS	GR + FR + HIS		Phylum	Class	Order	Family	Genus
199	2.0×10^{-4}	2.6×10^{-4}	1.2×10^{-3}	3.4×10^{-4}	1.7×10^{-4}	grain+fructose	Bacteroidetes	Bacteroidia	Bacteroidales	Unassigned	Unassigned
205	4.5×10^{-4}	5.5×10^{-4}	1.3×10^{-3}	1.7×10^{-4}	3.0×10^{-4}	grain+fructose	TM7	TM7-3	CW040	F16	Unassigned
462	2.1×10^{-4}	5.6×10^{-4}	7.4×10^{-4}	5.5×10^{-4}	0.0[3]	grain+fructose	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
1075	2.5×10^{-4}	5.8×10^{-4}	1.8×10^{-3}	9.2×10^{-4}	4.4×10^{-4}	grain+fructose	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
4102	2.4×10^{-4}	1.3×10^{-3}	5.7×10^{-3}	1.2×10^{-3}	2.5×10^{-3}	grain+fructose	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
50	5.8×10^{-4}	2.9×10^{-3}	4.6×10^{-4}	4.0×10^{-3}	1.3×10^{-3}	grain+histidine	TM7	TM7-3	CW040	F16	Unassigned
149	3.5×10^{-4}	3.1×10^{-4}	9.0×10^{-4}	1.8×10^{-3}	5.8×10^{-4}	grain+histidine	Firmicutes	Clostridia	Clostridiales	ClostridialesFamily XIII.IncertaeSedis	Unassigned
195	1.8×10^{-4}	3.6×10^{-4}	2.6×10^{-4}	1.1×10^{-3}	2.2×10^{-4}	grain+histidine	Firmicutes	Clostridia	Clostridiales	Unassigned	Unassigned
197	9.8×10^{-5}	4.5×10^{-5}	3.8×10^{-5}	2.4×10^{-3}	8.8×10^{-4}	grain+histidine	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
208	2.9×10^{-4}	1.5×10^{-4}	5.3×10^{-4}	1.3×10^{-3}	4.8×10^{-4}	grain+histidine	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unassigned
288	1.6×10^{-4}	1.5×10^{-4}	4.2×10^{-4}	8.8×10^{-4}	3.1×10^{-4}	grain+histidine	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
402	3.3×10^{-5}	2.0×10^{-4}	3.2×10^{-4}	9.6×10^{-4}	5.5×10^{-5}	grain+histidine	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Unassigned
1443	4.2×10^{-5}	6.6×10^{-5}	4.3×10^{-4}	1.3×10^{-3}	1.3×10^{-4}	grain+histidine	Firmicutes	Clostridia	Clostridiales	Unassigned	Unassigned
3852	1.9×10^{-4}	5.6×10^{-4}	9.5×10^{-4}	2.1×10^{-3}	7.3×10^{-4}	grain+histidine	TM7	TM7-3	CW040	F16	Unassigned
230	6.4×10^{-5}	3.0×10^{-4}	2.0×10^{-4}	2.4×10^{-4}	1.6×10^{-3}	grain+fructose+histidine	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio
270	8.4×10^{-5}	3.6×10^{-5}	2.2×10^{-4}	3.0×10^{-4}	1.2×10^{-3}	grain+fructose+histidine	Firmicutes	Clostridia	Clostridiales	Unassigned	Unassigned
1524	7.7×10^{-4}	5.6×10^{-4}	3.7×10^{-4}	2.7×10^{-4}	1.3×10^{-3}	grain+fructose+histidine	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Unassigned

[1]Control (no grain); GR = (crushed triticale 1.2% of BW DMI); GR + FR = grain (0.8% of BW DMI) + fructose (0.4% of BW DMI); GR + HIS = Grain (1.2% of BW DMI) + histidine (6 g/head); GR + FR + HIS = Grain (0.8% of BW DMI) + fructose (0.4% of BW DMI) + histidine (6 g/head).
[2]Treatment group to which the OTU is associated.
[3]Zero or not detected.

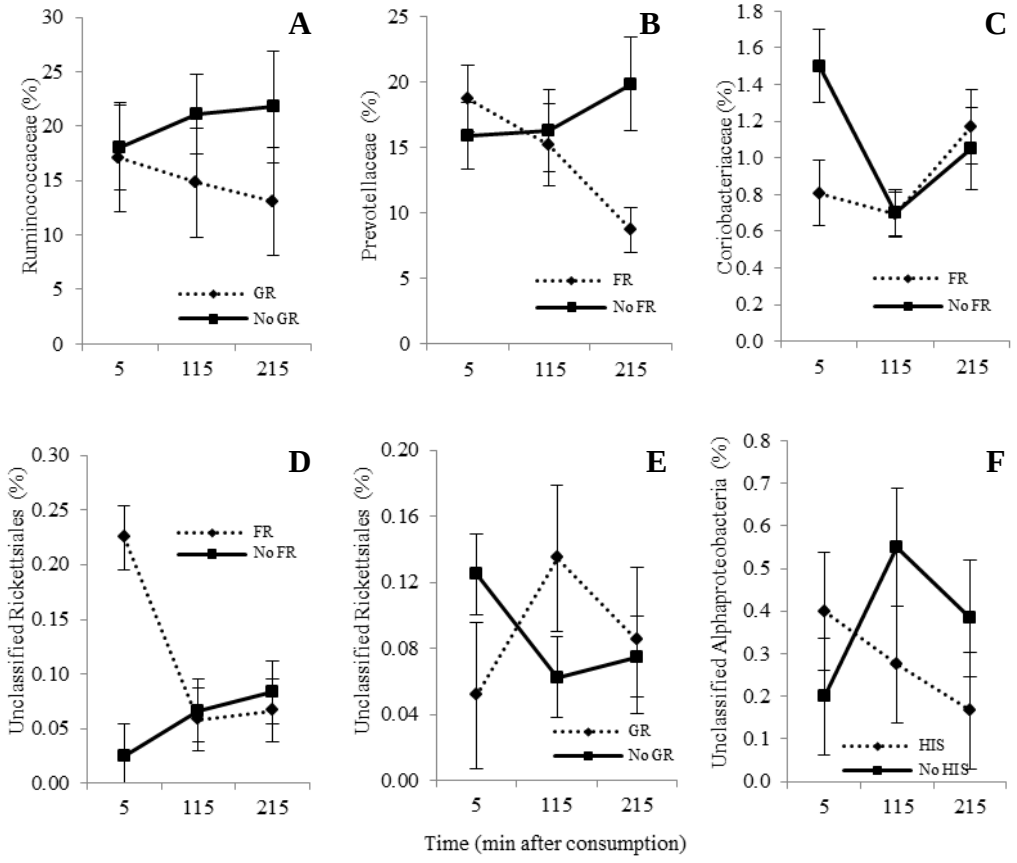

Figure 5. Predicted mean ± SEM percentage of relative abundance of the following ruminal bacterial families 5, 115, and 215 min after consumption of their challenge rations: Ruminococcaceae in grain and non-grain-fed (control) heifers (A); Prevotellaceae in fructose- and non-fructose fed heifers, (B); Coriobacteriaceae in fructose and non-fructose-fed heifers (C); Unclassified Rickettsiales in fructose- and non-fructose-fed heifers (D); Unclassified Rickettsiales in grain- and non-grain (control) fed heifers (E) and; Unclassified Alphaproteobacteria in histidine- and non-histidine-fed heifers. GR = grain (no GR is the control); FR = fructose; HIS = histidine.

DISCUSSION

This study showed that changes in BCC occurred in forage-fed cattle within approximately 3.6 h of exposure to a single, substantial, non-life-threatening challenge with starch, fructose, or histidine, and combinations of these, and were associated with ruminal fermentation measures. This novel comparison of ruminal bacterial community change over the initial period after consumption of challenge rations shows the rumen ecosystem can rapidly respond to and buffer abrupt changes in readily fermentable substrate.

The treatment group challenge rations were designed to represent the dietary changes that dairy cattle may be exposed to during the transition period. Effects of the treatment groups on ruminal fermentation, endotoxin, and oxidative stress measures are discussed in Golder et al. (2012) and Golder et al. (2013). Collection of ruminal fluid using a stomach tube is not likely to have influenced bacterial variation, as sampling site and method had little effect on bacterial diversity assessment in other studies (Li et al., 2009; Lodge-Ivey et al., 2009), and all treatment groups were exposed to the same method of collection. However, the authors Li et al. (2009) and Lodge-Ivey et al. (2009) used PCR denaturing gradient gel electrophoresis. Different responses may occur in particulate bacteria as opposed to those in the liquid phase of the rumen examined in our study.

The bacteria *Lactobacillus* spp., and *Megasphaera elsdenii* that are commonly associated with onset or prevention of ruminal acidosis and belong to the Firmicutes phylum (Hungate, 1966; Bergey et al., 2011), were not identified as one of the 31 OTU's that differed in the FDR analysis. However, S. bovis was associated and became more prevalent in heifer fed fructose, indicating that S. bovis rapidly responds diet and forms the initial changes to the ruminal microbiome when unadapted cattle are fed single, abrupt exposures to readily fermentable carbohydrates.

The large among- and within-group variation in bacterial communities, indicated in the constrained between group PCoA, is consistent with the considerable diversity in bacterial populations in ruminants fed the same diets (Brulc et al., 2009; Li et al., 2009; Chen et al., 2012). However, the bacterial phyla and families that dominated the rumen were consistent among treatment groups. This supports the concept that the rumen ecosystem is host specific but is comprised of a 'core rumen microbiome' (Hernandez-

Sanabria et al., 2010; Jami and Mizrahi, 2012) that has a unique ability to adapt to different substrates and may contribute to a host's individual susceptibility to disorders such as ruminal acidosis. Further, Weimer et al., (2010) showed that when >95% of ruminal fluid with differing pH, total VFA concentration, and BCC from 2 cows fed the same diet was exchanged, the ruminal pH, total VFA, and bacterial biomes of the 2 cows returned to their original profiles within 24 h. While significant changes in relative abundances in our study occurred among treatment groups for some bacterial phyla and families, the variation resulted in only numerical changes for others. Variation in bacterial communities between cattle may pose difficulties for control of ruminal acidosis and emphasizes the need for large numbers of cattle for *in vivo* studies. Chen et al. (2012) identified different biodiversity and differing total bacterial copy number of 16S rRNA genes between ruminal acidosis resistant and susceptible cattle. Those findings (Chen et al., 2012) and findings of this study suggest that opportunities may exist to select for cattle with particular BCC to, for example, reduce the risk of ruminal acidosis. as in order to for example reduce the risk of ruminal acidosis. The variation also suggests diets and preventive strategies may be most effective when tailored to individual cattle.

Bacteria from the Bacteroidetes (predominately gram negative) and Firmicutes phyla (predominately gram positive) dominated the core microbiome in all cattle, consistent with other studies (Tajima et al., 2000; Kong et al., 2010). The most well-studied members of the Bacteroidetes are those from the Bacteroidia class and include the *Prevotella* genus, which were the dominant genus in the current study, consistent with other studies feeding various forages and concentrates at different ratios (Tajima et al., 2000; Fernando et al., 2010; Jami and Mizrahi, 2012). The large genetic diversity of *Prevotella* and their capacity to selectively utilize a large variety of substrates; sugars, starches, hemicellulose, pectin, proteins, amino acids, and peptides allows them to dominate a range of diets and to thrive in the predominately liquid phase of the rumen that was collected.

The presence of the gram positive candidate phylum, TM7, as the third most prevalent phyla is of interest. It had a raw mean relative abundance of >2.5-fold higher in the grain + histidine-fed cattle, compared to controls; however, its relative abundance was not affected by specific substrates in the partial factorial analysis. TM7 candidate phyla

members are found in many ecosystems but, to date, cannot be cultured; they were originally described in soil, aquatic environments, sludge reactors, and, more recently, in periodontitis and inflammatory bowel disease (Rheims et al., 1996; Hugenholtz et al., 2001; Brining et al., 2003; Kuehbacher et al., 2008). The TM7 members have also been identified in dairy cattle studies (Khafipour et al., 2009; Kong et al., 2010; Hook et al., 2011; Golder, et al., 2014). Knowledge of TM7's substrate utilization and end products are limited but recent genome assemblies from metagenomic data suggest a limited fermentative pathway producing lactate and acetate (Albertsen et al., 2013). Lactate values in the heifers fed histidine were not elevated even though TM7 numbers increased >2.5 fold. Genomes assembled for TM7 are small and suggest either genome reduction or a reliance on other organisms for key biosynthetic pathways (Kantor et al., 2013). Six Clostridiales and a Bacteroidales bacteria also associated with the histidine treatment, showed a similar pattern in abundance changes between the treatments to that of the TM7 OTU's. Correlation networks to further investigate this will be undertaken and may provide some evidence to a symbiotic relationship.

The Proteobacteria were lower in relative abundance than generally reported in dairy cattle (Tajima et al., 1999; Tajima et al., 2000). Abundances of Proteobacteria can be very variable (Jami and Mizrahi, 2012) and differences in diet between this and other studies may account for the relatively low abundance of this gram negative phylum. The increase in relative abundance of the Proteobacteria in the fructose-fed heifers is consistent with the association between dietary sugar and the relative abundance of the genus, *Desulfobulbus,* belonging to the Proteobacteria phylum (Thoetkiattikul et al., 2013). *Escherichia coli,* a member of the Proteobacteria, which can be responsible for endotoxin release and has been associated with subacute ruminal acidosis induced by grain or alfalfa hay (Khafipour et al., 2009), were not identified (or were at levels below those detected by pyrosequencing), which is consistent with the lack of effect of treatment group on ruminal endotoxin concentrations in these heifers (Golder et al., 2013).

The phylum Fibrobacteres, considered to be critical for fiber degradation, had a relative abundance below our reported threshold. Fibrobacter species are often underrepresented in 16S rDNA libraries from rumen environments (Tajima et al., 1999, 2000, 2001). An absence of this bacterial phylum in beef steers on a restricted diet of medium-quality

grass-legume hay (Brulc et al., 2009) and detection in only half of the samples from cattle fed on a prolonged diet of 30% roughage and 70% concentrate (Jami and Mizrahi, 2012), supports our findings. With reduced forage material in the rumen due to the low NDF of our challenge diets and the 14-h period of diet withholding before challenge, the rumen samples collected contained only small particulate matter and predominately fluid. It is plausible that Fibrobacter species would be in higher relative abundance on a targeted investigation of the solid material remaining in the rumen.

The large percentage of bacteria from the Clostridiales and Bacteroidales orders that were not classified to the family level is consistent with others (de Menezes et al., 2011; Thoetkiattikul et al., 2013). It emphasizes that despite the rapidly advancing field of rumen microbiology, the diversity of bacteria that remain uncultured and unidentified is large and it is very possible that several of these bacteria are involved in ruminal acidosis.

The predominance of members of the Prevotellaceae, Ruminococcaceae, and Lachnospiraceae families is consistent with other bovine studies (de Menezes et al., 2011; Thoetkiattikul et al., 2013). The members of the Prevotellaceae family are gram negative bacteria that belong to the Bacteroidetes phylum and can utilize a range of substrates (Boone et al., 2011), whereas members of the Ruminococcaceae, and Lachnospiraceae families belong to the Firmicutes phylum and are largely cellulolytic and fibrolytic bacteria, respectively (Thoetkiattikul et al., 2013). The trend toward a decrease in relative abundance of Lachnospiraceae in the fructose-fed heifers is not consistent with the large increase in abundance of this bacterial family between cattle with mild and severe grain-induced ruminal acidosis (Khafipour et al., 2009). This difference may reflect substrate differences between our study and that of Khafipour et al. (2009) and our short 3.6-h sampling period after consumption of the challenge ration.

Ruminal BCC was associated with ruminal fermentation, consistent with others (Hernandez-Sanabria et al., 2010; Carberry et al., 2012). However, dietary effects on BCC have not always occurred, even when production effects were evident (Mullins et al., 2013). Mohammed et al. (2012) reported that shifts in BCC were not related to total VFA concentrations or individual proportions of VFA, milk yield, and milk composition results, DMI, or severity of ruminal acidosis in cows in the transition period. However, dietary changes in the study by Mohammed et al. (2012) were more subtle than those in

the current study. The association between the bacterial communities in the fructose-fed heifers and increased concentrations of ruminal butyrate and lactate suggest that fructose promotes bacterial communities that lead to less favorable ruminal conditions and an increased risk of ruminal acidosis within a short time after exposure. The grain was likely to have been fermented more slowly relative to the fructose (Firkins, 2011; Golder et al., 2012) by existing bacterial communities associated with increased concentrations of ruminal valerate, ammonia, and histamine, as minimal effects of the relative abundance of bacterial phyla and families were observed between the grain-fed heifers and the controls. Limited literature exists on the effects of sugars on bacterial composition; however, approximately half the amount of glucose is required to induce clinical ruminal acidosis compared to grain (Nagaraja and Titgemeyer, 2007). The relative abundances of gram negative bacteria are proposed to be replaced with abundances of gram positive bacteria during ruminal acidosis (Nagaraja and Titgemeyer, 2007), which may explain the increase in the Firmicutes to Bacteroidetes ratio in the fructose-fed heifers and the absence of effect in the grain-fed heifers. The rumen appears to be better adapted to changes in grain than sugar and diets with a high sugar content should be approached with caution.

Fructose is a substrate for fermentation for most heterofermentative *Lactobacilli* spp., which produce at least half of their end product carbon as lactate (Schleifer, 2009); hence, the relative abundance of the Lactobacillaceae family was anticipated to increase in the fructose-fed heifers, which did not occur. Similarly, *Lactobacillus* spp. were not identified as similarity matches to OTU's that differed among groups. The decline in lactate production over the sampling period (Golder et al., 2012) and ruminal pH >6.0 may indicate that ruminal conditions were not conducive to the growth of *Lactobacillus*, as proliferation generally occurs at pH <5.0 (Schleifer, 2009). The short time frame during which sampling occurred after consumption of the challenge ration may have also contributed to lack of change and could suggest *Lactobacilli* do not have a major role in lactate production in the initial hours after substrate intake in unadapted cattle. Other bacteria that can produce lactate, such as *Levilinea*, were identified as shifting in relative abundance, and could have a role in the pathogenesis of ruminal acidosis.

The increase in relative abundance of the Streptococcaceae in the fructose-fed heifers and the identification of OTU 4102, which is closely related to *S. bovis*, is consistent

with the increase in concentrations of ruminal D- and L-lactate and ruminal pH above 6 in these heifers and increased abundances of this bacterial family in dairy cattle during the transition period (Wang et al., 2012). The increase in relative abundance of the Streptococcaceae in the fructose-fed heifers over time is consistent with the known rapid growth rate of *S. bovis* when readily fermentable carbohydrates are available (Russell and Robinson, 1984). *Streptococcus bovis* has been suggested to be the causative agent of ruminal acidosis; however, *S. bovis*, has not always increased or even been identified in grain-fed cattle (Tajima et al., 2000; Klieve et al., 2003), and is not always the main cause of ruminal acidity (Hungate, 1966). It has also not been studied when sugars are fed.

The mesophilic *Levilinea saccharolytica* strain KIBI-1^T, to which OTU No. 1075 from the grain + fructose group is related, can utilize fructose and produce acetate, pyruvate, hydrogen, and small amounts of lactate as its major fermentation products on a medium with glucose (20 m*M*) and yeast extract (0.1%; Yamada et al., 2006). Hence, this organism may have contributed to the increase in concentrations of ruminal D- and L-lactate and the trend toward increased concentrations of ruminal acetate (P = 0.065) in the fructose groups and the highest D- and L-lactate concentrations in the grain + fructose group of heifers (Golder et al., 2012). However, OTU No. 1075, identified as increasing, is only distantly related to the *Levilinea* genus.

The Victivallaceae family require sugars to grow (Janssen and Hedlund, 2011), which explains their increase in relative abundance in the fructose-fed heifers. The sole genus of this family, *Victivallis*, can use fructose as its only energy and carbon source and produces acetate, ethanol, H_2, and bicarbonate as fermentation products from glucose (Janssen and Hedlund, 2011).

The increase in relative abundance of bacteria from the Veillonellaceae family in the fructose-fed heifers and these heifers over time was anticipated, given that these heifers had the highest concentrations of ruminal lactate (Golder et al., 2012). Some species belonging to this family, such as *Megasphaera elsdenii*, *Selenomonas ruminantium,* and *Veillonella parvula,* utilize lactate (Stewart et al., 1997); however, the *Megasphaera* and *Veillonella* genera were not detected and *Selenomonas* were present in a very low abundance. These lactate utilizing species increase with adaptation to readily fermentable carbohydrate diets (Huber, 1976), which may explain their absence from

our samples that were collected from unadapted cattle. These lactate-utilizing species are relatively slow growing and increase with adaptation to readily fermentable carbohydrate diets (Huber, 1976), which may explain their absence from our samples that were collected only 3.6 h after consumption of readily fermentable carbohydrates in unadapted cattle. Thus, unadapted cattle are likely to be at a higher risk of accumulation of lactate in the rumen and risk of ruminal acidosis than those adapted to substrates.

The changes in bacterial community in the histidine-fed heifers were greater than suggested from the minimal responses in ruminal fermentation measures. The increase in the relative abundance of the Unclassified Clostridiales appeared responsible for the shift in bacterial community within these heifers. Perhaps several bacteria from this order can utilize histidine to a range of fermentation products, which are subsequently utilized by other bacteria, explaining the absence of effects on ruminal fermentation measures. It is known histidine can be fermented to histamine (Garner et al., 2002), acetate, and butyrate (Chen and Russell, 1989). The decrease in relative abundance of bacteria from the Lentisphaerae phylum in the histidine-fed groups may reflect an inability of these bacteria to utilize amino acids (Cho et al., 2004).

An increase in histamine-producing bacteria was anticipated, as ruminal histamine concentrations approached a significant increase ($P = 0.054$) in the histidine-fed heifers and were approximately 2-fold higher than those of the controls (Golder et al., 2012). The absence of identification of *Allisonella histaminiformans*, which solely utilizes histidine as an energy source (Garner et al., 2002), and absence or very low abundance of several other bacteria such as *Lactobacillus* spp., *Bacterium coli, Clostridium* spp., and *Proteus* spp. which produce histidine decarboxylase that decarboxylates histidine to histamine (Gale, 1940; Gale et al., 1941; Schelp et al., 2001), suggests these may not be key bacteria involved in histamine generation over the challenge. It is possible that other bacteria are capable of histamine generation and production of histamine is a sufficiently more widely distributed trait and the role of any single species is limited.

The stronger relationship between histamine concentration and bacterial composition in the grain compared with either histidine-fed group suggests that generation of histamine from grain had a greater influence on BCC than the supplemented histidine over the time period. This is consistent with increased concentrations of ruminal histamine in concentrate-, compared to hay-fed cattle (Fuquay et al., 1969). The increase in relative

abundance of bacteria from the Anaerolinaceae family in the histidine-fed heifers may be of interest. Information on this bacterial family in ruminants is limited; however, casamino acids supported only weak growth of mesophilic strains isolated from an anerobic sludge blanket (Yamada et al., 2006).

The *Anaerovorax* genus to which OTU No. 149 is closely related, from the grain + histidine group, are strictly anaerobic chemo-organotrophic bacteria that prefer amino acid derivatives as substrates (Schink, 2009) and have been identified in ruminal samples (Kim et al., 2011). All strains from this genus identified to date fermented only putrescine, 4-aminobutyrate, or 4-hydroxybutyrate as substrates to acetate, butyrate, molecular hydrogen, and ammonia (Matthies et al., 1989; Matthies et al., 2000). As *Anaerovorax* spp. from a non-rumen origin are associated with amino acid degradation and the *Anaerovorax* genus has been identified from ruminal samples, we propose it may be associated with histidine degradation in the rumen.

Feeding cycle has been suggested to be the most important factor influencing bacterial community structure, during which there are intermittent supplies of fermentable energy exist for bacterial growth and catabolism (Welkie et al., 2010). Bacterial populations normally increase after feeding (Bryant and Robinson, 1968) and decline as substrate availability declines as the subsequent feeding approaches (Mullins et al., 2013). Sampling time was a very small vector in the co-inertia analysis, suggesting it did not have as large an influence on BCC as the fermentation measures. Populations of bacteria and growth activity of bacteria may have changed but were not quantified in terms of absolute abundance in our study.

Although it is a challenge to compare, BCC and relative abundance of bacteria differed from those in longer-term challenge studies where cattle were adapted to diets (Khafipour et al., 2009; Callaway et al., 2010; Golder et al., 2014). It is evident that the BCC of the rumen is capable of rapid responses to abrupt exposures to substrates. We hypothesize that ruminal bacteria involved in the initial onset of the pathogenesis of ruminal acidosis may differ from those involved in the later stages of ruminal acidosis and are likely to differ between adapted and unadapted cattle. Differences in BCC may occur between lactating cows and the heifers such as those in this study.

CONCLUSION

This study uniquely examined short-term changes in ruminal bacteria in cattle offered a single, substantial readily fermentable carbohydrate challenge. It exemplifies the dynamic ability of the rumen to cope with abrupt exposures to large amounts of readily fermentable carbohydrates. Our hypothesis was supported that distinct ruminal bacterial communities would begin to develop over 3.6 h after a single challenge feed among heifers fed combinations of grain, fructose, and histidine, and reflect ruminal fermentation measures. Although heifers shared a common core microbiome, variation among heifers was large, suggesting cattle have distinct ruminal bacterial communities that may influence their ability to cope with changes in substrate type and amount. Bacterial community composition of the fructose-fed heifers was more diverse and was associated with ruminal fermentation measures that may pose an increased risk of ruminal acidosis relative to grain. Bacterial communities of grain-fed heifers were associated with increased concentrations of ruminal valerate, ammonia, and histamine. We hypothesize that a large number of bacteria may utilize histidine as a substrate. An OTU identified in the fructose-fed cattle was closely associated with *S. bovis*. We did not identify OTU's with similarity to Lactobacillus spp., and *Megasphaera elsdenii* that differed among treatment groups in this abrupt, single feeding of readily fermentable carbohydrates.

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CHAPTER 5

Effects of Partial Mixed Rations and Supplement Feeding Amounts on Milk Production and Composition, Ruminal Fermentation, Bacterial Communities, and Ruminal Acidosis

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OVERVIEW OF CHAPTER 5

In Chapter 4, ruminal distinct bacterial community composition was observed within the initial 3.6 h after feeding among treatment groups in unadapted cattle challenged with a single feed and these were associated with ruminal fermentation measures. Bacteria closely related to *Streptococcus bovis* were identified in heifers fed grain + fructose in Chapter 4; however, other key bacteria associated with ruminal acidosis in literature were not identified as bacteria that shifted in relative abundance. It was hypothesized that the single challenge feed and short timeframe of sampling may have contributed to the absence of, or absence in change of these bacteria. I was fortunate to be given ruminal samples and access to dry matter intake, ruminal fermentation, and milk production data from the Flexible Feeding System Project (Future Farming Systems Research Division, Department of Environment and Primary Industries, Ellinbank, Victoria, Australia) to test this hypothesis and subsequent hypotheses in Chapter 5.

ABSTRACT

Late lactation Holstein cows (n = 144) that were offered 15 kg dry matter (**DM**)/cow per d of perennial ryegrass to graze were randomized into 24 groups of 6. Each group contained a fistulated cow and groups were allocated to 3 feeding strategies: (1) control (10 groups): cows were fed crushed wheat grain twice daily in the milking parlor and ryegrass silage at pasture; (2) partial mixed ration (**PMR**; 10 groups): PMR that was iso-energetic to the control diet and fed twice daily on a feed pad; (3) PMR+Canola (4 groups): a proportion of wheat in the PMR was replaced with canola meal to produce more estimated metabolizable protein than other groups. Supplements were fed to the control and PMR cows at 8, 10, 12, 14, or 16 kg of DM/d, and the PMR+Canola cows at 14 or 16 kg of DM/d. The PMR-fed cows had a lower incidence of ruminal acidosis compared to controls, and ruminal acidosis increased linearly and quadratically with supplement fed. Yield of milk fat was highest in the PMR+Canola cows fed 14 or 16 kg of total supplement DM/d, followed by the PMR-fed cows, and was lowest in controls fed at these amounts; a similar trend was observed for milk fat percentage. Milk protein yield was higher in the PMR+Canola cows fed 14 or 16 kg of total supplement DM/d. Milk yield and milk protein percentage were not affected by feeding strategy. Milk, energy-corrected milk, and milk protein yield increased linearly with supplement fed; while, milk fat percentage decreased. Ruminal butyrate and D-lactate concentrations, and acetate to propionate ratio, (acetate+butyrate)/propionate, and pH increased in PMR-fed cows, compared to controls for all supplement amounts, whereas propionate and valerate concentrations decreased. Ruminal acetate, butyrate, and ammonia concentrations, the acetate to propionate ratio, (acetate+butyrate)/propionate, and pH linearly decreased with amounts of supplement fed. Ruminal propionate concentration linearly increased and valerate concentration linearly and quadratically increased with supplement feeding amount. The Bacteroidetes and Firmicutes were the dominant bacterial phyla identified. The Prevotellaceae, Ruminococcaceae, and Lachnospiraceae were the dominant bacterial families, regardless of feeding group, and were influenced by feeding strategy, supplement feeding amount, or both. The Veillonellaceae family decreased in relative abundance in PMR-fed cows, compared to controls, and the Streptococcaeae and Lactobacillaceae families were present in only minor relative abundances, regardless of feeding group. Despite large among- and within-group variation in bacterial community composition, distinct bacterial communities occurred

among feeding strategies, supplement amounts, and sample times and were associated with ruminal fermentation measures. Control cows fed 16 kg of DM of total supplement per day had the most distinct ruminal bacterial community composition. Bacterial community composition was most significantly associated with supplement feeding amount and ammonia, butyrate, valerate, and propionate concentrations.

Feeding supplements in a PMR reduced the incidence of ruminal acidosis and altered ruminal bacterial communities, regardless of supplement feeding amount, but did not result in increased milk measures, compared to iso-energetic control diets component-fed to late-lactation cows.

Keywords: bacterial community composition, partial mixed ration, protein, ruminal acidosis, supplements

INTRODUCTION

A TMR fed to cattle on a feed pad between grazing periods is termed a partial mixed ration (**PMR**; Bargo et al., 2002b; Auldist et al., 2013). This feeding strategy increased milk yield and milk fat and protein percentage (Bargo et al., 2002a) and improved marginal milk responses and increased yields of milk fat (Auldist et al., 2013) over those of pasture-fed cows supplemented with grain in the milking parlor and conserved forage fed in the pasture.

Auldist et al. (2013) proposed that a well-formulated PMR that is consumed over a longer period of time could lead to more stable ruminal fermentation compared with when grain is fed in the parlor. The risk of ruminal acidosis may then be reduced, a hypothesis that is supported by a higher ruminal pH in PMR-fed, compared to control cows fed grain in the parlor, silage, and fresh cut pasture (Greenwood et al., 2014).

The substitution of some of the wheat for canola meal in a PMR also increased energy corrected milk (**ECM**) and was associated with a higher concentration and yield of milk fat, and higher pasture dry matter intake (**DMI**; Auldist et al., 2014). Other protein supplements, such as canola meal, have also increased milk yield (Oldham, 1984; Huhtanen et al., 2011; Martineau et al., 2013). Allen et al. (2006) proposed that high protein feeds have a buffering capacity in the rumen, which is consistent with the report of Auldist et al. (2014), in which substituting wheat for canola meal in a PMR decreased the amount of time pH was under 6.0 and increased daily ruminal pH, despite having no influence on volatile fatty acids (**VFA**; Auldist et al., 2014).

Understanding the complex and dynamic ruminal microbial ecosystem (Fernando et al., 2010) is essential to the development of feed management practices that promote optimal production efficiency (de Menezes et al., 2011). Ruminal acidosis is an important example of an interaction between ruminal microbial metabolism and diet that can impair health and production (Tajima et al., 2000; Khafipour et al., 2009). Weimer et al. (2010) suggested that the ruminal microbiome is reasonably resistant to dietary changes, and a change in the microbiome is not always related to the severity of ruminal acidosis (Mohammed et al., 2012). Early understandings of rumen microbiology and bacteria believed to be involved with ruminal acidosis were based on bacterial cultures. More recently, molecular techniques, which are rapidly improving and becoming less

expensive, have been adopted in evaluations of rumen microbiology. Integration of knowledge obtained from classical culture-based microbiology and modern molecular techniques is rapidly increasing our understandings of the rumen microbiome and its functions in general (Pers-Kamczyc et al., 2011). This knowledge will potentially facilitate optimal dietary management and reduce the incidence of nutritional disorders such as ruminal acidosis, among other benefits. Despite constant evolution of methods for studying the rumen ecosystem, only approximately 10% of the rumen microbiome is known (Pers-Kamczyc et al., 2011). This limited knowledge impedes understanding of the importance of changes in ruminal microbial populations observed during rumen perturbation and ruminal acidosis.

Recent work suggests that the rumen has a core bacterial microbiome that consists primarily of bacteria from the Firmicutes and Bacteroidetes phyla, which appear to change in cattle fed various ruminal acidosis induction diets or increasing amounts of grain (Khafipour et al., 2009; Callaway et al., 2010; Fernando et al., 2010; de Menezes et al., 2011). Bacteria from the Proteobacteria phyla appear to be the third most dominant in the rumen in several (Khafipour et al., 2009; Jami and Mizrahi, 2012), but not all, ruminant studies (Golder et al., 2014b). Increases in Proteobacteria have been reported in TMR-fed compared to pasture-fed cattle (de Menezes et al., 2011) and cattle with in grain-induced, compared to alfalfa pellet-induced, subacute acidosis (Khafipour et al., 2009). Increases in *Streptococcus* and *Lactobacillus* were associated with ruminal acidosis in early work (Hungate et al., 1952; Hungate, 1966); however, as only a small portion of the rumen microbiome is known, other rumen bacteria may have prominent roles in acidosis.

The objectives of this study were to examine the relationships between milk measures, ruminal fermentation measures, ruminal acidosis, and ruminal bacterial community composition in lactating cows offered a restricted pasture allowance and fed supplements using different feeding strategies and at linearly increasing supplement amounts. The aim was to identify feed management systems that improve milk measures, promote optimal conditions in the rumen for digestion, and reduce ruminal acidosis.

The hypotheses tested were (1) that cows fed supplements as a PMR, with or without canola meal, would have increased milk measures, altered ruminal measures, distinct

bacterial community composition (**BCC**), and decreased ruminal acidosis, compared to control cows fed iso-energetic diets as grain in the parlor and forage fed on the pasture; (2) that a linear increase in the amount of supplement fed (or increase when 14 and 16 kg of supplement DM are fed) would alter milk and ruminal fermentation measures, create a distinct ruminal BCC, and increase ruminal acidosis.

MATERIALS AND METHODS

The experiment was conducted in late fall at the Department of Environment and Primary Industries (DEPI), Ellinbank Centre, Victoria (**VIC**), Australia (38°14'S, 145°56'E). All experimental procedures were approved by the DEPI Agricultural Research and Extension Animal Ethics Committee (AEC 2010-18).

Animals and Experimental Design

This prospective randomized controlled cohort experiment with a partial factorial design included 3 supplement feeding strategies and 5 supplementary feeding amounts offered to 144 Holstein-Friesian cows. These cows were seasonally calving, multiparous cows of mixed ages with a mean bodyweight of 625 ± 45 kg and were 272 ± 17 days in milk. Twenty-four of these cows were rumen fistulated. The cows were milked twice daily at approximately 7:00 and 15:00 h. The experiment consisted of a 14-d pre-experimental period, during which cows were adapted to the amount of supplement, and a 16-d measurement period, during which DMI and milk production were measured. Ruminal fluid samples were collected on d 7 of the measurement period.

Supplement Feeding Strategies

All 144 cows had been fed either a control or PMR diet before the experiment and were allocated into 24 groups; each group of 6 cows was an experimental unit. Each group was allocated to one of the following feeding strategies:

(1) In the control strategy (10 groups), cows grazed a perennial ryegrass (*Lolium perenne L.*) pasture supplemented with crushed wheat grain individually hand-fed twice daily in the milking parlor and ryegrass silage fed under an electric wire on the pasture. The ratio of ryegrass silage:wheat grain fed as supplement was 27:73 (DM-basis). The pasture allowance was

kept constant at 15 kg of DM/cow per d measured to ground level, to provide a target intake of approximately 8 kg of DM/cow per day.

(2) In the PMR strategy (10 groups), cows grazed perennial ryegrass pasture twice daily at the same allowance as the control cows, were fed approximately 45% of their daily allocation of crushed wheat in the milking parlor, and were fed a PMR in equal proportions on a concrete feed pad immediately after each milking. The PMR comprised crushed wheat grain, crushed maize grain, maize silage, and pasture silage mixed and chopped in a feed wagon (model K160, Richard Keenan and Co. Ltd., Co. Carlow, Ireland; Table 1). Water was added to the ration such that the final DM content of the ration approximated 50%. The PMR provided the same estimated metabolizable energy (**ME**) intake as the supplements fed to the control cows and had the same ratio of forage to grain, but was formulated to ferment more slowly.

(3) In the PMR+Canola strategy (4 groups), cows were fed and managed the same as the PMR cows, except that the ration contained alfalfa hay instead of ryegrass silage and 16% DM of the crushed wheat grain was replaced with solvent-extracted canola meal (Table 1). This ration provided the same estimated metabolizable energy as the control and PMR diets, but had estimated amounts of metabolizable protein that exceeded requirements (NRC, 2001).

Amount of Supplement

Each group of 6 cows was randomly allocated within their feeding strategy to receive different amounts of supplement, and each group contained a rumen-fistulated cow (n = 2 fistulated cows/feeding strategy per supplement feeding amount; Tables 1). For the control and PMR cows, 2 groups were allocated to receive 8, 10, 12, 14, or 16 kg of total supplement DM/cow per day (**SDM**). For the PMR+Canola cows, 2 groups were allocated to receive either 14 or 16 kg of SDM. Cows fed the 14 and 16 kg of SDM amounts were introduced gradually to dietary regimes over the first 7 d of the pre-experimental period.

As part of their supplement, all cows received a vitamin and mineral pellet (Nutrifeed Hi-Milker, Debenham Australia Pty Ltd., Leongatha, VIC, Australia) that contained monensin (110 mg/100 g pellets) and tylosin (110 mg/100 g pellets; Table 1). Cows at the highest amount of supplementation (16 kg of SDM) received this supplement at the amount recommended by the manufacturers (250 g pellet/cow per day), whereas cows receiving lower amounts of supplement received proportionally less (e.g, cows fed 8 kg of SDM received 125 g/cow per d of the vitamin and mineral pellet). Control cows received their vitamin and mineral pellets mixed with their grain at milking time, whereas cows fed PMR and PMR+Canola received their pellets mixed into their PMR.

All cows remained in their groups of 6 during feeding and grazing and had several opportunities each day to access water, *ad libitum*. Control cows had access to pasture immediately after each milking, whereas those fed the PMR and PMR+Canola diets had access after they had consumed their ration on the feed pad. Each group of 6 cows grazed adjacent areas separated by electric tapes and were prevented from re-grazing areas from the previous days.

