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Exploring the potential of phytogenic compounds to modulate ruminal fermentation and mitigate subacute ruminal acidosis in dairy cattle

PhD thesis submitted for the fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (PhD)

University of Veterinary Medicine Vienna

submitted by

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Vienna, May 2023

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List of original publications included in the PhD thesis

Ricci, S.; **Rivera-Chacon, R.**; Petri, R.M.; Sener-Aydemir, A.; Sharma, S.; Reisinger, N.; Zebeli, Q.; Castillo-Lopez, E. Supplementation with phytogenic compounds modulates salivation and salivary physico-chemical composition in cattle fed a high-concentrate diet. Front Physiol. 2021; 12:645529.

Rivera-Chacon, R.; Castillo-Lopez, E.; Ricci, S.; Petri, R.M.; Reisinger, N.; Zebeli, Q. Supplementing a phytogenic feed additive modulates the risk of subacute rumen acidosis, rumen fermentation and systemic inflammation in cattle fed acidogenic diets. Animals (Basel). 2022; 12(9):1201

Rivera-Chacon, R.; Ricci, S.; Petri, R.M.; Haselmann, A; Reisinger, N.; Zebeli, Q.; Castillo-Lopez, E. Effect of duration of high-grain feeding on chewing, feeding behavior, and salivary composition in cows with or without a phytogenic feed supplement. Animals (Basel). 2022; 12(15):2001

List of original publications published within the PhD work (not included in the thesis)

Castillo-Lopez, E.; Petri, R.M.; Ricci, S.; **Rivera-Chacon, R.**; Sener-Aydemir, A.; Sharma, S.; Reisinger, N.; Zebeli, Q. Dynamic changes in salivation, salivary composition, and rumen fermentation associated with duration of high-grain feeding in cows. J Dairy Sci. 2021; 104(4):4875-4892.

Castillo-Lopez, E.; **Rivera-Chacon, R**.; Ricci, S.; Petri, R.M.; Reisinger, N.; Zebeli, Q. Short-term screening of multiple phytogenic compounds for their potential to modulate chewing behavior, ruminal fermentation profile, and pH in cattle fed grain-rich diets. J. Dairy Sci. 2021; 104(4):4271-4289.

Castillo-Lopez, E.; **Rivera-Chacon, R.**; Ricci, S.; Reisinger, N.; Zebeli, Q. Changes in fermentation profile of the reticulorumen and hindgut, and nutrient digestion in dry cows fed concentrate-rich diets supplemented with a phytogenic feed additive. J. Dairy Sci. 2022; 105(7):5747-5760.

Pacífico, C.; Ricci, S.; Sajovitz, F.; Castillo-Lopez, E.; **Rivera-Chacon, R.**; Petri, R.M.; Zebeli, Q.; Reisinger, N.; Kreuzer-Redmer, S. Bovine rumen epithelial miRNA-mRNA dynamics reveals post-transcriptional regulation of gene expression upon transition to high-grain feeding and phytogenic supplementation. Genomics. 2022; 110333.

Ricci, S.; Pacífico, C.; Castillo-Lopez, E.; **Rivera-Chacon, R.**; Schwartz-Zimmermann, H.E.; Reisinger, N.; Berthiller, F.; Zebeli, Q.; Petri, R.M. Progressive microbial adaptation of the bovine rumen and hindgut in response to a step-wise increase in dietary starch and the influence of phytogenic supplementation. Front Microbiol. 2022; 13:920427.

Ricci, S.; Petri, R.M.; Pacífico, C.; Castillo-Lopez, E.; **Rivera-Chacon, R.**; Sener-Aydemir, A.; Reisinger, N.; Zebeli, Q.; Kreuzer-Redmer, S. Characterization of presence and activity of microRNAs in the rumen of cattle hints at possible host-microbiota cross-talk mechanism. Sci Rep. 2022; 12(1):13812.

Castillo-Lopez, E.; **Rivera-Chacon, R.**; Ricci, S.; Khorrami, B.; Haselmann, A.; Reisinger, N.; Zebeli, Q. Dynamics of chewing and eating behavior, lying behavior, and salivary characteristics associated with duration of high grain feeding in cows with or with no phytogenic supplement. Appl. Anim. Behav. Sci. 2023; 261:105877.

Castillo-Lopez, E.; Pacífico, C.; Sener-Aydemir, A.; Hummel, K.; Nöbauer, K.; Ricci, S.; **Rivera-Chacon, R.**; Reisinger, N.; Razzazi-Fazeli, E.; Zebeli, Q.; Kreuzer-Redmer, S. Diet and phytogenic supplementation substantially modulate the salivary proteome in dairy cows. J. Proteom. 2023; 104795

Dedication

To my parents Melina and Ysaul who always supported me during this entire journey.

Acknowledgments

First, I would start thanking Univ. -Prof Dr.sc.agr. Qendrim Zebeli for his guidance from the start of my PhD. He was extremely patient explaining me how to improve my writing and presentation skills.

I would like to thank PhD Ezequias Castillo-Lopez for his countless explanations about statistical analysis, conducting animal experiments, writing manuscripts, preparing oral and poster presentations. I hope all the time he invested in me is worth it.

I would like to also thank PhD Renee M. Petri. Her insights during the conduction of the experiment as well as her recommendations during the manuscript preparations are highly appreciated.

I would like to thank PhD Andreas Haselmann for his help on the data processing related with lying behavior. I would also like to thank PhD Cátia Pacífico, and my dear PhD sister Sara Ricci, both helped me immensely during my PhD work and I learned a lot from them.

I am also thankful of having had the support of Lab members including Anita Dockner, Manfred Hollmann, Sabine Leiner, Suchitra Sharma, and Arife Sener-Aydemir. I would also like to thank two diploma students Boris Sariciftci and Silvia Nöbauer for their help during laboratory work.

I cannot finish this section without thanking Univ.-Prof. Dr.med.vet. Reinhold Erben as well for his supervision, guideline, and criticism during my interim evaluations; PhD Nicole Reisinger for her input on the manuscripts and abstract preparations and Mrs. Regina Kadi for her constant help navigating with the documentation requirements and assisting me for the courses enrolling every semester.

I am grateful to the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock and BIOMIN Holding GmbH for providing with the necessary funding to conduct and complete this PhD project.

Finally, I would like to thank my fellow graduate students and undergraduate students that helped me during the intensive sampling days at the farm including Anna Kaltenegger, Mubarik Mahmood, Felipe Penagos-Tabares, Claudia Lang, Parisa Kheirandish, Floriana Sajovitz and Svenja Johanns. Additionally, I would like to express my gratitude to Thomas Enzinger and Elmar Draxler at the VetFarm of the Vetmeduni in Pottenstein and to visiting scholars PhD Behzad Khorrami and PhD Elsayed Mickdam.

CONTRIBUTION OF AUTHORS

Dynamic changes in salivation, salivary composition, and rumen fermentation associated with duration of high-grain feeding in cows. Journal of Dairy Science. 104(4):4875-4892. Impact factor: 4.225

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R. Rivera-Chacon	Conducting animal experiment. Laboratory work.
A. Sener-Aydemir	Laboratory work.
S. Sharma	Laboratory work.
N. Reisinger	Funding acquisition, conceptualization, and study design.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.

Short-term screening of multiple phytogenic compounds for their potential to modulate chewing behavior, ruminal fermentation profile, and pH in cattle fed grain-rich diets. Journal of Dairy Science. 104(4):4271-4289.
Impact factor: 4.225

E. Castillo-Lopez	Supervising and conducting animal experiment, statistical analysis, writing the manuscript.
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S. Ricci	Conducting animal experiment.
R.M. Petri	Funding acquisition, conceptualization, and study design, supervising and conducting animal experiment.
N. Reisinger	Funding acquisition, conceptualization, and study design.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.

Supplementation with phytogenic compounds modulates salivation and salivary physico-chemical composition in cattle fed a high-concentrate diet. Front in Physiology. 2021; 12:645529.
Impact factor: 4.755

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Impact factor: 4.225

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Bovine rumen epithelial miRNA-mRNA dynamics reveals post-transcriptional regulation of gene expression upon transition to high-grain feeding and phytogenic supplementation. Genomics. 2022; 110333.
Impact factor: 5.736

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Supplementing a phytogenic feed additive modulates the risk of subacute rumen acidosis, rumen fermentation and systemic inflammation in cattle fed acidogenic diets. Animals (Basel). 2022; 12(9):1201
Impact factor: 2.752

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R.M. Petri	Funding acquisition, conceptualization, and study design.
N. Reisinger	Funding acquisition, conceptualization, and study design.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.

Progressive microbial adaptation of the bovine rumen and hindgut in response to a step-wise increase in dietary starch and the influence of phytogenic supplementation. Frontiers in Microbiology. 2022; 13:920427.
Impact factor: 6.064

S. Ricci	Conducting animal experiment, laboratory work, statistical analysis, bioinformatics, writing the manuscript.
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R.M. Petri	Funding acquisition, conceptualization, and study design.

Effect of duration of high-grain feeding on chewing, feeding behavior, and salivary composition in cows with or without a phytogenic feed supplement. Animals (Basel). 2022; 12(15):2001
Impact factor: 2.752

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N. Reisinger	Funding acquisition, conceptualization, and study design.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.
E. Castillo-Lopez	Supervising and conducting animal experiment, statistical analysis.

Characterization of presence and activity of microRNAs in the rumen of cattle hints at possible host-microbiota cross-talk mechanism. Sci Rep. 2022; 12(1):13812.
Impact factor: 4.996

S. Ricci	Conducting animal experiment, laboratory work, statistical analysis, bioinformatics, writing the manuscript. Developing the study design for miRNA-microbiota analyses.
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Dynamics of chewing and eating behavior, lying behavior, and salivary characteristics associated with duration of high grain feeding in cows with or with no phytogenic supplement. Appl. Anim. Behav. Sci. 2023; 261:105877.
Impact factor: 2.448

E. Castillo-Lopez	Supervising and conducting animal experiment, statistical analysis writing the manuscript.
R. Rivera-Chacon	Conducting animal experiment, laboratory work, statistical analysis.
S. Ricci	Conducting animal experiment.
B. Khorrami	Conducting animal experiment.
A. Haselmann	Statistical analysis
N. Reisinger	Funding acquisition, conceptualization, and study design.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.

Diet and phytogenic supplementation substantially modulate the salivary proteome in dairy cows. J. Proteom. 2023; 104795.
Impact factor: 3.537

E. Castillo-Lopez	Supervising and conducting animal experiment, statistical analysis, data analysis, interpretation, writing the manuscript.
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K. Hummel	Laboratory work.
K. Nöbauer	Laboratory work.
S. Ricci	Conducting animal experiment, bioinformatics.
R. Rivera-Chacon	Conducting animal experiment, samples preparation.
N. Reisinger	Funding acquisition, conceptualization, and study design.
E. Razzazi-Fazeli	Laboratory work.
Q. Zebeli	Funding acquisition, conceptualization, and study design, supervising animal experiment.
S. Kreuzer-Redmer	Conceptualization, study design, data analysis and interpretation

Declaration

I confirm that I have followed the rules of good scientific practice in all respects.

Contents

ABBREVIATIONS

NEB	Negative energy balance
FAO	The food and agriculture organization
NRC	The national research council
Mcal/kg	Mega calories per kilogram
DM	Dry matter
DMI	Dry matter intake
MJ/kg	Mega joule per kilogram
NEL	Net energy of lactation
GfE	Gesellschaft für Ernährungsphysiologie
SCFA	Short chain fatty acids
SARA	Subacute ruminal acidosis
peNDF	Physically effective neutral detergent fiber
LPS	Lipopolysaccharide
HC	High-concentrate
SEM	Standard error of the mean
TMR	Total mixed ration
h/d	Hours per day
g/g	Gram per gram
pK_a	Acidity constant
PHY	Phytogenic compounds
PAS	Phytogenic additive supplementation
CO_2	Carbon dioxide
CoA	Coenzyme A

SAA	Serum amyloid A
Hp	Haptoglobin
GLDH	Glutamate dehydrogenase
GGT	Gamma-glutamyl transferase
ALP	Alkaline phosphatase
AST	Aspartate aminotransferase

1. SUMMARY

1.1. English

Subacute ruminal acidosis (SARA) is a health disorder commonly reported in cows fed acidogenic diets like under intensive dairy farm operations. Typical acidogenic diets are those rich in starchy concentrates. Cows normally experience SARA when the mean ruminal pH descends intermittently through several hours below 5.8 during the day. Furthermore, SARA is associated with inflammation and liver tissue damage. In this regard, phytogenic compounds are herbal-derived products rich in secondary plant metabolites like essential oils. These compounds have lately received renewed interests in the animal production industry as feed additives replacing various conventional feed additives. In dairy cattle, there is an interest to use phytogenic additives as a tool to alleviate the negative impacts of acidogenic diets including changes in rumen buffering, rumen acidosis and dysbiosis as well as the related systemic effects such as inflammation.

The first main objective of this thesis was to evaluate whether the supplementation with single phytogenic compounds (PHY) will influence saliva composition and dynamics in cattle fed acidogenic diets. The second main objective was to assess the duration of high-concentrate feeding challenge on ruminal fermentation, inflammation, salivary composition, eating, chewing and lying behavior in cattle fed a phytogenic additive supplementation (PAS). For this purpose, two separate experiments were conducted. The first one evaluated 9 different PHY supplemented separately in a 65% concentrate diet (DM basis), during a short-term period of 4 hours. Whereas the second one focused on evaluating the dietary supplementation of a PAS including L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) in a diet with the same concentrate inclusion level as in the first study,

but fed during 4 consecutive weeks. For experiment one, multiple samples were collected daily to evaluate short-term responses, whereas for experiment two the sampling was done weekly.

The first experiment demonstrated that certain PHY hold potential to influence saliva and feed ensalivation to a different extent than others. For example, compared to a control diet without PHY, supplementation of thyme oil increased unstimulated salivary buffer capacity. In addition, garlic oil increased salivary osmolality and phosphate concentration; thymol and capsaicin increased the buffer capacity; gentian root increased buffer capacity of the saliva secretion during eating. Thymol and ginger increased salivation rate, while capsaicin increased the saliva flow.

The second experiment proved that we were able to induce SARA in cows, but also demonstrated that during weeks 3 and 4 of high-concentrate feeding, the PAS reduced the risk of SARA. Phytogenic supplementation increased acetate, butyrate, isobutyrate and isovalerate concentration but decreased propionate in week 2 compared to control with no changes in mean ruminal pH or the time that ruminal pH was below 5.8, as well as the acetate to propionate ratio on the same week. Inflammation response seemed to be suppressed by PAS during weeks 3 and 4 as indicated by reduced haptoglobin and serum amyloid A. Feed ensalivation was not influenced by PAS, only by high-concentrate diet compared to a forage only diet. Rumination time decreased as high-concentrate feeding weeks progressed. Also, PAS increased sorting behavior in favor of physically effective fiber on week 2 and increased saliva pH in week 4. Additionally, cows ate with a greater pace (meal size and eating rate) with advanced duration of high-concentrate diet, which may explain the increased risk of acidosis. Nevertheless, PAS reduced meal size in week 2, which may explain the reduced risk of SARA for cows on the following weeks within the trial. To conclude, the experiments conducted are evidence that supplementation of PAS containing L-menthol, thymol, eugenol, mint oil and cloves powder

hold potential to positively modulate saliva and ruminal fermentation in dairy cattle challenged with a long-term high-concentrate diet.

1.2. German

Die subakute Pansenazidose (SARA) ist eine Erkrankung, die häufig bei Kühen auftritt, die mit acidogenen Rationen gefüttert werden, wie es in intensiven Milchproduktionssystemen gängig ist. Typische acidogene Rationen sind solche, die reich an stärkehaltigen Kraftfuttern sind. Bei Kühen tritt SARA normalerweise auf, wenn der durchschnittliche pH-Wert im Pansen über mehrere Stunden des Tages unter 5,8 sinkt. Außerdem wird SARA mit Entzündungen und Lebergewebeschäden in Verbindung gebracht. Phytogene Verbindungen sind pflanzliche Stoffe, die reich an sekundären Pflanzenmetaboliten wie ätherischen Ölen oder anderen Komponenten sind und in letzter Zeit als Futtermittelzusatzstoffe, die verschiedene konventionelle Futtermittelzusatzstoffe ersetzen, wieder an Interesse in der Tierproduktion gewonnen haben. Beim Milchvieh besteht ein Interesse daran, phytogene Verbindungen zur Linderung negativer Auswirkungen von acidogenen Rationen bei Milchkühen einzusetzen, einschließlich Veränderungen der Pansenpufferung, Pansenazidose und Dysbiose sowie der damit verbundenen systemischen Auswirkungen wie beispielsweise Entzündungsgeschehen.

Das erste Hauptziel dieser Arbeit bestand darin, zu untersuchen, ob die Ergänzung mit einzelnen phytogenen Verbindungen (PHY) die Speichelzusammensetzung und -dynamik bei Rindern beeinflusst, die mit acidogenen Rationen gefüttert werden. Das zweite Hauptziel bestand darin, die Dauer einer kraftfutterreichen Fütterung auf die Pansenfermentation, Entzündungen, Speichelzusammensetzung, Fress-, Kau- und Liegeverhalten bei Rindern zu untersuchen, die mit einem phytogenen Zusatzstoff (PAS) gefüttert wurden. Zu diesem Zweck wurden zwei getrennte Versuche durchgeführt. Im ersten Versuch wurden Kühe über einen kurzen Zeitraum von 4 Stunden untersucht, die eine Ration mit 65% Kraftfutter erhielten, das

mit 9 verschiedenen PHY ergänzt wurde. Der zweite Versuch konzentrierte sich auf die Bewertung der Supplementierung mit einer PAS-Mischung aus L-Menthol, Thymol, Eugenol, Minzöl (Mentha arvensis) und Gewürznelkenpulver (Syzygium aromaticum) bei gleichem Kraftfutteranteil über vier aufeinanderfolgende Wochen. Im ersten Versuch wurden täglich mehrere Proben entnommen, um die kurzfristigen Reaktionen zu bewerten, während die Probennahme im zweiten Versuch wöchentlich erfolgte.

Unser erster Versuch zeigte, dass bestimmte PHY das Potenzial haben, den Speichel und die Futtereinspeichelung in anderem Ausmaß zu beeinflussen als andere. Beispielsweise erhöhte die Zugabe von Thymianöl die Pufferkapazität unstimulierten Speichels. Des Weiteren erhöhte Knoblauchöl die Speichelosmolalität und die Phosphatkonzentration; Thymol und Capsaicin erhöhten die Pufferkapazität; Enzianwurzel erhöhte die Pufferkapazität der Speichelsekretion während des Fressens. Thymol und Ingwer erhöhten die Speichelflussrate, während Capsaicin den Speichelfluss erhöhte.

Unser zweiter Versuch zeigte, dass wir in der Lage waren, SARA bei Kühen auszulösen, aber ebenso, dass die PAS-Supplementierung das SARA-Risiko in den Wochen 3 und 4 der kraftfutterreichen Fütterung reduzierte. Die phytogene Supplementierung erhöhte die Pansenfermentationsparameter einschließlich Acetat, Butyrat, Isobutyrat und Isovalerat, verringerte aber Propionat im Vergleich zur Kontrolle in Woche 2, ohne dass sich der mittlere Pansen-pH-Wert oder die Dauer, in der der ruminale pH-Wert unter 5,8 blieb, sowie das Acetat-Propionat-Verhältnis in derselben Woche änderten. Die Entzündungsreaktion, einschließlich Haptoglobin und Serum Amyloid A, schien durch die PAS-Gabe in den Wochen 3 und 4 unterdrückt zu werden. Die Futtereinspeichelung wurde durch PAS nicht beeinflusst, nur durch die kraftfuttereiche Fütterung im Vergleich zur reinen Fütterung mit Raufutter. Die Wiederkauzeit verringerte sich über die Zeit mit fortschreitender Fütterung der Ration mit

hohem Kraftfutteranteil. Außerdem steigerte PAS das Sortierverhalten zugunsten physikalisch effektiver Faser in Woche 2 und erhöhte den Speichel-pH-Wert in Woche 4. Zusätzlich fraßen die Kühe mit zunehmender Dauer der kraftfutterreichen Fütterung schneller (Mahlzeitengröße und Fressgeschwindigkeit), was das erhöhte Azidoserisiko erklären könnte. Nichtsdestotrotz reduzierte PHY die Mahlzeitgröße in Woche 2, was das geringere SARA-Risiko der Kühe in den darauffolgenden Wochen erklären könnte. Zusammenfassend kann gesagt werden, dass die durchgeführten Experimente belegen, dass die Supplementierung mit PAS wie L-Menthol, Thymol, Eugenol, Minzöl und Gewürznelkenpulver das Potenzial hat, den Speichel und die Pansenfermentation bei Milchkühen, die über einen längeren Zeitraum kraftfutterreich gefüttert werden, positiv zu beeinflussen.

2. INTRODUCTION

2.1. Milk production

Cattle domestication evidence shows its occurrence between 10,300-10,800 years ago (Helmer et al., 2005; Vigne et al., 2011). Since then, selection and crossbreeding allowed the development of different specialized breeds developed with the specific purpose of providing milk or meat for human consumption (O'Neill et al., 2010). The Holstein breed, for instance, originated in 1790 (Bewick, 1807). During the last century, dairy production systems in the world have evolved towards higher volumes of milk, highly efficient dairy cows, greater daily dry matter intakes (DMI) and energy-denser diets. As essential part of the milk industry, diet formulation in dairy cattle plays a key role to reach the true genetic potential in terms of milk yield.

Milk production in the world has evolved exponentially in the last 50 years. Since 1980, world milk production has increased by nearly 30%. In the USA, average milk production per cow has increased from 2500 to 10150 kg from 1945 to 2014, respectively (Brit et al., 2017); meanwhile in Austria, a similar trend occurred with an increase from 5500 to 8000 kg from 1988 to 2006, respectively (Knaus et al., 2008). In the same direction, Alexandratos and Bruinsma (2012) estimated that by 2067 the average dairy products consumption will increase up to 119 kg per person compared to 87 during 2017. More specifically, calculations done by Brit et al. (2017) estimate that by 2067 the average milk production per cow will nearly duplicate in the USA from 10000 to 18000 kg per year. If this will hold true, this increase of milk production will constitute an enormous challenge for the cow physiology to meet its nutrient demands in a healthy and sustainable way.

A common strategy to meet the energy demands is to increase the concentration of easily fermentable carbohydrate sources (corn, wheat, barley, triticale) and using highly digestible feeds such as pellets or extruded feed. Unfortunately, the progress in milk production potential

of cows came with a trade-off with cow health. In fact, metabolic issues often arise, mainly due to the high energy and nutrient demands for milk production, which are higher and difficult to meet especially at the beginning of lactation, often leading to a negative energy balance (NEB) (Schingoethe, 2017; Overton et al., 2017). The NEB provokes massive tissue mobilization that can be partly counteracted by increasing DMI in cows after parturition (McCarthy et al., 2016) or by providing nutrient denser or highly digestible diets.

2.2. Nutrient requirements in dairy cows

Different energy requirement systems have been developed in the world depending on the geographic location and climate conditions. With the increase of milk production, dairy cows have high requirements in energy and nutrients to support the maintenance and most importantly to sustain the milk yield level. As an example, according to National Research Council (NRC) guidelines (2001), a cow with 680 kg body weight that produces around 55 kg of milk with 3.5% fat and 3.2% protein will require a DMI of approximately 30 kg, with 51 mega calories (Mcal) of net energy of lactation (NEL) and approximately 3.5 kg of metabolizable protein per day. This level is very close to the German system that estimates 7.1 MJ NEL/kg of DM with similar high DMI of the cows (GfE, 2009). To meet this level of NEL, the diet of dairy cows should contain highly palatable ingredients of high energy density such as starch and sugar-rich ingredients. As an example, corn silage of high quality, with a high starch content can reach a level of 6.6 to 7.0 MJ NEL/kg DM, whereas concentrates or grains can be around 8 MJ NEL/kg DM. Thus, the level of concentrate in the diets of early lactating cows should contain at least 50% in DM to reach that assumed level of 7.1 MJ NEL/kg DM according to GfE (2009). Certainly, the lower the energy content of the forages, the higher is the required level of concentrate in the diet to reach the necessary energy content for dairy cows. Yet, the concentrate inclusion in the diet occurs at the expense of forages, which are the source of physically effective fiber (peNDF) of the diet (Klevenhusen and Zebeli, 2021).

An important issue in dairy cattle nutrition includes the NEB, a common metabolic condition in fresh cows after parturition that can also persist for a few months (Doepel et al., 2002; Ferris et al., 2002). The cows with NEB usually have also a decreased DMI, and this is the reason why diets of early lactating cows in general are richer with highly fermentable starch. Early lactating cows generally experience enormous metabolic stress including massive body loss and have a weaker immune capacity (Esposito et al., 2014). Furthermore, the rumen needs to adapt to a new starch-denser diet, and its papillae will gradually grow in surface area to uptake and process the massive build-up of short chain fatty acids (SCFA) (Nordlund et al., 1995). All this makes early lactation cows more susceptible to experience subacute ruminal acidosis (SARA) insults, and this aspect will be explained later in more detail. Briefly, SARA occurs when rumen pH reaches values of 5.8 for over 5 hours per day (Zebeli et al., 2008), mainly in cows fed high grain diets because of imbalances between microbial activity and host absorption of SCFA. This occurs because the conversion of carbohydrates to SCFA exceeds the absorption rate and buffering outflow capacity of the rumen (Plaizier et al., 2008) and it can also be related with reduction in feed intake (Gozho et al., 2005).

In order to prevent SARA, the diet of early lactating cows should contain a healthy balance between starch and peNDF. The latter combines the physical characteristics of the diet such as particle size with the chemical properties such as fiber (Mertens, 1997). Yet, with the level of concentrate and starch of the diets commonly fed to dairy cattle, it is hard to meet the requirements of dairy cows for peNDF. For instance, Khorrami et al (2021) revealed that 15-18% peNDF > 8mm is a safe level to prevent SARA through diet formulation, as long as starch is kept within the range of 20-25%. However, the level of starch in the diets of high-yielding dairy cows during most of the lactation is typically above 25%. This again increases the odds of ruminal dysfermentation and development of SARA.

2.3. Rumen fermentation

The rumen fermentation is a complex process that consists in the degradation of fiber, starch, sugars and protein to generate carbon dioxide, methane, ammonia and SCFA, through the activity of the rumen microbes. The main SCFA in the rumen include acetic, propionic, and butyric acid with a molar proportion in the range of 45 to 70%, 15 to 40%, and 5 to 20%, respectively (Bergman, 1990; Kristensen et al., 1996; Penner et al., 2009; Udén, 2010). Higher percentages of propionate and butyrate, at the expense of acetate, are observed when diets contain high amounts of fermentable carbohydrates. In the ruminal fluid, these three SCFA make a cumulative concentration from 60 to 150 mmol/L, representing approximately 95% of all fermentation acids (Bergman, 1990). Following simple stoichiometry, 1 mol of glucose can be converted into 2 mol of acetate, or 2 mol of propionate, or 1 mol of butyrate (Baldwin, 1995; Bannink et al., 2006), which are used as various energy sources from the host. Therefore, in terms of meeting the energy requirements, high SCFA concentration in the rumen is a desirable outcome in ruminants. More in detail, the main product of glycolysis is pyruvate. Among the two main routes of pyruvate to acetate conversions, the pyruvate-formate lyase system produces formate and Acetyl-CoA as immediate metabolites (Hungate, 1966). Whereas the second one is pyruvate-ferrodoxin oxidoreductase, synthesizing reduced ferredoxin, CO_2 and acetyl CoA from pyruvate (Baldwin and Allison, 1983). Pyruvate conversion to propionate is part of the dicarboxylic acid pathway (Baldwin, 1965) with the use of three enzymes: phosphoenolpyruvate carboxykinase, pyruvate carboxylase, and methylmalonyl-CoA carboxyltransferase. Alternatively, there is one metabolic pathway of pyruvate conversion, denominated as the acrylate pathway, which includes lactate formation and its reduction to propionyl-CoA and it's knowingly performed by *Megasphaera elsdenii* and *Bacteroides ruminicola* (Baldwin and Milligan, 1964; Counotte et al., 1981). However, this happens under normal rumen fermentation conditions and when rumen pH doesn't decrease below 5. For

instance, under normal conditions and according to Counotte et al. (1983), more than 80% of the lactate in the rumen is fermented by *M. elsdenii* to SCFA. Going into specifics, only 16% of L-lactate is fermented to propionate, whereas 75% of D-Lactate is converted into propionate. Nevertheless, rumen fermentation faces some challenges with high-concentrate diets due to the saturation of protons in the rumen (Gäbel and Aschenbach, 2006).

During SARA conditions, there is an increased SCFA (and to a lower extent lactate) due to increased fermentation rate in the rumen that can cause acid accumulation. When lactate accumulates in the rumen it decreases rumen pH more rapidly, due to its low dissociation constant (pKa) compared with other SCFA (3.9 vs. 4.8) (Aschenbach et al., 2011). The other SCFA shift to the undissociated form rapidly due to their higher pKa compared with lactate. Lactate, on the other hand, decreases rumen pH by 1 unit below in a balanced SCFA rumen (Stone, 2004).

Literature has demonstrated that 50-85% of SCFA produced in the rumen are directly absorbed across the reticulo-rumen wall allowing only the remaining 15-50% to pass to the distal part of the digestive tract (Aschenbach et al., 2011). Recent research has demonstrated that rumen microbiome as well as papillae structure need 2 to 3 weeks to fully adapt to the rich-grain diets and therefore to optimize the metabolization and absorption of the high concentration of SCFA (Hook et al., 2011; Saleem et al., 2012; Ghaffari et al., 2017). Nevertheless, it seems that cows can adapt to acidogenic diets as demonstrated by Castillo-Lopez et al. (2021a). In fact, in an experiment in which cows were fed for over 3 weeks a high grain diet, total SCFA decreased, while propionate increased in the rumen compared with the initiation of the high grain feeding, indicating that with a longer high-concentrate (HC) challenge there is an enhancement in SCFA absorption. The increase in the absorption rate may be explained by the increase of ruminal

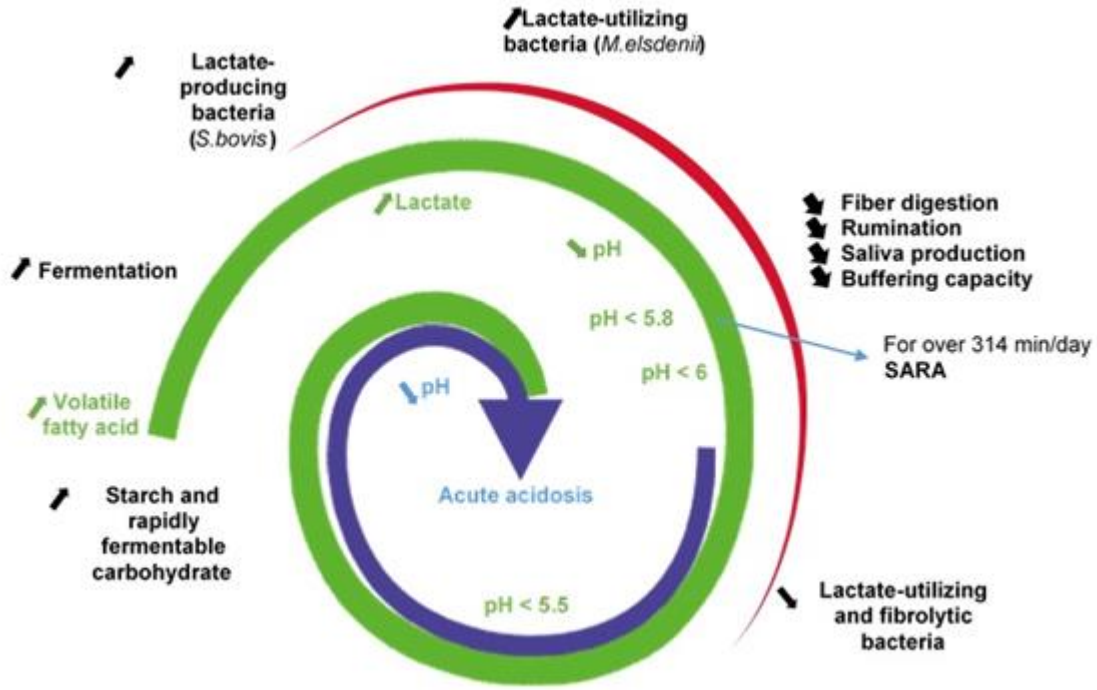
epithelium surface area as certain physical changes in terms of rumen papillae including length and width are observed (Steele et al., 2011a; Malhi et al., 2013).