Table 1. Target dry matter intake (DMI) of ration composition and actual DMI of feeding groups[1]

Ration component (kg of total supplement DM/cow per d)	Feeding strategy and supplement feeding amount (kg of total supplement DM/cow per day)											
	Control					PMR					PMR+Canola	
	8	10	12	14	16	8	10	12	14	16	14	16
Target DMI												
Crushed wheat[2]	5.8	7.3	8.8	10.4	11.7	3.0	3.8	4.6	5.4	6.2	3.1	3.5
Crushed maize	-	-	-	-	-	1.6	2.0	2.4	2.8	3.2	2.8	3.2
Alfalfa hay	-	-	-	-	-	1.0	1.2	1.4	1.6	1.8	1.7	1.9
Maize silage	-	-	-	-	-	2.4	3.0	3.6	4.2	4.8	4.2	4.8
Canola meal	-	-	-	-	-	-	-	-	-	-	2.2	2.6
Pasture silage	2.2	2.7	3.2	3.6	4.3	-	-	-	-	-	-	-
Pasture	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Total	16.0	18.0	20.0	22.0	24.0	16.0	18.0	20.0	22.0	24.0	22.0	24.0
Actual DMI												
Crushed wheat	5.3±0.0	6.6±0.0	7.7±0.0	8.8±0.2	10.0±0.3	1.4±0.0	1.8±0.1	2.0±0.1	2.2±0.0	2.6±0.0	2.3±0.0	2.6±0.0
Pasture silage	2.5±0.0	3.1±0.0	3.8±0.0	4.4±0.0	5.0±0.0	-	-	-	-	-	-	-
PMR	-	-	-	-	-	7.1±0.2	9.2±0.3	11.1±0.1	12.9±0.1	14.6±0.1	13.2±0.0	14.9±0.0
Pasture	8.8±0.0	9.1±0.3	8.3±0.5	9.0±0.0	7.9±0.2	8.6±0.4	8.7±0.6	7.9±0.5	8.0±0.0	7.8±0.1	8.2±0.3	8.0±0.1
Total supplement	7.8±0.0	9.8±0.0	11.4±0.0	13.2±0.2	15.1±0.3	8.4±0.2	11.0±0.1	13.1±0.0	15.2±0.1	17.2±0.1	15.5±0.0	17.5±0.1
Total	16.6±0.0	18.9±0.3	19.7±0.5	22.2±0.2	23.0±0.5	17.1±0.5	19.8±0.7	21.0±0.5	23.1±0.1	25.0±0.0	23.8±0.2	25.5±0.0

PMR= partial mixed ration; DM = dry matter; DMI = dry matter intake.

[1]Rations also contained a vitamin and mineral mix (Nutrifeed Hi-Milker pellets; Debenham Australia Pty Ltd., Leongatha, Victoria, Australia) that contained (110 mg/100 g pellets) of monensin and (110 mg/100 g pellets) of tylosin. Cows at the highest amount of supplementation (16 kg of DM/cow per day) received this supplement at the amount recommended by the manufacturers (125 g pellet/cow per day), while cows receiving lower amounts of supplement received proportionally less (eg. cows offered 8 kg of DM supplement/d received 71 g/cow per d of the vitamin and mineral pellet).

[2]Approximately 45% of the target DMI for crushed wheat for the PMR-fed cows was offered in the milking parlor and the remainder was included in the PMR.

Nutritive Characteristics and Supplement and Pasture Intakes

All ration components were analyzed by near-infrared spectroscopy (**NIR**; method 989.03; AOAC International, 2000; Dairy One Inc., Forage Testing Laboratory, Ithaca, NY; Table 2) and ration chemical composition was estimated in CPM Dairy Ration Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 3). Rations offered and refused were weighed every day of the measurement period and subsamples of ration residuals were collected and analyzed for DM and nutritive characteristics to allow the calculation of daily DMI and estimated ME intake for each group.

Pre- and post-grazing mass was measured daily (Pasturemeter XP1; C-Daz Ltd., Palmerston North, New Zealand) to calculate average pasture DMI for each group. Pre- and post-grazing pasture samples were collected from each new pasture break grazed by cutting pasture at ground level using electric shears. Samples were washed, freeze-dried, and ground through a 0.5-mm sieve, and DM digestibility was analyzed by NIR (Dairy One Inc.) to estimate ME.

Table 2. Chemical composition of feed components and pasture[1]

Item (% of DM)	Feed component						
	Crushed wheat	Crushed maize	Alfalfa hay	Maize silage	Canola meal	Pasture silage	Pasture consumed
CP	13.8	10.0	18.6	10.0	37.4	13.3	22.2
ADF	5.3	4.5	36.3	25.7	21.9	40.1	27.5
NDF	12.9	13.3	44.9	46.0	30.2	61.3	49.9
Lignin	0.7	0.6	10.9	3.3	10.5	3.7	3.4
NFC	74.4	70.8	28.0	36.8	25.5	14.8	17.1
Starch	54.2	59.8	2.6	28.4	1.2	0.7	1.5
Crude fiber	2.0	5.5	2.1	4.0	6.6	3.9	5.8
Ash	1.7	1.9	11.3	4.9	8.9	9.0	10.0
ME (MJ/kg DM)	14.7	14.6	8.2	10.8	12.7	9.7	11.5

CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC = non-fiber carbohydrates; ME = metabolizable energy.
[1]Analyzed by near-infrared spectroscopy (Dairy One Inc, Forage Testing Laboratory, Ithaca, NY).

Table 3. Estimated chemical composition of consumed rations[1]

Item (% of DM)	Feeding strategy and feeding supplement feeding amount (kg of DM/cow per day)											
	Control					PMR					PMR+Canola	
	8	10	12	14	16	8	10	12	14	16	14	16
DM	77.6	76.5	74.9	74.5	72.6	75.9	73.9	71.4	70.1	68.6	70.2	68.6
CP	18.1	17.7	17.1	17.0	16.5	17.4	16.7	16.0	15.7	15.4	18.2	18.1
RUP (% of CP)	26.9	27.7	27.6	28.8	28.5	27.5	28.7	29.1	30.1	30.9	35.2	34.6
RDP (% of CP)	73.1	72.3	72.4	71.2	71.5	72.5	71.3	71.0	69.9	69.1	64.8	65.4
RDP	13.2	12.8	12.4	12.1	11.8	12.6	11.9	11.4	11.0	10.7	11.8	11.8
Soluble protein (% of CP)	35.7	36.1	36.9	37.1	37.8	35.2	35.7	36.2	36.5	36.8	35.1	35.2
ADF	22.2	21.6	21.1	21.0	20.4	21.4	20.5	19.6	19.2	18.7	21.0	20.7
NDF	39.6	38.6	37.4	37.2	36.0	38.5	37.0	35.4	34.6	33.8	36.5	35.9
Forage NDF (% of NDF)	89.5	88.1	86.5	86.1	84.2	65.5	59.6	53.1	49.8	46.1	81.7	43.6
Forage NDF (% of DM)	35.4	34.0	32.4	32.1	30.3	25.2	22.0	18.8	17.2	15.6	29.9	15.7
Physically effective NDF	27.8	27.1	26.4	26.3	25.4	27.7	26.7	25.7	25.1	15.6	26.0	25.6
Lignin	2.57	2.48	2.39	2.37	2.27	3.07	3.00	2.93	2.89	2.85	3.80	3.83
NFC[2]	33.4	35.1	37.0	37.4	39.5	35.3	37.8	40.4	41.7	43.0	36.8	37.7
Silage acids	0.10	0.10	0.12	0.13	0.14	0.74	0.84	0.94	0.98	1.03	0.98	1.03
Sugar	9.86	9.44	8.85	8.73	8.18	9.23	8.58	7.92	7.60	7.26	8.28	8.01
Starch	17.6	19.3	21.3	21.7	23.8	20.2	22.9	25.6	27.0	28.4	21.3	22.2
Soluble fiber	5.89	6.26	6.74	6.84	7.31	5.15	5.52	5.90	6.08	6.27	6.28	6.47
Total ether extract	4.29	4.14	3.94	3.90	3.71	4.57	4.42	4.25	4.17	4.09	4.65	4.61
Total LCFA	2.71	2.63	2.52	2.50	2.40	3.11	3.06	3.00	2.98	2.95	3.33	3.34
Ash	7.55	7.34	7.05	7.00	6.70	7.39	7.06	6.68	6.50	6.31	7.29	7.16
DCAD (mEq/100g)	50.1	45.8	40.0	38.7	33.2	51.3	45.7	40.0	37.3	34.4	37.5	34.6
Minerals (mg/kg)												
Chloride	6,800	6,900	7,200	7,300	7,400	4,200	4,100	3,900	3,800	3,700	3,700	3,600
Calcium	4,600	4.800	5,000	5,100	5,200	4,100	4,200	4,200	4,200	4,200	5,000	5,100
Copper	9.38	9.68	9.88	9.95	10.1	9.78	10.2	10.4	10.5	10.6	10.4	10.5
Iron	154	155	156	157	157	157	158	158	157	157	182	183
Phosphorus	5,500	5,400	5,200	5,200	5,000	5,200	5,000	4,800	4,700	4,600	5,600	5,500
Potassium	30,300	28,400	25,900	25,400	23,000	29,300	26,600	23,900	22,600	21,300	23,700	22,500
Magnesium	2,100	2,200	2,200	2,200	2,200	2,200	2,200	2,200	2,300	2,300	2,600	2,700
Manganese	46.5	46.4	45.8	45.7	45.3	43.2	42.7	41.8	41.4	41.0	43.8	43.6
Sodium	2,000	2,000	2,200	2,200	2,300	1,000	1,000	1,000	900	900	1,000	1,000
Sulfur	2,700	2,600	2,500	2,500	2,400	2,600	2,400	2,300	2,300	2,200	2,700	2,700
Zinc	44.7	45.7	46.3	46.5	47.1	41.8	42.6	42.8	42.9	43.0	44.9	45.2

PMR = partial mixed ration; DM = dry matter; CP = crude protein; RUP = rumen undegradable protein; RDP = rumen degradable protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC =non-fiber carbohydrates; LCFA = long-chain fatty acids; DCAD = dietary cation-anion difference.

[1]Estimations were performed using CPM Dairy Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) using dry matter intake data from Table 2 and chemical composition of feed ration components in Table 3 and were based on 625-kg of bodyweight cows, 272 days in milk with a body condition score of 3.00 and mean milk yield, milk fat and protein percentage for each group.

[2]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash]. NDICP = neutral detergent insoluble crude protein.

Milk Sampling Procedure and Laboratory Analysis

Milk yield of every cow was measured at each milking during both the pre-experimental and measurement periods using the DeLaval Alpro milk metering system (DeLaval International AB, Tumba, Sweden). Fat and protein concentrations of the daily milk from each cow were determined twice a week during both periods using inline milk meters (DeLaval International AB) and an infrared milk analyzer (model 2000; Bentley Instruments Inc., Chaska, MN).

Ruminal Fluid Sampling Procedure

On d 7 of the measurement period, ruminal fluid was collected from each rumen-fistulated cow (2 cows per feeding group) at approximately 2.4-h intervals over a 24-h period spanning 2 d, totalling 10 samples per cow. This was done by restraining the cows in temporary yards set up in the pasture or permanent yards next to the feed pad and milking parlor. The first sample was collected after morning milking at approximately 8:20 h. Samples were collected per fistulae using a 100-mL plastic syringe connected to a brass pipe inserted into the rumen. Fluid was collected from several sites within the rumen and immediately tested for pH (Hannah HI9023 pH meter, Hannah Instruments, Keysborough, VIC, Australia). An additional untreated sample was stored at –20^{o}C for ruminal bacterial analysis. Ruminal data from sample 8 were excluded from the dataset due to sampling error.

Ruminal Fluid Laboratory Analysis

For VFA analysis, an aliquot of 4 mL of ruminal fluid was dispensed into a tube containing 1 mL of 25% metaphosphoric acid before being stored at –20°C until subsequent analysis. Concentrations of VFA were determined by capillary gas chromatography using the method of Packer et al. (2011). Sample VFA peaks were identified by comparing their retention time with those of a standard mixture of VFA (Sigma-Aldrich Pty Ltd., Castle Hill, NSW, Australia) and quantified using Shimadzu class GC10 version 1.62 software (Shimadzu Scientific Instruments, Rydalmere, NSW, Australia) with 4-methylvaleric acid as the internal standard.

For ammonia analysis, an aliquot of 10 mL of ruminal fluid was dispensed into a tube containing 10 mL of 0.1 *M* HCl before being stored at −20°C until analysis.

Concentrations of ammonia were assayed by a direct enzymatic procedure using a commercially available kit (Boehringer Mannheim; R-Biopharm Laboratory Diagnostics Pty Ltd., Taren Point, NSW, Australia) and a Cobas Mira S autoanalyzer (Roche, Montclair, NJ).

D-lactate was analyzed using a UV method for D-lactate determination in ruminal fluid using a Boehringer Mannheim kit (catalog no. 11 112 821 035; R-Biopharm-Laboratory Diagnostics Pty. Ltd.) after deproteinization with perchloric acid according to kit instructions on an Olympus AU400 Autoanalyzer. L-lactate was analyzed using a UV method for L-lactate determination in ruminal fluid using a Beckman Coulter kit (catalog no. OSR 6193; Beckman Coulter Australia Pty. Ltd) on an Olympus AU400 Autoanalyzer (AHL NTM-56; Olympus, Mount Waverly, VIC, Australia).

DNA Extraction

Bacterial community composition was determined on ruminal fluid collected at approximately 08:20, 12:00, and 16:40 h (sampling times 1, 3, and 5) on d 7 of the measurement period. Ruminal fluid samples (n = 72) were thawed at room temperature and a 1 mL aliquot was centrifuged at 10,000 × *g* for 1-min and the supernatant discarded. The pellet was resuspended by vigorous vortexing in 200 μL ATL buffer (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany) together with 200 mg silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK). The mixture was homogenized in a FastPrep-24 (MP Biomedicals, Seven Hills, New South wales, Australia) at maximum speed for 1-min, twice; heated at 70^{o}C for 15 min and centrifuged at 10,000 × *g* for 5-min. Supernatant (180 μL) was removed for digestion with proteinase K at 50^{o}C for at least 3 h, and DNA was extracted according to the QIAmp DNA mini kit protocol (Qiagen GmbH). DNA was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA).

PCR Amplification of 16S Ribosomal DNA Gene Sequences

Genomic DNA from each sample was diluted 1:30 with water and the 16S rRNA gene spanning V1 to V3 was PCR amplified using Platinum taq polymerase (Invitrogen, Carlsbad, CA) as follows: 1 cycle at 94^{o}C for 2 min, followed by 30 cycles of 94^{o}C for 10 s, 55^{o}C for 45 s, 72^{o}C for 45 s, with a final extension of 72^{o}C for 10-min. Primers used in the reaction were modified universal 8F (Snell-Castro et al., 2005) and 515R

(Lane, 1991) primers that included 454 sequencing adapters B and A, respectively. In addition, a unique 8 base pair barcode was included in the reverse primer of each amplicon, so that DNA sequence reads can be assigned accurately to each originating sample. The PCR products were visualized on agarose gels and equal amounts of PCR product were pooled and gel extracted (Qiaex gel extraction kit, Qiagen). Approximately 3 μg of pooled amplicon (~40 ng/μL) was dispatched to Macrogen (Seoul, Korea) for 454 DNA sequencing using a 454 GS FLX Sequencer with titanium chemistry (Roche, Branford, CT).

Sequence Analyses of Gene Amplicons

Sequence data was processed using the quantitative insights into microbial ecology (QIIME) software package (Caporaso et al., 2010). Recovered sequences were assigned to their originating sample based on the attached barcode and filtered based on quality and length parameters. Error correction of 454 data was performed using R software (package Acacia; Bragg et al., 2012). Clustering of recovered sequences to an operational taxonomic unit (**OTU**) at a 0.97 distance threshold was used. Taxonomic identification was based on similarity to the Greengenes Database (http://greengenes.lbl.gov). The OTU table was subjected to alpha and beta diversity measures using QIIME and passed through R (package Ade4; Dray and Dufour, 2007) for principal coordinates between group analysis and co-inertia analysis.

Statistical Analysis

Dry Matter Intake

Dry matter intake data were derived from averaging daily group measures over the measurement period, and data from all feeding amounts (n = 144 cows) were analyzed using a linear mixed model in Genstat (14th edition, VSN International Ltd., Hemel, Hempstead, UK), using model (1), with the modification of removal of time and interactions with time from the model. Orthogonal polynomial linear and quadratic contrasts were included for feeding amount. The correlation structure of the random error terms of the model was independent.

Model (1): $Y_{ijklmn} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\alpha\delta)_{il} + (\alpha\gamma\delta)_{ikl} + (\beta\gamma)_{jk} + (\beta\delta)_{jl} + (\beta\gamma\delta)_{jkl} + (\alpha\beta\gamma)_{ijk} + (\alpha\beta\delta)_{ijl} + (\alpha\beta\gamma\delta)_{ijkl} + X_m + (XY)_{mn} + \varepsilon_{ijklmn,}$

where Y_{ijklmn} = response at time i (i = 1 to 9, or 1 to 3), to protein j (yes or no), for feeding strategy k (k = 1 or 2), at supplement feeding amount l (l = 1 to 5), for herd m (m = 1 to 5), in group n (n = 1 to 5); μ = overall mean; α = time; β = protein; γ = feeding strategy nested within time and protein; δ = supplement feeding amount nested within time and protein; X = herd; Y = group nested within herd; ε = random error.

To incorporate the effect of protein, data from the groups fed total supplement at amounts of 14 and 16 kg of DM/cow per day were termed the high supplement feeding amounts and were analyzed using model (2), with the modification of removal of time and interactions with time from the model. Orthogonal polynomial linear and quadratic contrasts were not included in this analysis as the high supplement feeding amounts comprised only 2 feeding amounts. The correlation structure of the random error terms of the model was independent.

Model (2): $Y_{ijklm} = \mu + \alpha_i + \gamma_j + \delta_k + (\gamma\delta)_{jk} + (\alpha\gamma)_{ij} + (\alpha\delta)_{ik} + (\alpha\gamma\delta)_{iik} + X_m + (XY)_{mn} + \varepsilon_{ijklm,}$

where Y_{ijklm} = response at time i (i = 1 to 9), for feeding strategy j (j = 1 to 3), at supplement feeding amount k (k = 1 to 5), for herd l (l = 1 to 5), in group m (m = 1 to 5); μ = overall mean; α = time; γ = feeding strategy; δ = supplement feeding amount; X = herd; Y = group nested within herd; ε = random error.

Milk Measures

Milk measures (Table 4) were derived from averaging daily individual measures over the measurement period and were analyzed for the herd (n = 144 cows) and for the fistulates only (n = 24 cows) for all supplement feeding amounts and the high supplement feeding amounts, using models (1) and (2), respectively, with the removal of time and interactions with time from each model. Orthogonal polynomial linear and quadratic contrasts were included for supplement feeding amount in model (1).

Ruminal Measures

Ruminal data (Table 5) from 9 sampling times from the fistulated cattle were analyzed for all supplement feeding groups (n of samples = 216) and the high supplement feeding amount groups (n of samples = 108) using models (1) and (2), respectively. Orthogonal polynomial linear and quadratic contrasts were included for feeding amount in model (1).

Bacterial Prevalence

The relative abundance of bacterial families in ruminal fluid collected at approximately 8:20, 12:00, and 16:40 h (sampling times 1, 3, and 5) with a mean relative abundance of >0.2% for at least one feeding strategy were analyzed for all supplement feeding amounts (n of samples = 72) and the high supplement feeding amounts (n of samples = 36) using the same linear mixed models used for the ruminal data. The relative abundance of the following bacterial genera were also analyzed using the same models: *Prevotella*, *Lactobacillus*, *Butyrivibrio*, *Megasphaera*, *Selenomonas*, *Veillonella*, and *Streptococcus*.

Evaluation of Ruminal Acidosis

The incidence of ruminal acidosis was diagnosed using eigenvalues obtained using discriminant analysis of standardized variates of ruminal concentrations of the individual VFA: acetate, butyrate, propionate, valerate, iso-valerate, iso-butyrate, and caproate, and total lactate and concentrations of ruminal ammonia and ruminal pH defined according to the methods of Bramley et al. (2008). Acidosis eigenvalues were based on the statistical distance of each sample from the centroid for known cases of ruminal acidosis identified in the dataset of Bramely et al. (2008). Eigenvalues that approached 1 are in the center of the acidosis category and those approaching 0 are not acidotic.

RESULTS

A linear decrease in milk fat percentage with increased supplement amount fed was a clinical sign consistent with ruminal acidosis; however, diarrhea and lameness were not observed in any of the cows during the experiment. Acidosis eigenvalues showed control cows fed 16 kg of SDM had ruminal acidosis (Table 5; Figure 1). Milk data were not recorded for a control cow fed 12 kg of SDM during the measurement period, as she ceased production.

Dry Matter Intake

Total DMI was within 1 kg of DM of the target total DMI for the controls but was up to 1.8 kg of DM higher than the target DMI in the PMR and PMR+Canola cows (Table 1). Differences between target and actual DMI of total supplement did not exceed 1 kg of DM for the controls, regardless of supplement feeding amount. The DMI of total supplement for the PMR and PMR+Canola cows exceeded the target intakes for all supplement feeding amounts by between 0.4 to 1.5 kg of DM/cow per d. Pasture DMI for the controls was higher for all supplement feeding amounts, except the 12 and 16 kg of DM feeding groups, compared with the target DMI and was similar to the target DMI for pasture in the PMR and PMR+Canola cows. The DMI of crushed wheat was lower than the target DMI for all control cows, regardless of supplement feeding amount. The controls had from 0.3 to 0.8 kg of DM higher DMI of ryegrass silage than the target DMI (Table 1).

All Supplement Feeding Amounts

Pasture DMI was not influenced by feeding strategy; however, the total daily DMI and DMI of total supplement were higher in the PMR compared to control cows. Pasture DMI, total supplement DMI, and total daily DMI increased linearly with supplement feeding amount (Table 4).

High Supplement Feeding Amounts

Pasture DMI was higher in the control compared to PMR and PMR+Canola cows, highest in the control cows fed 14 kg of SDM, and lower in the cattle fed 16 kg of SDM. The DMI of total supplement was lower in the control compared to the PMR and PMR+Canola cows and higher in cows fed 16 compared to 14 kg of SDM. Total daily DMI was lower in the control compared to the PMR cows and highest in the PMR+Canola cows, and was higher in the cows fed 16 compared with 14 kg of SDM (Table 4).

Milk Measures

Higher variation in milk measures occurred in the fistulates compared to the herd (data not shown). The interaction of feeding strategy × supplement feeding amount was not significant for any milk measures (Table 4).

All Supplement Feeding Amounts

Yields of fat tended to increase in the PMR cows compared with control cows but feeding strategy had no other effects on milk measures. Yield of milk, ECM, and protein linearly increased with supplement feeding amount, and fat percentage linearly decreased with supplement feeding amount (Table 4).

High Supplement Feeding Amounts

Yield of energy-corrected milk and protein were higher in the cows fed PMR+Canola, compared with the control and PMR cows. Fat yield was higher in the PMR than the control cows and highest in the PMR+Canola-fed cows. A similar trend was observed for fat percentage. Protein percentage was higher in the cows fed 16 compared with 14 kg of SDM (Table 4).

Table 4. Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and means ± SED of feeding strategy for dry matter intake, milk yield, and milk composition of the herd including fistulates (n = 144 cows)

Item	Amount[1]	Feeding strategy means			SED	*P*-value			
		Control	PMR	PMR+ Canola		Strategy (S)	Amount (A)		S × A
							Lin	Quad	
Dry matter intake (kg/d)									
Pasture	All	8.62	8.21		0.20	0.068	0.011	0.495	0.666
	High	8.44[a]	7.89[b]	8.10[b]	0.14	0.024	0.004		0.041
Total Supplement	All	11.5[a]	13.0[b]		0.07	<0.001	<0.001	0.076	<0.001
	High	14.2	16.2	16.5	0.15	<0.001	<0.001		0.696
Total	All	20.1[a]	21.2[b]		0.24	<0.001	<0.001	0.266	0.340
	High	22.6[a]	24.1[b]	24.6[c]	0.22	<0.001	<0.001		0.088
Milk									
Yield (kg/cow/d)	All	17.0	17.1		0.65	0.847	<0.001	0.152	0.995
	High	19.7	19.6	22.6	1.40	0.120	0.673		0.556
Energy corrected milk (kg/cow/day)[2]	All	17.5	18.3		0.50	0.179	<0.001	0.055	0.534
	High	19.0[a]	20.0[a]	24.0[b]	1.14	<0.001	0.542		0.537
Fat (%)	All	4.17	4.41		0.15	0.137	<0.001	0.958	0.403
	High	3.56	4.02	4.32	0.24	0.052	0.854		0.422
Fat (kg/cow/day)	All	0.68	0.74		0.03	0.058	0.221	0.092	0.270
	High	0.68[a]	0.76[b]	0.96[c]	0.05	0.003	0.324		0.481
Protein (%)	All	3.76	3.75		0.04	0.654	0.505	0.477	0.198
	High	3.73	3.75	3.71	0.06	0.795	0.024		0.090
Protein (kg/cow/day)	All	0.63	0.64		0.02	0.844	<0.001	0.132	0.804
	High	0.73[a]	0.73[a]	0.84[b]	0.04	0.025	0.948		0.551

PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal; Lin = linear polynomial contrast; Quad = quadratic polynomial contrast; Total = dry matter intake of pasture and total supplement.

[a–c]Means within a row not sharing a common superscript differ significantly ($P < 0.05$).

[1]All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

[2]Standardized to 4.0% fat and 3.3% protein, was calculated using the following formula: Energy corrected milk (kg/cow per day) = milk yield kg × (376 × fat% + 209 × protein% + 948)/3138 (Tyrell and Reid, 1965).

Ruminal Measures

For all supplement feeding amounts, the high supplement feeding amounts, total and individual VFA and ammonia concentrations had a biphasic pattern over time, with peaks in concentrations occurring at times 3 and 6. The opposite pattern was observed for ruminal pH over time. Cconcentrations of D-lactate were relatively stable over time with the exception of an increase in concentration in the PMR and PMR+Canola cows at time 5 (the first sampling time after the afternoon feed).

All Supplement Feeding Amounts

Acidosis eigenvalues were lower in the PMR-fed cows by 0.2 ± 0.07 units in the PMR-fed cows, compared to the controls, and had a linear and quadratic increase with supplement feeding amount (Table 5). Eigenvalues were higher in the controls from times 3 to 9 (Figure 1B) and were higher in the cattle fed 16 kg of SDM at times 2 to 10, and the cattle fed 14 kg of SDM at times 3 and 4, compared to those fed 8 to 12 kg of SDM (Figure 1C). Peaks in acidosis eigenvalues occurred at times 4 and 7 (Figure 1B).

Concentrations of D-lactate and butyrate, the acetate to propionate ratio, the lipogenic to gluconeogenic VFA ratio [(acetate+butyrate)/propionate], and pH were higher in the cows, compared to the controls, whereas propionate and valerate concentrations were lower in the PMR, compared to control cows. Total VFA, acetate, and ammonia concentrations were not influenced by feeding strategy (Table 5).

The acetate to propionate ratio, the lipogenic to gluconeogenic VFA ratio, pH, and acetate, butyrate, and ammonia concentrations linearly decreased with supplement feeding amount. Propionate concentration linearly increased with supplement feeding amount and valerate concentration linearly and quadratically increased with supplement feeding amount (Table 5).

Table 5. Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and sample time (T) and means ± SED of feeding strategy for acidosis eigenvalues and ruminal measures

Item	Amount[1]	Feeding strategy means			SED	*P*-value							
		Control	PMR	PMR+ Canola		Strategy (S)	Amount (A)		Time (T)	S × A	S × T	A × T	S × A × T
							Lin	Quad					
Acidosis eigenvalue	All	0.26[a]	0.06[b]		0.07	0.012	<0.001	0.014	<0.001	0.075	0.030	<0.001	0.154
	High	0.63	0.14	0.06	0.16	0.066	0.104		<0.001	0.654	0.027	0.259	0.393
Rumen (m*M*)													
Total VFA	All	120.4	113.9		3.51	0.088	0.389	0.679	<0.001	0.205	<0.001	0.007	0.439
	High	123.0	113.4	122.7	6.47	0.313	0.846		<0.001	0.902	0.022	0.098	0.175
Acetate (A)	All	71.4	70.3		2.02	0.601	0.021	0.085	<0.001	0.045	0.034	0.152	0.624
	High	67.2	67.8	74.9	3.50	0.129	0.107		<0.001	0.801	0.236	0.474	0.215
Propionate (P)	All	30.2[a]	22.3[b]		1.79	<0.001	<0.001	0.110	<0.001	0.098	<0.001	<0.001	0.019
	High	39.9	26.2	26.9	3.58	0.071	0.049		<0.001	0.606	<0.001	<0.001	0.197
A:P	All	2.74[a]	3.39[b]		0.16	0.002	<0.001	0.186	<0.001	0.083	<0.001	0.002	0.742
	High	1.84	2.82	3.01	0.30	0.067	0.031		<0.001	0.438	<0.001	0.063	0.038
Butyrate (B)	All	13.5[a]	16.2[b]		0.80	0.006	<0.001	0.197	<0.001	0.025	<0.001	0.640	0.865
	High	10.3	14.5	15.4	1.57	0.125	0.143		<0.001	0.855	0.025	0.836	0.619
(A+B)/P	All	3.28[a]	4.17[b]		0.22	0.001	<0.001	0.218	<0.001	0.097	<0.001	<0.001	0.816
	High	2.11	3.41	3.63	0.40	0.077	0.033		<0.001	0.467	<0.001	0.053	0.126
Valerate	All	2.29[a]	1.77[b]		0.16	0.007	<0.001	0.006	<0.001	0.018	<0.001	<0.001	<0.001
	High	2.94	2.00	2.14	0.35	0.070	0.013		<0.001	0.067	<0.001	<0.001	0.001
D-lactate	All	0.06[a]	0.44[b]		0.09	0.002	0.377	0.194	<0.001	0.637	<0.001	0.988	0.990
	High	0.04[a]	0.60[b]	0.29[a]	0.14	<0.001	0.132		<0.001	0.657	<0.001	0.406	0.948
Ammonia	All	7.07	8.02		0.99	0.358	0.001	0.221	<0.001	0.255	<0.001	<0.001	0.051
	High	3.79[a]	5.59[a]	11.36[b]	1.34	0.011	0.071		<0.001	0.566	<0.001	0.099	0.104
pH	All	6.07[a]	6.25[b]		0.06	0.009	0.007	0.473	<0.001	0.645	<0.001	<0.001	0.926
	High	5.94	6.18	6.13	0.09	0.092	0.893		<0.001	0.935	<0.001	0.108	0.840

PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal; Lin = linear polynomial contrast; Quad = quadratic polynomial contrast;
[1]All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

Propionate and valerate concentrations were the only ruminal measures with a 3-way interaction between feeding strategy, supplement feeding amount, and sample time. Acetate concentration was higher in the control cows fed 8 and 10 kg of SDM and lower in control cows fed 12 kg of SDM, compared with the PMR cows. Butyrate concentrations were increased in the PMR cows fed 12 to 16 kg of SDM, compared to the controls. Valerate concentration was higher in the control cows fed 16 kg of SDM compared with others (Table 5).

Total VFA (Figure 2A), acetate, propionate, and valerate concentrations were higher in the control cows at times 3 and 6 and lower at time 5. The acetate to propionate ratio (Figure 2C), the lipogenic to gluconeogenic VFA ratio, pH, and butyrate concentrations were greater over time in the PMR-fed cattle (Table 5).

Total VFA concentrations were lower in cows fed 16 kg of SDM at times 9 and 10, compared to cows fed 8 and 12 kg of SDM, and were higher in cows fed 16 compared to 8 and 10 kg of SDM at time 6 (Figure 2B). Propionate and valerate concentrations were higher for the cows fed 16 kg of SDM than all other supplement feeding amounts at times 3 to 6. The acetate to propionate (Figure 2E) and lipogenic to gluconeogenic VFA ratios decreased with supplement feeding amount across time, with the highest ratios at times 1 and 10. Ruminal pH decreased with supplement feeding amount and was lowest at times 3 and 6. Ammonia concentration was greater in the PMR cows at times 2, 3, and 5, and lower at time 7.

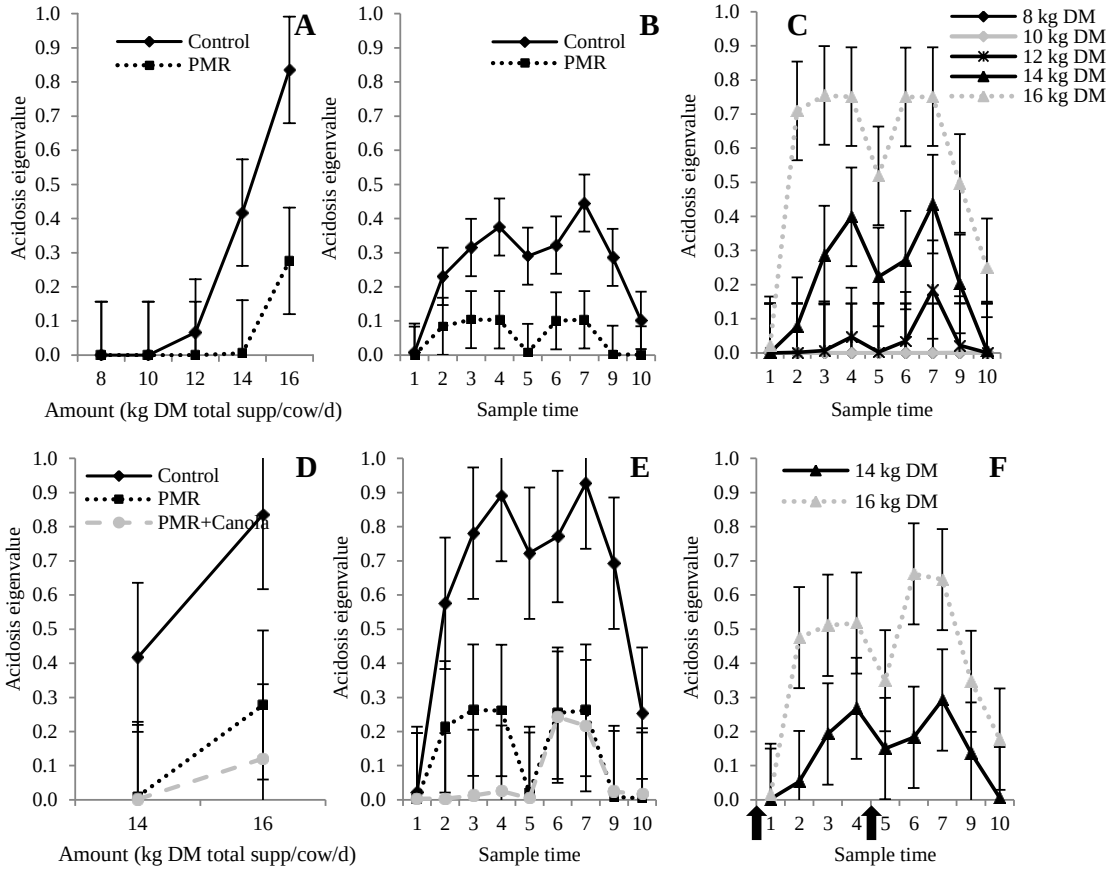
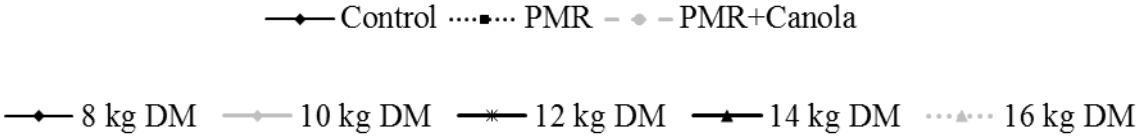


Figure 1. Mean (±SEM) acidosis eigenvalues for dairy cows from all feeding groups showing interactions between (A) feeding strategy and supplement feeding amount, (B) feeding strategy and sample time, and (C) supplement feeding amount and sample time. Mean (±SEM) acidosis eigenvectors for dairy cows from the high supplement feeding amount groups only (14 and 16 kg of DM of total supplement/cow per day) showing interactions between (D) feeding strategy and supplement feeding amount, (E) feeding strategy and sample time, and (F) supplement feeding amount and sample time. Sample times were approximately 2.4 h apart over a 24-h period. Sample time 1 was approximately 8:20 h and milking was at 7:00 and 15:00 h (black arrows). PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal; Amount = kg of DM of total supplement/cow per day.



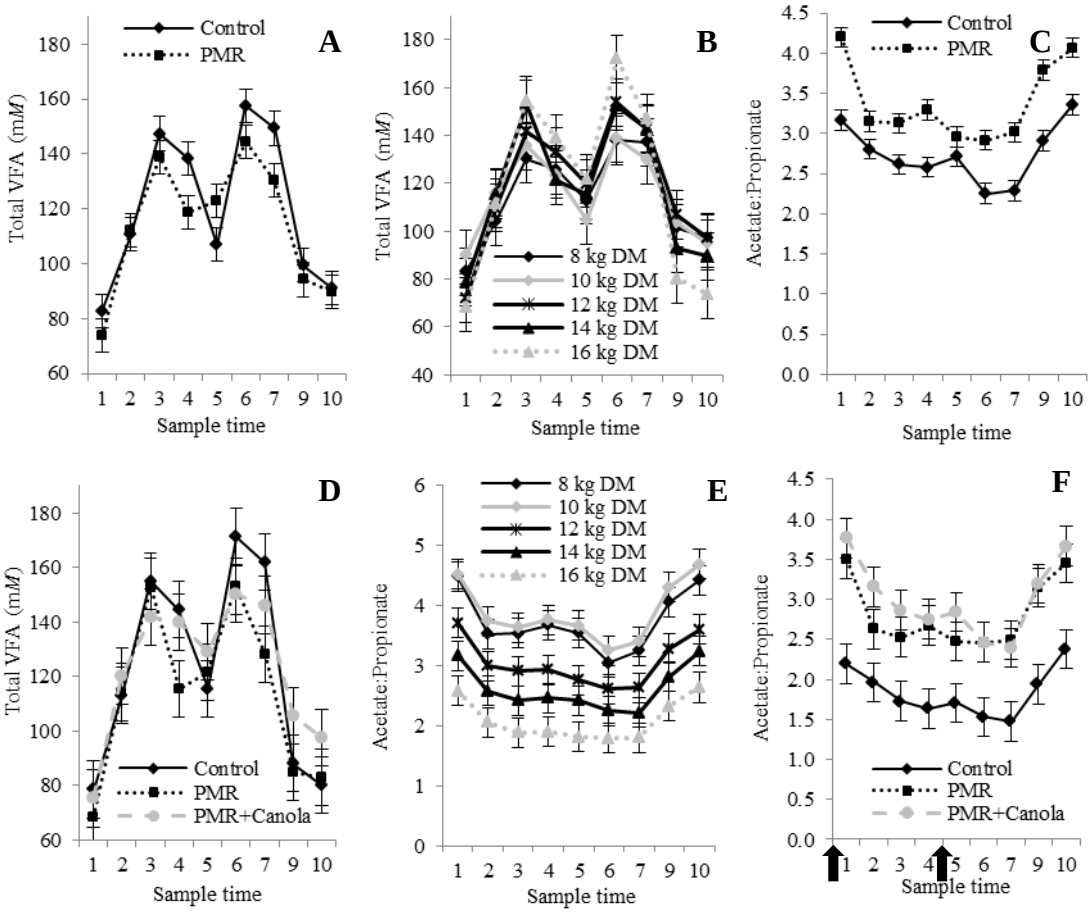


Figure 2. Mean interactions for (A) total volatile fatty acid (VFA) concentrations between feeding strategy and sample time from all feeding groups (n = 24 cows), (B) total VFA concentrations between supplement feeding amounts and sample time from all feeding groups (n = 24 cows), (C) acetate to propionate ratio between feeding strategy and sample time from all feeding groups (n = 24 cows), (D) total VFA concentrations for between feeding strategy and sample time from cows fed only 14 and 16 kg of total supplement/cow per day (n = 12 cows), (E) acetate to propionate ratio between feeding rate and sample time from all feeding groups (n = 24 cows), and (F) acetate to propionate ratio between feeding strategy and sample time from cows fed only 14 and 16 kg of total supplement/cow per day (n = 12 cows). Sample times were approximately 2.4 h apart over a 24 h period. Sample time 1 was approximately 8:20 h and milking was at 7:00 and 15:00 h (black arrows). PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal; Amount = kg of DM of total supplement/cow per day.

High Supplement Feeding Amounts

Acidosis eigenvalues were higher in the control compared to PMR and PMR+Canola cows between times 3 and 9 (Figure 1E) and peaked at times 4 and 7. D-lactate concentration was increased in the PMR compared to the control and PMR+Canola cows, and ammonia concentration was increased in the PMR+Canola cows. Propionate and valerate concentrations were increased in the cows fed 16, compared to 14 kg of SDM, and the acetate to propionate and lipogenic to gluconeogenic VFA ratios were decreased in the cows fed 16 kg of SDM, compared to 14 kg of SDM. A 3-way interaction of feeding strategy × supplement feeding amount × time occurred for valerate concentration, but no 2-way interaction of feeding strategy × supplement feeding amount was significant for any ruminal measures (Table 5).