2.4. Ruminal pH regulation

Ruminal pH can have wide oscillations throughout the day with drops particularly a few hours after the main meal. Furthermore, there are multiple ruminal pH regulation mechanisms highlighting how dissociated SCFA pass through the epithelium, and one of them includes the level of bicarbonate secretion into the lumen (Aschenbach et al., 2009; Dijkstra et al., 2012). Ruminal acidosis usually occurs in cattle fed high-concentrate diets due to the increased acid production exceeding the rate of salivary buffering and impairing rumen epithelium ion exchange capacity (Allen, 1997; Aschenbach et al., 2009). The ruminal acidosis terminology has been used since 1970 as suggested by Dirksen (1970): initially it was described as acute indigestion caused by excessive production of lactic acid (Dirksen, 1985) and with pH below 5 (Dirksen, 1983) or acute lactic acidosis characterized by an increased proliferation of Gram-positive bacteria (Dirksen, 1977; Nagaraja et al., 1998). Acute acidosis conditions cannot be easily reversed due to reduced salivation, decreased intestinal motility and ingesta turnover, increased dehydration, damage in rumen and liver tissues (Slyter, 1976). Subacute ruminal acidosis has been studied in the past three decades, with certain discrepancies on how it should be characterized. Some authors agree that it is diagnosed when ruminal pH is below 5.8 for over 314 min/day or when there is a daily mean ruminal pH below 6.16 (Zebeli et al., 2008). Meanwhile, Kleen et al. (2004) establish SARA occurrence when ruminal pH shows a transient fall below 5.5, although this threshold was measured with rumenocentesis. It is necessary to clarify that SARA occurs when the drop in ruminal pH is intermittent during the day with lactic acid concentrations of 0-5 mM; whereas in the acute conditions, the pH drop below 5 is persistent during the day and with higher concentrations of lactic acid (between 50-120 mM, mainly the D-form) (Nagaraja et al., 1998) as represented in Figure 1. Nonetheless, different

authors agree that SARA is closely related with multiple indicators measured as duration of ruminal pH or area below specific thresholds considering how rumen pH varies within a day (Plaizier et al., 2008; Zebeli et al., 2008; Petri et al., 2013). Subacute ruminal acidosis leads to high economical losses for dairy producers around the world including reduced milk yield and higher culling rates (Kleen et al., 2003). This health disorder has been predominantly demonstrated to occur in intensive confined systems up to 19% of early lactation and 26% of mid-lactation dairy population in the USA (Garret et al., 1997).

Figure 1: Changes in rumen pH during SARA and acute ruminal acidosis (adapted from Nocek, J.E. 1997)



2.5. Chewing and saliva secretion

Chewing is an essential physiological process in ruminants, needed for feed particle comminution and also to stimulate salivary secretions. Cattle chew while eating (6-1000 times) and ruminating (30-60 times) in series of successive jaw movement (Nørgaard, 1994).

Literature reports a mean eating time of 284 minutes per day, while a mean ruminating time of 436 minutes per day (White et al., 2017), highlighting the high variability in ruminating time oscillating from 236 to 610 minutes per day on the basis of the physical-chemical diet composition. For chewing, ruminants require peNDF: too little fiber in the diet will cause decrease in chewing activity, less salivary secretion, reduced ruminal pH, and alterations on the rumen fermentation patterns, ultimately decreasing acetate to propionate ratio resulting in decreased fat proportion in the milk (Mertens et al., 1997). In the same direction of research, Maekawa et al. (2003) demonstrated that primiparous and multiparous lactating cows reduce total chewing time and saliva secretion as forage in the diet is decreased. Furthermore, the potential beneficial impacts of increased chewing activity are not limited to more saliva production and inflow into the reticulum and rumen, but also faster particle size reduction (López-Paredes et al., 2020; Watt et al., 2015).

The three most important salivary glands include the parotid, the submaxillary and the sublingual (Church, 1988). Among them, the parotid is the largest and most important gland for rumen function, since it secretes important amounts of phosphate and bicarbonate (Kay, 1966). For most animals and particularly in ruminants, saliva is important for feed swallowing, feed intake and utilization (Meyer et al., 1964) and buffering of the ruminal pH (Kay, 1966). A parasympathetic reflex mediates saliva secretion. Therefore, taste, mastication and other stimuli bring reflex salivary secretion through gustatory receptors, mechanoreceptors, nociceptors, and olfactory receptors (Hector and Linden, 1999). Additionally, saliva has a buffering capacity because its composition includes bicarbonates and phosphates, which are used as important proton removal mechanisms in the rumen (Aschenbach et al., 2011). In addition, saliva represents a nitrogen source for the microbial population (McDonald, 1948; Phillipson, 1959).

Bailey and Bach (1961b) demonstrated that an only-forage diet had the highest saliva secretion rate in comparison with grain diets; however, based on the sampling outline, they were not able to explain a relationship connecting reticulo-rumen fluid pH (sampled at only 4 time points in a day) and salivary rate of secretion. Furthermore, saliva secretion includes a resting phase, which is not associated with eating or ruminating, mostly for protective reasons. Meanwhile, during eating, there are extra reflex stimulations by the taste and chewing of food reaching up to ten-fold increments for short periods of time (Emmelin, 1972). Resting and eating salivation did not show difference in an experiment conducted by Cassida and Stokes (1986). Later, experiments evaluating not only different forages source, but also the grain in the diet while assessing ensalivation proved that saliva secretion decreased 5 times compared with only-forage diet (Beauchemin et al., 2008).

Research from a few decades ago (Bailey, 1961) showed that saliva flow is strongly influenced by eating, diet composition and rumination. Some recent studies have also demonstrated that resting and eating saliva flow can be impacted by diet, duration of high grain feeding or phytogenic compounds. For instance, Kröger et al. (2019) reported an increased rumination using a mixture of phytogenic compounds, suggesting that salivation was increased, as rumen pH was also improved. Thus, there may be another way of stimulating chewing and salivation besides physically effective fiber. Castillo-Lopez et al. (2021a) proved that resting saliva osmolality increased when cows were fed high grain diets for 23 days. At the same time, the dietary regime decreased feed ensalivation and lowered the stimulated saliva pH, but increased buffer capacity compared to the beginning of the high grain feeding. As mentioned before, research in saliva has not been looked further in the last years and there is a gap of knowledge that this thesis intends to fill by studying changes in salivary composition/dynamics after the inclusion of phytogenic compounds.

2.6. SARA effects on cow health

Cows under SARA reduce DMI and body condition score, experience laminitis and milk fat depression (Plaizier et al., 2008; Enemark, 2008). Initial research demonstrated that milk fat depression happened due to the reduction of acetate to propionate ratio and increased insulin (Byers and Schelling, 1988; Khorasani and Kenelly, 2001; Bauman and Griinari, 2003; O'Grady et al., 2008). Following the same direction, Griinari et al. (1998) and Baumann and Griinari (2003) confirmed that low ruminal pH as a result of cows eating low fiber diets causes incomplete biohydrogenation of fatty acids increasing trans-octadecenoic acids, and specifically the trans-10 isomer of trans-octadecenoic acid main cause of milk fat depression. Plaiziet et al. (2008) also supports that milk fat depression occurs as a consequence of rumen acidity, triggering biohydrogenation of fatty acids in the rumen.

During SARA, due to the large amount of rapid digestible carbohydrates ingested, there is an increase in growth rate of certain rumen bacteria (Nocek,1997) accompanied by major fermentation that includes increasing amounts of acetate, propionate, and butyrate (Plaizier et al., 2009). The low rumen pH can cause a reduction of microbial diversity as well as of cellulolytic bacteria (Nagaraja et al.,1978; Goad et al., 1998; Mao et al., 2013). These intermittent drops of ruminal pH in cows susceptible to SARA show increased relative abundances of determined taxa, such as *Prevotella spp.* (starch-degrading bacteria) and decreased unclassified *Ruminococcaceae* spp*., Ruminococcus* spp*., Papillibacter,* and *Firmicutes* unclassified Family XIII (fiber-degrading bacteria) compared with tolerant to SARA (Zhang et al., 2022). This drop of pH altogether with the changes in microbial composition aggravates due to the accumulation of lactic acid and the additional accumulation of SCFA in the rumen (Nocek, 1997; Nagaraja and Titgemeyer 2007), and, if the conditions worsen, ulceration and ruminitis damaging the rumen epithelium can occur (Kleen et al., 2003; Enemark 2008; Plaizier et al. 2008). Ulcerations in the epithelium are common results of low

ruminal pH (Aslan et al., 1995) leading to gastrointestinal epithelial barrier dysfunction (Penner et al., 2011; Klevenhusen et al., 2013).

Rumen microbial health remains an important factor to preserve cows' health (Grove-White, 2004), and there is evidence linking dysbiosis in SARA to the acute inflammatory response. The inflammation occurs as result of a diet-induced disruption of rumen epithelial barrier function (Liu et al., 2013), which causes increased permeability of rumen epithelium (Klevenhusen et al., 2013). These changes allow microbes and immunogenic compounds to migrate to portal circulation (Khafipour et al., 2009). Lipopolysaccharide (LPS) is an endotoxin that is part of Gram-negative bacteria cell wall and that has been directly linked to rumen epithelial damages (Gao et al., 2022). LPS translocation from the rumen to peripheral circulation occurs through two pathways, with the lymphatic ducts and the portal vein, reaching systemic circulation (Zebeli and Metzler-Zebeli, 2012). LPS binding protein is activated by microbial infections, and its function is to help neutralizing LPS and to trigger the release of pro-inflammatory cytokines including tumor necrosis factor-α, interleukin-6 and interleukin-1β (De Nardi et al., 2014; Jia et al., 2014; Zhou et al., 2014). These cytokines are responsible for the production of acute phase proteins in the liver, which, in turn, activate the acute response in the body (Guo et al., 2017). Initially acute phase response can be seen as a host response to reduce agents causing stress or alteration of the homeostasis and re-establish the latter (Gabay and Kushner, 1999). Yet, sustained levels of inflammation are believed to be harmful for the host, as they may increase the risk for systemic disorders and divert energy and nutrients for non-productive processes (Zebeli et al., 2015; Plaizier et al., 2018). Additionally, cows show feed intake suppression (Kleen et al., 2003; Oetzel, 2003) and the reduction of DMI seems to be related to inflammation of cow's organs (Weingarten, 1996; Andersen et al., 2000).

Another health parameter affected by SARA includes the fecal scores (Ireland-Perry and Stallings, 1993), and these changes in fecal consistency and diarrhea are due to variations in osmotic pressure and to the water movement towards the digestive tract, with hypertonic digesta compared to blood plasma (Huber, 1976). Furthermore, it can also be explained by the extensive hindgut fermentation of fermentable substrates escaping the rumen (Nordlund et al., 2004). Diarrhea can compromise the water balance in the cow and reduce feed intake and milk production. Additionally, laminitis has been also recorded in cows challenged with SARA (Nordlund et al., 1995); as well as liver abscesses (Oetzel, 2003).

2.7. Animal behavior influenced by SARA

Normally dairy cattle spend between 4 and 7 h/d eating (Albright, 1992). During eating, cows might select for the more palatable portions of the diet in a process defined as sorting, thus influencing nutrient intake. Cows experiencing SARA change their feeding behavior, and in particular the sorting of feed particles of the diet. Even though cows eat a total mixed ration (TMR), ruminants can select within parts of the plant (Methu et al., 2001) and for grains, unbalancing their nutrient intake and reducing the nutritive value of the diet planned for the day (DeVries et al., 2005). Nevertheless, different literature shows inconsistent results: for instance, Gao and Oba (2014) demonstrated that SARA susceptible animals sorted to a greater extent against long feed particles (19 mm), while tolerant animals did not sort in the same direction. Supporting these findings, Stauder et al. (2020) and Kaltenegger et al. (2020) demonstrated that multiparous and primiparous cows fed a 60 and 50% grain diet, respectively, sorted in favor of long feed particle size. In another experiment, Greter and DeVries (2010) found that with different feed refusals and with a 46% grain diet cows sorted against long particles and in favor of short ones.

Animal behavior during feeding has been studied for decades (Baile and Della-Fera, 1984; Jacobs, 1992). Furthermore, rumination represents an important part of cows' behavior including regurgitation, re-mastication, and re-swallowing of the bolus (partially digested feed). Paudyal et al. (2017) reported that healthy high yield lactating cows ruminate approximately 459 min/day, while sick cows, mostly related with digestive disorders, reached 335 min/day. Additionally, periods of rumination are associated with lying time (Schirmann et al., 2012). Rumination is also inhibited with low pH, high osmotic pressure, and high SCFA concentration in the rumen (Focat et al., 1979; Welch, 1982; Wever et al., 1990), and this is the reason why rumination is often used as an indicator of rumen and animal health. In addition, rumination time decreased when particle size of diet is reduced (Grant et al., 1990). Likewise, when the fiber composition of the diet is reduced, rumination is reduced. Diets with higher inclusion levels of grains can not only reduce rumination time, but also DMI, and modify the meal patterns of the cow during the day (Zhang et al., 2017).

Research in eating behavior has also been conducted in dairy cattle and has demonstrated to change closely influenced by physiological status (DeVries et al., 2003). However, the impact of rumination and eating behavior on SARA development cannot be evaluated without considering important details on the cows meal and eating pace. This is because the rapid built-up of SCFA concentration in the rumen, which is influenced by the nature of cows meal and eating pace is one of the causes of SARA. Research demonstrated that eating time was increased when cows were fed ingredients separately in comparison with a TMR, demonstrating cow's preference due to higher grains palatability (Maekawa et al., 2002). Nevertheless, inconsistent results are reported in literature regarding faster eating rates when cows ate greater forage diets compared with low fiber diets (Johnson and Combs, 1992; DeVries et al., 2007) and similar eating times for diets with 44 and 67% forage according to Voelker et al. (2002).

Another important parameter of cow welfare includes lying behavior, and this is closely related with the rumination behavior. In relation with lying behavior, on average cows lie down for around 12 h/d (Jensen et al., 2005). Some studies dividing groups of cows with different diets at low-risk and high-risk of SARA with a 40 and 55% concentrate, respectively, demonstrated no difference in terms of standing, lying or eating behavior. However, rumination time decreased in the high-risk cows (DeVries et al., 2009). Fregonesi et al. (2007) support the idea that lying times in lactating dairy cattle predominantly follows a diurnal pattern, which opposes the pattern of feeding behavior. With respect to lying side, there are inconclusive results regarding cow's preference for laterality when lying down. Some preliminary studies suggest that cows prefer to lie down on the left side after eating to balance the additional weight in the rumen (Wagnon and Rollins, 1972); however, the strength of such study is today questionable.

Therefore, data on animal behavior have delivered inconclusive or contrasting results so far. In this thesis, the lying behavior of cows fed an acidogenic diet will be paired with data on chewing behavior, in an attempt to elucidate whether the two parameters show any correlations as physiological response to the induced SARA.

2.8. Phytogenic compounds as an alternative to alleviate impacts of SARA

Phytogenic compounds are plant-derived natural bioactive substances including several botanical, herbal extracts, tannins, and essential oils (Puvaca et al., 2013). Different phytogenic compounds possess antioxidant, antifungal, antimicrobial and antiviral properties (Brenes and Roura, 2010). Furthermore, studies have tested anise oils (*Pimpinella anisum*) as an alternative to increase DMI in high-grain:forage ratio diets for heifers (Cardozo et al., 2006). Similarly, research focusing on capsicum has demonstrated an increased saliva secretion rate in humans (Kono et al., 2018) while ginger increased saliva secretion in rats (Chamani et al. 2010). In ruminants, phytogenic compounds have demonstrated an antimicrobial mechanism in the

rumen (perturbation of cell membrane, modulation of gene expression pathways, inhibition of bacteria colonization) according to Bodas et al., (2012) and Reddy et al., (2020). Phytogenic compounds can modulate rumen microbial metabolism with the increase of cell membrane permeability of a few rumen bacteria (Omojate et al., 2014; Patra et al., 2019a). Literature has proved that their mode of action also includes decreasing the starch degradation rate or stimulation of the growth of lactate-utilizers in the rumen (Jouany, 2006). In this context, there is research focused on secondary plant compounds and their effects on volatile fatty acids production with inconsistent results so far (Benchaar et al., 2008, Broudiscou et al., 2000, Benchaar et al., 2007). Also, plant derivatives have proved in *in vitro* experiments to potentially impact gut health, protecting the rumen from reductions in pH due to excess of lactic acid (Hutton et al., 2009) and during HC feeding in dairy cattle (Kröger et al., 2017). Additionally, phytogenic compounds have demonstrated to modulate rumination (Kröger et al., 2017, Castillo-Lopez et al., 2021b). This effect is achieved due to their bioactive properties with potential to modulate the rumen microbiota and therefore fermentation (Cardozo et al., 2005; Neubauer et al., 2018). Another example includes compounds such as menthol, levomenthol, β-linalool, anethole, hexadecanoic acid and p-menthane, which have demonstrated to have similar effects and potential for increasing rumen fluid pH in cows fed a 50:50 concentrate to forage ratio compared to a control diet (Kholif and Olafadehan, 2021). Moreover, Ando et al. (2003) demonstrated that peppermint supplementation in cattle increased nutrient digestibility. Whereas at the systemic level, evaluations including phytogenic substances as supplement alternatives have also suppressed oxidative stress, improve blood profile and immune status in different species (Karaskova et al., 2015; Upadhaya and Kim, 2017; Li et al., 2018). Other report including tannins in the diet revealed lower haptoglobin in cows fed 39% grain (Rodrigues et al., 2019).

A large proportion of the cited studies has relied on *in vitro* systems to evaluate the effects of the tested phytogenic substances. However, such conclusions and indications can provide very different results in *in vivo* experiments, and especially in ruminants. In this thesis, the effects of several secondary phytogenic compounds were evaluated on a wide variety of physiological parameters in cattle, in order to assess their potential application in the modulation of the animal response to an acidogenic diet.

Furthermore, this work tries to elucidate whether phytogenic compounds can modulate rumen fermentation and counteract the negative effects of SARA in a long-term period, considering that only a few studies have focused on the long-term effects its implications and provided divergent results.

3. AIMS AND HYPOTHESIS OF THE STUDY

The first aim of this thesis was to evaluate the short-term effects of nine PHY on saliva composition, salivation, and ingested feed boli characteristics in cattle fed a HC diet. The underlying hypothesis was that phytogenic compounds can modulate saliva production and physico-chemical composition, thus displaying potential benefits on ruminal health in cattle fed HC diets. After this screening trial, the second experiment looked to assess the effect of a PAS in cattle transitioned from a forage to HC diet on ruminal pH, SARA risk, SCFA profile, lactate, and several biomarkers of inflammation and liver health; and to evaluate the effect of duration of a HC feeding challenge on chewing activity, eating, and lying behavior, salivary composition, and saliva production in dairy cows without or with PAS. Our hypothesis stated that PAS will mitigate the negative impacts of HC feeding by modulating ruminal pH and reduce lactate production, thus reducing the risk of SARA and associated systemic inflammation. We also hypothesized that advanced duration of the HC challenge would exacerbate negative effects on rumination, eating and lying behavior, and salivary production and composition. Another hypothesis was that PAS would alleviate the depression in chewing activity and improve feed sorting as well as salivary properties.

4. PUBLICATIONS

4.1.Publication 1

Supplementation with phytogenic compounds modulates salivation and salivary physico-chemical composition in cattle fed a high-concentrate diet.

Ricci, S.; Rivera-Chacon, R.; Petri, R.M.; Sener-Aydemir, A.; Sharma, S.; Reisinger, N.; Zebeli, Q.; Castillo-Lopez, E.

Front Physiol. 2021. 12:645529.

doi.org/10.3389/fphys.2021.645529

frontiers in Physiology

ORIGINAL RESEARCH
published: 03 June 2021
doi: 10.3389/fphys.2021.645529

Supplementation With Phytogenic Compounds Modulates Salivation and Salivary Physico-Chemical Composition in Cattle Fed a High-Concentrate Diet

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Saliva facilitates feed ingestion, nutrient circulation, and represents an important pH buffer for ruminants, especially for cattle fed high-concentrate diets that promote rumen acidification. This experiment evaluated the short-term effects of nine phytogenic compounds on salivation, saliva physico-chemical composition as well as ingested feed boli characteristics in cattle. A total of nine ruminally cannulated Holstein cows were used. Each compound was tested in four of these cows as part of a high-concentrate meal (2.5 kg of total mixed ration in dry matter basis for 4 h) in low or high dose, and was compared to a control meal without compound. Saliva was sampled orally (unstimulated saliva) for physico-chemical composition analysis. Composition of the ingested saliva (stimulated saliva), salivation and feed boli characteristics were assessed from ingesta collected at the cardia during the first 30 min of the meal. Analysis of unstimulated saliva showed that supplementation with capsaicin and thyme oil increased buffer capacity, while supplementation with thymol, L-menthol and gentian root decreased saliva pH. In addition, supplementing angelica root decreased saliva osmolality. Regression analysis on unstimulated saliva showed negative associations between mucins and bicarbonate as well as with phosphate when garlic oil, thyme oil or angelica root was supplemented. Analysis of stimulated saliva demonstrated that supplementation with garlic oil increased phosphate concentration, thyme oil tended to increase osmolality, capsaicin and thymol increased buffer capacity, and ginger increased phosphate content. Furthermore, salivation rate increased with ginger and thymol, and tended to increase with garlic oil, capsaicin, L-menthol and mint oil. Feed ensalivation increased with capsaicin. A positive association was found between feed bolus size and salivation rate when any of the phytogenic compounds was supplemented. Overall, our results demonstrate positive short-term effects of several phytogenic compounds on unstimulated and stimulated saliva physico-chemical properties, salivation or feed boli characteristics. Thus, the phytogenic compounds enhancing salivary physico-chemical composition have the potential to contribute to maintain or improve ruminal health in cattle fed concentrate-rich rations.

Keywords: saliva composition, salivation, cattle, phytogenic compound, high-concentrate

OPEN ACCESS

Edited by:
Stephen J. Pandol, Cedars-Sinai Medical Center, United States

Reviewed by:
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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *23 December 2020*
Accepted: *07 May 2021*
Published: *03 June 2021*

Citation:
Ricci S, Rivera-Chacon R, Petri RM, Sener-Aydemir A, Sharma S, Reisinger N, Zebeli Q and Castillo-Lopez E (2021) Supplementation With Phytogenic Compounds Modulates Salivation and Salivary Physico-Chemical Composition in Cattle Fed a High-Concentrate Diet. Front. Physiol. 12:645529. doi: 10.3389/fphys.2021.645529

INTRODUCTION

Salivary secretion is an important body fluid, rich in buffers like bicarbonate, nitrogen compounds and phosphate, and contains several important bioactive compounds, making saliva essential for food perception and ingestion, as well as the oral and gastrointestinal health (Humphrey and Williamson, 2001). Digestive physiology of cattle relies on large amounts of saliva that exceed 150 l/day, which is produced during their long chewing periods (Allen, 1997). Besides being essential for bolus formation, swallowing, nutrient release and circulation, saliva acts in cattle as a buffer that protects the rumen against a drop in pH, which commonly occurs in response to high concentrate feeding (Hofmann, 1989; Allen, 1997). Thus, saliva has a crucial role in rumen function and health in cattle (Fouhse et al., 2017).

Saliva has been extensively studied in humans (Hofman, 2001), and can provide valuable insights as a diagnostic tool in animals as well (Prickett and Zimmerman, 2010). Its composition and the dynamics of secretion have been the target of research for decades in various species (Bailey and Balch, 1961a,b; Carr, 1984). It is well established that diet is the most influencing factor on saliva flow by altering the chewing behavior of cows (Emery et al., 1960; Zebeli et al., 2012; Humer et al., 2018). However, despite its importance for digestion and health, studies evaluating the effect of diet on composition of unstimulated and eating-stimulated salivary secretions are limited in cattle. Research has proven that salivation rate and feed ensalivation are reduced in cattle fed a high-concentrate diet (Chibisa et al., 2016). Additionally, our group recently demonstrated that high-concentrate diet affects ensalivation and the physico-chemical characteristics of saliva including osmolality, mucin, lysozyme activity and main buffers such as bicarbonate and phosphate. These effects were observed either in unstimulated or stimulated saliva with implications for the regulation of ruminal pH of the cows (Castillo-Lopez et al., 2021a).

Apart from mechanical stimuli, research in other species has shown that taste and smell play an important role in the activity of salivary glands (Proctor, 2016). Phytogenic compounds have strong organoleptic properties and have a stimulatory effect on saliva secretion in humans (Nasrawi and Pangborn, 1990; Gardner et al., 2020). In a study evaluating the effects of different phytogenic compounds on chewing activity and ruminal fermentation in dairy cows, we found that certain compounds increased chewing time or influenced rumen fermentation profile, which has the potential to modulate ruminal pH (Castillo-Lopez et al., 2021b). Nonetheless, the effects of these phytogenic compounds on salivation and the physico-chemical properties of saliva in cattle fed high-concentrate diets need to be investigated. Therefore, the aim of the present study was to evaluate the short-term effects of nine phytogenic compounds on saliva composition, salivation and ingested feed boli characteristics in cattle fed a high-concentrate diet. Our hypothesis is that phytogenic compounds can modulate saliva production and physico-chemical composition, thus displaying potential benefits on ruminal health in cattle fed high-concentrate diets.

MATERIALS AND METHODS

Animals and Housing

The methods and protocols followed in this experiment were approved by the Institutional Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to the law for animal experiments (protocol number: BMNWF- 68.205/0003-V/3b/2019).

This study was part of a larger research elucidating the role of phytogenic compounds on chewing and salivary composition as well as rumen health in cattle fed concentrate-rich diets. Results of feed intake, chewing and rumen variables have been reported elsewhere (Castillo-Lopez et al., 2021b). In this trial, nine ruminally cannulated, non-lactating Holstein cows (887 ± 72.4 kg) were group-housed at the research dairy farm of the University of Veterinary Medicine, Vienna. Before the start of the experiment, cows were fed a forage diet and then transitioned during 1 week to a high concentrate diet containing on dry matter (DM) basis 26.25% grass silage, 8.75% corn silage, 26% rolled wheat, and 39% pelleted concentrate mixture. The diet had 32.8% starch, 41.9% non-fiber carbohydrates and 12.9% physically effective Neutral Detergent Fiber (NDF) (peNDF > 8 mm) (**Supplementary Table 1**), a diet considered with high acidogenic potential in cattle (Khorrami et al., 2021). All nutrients requirements for adult dairy cattle were met or exceeded. For sampling and animal management purposes, cows were blocked based on body weight in two groups with 5 and 4 cows, respectively. Cows were housed in a free-stall barn with individual deep cubicles (2.6 × 1.25 m) and straw bedding. Free choice mineral blocks and water were available *ad libitum*, except during the treatment meal consumption. Diet was mixed once a day using an automated feeding system (Trioliet Triomatic T15, Oldenzaal, Netherlands), and offered to cows as total mixed ration (TMR) in individual feed bunks equipped with electronic weigh scales (Insentec B.V., Marknesse, Netherlands). In order to increase palatability and reduce sorting, water was added to the TMR during mixing to target 46% DM content.

Treatments and Experimental Design

Details on phytogenic compound production and extraction as well as the strategy followed for the estimation of minimum number of animals needed for adequate statistical power have been reported in our companion paper (Castillo-Lopez et al., 2021b). Briefly, the nine phytogenic compounds evaluated were angelica root, capsaicin, garlic oil, gentian root, ginger, L-menthol, mint oil, thyme oil, and thymol (**Supplementary Table 2**). The compounds were in powder form and were mixed in either low (1×, LOW) or high dosage (10×, HIGH) with 50 g of a carrier consisting of silica wheat. This mixture was then combined with 2.5 kg of TMR (DM basis). The control meal (CON) was prepared by combining 50 mg of the carrier with 2.5 kg of TMR. Treatments and control were offered in the feed bunks and cows were allowed to eat during a 4-h meal, then orts were weighed and discarded. The experimental design included 4 cows per treatment, meaning that each substance at each dosage

was tested on four of the nine cows, using an incomplete Latin Square experimental design. Short-term effects were investigated, so that response variables for each evaluation were assessed on a single day, with four compounds tested per experimental day. Compounds were randomly allocated to the cows, so that each animal consumed both dosages of the corresponding phytogenic compound, and the treatment sequence was balanced for carry-over effects. The two dosages of each compound were tested in different days, with the LOW dosage being tested first. After each treatment assessment, there was a washout day in which cows consumed the CON diet. Each cow served as its own control, and the control measurements were performed at the beginning and at the end of the experiment, when the animals received the CON diet. Before offering the 4-h treatment meal, feed was automatically restricted for 9 h in order to promote feed consumption.

Feed Chemical Analyses

Feed samples were collected daily and pooled by week for chemical composition analyses. After drying samples at 65°C in a forced-air oven for 48 h, they were ground through a 0.5 mm screen (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany). Ash was analyzed by combustion overnight at 580°C. Crude protein (CP) was analyzed with the Kjeldahl method (VDLUFA, 2012) and ether extract with the soxhlet system (Extraction System B-811, Büchi, Flawil, Switzerland). NDF and Acid Detergent Fiber (ADF) contents were determined with sodium sulfite following the official analytical methods of VDLUFA (2012), and using the Fiber Therm FT 12 (Gerhardt GmbH and Co., KG, Königswinter, Germany) with heat-stable α-amylase for NDF. Starch content was measured with the K-TSTA kit (Megazyme Ltd.). Non-fiber carbohydrates were calculated as 100 – (% CP + %NDF + % ether extract + % ash).

Saliva Sampling and Analysis

Unstimulated Saliva Collection

Cows were tied using a halter and unstimulated saliva was sampled directly from the mouth (in the space between the teeth and the cheek) using a vacuum-pump with a maximum suction power of – 80 kPa (model Kataspir 30, MEDUTEK, GmbH and Co., KG., Bremen, Germany). Saliva was collected immediately before and 4 h after offering the meal (**Figure 1**). Approximately 100 mL of saliva were collected at each sampling time, divided in ten aliquots, and stored in 15 mL vials. Saliva pH was measured immediately after sampling, with a portable pH meter (Mettler-Toledo, AG; Analytical CH; Schwerzenbach, Switzerland), and the samples were frozen at −20°C for further analysis. The saliva container of the pump was washed and dried between collections.

Stimulated Saliva Collection and Measurement of Salivation

Ingested feed boli collections were conducted following the protocols of Maekawa et al. (2002), with minor modifications. First, the rumen was partially emptied and the digesta was stored in an insulated and pre-warmed 50 L polyethylene barrel, in order to keep it warm. Once the anterior pillar of the rumen was exposed and the cardia could be reached without interference of reticular digesta, the meal with either phytogenic treatment or control was offered. Then, swallowed feed boli were collected from the cardia while the cows were eating by inserting an arm through the ruminal cannula and using a plastic bag (17 × 30 cm) secured on the wrist with a rubber band. Up to five feed boli were collected from each cow over a 30-min time frame (2 min

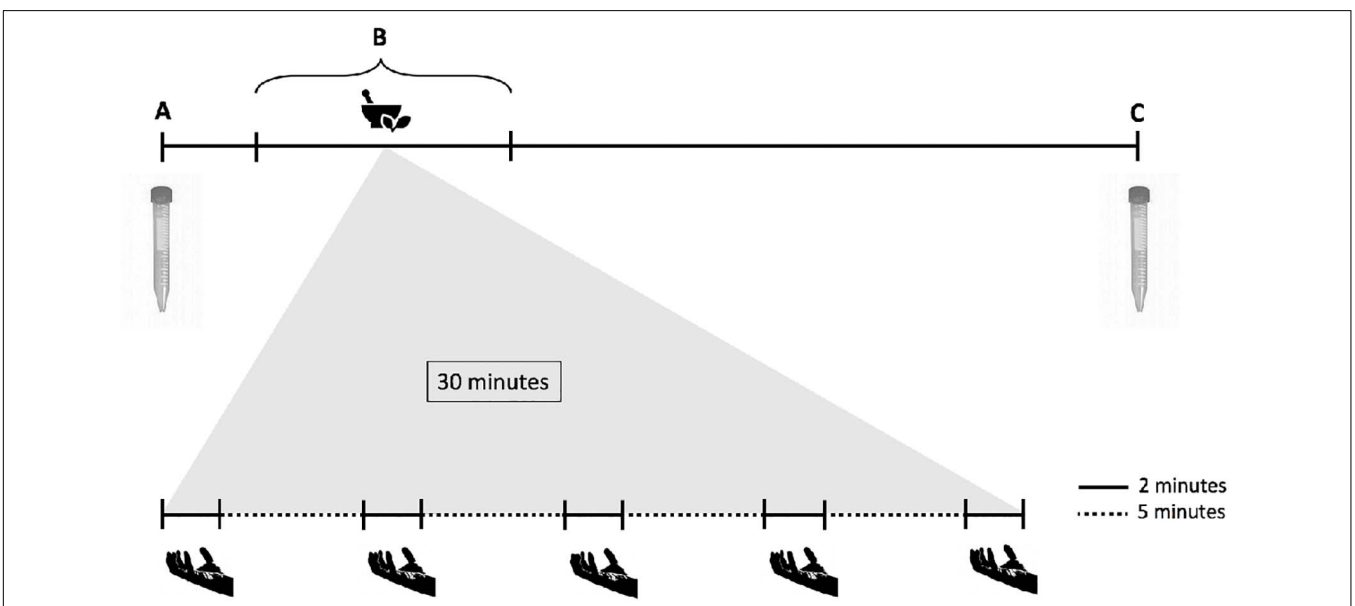

FIGURE 1 | Schematic illustration of the sampling procedure timeline. **(A)** Unstimulated saliva was collected from the mouth, then the rumen was partially emptied. **(B)** The cows were offered the controlled meal with phytogenic compounds or control. Feed boli sampling from the cardia (collection of stimulated saliva) was performed over the first 30 min of the meal, with 2 min for collection alternated with 5 min of rest. **(C)** Unstimulated saliva sampling, and end of the 4-h meal.

of sampling, followed by 5 min of break during which cows were left undisturbed) (**Figure 1**). Each sampled bolus was weighed and then strained using four layers of gauze to collect stimulated saliva. The fluid was collected in a 50 mL vial and pH was measured. At the end of the sampling procedure, ruminal content was returned into the rumen. Saliva and feed boli were then frozen at −20°C for further analysis. Saliva content of feed boli was calculated as the difference in moisture content between the feed offered and the collected ingesta samples. The DM content of feed boli were determined by oven drying at 55°C for 72 h. Feed ensalivation was calculated as the amount of saliva added to each gram of feed ingested (DM basis). Salivation rate (g/min) calculations were conducted by dividing the content of saliva from each bolus by collection time (2 min). In addition, feed ensalivation per unit of metabolic weight of cows (live weight)$^{0.75}$ was calculated. Conversion of saliva weight to volume was performed according to Maekawa et al. (2002) assuming that 1 g of saliva equals 1 mL, because the DM of saliva has been shown to be minimal (Bailey and Balch, 1961a).