Total VFA (Figure 2D), propionate, and valerate concentrations, the acetate to propionate ratio (Figure 2F), the lipogenic to gluconeogenic VFA ratio, and pH had similar patterns across time for each feeding strategy to when all supplement feeding amounts were analyzed. The PMR+Canola cows produced similar results to the PMR cows over time for these measures, except for an increase in pH in the PMR+Canola, compared with control and PMR cows, at time 3. Ammonia concentrations were higher in the PMR+Canola cows from times 1 to 7, compared to the control and PMR cows, and peaked at times 3 and 6. Propionate and valerate concentrations were higher in the cows fed 16 kg of SDM over time compared with those fed 14 kg of DM, similar to when all supplement feeding amounts were analyzed (Table 5).

Bacterial Diversity Analysis

Rarefaction analysis (Hughes et al., 2001), which is used to estimate the depth of coverage of diversity of ruminal bacteria within ruminal fluid samples (Kim et al., 2011), indicated coverage of bacterial diversity appeared to be sufficient to evaluate BCC. Sampling saturation of OTU had not yet been met. The PMR cows fed 10 kg of SDM had the highest number of observed OTU (722 ± 50) and the control cows fed 16 kg of SDM had the lowest (254 ± 29; Figure 3).

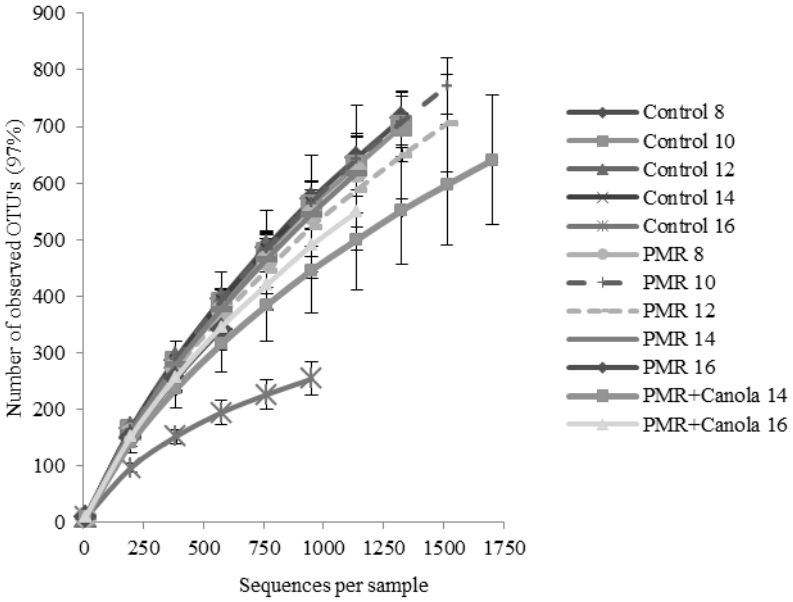


Figure 3. Rarefaction curves for each feeding group consisting of the mean number of observed operational taxonomic units (OTU) ± SEM**.** The OTU were calculated with a 3% disparity. Numbers 8, 10, 12, 14, and 16 denote the number of kg of dry matter of total supplement/cow per d offered. PMR = partial mixed ration; PMR+Canola = partial mixed ration + canola meal.

The overall microbiomes of the cows were not distinctly different in their BCC, with only 6 and 4.5% of the variance being explained, which could not be attributed to any of the groupings (data not shown). However, distinct variation (Monte Carlo $P < 0.001$) in the 16S rDNA-estimated BCC occurred among feeding strategies at the species level in a between-group constrained principal component analysis (**PCoA**) that captured 100% of the sample variation (Figure 4A). The first principal component (**PC**) of the between-group analysis accounted for 57.8% of the variation between feeding strategies, and the second PC accounted for 42.2% (Figure 4A). Bacterial community composition was the most distinct between the control and PMR+Canola fed cows. Spatial heterogeneity among cows was relatively large and was greatest in the control-fed cows (Figure 4A).

A between group constrained PCoA identified variation in the 16S rDNA estimated BCC among groups from different feeding strategy and supplement feeding amount combinations (Monte Carlo $P < 0.001$). This variation was not as distinct as that between feeding strategy groups alone, which accounted for 37.4% of the variation (Figure 4B). The first PC accounted for 22.4% of the variation between feeding strategies, and the second PC accounted for 15.0% (Figure 4B). Bacterial community composition was not different among the control cows fed 8 and 10, PMR cows fed 8, 10, 12, and 14, and the PMR+Canola cows fed 14 kg of SDM (Figure 4B); however, the BCC of these tightly clustered groups differed from that of the remaining groups. Bacterial community composition was similar between the PMR cows fed 16 and PMR+Canola cows fed 16 kg of SDM, and these 2 groups had the greatest spatial heterogeneity (Figure 4B). The control cows fed 12 and 14 kg of SDM also had a relatively large spatial heterogeneity and similarity in BCC. The control cows fed 16 kg of SDM had the most distinct BCC with, uniquely, no overlap of composition observed (Figure 4B).

Distinct BCC occurred among each of the 3 ruminal fluid sample times (Figure 4C; Monte Carlo $P < 0.001$). A total of 100% of the variation was accounted for by the first 2 PC: PC 1 accounted for 63.9% and PC 2 accounted for 36.2% of the variation (Figure 4C).

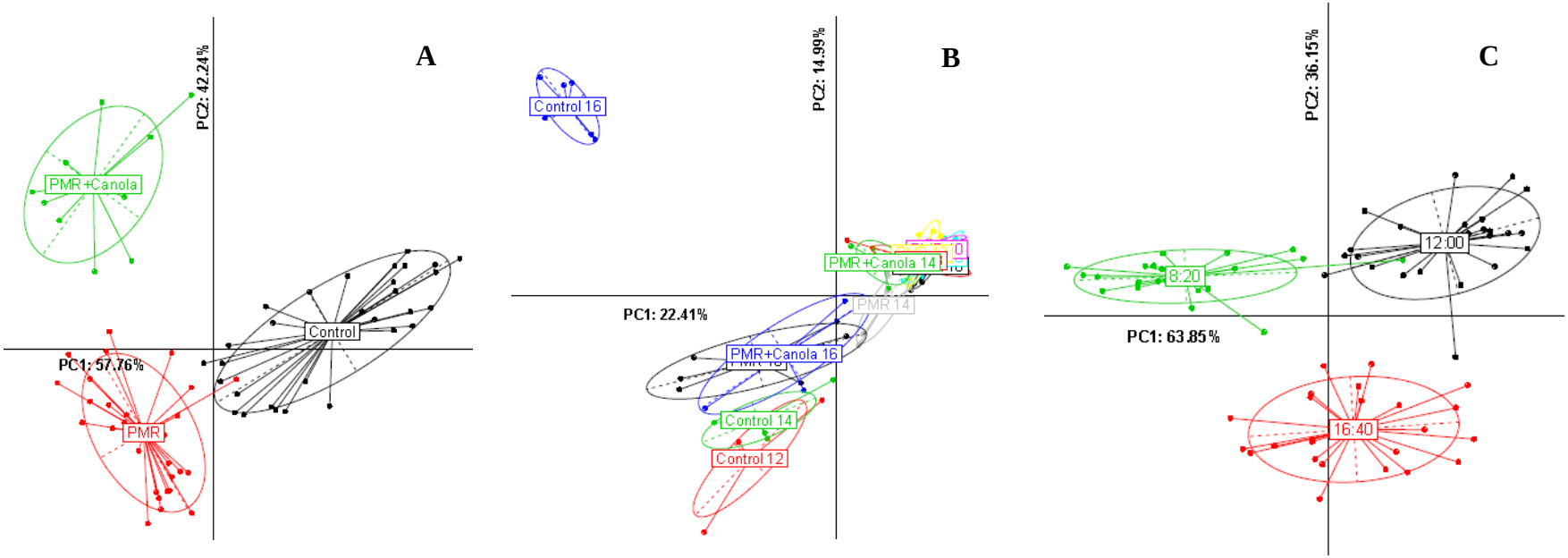


Figure 4. Constrained between-group principal coordinates analysis of bacterial 16S rDNA gene sequences at the species level from ruminal samples from dairy cattle (A) fed 1 of 3 feeding strategies: control (n = 10 cows; n = 30 samples), partial mixed ration (PMR; n = 10 cows; n = 30 samples), or PMR+Canola (PMR+Canola meal n = 4 cows; n = 12 samples)**,** (B) fed 1 of 3 feeding strategies: control (n = 10 cows), PMR (n = 10 cows), or PMR+Canola (n = 4 cows) at 1 of the following supplement feeding amounts: 8, 10, 12, 14, or 16 kg of DM of total supplement/cow per day (2 cows per rate from each feeding strategy; n = 6 samples per supplement feeding amount from each feeding strategy), and (C) collected at 1 of 3 sample times: 8:20, 12:00, and 16:40 h. All ruminal samples were collected on the 19th day of diet supplementation. Each point on the plot represents the bacterial community composition of a single sample (cow by feeding strategy by supplement feeding amount by sample time combination) with a greater distance between points indicating a greater difference in bacterial community composition.

Bacterial Prevalence

A total of 12 phyla and 1 candidate phyla were identified within the ruminal bacterial population from 16S rDNA gene pyrosequences. The majority of sequences were represented by the Firmicutes or Bacteroidetes phyla, accounting for a combined total of 84.2% of sequences; 49.9 and 34.3% of total sequences on average, respectively. The candidate phyla, TM7, represented 6.3% and the phylum, Tenericutes, represented 5.4% of the total sequences on average. The remaining microbiota was composed of phyla with low relative abundances. A total of 44 bacterial families were identified and the Prevotellaceae, Ruminococcaceae, and Lachnospiraceae families and the Unclassified Clostridiales had the largest relative abundances at 30.9, 21.3, 11.3, and 11.7%, respectively. In total, 64 genera were identified, with *Prevotella* being the predominant genus. These represented approximately 30.1% of the relative abundance of bacterial sequences, ranging from an average of 13.0 to 50.7% among groups.

When all supplement feeding groups were analyzed, members of the *Prevotella* genus were lower in relative abundance in the PMR, compared with control cows (mean ± SED of relative abundance = 30.0 and 38.6% ± 4.77; P =0.03). Relative abundance of members of the *Lactobacillus, Streptococcus,* and *Butyrivibrio* genera were higher in the PMR (0.12 ± 0.03, 0.05% ± 0.01, and 3.42 ± 0.57, respectively), compared with control cows (0.00% ± 0.03, 0.01% ± 0.01, and 1.52 ± 0.57, P = 0.001, 0.001, and 0.006, respectively). The relative abundance of the *Megasphaera, Selenomonas,* and *Veillonella* genera were not influenced by feeding strategy. The *Butyrivibrio* linearly increased in relative abundance with supplement feeding amount (P = 0.05); however, feeding amount had no effect on the relative abundance of the *Prevotella, Lactobacillus, Streptococcus*, *Megasphaera*, *Selenomonas,* and *Veillonella* genera. When only the high supplement feeding amount groups were analyzed, feeding strategy and supplement feeding amount did not affect the *Prevotella, Lactobacillus*, *Butyrivibrio*, *Megasphaera*, *Selenomonas,* and *Veillonella* genera, but *Streptococcus* was lower in control cows, compared to the PMR- and PMR+Canola-fed cows (0.06, 0.05, and 0.00% ± 0.02, respectively, P = 0.04).

Table 6. Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and sample time (T), and their interactions, and means ± SED for feeding strategy for bacterial families identified from 16S rDNA sequences with mean relative abundances of >0.2% in at least one feeding strategy

Family	Feeding strategy means (relative abundance, %)					*P*-value						
	Amount[1]	Control	PMR	PMR+ Canola	SED	Strategy (S)	Amount (A)		Time (T)	S × A	S × T	A × T	S × A × T
							Lin	Quad					
Prevotellaceae	All	38.9[a]	27.2[b]		4.81	0.032	0.178	0.361	<0.001	0.123	0.018	0.063	0.324
	High	46.8	29.6	22.6	8.28	0.122	0.061		<0.001	0.811	0.764	0.648	0.108
Ruminococcaceae	All	18.0[a]	24.5[b]		2.58	0.026	0.001	0.010	<0.001	0.027	0.682	0.017	0.211
	High	8.73	18.8	20.1	4.43	0.264	0.014		0.002	0.943	0.223	0.030	0.059
Unclassified Clostridiales	All	10.35[a]	13.20[b]		1.13	0.026	<0.001	0.038	<0.001	0.009	0.251	0.148	0.440
	High	4.66	12.77	10.86	2.11	0.114	0.044		<0.001	0.077	0.124	0.059	0.014
Lachnospiraceae	All	12.9	10.6		2.13	0.296	0.001	0.074	0.001	0.877	0.002	0.018	0.009
	High	17.8	14.9	9.24	4.69	0.369	0.082		0.001	0.671	0.004	0.078	0.019
F16	All	2.30	6.89		2.52	0.094	0.265	0.882	0.041	0.876	0.222	0.410	0.727
	High	3.27[a]	5.85[ab]	12.4[b]	3.88	0.009	0.074		0.077	0.357	0.173	0.222	0.357
Erysipelotrichaceae	All	5.16[a]	3.06[b]		0.73	0.019	<0.001	0.003	0.061	<0.001	<0.001	0.385	0.813
	High	8.86	3.53	6.68	2.02	0.051	0.003		0.143	0.009	0.499	0.119	0.709
Veillonellaceae	All	4.63[a]	2.60[b]		0.61	0.006	0.771	0.253	<0.001	0.043	0.007	0.118	0.220
	High	4.98[a]	2.25[b]	1.58[b]	1.07	0.042	0.424		<0.001	0.061	<0.001	0.150	0.458
Unclassified Bacteroidales	All	3.27	3.52		0.47	0.603	0.029	0.430	0.002	<0.001	0.002	0.113	0.193
	High	3.89[a]	4.85[b]	2.91[a]	0.86	0.044	0.701		0.598	<0.001	0.030	0.669	0.156
Coriobacteriaceae	All	0.97	2.28		0.48	0.272	0.614	0.300	<0.001	0.082	0.001	0.574	0.178
	High	0.32	1.96	4.15	2.63	0.438	0.244		0.062	0.328	0.229	0.395	0.492
Anaerolinaceae	All	0.87[a]	1.39[b]		0.23	0.044	<0.001	0.509	0.002	0.035	0.116	0.911	0.581
	High	0.09	0.82	1.25	0.45	0.164	0.005		0.028	0.209	0.229	0.783	0.835

PMR = partial mixed ration; PMR+Canola = PMR+Canola meal; Lin = linear polynomial contrast; Quad = quadratic polynomial contrast.

[1]All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

Table 6 (continued). Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and sample time (T), and their interactions, and means ± SED for feeding strategy for bacterial families identified from 16S rDNA sequences with mean relative abundances of >0.2% in at least one feeding strategy

Family	Feeding strategy means (relative abundance, %)					*P*-value							
	Amount[1]	Control	PMR	PMR+ Canola	SED	Strategy (S)	Amount (A)		Time (T)	S × A	S × T	A × T	S × A × T
							Lin	Quad					
Clostridiales Family XIII Incertae Sedis	All	0.82a	1.34b		0.22	0.035	0.262	0.615	<0.001	0.218	0.032	0.876	0.606
	High	0.45	1.28	1.59	0.44	0.253	0.581		0.083	0.691	0.579	0.731	0.746
Unclassified RF39	All	0.39	0.64		0.16	0.155	0.709	0.578	0.583	0.775	0.373	0.881	0.885
	High	0.31[a]	0.65[a]	1.38[b]	0.38	0.031	0.409		0.632	0.179	0.840	0.770	0.669
Catabacteriaceae	All	0.49	0.48		0.14	0.945	0.030	0.924	0.047	0.228	0.020	0.033	0.452
	High	0.33	0.36	0.42	0.20	0.904	0.051		0.022	0.184	0.010	0.021	0.009
Bacillaceae	All	0.05[a]	0.46[b]		0.15	0.018	0.381	0.816	0.001	0.688	0.014	0.576	0.771
	High	0.02	0.52	0.44	0.35	0.504	0.503		0.016	0.792	0.240	0.291	0.864
Fibrobacteraceae	All	0.27[a]	0.08[b]		0.08	0.039	0.008	0.275	0.037	0.275	0.116	0.270	0.070
	High	0.56	0.13	0.10	0.18	0.084	0.600		0.074	0.843	0.122	0.774	0.267
Acetobacteraceae	All	0.00[a]	0.28[b]		0.05	<0.001	<0.001	0.043	<0.001	0.005	<0.001	<0.001	0.002
	High	0.00[a]	0.55[b]	0.32[b]	0.12	0.019	0.006		<0.001	0.094	<0.001	0.004	0.101

PMR = partial mixed ration; PMR+Canola = PMR+Canola meal; Lin = linear polynomial contrast; Quad = quadratic polynomial contrast.

[1]All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

The main effects and the interactions of feeding strategy, supplement feeding amount, and sample time for bacterial families with a mean relative abundance of >0.2% in at least one feeding strategy for all cows are displayed in Table 6. These main effects and interactions are also displayed for cows fed 14 and 16 kg of SDM, incorporating the effect of canola substitution (Table 6). The Streptococcaceae and Lactobacillaceae were among the bacterial families that had a mean relative abundance of >0.2% in at least one feeding strategy.

All Supplement Feeding Amounts

Relative abundances of the following bacterial families were increased in the control compared to PMR cows: Prevotellaceae, Erysipelotrichaceae, Veillonellaceae, and Fibrobacteraceae. Relative abundances of the following bacterial families decreased in the control compared to the PMR cows: Ruminococcaceae, Unclassified Clostridiales, Anaerolinaceae, Clostridiales Family XIII Incertae Sedis, Bacillaceae, and Acetobacteraceae. Relative abundances of Lachnospiraceae, Unclassified Bacteroidales, F16, Coriobacteriaceae, Unclassified RF39, and Catabacteriaceae were not influenced by feeding strategy.

The relative abundance of Ruminococcaceae and Unclassified Clostridiales linearly and quadratically decreased with supplement feeding amount. The relative abundance of Lachnospiraceae, the Unclassified Bacteroidales, Fibrobacteraceae, and Acetobacteraceae linearly increased with supplement feeding amount, whereas that of Erysipelotrichaceae was both linearly and quadratically increased. Anaerolinaceae and Catabacteriaceae linearly decreased in relative abundance with supplement feeding amount.

The relative abundances of F16, Coriobacteriaceae, Anaerolinaeceae, Clostridiales Family XIII Incertae Sedis, Bacillaceae, and Acetobacteraceae increased, whereas Veillonellaceae and Fibrobacteraceae decreased over the sample times. The relative abundance of Ruminococcaceae, Unclassified Clostridiales, Lachnospiraceae, and Catabacteriaceae increased at 12:00, compared to 8:20 and 16:40 h, and the inverse occurred for Prevotellaceae and Unclassified Bacteroidales.

The Ruminococcaceae linearly and quadratically decreased in relative abundance with supplement feeding amount in the control and PMR cows, respectively. The relative

abundance of the Unclassified Clostridiales and Veillonellaceae linearly and quadratically decreased in the controls with supplement feeding amount, respectively. The Erysipelotrichaceae increased linearly and quadratically in relative abundance in the controls with supplement feeding amount. The relative abundance of the Unclassified Bacteroidales linearly and quadratically increased in the PMR cows and quadratically decreased in the controls with supplement feeding amount. The Anaerolineaceae linearly and quadratically decreased in relative abundance with supplement feeding amount in the control and PMR cows, respectively. The Acetobacteraceae linearly and quadratically increased in the PMR cows with supplement feeding amount (Table 8).

High Supplement Feeding Amounts

The relative abundance of F16 was higher in the PMR and PMR+Canola cows and similar between the control and PMR cows. Veillonellaceae and Acetobacteraceae increased and decreased in relative abundance in the control, compared to PMR and PMR+Canola cows, respectively. The relative abundance of the Unclassified Bacteroidales was higher in the PMR, compared with control and PMR+Canola cows. Relative abundances of Ruminococcaceae, Unclassified Clostridiales, and Anaerolinaceae decreased in cows fed 16 kg of SDM, and increases in Erysipelotrichaceae and Acetobacteraceae occurred for these cows.

Prevotellaceae, Ruminococcaceae, Lachnospiraceae, Unclassified Clostridiales, Anaerolinaceae, Bacillaceae, and Acetobacteraceae had a consistent pattern of relative abundance over time, compared to when all supplement feeding amounts were analyzed. The relative abundance of Veillonellaceae was decreased at 12:00, compared to 8:20 and 16:40 h and the Catabacteriaceae were decreased in relative abundance at 16:40 h.

The Erysipelotrichaceae increased in relative abundance in the controls fed 16 kg of SDM, compared with the PMR and PMR+Canola cows fed 14 or 16 kg of SDM, and relative abundance of this bacterial family decreased in the PMR cows fed 16 kg of SDM, compared with control and PMR+Canola cows fed 16 kg of SDM. The Unclassified Bacteroidales were increased in relative abundance in the controls fed 14 kg of SDM, compared with the other feeding strategies fed at this amount and the control and PMR+Canola cows fed 16 kg of SDM. The relative abundance of Unclassified Bacteroidales was lower in the control cows fed 16 kg of SDM, compared

to cows fed by all feeding strategies at 14 and 16 kg of SDM, except the PMR+Canola cows fed 14 kg of SDM (Table 8).

Co-inertia Analysis

Co-inertia analysis showed that supplement feeding amount had the largest association with BCC. Bacterial community composition had the largest association with concentrations of the fermentation products ammonia, butyrate, valerate, and propionate, whereas time, pH, and total VFA concentration were less associated (Figure 5). Bacterial community composition in the PMR+Canola cows fed 14 kg of SDM and PMR cows fed 12 kg of SDM was associated with concentrations of ruminal ammonia, butyrate, lactate, acetate, and pH. An association was also observed between BCC of the controls fed 14 or 16 kg of SDM and ruminal propionate concentration. Valerate concentration was associated with BCC of controls fed 16 kg of SDM (Figure 5).

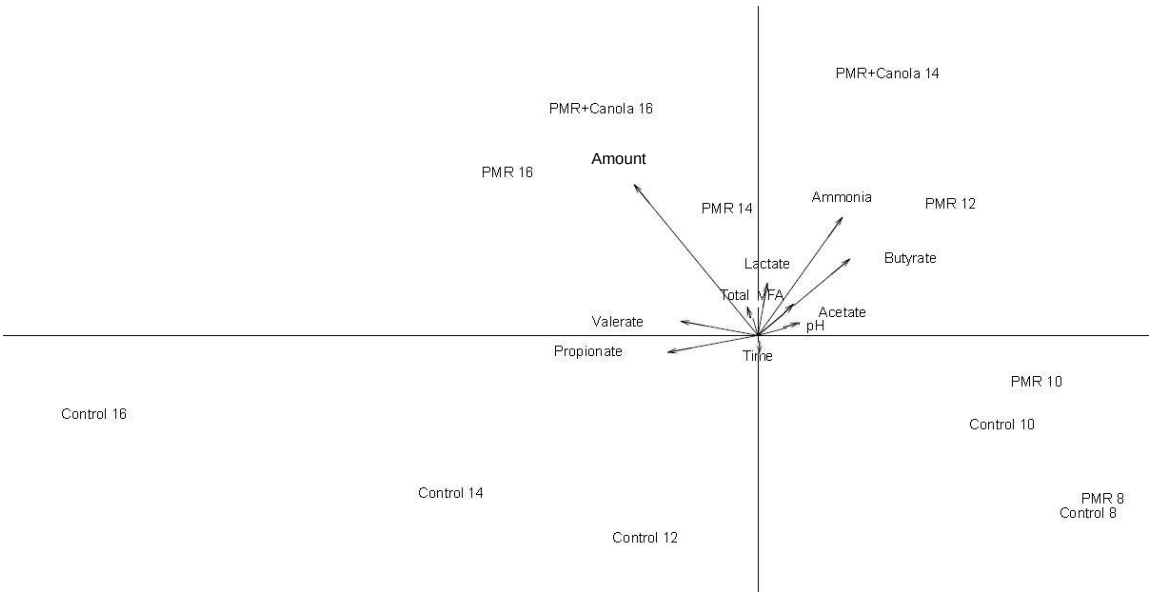


Figure 5. Duality diagram of co-inertia analysis of ruminal bacterial communities from 16S rDNA 454 pyrosequences, measures of ruminal fermentation, sample time, and amount of total supplements fed in dairy cattle fed 1 of 3 feeding strategies: control (n = 10 cows), partial mixed ration (PMR; n = 10 cows), or PMR+Canola (PMR+Canola meal n = 4 cows) at amounts 8, 10, 12, 14, or 16 kg of DM of total supplement/cow per day (2 cows per supplement feeding amount at 3 times from each feeding strategy). On the bi-plot the ruminal fermentation measures are represented as arrows. The direction of the arrow of each ruminal fermentation measure indicates an increasing magnitude of that measure. The angle between the arrows indicates their degree of correlation. The magnitude of the arrows indicates the importance of a measure on bacterial community composition. Measures with long arrows are more strongly correlated with the ordination axes than short arrows and have a greater influence on the pattern of variation (Carberry et al., 2012).

DISCUSSION

This study examined the effects of PMR and linearly increasing supplement feeding amounts on milk production, milk composition, and ruminal fermentation, and ruminal measures, ruminal acidosis, and ruminal BCC and their associations in late-lactation cows. Many of the feeding strategies and supplement feeding amount combinations evaluated in this study are consistent with those fed on commercial farms (Bramley et al., 2012). The control feeding strategy mimics traditional twice-daily feeding of concentrates in the milking parlor, which is the dominant supplement feeding system on dairy farms in south eastern Australia (Bramley et al., 2012) and many other countries. Feeding supplements as a PMR is the second most dominant feeding system for dairy farming in this region (Bramley et al., 2012), and is designed to provide energy over a longer period, compared with feeding an iso-energetic component-fed control diet (Bargo et al., 2003; Auldist et al., 2013).

It should be noted that while the control and PMR feeding strategies evaluated in this current experiment were iso-energetic, they contained different feed components. The substitution of some wheat for canola meal in the PMR was designed to increase metabolizable protein supply above NRC (2001) requirements. The removal of starch and addition of protein, some of which provides ammonia and peptides, may provide better ruminal conditions and increased milk production. Although cows in the current study consumed a similar total DMI to target intakes, the controls consumed less wheat and more ryegrass silage than targeted and were at a lower risk of ruminal acidosis than anticipated. These interventions were initiated in very late lactation, and milk production levels are consequently low.

The effects of feeding groups on the incidence of ruminal acidosis was assessed by the generation of acidosis eigenvalues using the model described by Bramley et al. (2008), which has been used to assess other datasets (O'Grady et al., 2008; Golder et al., 2012; Golder et al., 2014a). The eigenvalues are based on ruminal fermentation measures associated with health and production measures from 800 cows from 100 dairies that included pasture only, pasture and concentrate, PMR, and TMR feeding systems (Bramley et al., 2008; Bramley et al., 2012). Thus acidosis eigenvalues are a more comprehensive measure to diagnose ruminal acidosis in comparison to single measures such as ruminal pH. The acidosis eigenvalues indicate ruminal acidosis or rumen

perturbation was present in different severities in the feeding groups. The acidosis eigenvalues in this study are consistent with expected physiological outcomes for the feeding groups and are consistent with the milk measures. The co-inertia analysis also supports the Bramley et al. (2008) model, with the strength and direction of relationships between feeding groups, ruminal fermentation, and ruminal BCC data coinciding with eigenvalues for feeding groups.

The acidosis eigenvalues of the control cows fed 14 or 16 kg of SDM indicate these cows had the greatest incidence of ruminal acidosis. The control cows fed 16 kg of SDM also had the most distinct BCC in the constrained PCoA and co-inertia analysis and the lowest number of observed OTU in the rarefaction analysis. The linear and quadratic increase in acidosis eigenvalues with feeding amount is not surprising, as increasing feeding amount provided an increase in dietary starch content. The quadratic decrease in yield of milk fat with feeding amount provides further support for this. Supplement feeding amount also had the largest influence on BCC of the measures tested in the co-inertia analysis, supporting these findings.

The PMR+Canola cows had very low eigenvalues in cows fed both 14 and 16 kg of SDM, suggesting that these cows had the most favourable ruminal conditions of all the feeding groups at higher feeding amounts. This favorable environment coincided with the increased yield of ECM and milk fat in these groups of cows. Increased formation of metabolizable protein may have contributed to the increase in yield of milk fat and ECM in the PMR+Canola cows; however, to contrast, yields of milk and milk fat were not influenced by RUP content in the majority of studies quantitatively reviewed (Bargo et al., 2003). The involvement of dietary protein in ruminal acidosis and protein and carbohydrate synchrony require further investigation.

The lower eigenvalues in cows fed the PMR, compared to the controls indicate more favourable ruminal conditions and a lower incidence of ruminal acidosis in the PMR-fed cows. This finding may in part reflect the slower rate of fermentation of maize starch fed in the PMR, compared to the rapid rate of fermentation of wheat starch, which predisposes cows to digestive disorders (Sutton, 1984; Khorasani et al., 2001). The more stable fermentation pattern that occurred in the PMR cows across the day, which may be more favorable, may also be partly because the maize grain and maize silage included in the PMR were consumed over a longer time period than the wheat consumed by the

controls. Partial mixed ration feeding also allows simultaneous delivery of fermentable carbohydrates and physically effective fiber and better synchronization of starch and protein fermentation, compared with feeding concentrates in the milking parlor followed by silage access on the pasture.

The effects of feeding strategy (Bargo et al., 2002b; Auldist et al., 2013; Auldist et al., 2014) and increasing supplementary feeding amount (Stockdale et al., 1987; Leddin et al., 2010; Auldist et al., 2013) on ruminal pH, total and individual VFA measures, and ammonia concentrations were generally consistent with similar studies. However, in contrast propionate and valerate concentrations decreased, and acetate to propionate ratio, and butyrate concentrations increased in the PMR compared to control cows in our study. Ruminal pH was decreased with increased supplement feeding amount in the current study, but was not influenced in other studies (Stockdale et al., 1987; Leddin et al., 2010); however, those authors fed supplements only up to 10 kg of DM. Supplementation of the ionophore, monensin and antibiotic, tylosin, in the current study to all cows may also have reduced the generation of lactic acid (Dennis et al., 1981; Weimer et al., 2008) and influenced BCC. The spike in D-lactate concentration at sample time 5 in the PMR cows likely corresponded to the feeding of maize silage containing preformed lactic acid.

Concentrations of ammonia, butyrate, valerate, and propionate had a large influence on ruminal BCC, after feeding amount, supporting Bramley et al. (2008), who found that these are good predictors of ruminal acidosis. Ruminal pH had little influence on BCC in the co-inertia analysis (Figure 5), consistent with findings that cows with different pH profiles had similar BCC (Palmonari et al., 2010). In contrast, ruminal pH was a large vector in the study by Carberry et al. (2012) which examined associations between bacterial community diversity patterns and ruminal fermentation measures.

Protein supplementation did not influence total VFA concentration and molar proportion of acetate, propionate, and butyrate in the majority of studies in a quantitative review (Bargo et al., 2003), a finding consistent with this study. A higher ammonia concentration was associated with BCC in the PMR+Canola cows fed 14 kg of SDM in the co-inertia analysis (Figure 5). These findings may have resulted from an increase in RUP availability and increased microbial growth in the PMR+Canola cows; however, these measures were not directly assessed in the current study.

A decrease in the lipogenic to gluconeogenic VFA ratio (Auldist et al., 2013) and increases in *Butyrivibrio fibrisolvens*, and *Megasphaera* spp., that are regarded as major biohydrogenating bacteria in the rumen (Or-Rashid et al., 2009), have been associated with lower yield of milk fat. The relative abundance of *Butyrivibrio* spp. increased and the lipogenic to gluconeogenic VFA ratio decreased with increased feeding amount, along with decreased milk fat percentage, which supports this association, but the relative abundance of *Megasphaera* spp. was not influenced by feeding amount. We hypothesized that *B. fibrisolvens*, and *Megasphaera* spp. would be increased in relative abundance in the controls fed 14 or 16 kg of SDM, compared to the PMR and PMR+Canola cows fed at these amounts, as they had a lower yield of milk fat and tended to have a lower milk fat percentage. However, the relative abundance of *Butyrivibrio* and *Megasphaera* spp. was not different in these cows. Ramirez et al. (2012) found no difference in biohydrogenating bacteria in the rumen, despite differences in milk fat percentage and yield. Perhaps these bacteria do not always have the most substantial role in biohydrogenation.

The large among- and within-group variation in BCC, which is evident in the constrained between group PCoA, is consistent with considerable diversity in bacterial populations in ruminants fed identical diets (Brulc et al., 2009; Li et al., 2009; Chen et al., 2012). The apparent host specificity of ruminal bacteria may pose a challenge when examining interventions or management changes that affect the rumen and emphasizes the need for large sample sizes for in vivo studies.

The ruminal BCC in the current study was consistent with known bacterial communities in dairy cattle, as bacteria from the Bacteroidetes (predominately gram negative) and Firmicutes phyla (predominately gram positive) appeared to dominate the core bacterial microbiome, regardless of feeding group, consistent with other studies (Khafipour et al., 2009; de Menezes et al., 2011; Jami and Mizrahi, 2012). Similarly, the *Prevotella* were the dominant ruminal bacterial genus in the current study and others (Tajima et al., 2000; Fernando et al., 2010; Jami and Mizrahi, 2012). However, the relative abundance of bacteria from the Proteobacteria phylum, which have highly diverse metabolic functions (Garrity et al., 2005), was much lower in the current study than generally reported in dairy cows. Knowledge of the substrate utilization and end products of members of the candidate phylum, TM7, which had the third highest relative abundance

in the current study, is limited, but members have been identified in dairy cows (Khafipour et al., 2009; Kong et al., 2010; Hook et al., 2011).

The predominance of the Prevotellaceae, Ruminococcaceae, and Lachnospiraceae bacterial families, regardless of feeding group, is consistent with other studies (de Menezes et al., 2011; Thoetkiattikul et al., 2013; Zened et al., 2013); however, Thoetkiattikul et al. (2013) also reported a large relative abundance of Flavobacteriaceae, which were not observed in the current study. The members of the Prevotellaceae family are gram negative bacteria that belong to the Bacteroidetes phylum and can utilize a range of substrates (Boone et al., 2011), whereas members of the Ruminococcaceae and Lachnospiraceae families belong to the Firmicutes phylum and are largely cellulolytic and fibrolytic bacteria, respectively (Thoetkiattikul et al., 2013).

Nagaraja and Titgemeyer (2007) state that the relative abundances of gram negative bacteria are replaced with abundances of gram positive bacteria during acute ruminal acidosis. However, in our study, the relative abundance of the Prevotellaceae, which represent the dominant gram negative bacteria, was increased, whereas the abundance of the Ruminococcaceae and the Unclassified Clostridiales (gram positive bacteria) were lower in the control compared to PMR cows. Zened et al. (2013) also reported a dietary effect on BCC of members of the Prevotellaceae and Ruminococcaceae families, but in contrast to the current study, those authors reported an influence on Lachnospiraceae in cows fed maize silage diets with high or low starch content. A higher relative abundance of Prevotellaceae has been reported in cows fed pasture compared with TMR (de Menezes et al., 2011), a finding consistent with their higher relative abundance in the controls in the current study. Khafipour et al. (2009) reported a decrease in *Prevotella* spp. with induced subacute ruminal acidosis, whereas in the current study, *Prevotella* were higher in relative abundance in the control cows, which had higher acidosis eigenvalues, compared to the PMR cows; Mohammad et al. (2012) found *Prevotella* were not directly associated with ruminal acidosis.

The relative abundance of the ruminal bacteria *Streptococcus,* and *Lactobacillus,* which are associated with ruminal acidosis, and *Megasphaera,* which are associated with protection against ruminal acidosis, were <1% in the current study. The involvement of *Streptococcus bovis* in ruminal acidosis is unclear; other studies have reported varied

effects of starch on the relative abundance of or total bacterial rDNA copies contributed by *S. bovis* (Tajima et al., 2000; Fernando et al., 2010; Palmonari et al., 2010). The cows in the current study were adapted to their feeding groups before rumen sampling and were also supplemented with monensin and tylosin, which may account for the low abundance of *Streptococcus* and *Lactobacillus*.

The higher relative abundance of the Veillonellaceae family in the controls is similar to the higher abundance of these bacteria identified in pasture-fed, compared with TMR-fed cows (de Menezes et al., 2011). The Veillonellaceae family includes members that produce propionate as their major fermentation product (Strobel and Russell, 1991), which is consistent with the current study. Some bacterial species belonging to the Veillonellaceae family utilize lactate (Stewart et al., 1997).

The Erysipelotrichaceae family, that had a higher relative abundance in the control cows, is more abundant in pasture-fed, compared to TMR-fed cows (de Menezes et al., 2011), possibly reflecting the higher pasture DMI of the control cows in the current study, although the role of this family in ruminal microbial fermentation is unknown (Verbarg et al., 2004; de Menezes et al., 2011). Literature on the involvement of bacteria from the following families in ruminal acidosis is limited: F16, Coriobacteriaceae, Anaerolinaceae, Clostridiales Family XIII Incertae Sedis, Catabacteriaceae, Bacillaceae, and Acetobacteraceae, but these ruminal bacteria warrant further investigation.

The increase in dietary starch content with increasing supplement feeding amount was expected to decrease the number of fibrolytic bacteria, including members of the Ruminococcaceae and Fibrobacteraceae families (Tajima, 2001; Zened et al., 2013). This finding was consistent with observations for the Ruminococcaceae, but in contrast to the increase in relative abundance of bacteria from the Fibrobacteraceae family.

Studies of the effects of protein on ruminal BCC are limited. The relative abundance of bacteria belonging to the *Prevotella*, *Butyrivibrio*, and *Streptococcus* genera that contain proteolytic bacteria were not affected by increased dietary protein in the current study. The only ruminal bacterial families that were influenced in the high supplement feeding amounts analysis that incorporated the effect of protein were the F16, Veillonellaceae, and Acetobacteraceae, and further research is required to investigate their involvement in ruminal function.

Assessment of the microbiome by meta-transcriptomics, as suggested by Mullin et al. (2013), would allow examination of metabolic shifts occurring during dietary changes and may be beneficial to broadening our understandings of ruminal responses to dietary changes. Investigations into the interactions of ruminal bacteria, protozoa, archaea, bacteriophages, and fungi, and associations with ruminal acidosis may be beneficial.

CONCLUSION

Firstly, as hypothesized, cows fed diets as a PMR, with or without canola meal, had improved ruminal measures, distinct ruminal BCC, and a decreased incidence of ruminal acidosis compared to controls fed iso-energetic diets as individual feed components. However, milk measures were only increased in the cows fed PMR+Canola. Second, as hypothesized, a linear increase in supplement feeding amount between 8 to 16 kg of DM/cow per day of total supplement altered milk and ruminal measures, created a distinct ruminal BCC, and increased the incidence of ruminal acidosis. These measures were also associated with each other. In conclusion, feeding diets as a PMR may benefit ruminal conditions and reduce the incidence of ruminal acidosis, regardless of feeding amount up to 16 kg of DM of total supplement/cow per day, but did not result in increased milk measures, compared to iso-energetic control diets fed as components in late-lactation cows. Feeding protein above dietary requirements may provide milk production and milk composition benefits; however, further research is warranted.

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CHAPTER 6

Effects of Feed Additives on Ruminal pH, Fermentation Products and Oxidative Stress Responses in Dairy Heifers Challenged with Grain

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OVERVIEW OF CHAPTER 6

The critical review in Chapter 1 showed that *in vivo* information on the effects of feed additives and their combinations in ruminal fermentation measures is limited and inconsistent. We hypothesized that supplementation with Fermenten®, monensin, flavophospholipol, and tylosin would modify ruminal fermentation measures and plasma oxidative stress responses in dairy heifers compared to unsupplemented control heifers. We further hypothesized combinations of these feed additives would have synergistic effects on ruminal measures.

ABSTRACT

The efficacy of feed additives and their combinations on ruminal pH, fermentation products, and plasma oxidative stress responses was evaluated in Holstein-Friesian heifers fed a single grain challenge in 2 randomized block studies (study 1 and study 2). In study 1, heifers (n = 42) were allocated to 14 isoenergetic groups in a randomized block partial factorial arrangement (n = 3 heifers/group): (1) grain only, (2) Fermenten® (**FE**), (3) monensin (**M**), (4) flavophospholipol (**FL**), (5) tylosin (**T**), (6) FE+M, (7) FE+FL, (8) FE+T, (9) M+FL, (10) M+T, (11) FL+T, (12) FE+M+FL, (13) FE+M+T, and (14) FE+FL+T. In study 2, heifers (n = 18) were allocated to 3 groups in a randomized block design (n = 6 heifers/group): (1) grain only, (2) M, and (3) M+FL. All heifers were fed their additives for a minimum of 7 d before a single 1.2% dry matter of bodyweight grain challenge. Ruminal samples were collected using a stomach tube 5, 65, 115, 165, and 215 min, and blood samples 5 and 215 min after consuming the challenge ration. Ruminal data from both studies was combined to increase statistical power. All ruminal measures increased in concentration over the 3.6 h sampling besides a decrease in ruminal pH and D- and L-lactate concentrations. Fermenten® decreased valerate and increased ammonia concentrations compared to controls. Monensin decreased total volatile fatty acid, acetate, propionate, and valerate concentrations relative to controls; whereas, FL had no effects on ruminal measures. Tylosin increased butyrate and caproate, and decreased L-lactate concentrations relative to controls. Plasma D-lactate concentrations were decreased by the interaction of FE, M, and T and plasma urea was increased in FE heifers relative to controls in study 1. Plasma oxidative stress and ruminal histamine measures were not influenced by feed additives in study 2. The effects of combinations of feed additives on ruminal and plasma responses were generally not synergistic or additive. Monensin and sources of ruminally degradable amino acids, peptides, and non-protein nitrogen may be beneficial in controlling starch-based ruminal acidosis.

Keywords: Fermenten®, flavophospholipol, monensin, ruminal acidosis, tylosin

INTRODUCTION

Feeding grain is routinely practiced in beef and dairy industries to increase productivity by providing fermentable energy. Consumption of large amounts of grain or other sources of readily fermentable carbohydrates and insufficient physically effective fiber can cause disorders such as ruminal acidosis, rumenitis, or liver abscesses (Nagaraja and Titgemeyer, 2007) and is associated with a lower milk fat to protein ratio (Bramley et al., 2008), and an increase in lameness (Bramley et al., 2005). These disorders can have a substantial economic impact on both beef and dairy industries (Nagaraja et al., 1999; Stone, 1999). A number of feed additives with different modes of action are available that may reduce the risk of these disorders and improve health and productivity. Commercially available feed additives that can affect rumen function include: antibiotics, ionophores, yeasts, enzymes, direct-fed microbials, buffers, and neutralizing agents. There is a potential for synergistic or antagonistic effects on rumen function when feed additives, particularly those with different modes of action, are fed in combination. Studies on the combined effects of feed additives are limited and have not been examined between Fermenten® (Church & Dwight Co. Inc., Princeton, NJ), monensin, flavophospholipol, and tylosin, with the exception of the combination of monensin and tylosin.

Fermenten® is a byproduct of lysine production and is a source of ruminally degradable amino acids, peptides, and non-protein nitrogen (**NPN**) that provides a substrate for microbial protein (Cooke et al., 2009). A meta-analysis of Fermenten® or Biochlor® (Biovance Technologies, Omaha, NB), a source of amino acids and peptides, used in 15 continuous culture fermenter trials increased acetate to propionate ratio, microbial protein production, and organic matter, crude protein and non-structural carbohydrate digestibility above controls (Lean et al., 2005). Decreased propionate and ammonia nitrogen concentrations above controls were observed (Lean et al., 2005). Fermenten® has also numerically increased milk fat yield, 4% fat corrected milk yield, and milk energy output in dairy cattle (Penner et al., 2009).

Monensin is a carboxylic polyether ionophore produced by a naturally occurring strain of *Streptomyces cinnamonensis* (Haney and Hoehn, 1967). It favors increased ruminal propionate, and decreased acetate, butyrate, and methane percentages (Richardson et al., 1976), and decreased lactate concentrations (Dennis et al., 1981) by selective inhibition

of gram positive bacteria. An increased efficiency of nitrogen use and energy utilization, and reduced risk of bloat and ketosis and associated clinical diseases can result (Duffield et al., 2002). A meta-analysis showed monensin supplemented dairy cattle had improved body condition score, bodyweight, milk and milk protein yields by 0.03, 0.06 kg/d, 2.5%, and 0.016 kg/d, respectively (Duffield et al., 2008). Milk fat and protein percentages were decreased by 0.13% and 0.03%, respectively, and dry matter intake (**DMI**) was reduced by 0.7 kg (Duffield et al., 2008).

Flavophospholipol is a phosphoglycolipid antimicrobial produced by strains of *Streptomyces* spp. (Gallo et al., 2010) which inhibits cell wall synthesis in gram positive bacteria and has some activity against *Salmonella* spp. and *Escherichia coli* (Butaye et al., 2003). No effects of flavophospholipol on total and individual volatile fatty acid (**VFA**) concentrations in cattle have been demonstrated (Albert et al., 1991; Mogentale et al., 2010), except a decrease in total VFA in bulls (Alert et al., 1993). Flavophospholipol has improved average daily gain (**ADG**) in a number of feedlot studies (Galbraith et al., 1983; Scott et al., 1984; Rowland et al., 1999) and milk yield (Arana et al., 1992; Blaziak et al., 1992), and milk protein and fat yields in dairy cattle (Bahrecke et al., 1984).

Tylosin is a macrolide antibiotic produced by *Streptomycetes fradiae* that inhibits protein biosynthesis in gram positive bacteria (Liu and Douthwaite, 2002). It is effective against the gram negative opportunistic bacteria, *Fusobacterium necrophorum*, the etiological agent in liver abscess (Nagaraja et al., 1999). Tylosin increased total VFA and butyrate concentrations and tended to decrease plasma lactate concentrations in lactating dairy cattle during a ruminal acidosis challenge (Lean et al., 2000). In feedlot cattle tylosin reduced the risk of liver abscess to 8% compared with 30% in unsupplemented cattle, but benefits to ADG, DMI, and feed efficiency were not consistent (Wileman et al., 2009).

Understanding mechanisms by which these feed additives influence health through evaluation of ruminal and blood measures will aid in development of prudent use protocols. The aim of this study was to evaluate the individual and combined effects of 4 feed additives: Fermenten®, monensin, flavophospholipol, and tylosin on ruminal pH, fermentation products, and plasma oxidative stress responses in comparison to unsupplemented controls in the context of production implications. We hypothesized

that supplementation would modify ruminal fermentation measures and plasma oxidative stress responses in dairy heifers compared to unsupplemented control heifers. We further hypothesized combinations of the feed additives would have synergistic effects on ruminal measures.

MATERIALS AND METHODS

All experimental procedures were approved by the Bovine Research Australasia (**BRA**) Animal Ethics Committee (BRA 0609-0610 and AEC 0405-0406). Two randomized block intervention studies were conducted at Camden, New South Wales (**NSW**), Australia on nonpregnant Holstein-Friesian heifers <18 mo of age from a commercial dairy herd. The starch grain-based challenge model used in both studies was similar to that described by Lean et al. (2013) and Golder et al. (2012).

Study 1

Animals and Experimental Design

Forty two Holstein-Friesian heifers [370 ± 11 kg of bodyweight (**BW**)] were enrolled in a randomized blocked intervention study with a partial factorial arrangement consisting of a pre-study acclimatization period (3 wk), adaptation period (minimum of 7 d), a grain challenge (single morning feed), and postchallenge monitoring period (2 d). The study was conducted in 2 blocks, 13 d apart (n = 21 heifers/block). Heifers were housed on a dry lot and individually fed all feed on a feed pad with individual head stanchions throughout the study. During the pre-study period heifers were accustomed to the feed pad and fed *ad libitum* ryegrass silage (*Lolium multiflorum*).

At the end of the pre-study period, heifers were paired for BW and randomly allocated to a block, day, and 1 of 14 isoenergetic feed additive groups using Stata v.11 (StataCorp. LP, College Station, TX). The groups (n = 3 heifers/group) were as follows: (1) grain only, (2) Fermenten® (**FE**), (3) monensin (**M**), (4) flavophospholipol (**FL**), (5) tylosin (**T**), (6) FE+M, (7) FE+FL, (8) FE+T, (9) M+FL, (10) M+T, (11) FL+T, (12) FE+M+FL, (13) FE+M+T, and (14) FE+FL+T (Table 1). Sample size was based on a previous method development study (Lean et al., 2013). The farm staff were not blinded to feed allocations due to the characteristics of some of the additives and risk of error in allocation.

Table 1. Feed additive groups from studies 1 and 2 and the number of heifers in each group (n)

		Feed additive			
Group	n	FE	M	FL	T
Grain[1]	9	–	–	–	–
FE	3	+	–	–	–
M[1]	8	–	+	–	–
FL	3	–	–	+	–
T	3	–	–	–	+
FE+M	3	+	+	–	–
FE+FL	3	+	–	+	–
FE+T	3	+	–	–	+
M+FL[1]	9	–	+	+	–
M+T	3	–	+	–	+
FL+T	3	–	–	+	+
FE+M+FL	3	+	+	+	–
FE+M+T	3	+	+	–	+
FE+FL+T	3	+	–	+	+
M+FL+T[2]	3	–	+	+	+
FE+M+FL+T[2]	3	+	+	+	+

FE = Fermenten® (3.0% of dry matter intake; Church & Dwight Co. Inc., Princeton, NJ); M = monensin (160 mg/heifer per d; Moneco 100; International Animal Health, Sydney, Australia); FL = flavophospholipol (25 mg/heifer per d; FLAVECO 5; International Animal Health,); T = tylosin (90 mg/heifer per d; Tyleco 50; International Animal Health); (+) = present; (–) = absent.
[1]Included heifers from studies 1 and 2. Heifers from study 2 in the M or M+FL groups received monensin at a dose rate of 135 mg/heifer per d (Rumensin 100; Elanco, Macquarie Park, Australia) and (or) flavophospholipol at a dose rate of mg/heifer per d (FLAVECO 5, International Animal Health).
[2]Group not tested.

All feed additives (except FE) were pelleted with wheat using a cold pellet machine. Sodium monensin was fed at a dose rate of 160 mg/heifer per d (Moneco 100; International Animal Health), FL was fed at a dose rate of 25 mg/heifer per d (FLAVECO 5; International Animal Health, Sydney, Australia), and T was fed at a dose rate of 90 mg/heifer per d (Tyleco 50; International Animal Health). Fermenten® (Church & Dwight) was administered as a top dress powder at 3.0% of individual estimated DMI for each heifer. Dry matter intake was estimated at 2.25% of BW dry matter (**DM**), based on the maintenance requirement of cattle.

During the adaptation period heifers were offered ryegrass silage in the morning and afternoon and approximately 1 kg (as-fed basis) of rolled barley grain hand mixed with 50 g of feed additive pellets or 3.0% of estimated individual DMI of FE in the morning daily. On the first day of the adaptation, cattle were offered 25 g of their allocated feed additives or 1.5% of estimated DMI of FE. The non-FE groups were supplemented with

millrun at 1.5% of estimated individual DMI on the first day of adaptation and 3.0% of estimated individual DMI for the remainder of the adaptation period to create 14 isoenergetic groups.

The target daily feed intake during the adaptation period was 8 kg/d DM ryegrass silage and 1 kg/d (as-fed basis) of grain. The chemical composition of this diet was estimated (CPM Dairy Ration Analyzer; version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 2) using feed analysis results of the ryegrass silage (Table 3) and barley grain (Table 4). The estimated chemical composition was based on a 370-kg heifer, with a body condition score (**BCS**) of 3.50 and growth rate of 0.19 kg/d.

Challenge Procedure

Each block of heifers was challenged on 1 of 2 consecutive days with up to 11 heifers sampled a day. Feed was withheld for 14 h (18:00 to 8:00 h) prior to the challenge feed. All heifers were then offered 350 g (as-fed basis) of ryegrass silage. We found that feeding a small proportion of hay or silage immediately before feeding the challenge ration prevented cattle from salivating excessively before sampling. Immediately after ryegrass silage consumption the heifers were offered 1.2% of their BW DM of wheat hand mixed with their allocated feed additives. Orts were weighed to calculate the percentage of ration consumed. The chemical composition of the challenge ration was estimated (CPM Dairy Ration Analyzer; Table 2) using feed analysis results of the ryegrass silage (Table 3) and wheat (Table 4).

A 500 mL ruminal fluid sample was collected using a custom-designed stomach pump and 3 m tube inserted to a length of >2 m at 5, 65, 115, 165, and 215 min after consumption of the challenge ration. Each sample was scored for saliva contamination as described by Bramley et al. (2008) using a 3 point scoring system (3 being the highest level of contamination). No ruminal samples retained for analysis had saliva scores >1. Ruminal pH was measured immediately using a pH meter (pHTestr 30, Oakton Instruments, Vernon Hills, IL). The ruminal fluid samples were kept on ice and later centrifuged at 1512 × *g* at 5^{o}C for 15 min. The supernatant was dispensed into polypropylene tubes and stored at –20^{o}C for VFA, ammonia, and D- and L-lactate analysis. Jugular blood was collected into 10 mL lithium heparin (for urea analysis) and 10 mL fluoride oxalate collection tubes (for D- and L-lactate analysis) immediately after

the 5- and 215-min ruminal fluid collections, centrifuged at 1512 × *g* for 15 min at 5°C and plasma was decanted off and stored at –20°C.

Table 2. Estimated chemical composition (CPM Dairy Ration Analyzer version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY) of the diet fed during the adaptation period and on challenge morning for studies 1[1] and 2[2]

	Study 1		Study 2	
Item (% of DM)	Adaptation	Challenge	Adaptation	Challenge
DM	61.0	87.3	73.0	76.3
CP	11.5	14.7	16.7	14.5
RUP (% of CP)	29.7	15.1	25.7	14.5
RDP (% of CP)	70.3	84.9	74.3	85.5
RDP	8.09	12.5	12.4	12.4
Soluble protein (% of CP)	42.9	30.4	51.4	30.9
ADF	30.4	3.82	30.0	4.86
NDF	48.0	13.8	42.3	12.9
Forage NDF (% of NDF)	95.5	13.7	97.3	15.1
Forage NDF (% of DM)	45.9	1.89	41.1	1.94
Physically effective NDF	41.4	6.48	39.2	5.73
Lignin	4.56	0.92	5.57	0.84
NFC[3]	30.4	67.9	30.2	67.3
Silage acids	4.50	0.19	6.54	0.00
Sugar	16.3	1.14	6.98	3.04
Starch	6.99	65.0	7.73	60.9
Soluble fiber	2.59	1.61	8.93	3.31
Total ether extract	3.23	1.95	4.59	2.05
Total LCFA	1.57	1.71	1.41	1.78
Ash	9.94	2.32	8.93	3.98
DCAD (mEq/100g)	8.18	-1.83	23.8	1.69
Minerals (mg/kg)				
Chloride	17,200	1,600	14,700	1,500
Calcium	5,500	700	6,600	4,900
Copper	84	7	6	7
Iron	184	75	281	140
Phosphorus	3,200	4,200	3,000	4,100
Potassium	25,700	5,000	20,900	5,900
Magnesium	1,900	1,600	2,200	4,000
Manganese	46	42	108	42
Sodium	600	200	6,100	400
Sulfur	1,900	1,700	2,400	1,700
Zinc	40	43	22	42

DM = dry matter; CP = crude protein; RUP = rumen undegradable protein; RDP = rumen degradable protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC = non-fiber carbohydrate; LCFA = long chain fatty acid; DCAD = dietary cation-anion difference.

[1]The adaptation diet consisted of ryegrass silage fed *ad libitum* with a target intake of 8.0 kg/d dry matter (DM) of ryegrass silage, and 1 kg/d (as-fed basis) of barley grain once daily. The challenge diet consisted of 350 g of alfalfa hay and 1.2% of bodyweight (BW) DM of wheat grain.

[2]The adaptation diet consisted of ryegrass silage and alfalfa hay fed *ad libitum* with a target intake of 7.2 kg/d DM of ryegrass silage, 2 kg/d DM of alfalfa hay, and 1 kg/d (as-fed basis) of triticale grain cultivar Berkshire once daily. The challenge diet consisted of 200 g of alfalfa hay and 1.2% of BW DM of triticale grain cultivar 'Berkshire'.

[3]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash]. NDICP = neutral detergent insoluble crude protein

Table 3. Chemical composition of ryegrass silage fed in study 1 during the adaptation period and on challenge morning and alfalfa hay and ryegrass silage fed during the adaptation period, and triticale grain cultivar 'Berkshire' fed on challenge morning for study 2[1]

	Study 1	Study 2		
Item (% of DM)	Ryegrass silage	Alfalfa hay	Ryegrass silage	Triticale grain
DM	58.9	12.3	23.8	11.2
CP	11.4	20.7	17.7	16.7
Soluble protein (% of CP)	44.5	43	40.5	26.5
Crude fat	3.3	2.5	2.6	1.5
Ash	10.7	9.0	10.4	2.4
Lignin	4.8	6.8	6.5	2.3
ADF	32.9	33.6	35.6	5.3
NDF	50.9	45.9	52.3	22.1
ADICP	0.6	1.2	1.5	0.3
NDICP	3.3	3.5	5.6	2.9
NFC[2]	27.0	25.5	22.8	60.0
Available protein	10.9	19.5	16.2	16.5
Degradable protein (% of CP)	66.5	69	64	70
Starch	2.0	2.5	1.7	51.7
NSC (%)	19.8	-	-	-
Sugar (%)	17.8	-	-	-
WSC	-	7.2	7.5	-
ESC (simple sugars)	-	4.9	7.3	3.8
DCAD (mEq/100g)	-	20	5	1
Minerals (mg/kg)				
Calcium	6,000	10,002	9,828	357
Phosphorus	3,000	3,400	2,700	3,000
Magnesium	2,000	2,906	3,608	1,300
Potassium	28,000	24,170	19,730	6,625
Sulfur	2,000	3,000	3,400	1,900
Chloride ion	19,000	9,388	16,367	1,358

DM= dry matter; NDF = neutral detergent fiber; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; ADICP acid detergent insoluble crude protein; NDICP = neutral detergent insoluble crude protein; NFC = non-fiber carbohydrate; NSC = non-structural carbohydrate; WSC = water-soluble carbohydrate; ESC = ethanol-soluble carbohydrate; DCAD = dietary cation-anion difference.

[1]Values are means obtained from near-infrared spectroscopy (AOAC 2000) and wet chemistry (George Weston Technologies, Sydney, NSW, Australia; Golder et al., 2012b).

[2]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash].

Table 4. Chemical composition of barley and wheat grain offered in the adaptation and challenge periods, respectively in study 1.

Item (% of DM)	Feed	
	Barley	Wheat
Englyst NDF	20.9	12.4
CP	14.0	14.8
Crude fiber	4.4	2.5
ADF	5.1	2.7
Total starch	62.1	70.4
Total insoluble NSP	9.2	6.9
Total soluble NSP	3.3	0.6

CP = crude protein; ADF = acid detergent fiber; NSP = non-starch polysaccharide.

Heifers were examined by the study veterinarian on the 2 d after challenge for any abnormal behavior or signs of illness. Clinical observation was used to diagnose ruminal acidosis and supported by ruminal pH, VFA, ammonia, and lactate and plasma lactate, and urea measures.

Laboratory Analysis

Near infrared spectroscopy (AOAC, 2000) and wet chemistry analysis (George Weston Technologies, Sydney, NSW, Australia; Golder et al., 2012b; Table 3) were carried out on pooled ryegrass silage samples collected and frozen at –20^{o}C from each bale opened and fed during the adaptation and challenge periods.

The barley and wheat grain offered during the adaptation and challenge, respectively were analyzed by ChemCentre (Bentley, Western Australia, Australia) for the following chemical components (Table 4): Englyst NDF (RACI, 1995, method 03-02), crude protein (**CP**) (Dumas Nitrogen with nitrogen value × 6.25; AOAC, 1995, method 4.2.04), crude fiber (AOAC, 1995, method 4.6.01), and acid detergent fiber (**ADF**) (AOAC, 1995, method 4.6.03). Total starch (Megazyme amyloglucosidase/α-amylase method; McCleary et al., 1997; AOAC, 1999, method 996.11; AACC, 1976, method 76.13), total insoluble non-starch polysaccharides (**NSP**), and total soluble NSP [modification of Englyst and Hudson (1993) and, Theander and Westerlund (1993)] were analyzed at the Animal Science Laboratory (University of New England, Armidale, NSW, Australia). See equations:

$$\text{Total insoluble NSP} = \text{insoluble } [(\text{rhamnose} + \text{fuctose} + \text{ribose}) \times 0.89] + [(\text{arabinoxylose} + \text{xylose}) \times 0.88] + [(\text{mannose} + \text{galactose} + \text{glucose}) \times 0.9]$$

$$\text{Total soluble NSP} = \text{soluble } [(\text{rhamnose} + \text{fuctose} + \text{ribose}) \times 0.89] + [(\text{arabinoxylose} + \text{xylose}) \times 0.88] + [(\text{mannose} + \text{galactose} + \text{glucose}) \times 0.9] + \text{beta-glucan}$$

Ruminal VFA concentrations were analyzed by an Agilent series gas chromatograph with HP 6890 injection and HP-FFAP 30 mm × 0.53 mm × 1.0 μm capillary column (HP Part No. 199095F-123; Agilent Technologies Inc., Wilmington, DE), and Chemstation software (Agilent Technologies Inc.) based on methodology from Supelco Inc. (1975). A standard and blank were included with every run of 100 samples. The inter-assay coefficient of variation (**CV**) of acetate, propionate, iso-butyrate, butyrate,

iso-valerate, valerate, and caproate were 5.1, 3.8, 3.2, 4.0, 3.4, 3.7, and 3.2% respectively. D- and L-lactate concentrations in ruminal fluid and plasma (no plasma D-lactate determination in study 2) were analyzed using a Boehringer Mannheim kit (Cat. no. 11 112 821 035; Arrow Scientific, Lane Cove, NSW, Australia) on a Cobra Mira S autoanalyzer at 340 nm (Roche, Dee Why, NSW, Australia). The inter-assay CV of D- and L-lactate was dependent on the D- and L-lactate concentrations with a CV of 22.7 and 7.9% for concentrations <1 m*M*, respectively, and 2 and 1.4% for concentrations >1 m*M*, respectively. Ruminal ammonia was analyzed by the direct enzymatic method (Pesce and Kaplan, 1996) using a Boehringer Mannheim kit (catalog no. 11112 732035; Arrow Scientific) on a Cobra Mira S autoanalyzer at 340 nm. Plasma urea concentrations were measured using an Olympus kit [catalog no: OSR 6134; Mt Waverly, Victoria (**VIC**), Australia] based on the methods described by Tiffany et al. (1972). The inter-assay CV was 2.23%.

Study 2

Experimental Design

A total of 18 Holstein-Friesian heifers (361 ± 8 kg of BW) were enrolled in a randomized block intervention study consisting of a pre-study acclimatization period (2 wk), adaptation period (8 d), starch grain-based challenge (single morning feed), and postchallenge monitoring period (2 d). Heifers were housed on a dry lot and individually fed all feed on a feed pad with individual head stanchions throughout the study. During the pre-study period heifers were accustomed to the feed pad and offered *ad libitum* ryegrass silage (*Lolium multiflorum*) and alfalfa hay (*Medicago sativa*). At the end of the pre-study period they were paired for BW and randomly allocated to 1 of 4 d and 1 of 3 feed additive groups using Stata v.11 (n = 6 heifers/group). Groups were as follows: (1) grain only, (2) M, and (3) M+FL. Study investigators had knowledge of the feed additive groups throughout the trial. The sodium monensin was fed in granular form at a dose rate of 135 mg/heifer per d (Rumensin 100; Elanco, Macquarie Park, Australia) and the FL was fed as a powder at a dose rate of 45 mg/heifer per d (FLAVECO 5, International Animal Health).

During the adaptation period heifers were offered ryegrass silage and alfalfa hay twice daily in individual head stanchions and 1 kg (as-fed basis) of rolled barley grain hand

mixed with respective feed additives in the morning daily. The target daily feed intake during this period was 2 kg/d DM alfalfa hay, 7.2 kg/d ryegrass silage DM, and 1 kg/d (as-fed basis) of grain. The chemical composition of the diet was estimated (CPM Dairy Ration Analyzer; Table 2) using feed analysis results of the ryegrass silage and alfalfa hay (Table 3). The estimated chemical composition was based on a 400-kg heifer, with a BCS of 3.25 and growth rate of 0.73 kg/d.

Challenge Procedure

Heifers were challenged on 1 of 4 consecutive days for ease of sampling. Feed was withheld for 14 h prior to challenge as described in study 1. On the day of challenge each heifer was offered and ate 200 g of alfalfa hay to reduce saliva contamination of the ruminal samples. Immediately after hay consumption, heifers were fed 1.2% of their BW DM of triticale grain cultivar Berkshire with their respective feed additives. The chemical composition of the challenge ration was estimated (CPM Dairy Ration Analyzer; Table 2) using feed analysis results of the alfalfa hay and triticale grain (Table 3).

Ruminal fluid samples were collected and scored for saliva at 5, 65, 115, 165, and 215 min after challenge ration consumption by a stomach tube and custom-designed stomach pump as described in study 1. No ruminal samples retained for analysis had saliva scores >1. Blood samples were taken via jugular venipuncture into 10 mL lithium heparin blood collection tubes (BD Vacutainer, Devon, UK), immediately after the 5- and 215-min ruminal fluid sample collections and processed for plasma L-lactate analysis as described in study 1. Orts were weighed to calculate the percentage of allocated ration consumed. Heifers were monitored for 2 d after challenge as per study 1.

Laboratory Analysis

Pooled ryegrass silage and pooled alfalfa hay samples collected and frozen at –20^{o}C from each bale opened and fed during the adaptation and challenge periods and a sample of triticale Berkshire were analyzed by NIR (George Weston Technologies) and wet chemistry analysis as described in study 1 (George Weston Technologies and Dairy One Inc, Forage Testing Laboratory, Ithaca, NY; Table 3).

Ruminal fluid samples were analyzed for pH immediately after collection using a pH meter (Merck Pty Ltd., Kilsyth, Australia). Ruminal VFA, ammonia, and D- and L-lactate, and plasma L-lactate concentrations were measured as described in study 1. Ruminal histamine concentrations were analyzed using a human histamine ELISA kit (IBL International, Hamburg, Germany) according to manufacturer instructions for human plasma samples. Ruminal fluid was passed through a 0.22 μm syringe filter (Millipore, Carrigtwohill, County Cork, Ireland) prior to analysis. The kit was validated for bovine ruminal and plasma histamine by Rabiee et al. (2009) as described by Golder et al. (2012b).

Measures of oxidative stress biomarkers were performed as described by Golder et al. (2013). Briefly, the concentrations of plasma derivatives of reactive oxygen metabolites (**dROM**) and biological antioxidant potential (**BAP**) were measured using the d-ROMs and BAP colorimetric assays, respectively (Diacron International, Grosseto, Italy). The extent of oxidative stress was expressed as an oxidative stress index estimated by [(dROM/BAP) × 100] as suggested by Celi (2011). Advanced oxidation protein products (**AOPP**) were measured according to the methods of Witko-Sarsat et al. (1998) at 340 nm using a POLARstar Optima (BMG Labtech, Melbourne, Australia). The concentrations of plasma glutathione peroxidase (**GSH-Px**) were measured based on a spectrometric method using a Cayman kit (Item no. 703102; Cayman, Ann Arbor, MI) on a POLARstar Optima. Plasma ceruloplasmin concentrations were determined according to the methods described by Sunderman and Nomoto (1970) with the exception that absorbance was read at 510 nm (POLARstar Optima).

Statistical Analysis

All models were fitted using R software (version 2.14.1; R Development Core team, 2005). A residual analysis was performed for each response measure, testing for the distributional assumption, homogeneity of the variance, and influential observations using residual and deviance plots. To increase statistical power the ruminal data from study 1 and study 2 were combined. A generalized estimating equations (**GEE**) model with repeated measures as a partial factorial arrangement was fitted with grain as a reference group using the R package geepack (Yan and Fine, 2004; Halekoh et al., 2006). Block and day were not significant and were removed from the final model.

Valerate and isovalerate were transformed using a natural logarithm to achieve a normal distribution of residuals. The model used was:

$$Y_{ijklmno} = \mu + \alpha_i + \beta_j + \omega_k + \delta_l + \gamma_m + \rho_n + (\alpha\beta)_{ij} + (\alpha\omega)_{ik} + (\alpha\delta)_{il} + (\beta\omega)_{jk} + (\beta\delta)_{jl} + (\omega\delta)_{kl} + (\alpha\gamma)_{im} + (\beta\gamma)_{jm} + (\omega\gamma)_{km} + (\delta\gamma)_{lm} + (\alpha\beta\omega)_{ijk} + (\alpha\beta\delta)_{ijl} + (\alpha\omega\delta)_{ikl} + (\alpha\beta\gamma)_{ijm} + (\alpha\omega\gamma)_{ikm} + (\alpha\delta\gamma)_{ilm} + (\beta\omega\gamma)_{jkm} + (\beta\delta\gamma)_{jlm} + (\omega\delta\gamma)_{klm} + (\alpha\beta\omega\gamma)_{ijkm} + (\alpha\beta\delta\gamma)_{ijlm} + (\alpha\omega\delta\gamma)_{iklm} + \varepsilon_{ijklmno,}$$

Y_{ijkl} = response to FE i (i = 1 or 2), M j (j = 1 or 2), FL k (k = 1 or 2), and T 1 (l = 1 or 2), at m time (m = 1 to 5) from study n (n = 1 or 2) by heifer o (o = 1 to 59);

μ = overall mean; α_i = fixed effect of FE; β_j = fixed effect of M; ω_k = fixed effect of FL; δ_l = fixed effect of T; γ_m = fixed effect of time; ρ_n = fixed effect covariate of study (represents the difference in season, ration, feed additive manufacturer, and feed additive dose rate); $\varepsilon_{ijklmno}$ = random residual error at time m from study n by heifer o using a first-order autoregressive correlation pattern (**AR1**). *P*-values < 0.05 were considered significant.

Plasma measures from study 1 were analyzed using the same model as the ruminal measures; however, study was removed from the model. Plasma measures and ruminal histamine concentrations from study 2 were fitted using a GEE model with repeated measures with grain as a reference group using geepack. The variables OSI and ceruloplasmin were transformed using a logarithm base 10 to achieve a normal distribution of residuals and L-lactate using a natural logarithm. The model used was:

$$Y_{ijk} = \mu + \alpha_i + \gamma_j + (\alpha\gamma)_{ij} + \varepsilon_{ijk,}$$

where Y_{ijk} = response to feed additive group i (i = 1 or 2), at time j (j = 1 to 5), in heifer k (k = 1 to 17), μ = overall mean; α_i = fixed effect of feed additive group; γ_j = fixed effect of time; $(\alpha\gamma)_{ij}$ = feed additive by time interaction; ε_{ijk} = random residual error adjusted for repeated measures within feed additive group i at time j for heifer k using an AR1 correlation pattern. A Pearson's correlation was performed to determine the relationship between ruminal and plasma lactate measures from both studies 1 and 2 at the 5- and 215-min samplings individually.

Table 5. Coefficient estimates (±SEM) and *P*-values for the main effects of time, Fermenten® (FE), flavophospholipol (FL), tylosin (T) and monensin (M) and their interactions for ruminal measures from studies 1 and 2 (n of heifers = 71; n of samples = 355)

Item (m*M*)	Coefficient ± SEM (*P*-value)													
	Time	FE	M	FL	T	FE × M	FE × FL	FE × T	M × FL	M × T	FL × T	FE × M × FL	FE × M × T	FE × FL × T
Total VFA	7.86±1.15 (<0.001)	5.27±5.41 (0.331)	-10.60±5.24 (0.043)	1.99±7.80 (0.799)	14.19±7.87 (0.071)	7.85±5.69 (0.168)	-16.34±8.35 (0.050)	-21.07±9.24 (0.023)	0.71±8.26 (0.932)	-9.61±9.74 (0.324)	-11.91±10.22 (0.244)	15.35±9.44 (0.104)	20.31±12.01 (0.091)	37.13±12.23 (0.002)
Acetate	4.27±0.77 (<0.001)	4.09±3.67 (0.265)	-7.70±3.15 (0.015)	1.21±4.57 (0.792)	8.40±5.61 (0.135)	5.31±3.78 (0.160)	-11.07±5.13 (0.031)	-14.74±6.10 (0.016)	0.48±4.90 (0.922)	-5.85±7.15 (0.414)	-8.07±6.88 (0.241)	8.59±5.79 (0.138)	13.52±7.83 (0.084)	25.36±7.99 (0.002)
Propionate	1.85±0.21 (<0.001)	1.39±1.16 (0.234)	-2.12±1.08 (0.050)	0.95±1.80 (0.599)	1.74±1.31 (0.184)	1.84±1.24 (0.137)	-3.40±2.10 (0.108)	-3.28±1.92 (0.088)	-0.24±1.87 (0.898)	-0.14±1.80 (0.939)	-1.97±1.99 (0.323)	3.11±2.35 (0.187)	2.84±2.74 (0.300)	6.64±2.86 (0.020)
Acetate:propionate	-0.20±0.03 (<0.001)	-0.21±0.11 (0.073)	-0.04±0.13 (0.788)	-0.27±0.17 (0.118)	-0.03±0.16 (0.862)	-0.07±0.16 (0.641)	0.24±0.27 (0.381)	0.05±0.34 (0.882)	0.17±0.19 (0.385)	-0.30±0.21 (0.146)	0.10±0.26 (0.709)	-0.28±0.35 (0.419)	0.13±0.42 (0.749)	-0.10±0.60 (0.864)
Butyrate	0.95±0.13 (<0.001)	-0.38±1.11 (0.735)	-0.78±1.05 (0.457)	-0.37±1.52 (0.807)	4.06±1.20 (0.001)	0.69±1.36 (0.610)	-1.16±1.95 (0.552)	-2.90±2.35 (0.216)	0.91±1.61 (0.570)	-3.95±1.64 (0.016)	-2.13±2.34 (0.362)	2.56±2.29 (0.264)	4.29±3.01 (0.153)	4.51±3.79 (0.233)
Ln valerate	0.20±0.02 (<0.001)	-0.20±0.09 (0.033)	-0.29±0.12 (0.017)	-0.18±0.16 (0.254)	0.02±0.14 (0.868)	0.48±0.14 (<0.001)	-0.13±0.17 (0.445)	0.12±0.20 (0.554)	0.28±0.19 (0.144)	-0.11±0.24 (0.647)	-0.04±0.25 (0.866)	-0.02±0.23 (0.946)	-0.23±0.30 (0.436)	0.33±0.33 (0.309)
Caproate	0.10±0.02 (<0.001)	0.02±0.03 (0.501)	-0.03±0.03 (0.289)	0.09±0.05 (0.056)	0.19±0.04 (<0.001)	0.01±0.03 (0.830)	-0.09±0.06 (0.099)	-0.21±0.04 (<0.001)	-0.09±0.05 (0.086)	-0.09±0.07 (0.169)	-0.06±0.14 (0.654)	0.27±0.11 (0.019)	0.09±0.07 (0.221)	0.11±0.15 (0.451)
Isobutyrate	0.09±0.02 (<0.001)	0.06±0.06 (0.333)	0.06±0.06 (0.300)	0.12±0.07 (0.094)	0.12±0.07 (0.094)	0.02±0.07 (0.826)	-0.32±0.14 (0.024)	-0.12±0.09 (0.176)	-0.20±0.12 (0.089)	-0.03±0.11 (0.790)	-0.10±0.09 (0.248)	0.34±0.18 (0.060)	0.00±0.15 (0.976)	0.40±0.17 (0.016)
Ln Isovalerate	0.10±0.01 (<0.001)	-0.15±0.09 (0.100)	0.07±0.06 (0.208)	-0.09±0.11 (0.422)	-0.09±0.07 (0.202)	-0.09±0.17 (0.599)	-0.10±0.15 (0.515)	0.30±0.15 (0.044)	-0.04±0.14 (0.762)	-0.02±0.17 (0.922)	0.20±0.18 (0.270)	0.15±0.22 (0.488)	-0.09±0.27 (0.749)	-0.09±0.25 (0.710)
Total lactate	-0.06±0.03 (0.024)	-0.09±0.08 (0.260)	0.13±0.16 (0.436)	-0.01±0.10 (0.934)	-0.06±0.08 (0.442)	-0.10±0.17 (0.552)	0.03±0.11 (0.773)	0.42±0.29 (0.151)	0.11±0.23 (0.630)	-0.14±0.16 (0.409)	0.41±0.20 (0.034)	-0.13±0.23 (0.560)	-0.25±0.33 (0.449)	-0.76±0.34 (0.028)
D-lactate	-0.05±0.02 (0.053)	-0.06±0.07 (0.344)	0.13±0.13 (0.349)	0.00±0.08 (0.956)	-0.02±0.07 (0.729)	-0.10±0.14 (0.457)	0.03±0.08 (0.763)	0.18±0.13 (0.157)	0.09±0.19 (0.642)	-0.15±0.14 (0.282)	0.25±0.13 (0.061)	-0.11±0.19 (0.569)	-0.03±0.18 (0.025)	-0.39±0.17 (0.878)
L-lactate	-0.02±0.01 (0.004)	-0.02±0.02 (0.198)	0.00±0.03 (0.966)	0.00±0.03 (0.877)	-0.04±0.02 (0.047)	0.00±0.04 (0.925)	0.01±0.03 (0.855)	0.24±0.17 (0.169)	0.02±0.04 (0.626)	0.01±0.03 (0.774)	0.17±0.07 (0.021)	-0.03±0.05 (0.590)	-0.22±0.18 (0.205)	-0.37±0.19 (0.047)
Ammonia	0.97±0.46 (0.035)	9.43±1.35 (<0.001)	1.72±1.45 (0.236)	-0.89±2.64 (0.736)	-2.41±1.54 (0.118)	-4.19±2.52 (0.841)	-0.02±2.91 (0.995)	-0.24±3.22 (0.940)	-1.25±3.37 (0.710)	0.86±2.26 (0.705)	0.65±3.21 (0.096)	0.60±4.25 (0.888)	-0.12±4.54 (0.979)	1.83±6.00 (0.761)
pH	-0.04±0.02 (0.021)	-0.11±0.06 (0.076)	0.03±0.08 (0.698)	-0.04±0.07 (0.534)	-0.03±0.09 (0.722)	0.10±0.08 (0.256)	0.18±0.08 (0.023)	0.08±0.11 (0.431)	0.09±0.09 (0.333)	0.08±0.11 (0.484)	-0.03±0.12 (0.766)	-0.29±0.11 (0.008)	-0.19±0.13 (0.132)	-0.17±0.14 (0.218)

VFA = volatile fatty acid.

RESULTS

No heifers developed clinical signs of ruminal acidosis during either study. Heifers allocated to the FE combinations in study 1 took a longer time period to adapt to these rations than other heifers. Two heifers from block 2 in study 1 were replaced on the third day of adaptation with 2 from block 1 after they did not consume an adequate quantity of their rations. One M heifer from study 2 consumed none of her challenge ration and was removed. A heifer from the FE+FL+T group from study 1 displayed estrus on challenge day and ate approximately 50% of her ration. She was drenched with the remainder of her ration and ~15 L of water. Greater than 95% of the 1.2% DM of BW challenge rations were consumed for 12 of the 14 groups in study 1, and 92% ± 8 for the Fe+T and 81% ± 16 for the Fe+FL+T groups. Heifers from all 3 groups consumed >98% of the 1.2% of BW DM challenge rations in study 2.

The main effects of time, FE, M, FL, and T, their 2 and 3 way interactions, and mean coefficients ± SE for the ruminal measures of studies 1 and 2 combined are presented in Table 5. The means ± SE at each of the 5 time samplings for ruminal total VFA, acetate, propionate, acetate:propionate, butyrate, valerate, ammonia, and total lactate concentrations, and pH are displayed in Figure 1. All ruminal measures increased in concentration across the sampling period on the challenge day, with the exception of ruminal pH and D- and L-lactate concentrations that declined (Table 5 and Figure 1I).