Composition Analyses for Unstimulated and Stimulated Saliva

The parameters evaluated to assess unstimulated saliva composition were pH, bicarbonate concentration, phosphate concentration, total proteins, mucin concentration, buffer capacity, osmolality, and lysozyme activity. Saliva pH was measured using a manual pH meter (Mettler-Toledo, AG; Analytical CH; Schwerzenbach, Switzerland). Bicarbonate concentration was estimated according to an enzymatic colorimetric assay (Zabokova-Bilbilova et al., 2013) and following the Diagnostic Systems Kit (DiaSys GmbH, # 109509910021, Holzheim, Germany). Phosphate concentration was assessed with a colorimetric method (Fiyaz et al., 2013) using the Malachite Green Phosphate Assay Kit (MAK307, Sigma-Aldrich, Austria). Total proteins were measured according to Bradford (1976), also used by Roa et al. (2008) and Kejriwal et al. (2015) with minor modifications. Mucin concentration was measured according to the protocol described by Kilcoyne et al. (2011) with minor modifications. Buffer capacity was measured according to the method utilized by Pinto Antunes et al. (2015). Osmolality was determined according to the method used by Villiger et al. (2018) with minor modifications. Lysozyme activity was determined according to the procedure used by Helal and Melzig (2008) and following the Lysozyme Detection Kit (Sigma-Aldrich, # LY0100, Austria) with minor modifications. For stimulated saliva, samples were thawed on ice, centrifuged at 4°C for 20 min at 15,000 rpm and aliquots were prepared to measure pH, phosphate concentration, buffer capacity, and osmolality. Due to the dark appearance of the stimulated saliva, it was not possible to measure bicarbonate concentration, total proteins, mucin concentration, and lysozyme activity in these samples. Minor modifications for the listed protocols used are described in Castillo-Lopez et al. (2021a).

Statistical Analysis

Data were analyzed with the Proc Mixed of SAS (version 9.4; SAS Institute, Cary, NC, United States) with treatment dose as fixed effects and cow within experimental block as random effect, linear and quadratic effects were evaluated. Data on unstimulated saliva composition prior to treatment or control meal intake were used as covariate measurements. Data of feed boli from the same cow within treatment dose or control in different times were processed as repeated measures in the analysis with a first order variance-covariance structure matrix taking into consideration that the covariance decays with time. Data were also checked for normal distribution using Proc Univariate followed by the normal and plot options. The largest standard error of the mean (SEM) is reported. Statistical significance was declared when $P \leq 0.05$ and tendency was mentioned if $0.05 < P \leq 0.10$. Correlation heatmaps were constructed with (R Core Team., 2020) using the corrplot package (Wei and Simko, 2017). Then, based on results from correlation analysis, linear regressions were performed with SAS to evaluate the association among salivary components, salivation dynamics and chewing index. Additionally, *a posteriori* statistical power analysis was conducted according to Stroup (1999) and Kononoff and Hanford (2006), which displayed an acceptable statistical power with an average of 0.812 with $\alpha = 0.05$.

RESULTS

Unstimulated Saliva Composition

Data listed in **Table 1** indicate that supplementing garlic oil tended to linearly enhance bicarbonate concentration and the buffer capacity of saliva ($P = 0.06$), while the low dose tended to reduce total proteins ($P = 0.09$). Mucins concentration tended to decrease after cows received the meal with garlic oil ($P = 0.06$). Thyme oil linearly increased the buffer capacity ($P < 0.05$) and tended to quadratically affect ($P = 0.10$) mucin concentration (**Table 2**). The regression analysis revealed negative associations between mucin concentration and bicarbonate as well as phosphate in the unstimulated salivary secretions when garlic oil, thyme oil or angelica root were supplemented (**Figure 2**). Accordingly, for every mg/mL increment in mucin concentration, bicarbonate and phosphate concentration decreased by 19.9 and 2.6 mM, respectively. Additionally, the unstimulated saliva pH generally dropped after supplementation with five phytogenic compounds, especially when using a high dosage. For example, the high dosages of thyme oil and capsaicin tended ($P = 0.09$) to lower salivary pH (**Tables 2**, **3**), while thymol ($P < 0.05$) and L-menthol ($P < 0.05$) significantly decreased this variable (**Tables 4**, **5**). Supplementation with angelica root showed a quadratic effect on saliva osmolality ($P < 0.01$) (**Supplementary Table 3**). Gentian root decreased salivary pH ($P < 0.05$) (**Supplementary Table 4**). Mint oil and ginger did not affect any of the unstimulated saliva composition parameters.

Stimulated Saliva Composition

Low dose of garlic oil increased stimulated saliva osmolality and phosphate ($P < 0.01$), but reduced pH and buffer capacity ($P < 0.05$) (**Table 1**). Low dose of thyme oil decreased pH ($P < 0.01$) and tended to increase phosphate concentration

TABLE 1 | Effect of supplementation with garlic oil on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]			*P*-value		
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.82	8.78	8.73	0.062	0.25	0.99
Bicarbonate, mM	71.26	93.34	95.16	8.620	0.06	0.32
Phosphate, mM	9.71	10.88	11.76	1.100	0.15	0.91
Total proteins, μg/mL	380.9	184.7	312.9	71.81	0.47	0.09
Buffer capacity, mol of HCl/L/ΔpH	0.014	0.016	0.018	0.0015	0.06	0.82
Osmolality, mOsm/kg	246.8	251.7	251.3	10.33	0.73	0.83
Lysozyme activity, U/mL/min	33.88	36.93	43.56	6.350	0.23	0.82
Mucins, mg/mL	1.56	1.01	0.93	0.195	0.06	0.38
Stimulated saliva[3]						
pH	6.79	6.55	6.71	0.0709	0.25	0.01
Phosphate, mM	12.52	17.35	14.84	1.287	0.03	<0.01
Buffer capacity, mol of HCl/L/ΔpH	0.035	0.026	0.036	0.0034	0.66	0.01
Osmolality, mOsmol/kg	402.45	546.81	437.79	66.009	0.38	<0.01
Saliva dynamics						
Salivation rate, g/min	67.40	83.24	79.81	5.578	0.06	0.14
Ensalivation, g/g DM feed	5.05	3.62	4.39	0.509	0.33	0.13
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.033	0.023	0.028	0.004	0.21	0.09
Bolus size (as is), g	213.29	272.78	284.77	21.475	0.12	0.08
Bolus size (DM), g	34.24	49.78	41.58	5.573	0.20	0.05

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 0.3 ppm of garlic oil in 2.5 kg (DM) of TMR; HIGH: 3 ppm of garlic oil in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

TABLE 2 | Effect of supplementation with thyme oil on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]			*P*-value		
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.81	8.81	8.70	0.0520	0.09	0.37
Bicarbonate, mM	76.71	77.61	83.76	10.734	0.56	0.82
Phosphate, mM	10.45	9.32	10.12	1.532	0.87	0.59
Total proteins, μg/mL	352.7	200.6	397.2	93.87	0.69	0.15
Buffer capacity, mol of HCl/L/ΔpH	0.013	0.015	0.018	0.0013	0.01	0.97
Osmolality, mOsm/kg	243.5	238.6	261.4	12.81	0.17	0.25
Lysozyme activity, U/mL/min	31.27	42.35	34.15	5.539	0.63	0.13
Mucins, mg/mL	1.53	0.95	1.26	0.193	0.36	0.10
Stimulated saliva[3]						
pH	6.86	6.63	6.89	0.131	0.66	<0.01
Phosphate, mM	11.67	13.49	11.66	1.533	0.99	0.06
Buffer capacity, mol of HCl/L/ΔpH	0.029	0.022	0.025	0.0038	0.32	0.14
Osmolality, mOsmol/kg	409.69	533.76	488.76	53.521	0.09	0.07
Saliva dynamics						
Salivation rate, g/min	60.15	80.40	73.75	12.310	0.23	0.17
Ensalivation, g/g DM feed	4.61	4.47	5.99	1.261	0.07	0.20
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.719	0.954	0.887	0.139	0.21	0.20
Bolus size (as is), g	195.90	246.94	239.54	38.867	0.12	0.22
Bolus size (DM), g	29.46	42.63	41.47	9.623	0.03	0.14

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 9.4 ppm of thyme oil in 2.5 kg (DM) of TMR; HIGH: 94 ppm of thyme oil in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

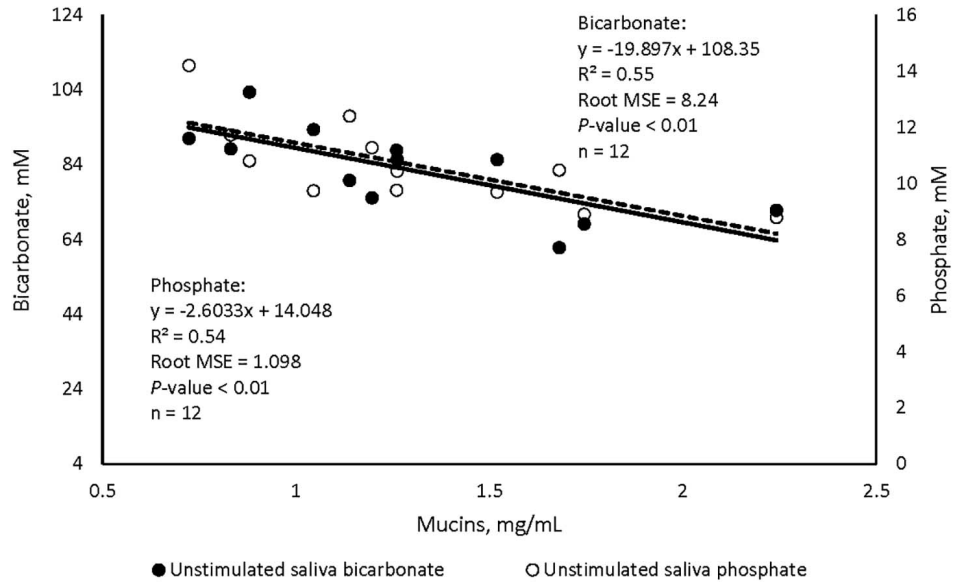

FIGURE 2 | Regression plots illustrating the associations between mucin concentration and bicarbonate (●), and phosphate (-○-) in the unstimulated salivary secretions of cattle, plots include average values across angelica root, garlic oil, and thyme oil.

TABLE 3 | Effect of supplementation with capsaicin on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.83	8.84	8.75	0.047	0.09	0.22
Bicarbonate, mM	74.12	81.17	75.52	5.758	0.84	0.37
Phosphate, mM	11.15	11.98	14.08	1.756	0.23	0.74
Total proteins, μg/mL	275.1	267.7	263.6	128.84	0.93	1.00
Buffer capacity, mol of HCl/L/ΔpH	0.013	0.015	0.014	0.0014	0.58	0.50
Osmolality, mOsm/kg	242.2	247.8	231.2	11.42	0.31	0.30
Lysozyme activity, U/mL/min	34.11	38.95	26.51	7.916	0.45	0.39
Mucins, mg/mL	1.52	1.64	1.48	0.465	0.94	0.81
Stimulated saliva[3]						
pH	6.97	6.71	6.90	0.158	0.58	0.09
Phosphate, mM	10.25	11.18	12.62	1.408	0.07	0.86
Buffer capacity, mol of HCl/L/ΔpH	0.036	0.050	0.118	0.0447	0.03	0.56
Osmolality, mOsmol/kg	365.91	440.62	312.59	81.004	0.44	0.13
Saliva dynamics						
Salivation rate, g/min	69.43	92.79	56.27	10.778	0.06	<0.01
Ensalivation, g/g DM feed	5.87	4.00	8.02	1.562	0.03	0.01
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.036	0.024	0.049	0.010	0.03	0.02
Bolus size (as is), g	203.25	293.42	149.78	39.367	0.02	<0.01
Bolus size (DM), g	29.94	50.04	17.37	8.947	<0.01	<0.01

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 10 ppm of capsaicin in 2.5 kg (DM) of TMR; HIGH: 100 ppm of capsaicin in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

(P = 0.06) and osmolality (P = 0.07) (**Table 2**). Low dose of thymol decreased pH (P < 0.01), but this compound linearly increased buffer capacity (P < 0.05) (**Table 4**). The low dose of capsaicin showed a trend to reduce pH (P = 0.09) and a significant linear increase of buffer capacity (P < 0.05) (**Table 3**). Supplementation with ginger reduced salivary pH (P < 0.01), but increased phosphate concentration (P < 0.01) (**Table 6**). Low dosage of L-menthol showed a trend to increase buffer

TABLE 4 | Effect of supplementation with thymol on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.86	8.80	8.71	0.052	0.04	0.82
Bicarbonate, mM	81.81	83.99	88.22	7.412	0.34	0.86
Phosphate, mM	9.85	9.71	12.53	1.735	0.22	0.42
Total proteins, μg/mL	362.0	295.5	309.2	93.45	0.70	0.72
Buffer capacity, mol of HCl/L/ΔpH	0.014	0.017	0.016	0.0014	0.31	0.22
Osmolality, mOsm/kg	253.1	244.5	253.5	6.35	0.97	0.22
Lysozyme activity, U/mL/min	39.56	34.68	38.05	8.833	0.88	0.63
Mucins, mg/mL	1.52	1.38	1.52	0.503	0.99	0.80
Stimulated saliva[3]						
pH	6.83	6.44	6.72	0.099	0.31	<0.01
Phosphate, mM	12.11	14.49	12.00	2.336	0.95	0.13
Buffer capacity, mol of HCl/L/ΔpH	0.041	0.056	0.133	0.0335	0.01	0.24
Osmolality, mOsmol/kg	377.58	413.25	379.95	68.880	0.95	0.35
Saliva dynamics						
Salivation rate, g/min	71.25	71.81	96.33	8.996	0.04	0.24
Ensalivation, g/g DM feed	4.96	4.54	3.97	0.857	0.35	0.95
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.032	0.028	0.025	0.001	0.25	0.96
Bolus size (as is), g	226.61	231.40	309.02	35.781	0.04	0.29
Bolus size (DM), g	36.44	40.55	53.33	9.025	0.06	0.58

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 5 ppm of thymol in 2.5 kg (DM) of TMR; HIGH: 50 ppm of thymol in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

TABLE 5 | Effect of supplementation with L-menthol on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.78	8.76	8.59	0.067	0.01	0.25
Bicarbonate, mM	77.82	76.04	85.20	8.960	0.42	0.52
Phosphate, mM	10.99	9.83	11.11	1.285	0.94	0.40
Total proteins, μg/mL	353.2	374.5	449.1	131.27	0.55	0.87
Buffer capacity, mol of HCl/L/ΔpH	0.013	0.014	0.016	0.0014	0.15	0.82
Osmolality, mOsm/kg	244.5	245.0	241.7	12.62	0.86	0.90
Lysozyme activity, U/mL/min	31.38	36.26	27.45	6.428	0.59	0.34
Mucins, mg/mL	1.39	1.46	1.91	0.352	0.33	0.67
Stimulated saliva[3]						
pH	6.88	6.76	6.80	0.103	0.15	0.13
Phosphate, mM	11.73	11.88	12.57	1.374	0.47	0.80
Buffer capacity, mol of HCl/L/ΔpH	0.033	0.055	0.033	0.0120	0.97	0.09
Osmolality, mOsmol/kg	381.88	432.18	427.36	46.839	0.25	0.44
Saliva dynamics						
Salivation rate, g/min	61.14	72.65	72.98	8.508	0.08	0.29
Ensalivation, g/g DM feed	6.13	6.92	5.61	1.900	0.64	0.30
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.037	0.042	0.035	0.011	0.70	0.31
Bolus size (as is), g	177.75	216.51	218.23	34.506	0.06	0.33
Bolus size (DM), g	25.58	33.67	33.47	7.963	0.07	0.29

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 6.7 ppm of L-menthol in 2.5 kg (DM) of TMR; HIGH: 67 ppm of L-menthol in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

TABLE 6 | Effect of supplementation with ginger on salivary physico-chemical properties, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.77	8.72	8.71	0.061	0.48	0.73
Bicarbonate, mM	82.53	82.82	84.81	11.055	0.87	0.95
Phosphate, mM	10.33	10.14	11.15	0.909	0.46	0.57
Total proteins, μg/mL	348.5	316.5	396.4	111.47	0.75	0.66
Buffer capacity, mol of HCl/L/ΔpH	0.014	0.016	0.014	0.0011	0.84	0.15
Osmolality, mOsm/kg	248.7	257.2	246.0	11.18	0.84	0.46
Lysozyme activity, U/mL/min	33.70	37.28	31.08	4.783	0.66	0.39
Mucins, mg/mL	1.04	1.13	1.18	0.288	0.74	0.95
Stimulated saliva[3]						
pH	6.76	6.61	6.58	0.056	<0.01	0.34
Phosphate, mM	12.99	14.94	17.73	1.044	<0.01	0.71
Buffer capacity, mol of HCl/L/ΔpH	0.038	0.033	0.032	0.0041	0.24	0.75
Osmolality, mOsmol/kg	368.25	452.58	433.35	55.700	0.13	0.22
Saliva dynamics						
Salivation rate, g/min	68.95	86.35	93.15	6.520	0.01	0.46
Ensalivation, g/g DM feed	4.33	4.04	3.86	0.469	0.32	0.91
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.027	0.025	0.024	0.003	0.29	0.94
Bolus size (as is), g	214.24	272.47	292.18	25.487	<0.01	0.50
Bolus size (DM), g	35.37	45.65	49.01	6.829	0.05	0.65

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 40 ppm of ginger in 2.5 kg (DM) of TMR; HIGH: 400 ppm of ginger in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

capacity ($P = 0.09$) (**Table 5**). Low dose of mint oil decreased pH ($P < 0.05$), but increased salivary phosphate concentration ($P < 0.01$). However, mint oil tended to linearly decrease osmolality ($P = 0.09$) (**Table 7**). Supplementing angelica root tended to quadratically decrease pH ($P = 0.09$) and osmolality ($P = 0.07$) (**Supplementary Table 3**). Low dose of gentian root reduced salivary pH ($P < 0.05$), but increased phosphate concentration ($P < 0.05$) and the buffer capacity ($P = 0.05$) (**Supplementary Table 4**). Regression analysis showed that for every unit increment in chewing index during the meal, stimulated saliva pH increased by 0.005 when capsaicin or thyme oil were supplemented (**Figure 3A**). Additionally, a positive association was found between stimulated saliva pH and feed ensalivation when supplementing garlic oil, capsaicin, mint oil or gentian root. As shown in the regression plot, for every gram increment in feed ensalivation, salivary pH increased by 0.28 units (**Figure 3B**).