Fermenten® decreased valerate and increased ammonia concentrations compared to controls. Monensin decreased total VFA, acetate, propionate, and valerate concentrations relative to controls. Flavophospholipol had no significant effects on ruminal measures. Tylosin increased butyrate and caproate concentrations, and decreased L-lactate concentrations compared to controls (Table 5 and Figure 1).

Table 6. Coefficient estimates (±SEM) and *P*-values for the main effects of time, Fermenten® (FE), flavophospholipol (FL), tylosin (T) and monensin (M) and their interactions for plasma measures from study 1 (n of heifers = 42; n of samples = 84)

Item (m*M*)	Coefficient ± SEM (*P*-value)													
	Time	FE	M	FL	T	FE × M	FE × FL	FE × T	M × FL	M × T	FL × T	FE × M × FL	FE × M × T	FE × FL × T
D-lactate ($\times 10^3$)	0.83±1.1 (0.465)	-3.33±3.2 (0.302)	-3.33±3.2 (0.302)	-2.36E-15±4.6 (1.000)	-3.33±3.2 (0.302)	6.67±4.6 (0.144)	10.0±11.0 (0.350)	6.67±4.6 (0.144)	1.72E-15±4.6 (1.000)	6.67±4.6 (0.144)	3.12E-15±4.6 (1.000)	-10.0±11.6 (0.390)	-13.3±6.45 (0.039)	-3.33±12.5 (0.790)
L-lactate	0.00±0.05 (0.987)	-0.03±0.45 (0.948)	0.08±0.18 (0.664)	0.25±0.26 (0.341)	0.31±0.16 (0.053)	0.58±0.54 (0.287)	-0.01±0.53 (0.989)	0.31±0.58 (0.594)	-0.34±0.29 (0.239)	0.03±0.35 (0.925)	-0.32±0.28 (0.250)	-0.35±0.61 (0.571)	-1.21±0.71 (0.089)	-0.16±0.65 (0.800)
Total Lactate	0.00±0.05 (0.974)	-0.03±0.44 (0.943)	0.08±0.19 (0.680)	0.25±0.26 (0.347)	0.31±0.16 (0.059)	0.58±0.53 (0.268)	0.00±0.52 (0.998)	0.31±0.56 (0.582)	-0.34±0.30 (0.244)	0.04±0.35 (0.910)	-0.32±0.28 (0.255)	-0.36±0.60 (0.554)	-1.22±0.70 (0.081)	-0.17±0.63 (0.789)
Urea	0.28±0.05 (<0.001)	0.63±0.17 (<0.001)	1.20±0.65 (0.064)	-0.40±0.29 (0.166)	0.20±0.37 (0.592)	-0.97±0.69 (0.159)	-0.10±0.37 (0.785)	-0.13±0.59 (0.820)	-1.17±0.73 (0.107)	-1.00±0.79 (0.203)	0.20±0.53 (0.708)	1.13±0.79 (0.152)	0.23±0.93 (0.802)	-0.20±0.76 (0.792)

Table 7. *P*-values for the main effects of feed additive group and time and their interaction for plasma measures and ruminal histamine from study 2 (n of heifers = 17; n of samples = 34)

Item	*P*-value		
	Group (G)	Time (T)	G × T
Ln L-lactate	0.740	0.240	0.110
dROM	0.590	0.320	0.370
BAP	0.910	0.540	0.380
Log_{10} OSI	0.910	0.920	0.830
AOPP	0.355	0.083	0.322
GSH-Px	0.871	0.014	0.715
Log_{10} ceruloplasmin	0.062	<0.001	0.958
Ruminal histamine	0.340	0.150	0.290

dROM = reactive oxygen metabolites; BAP = biological antioxidant potential; Log_{10} = logarithm base 10; OSI = oxidative stress index [(dROM/BAP) × 100]; AOPP = advanced oxidation protein products; GSH-Px = glutathione peroxidase.

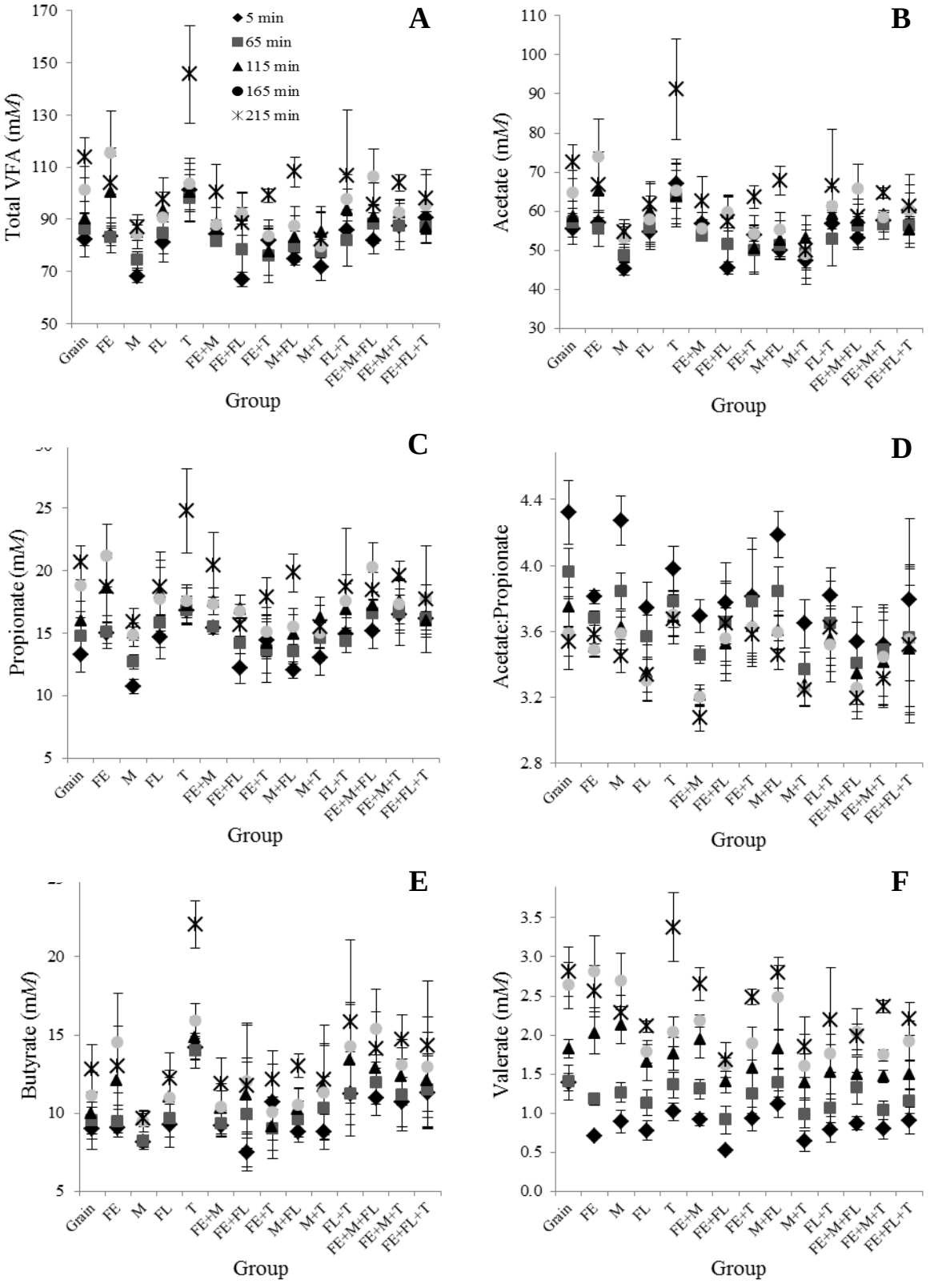

Figure 1. Mean (±SEM) ruminal concentrations of total volatile fatty acids (VFA) (A); acetate (B); propionate (C); acetate:propionate (D); butyrate (E); valerate (F); total lactate (G); ammonia (H); and pH (I) for 14 feed additive groups taken 5, 65, 115, 165, and 215 min after consumption of the challenge ration. FE = Fermenten®; M = monensin; FL = flavophospholipol; T = tylosin.

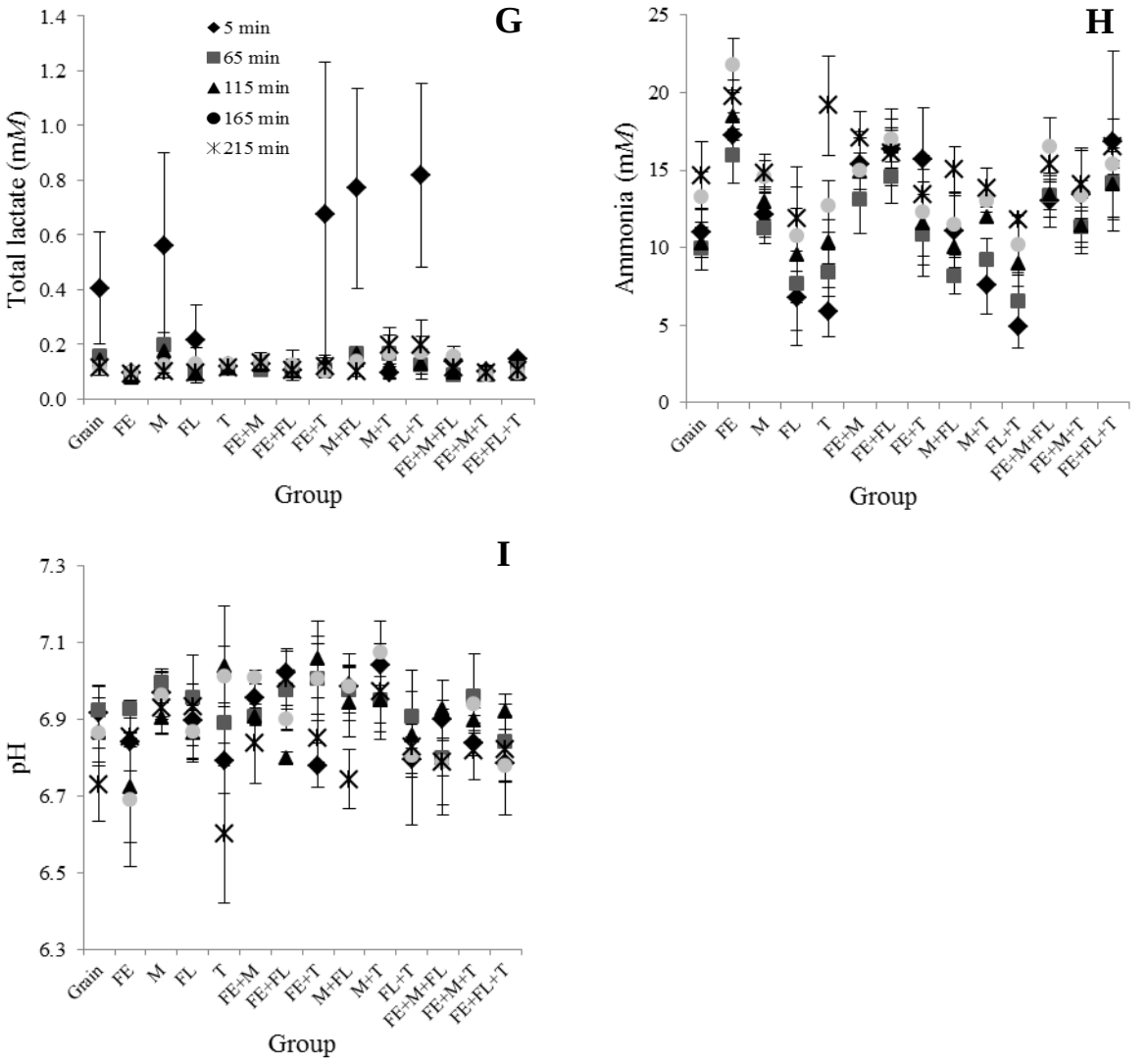

Figure 1 (continued). Mean (±SEM) ruminal concentrations of total volatile fatty acids (VFA) (A); acetate (B); propionate (C); acetate:propionate (D); butyrate (E); valerate (F); total lactate (G); ammonia (H); and pH (I) for 14 feed additive groups taken 5, 65, 115, 165, and 215 min after consumption of the challenge ration. FE = Fermenten®; M = monensin; FL = flavophospholipol; T = tylosin.

The interaction of FE and M increased valerate concentrations compared to controls. The interaction of FE and FL largely reduced total VFA, acetate, and isobutyrate concentrations and had the highest ruminal pH. Total VFA, acetate, and caproate concentrations were decreased, and isovalerate concentrations were increased when FE interacted with T compared to controls. The interaction of M and FL had minimal effects on ruminal measures relative to controls. Monensin interacted with T to decrease butyrate concentrations. The interaction of FL and T increased L-lactate and tended to increase D-lactate concentrations (P = 0.061). The 3 way interaction between FE, M, and FL decreased ruminal pH and increased caproate concentrations. D-lactate concentrations were decreased by the 3 way interaction between FE, M, and T relative to controls. The interaction between FE, FL, and T produced a large increase in total VFA, acetate, propionate, and isobutyrate concentrations and decreased L-lactate concentrations compared to controls (Table 5 and Figure 1).

The main effects of time, FE, M, FL, and T, their 2 and 3 way interactions, and mean coefficients ± SEM for the plasma measures from study 1 are presented in Table 6. The 3 way interaction of FE, M, and T decreased plasma D- lactate and tended to decrease L-lactate (P = 0.089) and total lactate concentrations in study 1 (P = 0.081, Table 6). Plasma urea concentrations were increased over time by FE and approached a significant increase in the M fed heifers (P = 0.064; Table 6). Total and L-lactate ruminal and plasma lactate concentrations at the 5 min sampling in study 1 were correlated (r = 0.47, P = 0.002 and r = 0.54, P < 0.001, respectively).

The main effects of feed additive group and time, and their interaction are displayed in Table 7. Plasma concentrations of GSH-Px and ceruloplasmin decreased over the 5 to 215 min sampling period in study 2 and ceruloplasmin concentrations approached a group difference (P = 0.062; Table 7). No group, time, or group by time interaction was observed for plasma L-lactate, dROM, BAP, OSI, AOPP, or ruminal histamine measures in study 2 (Table 7).

DISCUSSION

The starch-based grain challenge was adequate to evaluate the efficacy of the 4 feed additives and their combinations on ruminal fermentation measures, as total VFA, D- and L-lactate, and individual VFA concentrations were altered from those of unsupplemented controls. However, the challenge was relatively modest, as ruminal perturbation did not induce clinical signs of ruminal acidosis or substantial plasma changes. The complexity of interactions and differences in responses highlight the dynamic metabolic capabilities of the rumen. Ruminal fermentation measures indicated that feed additives modified the rumen by different mechanisms supporting our primary hypothesis, but had in combination only minor synergistic or antagonistic responses.

Starch-based grain challenges fed at 1.2% DM of BW, similar to that of this study, have induced ruminal changes in dairy heifers of comparable age and management (Golder et al., 2012b; Lean et al., 2013). The challenge ration fed in this study is comparable to some dairy transition rations and those that can be received by beef cattle at feedlot entry.

The relatively high ruminal pH's throughout both studies may reflect the high physically effective NDFs and relatively low NFC contents of the adaptation diets. Ruminal fluid collection site and method can influence ruminal pH and fermentation measures (Duffield et al., 2004; Shen et al., 2012) and should be considered when comparing ruminal results. Saliva contamination may occur when ruminal fluid is collected by a stomach tube (Duffield et al., 2004); however, this can be overcome by correct insertion technique (Lodge-Ivey et al., 2009; Shen et al., 2012). The absence of detectable saliva contamination in ruminal samples in this study as assessed using a 3 point scoring system in each ruminal fluid sample (Bramley et al., 2008) indicated saliva contamination was unlikely to be a concern.

Fermenten® supplementation had minimal effects on ruminal fermentation, a similar result to the lack of changes in total or individual VFA observed in a lactating dairy study (Penner et al., 2009). However, the trend toward a decrease in acetate to propionate ratio in the current study (P = 0.073) contrasts with the decrease in propionate and increase in acetate to propionate ratio in a meta-analysis of continuous culture fermenter FE or Biochlor® studies compared to control cultures (Lean et al.,

2005). The decrease in valerate concentrations in heifers fed FE in the current study is consistent with a trend observed in this meta-analysis (Lean et al., 2005). Valerate is a good diagnostic measure for ruminal acidosis (Bramley et al., 2008; Golder et al., 2012a); thus, may indicate reduced ruminal acidosis risk in the heifers fed FE.

The effects of FE on ruminal and plasma lactate concentrations in cattle studies have not been examined and it appears FE did not influence these measures. Ruminal pH was anticipated to increase in heifers fed FE due to FE's capacity to increase microbial N efficiency (Lean et al., 2005), which should result in the use of more organic matter for microbial cell synthesis (Penner et al., 2009). Ruminal pH was not affected in this study, a lactating dairy cow study (Penner et al., 2009), or continuous cultures with FE or Biochlor® (Lean et al., 2005).

The increased ammonia concentrations for the heifers fed FE were anticipated from the high rumen degradable amino acid, peptide, and NPN content of FE, and consistent with observed increases in ammonia nitrogen concentrations in continuous culture fermenter FE or Biochlor® studies (Lean et al., 2005). Similarly, ammonia nitrogen release initially increased *in vitro* up to 4 h into incubation with FE (Cooke et al., 2009), and there was a trend toward an increase in ammonia concentrations in dairy cattle fed FE (Penner et al., 2009).

The observed potential benefits of FE to ruminal fermentation may not translate to *in vivo* production benefits as these were not observed in dairy or beef studies (Cooke et al., 2009; Penner et al., 2009). The heifers in the current study took longer to adapt to the FE diets, which could indicate FE may not be as palatable as other feed inclusions. There may be potential benefits of FE combined with FL or T, or from a combination of all 3 of these additives. However, their 3 way interaction produced an increase in total VFA and acetate concentrations compared to a decrease in concentrations of these measures when FE and FL, and FE and T interacted. Production effects would need to be evaluated to establish if these proposed combinations are synergistic.

Monensin appeared to have an impact on a greater number of ruminal measures compared to the other feed additives and is the most extensively studied of the feed additives evaluated. The increased total VFA concentrations in heifers fed M in this study contrast with an absence of effects in lactating dairy cows fed M (Benchaar et al.,

2006; Martineau et al., 2007; da Silva-Kazama et al., 2011). The decrease in propionate concentrations in cattle fed M is also not consistent with the lack of effects (Haïmoud et al., 1995; Mutsvangwa et al., 2002) and increase in other studies (Ramanzin et al., 1997). However, lower acetate concentrations in heifers fed M is consistent with other M dairy studies (Ramanzin et al., 1997; Green et al., 1999).

Propionate and valerate are good diagnostic measures of ruminal acidosis (Bramley et al., 2008; Golder et al., 2012a); hence the decrease in both these VFA in the heifers fed M may indicate that M reduced the risk of ruminal acidosis. These VFA can act as safe sinks for hydrogen and electrons; therefore, depending on the complex interactions in the rumen and modes of action of feed additive interventions, increases in these VFA may also indicate a reduction in ruminal acidosis risk. A proposed action of monensin is an ability to inhibit lactate producing bacteria *in vitro*, without affecting most lactate utilizing bacteria (Dennis et al., 1981); however, lactate concentrations are rarely reported in *in vivo* cattle studies with M. This proposed mode of action is supported by decreased D- and L-lactate concentrations in cattle intra-ruminally administered ground corn, corn starch, and M (Nagaraja et al., 1985). Lactate concentrations were unchanged in the current study and may reflect the modest nature of the challenge.

Monensin has a 'protein sparing' effect that results in a decrease in ruminal ammonia concentrations (Haïmoud et al., 1995; Ghorbani et al., 2011). Ammonia concentrations were not effected in this current study or other studies in M supplemented dairy cattle (Ramanzin et al., 1997; da Silva-Kazama et al., 2011). The lack of effect of M on ruminal pH in this study is consistent with a number of previous dairy studies (Haïmoud et al., 1995; Ramanzin et al., 1997; Mutsvangwa et al., 2002).

The disparity in ruminal fermentation responses between discussed studies may result from differences in dose rates of M, cattle management, physiological state of the cattle, and diet. Despite variation in fermentation responses to M, the meta-analyses of Duffield et al. (2008; 2012) demonstrated M produced beneficial production responses in both dairy and beef cattle. This study and literature suggest M may provide health and production benefits when starch-based rations are fed.

This study and others in cattle found no effects of FL on ruminal total VFA (Rowe et al., 1982) or total and individual VFA concentrations (Albert et al., 1991; Mogentale et al.,

2010); however, a decrease occurred in total VFA FL supplemented bulls (Alert et al., 1993). Although no studies have reported ruminal pH or lactate in FL fed cattle Morgentale et al. (2010) showed FL administered through a rumen fistula to cattle had no effect on ruminal pH as did this study. Flavophospholipol has been proposed to decrease ruminal proteolysis and increase protein flow from the rumen and amino acid absorption in the small intestine (Behrens et al., 1993). Ammonia was not affected in the current study supporting findings of other FL studies in cattle (Rowe et al., 1982; Albert et al., 1991; Mogentale et al., 2010).

Despite the lack of beneficial effects of FL on ruminal fermentation observed in cattle, FL supplementation in dairy cattle increased milk yield (Arana et al., 1992; Blaziak et al., 1992) and milk protein and fat yield (Bahrecke et al., 1984). In feedlot studies FL has produced ADG improvements (Galbraith et al., 1983; Scott et al., 1984; Rowland et al., 1999). Flavophospholipol has clear benefits to dairy and beef production; however, these benefits are not reflected in ruminal fermentation measures in *in vivo* studies. Investigation of FL's impact on the rumen microbiome may further elucidate its mode of action.

Tylosin appeared to have minimal effects on ruminal fermentation measures in this study. The increase in butyrate and trend toward an increase in total VFA concentrations ($P = 0.071$) in heifers fed T is consistent with increases in these measures in lactating dairy cattle supplemented with T compared to controls during a ruminal acidosis challenge study (Lean et al., 2000). However, no effect on butyrate or total VFA molar percentage (Horton and Nicholson, 1980) or concentrations was reported in T supplemented feedlot steers (Nagaraja et al., 1999). Consistent with this study, these authors also reported no effects of T on acetate, propionate or ammonia, and Horton and Nicholson (1980) reported no effects on ruminal pH. Nagaraja et al. (1999) reported no effects of T on ruminal lactate concentrations in steers in contrast with the decrease in ruminal L-lactate concentrations in the current study.

Tylosin supplementation reduced the risk of liver abscesses to 8% in a feedlot cattle meta-analysis compared with a 30% risk in unsupplemented control cattle (Wileman et al., 2009). However, benefits on ADG, DMI, or feed efficiency were not consistent in cattle fed T (Wileman et al., 2009) and the influence of severity of abscesses that may reduce performance was not incorporated in the model (Nagaraja and Titgemeyer,

2007). A study in 14 herds of beef cattle supplemented with T or M+T reported similar ADG and feed efficiencies between these and unsupplemented cattle (Potter et al., 1985). Tylosin appears to reduce the incidence of liver abscesses; however, its effects on ruminal measures and productivity require further investigation.

To the best of our knowledge, with the exception of M+T, there are no studies on the combinations of FE, M, FL, and T. The combination of M+T is used in certain countries for proposed synergistic effects that increase productivity through alterations of ruminal microflora, with T specifically included to control liver abscess.

While there was no effect of M+T on total VFA concentrations, these were increased in lactating dairy cattle fed M+T (Lean et al., 2000) and decreased in M+T fed feedlot cattle (Ives et al., 2002). The absence of interaction between M and T on propionate concentrations is consistent with the majority of feedlot studies (Morris et al., 1990; Clary et al., 1993; Nagaraja et al., 1999); however, propionate concentrations were decreased in M+T fed feedlot cattle (Ives et al., 2002) and increased in dairy cattle fed M+T (Lean et al., 2000). The decrease in butyrate and absence of effect on acetate concentrations when M and T interacted in this study is not consistent with increases in dairy cattle fed M+T (Lean et al., 2000).

Ruminal lactate was not measured in the majority of M+T studies but was not altered in this study nor in feedlot steers (Nagaraja et al., 1999). Plasma D- and L- lactate concentrations were not affected by M+T in dairy cattle (Lean et al., 2000) or in feedlot steers (Morris et al., 1990; Clary et al., 1993; Nagaraja et al., 1999). The lack of interaction of M and T on ruminal ammonia concentrations is consistent with M+T-fed feedlot cattle (Morris et al., 1990; Coe et al., 1999; Ives et al., 2002).

The lack of M and T interaction on ruminal pH in the current study is consistent with that for M+T supplemented feedlot steers (Morris et al., 1990; Clary et al., 1993). However, is not consistent with increases in M+T supplemented feedlot steers (Ives et al., 2002) and Holstein steers (Coe et al., 1999), and a decrease in M+T fed dairy cattle (Lean et al., 2000).

The combination of M and T has improved ADG and feed efficiency (Clary et al., 1993), and improved feed efficiency, and decreased DMI in some feedlot studies (Zinn, 1987) but had no effect on DMI, ADG, or feed efficiency in another (Morris et al.,

1990). This inconsistency in ruminal fermentation and productivity measures indicates a need for further investigation to elucidate M+T's mode of action and potential benefits.

Increases in ruminal histamine have occurred in ruminal acidosis challenge studies (Ahrens, 1967; Golder et al., 2012b); therefore, it was hypothesized that ruminal histamine concentrations may be decreased in heifers fed feed additives compared to the heifers fed grain only in study 2. This hypothesis was not supported but is consistent with the lack of clinical signs of ruminal acidosis and other ruminal measures.

The lack of significant effects on oxidative stress measures may reflect the relatively mild nature of this single feeding challenge or indicate that oxidative stress is not affected during these feeding conditions. High levels of starch fed to dairy cows at 80 days in milk has increased oxidative stress (Gabai et al., 2004) and rations fed to achieve restricted or high milk production were associated with oxidative stress (Pedernera et al., 2010). The decrease in ceruloplasmin concentrations over the 3.6 h sampling was consistent with that in heifers subjected to the same challenge protocol (Golder et al., submitted). A more extreme readily fermentable carbohydrate challenge may induce greater oxidative stress responses; thus, further evaluation of oxidative stress responses to feed additives are required during different readily fermentable carbohydrate challenges.

The highest plasma urea concentrations observed in the heifers fed FE was anticipated as FE is a source of amino acids, peptides, and NPN. Penner et al. (2009) however, reported unchanged plasma urea nitrogen concentrations in dairy cattle fed FE. Monensin given in a control release capsule resulted in increased plasma urea concentrations (Hayes et al., 1996; Duffield, 1997; Green et al., 1999), in line with the trend toward an increase in the heifers fed M in this study ($P = 0.064$). Similar to the findings of the current study, no effects on plasma urea were reported for T or M+T supplemented dairy cattle (Lean et al., 2000).

Feed additives did not appear to have substantial beneficial or detrimental interactions. The complex responses that are likely to occur between ruminal microbes as a result of feed additive combinations may reduce their effects on ruminal fermentation. Evaluation of production responses may provide an improved indication of the potential benefits of feed additive combinations. Alternatively, more severe rumen perturbations induced by

a larger dose of readily fermentable carbohydrates may amplify accumulative feed additive effects.

A portion of the large differences in responses to feed additives between different studies may be explained by the large amount of animal variation in microbial composition (Fernando et al., 2010; Jami and Mizrahi, 2012) and differences among cattle in responses to readily fermentable carbohydrates (Dougherty et al., 1975; Brown et al., 2000). This variability among cattle makes the study of feed additives difficult. The ruminal bacterial community has the ability to re-establish its characteristic ruminal pH and total VFA concentrations after a dramatic perturbation of its ruminal bacterial community (Weimer et al., 2010). This shows the rumen ecosystem is capable of handling a significant degree of disturbance and presents a further difficulty in studying the pathophysiological responses to feed additives.

CONCLUSIONS

In conclusion, our hypothesis that feed additive supplementation would modify ruminal fermentation measures in comparison to unsupplemented control heifers was supported; however, modification of oxidative stress responses by feed additive supplementation was not supported. Our secondary hypothesis was equivocal as the effects of combinations of the feed additives were primarily not synergistic. Monensin and sources of ruminally degradable amino acids, peptides, and non-protein nitrogen may be beneficial in controlling starch-based ruminal acidosis. This study highlights the dangers in assuming additive or synergistic effects of feed additives. Feed additives have an important role in ruminant health and future directions could include research into other feed additive combinations, dose rates required for feed additives fed in combination, evaluation of production responses, and metagenomic effects of readily fermentable carbohydrate induced rumen perturbations.

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CHAPTER 7

Effects of Feed Additives on Ruminal and Blood Measures During a Grain and Fructose Challenge

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OVERVIEW OF CHAPTER 7

Effects of feed additives on ruminal fermentation measures were observed in Chapter 6 when unadapted cattle were fed a single challenge feed of grain. However, combinations of feed additives generally did not have synergistic or additive effects on ruminal and plasma responses in Chapter 7. In Chapter 7, the effects of different combinations of feed additives on ruminal fermentation measures were evaluated in cattle that had been fed a 62:38 forage:concentrate total mixed ration before a single challenge feed of grain and fructose. The intention of Chapter 7 was also to further examine the pathophysiology and clarify definitions of ruminal acidosis.

ABSTRACT

We evaluated the effect of feed additives on the risk of ruminal acidosis in Holstein heifers (n = 40) fed starch and fructose in a challenge study. Heifers were randomly allocated to feed additive groups (n = 8 heifers/group): (1) control (no additives); (2) virginiamycin (**VM**); (3) monensin + tylosin (**MT**); (4) monensin + live yeast (**MLY**); and (5) sodium bicarbonate + magnesium oxide (**BUF**). Heifers were fed 2.5% of body weight (**BW**) dry matter intake (**DMI**) per day of total mixed ration (62:38 forage:concentrate) and feed additives for a 20-d adaptation period. Fructose (0.1% of BW/d) was included for the last 10 d of the adaptation period. On d 21, heifers were fed to target a DMI of 1.0% of BW wheat, 0.2% of BW fructose, and their feed additives. Ruminal fluid samples obtained by stomach tube and blood samples were collected weekly as well as during a 3.6-h period on challenge day (d 21). Virginiamycin and BUF groups maintained a consistently high DMI across the 20-d adaptation period. The MLY heifers had low DMI of the challenge ration. Average daily gain and feed conversion ratio were not affected by feed additives. All ruminal and plasma measures changed weekly over adaptation and over the challenge sampling period with the exception of ruminal total lactate and histamine concentrations, plasma oxidative stress index, and ceruloplasmin. Substantial within- and between-group variation was observed in ruminal and plasma measures at challenge sampling. No significant feed additive group changes were observed in ruminal total volatile fatty acids, propionate, acetate to propionate ratio, isobutyrate, caproate, isovalerate, total lactate, D- and L-lactate, and pH measures on challenge day. Acetate concentration was increased in the BUF and control groups on challenge day. Butyrate concentration was lower in the MLY and MT groups compared with other feed additive groups at challenge. Valerate concentrations were lowest in the control, VM, and BUF groups and lactate concentrations were numerically lower in the MLY, VM, and BUF groups. Total lactate concentrations were >10 m*M* for each feed additive group throughout the challenge. Ammonia concentrations were lower in the MLY and MT groups. Histamine concentrations were decreased in MLY and increased in the VM and BUF groups. Plasma oxidative stress measures were not influenced by feed additives weekly or on challenge day, except for an increase in biological antioxidant potential in the control, VM, and MT groups on challenge day. Despite the large within animal variation, all

feed additives modified rumen function and may influence the risk of ruminal acidosis by different mechanisms; however, none stabilized the rumen in all heifers.

Key words: feed additive, fructose, lactic acid, ruminal acidosis

INTRODUCTION

Ruminal acidosis is a complex nutritional disorder. It is caused by the accumulation of organic acids initiated by the combination of consumption of large amounts of readily fermentable carbohydrates and insufficient intake of physically effective fiber (Nagaraja and Titgemeyer, 2007; Bramley et al., 2008). Periods of high risk for ruminal acidosis occur when dairy cattle are fed substantially more concentrate close to calving or when beef cattle enter the feedlot. The complex can occur from a relatively mild form where symptoms are subclinical to the peracute, resulting in death. Clinical signs include losses in production performance, diarrhoea, dehydration, lameness, and decreased appetite (RAGFAR, 2007; Plaizier et al., 2008). Clinical definitions of ruminal acidosis, largely based on ruminal pH, are inconsistent and can create confusion, leading to inaccurate diagnosis of ruminal acidosis (Kleen et al., 2003; Nagaraja and Titgemeyer, 2007; Plaizier et al., 2008). We largely concur with the view of Britton et al. (1989) that "acidosis is not one disease, but rather a continuum of degrees of ruminal acidity". Perhaps this description could be reworded to 'degrees of safe sequestration of hydrogen'.

Inclusion of feed additives is one practice of several used to reduce ruminal acidosis risk in the dairy and beef industries. A substantial body of evidence exists that supports the use of feed additives in cattle. However, relatively few papers exist that examine the effects of combinations of these on ruminal measures *in vivo* in dairy cattle (Clayton et al., 1999; Lean et al., 2000). Scientific evaluation of the effects of feed additives will allow producers, nutritionists, and veterinarians to make informed management decisions when considering their use and assist in the development of the most prudent use strategies for antimicrobial and other agents that modify rumen function.

Our primary aim was to evaluate the efficacy of the following feed additives to reduce ruminal acidosis risk during a non-life-threatening, but substantial, starch and fructose challenge: virginiamycin, combinations of monensin and tylosin, monensin and yeast, and sodium bicarbonate and magnesium oxide. We hypothesized that feed additives would reduce ruminal acidosis risk in cattle compared to unsupplemented control cattle, as indicated by production, ruminal, inflammation, and oxidative stress measures. We also intended to further examine the pathophysiology and clarify definitions of ruminal acidosis.

MATERIALS AND METHODS

Animals and Housing

The study was conducted on 36 pregnant and 4 nonpregnant Holstein-Friesian heifers from a commercial dairy herd (n = 40). All heifers were between 15 to 21 mo of age and had a mean bodyweight (**BW**) of 383 ± 49 kg on arrival at the study site located at Cobbitty [New South Wales (**NSW**), Australia]. For the duration of the study, all heifers, when not being fed or sampled, were kept as 1 herd in a paddock containing dormant kikuyu (*Pennisetum clandestinum*) with no available pasture and *ad libitum* water access. All experimental procedures were approved by the SBS*cibus* Animal Ethics Committee (SBS*cibus* 0512-0513).

Experimental Design

Each heifer was enrolled in the study for a period of 29 d, consisting of 5 experimental periods: (1) preadaptation (d –2 to 0), (2) adaptation I (d 1 to 10), (3) adaptation II (d 11 to 20), (4) challenge (d 21), and (5) postchallenge (d 22 to 26; Figure 1). Heifers were randomly allocated by identification number to 1 of 5 feed additive groups (n = 8 heifers/group) and 1 of 4 blocks (A to D; n = 10 heifers/block), with 2 heifers/group allocated to each block using a random numbers table generated from Stata v.11 (StataCorp. LP, College Station, TX). Enrolment into the study was staggered, with heifers allocated to each block entering the study 1 d after the previous block to allow sampling of 10 heifers/d only, over 4 consecutive days. Sample sizes were based on previous studies in which significant differences in fermentation characteristics were observed (Golder et al., 2012; Lean et al., 2013). To ensure that feeds were allocated correctly farm workers were not blinded to feed additive groups.

Feed Additive Groups

The feed additive groups were as follows: (1) control (no additives); (2) virginiamycin (**VM**); (3) monensin + tylosin (**MT**); (4) monensin + live yeast (**MLY**); and (5) sodium bicarbonate + magnesium oxide (**BUF**). The feed additives (Table 1) were incorporated into wheat pellets mixed on top of each heifer's total mixed ration (**TMR**), with the exception of the yeast, sodium bicarbonate, and magnesium oxide, which were weighed out separately in individual feeding portions and mixed on top of the TMR. All heifers

received the same amount of wheat pellets (Figure 1); however, those received by the control and BUF heifers contained no feed additives.

Diet

The rations offered in each of the experimental periods are detailed in Figure 1. The estimated chemical composition of the rations offered during the adaptation I, adaptation II, and challenge periods were calculated using CPM Dairy Ration Analyzer (version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 2) from ration components analyzed by wet chemistry (Dairy One Inc, Forage Testing Laboratory, Ithaca, NY; Table 3). Wet chemistry methods are described in Golder et al. (2012). Samples of the forages were taken by a forage core sampler (Best Harvest, Saint Petersburg, FL). Three cores from each bale of hay used in the TMR were pooled for both the wheaten and alfalfa hay.

Feeding Procedure

All heifers were fed half their daily rations at 7:00 and 14:00 h in individual concrete floor feeding pens. The order that heifers were fed and the feeding pen number were *ad hoc* at each feeding session. Pens were cleaned between heifers and feeding sessions to avoid cross-contamination of feed additives. Each heifer had a single labeled feed bin to prevent feed contamination. The fructose (CornSweet Crystalline Fructose, ADM Corn Processing, Decatur, IL) and urea [Incitec Pivot Ltd., Melbourne, Victoria (**VIC**), Australia], when incorporated in the ration (Figure 1), were weighed out daily based on individual BW and mixed on top of the TMR. Observed total eating time and manually weighed orts were recorded for each heifer at each feeding session. No water access was available in the feeding pens or holding yards during the feeding sessions. Heifers were subsequently returned to the paddock with no access to feed until the next feeding session.

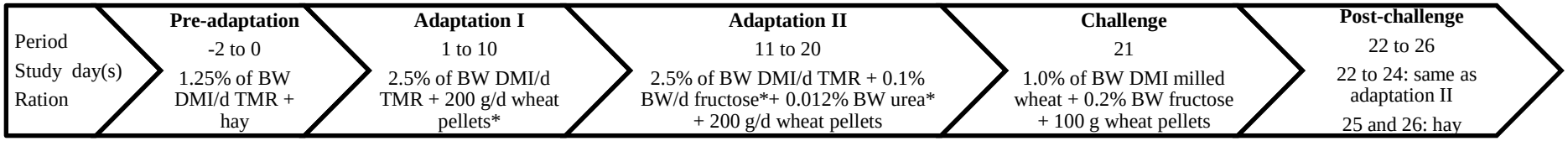

Figure 1. Experimental periods and their corresponding study days and rations offered during the study. The rations were offered in equal proportions twice daily, with the exception of the challenge period. Ruminal and blood samples were collected on d 0, 7, 14, and 21 during their respective experimental periods. Wheat pellets contained respective feed additives for their groups as indicated in Table 1. Heifers in the monensin + live yeast (MLY) group received yeast and those in the sodium bicarbonate + magnesium oxide (BUF) group received sodium bicarbonate and magnesium oxide in addition to wheat pellets. *Introductory doses were offered for the initial days before the full rate was offered. BW = bodyweight; DMI = dry matter intake; TMR = total mixed ration (62:38 forage:concentrate, consisting of 31.5% wheaten hay, 30.5% alfalfa hay, and 38% milled wheat).

Table 1. Feed additives administered to each feed additive group

Group	Feed additives					
	Active constituent	Commercial name	Manufacturer	Delivery form	Active (mg)	Dose rate (g/head/d)
Control[1]	–	–	–	–	–	–
Virginiamycin (VM)	Virginiamycin	Eskalin	Phibro Animal Health, Girraween, NSW, Australia	Pellet[2]	200	10
Monensin + tylosin (MT)	Sodium monensin Tylosin	Rumensin 100 Tylan	Elanco Animal Health, West Ryde, NSW, Australia Elanco Animal Health	Pellet containing both additives[2]	200 110	2.2 0.44
Monensin + live yeast (MLY)	Sodium monensin *Saccharomyces cerevisiae* CNCM I-1077	Rumensin 100 Levucell SC® Direct	Elanco Animal Health Lallemand Animal Nutrition, Maroochydore, QLD, Australia	Pellet[2] Dry active yeast	220 500[3]	2.5 25
Sodium bicarbonate + magnesium oxide (BUF)[1]	Sodium bicarbonate Magnesium oxide	Sodium bicarbonate Causmag	Penice Soda Products Pty Ltd, Osborne, SA, Australia Causmag International, Young, NSW, Australia	Powder Fine granules (mean particle size 0.85 mm)	– –	200 30

NSW = New South Wales, QLD = Queensland, SA = South Australia.
[1]Wheat pellets were given containing no feed additives.
[2]Pellets comprised respective feed additives, disc-milled wheat and 2.5 g/head/d of mineral premix (Cows R Us Base, DSM Nutritional Products, Wagga Wagga, NSW, Australia) and were pelleted using a cold pellet press.
[3]10 billion coliform forming units (CFU)/head/d.