Salivation and Feed Bolus Dynamics

Supplementation with garlic oil tended to increase salivation rate ($P = 0.06$), and the low dose increased ($P = 0.05$) bolus size; garlic oil also tended ($P = 0.09$) to display a quadratic effect on feed ensalivation per unit of metabolic body weight (**Table 1**). Supplementation with thyme oil showed a trend toward a linear increment of feed ensalivation ($P = 0.07$), while causing an increment ($P < 0.05$) of feed boli size on a DM basis (**Table 2**). Thymol increased salivation rate ($P < 0.05$) and feed boli weight as is ($P < 0.05$) (**Table 4**). Capsaicin had a strong influence on saliva dynamics. Interestingly, the low dose of capsaicin increased both saliva flow to the rumen ($P < 0.01$) and the bolus size ($P < 0.01$). On the contrary, the high dosage of capsaicin reduced the weight of the feed boli and the flow of saliva to the rumen, while increasing ($P < 0.05$) feed ensalivation. Capsaicin also had a significant quadratic effect on feed ensalivation per unit of metabolic body weight (**Table 3**). Ginger increased salivation rate ($P < 0.05$) and feed bolus size, both as is ($P < 0.01$) and on DM basis ($P = 0.05$) (**Table 6**). L-menthol showed a trend toward an increment of boli size ($P = 0.07$), and tended to linearly increase ($P = 0.08$) salivation rate (**Table 5**). Mint oil tended to increase salivation rate ($P = 0.08$), and its low dosage reduced feed ensalivation ($P < 0.05$), likely due to the increase ($P < 0.05$) in feed bolus size with this dosage; this compound also displayed a quadratic effect on feed ensalivation per unit of metabolic body weight (**Table 7**). Gentian root tended to increase salivation rate ($P = 0.08$) (**Supplementary Table 4**). The relationship between the bolus size and salivation rate across all phytogenic compounds is shown in **Figure 4**. The regression revealed a strong positive effect of bolus size on the salivation rate ($R^2 = 0.97$). For every gram increment in feed boli weight, salivation rate increased by 0.31 g/min.

DISCUSSION

Salivation and saliva composition in cattle have received very little attention, despite their importance for digestion and rumen health. Saliva is the result of continuous secretion and mixing

TABLE 7 | Effect of supplementation with mint oil on salivary physico-chemical properties, salivation, and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	**CON**	**LOW**	**HIGH**	**SEM[4]**	**Linear**	**Quadratic**
pH	8.85	8.81	8.75	0.064	0.15	0.80
Bicarbonate, mM	82.03	71.28	82.21	11.714	0.99	0.38
Phosphate, mM	11.82	9.25	7.64	1.893	0.15	0.80
Total proteins, μg/mL	384.1	434.9	518.0	97.00	0.28	0.89
Buffer capacity, mol of HCl/L/ΔpH	0.013	0.013	0.014	0.0011	0.61	0.73
Osmolality, mOsm/kg	241.6	224.4	236.1	16.39	0.77	0.44
Lysozyme activity, U/mL/min	30.39	37.56	50.06	13.757	0.20	0.87
Mucins, mg/mL	1.28	1.51	2.69	0.595	0.17	0.55
Stimulated saliva[3]						
pH	6.95	6.57	6.79	0.142	0.20	0.02
Phosphate, mM	11.13	16.24	9.97	2.325	0.55	<0.01
Buffer capacity, mol of HCl/L/ΔpH	0.033	0.030	0.030	0.0096	0.78	0.92
Osmolality, mOsmol/kg	373.74	316.92	301.92	69.190	0.09	0.62
Saliva dynamics						
Salivation rate, g/min	68.62	86.39	91.54	13.396	0.08	0.64
Ensalivation, g/g DM feed	5.37	4.17	5.58	1.058	0.69	0.05
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.032	0.025	0.034	0.007	0.63	0.02
Bolus size (as is), g	197.31	290.43	273.73	40.663	0.05	0.16
Bolus size (DM), g	28.57	52.20	39.67	8.976	0.12	0.02

[1] *CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 15.3 ppm of mint oil in 2.5 kg (DM) of TMR; HIGH: 153 ppm of mint oil in 2.5 kg (DM) of TMR.*
[2] *Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.*
[3] *Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.*
[4] *The largest standard error of the mean.*

of parotid, mandibular, sublingual, and ventral buccal salivary glands (Kay, 1960; Hofmann, 1989) and its secretion depends on many factors, such as gland type, stimuli, activity of the nervous system, individual animal variation, diet composition and organoleptic properties of the feed (Emery et al., 1960; Larsen et al., 1999; Nieuw Amerongen et al., 2007; Thomas et al., 2009). In agreement with our hypothesis, the present study demonstrated that phytogenic compounds are able to positively modulate salivation and saliva composition. Although we did not investigate the mode of action of the substances, we speculate that they exerted organoleptic effects or targeted stimuli resulting in enhancement of salivation or changes of salivary physico-chemical properties. It is also important to note that this experiment included dry cows, with dry matter intake and metabolic activity likely being different compared to lactating cows. However, findings from this experiment contribute to improve the current knowledge on salivation dynamics affected by concentrate-rich diets and may serve as a foundation toward further investigation in lactating cows under similar intensive feeding systems.

Salivary buffer capacity and buffer content are important properties contributing to rumen health in cattle (Bailey and Balch, 1961a), which become even more crucial when the amount of concentrates in the diet is high, as in our study. Findings from this trial show that these properties were enhanced by several phytogenic compounds; in particular, capsaicin increased buffer capacity and tended to increase phosphate content of stimulated saliva. Interestingly, capsaicin increased eating time and its low dose increased rumination time, as shown in the companion study (Castillo-Lopez et al., 2021b), indicating the positive influence of chewing activity for the enhancement of salivary properties in cattle fed high-concentrate diets. In addition, feeding garlic oil had a positive influence on buffer capacity of unstimulated saliva, bicarbonate content, and salivation. These changes may have contributed to lower the concentration of reticular volatile fatty acids, and to enhance ruminal pH post consumption (Castillo-Lopez et al., 2021b). However, we found that higher concentrations of bicarbonate and phosphate were associated with lower content of mucins in unstimulated saliva, and that mucins tended to decrease with garlic oil supplementation. Mucins contribute to dental health and give saliva its viscosity and elasticity to maintain the structure of feed boli (Nieuw Amerongen et al., 2007; Carpenter, 2013). The reduction of these proteins with garlic oil supplementation could be due to the sulfur-based molecules in allicin, one of the components of garlic, which can influence mucin-secreting cells and the proteins structure (Choong et al., 2004; Kudva et al., 2012; Dewi et al., 2017). Nevertheless, further research on the association between salivary buffers, mucin content and their impact on rumen function is needed.

Our results showed that high dose of thyme oil tended to decrease the pH of unstimulated saliva, and the low dose reduced stimulated saliva pH. Thyme oil, and in particular some of its components, such as carvacrol, have been demonstrated to have antimicrobial properties. For instance, Juven et al. (1994)

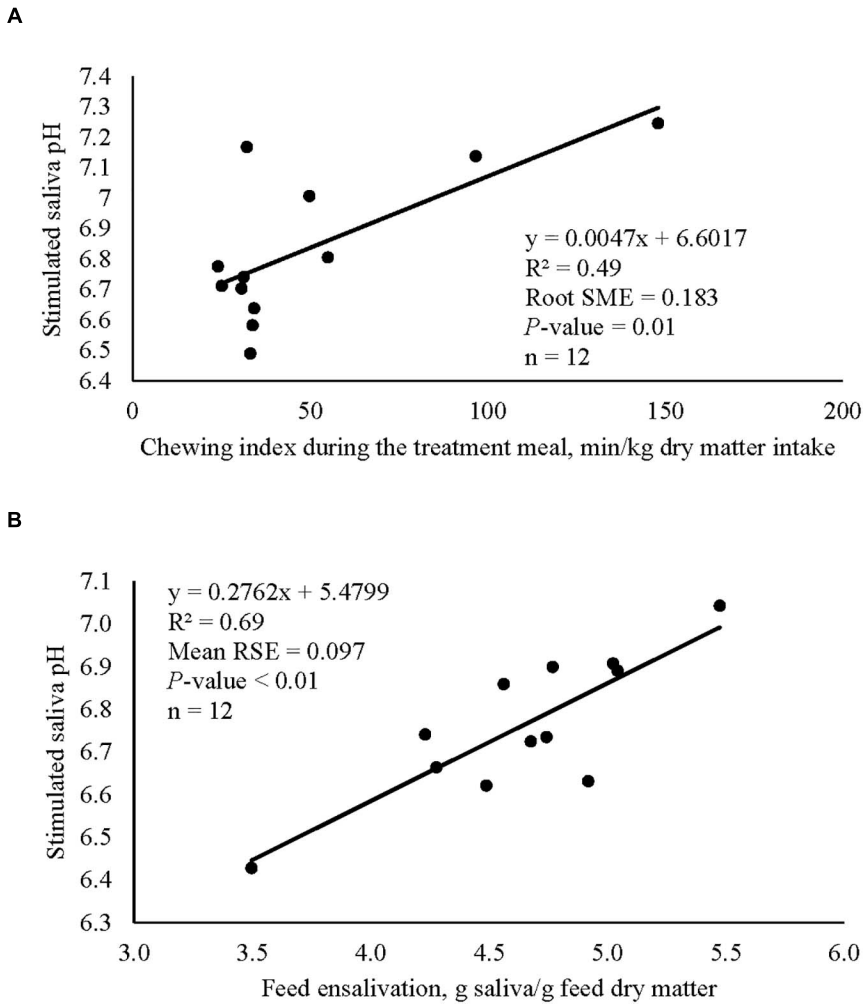

FIGURE 3 | Regression plots showing the association between stimulated salivary pH of cows and chewing index during the meal **(A)**, plot includes the averaged values of capsaicin and thyme oil, and the association between stimulated salivary pH and feed ensalivation **(B)**, plot includes the averaged values of capsaicin, garlic oil, gentian root, and mint oil.

reported the antimicrobial activity to be enhanced in anaerobic conditions and at pH as low as 5.5. Thymol, which is a component of thyme oil, showed a similar pattern and decreased both stimulated and unstimulated saliva pH. This compound has also been shown to have antimicrobial activity, especially in anaerobic conditions and lower pH (Juven et al., 1994; Evans and Martin, 2000; Nagoor Meeran et al., 2017). Thus, the reduction of saliva pH may have beneficial effect for the reinforcement of the antimicrobial activity of these components when fed to cattle. Other compounds that tended or significantly decreased unstimulated salivary pH were L-menthol, capsaicin and gentian root. This change in pH may confer additional benefits to saliva. In fact, saliva contains specific proteins, e.g., proline-rich proteins, amylases, histatins, and cystatins, that interact with plant polyphenols, such as tannins (Hofmann, 1989; Bennick, 2002; Lamy et al., 2011). These potentially harmful compounds are found in different plants, and can be bound only at certain pH levels (Bennick, 2002). Furthermore, Friedman and Jürgens (2000) demonstrated that some polyphenols have their structure irreversibly altered by variations in pH. Therefore, the phytogenic compounds in this study could have triggered a defensive reaction resulting in lowered unstimulated salivary pH (Lamy et al., 2011).

Many of the substances tested in our study influenced saliva flow, ensalivation, or ensalivation per unit of metabolic weight of cows. Overall, there was an augmentation in saliva flow rate to the rumen when the size of the ingested boli increased, as shown by the regression analysis. Capsaicin was responsible for the strongest effects on salivation dynamics. The low dose of capsaicin increased salivation rate and feed bolus size, suggesting that a low dose of this compound may promote the eating stimulus in ruminants. Conversely, the high dose reduced saliva flow, but increased feed ensalivation when expressed as g/g DM feed as well as per unit of metabolically active body tissue. Capsaicin is a compound that derives from chili peppers, and reacts with specific sensors, responsible for pain sensations

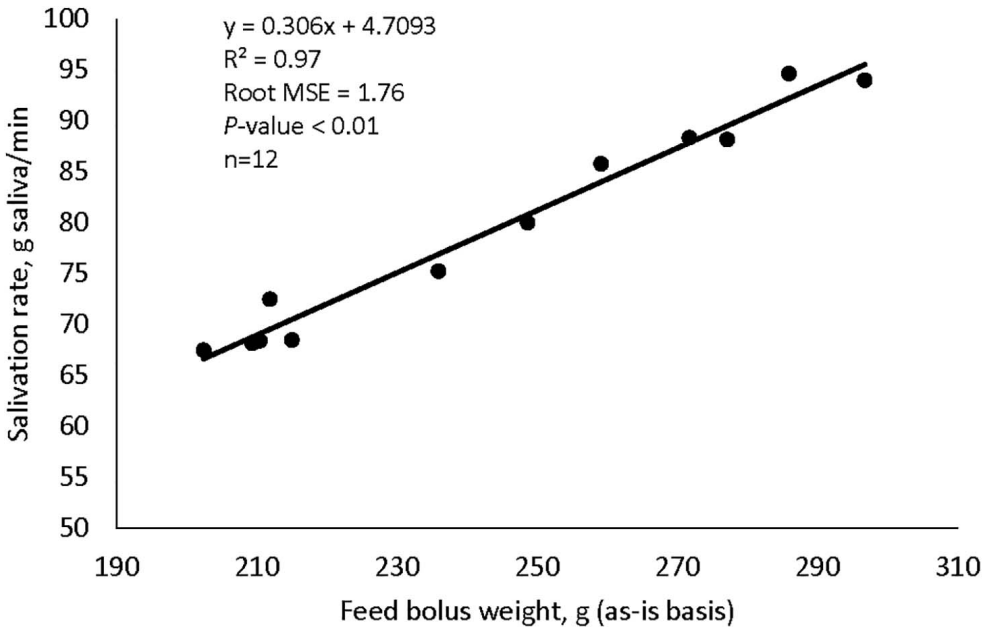

FIGURE 4 | The influence of feed bolus weight on the salivation rate in cattle fed concentrate-rich diets, plot includes values across all nine phytogenic compounds.

(Caterina et al., 1997); this could have contributed to reduce feed intake with the high dosage. Gardner et al. (2020) found a similar increment in saliva flow rate after stimulation with capsaicin in humans. However, the effect was limited to the time that the subjects kept the treatment in the mouth and vanished soon after. Thus, the finding implies that capsaicin exerts a stimulatory effect on salivation when present in the mouth or during mastication. This effect would be particularly important in cows given the time they spend chewing the regurgitated digesta contents during rumination. Therefore, results from this study suggest the potential of a low dose of capsaicin to improve not only salivation, but also physico-chemical properties of saliva, and hence, a potential benefit on rumen pH regulation in cattle fed high-concentrate diets. Saliva flow rate to the rumen was also enhanced by ginger extract. Ginger is a root widely studied and known for its beneficial properties to the gastrointestinal tract (Ghayur and Gilani, 2005; Nikkhah Bodagh et al., 2019; Wang et al., 2020). Mardani et al. (2017) demonstrated a positive effect of ginger on saliva production in human patients affected by xerostomia. Our results indicate that ginger, possibly by the action of its active ingredients such as gingerol and shogaol, may display a similar effect in cattle. Given its cholinergic agonist-like activity, ginger probably stimulates the parasympathetic receptors in salivary glands to increase salivation (Ghayur and Gilani, 2005; Carpenter, 2013). The concomitant increase in the size of ingested boli also suggests an influence of the amount of ginger ingested on salivation stimulus.