Table 2. Estimated chemical composition of the ration offered during the following adaptation I, adaptation II, and challenge periods[1]

Item (% of DM)	Period (study days)		
	Adaptation I (d 1 to 10)	Adaptation II (d 11 to 20)	Challenge (d 21)
DM	89.6	89.9	90.2
CP	13.4	14.1	9.8
RUP (% of CP)	27.6	21.3	11.8
RDP (% of CP)	72.4	78.7	88.2
RDP	9.70	11.1	8.60
Soluble protein (% of CP)	39.8	45.0	38.5
ADF	22.4	21.6	4.49
NDF	35.0	33.6	10.7
Forage NDF (% of NDF)	85.8	85.8	0.0
Forage NDF (% of DM)	30.0	28.8	0.0
Physically effective NDF	30.6	29.4	4.2
Lignin	3.6	3.5	0.9
NFC[2]	46.1	47.7	76.3
Silage acids	0.0	0.0	0.0
Sugar	9.4	12.6	19.1
Starch	27.5	26.4	54.1
Soluble fiber	9.1	8.7	3.2
Total ether extract	1.95	1.88	1.74
Total LCFA	1.39	1.34	1.57
Ash	5.98	4.94	1.82
DCAD (mEq/100g)	24.7	23.7	-0.25
Minerals (mg/kg)			
Chloride	4,400	4,300	1,000
Calcium	4,800	4,500	900
Copper	21	19	20
Iron	128	122	42
Phosphorus	3,000	2,900	2,800
Potassium	1,700	16,300	4,100
Magnesium	1,400	1,300	1,000
Manganese	75	70	58
Sodium	1,300	1,200	100
Sulfur	1,900	1,800	1,300
Zinc	64	58	62

DM = dry matter; CP = crude protein; RUP = rumen undegradable protein; RDP = rumen degradable protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC = non-fiber carbohydrate; LCFA = long chain fatty acid; DCAD = dietary cation-anion difference.

[1]Estimations were performed using CPM Dairy Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) based on a 400 kg BW heifer with a body condition score of 3.25 and a growth rate of 0.73 kg/d. The ration fed in adaptation I comprised 2.5% of bodyweight (BW) dry matter intake (DMI)/d total mixed ration (TMR) + 200 g/d wheat pellets. The ration fed in adaptation II comprised 2.5% of BW DMI/d TMR + 0.1% of BW/d fructose + 200 g/d wheat pellets. The ration fed during the challenge comprised 1.0% of BW DMI wheat + 0.2% of BW fructose + 100 g wheat pellets. The TMR (62:38 forage:concentrate) consisted of 31.5% wheaten hay, 30.5% alfalfa hay, and 38% disc milled wheat.

[2]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash].

Table 3. Chemical composition of the total mixed ration (TMR) components and their proportions within the TMR: wheaten hay (31.5%), alfalfa hay (30.5%), and milled wheat (38.0%)[1]

Item (% DM)	Wheaten hay	Alfalfa hay	Milled wheat
DM	88.3	91.5	88.5
CP	7.2	22.1	11.7
Soluble protein (% of CP)	40	41	39
Crude fat	1.6	2.2	2.1
Ash	6.83	9.82	1.89
Lignin	4.1	6.6	1.3
ADF	34.7	33.2	5.5
NDF	56.3	43.8	15
ADICP	0.7	1.4	1.1
NDICP	1.8	5.6	3.5
NFC[2]	29.8	27.6	72.8
Available protein	6.5	20.7	10.6
Starch	4.1	1.4	62.4
ESC (simple sugars)	17.5	9.2	3.8
DCAD (mEq/100g)	20	58	-1
Minerals (mg/kg)			
Chloride	2,800	8,000	1,200
Calcium	1,300	12,300	400
Copper	6	6	5
Iron	229	115	39
Phosphorus	1,700	3,800	3,200
Potassium	13,200	36,900	4,800
Magnesium	900	2,000	1,100
Manganese	84	33	41
Molybdenum	0.4	1.4	1
Sodium	170	1260	90
Sulfur	1,100	3,100	1,600
Zinc	10	20	16

DM = dry matter; CP = crude protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; ADICP = acid detergent insoluble protein; NDICP = neutral detergent insoluble crude protein; NFC = non-fiber carbohydrate; ESC = ethanol-soluble carbohydrate; DCAD = dietary cation-anion difference.

[1]Values are means obtained from near-infrared spectroscopy and wet chemistry from DairyOne (Dairy One Inc, Forage Testing Laboratory, Ithaca, NY).

[2]NFC = 100 – [(NDF-NDICP) + CP + crude fat + ash].

Sampling Procedure

A ruminal and blood sample was taken within 3 h of the morning feed from each heifer on d 0, 7, and 14 during the preadaptation, adaptation I, and adaptation II periods, respectively. Ruminal fluid was collected using a custom-designed stomach pump and a tube approximately 3 m in length with a multi-holed aluminum probe at one end into a 500 mL container. The tube was inserted to a minimum length of approximately 2 m. Ruminal fluid was scored for saliva contamination as described by Bramley et al. (2008) using a 3-point scoring system (3 being the highest level of contamination). Ruminal fluid collection was repeated up to 3 times if a saliva score of >1 was observed and the lowest scoring sample was used for pH measurement. No ruminal samples retained for analysis had a saliva score >2. Ruminal fluid samples were analyzed for pH immediately after collection using a pH meter (Merck Pty Ltd., Kilsyth, VIC, Australia). The samples were sieved to remove large particles and centrifuged (Allegra® X-12R; Beckman Coulter Australia Pty. Ltd., Gladesville, NSW, Australia) at 1,512 × *g* for 15 min at 5^{o}C. The supernatant was aliquoted into 1.5-mL collection tubes and stored at –20^{o}C for VFA, ammonia, L- and D-lactate, and histamine analysis.

Blood samples were taken by coccygeal venipuncture using heparinized blood collection tubes (BD Vacutainer, Devon, Plymouth, UK) and centrifuged at 1,512 × *g* for 15 min at 5^{o}C. The plasma was then aliquoted into 1.5-mL storage tubes, and stored at –20^{o}C for histamine and oxidative stress analysis.

Challenge Procedure

Each heifer was challenged once on d 21 with 1.0 and 0.2% DMI of their BW of milled wheat and fructose, respectively, and fed their morning allocation of feed additives. The non-fiber carbohydrate (**NFC**) of the challenge ration was estimated at 76.3% of dry matter (**DM**) (CPM Dairy Ration Analyzer; Table 2). The blocks of heifers were sampled over 4 consecutive days and feeding was staggered within each block with the first heifer fed at 7:00 h. Before the challenge rations were offered each heifer was offered 200 g of alfalfa hay to reduce saliva contamination of the ruminal samples. Previously, we found that feeding a small proportion of hay or silage immediately before feeding the challenge rations prevented cattle from salivating excessively before sampling. Immediately after consumption of the hay, heifers were offered their

challenge rations. Ration consumption time and weighed orts were recorded to calculate the average ration consumption time and percentage of ration consumed per feed additive group. Ruminal fluid samples were collected 5, 65, 115, 165, and 215 min after ration consumption using a stomach tube and pump. A blood sample was taken by coccygeal venipuncture 5 and 215 min after ration consumption. Ruminal and blood samples were processed as described under the Sampling Procedure section.

As a result of clinical ruminal acidosis in a control heifer from block A 10 h after challenge feeding, all control heifer from blocks B to D were drenched with 200 g/head of sodium bicarbonate (Penice Soda Products Pty Ltd, Osborne, South Australia, Australia) in 10 L of water within 2 h of the final sampling. All heifers were monitored closely in the afternoon of the challenge day and offered access to wheaten and millet hay. Heifers were returned to their adaptation II rations for the first 3-d after challenge and feed intake was recorded.

Locomotion Scoring

Heifers were locomotion scored on 7 occasions during the study, after ruminal and blood sampling in the preadaptation, adaptation I and II periods, on challenge day, and 1, 2, and 5 d after challenge. Scoring was conducted by 2 study investigators working in tandem as heifers were individually walked past them on a concrete surface using the 5-point scoring system developed by Sprecher et al. (1997), with 5 being severely lame. The scorers were blinded to previous scores. Scores <2 were considered normal.

Bodyweight and Physical Examination

Individual heifer BW was measured on arrival at the study site and weekly. Ration components based on BW were updated after weighing. All heifers were observed during each feeding session and were given a physical examination if low appetite or abnormal demeanor were observed. The physical examination included rectal temperature, rumen contractions, heart rate, ruminal pH measurement, and optional ruminal fluid and blood collection. Heifers with reduced feed intake or diarrhoea were closely observed and monitored at subsequent feeding sessions. Any potential health concerns were recorded.

Acidosis Diagnosis

Clinical ruminal acidosis was diagnosed based on a combination of clinical signs that included inappetence, dull demeanor, abnormal rectal temperature, increased respiratory rate, absence of rumen contractions, and lameness. Subclinical ruminal acidosis was diagnosed based on a combination of DMI and acidosis eigenvalues obtained from standardized ruminal pH, VFA, ammonia, and lactate concentrations, using the model by Bramley et al. (2008).

Laboratory Analysis

Ruminal VFA concentrations were analyzed by an Agilent series gas chromatograph with HP6890 injection, 30 mm × 0.53 mm × 1.0 μm capillary column (Agilent Technologies Inc., Wilmington, DE), and Chemstation software (Agilent Technologies Inc.) based on methodology from Supelco Inc. (1975). The inter-assay coefficients of variation (**CV**) for acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, and caproate were 7.7, 7.1, 6.8, 6.7, 13.7, 6.9, and 8.3% respectively.

Ruminal ammonia concentrations were analyzed by the direct enzymatic method (Pesce and Kaplan, 1996) using Infinity Ammonia Liquid Stable Reagent (catalog no. TR60101; Beckman Coulter Australia Pty. Ltd.), on an Olympus AU400 Autoanalyzer (AHL NTM-56, Mt Waverly, VIC, Australia). The inter-assay CV for ammonia samples was 10%. Ruminal D-lactate was analyzed using a UV method for D-lactate determination in ruminal fluid using a Boehringer Mannheim kit (catalog no. 11 112 821 035; R-Biopharm-Laboratory Diagnostics Pty. Ltd., Taren Point, NSW, Australia) after deproteinization with perchloric acid according to kit instructions on an Olympus AU400 Autoanalyzer. Ruminal L-lactate was analyzed using a UV method for L-lactate determination in rumen fluid using a Beckman Coulter kit (catalog no. OSR 6193; Beckman Coulter Australia Pty. Ltd) on an Olympus AU400 Autoanalyzer.

Ruminal histamine concentrations were analyzed in samples collected 5 and 215 min after challenge and plasma histamine in those collected 215 min after challenge ration consumption. Analysis was performed within 7 wk of sample collection using a human histamine ELISA kit (catalog no. RE59221; IBL International GMBH, Hamburg, Germany) according to the manufacturer's instructions for human plasma samples (Golder et al., 2012). Ruminal fluid was passed through a 0.22-μm polyethersulfone

membrane syringe filter (Millipore, Carrigtwohill, Cork, Ireland) and diluted 1:2 with assay buffer supplied within the kit before analysis. Absorbance was measured at 450 nm using a POLARstar Optima (BMG Labtech, Melbourne, VIC, Australia). The inter-assay CV was <17% and the intra-assay CV for ruminal fluid was <15% and <8% for plasma.

Plasma derivatives of reactive oxygen metabolites (**dROM**; d-ROMs Test, Diacron International, Grosseto, Italy) and biological antioxidant potential (**BAP**; Bap Test; Diacron International) were measured according to kit instructions. The extent of oxidative stress was expressed as an oxidative stress index (**OSI**), estimated using the ratio of (dROM/BAP) × 100 (Celi, 2011). Plasma ceruloplasmin concentration was determined according to the methods described by Sunderman and Nomoto (1970), with the exception that absorbance was read at 510 nm (POLARstar Optima, BMG Labtech).

Statistical Analysis

All data were analyzed using the following linear mixed model in Genstat (14th edition, VSN International Ltd., Hemel, Hempstead, UK), unless otherwise indicated:

$$Y_{ijkl} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + X_k + (XZ)_{kl} + \varepsilon_{ijkl,}$$

where Y_{ijkl} = response of feed additive group i (i = 1 to 5) at the jth period (j = 1 to 4; or time j = 1 to 5; or study day j = 1 to 20) from block k (k = A to D) by heifer l (l = 1 to 39); μ = overall mean; β_i = fixed effect of feed additive group; γ_j = fixed effect of period (or time); $(\beta\gamma)_{ij}$ = effect of feed additive group by period (or time, or study day) interaction; X_k = random effect of block; $(XZ)_{kl}$ = random effect of heifer nested within block; ε_{ijkl} = random residual error within heifer l at period (or time, or study day) j from block k. The covariance structure of the random error terms of the model was independent. This model was chosen following examination of other covariance structures, including first-order regressive structures using a likelihood ratio test to compare models and consequently all the correlation was modeled through the random terms. A residual analysis was performed for each response variable, testing for the distributional assumption, homogeneity of the variance, and influential observations using residual and deviance plots.

Preadaptation ruminal and plasma measures were analyzed without the fixed effect of period and the interaction of feed additive group × period (data not shown). The same model was used to test the effect of feed additive group for arrival, adaptation I, and challenge BW, and average daily gain (**ADG**) and feed conversion ratio (**FCR**) from adaptation I to challenge. Arrival BW was used as a covariate for adaptation I and challenge BW. Six BW measurements were rejected as implausible and replaced by points interpolated by calculating the average of the BW taken from the previous and subsequent week, including one arrival BW, estimated by extrapolation using a linear trendline.

Study day (j = 1 to 20) was used as a main effect in replacement of period for the analysis of DMI and ration consumption percentage over the 20-d adaptation. Dry matter intake and ration consumption percentage on challenge day were analyzed without the fixed effect of period and the interaction of feed additive group × period. Individual heifer ration consumption percentages within each feed additive group were graphed over the 20-d adaptation (data not shown). The difference in ration consumption percentage and DMI between the mean 3 d before and after challenge was analyzed using the fixed effects of feed additive group and period, and their interaction (j = 1 to 2; data not shown).

Ruminal acidosis risk at the preadaptation and adaptation I and II periods was assessed using eigenvalues based on the statistical distance of each sample from the centroid for known ruminal acidosis cases. Eigenvalues approaching close to 1 are in the center of the acidosis category and those approaching 0 are not acidotic. Acidosis eigenvalues were obtained using discriminant analysis and the acidosis categories defined according to the methods of Bramley et al. (2008). Heifers did not classify appropriately using this model for any of the 5 sample times on challenge day. The main effects of feed additive group and period (j = 1 to 3) and their interaction for the acidosis eigenvalues were analyzed using the linear model described. No covariates were used.

Weekly ruminal and plasma data measured were analyzed using the linear model described. Only measures from the final challenge sampling (215 min) were used in the data set for the fourth wk measurement (d-21). No covariates were significant. The variables ruminal valerate and isovalerate were transformed using a logarithm base 10

and ruminal acetate to propionate ratio, total lactate, and D- and L-lactate were transformed using a natural logarithm to achieve a normal distribution of residuals.

All ruminal and plasma data from the challenge period were analyzed with the fixed effects of feed additive group and time, and their interaction. Plasma was obtained at the 5- and 215-min samplings only (j = 1 to 2). The covariates, arrival BW, preadaptation (d 0) measures, mean DMI and ration consumption percentage over the 20-d adaptation, BW at challenge, DMI at challenge, consumption percentage of challenge ration, and time for challenge ration consumption were tested in the model, but none were significant. The variables ruminal valerate and isovalerate were transformed using a logarithm base 10 to achieve a normal distribution of residuals. Data from each heifer within each feed additive group was graphed over the challenge sampling period to identify heifers with unusual measures (data not shown).

A Pearsons correlation was performed to determine the relationship between ruminal and plasma histamine concentrations at the final challenge sampling, regardless of feed additive group. A count of locomotion scores ≥2 for each feed additive group and period was performed and no formal analysis was undertaken for these.

RESULTS

A heifer from the MT group was removed from the final analysis, as she consumed only 12% of her challenge ration. No differences in ruminal or plasma measures were obtained between feed additive groups in the preadaptation sampling (data not shown).

Clinical Observations During Adaptation

Abnormal watery, bubbly feces were observed for several heifers from different feed additive groups during the 20-d adaptation period. Six heifers (1 control, 1 VM, 3 MT, and 1 MLY) including a heifer later excluded from the study, were given a physical examination on d 4 after presenting with a dull demeanour and inappetence. Rectal temperatures were within the normal range, 2 heifers had elevated heart rates (98 and 100 beats/min), 1 had a ruminal pH of 5.6, and 2 had infrequent or no rumen contractions. All 40 heifers were subsequently given access to millet hay with their TMR for the following 3 d. None of these 6 heifers examined showed signs of lameness and resumed normal appetite within 2 to 5 d after examination.

Table 4. Predicted means (±SEM) of adaptation I and challenge bodyweight (BW), average daily gain (ADG) and feed conversion ratio (FCR) over a 23-d period from adaptation I to challenge; mean dry matter intake (DMI) and ration consumed over the 20-d adaptation period; and mean

Item	Group[1]						Main effects (*P*-value)	
	Control	VM	MT	MLY	BUF	SEM	Group	Study day
No. of heifers	8	8	7	8	8			
Arrival BW (kg)	353[a]	396[bc]	422[c]	358[ab]	381[ab]	15.4	0.024	–
Adaptation I BW (kg)[2]	388	380	387	383	385	4.26	0.457	–
Challenge BW (kg)[2]	423	418	420	423	426	6.31	0.803	–
ADG (kg/d)	1.47	1.71	1.48	1.66	1.78	0.22	0.748	–
FCR (kg DMI/kg gain)	9.44	14.49[3]	8.41	6.44	6.59	4.00	0.579[4]	–
Adaptation (20 d)								
DMI (kg/d)	9.34[b]	10.89[d]	9.97[bc]	8.49[a]	10.17[cd]	0.41	0.002	<0.001
Ration consumed (% of offered ration consumed/d)	90.5[b]	95.1[c]	80.7[a]	82.7[a]	92.9[bc]	2.02	<0.001	<0.001
Challenge								
DMI (kg)	4.60[b]	5.03[bc]	5.54[c]	3.27[a]	4.70[b]	0.41	0.007	–
Ration consumed (% of offered ration consumed)	90.8[b]	90.5[b]	93.1[b]	65.0[a]	87.8[b]	8.05	0.028	–
Consumption time (min)	59.3	49.13	57.7	80.4	68.9	8.73	0.059	–

[a-d]Means within a row not sharing a common superscript differ significantly ($P < 0.05$). BW = bodyweight; FCR = feed conversion ratio; DMI = dry matter intake

[1]Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY= monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide.

[2]Arrival BW was used as a covariate in analysis.

[3]When 1 heifer with an FCR of 70.6 kg of DMI/kg gain was removed from the VM group, the mean FCR was 6.50 kg of DMI/kg gain.

[4]When 1 heifer with an FCR of 70.6 kg of DMI/kg gain was removed from the VM group, the *P*-value was 0.385.

Bodyweight and DMI During Adaptation

Bodyweight at adaptation I and challenge, ADG, and FCR were not affected by feed additive group (Table 4). Over the 20-d adaptation, DMI was greatest in the VM and BUF groups, similar in the MT and BUF groups, and also similar in the control and MT groups; however, DMI was lowest in the MLY group (Table 4). Heifers had the lowest DMI and percentage of offered ration consumed on study d 3 and 4 and the highest DMI on study d 19 and 20 (Figure 2). Marked variation in ration consumption percentage among heifers within each feed additive group was observed but was greatest in the control, MT, and MLY groups. A cyclic feeding pattern was also observed for these groups (Figure 2).

Acidosis Classification

Acidosis eigenvalues showed a trend toward a feed additive group × period interaction (P = 0.086) and period effect (P = 0.067; Table 5). Eigenvalues increased weekly and were greatest for the MT and control groups (Table 5; Figure 3A). Three heifers (2 from the MT and 1 from the control group) were classified as acidotic based on eigenvalues and Bramley et al. (2008) acidosis group classification at the adaptation II sampling.

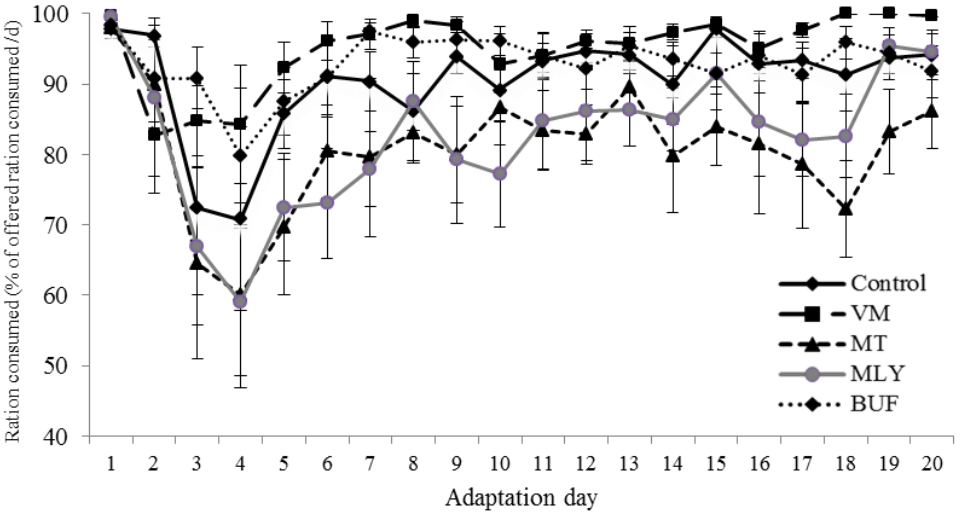

Figure 2. Mean (±SEM) percentage of ration consumed for each feed additive group over the 20-d adaptation period. Control = no feed additives; Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY = monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide.

Weekly Ruminal and Plasma Measures

Total VFA and acetate concentrations were influenced by the feed additive group × period interaction, with concentrations for all feed additive groups increasing from the preadaptation to the adaptation I sampling. Concentrations of total VFA and acetate were increased in the BUF group, decreased in the MT group, and were relatively similar to adaptation I concentrations for the other feed additive groups at the adaptation II sampling. Concentrations of these decreased for all feed additive groups, with the exception of an increase for the MT group at the challenge sampling (Table 5; Figure 3B and C, respectively).

Propionate concentrations increased between the preadaptation and adaptation I sampling, concentrations were relatively consistent between the adaptation I and II sampling, and decreased at the challenge sampling (Table 5; Figure 3D). The acetate to propionate ratio decreased between the preadaptation and adaptation I sampling and was relatively stable between the adaptation I and challenge samplings. The ratio increased in the BUF group at the challenge sampling (Table 5; Figure 3E). Butyrate concentrations increased for the majority of feed additive groups (Table 5; Figure 3F). Isobutyrate concentrations decreased for all feed additive groups from preadaptation to adaptation I and increased between adaptation I and II, except for a decrease for the MT group, before decreasing in all feed additive groups at the challenge sampling.

Table 5. Main effects (*P*-values) of feed additive group and period and their interaction for acidosis eigenvalues, and ruminal and plasma measures taken weekly from preadaptation (d 0) to the final sampling on challenge day (d 21; 215 min after ration consumption). n of samples = 156 (4 samples/heifer)

Item	*P*-value		
	Group (G)	Period (P)	G × P
Acidosis eigenvalue[1]	0.215	0.067	0.086
Ruminal (m*M*)			
Total VFA	0.081	<0.001	0.044
Acetate	0.001	0.006	0.005
Propionate	0.234	<0.001	0.392
Ln acetate:propionate	0.003	<0.001	0.500
Butyrate	0.106	<0.001	0.384
Isobutyrate	0.044	<0.001	0.342
Caproate	0.184	<0.001	0.009
Log_{10} valerate	0.349	<0.001	0.074
Log_{10} isovalerate	0.222	<0.001	0.010
Ln total lactate	0.083	<0.001	0.313
Ln D-lactate	0.070	<0.001	0.182
Ln L-lactate	0.041	<0.001	0.216
Ammonia	0.141	<0.001	0.195
pH	0.125	<0.001	0.096
Plasma			
dROM (Carr U)	0.334	<0.001	0.113
BAP (μ*M*)	0.431	<0.001	0.646
OSI (arbitrary units)	0.652	<0.001	0.236
Ceruloplasmin (mg/L)	0.971	0.030	0.830

VFA = volatile fatty acids; Ln = natural logarithm; Log_{10} = logarithm base 10; dROM = reactive oxygen metabolites; Carr U = Carratelli units; 1 Carr U = 0.08 mg/100 mL of hydrogen peroxide); BAP = biological antioxidant potential; OSI = oxidative stress index [(dROM/BAP) × 100].

[1]The acidosis eigenvalues were calculated from the preadaptation and adaptation I and II samplings (n of samples = 117).

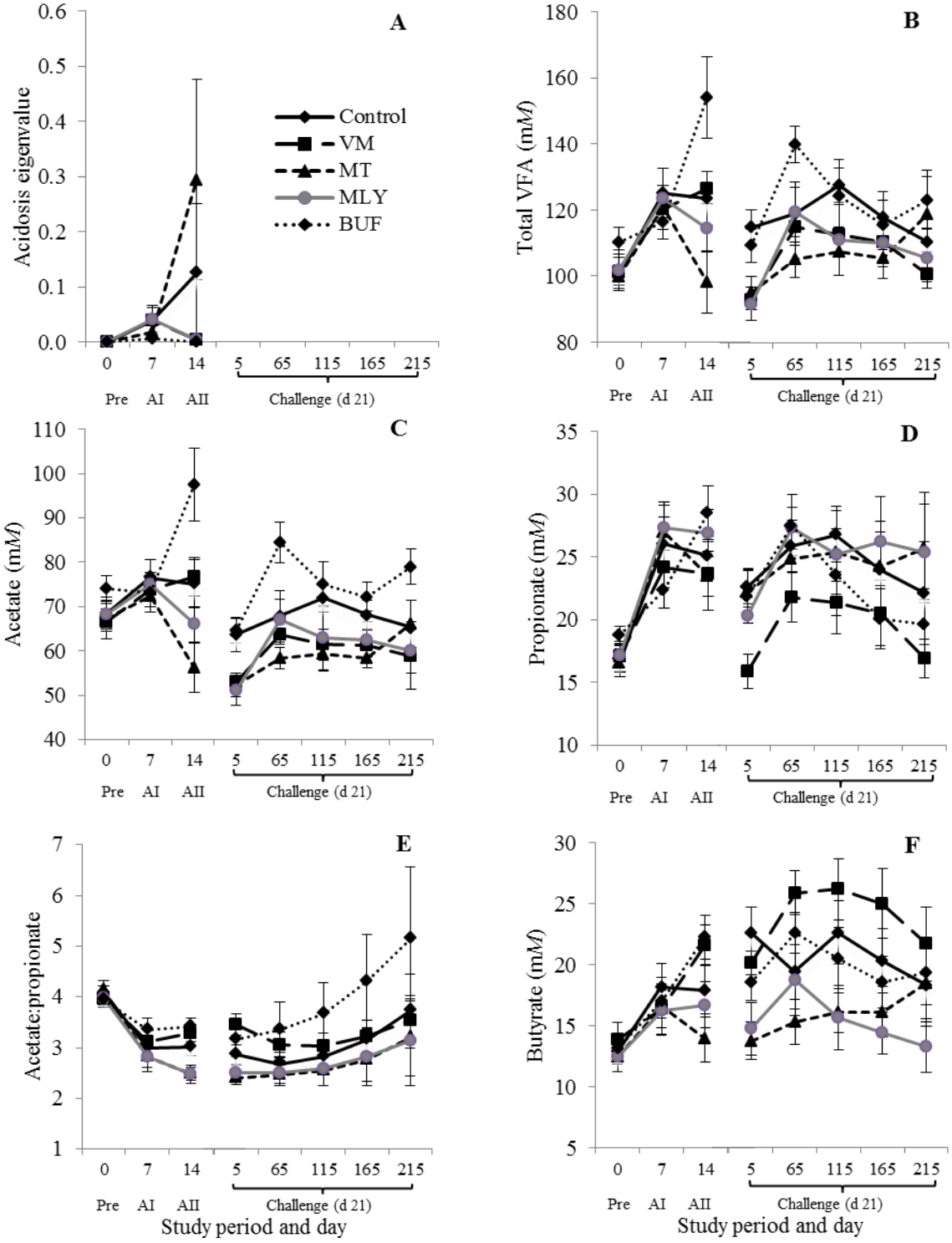

Figure 3. Mean (±SEM) acidosis eigenvalue (0 = not acidotic to 1 = acidosis; A); ruminal total volatile fatty acid (VFA) (B); acetate (C); propionate (D); acetate:propionate (E); butyrate (F); valerate (G); total lactate (H); D-lactate (I); L-lactate (J) ammonia (K); and ruminal pH (L) measures for feed additive groups collected during the 4 experimental periods on the study day in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); and challenge day (d 21). On the challenge day, ruminal fluid samples were collected 5, 65, 115, 165, and 215 min after challenge ration consumption. Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY = monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide.

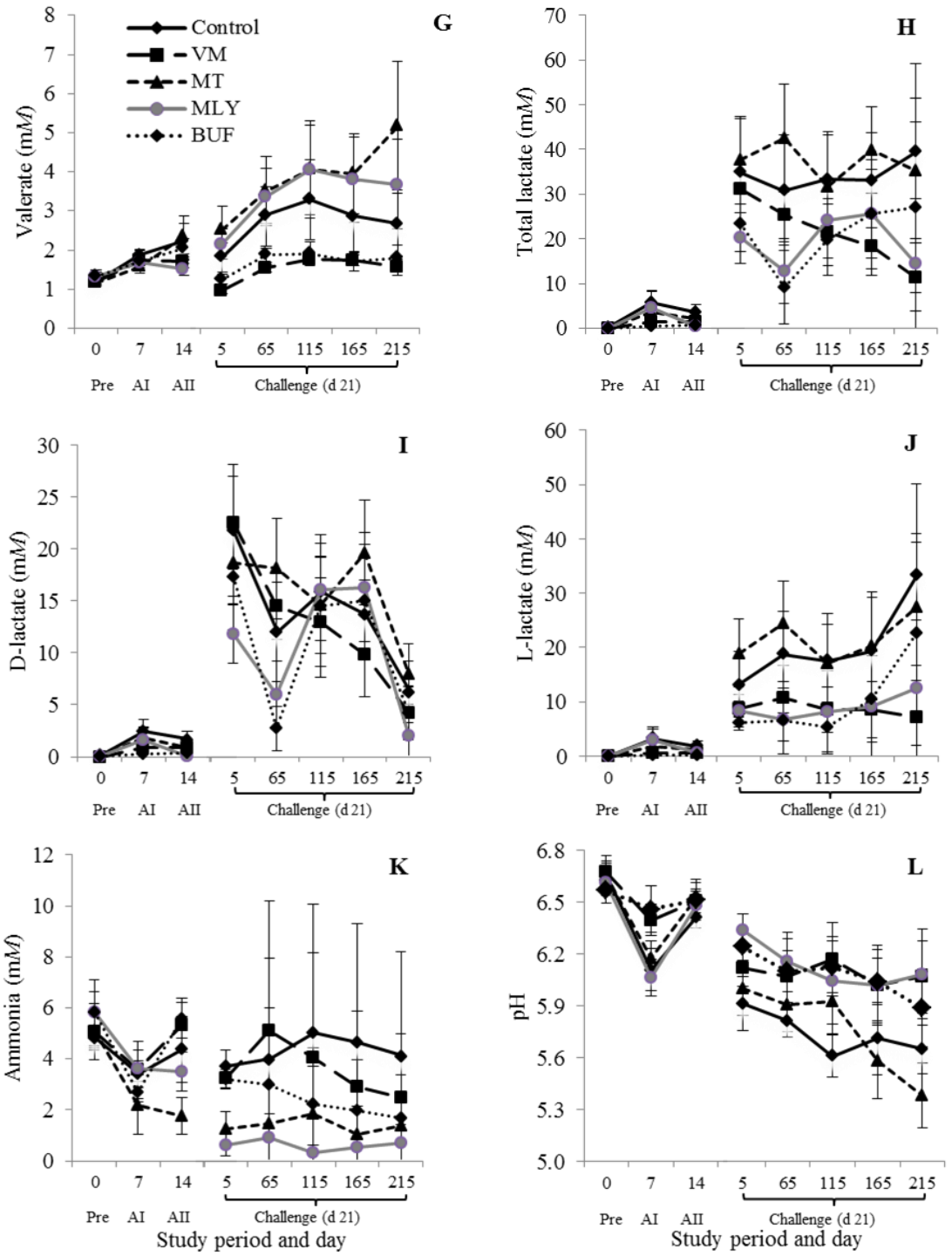

Figure 3 continued. Mean (±SEM) acidosis eigenvalue (0, not acidotic to 1, acidosis; A); ruminal total volatile fatty acid (VFA) (B); acetate (C); propionate (D); acetate:propionate (E); butyrate (F); valerate (G); total lactate (H); D-lactate (I); L-lactate (J) ammonia (K); and ruminal pH (L) measures for feed additive groups collected during the 4 experimental periods on the study day in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); and challenge day (d 21). On the challenge day. ruminal fluid samples were collected 5, 65, 115, 165, and 215 min after challenge ration consumption. Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY = monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide.

Caproate and valerate concentrations increased weekly (Table 5; Figure 3G). Isovalerate concentrations increased, except for the BUF group, from preadaptation to adaptation I, decreased in the VM and MT groups, and increased in the other feed additive groups between adaptation I and II. Isovalerate concentrations decreased in the MLY, control, and VM groups and remained relatively stable for the other feed additive groups between adaptation II and challenge (Table 4).

Total lactate concentrations increased from a mean of 0.09 ± 0.05 to 25.4 ± 7.0 m*M* from preadaptation to challenge day (Table 5; Figure 3H). D- and L-lactate concentrations increased markedly at challenge day (Table 5; Figure 3I and J, respectively). L-lactate concentrations were highest in the control group.

Ammonia concentrations decreased between the preadaptation and adaptation I sampling, increased in the majority of feed additive groups at the adaptation II sampling, and decreased on challenge day (Table 5; Figure 3K). Ruminal pH was not different between feed additive groups but was highest at the preadaptation sampling, lower at adaptation I, increased at adaptation II, and was the lowest at challenge sampling: 0.81 units lower than at preadaptation (Table 5; Figure 3L).

Plasma dROM, BAP, OSI, and ceruloplasmin concentrations were not influenced by the feed additive group × period interaction or feed additive group effect (Table 5). The concentration of dROM was similar at the preadaptation and challenge day, but increased between the preadaptation to adaptation I, and the adaptation I to adaptation II samplings (Table 5; Figure 4A). BAP concentrations were consistent at the preadaptation and challenge day samplings but were increased between these periods (Table 5; Figure 4B). The OSI was highest at the adaptation II sampling (Table 5; Figure 4C). Ceruloplasmin concentrations peaked at the adaptation I sampling and returned to a concentration comparable to that at preadaptation by the challenge sampling (Table 5; Figure 4D).

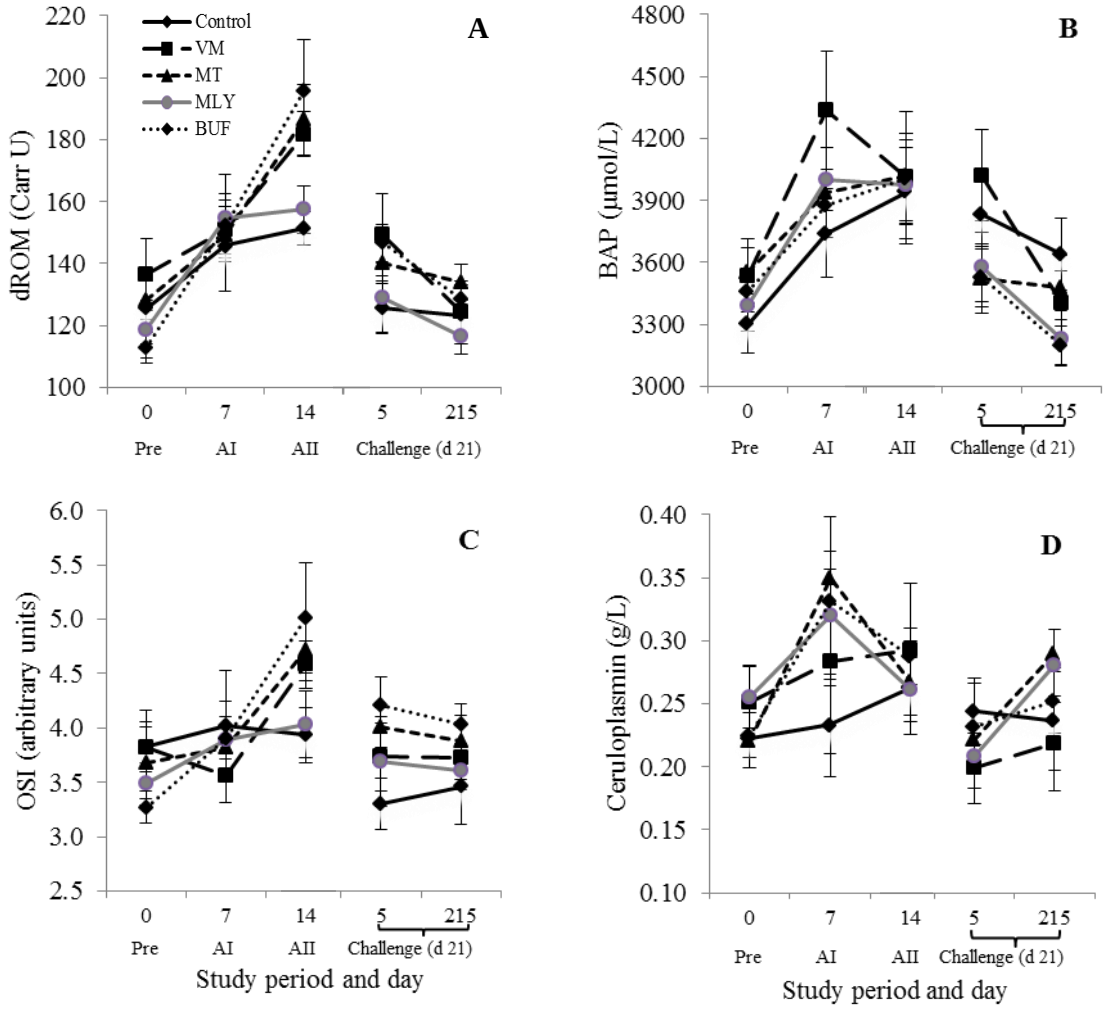

Figure 4. Mean (±SEM) measures of plasma reactive oxygen metabolites (dROM) (A); biological antioxidant potential (BAP) (B); oxidative stress index (OSI) (C), and ceruloplasmin (D) measures for feed additive groups collected during the 4 experimental periods on the study day in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); and challenge day (d 21). On the challenge day, blood was collected 5 and 215 min after challenge ration consumption. Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide. Carr U = Carratelli units; 1 Carr U = 0.08 mg/100 mL of hydrogen peroxide.

Clinical Signs and DMI on Challenge Day

No clinical signs of ruminal acidosis were observed during the sampling period. A control heifer in the first block of heifers challenged was diagnosed with acute ruminal acidosis 10 h after challenge feeding. She was drenched with 200 g of sodium bicarbonate in 20 L of water 24 h after challenge and had recovered by 48 h after challenge. A detailed clinical case report on this heifer will be available (Golder et al., 2014).

Dry matter intake was greatest for the MT and VM heifers and lowest for the MLY heifers, which, on average, consumed only 65% of their offered ration (Table 4). Ration consumption time approached significance (P = 0.059), with VM heifers, on average, consuming the ration the quickest (approximately 49 min), compared with the BUF heifers which took approximately 20 min longer (Table 4). No difference was observed in percentage of ration consumption and DMI 3 d before and after challenge (P = 0.720 and P = 0.831, respectively, data not shown).