L-menthol was another compound that tended to increase salivation in this study, and interestingly its low dose increased mean ruminal pH post feeding in our companion study (Castillo-Lopez et al., 2021b). Menthol has several isomers, and the L-(-) form has the strongest organoleptic properties and is the most common in nature (Eccles, 1994). Menthol is also the main component of mint essential oil. In our study, mint oil tended to increase salivation rate, and the low dose increased stimulated saliva phosphate. Mint flavor has been reported to increase saliva flow rate in humans (Davies et al., 2009). Specifically, *Mentha* species have been proven to inhibit acetylcholinesterase, an enzyme involved in the catabolism of neurotransmitters. This enzyme is present and active in bovine salivary glands, and its inhibition increases saliva flow rate (Liston et al., 2004; de Sousa Barros et al., 2015). Thus, results indicate that these phytogenic compounds may have a similar effect in cattle. It is suggested that menthol could have a direct effect on the olfactory-salivary reflex, given its strong aroma (Lee and Linden, 1992; Eccles, 1994; Haahr et al., 2004). However, the exact mechanism underlying the stimulation of saliva production by these compounds is not known yet, and in fact, the low dosage of mint oil reduced the ensalivation per unit of metabolic weight. Similarly, the tendency for the enhanced salivation with gentian root may be due to its organoleptic properties, and in particular to its bitterness. It is known that bitter taste stimulates specific neurons, that induce saliva flow (Matsuo et al., 2001; Travers and Geran, 2009; Mirzaee et al., 2017). In general, our findings for feed ensalivation are comparable with reported values in cattle (Beauchemin et al., 2008), but observations for salivation rate are lower compared to previous findings (Maekawa et al., 2002). Likely, differences in salivation may reflect dietary composition (Keesman et al., 2016), diet dry matter, animal age or animal physiological stage. For example, compared to our results, Chibisa et al. (2016) found greater flow rates of saliva with diets containing grater levels of dry matter, and using continental crossbred heifers as experimental unit.

The change in salivary osmolality when some of the compounds were supplemented may be due to their influence on ion channels. For example, angelica root contains components, such as ligustilide, that has been demonstrated to affect ion channels in other species (Huang et al., 2019). It is thus possible that this compound influenced the exchange channels in the oral cavity of the cows, disrupting the osmotic balance and consequently decreasing the ion content in saliva (Carr and Titchen, 1978). Nonetheless, long-term effects of the change in osmolality warrants further research.

CONCLUSION

Phytogenic compounds tested in this study influenced salivation and salivary composition. Overall, most phytogenic compounds showed potential to enhance saliva flow or physico-chemical properties, and some of these effects were consistent with observed ruminal fermentation profile reported in the companion study. Thus, our results suggest the efficacy of these substances to contribute to animal health and to modulate ruminal fermentation in cattle fed concentrate-rich diets. Further research is needed to better understand and identify the mode of action of the phytogenic compounds. In particular, it would be important to investigate the mechanism of action of those compounds displaying an enhancement in salivary physico-chemical properties or dynamics. Given the dose-dependent responses observed for some of the compounds, it would also be essential to test a combination of these phytogenic compounds at specific doses, to determine any synergistic effects while avoiding undesired outcomes. Additionally, research is needed to evaluate long-term effects on health as well as milk production in dairy cattle fed high-concentrate diets. Our findings can provide an important starting point for further research to elucidate saliva dynamics in animals with high dry matter intake and a more complex metabolic status, such as high-producing dairy cows.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Ethics and Animal Welfare Committee of the University of Veterinary Medicine Vienna and the Austrian National Authority.

AUTHOR CONTRIBUTIONS

QZ acquired funding and led the CD laboratory. QZ, RP, and NR designed the experiment, that was performed by SR, RR-C, RP, and EC-L. AS-A and SS performed the lab analysis. EC-L analyzed the data. SR and RR-C wrote the manuscript, that was read and approved by all authors.

FUNDING

This research received funding by Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development. Biomin GmbH supports financially the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock.

ACKNOWLEDGMENTS

We would like to thank the staff at the vetFARM for their help and assistance during the experiment. In particular, we thank E. Draxler and T. Enzinger for their appreciated help. We also thank A. Kaltenegger, F. Penagos-Tabares, and M. Mahmood for assisting during the experimental procedures. Our thanks go also to the laboratory staff at the institute of animal nutrition, A. Dockner and S. Leiner, and to B. Khorrami and M. Hollmann for their help with lab analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.645529/full#supplementary-material

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Conflict of Interest: NR was employed by the company BIOMIN Holding GmbH.

The authors declare that this study received funding from BIOMIN Holding GmbH. The funder had the following involvement with the study: processed and provided phytogenic compounds that were evaluated. The funder was not involved in collection, analysis, or interpretation of data.

4.2.Publication 2

Supplementing a phytogenic feed additive modulates the risk of subacute rumen acidosis, rumen fermentation and systemic inflammation in cattle fed acidogenic diets.

Rivera-Chacon, R.; Castillo-Lopez, E.; Ricci, S.; Petri, R.M.; Reisinger, N.; Zebeli, Q.

Animals (Basel). 2022; 12(9):1201

doi.org/10.3390/ani12091201

animals

MDPI

Article

Supplementing a Phytogenic Feed Additive Modulates the Risk of Subacute Rumen Acidosis, Rumen Fermentation and Systemic Inflammation in Cattle Fed Acidogenic Diets

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Simple Summary: The present study evaluated the hypothesis that phytogenic supplementation in the diet will reduce the negative impacts of subacute ruminal acidosis and modulate rumen fermentation. A control group of cows with no supplementation was compared to a group supplemented with 0.04% (DM basis) of a phytogenic feed additive. We observed that after the high-concentrate diet was implemented with the phytogenic blend based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*), the mean ruminal pH increased and the time for pH to reach below 5.8 decreased during the last two weeks of the experiment. Phytogenic feed supplementation also increased ruminal acetate and butyrate and reduced propionate, promoting more stable rumen fermentation compared to no supplementation (Control). Acute phase proteins decreased with the phytogenic feed additive from week 3 of high concentrate feeding. Nevertheless, liver enzymes did not seem to be affected by supplementation. Our study demonstrated that acidogenic diets supplemented with a phytogenic compound can reduce the risk of subacute ruminal acidosis.

Abstract: Feeding with high-concentrate diets increases the risk of subacute ruminal acidosis (SARA). This experiment was conducted to evaluate whether supplementing a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) (PHY) can amend the ruminal fermentation profile, modulate the risk of SARA and reduce inflammation in cattle. The experiment was designed as a crossover design with nine non-lactating Holstein cows, and was conducted in two experimental runs. In each run, cows were fed a 100% forage diet one week (wk 0), and were then transitioned stepwise over one week (0 to 65% concentrate, wk adapt.) to a high concentrate diet that was fed for 4 weeks. Animals were fed diets either with PHY or without (CON). The PHY group had an increased ruminal pH compared to CON, reduced time to pH < 5.8 in wk 3, which tended to decrease further in wk 4, reduced the ruminal concentration of D-lactate, and tended to decrease total lactate (wk 3). In wk 2, PHY increased acetate, butyrate, isobutyrate, isovalerate, and the acetate to propionate ratio compared to CON. Phytogenic supplementation reduced inflammation compared to CON in wk 3. Overall, PHY had beneficial effects on ruminal fermentation, reduced inflammation, and modulated the risk of SARA starting from wk 3 of supplementation.

Keywords: rumen pH; high concentrate; fermentation; cattle

Citation: Rivera-Chacon, R.; Castillo-Lopez, E.; Ricci, S.; Petri, R.M.; Reisinger, N.; Zebeli, Q. Supplementing a Phytogenic Feed Additive Modulates the Risk of Subacute Rumen Acidosis, Rumen Fermentation and Systemic Inflammation in Cattle Fed Acidogenic Diets. *Animals* **2022**, *12*, 1201. https://doi.org/10.3390/ani12091201

Academic Editor: Juana Catarina Cariri Chagas

Received: 22 March 2022
Accepted: 3 May 2022
Published: 6 May 2022

1. Introduction

Feeding energy-dense diets with high levels of concentrates is necessary to meet the energy requirements and support the production performance of dairy cattle. High concentrate diets typically contain elevated levels of starch and low physically effective fiber (peNDF) [1]. Large amounts of starch are rapidly fermented into lactate and other short-chain fatty acids (SCFA) such as propionate, which is an important glucogenic precursor [2,3]. These events coupled with decreased salivary secretions as a result of low dietary peNDF may lead to decrease ruminal pH. Ruminal pH is crucial for the sustained activity of rumen microbiota [4]. A regular and intermittent reduction in ruminal pH, that typically starts around 4–8 h after the main meal of the day and lasts for several hours a day increases the risk of subacute ruminal acidosis (SARA) [5]. SARA often leads to systemic inflammation and increased odds for various health disorders in cattle [6]. The mechanisms behind SARA-induced systemic inflammation are not well understood, but it is believed that the drop in the pH combined with the release of microbial-derived toxins in the rumen increases the permeability of the rumen epithelium [7]. Once in the host bovine systemic circulation, microbial-derived toxins activate the proinflammatory cascade, leading to an increased secretion of inflammation markers such as serum amyloid A (SAA) and haptoglobin (Hp) [6,7].

Over the years, research has aimed to develop nutritive prevention strategies against SARA [4,5,8]. Yet, with diets containing more than 25% starch, as typically fed during lactation, SARA prevention is extremely difficult [1,9]. In such dietary conditions, the inclusion of various feed additives including phytochemicals have been shown to influence rumen fermentation, regulate ruminal pH and alter systemic metabolism [10,11]. Specifically, phytogenic additives have shown several benefits, including the potential to modulate rumination and enhance salivary secretions [12–14]. Furthermore, research conducted by our group when testing nine pure phytogenic compounds at two inclusion levels helped to positively influence salivary composition and ruminal fermentation profile [13,14]. However, despite those findings, most research has been limited to short-term effects, and the effects of a long-term supplementation in cows under a high concentrate (HC) challenge remains largely unknown. Therefore, the objectives of this study were to assess the effect of a phytogenic additive supplementation in cattle transitioned from a forage to HC diet and fed for four consecutive weeks on ruminal pH, SARA risk, SCFA profile, ammonia, lactate, and several biomarkers of inflammation and liver health. Our hypothesis states that supplementation with the phytogenic additive will mitigate the negative impacts of HC feeding by modulating ruminal pH and reduce lactate production, thus reducing the risk of SARA and associated systemic inflammation.

2. Materials and Methods

2.1. Animals, Experimental Design, Treatments and Management

The experiment used nine rumen-cannulated (Bar Diamond, Parma, ID, USA) non-lactating Holstein (992 ± 72.6 kg and 10 ± 0.8 years) cows in a cross-over experimental design with two experimental runs. The animals were group-housed at the research dairy farm of the University of Veterinary Medicine, Vienna (Pottenstein, Lower Austria). Cows were blocked in two groups with four and five animals based on body weight and randomly assigned to either a control diet (CON) with no supplementation or a diet supplemented with 0.04% (DM basis) of a phytogenic feed additive in powder form based on a blend of spices, extracts and herbs including L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) (PHY; Digestarom®, DSM GmbH, Grenzach-Wyhlen, Germany). Prior to the start of the experiment, the cows were adapted to the feeders with a forage-only diet for two weeks. Each experimental run consisted of six weeks. During the first week of each run (wk 0), the cows were fed a forage-only diet (F) including grass silage, corn silage and hay (Table 1). Then, the cows were transitioned step-wise during one week by increasing the proportion of concentrate in the total mixed ration (TMR) by 10% daily. The high concentrate (HC) TMR contained (DM basis) 26.25%

grass silage, 8.75% corn silage, and 65% pelleted concentrate (Table 1), which was fed for four weeks. During wk 0 of forage feeding, the mineral and vitamin mix either with or without the phytogenic additive were dosed through the ruminal cannula of treatment cows. Throughout the adaptation (diet transition), the amount of supplementation dosed through the ruminal cannula was adjusted according to the level of dietary concentrate inclusion. Once cows consumed the high-concentrate diet, the mineral and vitamin mix was combined with the corresponding concentrate, and then integrated in the TMR. The level of inclusion of the PHY or CON supplementation was 0.04% of the TMR. The diets were formulated to meet or exceed the nutrient requirements of a dry cow (GfE, 2001). There was a washout period between experimental runs that lasted 4 weeks; during which, cows were fed only hay.

Table 1. Ingredients, chemical composition, particle size distribution of diets fed to cows during the week of forage feeding and the high concentrate feeding period.

Item	Diet and Treatment [1]		
	Forage Diet	High Concentrate Diet	
		CON	PHY
Ingredients (% of DM)			
Grass silage	75	26.25	26.25
Corn silage	15	8.75	8.75
Grass hay	10	0	0
CONTROL concentrate [1]	0 *	65	0
TREATMENT concentrate [2]	0 *	0	65
Chemical composition (% of DM unless stated)			
DM, % as fresh	32.4 ± 5.16	45.1 ± 0.83	44.0 ± 2.09
Crude protein (CP)	17.2 ± 1.08	19.6 ± 0.80	19.3 ± 1.15
Neutral detergent fiber (NDF)	50.4 ± 1.58	30.2 ± 2.09	31.6 ± 2.44
Acid detergent fiber (ADF)	36.6 ± 6.39	19.9 ± 2.12	20.0 ± 1.89
Starch	4.2 ± 1.3	28.9 ± 1.85	28.0 ± 1.59
Ether extract (EE)	2.9 ± 1.32	3.2 ± 0.16	3.2 ± 0.21
Non-fiber carbohydrates	18.4 ± 0.47	39.5 ± 1.85	39.0 ± 1.83
Ash	11.0 ± 1.87	6.8 ± 0.26	6.7 ± 0.25
Particle fraction (% retained) [3]			
Long	86.7	27.8 ± 4.95	29.2 ± 6.57
Medium	5.54	29.3 ± 1.74	29.7 ± 2.55
Short	7.30	20.3 ± 2.20	18.8 ± 3.21
Fine	0.50	1.4 ± 0.93	1.1 ± 0.80
pef [4] $_{>8\,mm}$	0.92	0.6 ± 0.02	0.6 ± 0.04
peNDF [5] $_{>8\,mm,}$ % of DM	47.5	17.3 ± 0.71	18.6 ± 0.25

[1] CON: The CON pelleted concentrate mixture contained: barley (30.22%), triticale (18.1%), bakery by-product (23.08%), rapeseed meal (24.0%), molasses (3.0%), mineral-vitamin premix for dairy cattle (0.8%), limestone (0.5%), and salt (0.3%); [2] PHY: The pelleted PHY concentrate mixture contained: barley (30.22%), triticale (18.04%), bakery by-product (23.08%), rapeseed meal (24.0%), molasses (3.0%), mineral-vitamin premix for dairy cattle (0.8%), limestone (0.5%), and salt (0.3%). In addition, it was formulated to provide 0.04% of a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) in the TMR; * 100 g of mineral and vitamin mix (16% Ca, 8% P, 11.5% Mg, 2.2% Na, 16.2 g Mn, 24 g Zn, 3.6 g Cu, 0.27 g Co, 0.54 g I, 0.13 g Se, 2300 kIU Vit A, 240 kIU Vit D, 5 g Vit E, 2 g Vit B1 per kg feed) without (CON) or with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) (PHY) was added in the rumen through the ruminal cannula before morning feeding; [3] Particle fractions determined by Penn State Particle Separator with a 19 mm screen (long), 8 mm screen (medium), 1.18 mm screen (short), and a pan (fine) according to Kononoff et al. [15]; [4] Physical effectiveness factor; [5] Physically effective NDF.

During the experiment, cows were housed in a free-stall barn with deep litter cubicles (2.6 × 1.25 m, straw litter) and free-choice mineral blocks. Water and feed was available for ad libitum consumption. The TMR was prepared once a day using an automatic feeding system (Trioliet Triomatic T15, Oldenzaal, The Netherlands), and offered in individual feeding troughs to the cows at approximately 0800 h. Individual feed intake was continuously controlled and recorded as feed bunks were equipped with electronic scales and

computer-regulated access gates (Insentec B.V., Marknesse, The Netherlands) and checked daily before discarding the refusals. With the purpose of increasing palatability, and due to the low proportion of moisture of the TMR, water was added during mixing with a targeted DM content of approximately 46%. The ethical consent number of the present experiment was BMBWF-68.205/0003-V/3b/2019.