Ruminal and Plasma Measures on Challenge Day

Caproate was the only ruminal measure with a feed additive group × time interaction (Table 6). Acetate concentrations were highest in the BUF group and also high in the controls. Acetate concentrations were similar in the VM, MT and, MLY groups. Acetate concentrations increased between the 5- and 65-min sampling and then plateaued (Table 6; Figure 3C). Butyrate concentrations were higher in the control and BUF groups compared with the MT and MLY groups (Table 6; Figure 3F). Butyrate concentrations were highest between the 65- to 165-min samplings and returned to concentrations similar to those at the initial sampling by 215 min (Figure 3F). Valerate concentrations were lowest in the VM and BUF groups and highest in the MT and MLY groups. Valerate concentrations for the control were similar to all other feed additive groups. Valerate concentrations increased between the 5- and 65-min sampling times and then plateaued (Table 6; Figure 3G). Ammonia concentrations were lowest in the MLY and MT groups. The MT and BUF groups had a comparable ammonia concentration, as did the BUF and VM, and VM and control groups. Ammonia concentrations were highest at the 65-min sampling (Table 6; Figure 3K).

No feed additive group effects were evident for ruminal total or other individual VFA, lactate, or pH measures; however, time effects were significant for each of these measures, with the exception of total lactate. Total VFA, propionate, and L-lactate concentrations increased between the 5- and 65-min sampling times and then plateaued (Table 6; Figure 3B, D, and J). The acetate to propionate ratio was highest at the final sampling (215 min; Figure 3E). D-lactate concentration decreased at the 65-min sampling, returned to a concentration similar to that at the first sampling over the 115- to 165-min period and decreased at the final 215-min sampling (Table 6; Figure 3I). Ruminal pH decreased after the 115-min sampling (Figure 3L).

Ruminal histamine concentrations were considerably lower in the MLY heifers compared with other feed additive groups and more than 4-fold lower than those of the VM group. The VM and BUF, BUF and control, and control and MT groups had comparable concentrations (Table 6; Figure 5). Ruminal and plasma histamine concentrations were not correlated (r = –0.07).

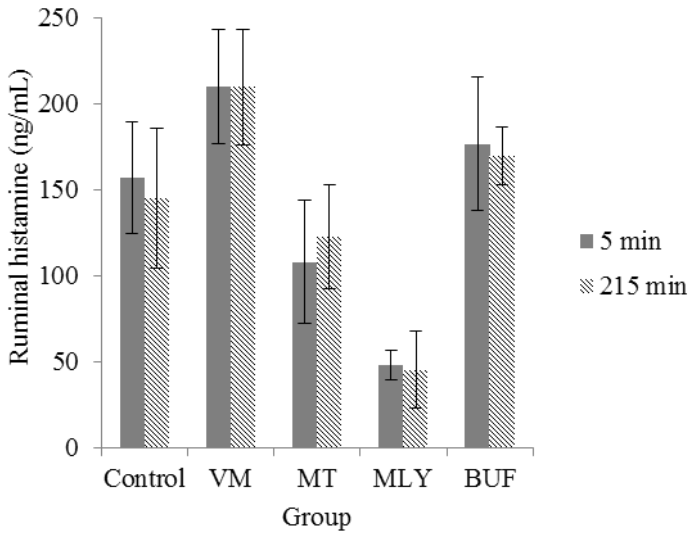

Figure 5. Mean (±SEM) ruminal histamine concentration for feed additive groups from ruminal fluid collected 5 and 215 min after consumption of the challenge ration. Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY = monensin + live yeast; BUF = sodium bicarbonate + magnesium oxide.

Table 6. Predicted means (±SEM), main effects of feed additive group and time, and their interaction for rumen measures taken 5, 65, 115, 165, and 215 min and plasma measures taken 5 and 215 min after consumption of challenge rations

Item	Group[1]						Effects and interactions (*P*-values)		
	Control	VM	MT	MLY	BUF	SEM	Group (G)	Time (T)	G × T
No. of heifers	8	8	7	8	8				
Ruminal (m*M*)									
Total VFA	118.1	106.5	106.3	107.8	122.6	5.66	0.107	<0.001	0.430
Acetate	67.28^{b}	59.64^{a}	58.84^{a}	60.71^{a}	75.06^{c}	2.99	0.002	<0.001	0.568
Propionate	24.28	19.28	24.57	24.89	22.53	2.28	0.340	<0.001	0.367
Acetate:Propionate	3.05	3.27	2.67	2.72	3.93	0.47	0.296	<0.001	0.737
Butyrate	20.62^{bc}	23.75^{c}	15.90^{a}	15.33^{a}	19.90^{b}	1.91	0.020	0.032	0.111
Isobutyrate	0.86	0.75	0.68	0.81	0.88	0.08	0.392	<0.001	0.331
Caproate	0.80	0.36	1.35	0.93	0.78	0.32	0.244	<0.001	0.002
Log_{10} valerate[2]	0.33^{ab}	0.16^{a}	0.47^{b}	0.43^{b}	0.18^{a}	0.10	0.035	<0.001	0.382
Log_{10} isovalerate[2]	0.12	0.05	0.01	0.10	0.21	0.07	0.369	<0.001	0.067
Total lactate	34.43	21.59	37.46	19.46	21.04	9.33	0.532	0.637	0.584
D-lactate	13.93	12.81	15.81	10.47	10.77	2.63	0.600	<0.001	0.508
L-lactate	20.50	8.78	21.65	8.99	10.27	7.15	0.532	0.020	0.875
Ammonia	4.29^{d}	3.57^{cd}	1.41^{ab}	0.63^{a}	2.43^{bc}	0.62	<0.001	0.037	0.080
Histamine (ng/mL)	151.2^{bc}	210.3^{d}	115.6^{b}	46.80^{a}	173.4^{cd}	26.25	0.001	0.903	0.983
pH	5.74	6.09	5.76	6.13	6.08	0.14	0.155	0.001	0.674
Plasma[3]									
dROM (Carr U)	125	137	137	123	138	7.4	0.405	0.002	0.354
BAP (μ*M*)	3738^{b}	3712^{b}	3509^{ab}	3405^{a}	3363^{a}	143	0.028	0.001	0.390
OSI (arbitrary units)	3.4	3.7	3.9	3.6	4.1	0.2	0.210	0.741	0.939
Ceruloplasmin (mg/L)	239	208	255	244	243	24	0.710	0.061	0.586
Histamine (ng/mL)[4]	0.24	0.16	0.08	0.22	0.29	0.05	0.075	–	–

[a-d]Means within the same row not sharing a common superscript differ significantly ($P < 0.05$). VFA = volatile fatty acids; Log_{10} = logarithm base 10

[1]Control = no feed additives; VM = virginiamycin; MT = monensin + tylosin; MLY = monensin + live yeast; BUF = sodium bicarbonate +magnesium oxide

[2]log^{-1}(y) of Log_{10} valerate: 2.14, 1.45, 2.95, 2.69, 1.51; Log_{10} isovalerate: 1.32, 1.12, 1.02, 1.26, 1.62.

[3]BAP = biological antioxidant potential; dROM = reactive oxygen metabolites; OSI = oxidative stress index [(dROM/BAP) × 100].

[4]Histamine was reported for the 215-min sample collection only.

The concentration of BAP declined over the challenge sampling and was similar in the control, VM and, MT groups. Concentrations were lower in the MLY and BUF groups which were comparable to that of the MT cattle (Table 6; Figure 4B). Concentrations of dROM, OSI, ceruloplasmin, and histamine in plasma were not affected by the interaction of feed additive group × time or the main effect of feed additive group (Table 6; Figure 4A, C and D). Concentrations of dROM decreased over the challenge sampling (Figure 4A) and a trend occurred toward an increase in ceruloplasmin concentrations ($P = 0.061$; Table 6; Figure 4D).

The relatively large SEM's in Table 6 and error bars in Figure 3 indicate considerable between heifer variation within all feed additive groups. For example, within the VM group, 2 heifers had considerably lower ruminal pH, and higher propionate and lactate concentrations than the other 6. One heifer's ruminal pH decreased from 5.9 to 5.1 and propionate increased from 23 to 36 m*M* between the 5- and 165-min samplings and her total lactate concentration peaked at 60 m*M* (65-min sampling). Similarly, in the MLY group, a heifer had a ruminal pH of 6.1 that decreased to 4.6 and total lactate and ammonia concentrations that increased from 25 to 116 and 0.2 to 5.7 m*M*, respectively, over the challenge sampling. Her acetate concentration was higher until the 115-min sampling, whereafter it and propionate and butyrate concentrations dropped. One heifer from the BUF group had a ruminal pH that decreased to 4.8 and a total lactate concentration of 148 m*M* at the final sampling.

Locomotion Scores

Of the total number of locomotion scores taken on 7 occasions throughout the study, only 4.8% had a score ≥2 and no scores were above 2.5. No difference in locomotion scores occurred between feed additive groups; however, no scores ≥2 were recorded in the preadaptation or 5-d-after challenge scoring sessions.

DISCUSSION

The starch and fructose challenge model was sufficient to evaluate the capacity of feed additives to reduce ruminal acidosis risk, given the substantial changes in ruminal conditions during the adaptation period and over the first 215 min after the challenge feed. Cyclic feeding patterns and clinical signs of ruminal acidosis in some heifers during the adaptation period and diagnosis of a clinical case of acute ruminal acidosis on challenge day further validates the severity of this challenge model.

None of the feed additives examined stabilized the rumen in all their heifers; however, positive responses to interventions were evident in all feed additive groups. The complex interactions within the rumen ecosystem and differences in modes of action of the feed additives are evident in the diverse responses of individuals within feed additive groups.

The challenge ration fed at 1.2% of BW is similar to the challenge rations of Golder et al. (2012) and Lean et al. (2013) fed to dairy heifers. Grain-based rations up to 1.5% of BW or diets that contained glucose at 0.3 to 0.6% of BW produced subacute ruminal acidosis with minimal risk of acute ruminal acidosis (Nagaraja and Titgemeyer, 2007). Ruminal and systemic acidosis has been induced in dairy heifers of comparable age and weights to those in this study by orally drenching with oligofructose at 0.17% of BW (Danscher et al., 2010). The TMR fed in the adaptation II period (NDF at 33.6, starch at 26.4, NFC at 47.7, and sugar at 12.6 % of DM) is representative of beef feedlot rations and some lactating dairy rations and thus should mirror effects in the field. Average daily gains were much higher than expected and consistent with those of feedlot cattle (Reinhardt et al., 2012), providing additional validation of the performance of the diets.

The challenge was large enough to induce a pro-oxidative response over the duration of the study, but not between feed additive groups, a finding consistent with another starch and fructose challenge (Golder et al., 2013). The effects of period and time on oxidative stress biomarkers support the finding by Gabai et al. (2004) that substrate changes influence oxidative stress.

Intake control and subsequent maintenance of optimum ruminal conditions may be a defense mechanism of animals or response to feed additives that prevent ruminal acidosis (Owens et al., 1998). The quantitative review of Nagaraja and Titgemeyer

(2007) suggests that responses in cattle that consume feed appear to be different to those that receive direct rumen challenges such as drenching or insertion of feed using fistulae. These observations support the decision to allow voluntary ration intake, which more closely mimics on-farm practices and ensures rations are within physiological limits that cattle can consume.

Ruminal acidosis definitions have largely been based on various ruminal pH cut points (Gozho et al., 2006; Plaizier et al., 2008); however, pH determinations vary according to heterogeneity of the rumen, ruminal fluid collection method, collection site, intake of fermentable carbohydrate, buffering capacity, rates of absorption, time of day, and the time between feeding and fluid collection (Duffield et al., 2004; Nagaraja and Titgemeyer, 2007; Shen et al., 2012). Saliva contamination may occur when ruminal fluid is collected by a stomach tube (Duffield et al., 2004); however, this can be overcome by correct insertion technique (Lodge-Ivey et al., 2009; Shen et al., 2012). It was our intention to define ruminal acidosis status based on a combination of standardized individual VFA, ammonia, ruminal pH, and lactate measures using the model by Bramley et al. (2008). The trend toward an increase in ruminal acidosis risk between the preadaptation and adaptation II samplings, as indicated by the acidosis eigenvalues, was anticipated due to the diet composition.

Ruminal fluid measures from the majority of ruminal samples collected from all heifers at all 5 sampling times on challenge day were not consistent with the Bramley et al. (2008) model or those from other studies fitted using this model (O'Grady et al., 2008; Golder et al., 2012; Lean et al., 2013). The model was based on ruminal measures collected from 800 cows from 100 dairy herds across South Eastern Australia that included predominately pasture-based herds, herds fed grain twice daily at milking, and lot fed herds (Bramley et al., 2008). Approximately 10% of these cattle were defined as acidotic and the majority of cows in that data set (Bramley et al., 2008) had lactate concentrations <1 m*M*. The high total lactate concentrations of >19 m*M* for the mean of each feed additive group on challenge day in our study and individual heifers with concentrations as high as 148 m*M* may explain why the samples did not fit the model.

The ruminal measures from the challenge day do not consistently fit within any of the definitions used for cattle with normal ruminal conditions or cattle with subacute or acute ruminal acidosis (Nordlund and Garrett, 1994; Kleen et al., 2003; Nagaraja and

Titgemeyer, 2007). Ruminal pH values were greater than those suggested for cattle with subacute or acute ruminal acidosis by those authors, and lactate concentrations were greater than those suggested for normal cattle. The inability to fit ruminal measures to the model of Bramley et al. (2008) or consistently diagnose ruminal acidosis in these heifers using current definitions of ruminal acidosis based on starch feeding indicates that these heifers had distinctly different ruminal measures to these descriptions. We consider that the majority of heifers in this study, regardless of feed additive group, experienced lactic ruminal acidosis throughout sampling on challenge day, possibly as a result of the fructose inclusion in the diet.

Large increases in lactate have previously only been associated with acute ruminal acidosis (Burrin and Britton, 1986; Nagaraja and Titgemeyer, 2007). Mean total lactate concentrations per feed additive group exceeded those (0 to 5 m*M*) defined by Nagaraja and Titgemeyer (2007) for normal cattle or those with subacute ruminal acidosis for all heifers throughout the challenge sampling regardless of feed additive group. However, these were less than concentrations defined by those authors for acute ruminal acidosis (50 to 120 m*M*). This suggests lactate accumulation can occur without clinical signs of ruminal acidosis, is not necessarily associated with clinical ruminal acidosis severity, and could be associated with dietary substrate.

Differences in bacterial communities may be responsible for the approximately equal proportions of lactate stereoisomers in the VM, MLY, and BUF heifers, and dominant L-lactate concentrations in the control and MT groups. However, the ratio of stereoisomers does not always reflect their production, as lactate racemase can inter-convert stereoisomers (Asanuma and Hino, 2002). D-lactate is metabolized more slowly and has been suggested to be associated with more severe ruminal acidosis than L-lactate (Dunlop and Hammond, 1965). Further research on the association between lactate stereoisomers and clinical ruminal acidosis is warranted.

Substrates and the length of exposure to substrate influence the risk of ruminal acidosis, primarily through changes in microbial communities and rumen papillae (Dirksen et al., 1985; Nagaraja and Titgemeyer, 2007). This current study supports a quantitative review (Nagaraja and Titgemeyer, 2007) that suggests substrates, particularly sugars, may be a greater risk factor for ruminal acidosis than length of substrate exposure, because lactate accumulation occurred on challenge day irrespective of feed additive. In

ruminal acidosis challenge models, approximately half the amount of glucose compared to grain is required to induce ruminal acidosis (Nagaraja and Titgemeyer, 2007). Higher lactic acid concentrations were found in direct comparisons between steers fed sugar or starch (Harmon et al., 1985; Heldt et al., 1999) and ruminal and systemic acidosis developed in cattle orally drenched with oligofructose after previous oligofructose exposure (Danscher et al., 2010). A single feed of dietary fructose included at 0.4% of BW combined with 0.8% of BW triticale increased D-lactate concentrations approximately 22 and 5 fold above control or triticale only-fed heifers, respectively (Golder et al., 2012). These studies suggest sugars produce higher amounts of lactate, pose a greater risk of ruminal acidosis than grain-based diets, and that the rumen is more capable of withstanding single starch-based grain exposures compared to exposure to sugars. Substrate may also influence the efficacy of feed additives, an observation that merits further investigation.

Relatively large variations in ruminal fermentation measures were observed among individual heifers both within- and between-groups, consistent with some other carbohydrate feeding studies and may reflect the capacity of individuals to utilize different substrates and cope with changes in diet (Brown et al., 2000; Bevans et al., 2005). However, variations are greater than observed in other carbohydrate feeding studies (Mutsvangwa et al., 2002; Golder et al., 2012). These observations emphasize the need for large numbers of experimental animals to test interventions (Nagaraja and Titgemeyer, 2007). The large variation in response can mask the effect of dietary manipulation (Yang and Russell, 1993), and may explain the lack of significant feed additive group effects on total VFA, propionate, lactate, and ruminal pH measures that are commonly associated with ruminal acidosis (Coe et al., 1999). Despite the large observed among- and within-group variation in ruminal measures, group differences in ruminal acetate, butyrate, valerate, ammonia, and histamine concentrations were evident.

Virginiamycin findings appeared to support the conclusion of Coe et al. (1999) that VM has the potential to moderate ruminal fermentation in situations that could lead to rapid production of lactic acid. Responses observed in our study were consistent with VM studies in Holstein steers (Coe et al., 1999), and dairy cattle (Clayton et al., 1999). The combination of increased DMI over the adaptation periods, increased butyrate and

ammonia, lower acetate and valerate, and numerically lower propionate and total lactate concentrations, and numerically higher ruminal pH in VM heifers at challenge samplings supports a role for VM in stabilizing fermentation.

Other cattle studies with VM found similar effects on the proportion of total VFA (Salinas-Chavira et al., 2009) and propionate (Clayton et al., 1999; Valentine et al., 2000) with concentration results to our study. However, numerically lower propionate concentrations in the VM heifers possibly conflict with the proposal that VM selects for propionate-producing bacteria (Dennis et al., 1981). The low valerate concentrations have not been observed in other VM studies (Nagaraja et al., 1987; Valentine et al., 2000).

The numerically lower total lactate concentrations, compared to control heifers, support a proposed mode of action for VM through inhibition of protein synthesis in gram positive bacteria (Cocito, 1979) and observations of reduced or no effect on ruminal lactate concentrations in Holstein steers fed VM (Coe et al., 1999; Salinas-Chavira et al., 2009). However, total lactate concentrations, regardless of feed additive group, were abnormally high throughout the challenge sampling, suggesting a degree of ruminal acidosis risk and a reduction, but not complete prevention, of lactate accumulation in the VM heifers.

The lack of difference in ammonia concentrations between the VM and control cattle is consistent with other cattle studies (Hedde et al., 1980; Coe et al., 1999; Ives et al., 2002); however, the decrease in ammonia concentrations over the challenge sampling in the VM heifers could support a protein-sparing effect of VM identified *in vitro* (Van Nevel et al., 1984). The highest histamine concentrations in the VM heifers, suggest VM favors histamine-producing bacteria. The lack of significant effect of VM on ruminal pH is both consistent with some findings (Salinas-Chavira et al., 2009) but contrasts with increases (Hedde et al., 1980; Coe et al., 1999) in Holstein steers fed VM.

Clinical observations, the highest acidosis eigenvalues, and variable DMI in the MT group before challenge day suggest these heifers had the greatest ruminal acidosis risk before challenge day. On challenge day, similar ruminal pH values to the controls, numerically lowest acetate to propionate ratio, and numerically highest lactate concentration suggest that a degree of risk of ruminal acidosis occurred in the MT

group. However, this group appeared to have beneficial effects of lower ruminal ammonia and histamine accumulation and increased valerate concentration.

The lack of effect of MT on total VFA concentration is not consistent with an increase (Lean et al., 2000) or decrease (Ives et al., 2002) observed in other MT cattle studies. The absence of effect of MT on propionate concentration is consistent with the majority of MT cattle studies (Morris et al., 1990; Clary et al., 1993); however, it is in contrast to its proposed mode of action of increasing propionate production. The reduction in acetate and butyrate concentrations in both monensin-containing groups is consistent with monensin's proposed mode of action, which may also include a possible reduction in protozoal populations (Poos et al., 1979). The highest valerate concentrations in both the monensin groups could indicate growth of bacteria, such as *Megasphaera elsdenii*, that can ferment lactate to valerate among other end products (Marounek et al., 1989). Valerate is a safe sink for electrons and high concentrations may indicate a lower ruminal acidosis risk. Valerate concentration also tended to be higher than controls in MT-fed lactating dairy cattle (Lean et al., 2000).

The low ammonia concentrations in both monensin groups are consistent with the protein-sparing effect and decreased ammonia concentrations reported in monensin-fed dairy cattle (Plaizier et al., 2000; Ghorbani et al., 2011) but no effects have also been observed (Ramanzin et al., 1997; da Silva-Kazama et al., 2011). Protein sparing can be beneficial to the host; however, as ammonia is required for microbial protein synthesis, a very low ammonia concentration could be detrimental, depending on the quality of protein spared.

The lowest concentrations of ruminal histamine in both monensin-containing groups may be a positive response to the feed challenge. However, the role of histamine in ruminal acidosis and its sequalae remains unclear despite its well-known vasoactive characteristics. Ruminal pH was not influenced by MT in the current study, consistent with findings in feedlot steers (Morris et al., 1990; Clary et al., 1993) but was increased in other MT feedlot studies (Coe et al., 1999; Ives et al., 2002).

The MLY combination showed potential to reduce ruminal acidosis risk, largely through reduced ruminal acetate, butyrate, and histamine accumulation, higher valerate concentrations, and protein sparing. The low DMI and marked variation in DMI for

MLY heifers throughout this study suggest MLY was not as effective as VM or BUF. Reduced voluntary feed intake could be a strategy to maintain rumen health and did not appear to influence ADG or FCR. In contrast to this study, no effects on DMI occurred in monensin-fed dairy cattle (Ramanzin et al., 1997; Mutsvangwa et al., 2002) or Levucell SC®-fed dairy cattle in a multi-study analysis (de Ondarza et al., 2010), suggesting that the combination of MLY may behave differently to monensin or Levucell SC® only.

Consistent with this study, Kowalik et al (2012) reported no influence of MLY supplementation on total VFA concentrations in Levucell SC®-supplemented dairy cattle; however, Zelvyte et al (2006) found that total VFA concentration increased in Levucell SC®fed dairy cattle. Contrary to the increase in acetate concentration in MLY heifers in our study, acetate concentrations were unchanged in Levucell SC®-supplemented dairy cattle (Thrune et al., 2009; Kowalik et al., 2012). The increase in butyrate concentration is consistent with that observed in dairy cattle fed Levucell SC® (Thrune et al., 2009). Lactic acid concentrations have not been reported in dairy cattle fed Levucell SC and we propose that enhanced growth of lactate-utilizing bacteria may have led to increased movement of electrons to the safe sinks, propionate and valerate. All but one MLY heifer in our study had markedly lower lactate concentrations than the majority of other cattle and concentrations were approximately half those of the MT or control group during challenge (Table 6).

Ammonia concentrations were not affected in dairy cattle fed Levucell SC® (Thrune et al., 2009), in contrast to the decrease observed in this study. The numerically highest ruminal pH in the MLY group is consistent with increased ruminal pH in dairy cows supplemented with Levucell SC® (Zelvyte et al., 2006; Thrune et al., 2009; Kowalik et al., 2012) and lower feed intake. Overall, aspects of response to MLY exist that differ markedly from monensin or Levucell SC® fed singly, suggesting a positive synergy.

In the BUF group, feed intake variation was reduced before challenge day and the rumen was stabilized on challenge day; however, the administered recommended inclusion rate of sodium bicarbonate and magnesium oxide was higher than rates fed in practice (Bramley et al., 2012). The increase in DMI before challenge day in the BUF-fed heifers contrasts with effects from quantitative reviews on sodium bicarbonate, magnesium oxide, or their combination (Erdman, 1988; Staples and Lough, 1989; Hu

and Murphy, 2005). The majority of studies included in these reviews fed maize silage which influenced results (Erdman, 1988) and emphasizes the importance of substrate in evaluating studies.

Consistent with our study, propionate and acetate to propionate ratio were not affected when maize was not included in the ration, except when >30% forage diets were fed (Erdman, 1988; Hu and Murphy, 2005). A lack of effect of sodium bicarbonate or magnesium oxide on acetate proportion identified in reviews (Staples and Lough, 1989; Hu and Murphy, 2005) differs from the large increase in acetate in the BUF group. The lack of difference in butyrate concentrations between the BUF and control heifers is similar to the absence of effect on butyrate proportions observed in other BUF dairy cattle studies (Stokes et al., 1986; Arambel et al., 1988). The low valerate concentrations in the BUF heifers may reflect the numerically lower lactate concentrations than controls; however, other studies found no changes in valerate proportion in BUF fed dairy cattle (Stokes et al., 1986; Arambel et al., 1988).

The lack of ruminal pH difference to controls for the BUF group is consistent with quantitative reviews (Erdman, 1988; Hu and Murphy, 2005); however, the pH was numerically increased in the majority of studies reviewed by Staples and Lough (1989) and lower for those that fed maize diets (Hu and Murphy, 2005).

Lactate concentrations were not effected in the BUF-fed heifers, consistent with sodium bicarbonate-fed dairy cattle (Kilmer et al., 1981; Kennelly et al., 1999), but were numerically lower than the control and MT groups. The decrease in ammonia concentration may suggest the BUF reduced protein degradation; however, ammonia concentrations were not affected in sodium bicarbonate-fed cattle (Solorzano et al., 1989; González et al., 2008).

CONCLUSIONS

In summary, our hypothesis that feed additives would reduce ruminal acidosis risk compared with unsupplemented control cattle was equivocally supported. All feed additives modified the rumen in ways that influence the risk of ruminal acidosis by different means. Some positive and negative ruminal fermentation responses to feed additive groups were evident, and no individual feed additive group was able to maintain completely favorable ruminal conditions in all heifers. The large variation

between animals suggests feed additives may not be capable of controlling ruminal acidosis under extreme challenge or in cattle with a high ruminal acidosis risk. Feeding behavior may be an important means by which cattle reduce risk of ruminal acidosis. Metagenomic assessment of samples from this study, alternative feed additive combinations, and dose rates of feed additives should be matters for further investigation.

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CHAPTER 8

Case Report: Ruminal Acidosis in 21-month-old Holstein Heifer

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OVERVIEW OF CHAPTER 8

In Chapter 7, a control heifer (fed no feed additives) was diagnosed with acute ruminal acidosis within 10 h of consuming her wheat and fructose ration on challenge day. Chapter 8 is a case report on this heifer and tracks her dry matter intake, clinical signs, and ruminal and blood biochemical measures from her enrolment in the study to her recovery and compares these to heifers in her cohort.

ABSTRACT

Ruminal and blood biochemical measures were monitored in 8 Holstein-Friesian heifers exposed to a carbohydrate feeding challenge. One of the heifers had clinical signs consistent with acute ruminal acidosis on the day of, and subsequent to, the challenge. Within 24 h of challenge, 6 of 7 ruminal volatile fatty acids measured were not detectable in this heifer and her ruminal total lactate concentration was > 70 m*M*.

INTRODUCTION

The objective of this report was to describe findings, especially ruminal conditions that were present in a heifer with clinical signs consistent with acute ruminal acidosis. Clinical definitions of ruminal acidosis are inconsistent and largely based on arbitrary ruminal pH cut points (Nagaraja and Titgemeyer, 2007; Plaizer et al., 2008). Lean et al. (2009) note the need for definitions of metabolic disease to be accurate, standardized, repeatable, and based on clinical outcomes including measurable changes in metabolism, morbidity, mortality, or production.

Sporadic disorders such as acute ruminal acidosis are only rarely documented in detail that provides a combination of clinical signs, feed intake, nutritional composition of the diet, and ruminal and blood measures; therefore, there is merit in presenting observations on a clinical case, particularly when detailed comparative data on other cattle were available.

We report feed intake, clinical signs, and ruminal and blood biochemical measures of a heifer exposed to a diet high in readily fermentable carbohydrates for 20 d preceding challenge with a ration containing 19.1% sugar and 54.1% starch on a dry matter (**DM**) basis. Her ruminal and blood biochemical measures are compared to those of 7 cohort heifers.

CASE DESCRIPTION

A 21-mo-old pregnant Holstein-Friesian heifer [Identification no. 1250; bodyweight (**BW**) 488 kg] showed clinical signs of acute ruminal acidosis within 10 h of consuming 4.8 kg of DM of milled wheat and 960 g DM of fructose (1.0 and 0.2% of her BW, respectively). Heifer 1250 was 1 of 8 control heifers (mean BW 382 ± 17 kg, excluding heifer 1250) enrolled in a 29 d readily fermentable carbohydrate challenge study evaluating the effectiveness of feed additives to reduce the risk of ruminal acidosis (Golder et al., 2014). The study was conducted at Cobbitty, New South Wales (**NSW**), Australia. All experimental procedures were approved by the SBS*cibus* Animal Ethics Committee (SBScibus 0512-0513).

The study consisted of 5 experimental periods: (1) preadaptation (d –2 to 0), (2) adaptation I (d 1 to 10), (3) adaptation II (d 11 to 20), (4) challenge (d 21), and (5)

postchallenge (d 22 to 26). Rations offered at each of these periods are detailed in Figure 1 and Table 1. The 20 d adaptation period was considered adequate to study rumen perturbation. Ruminal and blood samples were not collected from the cohort heifers during the postchallenge period. Heifers were randomly allocated to 1 of 4 blocks (A to D; 2 heifers/block), enrolment of each block of heifers in the study was staggered over 4 consecutive days. Heifer 1250 was allocated to block A.

All heifers were housed with the main study herd in a paddock containing dormant kikuyu (*Pennisetum clandestinum*) with no available pasture and *ad libitum* water access. An equal amount of feed was offered twice daily at 7:00 and 14:00 h in individual feed bins and separate feeding pens that prevented access between pens. The amount of ration that the heifers consumed before ceasing consumption and the time they took to consume this amount were recorded. Consumption time did not exceed 2 h. Heifers were subsequently returned to the paddock with no access to feed until the next feeding session.

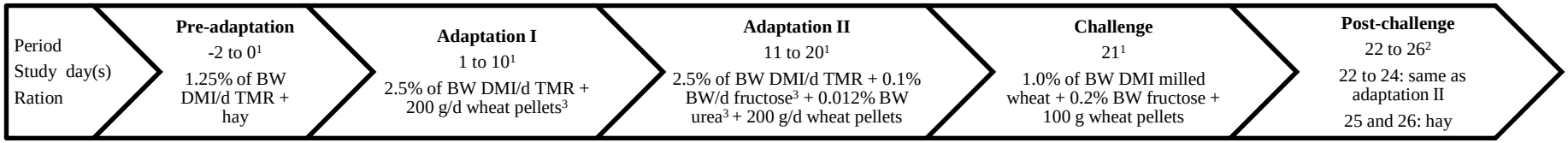

Figure 1. Experimental periods and their corresponding study days and rations offered for the study in which heifer 1250 and her cohort of heifers were enrolled. BW = bodyweight; DMI = dry matter intake; TMR = total mixed ration (62:38 forage:concentrate, consisting of 31.5% wheaten hay, 30.5% alfalfa hay, and 38% milled wheat).[1]Ruminal and blood samples were collected on d 0, 7, 14, and 21 during their respective experimental periods from both heifer 1250 and her cohort of heifers.[2]Ruminal and blood samples were collected from heifer 1250 on d 22 and 23. [3]Introductory doses were offered for the initial days before the full rate was offered.

Table 1. Estimated chemical composition of the ration offered during the adaptation I, adaptation II, and challenge periods[1]

Item (% DM)	Adaptation I (d 1 to 10)	Adaptation II (d 11 to 20)	Challenge (d 21)
DM	89.6	89.9	90.2
CP	13.4	14.1	9.8
RUP (% of CP)	27.6	21.3	11.8
RDP (% of CP)	72.4	78.7	88.2
RDP	9.70	11.1	8.60
Soluble protein (% of CP)	39.8	45.0	38.5
ADF	22.4	21.6	4.49
NDF	35.0	33.6	10.7
Forage NDF (% of NDF)	85.8	85.8	0.0
Forage NDF (% of DM)	30.0	28.8	0.0
Physically effective NDF	30.6	29.4	4.2
Lignin	3.6	3.5	0.9
NFC[2]	46.1	47.7	76.3
Silage acids	0.0	0.0	0.0
Sugar	9.4	12.6	19.1
Starch	27.5	26.4	54.1
Soluble fiber	9.1	8.7	3.2
Total ether extract	1.95	1.88	1.74
Total LCFA	1.39	1.34	1.57
Ash	5.98	4.94	1.82
DCAD (mEq/100g)	24.7	23.7	-0.25
Minerals (mg/kg)			
Chloride	4,400	4,300	1,000
Calcium	4,800	4,500	900
Copper	21	19	20
Iron	128	122	42
Phosphorus	3,000	2,900	2,800
Potassium	1,700	16,300	4,100
Magnesium	1,400	1,300	1,000
Manganese	75	70	58
Sodium	1,300	1,200	100
Sulfur	1,900	1,800	1,300
Zinc	64	58	62

DM = dry matter; CP = crude protein; RUP = rumen undegradable protein; RDP = rumen degradable protein; ADF = acid detergent fiber; NDF = neutral detergent fiber; NFC =non-fiber carbohydrates; LCFA = long-chain fatty acids; DCAD = dietary cation-anion difference.

[1]Estimations were performed using CPM Dairy Analyzer version 3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) based on a 400 kg BW heifer with a body condition score of 3.25 and a growth rate of 0.73 kg/d. The ration fed in adaptation I comprised 2.5% of bodyweight (BW) dry matter intake (DMI)/d total mixed ration (TMR) + 200 g/d wheat pellets. The ration fed in adaptation II comprised 2.5% of BW DMI/d TMR + 0.1% of BW/d fructose + 200 g/d wheat pellets. The ration fed during the challenge comprised 1.0% of BW DMI wheat + 0.2% of BW fructose + 100 g wheat pellets. The TMR (62:38 forage:concentrate) consisted of 31.5% wheaten hay, 30.5% alfalfa hay, and 38% disc milled wheat.

[2]NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash]. NDICP = neutral detergent insoluble crude protein.

Heifer 1250's dry matter intake (**DMI**) over the 20 d adaptation period, where she was offered 2.5% of her BW DM total mixed ration (**TMR**)/d (d 1 to 10) and additional 0.1% of her BW fructose (d 11 to 20), averaged 8.6 kg of DM/d (84 ± 3.3% of the offered ration/d), compared to 8.3 ± 0.3 kg of DM/d (91 ± 1.5% of the offered ration/d) for the cohort heifers. Qualitatively, she had a similar DMI pattern to the cohort heifers, but had more pronounced drops in DMI. She consumed only 4.9 kg of DM/d (50% of her allocated ration) on d 4 and a physical examination showed that her rectal temperature, rumen contractions, and heart rate (80 beats/min) were within normal ranges. Her DMI also decreased during the 4 d before challenge (d 17 to 20), to approximately 9.6 kg of DM/d (79% of her offered ration/d), from approximately 11.5 kg of DM/d (95% of her offered ration) for days 13 to 16. On d 17 to 20 the cohort heifers consumed on average 9.3 ± 0.3 kg of DM/d (95 ± 1.5% of their offered ration/d).

Ruminal fluid collected using a custom-designed stomach pump and tube, and blood samples were taken weekly before challenge on d 0 (preadaptation), d 7 (adaptation I), and d 14 (adaptation II). The stomach tube was inserted to a minimum length of 2 m and ruminal fluid was scored for saliva contamination (Bramley et al. 2008). Ruminal pH [Merck Pty Ltd., Kilsyth, Victoria (**VIC**), Australia; Figure 2I] was measured within 3 min of collection of ruminal fluid and all other measurements were done at a later date on samples immediately placed on ice and later stored at –20^{o}C. All ruminal and blood measures from heifer 1250 were compared to those of the 7 other heifers and are referred to as higher or lower if they are outside the mean ± SEM of these cohort heifers.

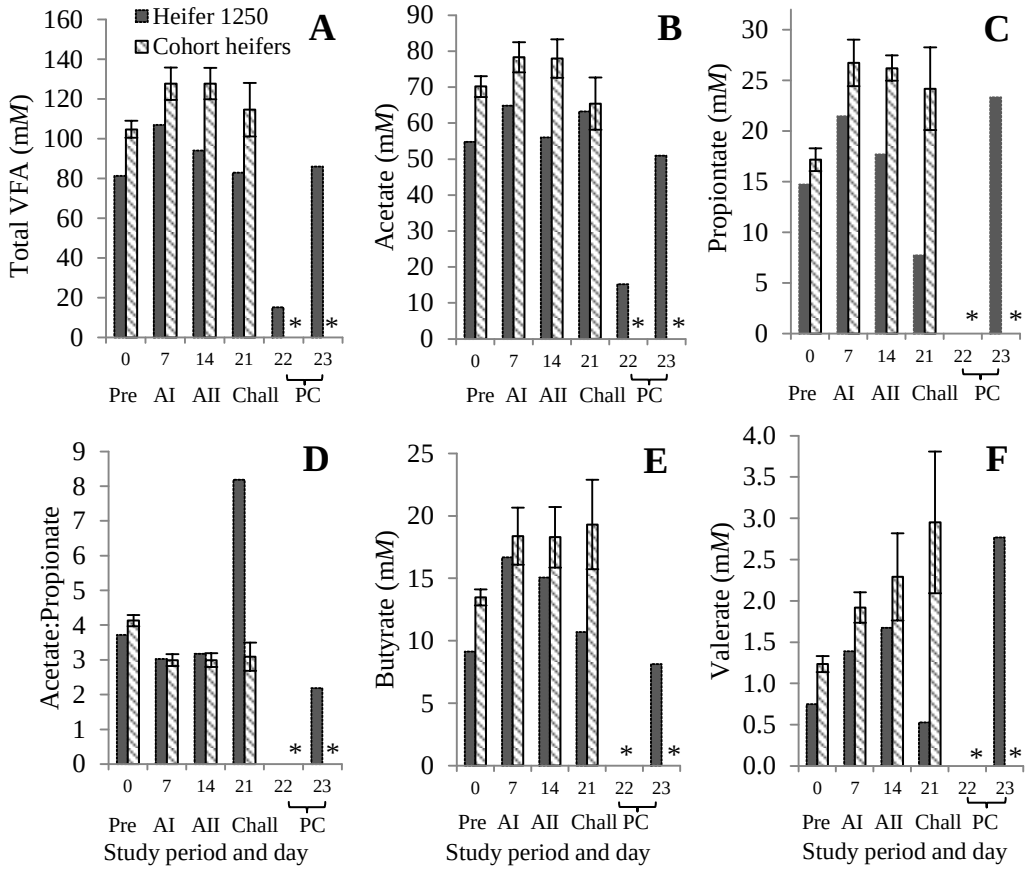

Figure 2. Ruminal concentrations of total volatile fatty acid (VFA) (A); acetate (B); propionate (C); acetate:propionate (D); butyrate (E); valerate (F); ammonia (G); and total lactate (H); and pH (I); plasma oxidative stress index (OSI) (J); plasma ceruloplasmin concentrations (K); and serum β-hydroxybutyrate concentrations (L) in heifer 1250 that was diagnosed with acute ruminal acidosis and the mean (±SEM) of 7 cohort heifers. Ruminal fluid samples were collected using a stomach tube over the following 5 experimental periods on the study day indicated in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); Chall = challenge (d 21); and PC = postchallenge (d 22 and 23). Refer to Figure 1 for rations offered during each experimental period. On challenge (d 21) ruminal fluid samples were collected approximately 3.6 h after the challenge rations were consumed. *Ruminal fluid was not collected from the 7 cohort heifers during the postchallenge period.

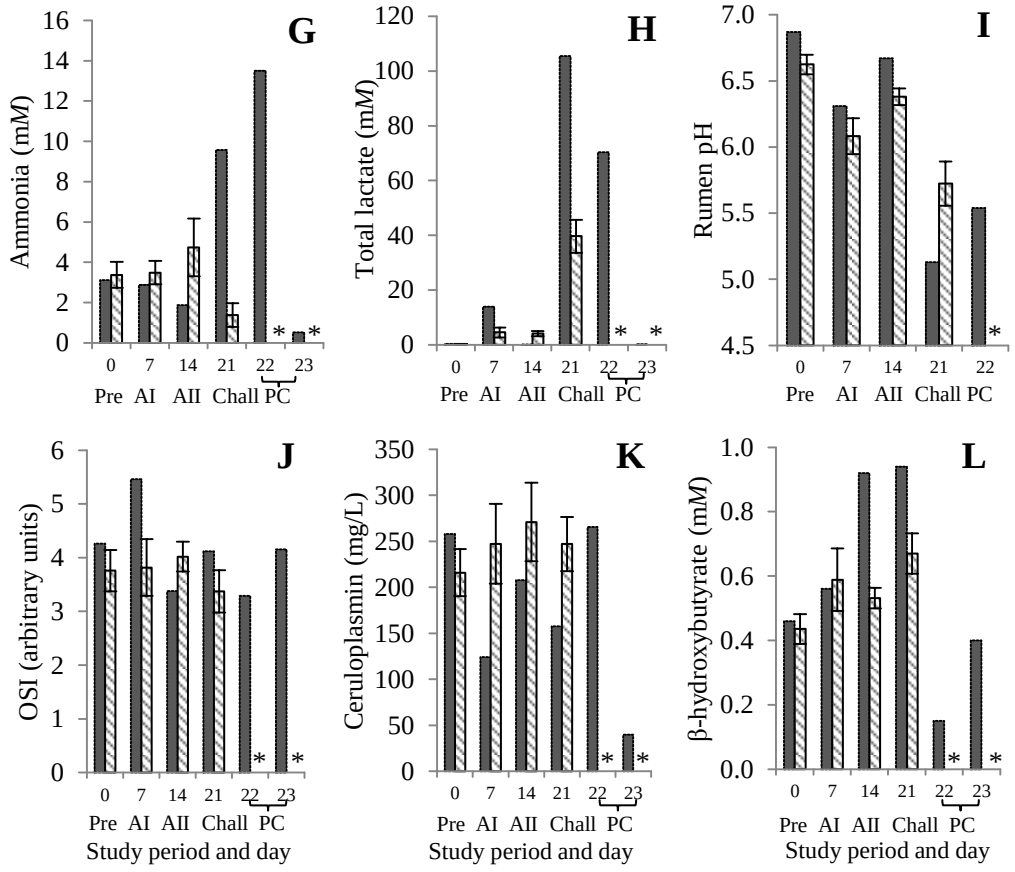

Figure 2 (continued). Ruminal concentrations of total volatile fatty acid (VFA) (A); acetate (B); propionate (C); acetate:propionate (D); butyrate (E); valerate (F); ammonia (G); and total lactate (H); and pH (I); plasma oxidative stress index (OSI) (J); plasma ceruloplasmin concentrations (K); and serum β-hydroxybutyrate concentrations (L) in heifer 1250 that was diagnosed with acute ruminal acidosis and the mean (±SEM) of 7 cohort heifers. Ruminal fluid samples were collected using a stomach tube over the following 5 experimental periods on the study day indicated in parentheses: Pre = preadaptation (d 0); AI = adaptation I (d 7); AII = adaptation II (d 14); Chall = challenge (d 21); and PC = postchallenge (d 22 and 23). Refer to Figure 1 for rations offered during each experimental period. On challenge (d 21) ruminal fluid samples were collected approximately 3.6 h after the challenge rations were consumed. *Ruminal fluid was not collected from the 7 cohort heifers during the postchallenge period.

Heifer 1250 had a higher ruminal pH (Figure 2I), 20 to 30 m*M* lower total volatile fatty acids (**VFA**), which comprised acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, and caproate measured by gas chromatography (Figure 2A), lower major individual VFA (Figures 2B, C, E and F), and a similar acetate to propionate ratio (Figure 2D) than the cohort heifers before challenge day. Her ammonia concentration measured by a direct enzymatic method was lower in the adaptation II sampling (Figure 2G), while her total ruminal lactate concentration (D- lactate; Boehringer Mannheim kit: catalog no. 11 112 821 035; R-Biopharm-Laboratory Diagnostics Pty. Ltd., Taren Point, NSW, Australia; L- lactate; catalog no. OSR 6193; Beckman Coulter Australia Pty. Ltd) was higher in the adaptation I sampling (Figure 2H).

Plasma reactive oxygen metabolites (**dROM**; d-ROMS Test, Diacron International, Grosseto, Italy) and biological antioxidant potential (**BAP**; Bap Test; Diacron International; Celi, 2011a) were increased in heifer 1250 compared to the cohort heifers at the adaptation I and II samplings, respectively (202 compared to 138 ± 14 Carratelli units, and 4351 compared to 3884 ± 280 μmol/L). Heifer 1250's plasma oxidative stress index (**OSI)** [(dROM/BAP) × 100] (Celi, 2011a) (Figure 2J) was increased in the preadaptation and adaptation I samplings. Her plasma ceruloplasmin (Sunderman and Nomoto, 1970) dropped 133.7 mg/L between the preadaptation and adaptation I samplings (Figure 2K).

Serum non-esterified fatty acid (**NEFA**) concentrations (catalog no. FA 115, Randox Laboratories, Crumlin, Antrim, UK) for heifer 1250 were similar to those of the cohort heifers before challenge. Her serum β-hydroxybutyrate concentration (catalog no. RB 1007, Randox Laboratories) was higher in adaptation II than the cohort heifers' (Figure 2L). Heifer 1250's serum glucose concentration (Infinity^{TM} Glucose Hexinase Liquid Stable Reagent; Product no. TR15421, Thermo Scientific, Scoresby, VIC, Australia) was lower in the adaptation II sampling than the cohort heifers' (4.3 compared to 4.8 ± 0.2 m*M*, respectively). Serum urea concentration for heifer 1250 (Infinity^{TM} Urea Liquid Stable Reagent; Product no. TR12421, Thermo Scientific) was lower than the cohort heifers' in the preadaptation (2.5 compared to 3.8 ± 0.2 m*M*, respectively) and higher in the adaptation II samplings (5.2 compared to 3.1 ± 0.1 m*M*, respectively).

Heifers were scored for locomotion using a 5-point scoring system (Sprecher et al., 1997) after ruminal and blood collection and heifer 1250 was scored as <2 (sound) during all scoring sessions leading up to challenge day, similar to her cohorts.

On the challenge day (d 21) each heifer was offered 200 g of alfalfa hay and immediately after its consumption each heifer was offered 1.0 and 0.2% DMI of their BW in milled wheat and fructose, respectively, and 200 g of wheat pellets. The challenge ration had a 19.1% DM sugar and 54.1% DM starch content (CPM Dairy Ration Analyzer version 3.10; Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 1). Feeding was staggered within each block with the first heifer fed at 7:00 h. Heifer 1250 consumed 100% of her offered 4.8 kg of DM of milled wheat, 960 g of DM of fructose, and 200 g of wheat pellets in 36 min (total intake 6.0 kg of DM). The mean DMI, percentage of offered ration consumed, and consumption time for the cohort heifers were 4.2 ± 0.4 kg of DM, 90 ± 9.6%, and 62 ± 9.8 min, respectively. At approximately 3.6 h after consumption of the ration a ruminal fluid sample and a blood sample were collected from all heifers.

Heifer 1250's ruminal pH was 5.1 on the challenge day, which was a decline of 1.7 pH units from her preadaptation sample pH. Mean ruminal pH of the cohort heifers on the challenge day was 5.7 (Figure 2I).

On the challenge day heifer 1250's total VFA concentration was lower (82.9 m*M*) compared to her cohort (114.7 ± 13.4 m*M*; Figure 2A). Her acetate concentration was similar to the cohort heifers' (Figure 2B); however, her propionate concentration was lower at 7.7 m*M* compared to 24.1 ± 4.1 m*M* for her cohort (Figure 2C), and her acetate to propionate ratio was 8.2 compared to 3.1 ± 0.4. Heifer 1250's butyrate concentration was lower (10.7 m*M*) than the cohort heifers' (19.3 ± 3.6 m*M*; Figure 2E). Her valerate concentration was lower at 0.5 m*M* compared to 3.0 ± 0.9 m*M* for the cohort heifers (Figure 2F), and ammonia concentration was over 6 fold higher than that of the cohort heifers (Figure 2G).

Heifer 1250's ruminal histamine concentration (human histamine ELISA kit; IBL International, Hamburg, Germany) was more than double that of the cohort heifers on challenge day, 291.6 and 124.2 ± 40.5 m*M*, respectively. Her dROM were higher over the challenge period than that of the cohort heifers (147 compared to 120 ± 10 Carratelli

units) and her OSI measure was 4.1 compared to 3.4 ± 0.4 for the cohort. Her ceruloplasmin concentrations were lower (157.5 mg/L) than those of the cohort heifers (247.1 ± 43.2 mg/L; Figure 2K).

Heifer 1250's β-hydroxybutyrate (Figure 2L) and urea concentrations were higher than those of the cohort heifers on the challenge day (4.1 compared to 2.9 ± 0.3 m*M*, respectively), while her glucose was lower (3.7 and 4.8 ± 0.3 m*M*, respectively) and her NEFA was similar (0.07 and 0.05 ± 0.02 m*M*, respectively).

In the afternoon after the challenge all heifers were scored for locomotion, and offered *ad libitum* access to wheaten and millet hay 7 h after the challenge with close monitoring of feed intake. Heifer 1250's locomotion score was 1.5, compared to 1.1 ± 0.1 for the cohort heifers.

The first clinical sign that heifer 1250 may have had acute ruminal acidosis was a different behaviour from her cohort heifers when hay was offered. The cohort heifers rushed to eat the hay, whereas 1250 showed no interest in eating. At 17:30, 20:00, and 22:30 h checks she stood at the rear of the paddock, continued to show no interest in hay, had very loose diarrhea, and had dull mentation, but was sufficiently alert to not allow contact within 3 m.

On the morning after challenge (d 22), heifer 1250 stood in an area surrounded by watery, bubbly diarrhea, was dull, and walked tentatively. Her heart rate was 66 beats/min, respiration rate was 18 breaths/min, and her rumen was static and distended, as assessed by auscultation and ballotment of the paralumbar fossa. Ruminal fluid and a blood sample were taken at 7:00 h and the immediate ruminal pH measure was 5.5. Later VFA analysis (in duplicate) revealed heifer 1250 had no detectable concentrations of propionate, butyrate, valerate, isobutyrate, isovalerate, or caproate (Figures 3A, C, E, and F). The only VFA detected was 15.2 m*M* of acetate (Figure 2B). Her ammonia concentration was 13.5 m*M* (Figure 2G) and total ruminal lactate concentration was 67% (70 m*M*; Figure 2H) and ruminal histamine concentration was 77% (224 m*M*), of the respective concentrations measured 20 h earlier. Her OSI had returned to a similar level to that reported at the adaptation II sampling (Figure 2J), but plasma ceruloplasmin was 266 mg/L (Figure 2K). Heifer 1250's β-hydroxybutyrate concentration was approximately 6 fold lower than 20 h earlier (Figure 2L). Her NEFA concentration was

58% higher, and her urea and glucose were approximately 53 and 9% lower, respectively, than 20 h earlier.

Heifer 1250 was treated by stomach tube with 200 g of sodium bicarbonate (Penice Soda Products Pty Ltd, Osborne, South Australia, Australia) in 20 L of water at 7:30 h (d 22), before being scored for locomotion. She was reluctant to walk; her gait was slow and tentative and was scored at 2.5, 1 score higher than the previous day. No other heifers in the cohort had locomotion scores above 1.5. Heifer 1250 was housed in a separate yard where she was offered wheaten and millet hay and water, none of which she consumed. Later she was offered TMR but there was no detectable intake. She was given Flunixin meglumine (Ilium Flunixil, Troy Laboratories Pty. Ltd., Smithfield, NSW, Australia), 2.2 mg/kg BW, IM, at 14:30 h on the same day. She was examined on the 2nd d after challenge (d 23), 48 h after challenge. At this time her appetite had resumed and she displayed no abnormal signs during a physical examination. She was subsequently returned to the main herd. Ruminal fluid and blood measures from samples collected at 14:00 h on the same day (d 23) revealed that her total VFA concentrations had returned to a similar concentration to those on the challenge day. Her acetate to propionate ratio (Figure 2D) was 2.2, valerate concentration was much higher (2.77 m*M*) than her previous recordings (Figure 2F), ammonia had dropped markedly to 0.53 m*M* (Figure 2G), and her total lactate concentration was very low (Figure 2H). Her ruminal histamine concentration had decreased from the previous sampling by 70%, OSI increased by 21% (Figure 2J), BAP decreased by 15%, and plasma ceruloplasmin had decreased dramatically from 265.9 to 40.0 mg/L (Figure 2K). Heifer 1250's NEFA concentrations decreased from 0.17 to 0.09 m*M*, while her β-hydroxybutyrate (0.15 to 0.40 m*M*; Figure 2L), glucose (3.4 to 4.1 m*M*), and urea concentrations (1.9 to 2.7 m*M*) increased compared to the previous day. Her DMI (11.1 ± 1.1 kg of DM; 91 ± 9.4% of offered ration) on the 2nd and 3rd d after challenge (d 23 and 24) was similar to the cohort heifers' (8.9 ± 0.6 kg of DM; 91 ± 6.2% of offered ration). Her locomotion score was normal on the 5th d after challenge (d 26) and consistent with that of the cohort heifers'.

Over the study, heifer 1250 gained 34 kg of BW and on average the cohort heifers gained 37 ± 4 kg of BW. After being returned to the farm of origin heifer 1250 calved

with no complications and the herd manager reported that she has been in excellent health since her return.

DISCUSSION

The clinical signs of diarrhea, inappetence, and reluctance to move observed within 10 h of consumption of readily fermentable carbohydrate are consistent with the diagnosis of acute ruminal acidosis (Plaizier et al., 2008). Total lactate concentrations were within the range for acute ruminal acidosis (Nagaraja and Titgemeyer, 2007), throughout the challenge and remained within this range 1 d after, until these returned to near zero 2 d after challenge. When considering the difference between ruminal pH values collected by stomach tube and fistula, the observed pH of 5.1, 3.6 h after challenge ration consumption is consistent with acute ruminal acidosis definitions (Duffield et al., 2004). However, ruminal pH was 5.5 when the clinical signs were observed. It is possible rumen pH continued to decline after 3.6 h; however, ruminal fluid collection at 2 to 5 h after the primary concentrate meal has been reported as the optimum sampling time (Nordlund and Garrett, 1994) and symptoms were most visible at the time pH was 5.5.

This is the first reported case in cattle, where concentrations of the individual VFA: propionate, butyrate, isobutyrate, isovalerate, valerate, and caproate, were not detected during acute clinical ruminal acidosis. An absence of propionate and butyrate concentrations was similarly noted in wethers within 14 h of being administered a sucrose solution at 15 g/kg of BW through a rumen fistula (Kezar and Church, 1979). Further, all wethers had acetate concentrations of <5 m*M* within 24 h of treatment and lactate concentrations were >70 m*M* (Kezar and Church, 1979). No detectable or very low concentrations of propionate, acetate, and valerate were reported in sheep as early as 10 h after 1.4 to 2.7 kg of cracked wheat was administered through a rumen fistula (Ryan, 1964). A total VFA concentration of <20 m*M* was reported in a wether 24 h after consuming 6.8 kg of mangolds (Scarisbrick, 1954). It has been suggested that in acute ruminal acidosis, VFA concentrations are initially increased and then decline below 100 m*M* (Nagaraja and Titgemeyer, 2007); however, these observations suggest a more dramatic decline is present.

This study was planned to induce a ruminal acidosis of non-life threatening severity based on feeding an amount of grain similar to percentages that are commonly fed to beef and dairy cattle and 0.2% DMI of BW of fructose which was designed to increase the risk of ruminal acidosis and mimic large exposures to sugar sources from access to high fructan or sucrose sources such as some fresh forages, brassicas, or citrus pulp, molasses, whey, or crystalline sugar. The fructose would have been rapidly fermented and may have increased ruminal fluid passage and hindgut fermentation. Thus, grain fed in the milking parlour in combination with sugar sources and inadequate effective fiber may place cattle at increased risk of ruminal acidosis.

Individual animals have different susceptibilities to ruminal acidosis (Brown et al., 2000). Heifer 1250 had different ruminal fermentation measures to her cohorts at study d 0 which may indicate an inherent increased risk of ruminal acidosis. Her reduction in DMI to approximately 9.6 kg of DM/d (79% of her offered ration/d) during the 4 d leading up to the challenge day may further indicate a greater susceptibility to ruminal acidosis compared to the cohort heifers before challenge. Her rapid consumption of 100% of the ration in approximately half the time of the cohort heifers may have also increased her risk of ruminal acidosis. As a consequence of heifer 1250 developing ruminal acidosis, the 6 heifers allocated to blocks B to D that were challenged on subsequent days were drenched with 200 g of sodium bicarbonate 4 h after the challenge.

Bramley et al. (2008) defined ruminal acidosis on the basis of a combination of individual VFA, ammonia, ruminal pH, and lactate measures. Definitions based on ruminal pH cut points alone are not consistent and need to be accurately defined to allow appropriate diagnosis and treatment. Definitions also need to be specific for ruminal fluid collection method to account for the heterogeneous nature of the rumen and also need to better accommodate single samples. This case study supports the work of Bramley et al. (2008) suggesting that ruminal pH is not the key indicator of ruminal acidosis. Although heifer 1250 had lower ruminal pH values than the cohort heifers, her pH was borderline for diagnosis of acute ruminal acidosis, whereas the very high lactate, and histamine and low VFA levels were suggestive of acute ruminal acidosis. This suggests that high ruminal lactate concentrations do not necessarily correspond to a very

low ruminal pH and cowside VFA tests may be important diagnostic tools for ruminal acidosis in the future.

The increases in histamine were consistent with the knowledge that histamine generation occurs after feeding and can accumulate in the rumen during acidotic conditions (Ahrens, 1967; Golder et al., 2012). The increase in locomotion score suggests an association with histamine. While net absorption of histamine from the rumen appears to be low, and it is inactivated either during or after absorption into the blood, low ruminal pH, and gut lesions may favor absorption (Aschenbach and Gabel, 2000).

The increase in OSI after challenge suggests heifer 1250 was subject to increased oxidative damage, an observation which is consistent with elevated oxidative stress measures in cattle fed high starch diets (Gabai et al., 2004). The decrease in ceruloplasmin and BAP concentrations 2 d after the challenge may suggest they are involved in the pathogenesis of ruminal acidosis (Celi, 2011b). The decrease in β-hydroxybutyrate, glucose, and urea and increase in NEFA concentrations 24 h after challenge support the presence of a metabolic disturbance. These measures may also be useful ancillary diagnostic measures for ruminal acidosis that could be developed for cowside use in the future.

In conclusion, this study emphasizes the variability in susceptibility to ruminal acidosis of individual cattle and the importance of detection of early clinical signs such as decreases in DMI, diarrhea, and change in demeanour. It also draws attention to the potential dangers of sugars in the diet, particularly in combination with grain. Clinicians should be aware that ruminal pH is not always an adequate diagnostic method for evaluating ruminal acidosis, especially when a single sample is obtained. Other ruminal measures including ruminal VFA, lactate, and ammonia can provide a greater understanding of rumen function. Recovery of rumen function can rapidly occur after treatment despite severe perturbation.

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GENERAL DISCUSSION

GENERAL DISCUSSION

The aim of this research was to increase our overall understanding of the pathogenesis of ruminal acidosis and control strategies for ruminal acidosis in dairy cattle. This was achieved through investigation of the role of substrates in rumen perturbation and evaluation of feeding systems and potential feed additive control agents for ruminal acidosis in 4 short or longer term randomized challenge studies. The following outcomes were evaluated throughout the thesis: products of ruminal fermentation, oxidative stress responses, ruminal bacterial community composition, production performance, clinical signs, dry matter intake, locomotion scores, and feed and ration composition. Outcomes were considered in the context of existing models of ruminal acidosis (Hungate, 1966; Baldwin and Allison, 1983; Nocek et al., 1997; Owens et al., 1998; RAGFAR, 2007) and applications in the field when relevant.

The overall hypothesis was supported that starch-, sugar-, and protein or amino acid-based feed substrates would produce different ruminal, blood, and ruminal bacterial community composition associated with different risks of ruminal acidosis. Oxidative stress responses were equivocal. Our secondary hypothesis, that partial mixed ration feeding strategies and feed additive control agents would promote favorable ruminal conditions and reduce the risk of ruminal acidosis was also supported; however, whether feed additive control agents reduced the risk of ruminal acidosis was equivocal.

The critical review (Chapter 1) identified that ruminal acidosis remains a prominent economic and welfare issue for the dairy industry worldwide that warrants further investigation to reduce its impact. It was evident ruminal acidosis can present with different clinical severities (Kleen et al., 2003), but definitions of ruminal acidosis lacked consistency and were largely based on ruminal pH measures (Kleen et al., 2003; Nagaraja and Titgemeyer, 2007; Plaizier et al., 2008). An integration of understandings of the pathogenesis of ruminal acidosis from this thesis suggests that ruminal acidosis occurs along a continuum of severity, reflecting hydrogen sequestration. It is clear the rumen is very dynamic and can adapt and recover from severe ruminal perturbation, providing opportunities for rumen manipulation and ruminal acidosis control. Not all cattle were able to be adequately categorized as having normal ruminal conditions, subacute or acute ruminal acidosis as defined in literature (Nagaraja and Titgemeyer, 2007; Chapters 2, 7, 8).

Accurate, consistent, and practical methods of diagnosing ruminal acidosis in large numbers of cattle were required to enhance investigation of the role of feed substrates or efficacy of control strategies. The acidosis model of Bramley et al. (2008) that defines ruminal acidosis based on a combination of ruminal measures was identified as the only model that linked a definition of ruminal acidosis to feed composition, health, and production data and this model was used to aid definition of ruminal acidosis in Chapters 2, 5, and 7. This model adequately defined starch-based ruminal acidosis in Chapter 5, providing validation for the use of this model; however, it could not adequately categorize heifers with sugar-based ruminal acidosis resulting from fructose in Chapter 7. This finding suggests that different definitions and models of ruminal acidosis are required for when very different feed substrates are fed. Simultaneous observations and assessment or monitoring of multiple measures including clinical observations, feed assessment, and feeding behavior supported by ruminal, milk, urine, blood, and other measures should be used to assess ruminal conditions and ruminal acidosis and were carried out throughout this thesis. Refinement or adoption of ruminal acidosis models such as that of Bramley et al. (2008) that use multiple indicators will aid in interpretation of results and diagnosis of ruminal acidosis. Use of validated reference values will also aid diagnosis of ruminal acidosis (Chapter 1).

Comparison of ruminal fluid collection techniques in the review (Chapter 1), concluded that the use of rumenocentesis, fistulates, and stomach tubes were all valid methods of ruminal fluid collection, each with their advantages and disadvantages and correction factors may need to be applied to compare values obtained using different techniques. Stomach tubing was used to allow repeated sampling of large numbers of heifers for ruminal fluid collection in Chapters 2, 6, 7, and 8, while fistulates were used in Chapter 5. Ruminal pH is not always an adequate diagnostic method for evaluating ruminal acidosis, especially when a single sample is obtained (Chapter 8), which is consistent with the findings of Bramley et al. (2008) and Lean and Rabiee (2012). Ruminal pH does not always correlate with ruminal lactate concentrations; however, valerate and propionate appear to be good diagnostic measures for ruminal acidosis (Chapters 1 and 5). This is consistent with the findings of Bramley et al. (2008), and there may be an opportunity to develop cowside kits in the future.

A variety of feedstuffs have been implicated in inducing ruminal acidosis (Underwood, 1992). A stepwise adaptation to feed changes is recommended (Elam, 1976) and target dietary non-structural carbohydrate, soluble fiber, starch and protein requirements have been reported (Chapter 1), but a greater understanding of the involvement of sugar, starch, and protein in rumen perturbation and ruminal acidosis is needed. The role of substrates in the pathogenesis of ruminal acidosis and risk of ruminal acidosis was investigated throughout the thesis. Substitution of fructose for grain in a single challenge feed lowered ruminal pH and increased volatile fatty acid and lactate concentrations (Chapter 2), suggesting that the risk of ruminal acidosis may be increased by feeding substrate sources with high sugar content such as brassicas, molasses, whey, citrus pulps, root crops, certain forages, or crystalline sugar. Producers need to be aware of overall sugar content of diets and variability in water-soluble carbohydrate content of substrates. The heifers in Chapter 2 were better able to adapt to a single pulse exposure with grain compared to abrupt exposure to the combination of grain and fructose (Chapter 2). Cows fed a partial mixed ration with an estimated metabolizable protein content above NRC (2001) requirements had lowered acidosis eigenvalues, compared to those fed an isoenergetic diet of crushed wheat fed in the milking parlor and pasture silage fed on the pasture or those fed a partial mixed ration, although not significantly (Chapter 5). This finding coincides with the effect of Fermenten® in promoting more favorable ruminal conditions (Chapter 6). However, milk measures were not increased in the cows fed a partial mixed ration, compared to those fed an isoenergetic diet as components (Chapter 5).

The existing models of the pathogenesis of ruminal acidosis (Hungate, 1966; Baldwin and Allison, 1983; Nocek et al., 1997; Owens et al., 1998; RAGFAR, 2007) were not consistent with ruminal fermentation responses when fructose was included in the diet in Chapters 2 and 7. Furthermore, key ruminal bacteria associated with ruminal acidosis in these models were not identified in heifers from the single challenge exposure in Chapter 2. This suggests that ruminal acidosis models in literature (Hungate, 1966; Baldwin and Allison, 1983; Nocek et al., 1997; Owens et al., 1998; RAGFAR, 2007) may not represent ruminal acidosis pathogenesis during all forms of ruminal acidosis and models may differ with substrate and length of substrate exposure. Other unknown factors and undefined micro-organisms are likely to be involved in ruminal acidosis (Chapter 4 and 5). In Chapter 8, the complete absence of propionate, butyrate, and

valerate, and high ammonia and lactate concentrations occurred in a heifer with acute clinical ruminal acidosis. These findings are consistent with observations in sheep studies (Ryan, 1965; Kezar and Church, 1979), but are the first such findings in cattle, further suggesting that current understandings of the pathogenesis of ruminal acidosis are incomplete.

Experimental induction of ruminal acidosis by voluntary feed intake is difficult to achieve (Dunlop and Hammond, 1965) and no standard method is used. Therefore, as a result of the marked changes in ruminal measures in the unadapted heifers fed a single challenge feed of grain at 0.8% dry matter intake (**DMI**) of bodyweight (**BW**), combined with fructose fed at 0.4% of BW in Chapter 2, a modification of this challenge model (1% DMI of BW grain and 0.2% of BW fructose) was fed in a longer term exposure study (Chapter 7) to evaluate the efficacy of feed additives to reduce the risk of ruminal acidosis. This challenge model produced acute clinical ruminal acidosis in a control heifer (Chapter 8) and changes in ruminal fermentation measures suggestive of ruminal acidosis in the remaining heifers (Chapter 7). This challenge model appears adequate to induce ruminal acidosis and evaluate interventions.

Lactate accumulation in the rumen is generally accepted to be associated with acute as opposed to subacute ruminal acidosis (Nagaraja and Titgemeyer, 2007). Lactate accumulated in concentrations substantially above the normal threshold of 5 m*M* (Nagaraja and Titgemeyer, 2007) in fructose supplemented heifers that did not show clinical signs of ruminal acidosis in Chapters 2 and 7. This may suggest lactate generation can occur without clinical signs of ruminal acidosis and is substrate driven, as opposed to being a result of stage in the pathogenesis or severity of ruminal acidosis. It is possible that lactate may not have a crucial role in the pathogenesis of ruminal acidosis and the involvement of other factors is required in conjunction with increased lactate concentrations to trigger clinical signs of ruminal acidosis.

The involvement of histamine in ruminal acidosis and possible links to lameness and laminitis remain unclear as histamine generation occurs after feeding and can accumulate in the rumen during acidotic conditions (Ahrens, 1967). Histamine was generated in grain, and grain and fructose-fed heifers, as well as histidine supplemented heifers in Chapter 2, grain-fed heifers in Chapter 6, and grain and fructose challenged heifers in Chapter 7, but translocation of histamine into the bloodstream was not evident

in these studies. Ruminal histamine concentration in the heifer with acute clinical ruminal acidosis on challenge day in Chapter 8 was greater than double that of heifers in her cohort and her gait was slow and tentative 24 h after challenge feeding (locomotion score 2.5). This supports the association between ruminal acidosis and lameness; however, it is unclear whether ruminal histamine or endogenous histamine release is essential to the pathogenesis of lameness. The bacteria *Allisonella histaminiformans* that utilizes histidine as its sole energy source (Garner et al., 2002) or other known bacteria capable of producing histamine as an end fermentation product were not identified in any heifers from the experiment in Chapter 2 reported in Chapter 4. This finding suggests that histamine generation may not be restricted to a small niche of bacteria.

Oxidative stress biomarkers were hypothesized to be influenced in carbohydrate challenged heifers (Chapters 3, 6, and 7). The lack of oxidative stress responses in these studies, suggests that oxidative stress is not associated with moderate rumen perturbations in the first 3.6 h after a single carbohydrate challenge. The increase in oxidative stress index in the control heifer with acute clinical ruminal acidosis suggests that oxidative stress responses occur in more severe rumen perturbations (Chapter 8). In support of this finding, oxidative stress responses have been reported in other ruminal acidosis (Wullepit et al., 2009) and starch-based studies (Gabai et al., 2004). Although oxidative stress responses were minimal in Chapters 3, 6, and 7, data from these studies contribute to the establishment of normal ranges for oxidative stress ranges in dairy cattle, which are currently not defined and measurement of oxidative stress responses in cattle fed feed additives is novel (Chapter 7).

Ruminal endotoxin concentrations were not influenced by carbohydrate challenge in Chapter 3, suggesting that endotoxin may not be involved in the initial phases of rumen perturbation 3.6 h after feeding. However, high concentrations of endotoxin in the rumen have been implicated in contributing to the nonspecific, acute phase response during subacute ruminal acidosis induced by feeding high levels of concentrate over several days (Gozho et al., 2007; Khafipour et al., 2009). Hence, the involvement of ruminal endotoxin in the pathogenesis of ruminal acidosis remains equivocal.

Understanding the complex and dynamic rumen microbial ecosystem (Fernando et al., 2010) is essential to the development of feed management practices that promote optimal production efficiency (de Menezes et al., 2011). Rapidly advancing molecular

techniques now allow a high-throughput, culture-independent exponential expansion of knowledge of the rumen ecosystem (McSweeney et al., 2007) which can be integrated with other animal measures. Combining the use of these new technologies with the foundations of rumen microbiology described by pioneers of this field such as Hungate et al. (1952) and Russell and Hespell (1981) allowed examination of bacterial community composition in both short and long term carbohydrate exposure studies when different substrates, feeding strategies, and supplement feeding amounts were imposed in Chapters 4 and 5. In these chapters, cattle appeared to have host specific bacterial community composition, but shared a common core microbiome, consistent with findings and suggestions (Jami and Mizrahi, 2010). Such host specificity in the rumen microbiome may explain the variation in ruminal fermentation measures and unique susceptibilities of individual cattle to ruminal acidosis in Chapter 7, and poses a challenge for developing ruminal acidosis control strategies. It also emphasizes the need for large numbers of cattle in experimental treatment groups (Nagaraja and Titgemeyer, 2007). A component of susceptibility to ruminal acidosis may be genetic, as breed of cattle had a greater influence than diet on bacterial profiles (Lee et al., 2012). Integration of animal genomes, rumen microbiomes, and ruminal fermentation measures in studies is warranted.

The Firmicutes and Bacteroidetes phyla and the *Prevotella* genus were the most predominant in relative abundance in the cattle from both Chapters 4 and 5, consistent with literature (Taijma et al., 2000; Kong et al., 2010), suggesting these form a large part of the core rumen microbiome. The candidate phylum, TM7, was the third most prevalent phylum in both these Chapters. Literature on bacteria from this phylum is limited and it possibly plays a role in the pathogenesis of ruminal acidosis, but further investigations are required to establish the role of this phylum.

Streptococcus bovis is often considered to be the causative agent in ruminal acidosis and an OTU that was closely related to *S. bovis* was identified in the grain + fructose heifers in Chapter 4. *S. bovis* appears to increase in the short term under certain feeding conditions, namely rapid changes to readily fermentable carbohydrates, and may contribute to the pathogenesis of ruminal acidosis (Hungate et al., 1952). However, proliferation of *S. bovis* does not appear to be essential for the aetiology of ruminal acidosis as a it has not always increased or been identified in grain-fed cattle (Taijma et

al., 2000; Golder et al., unpublished) and is not always the main cause of substantial ruminal acidity (Hungate, 1966). Numbers of *S. bovis* also decline in cattle adapted to a grain diet and it is outcompeted by other organisms such as *Lactobacillus* spp. by the release of bacteriocins and a decline in ruminal pH (Wells et al., 1997).

Although the relative abundance of the Veillonellaceae bacterial family was increased in the fructose-fed heifers in Chapter 4, *Megasphaera elsdenii* was not identified as key bacteria shifting in relative abundance in this study. The relative abundance of the Lactobacillaceae was not influenced despite increases in lactate concentrations in the fructose-fed heifers. Similarly, the Lactobacillaceae were only present in very low relative abundances in cattle from both Chapters 4 and 5.

Bacterial communities were associated with ruminal fermentation measures in Chapters 4 and 5, consistent with other studies (Hernandez-Sanabria et al., 2010; Carberry et al., 2012). Bacterial communities from the fructose-fed heifers in Chapter 2 were associated with lactate and butyrate concentrations. Grain-fed heifers had ruminal bacterial communities associated with ammonia, valerate, and histamine concentrations. In Chapter 5, bacterial communities from the cows identified with the highest acidosis eigenvalues, those fed control diets with 16 kg of dry matter (**DM**) of total supplement/cow per d that comprised crushed wheat and pasture silage were positively related to propionate concentration; while, those fed control or partial mixed ration diets at 8 and 10 kg of total supplement/cow per d had low acidosis eigenvalues. Amount of feeding total supplements, ammonia, butyrate, valerate, and propionate concentrations had large impacts on bacterial community composition (Chapter 5). These studies emphasize the importance of understanding the dynamic interactions within the rumen in response to substrates and feeding systems.

Operational taxonomic units that were identified as significantly changing in Chapter 4 were more diverse in the grain + fructose, grain + histidine, and grain + fructose + histidine heifers, compared to those identified from the control and grain groups. Diversity of bacteria was highest cows fed a partial mixed ration at 10 kg of total supplement/cow per d and lowest in cows fed wheat and pasture silage (controls) at 16 kg of DM of total supplement/cow per d.

Ruminal acidosis control involves many aspects of feed management and can be a balancing act between optimum feed efficiency and animal welfare (Chapter 1). Acidosis eigenvalues showed that ruminal acidosis was reduced in cows fed a partial mixed ration compared to cows that were component-fed an isoenergetic diet of crushed wheat in the milking parlor and pasture silage on the pasture and ruminal acidosis increased with supplement feeding amount (Chapter 5).

Feeding behavior may be an important means by which cattle reduce the risk of ruminal acidosis, with reduced feed intake being observed in heifers fed grain + fructose and grain + fructose + histidine in Chapter 2 and heifers fed monensin + live yeast in Chapter 7. Control cows fed 16 kg of total supplement/cow per d in Chapter 5 also appeared to lower their intake of crushed wheat and increase their intake of pasture silage. Allowing cattle access to *ad libitum* sources of physically effective fiber may allow them to reduce their risk of ruminal acidosis. The large variability in susceptibility to ruminal acidosis observed in Chapter 7 suggests that feed management options may offer a means for cattle to control their intake.

Ruminal responses to feed additives used to control ruminal acidosis varied in the literature and *in vivo* data to support proposed modes of action of feed additives that were based on *in vitro* ruminal responses were limited (Chapter 1). The review showed that additive effects of feed additives were largely unknown and there was a need to develop prudent use strategies for feed additive agents used to control ruminal acidosis. These matters were addressed in Chapters 6 and 7. Feed additives were hypothesized to reduce the risk of ruminal acidosis based on ruminal, inflammation, and oxidative stress measures in Chapters 6 and 7. Further, feed additive combinations were hypothesized to have synergistic effects on ruminal responses in Chapter 6. The feed additives evaluated in both these chapters appeared to modify the rumen by different mechanisms; however, whether these manipulations were positive or negative attributes was not always clear (Chapters 6 and 7). This highlights the continued need for research into the pathogenesis of ruminal acidosis and the complexity of this disorder. Ruminal fermentation measures were not always consistent with proposed modes of action of feed additives based on *in vitro* studies and suggest that feed additive responses measured *in vitro* may not correlate well with those *in vivo*. The findings also emphasize the importance of *in vivo* studies, in general.

The effects of feed additive combinations were not primarily synergistic; however, they may be synergistic under other experimental conditions. Despite mean beneficial effects of feed additives on ruminal fermentation responses to the grain and fructose challenge in Chapter 7, none of the feed additives were able to stabilize the rumen in all heifers within their group. This could suggest that perhaps no feed additive or feed additive combination will be capable of controlling ruminal acidosis under extreme challenge or in cattle with a high risk of ruminal acidosis. It is clear that animal variation in ruminal fermentation responses to substrate challenge and feed additives pose a challenge for feed additive control agents.

Although the subtherapeutic use of antibiotics for animal production is banned in regions such as the European Union, antibiotic control agents such as virginiamycin were effective ruminal acidosis control agents in Chapter 7 and monensin showed potential in Chapter 6. Careful consideration for both human and animal welfare should be given when evaluating the use of antibiotics in the animal industries. Prudent use strategies for antibiotic usage in the animal industries need to be implemented to ensure animal welfare and ruminal acidosis control while alternative cost effective ruminal acidosis control agents and management strategies are developed.

CONCLUSION

This thesis achieved its aim of increasing our understandings of the pathogenesis of ruminal acidosis and control strategies for ruminal acidosis in dairy cattle. It has identified a number of potential areas for further research into this complex nutritional and metabolic disorder. The findings of this thesis can help producers, nutritionists, veterinarians, and researchers with management of ruminal acidosis.

The key findings from this thesis are: (1) That the ruminal acidosis model by Bramley et al. (2008) adequately defines starch-based ruminal acidosis but is not suitable for diagnosis of sugar-based ruminal acidosis. Valerate and propionate are good diagnostic measures of ruminal acidosis; (2) That feed substrates have an important influence on the risk of ruminal acidosis and the rumen microbiome. Sugars increase the risk of ruminal acidosis, compared to grain fed when physically effective fiber is inadequate; (3) The involvement of ruminal histamine and endotoxin concentrations and plasma oxidative stress responses in the pathogenesis of ruminal acidosis remains unclear and

requires further investigation; (4) That individual cattle were observed to have host specific bacterial community composition that shares a common microbiome; (5) That feeding systems and feed additives have an important role in ruminal acidosis control, but require refinement and integrated prudent use strategies, respectively.

The key understandings developed from this thesis are that ruminal acidosis appears to occur along a continuum of hydrogen sequestration and clinical severity. Cattle are not always readily classified within definitions defined in literature of normal ruminal conditions, or subacute and acute ruminal acidosis. I recommend that ruminal acidosis be defined for different substrates and for each ruminal fluid collection technique. Diagnosis of ruminal acidosis is best based on a combination of ruminal pH, fermentation products, clinical signs, feeding management history, and production data when possible. That the increased risk of acidosis when sugars are fed could have implications for producers feeding brassicas, root crops, molasses, pastures with high water-soluble carbohydrate content, crystalline sugar, or whey if diets are not balanced with adequate physically effective fiber. Individual cattle have unique susceptibilities to ruminal acidosis and differing responses to dietary changes that have implications for ruminal acidosis control strategies. Feeding systems and feed additives may not be capable of controlling ruminal acidosis under extreme challenge or in cattle with a high risk of ruminal acidosis.

Examining genetic by environment interactions, development of ruminal acidosis models for different feed substrates, further examining modes of action of feed additives, and characterization of a greater proportion of microbiota in the rumen are only a few potential areas for continued research in this field.

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