2.2. Feed Sampling and Chemical Analysis

The dry matter concentration of the TMR was determined every day by drying samples at 100 °C for 24 h. Individual feed samples were collected at the beginning and at the end of each experimental run, and samples of TMR were collected once a week for chemical composition. All nutrient analyses of feed samples were evaluated in duplicate according to the German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA; Naumann and Bassler) [16]. The DM of wet feed samples was estimated by forced-air drying at 55 °C for 48 h and the residual water was subsequently analyzed by oven drying at 105 °C for 4 h (method 3.1). Ash was determined by combustion in a furnace oven at 580 °C overnight (method 8.1). Crude protein (CP) was estimated with the Kjeldahl method [16] and ether extracts (EE) using the soxhlet extraction system (Extraction System B-811, Büchi, Flawil, Switzerland). Similarly, for NDF and ADF (methods 6.5.1 and 6.5.2, respectively) concentrations were estimated with sodium sulfite and reported exclusive residual ash following the official analytical methods [16] using the Fiber Therm FT 12 (Gerhardt GmbH & Co. KG, Königswinter, Germany) with heat-stable α-amylase for NDF analysis. Starch content was measured with K-TSTA kit (Megazyme Ltd., Wicklow, Ireland). Non-fiber carbohydrates content was calculated as 100 − (% CP + % NDF + % EE + % ash). Acid detergent lignin was determined gravimetrically after ADF separation with 72% sulfuric acid.

Particle size distribution of the HC diets was determined according to Kononoff et al. [15] with a modified Penn State Particle Separator that included three screens (19.0, 8.0, and 1.18 mm) and a pan, with a minor adjustment. Briefly, because a certain portion of the concentrate pellets stayed on the 8 mm screen, after analysis for particle size distribution, an adjustment factor was applied to correct these values by hand picking the remaining pellets from the 8 mm screen and transferring them to the 1.18 mm screen. With this data, physically effective NDF (peNDF) and the physically effectiveness factor (pef) were calculated as outlined by Beauchemin and Yang [17]. The peNDF concentration of the diet was determined with the multiplication of NDF content of the diet by its pef. The pef (ranged from 0 to 1) was calculated as the sum of the proportion of particles retained on the corresponding sieves (19.0 and 8.0 mm sieves for pef > 8 mm).

2.3. Measurements of Ruminal pH and Monitoring of SARA

Ruminal pH was continuously measured using the Lethbridge Research Centre Ruminal pH Measurement System (LRCpH; Dascor Inc., Oceanside, CA, USA) placed in the rumen ventral sac as outlined by Penner et al. [18]. Ruminal pH data were downloaded, and systems were calibrated every week using a pH 4 and 7 solution and were programmed to record pH every 15 min. To monitor the risk of SARA, we calculated minimum, mean and maximum ruminal pH, the difference between maximum and minimum pH, the time at which ruminal pH was below 5.8 and 6.0, and the area below ruminal pH 5.8 and 6.0, as SARA indicators [5,6]. Additionally, a SARA index was calculated using two approaches. First, by calculating the time that ruminal pH was below 5.8 per kg DMI, and then by calculating the area for which pH was below 5.8 per kg of DMI [19].

2.4. Collection of Ruminal and Reticular Fluid and Analysis

Approximately 10 mL of reticular fluid was collected manually through the rumen cannula with a disposable 20 mL syringe. After the rumino-reticular fold was reached and passed, the sample was aspirated from the ventral region of the reticulum. Ruminal fluid samples were collected from the ventral sac of the rumen. Samples were transferred

to 15-mL vials and immediately frozen at −20 °C. At the end of experimental samplings, SCFA measurements were conducted following Qumar et al. [20] with minor modifications. Briefly, reticular and ruminal samples were thawed overnight at 4 °C, centrifuged at 3220× *g* for 20 min and the supernatant was used for further analysis. Then, 200 µL of distilled water, the internal standard 4-methylvaleric acid (Sigma-Aldrich, St. Louis, MO, USA) and 200 µL of 1.8 mol hydrochloric acid were added to 600 µL of supernatant. Samples were vortexed and then centrifuged at 20,000× *g* for 20 min at 4 °C. The clear supernatant was transferred into glass vials for the gas chromatograph. The analysis was conducted using a gas chromatography apparatus (Shimadzu GC Plus with FID detector) which was equipped with 30 m × 0.53 mm ID × 0.53 µm capillary column (Trace TR Wax, Thermo Fisher Scientific, Waltham, MA, USA). The injector and detector had temperatures of 170 °C and 220 °C, respectively. The gas used as carrier was Helium with a flow rate of 6 mL/min. Additionally, ruminal and reticular ammonia was determined using the indophenol reaction [21], and a lactate analysis was conducted following the Megazyme K-DATE assay (Megazyme Ltd., Wicklow, Ireland).

2.5. Blood Sampling and Analysis of Systemic Health Biomarkers

Blood samples were collected on a weekly basis before the morning feeding from the jugular vein; serum was obtained using 9-mL vacutainer tubes (Vacuette; Greiner Bio-One, Kremsmünster, Austria). Acute phase proteins concentration analyses including Hp and SAA were determined using a Tridelta phase range Multispecies SAA ELISA kit (Tridelta Development Ltd., Greystones, Co., Wicklow, Ireland), SAA serum samples were diluted 1:500 and samples with optical density values above the standard curve were diluted again (1:400 or 1:250) and analyzed once more. No dilution of serum was needed for Hp measurement. Liver enzymes including alkaline phosphatase (ALP), aspartate aminotransferase (AST), glutamate dehydrogenase (GLDH), and gamma-glutamyl transferase (GGT) were measured with a conventional large-scale analyzer for clinical chemistry at the laboratory of the Central Clinical Pathology Unit, University of Veterinary Medicine, Vienna. The standard enzymatic colorimetric analyses with a fully automated autoanalyzer for clinical chemistry (Cobas 6000/c501; Roche Diagnostics GmbH, Vienna, Austria) was used. The intra-assay variation was controlled by limiting the coefficient of variation to ≤10% for SAA and <5% for other blood variables.

2.6. Statistical Analysis

Statistical analyses were performed according to a crossover design [22] using the PROC MIXED of SAS (version 9.4; SAS Institute, Cary, NC, USA). Data were first checked for outliers using the Cook's D with a 0.08 threshold used for outliers, which were removed from further analyses. The normality of data was evaluated with the PROC UNIVARIATE followed by the normal and plot options. If the normality assumption was not met, PROC TRANSREG performing a Box-Cox was used to determine the transformation mode, which was performed before the ANOVA. The statistical model included the fixed effects of run, diet, and treatment supplementation, as well as the interactions diet × week × treatment. The cow within the run was considered as a random effect, whereas data obtained from the same cow in different times were processed as repeated measures in the ANOVA, with a first order variance-covariance structure matrix, taking into consideration that the covariance decays with time. Data are reported as LSM and the transformed data were transformed back after the ANOVA. The largest standard error of the mean (SEM) is reported. Statistical significance was declared when $p \leq 0.05$ and tendency is discussed if $0.05 < p \leq 0.10$. For a better visualization of the SCFA profile and fermentation pattern occurring in the rumen and reticulum, boxplot figures were constructed for data of ruminal and reticular individual SCFA with R [23] and using the ggplot2 package version 3.3.5 [24].

3. Results

3.1. Ruminal pH and SARA

Data showed a decreased ruminal pH after switching the diet from F to HC, and the ruminal pH depression was maintained throughout the experiment, as indicated by various SARA indicators measured (Table 2). However, in both wk 3 and 4 of HC feeding, the PHY supplementation reduced the risk of all SARA indices measured. In particular, supplementation increased mean and minimum ruminal pH compared to CON ($p < 0.05$). Furthermore, the supplemented cows showed a shorter time below 5.8 in both wk 3 and 4 of HC (148 vs. 287 min in wk 3, and 196 vs. 330 min in wk 4 for PHY and CON, respectively) and the area with pH < 5.8 tended to be lower for PHY compared to CON in wk 3 HC ($p = 0.10$). Consequently, the SARA index (time pH < 5.8/kg DMI) was lower for PHY compared to CON ($p < 0.05$) in wk 3 and 4 of HC feeding and the SARA index (area pH < 5.8/kg DMI) was lower for PHY compared to CON ($p < 0.05$) in wk 3 (Table 2). Additionally, PHY feed additive tended to decrease DMI compared to CON.

Daily ruminal pH oscillation was also impacted by diet composition, showing a larger variation for the HC compared to forage feeding ($p < 0.01$; Figure 1). During forage feeding, rumen pH oscillated between 6.72 and 6.34 at the time of feeding and 12 h later, respectively. During the fourth day of diet transition, ruminal pH ranged between 6.29 and 5.83 at 1 h prior and 13 h after feeding, respectively. The data showed that rumen pH peak was reached at the time of feeding whereas a low and relatively stable pH occurred 12 h after feeding. (Figure 1B–F). An improved response of ruminal pH in wk 3 and 4 of HC feeding was observed during the day (Figure 1E,F).

3.2. Ruminal and Reticular Short Chain Fatty Acids Profile

Data of SCFA profile indicated that there was an effect of the concentrate level ($p < 0.01$) on ruminal SCFA concentration (Table 3). Specifically, the total SCFA concentration increased by approximately 28% during HC feeding compared to wk 0 (forage feeding), with a maximum concentration of SCFA observed in wk 2 on HC diet consumption averaging 120.8 mM, independent of PHY supplementation ($p = 0.85$).

There was an increase ($p < 0.01$) in ruminal acetate, butyrate, isobutyrate, and isovalerate as well as a decrease ($p < 0.01$) in ruminal propionate with diet change. Interestingly, there was an interaction between diet, week of feeding and PHY supplementation on ruminal acetate, propionate, isobutyrate and isovalerate, with PHY supplementation displaying an increase in acetate from 52.6 to 55.4%, butyrate from 12.0 to 14.0%, isobutyrate from 0.80 to 1.0%, isovalerate from 1.43 to 1.90% and a decrease in propionate from 29.5 to 24.1% compared to CON in wk 2 of HC feeding. Moreover, PHY increased isovalerate from 1.65 to 2.08% in wk 3 compared to CON. Nevertheless, PHY increased the acetate to propionate ratio in wk 2 HC ($p < 0.05$) (Table 3).

Time post-feeding ($p < 0.05$) also showed an effect on total and individual proportions of SCFAs, with a reduction in acetate from 0 to 12 h after feeding, an increase in the proportion of propionate from 0 to 12 h post-feeding in wk 1 and 2 for both CON and PHY, wk 3 for CON and wk 4 for PHY. The proportion of butyrate increased with time post-feeding in wk 0 for both CON and PHY; this variable also increased post-feeding in wk 1 and 3 for CON and PHY, respectively (Figure 2).

Table 2. Effect of supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) on ruminal pH parameters in cows consuming a forage diet or a high concentrate diet [1].

Item	Forage Diet Week 0		High Concentrate Week 1		High Concentrate Week 2		High Concentrate Week 3		High Concentrate Week 4		*p*-Values [3]			
	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM [2]	D	T	I
DMI, kg	8.43	7.57	13.31	12.85	13.93	13.09	14.09	13.32	12.91	11.77	0.602	<0.01	0.08	0.23
Maximum pH	6.89	6.82	6.64	6.60	6.71	6.66	6.60	6.64	6.59	6.66	6.7×10^{-11}	<0.01	0.76	0.10
Minimum pH	6.30	6.31	5.61	5.60	5.50	5.44	5.52 ^{b}	5.66 ^{a}	5.51 ^{b}	5.67 ^{a}	9.2×10^{-10}	<0.01	0.12	<0.01
Mean pH	6.58	6.56	6.04	6.05	6.03	6.00	6.02 ^{b}	6.15 ^{a}	6.02 ^{b}	6.16 ^{a}	2.8×10^{-10}	<0.01	0.17	<0.05
Difference *	0.55 ^{a}	0.48 ^{b}	1.01	0.99	1.22	1.22	1.05	0.96	1.07	0.99	0.042	<0.01	<0.05	<0.01
Dur [4] 6.0, min *	2.45	0.31	581.2	651.9	620.5	662.4	538.3 ^{a}	364.2 ^{b}	653.1 ^{a}	410.9 ^{b}	2.12	<0.01	<0.05	<0.01
Dur [4] 5.8, min *	1.27	0.62	239.6	244.2	304.7	349.1	286.8 ^{a}	148.4 ^{b}	330.1 ^{x}	195.5 ^{y}	2.64	<0.01	0.07	<0.05
Area 6.0, min × pH *	1.13	0.01	143.6	115.6	169.0	184.3	108.3 ^{a}	49.9 ^{b}	146.9 ^{a}	69.67 ^{b}	1.48	<0.01	<0.05	<0.01
Area 5.8, min × pH *	0.20	0.00	68.06	38.89	92.14	89.48	51.87 ^{x}	23.80 ^{y}	70.85	42.72	1.29	<0.01	<0.05	<0.05
Acidosis index, area pH < 5.8/kg DMI *	0.01	0.00	5.16	3.18	7.21	7.44	4.75 ^{a}	1.75 ^{b}	6.99	3.08	0.16	<0.01	<0.05	<0.05
Acidosis index, time pH < 5.8/kg DMI *	0.09	0.02	17.88	18.60	22.82	27.00	20.92 ^{a}	10.83 ^{b}	30.93 ^{a}	13.20 ^{b}	0.25	<0.01	<0.05	<0.01

[1] CON: A control diet containing no phytogenic product; PHY: supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*); [2] The largest standard error of the mean; [3] *p*-Values for the effect of diet (D), phytogenic treatment (T) and the diet × week × treatment interaction (I); [4] Duration (weeks of high concentrate feeding); * Values were transformed using the root square function after checking for normal distribution, and were transformed back after the analysis; ^{a,b} Means with different superscripts indicate a significant difference ($p < 0.05$) between CON and PHY; ^{x,y} Means with different superscripts indicate a tendency for significant differences ($0.05 < p \leq 0.10$) between CON and PHY.

Table 3. Effect of supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) on ruminal short chain fatty acid profile, ammonia and lactate in cows consuming a forage diet or a high concentrate diet [1].

Item	Forage Diet Week 0		High Concentrate Week 1		High Concentrate Week 2		High Concentrate Week 3		High Concentrate Week 4		*p*-Values [3]			
	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM [2]	D	T	I
Total SCFA concentration, mM	86.0	84.0	105	108	116	118	106	98.0	112	114	4.98	<0.01	0.82	0.71
% of total SCFA														
Acetate	67.4	67.8	58.1	56.9	52.6 ^{b}	55.4 ^{a}	57.8	57.1	56.9	57.9	0.76	<0.01	0.57	<0.01
Propionate	15.6	15.1	20.5	21.6	29.5 ^{a}	24.1 ^{b}	23.9	23.3	23.7	23.2	0.70	<0.01	0.08	<0.01
Butyrate	10.5	10.8	16.0	16.7	12.0 ^{b}	14.0 ^{a}	12.6	12.9	12.8	12.8	0.60	<0.01	0.16	0.17
Isobutyrate	1.70	1.80	0.84	0.90	0.80 ^{b}	1.00 ^{a}	1.15	1.18	1.23	1.11	0.07	<0.01	0.49	0.01
Isovalerate	2.17	2.29	1.27	1.30	1.43 ^{b}	1.90 ^{a}	1.65 ^{b}	2.08 ^{a}	1.92	1.78	0.08	<0.01	<0.05	<0.01

Table 3. *Cont.*

Item	Forage Diet Week 0		High Concentrate Week 1		High Concentrate Week 2		High Concentrate Week 3		High Concentrate Week 4		*p*-Values [3]			
	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM [2]	D	T	I
Valerate	1.48	1.45	1.90	1.98	2.22	2.19	2.01	2.06	2.29	2.37	0.08	<0.01	0.70	0.87
Ratio of acetate to propionate	4.39	4.54	2.86	2.77	1.90 ^{b}	2.60 ^{a}	2.46	2.67	2.37	2.68	0.15	<0.01	0.20	<0.01
Ammonia, mg/dL	21.72	20.16	13.53	14.30	9.64 ^{b}	15.35 ^{a}	17.66 ^{a}	13.12 ^{b}	18.26	18.77	1.61	<0.01	0.88	<0.01
Lactate [4]														
D-lactate, mM	0.037 ^{b}	0.062 ^{a}	0.433	0.347	0.386	0.317	1.053 ^{a}	0.765 ^{b}	0.718	0.669	0.0027	<0.01	0.35	<0.01
L-lactate, mM	0.062	0.131	0.259	0.260	0.293	0.231	0.372	0.408	0.363	0.386	0.0010	<0.01	0.29	<0.01
Total lactate, mM	0.118	0.218	0.711	0.618	0.714	0.558	1.539 ^{x}	1.183 ^{y}	1.107	1.089	0.0033	<0.01	0.46	<0.01

[1] CON: A control diet containing no phytogenic product; PHY: supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*); [2] The largest standard error of the mean; [3] *p*-Values for the effect of diet (D), phytogenic treatment (T) and the diet × week × treatment interaction (I); [4] Values were transformed using the root square function after checking for normal distribution, and were transformed back after the analysis; [a,b] Means with different superscripts indicate a significant difference ($p < 0.05$) between CON and PHY; [x,y] Means with different superscripts indicate a tendency for significant differences ($0.05 < p \leq 0.10$) between CON and PHY.

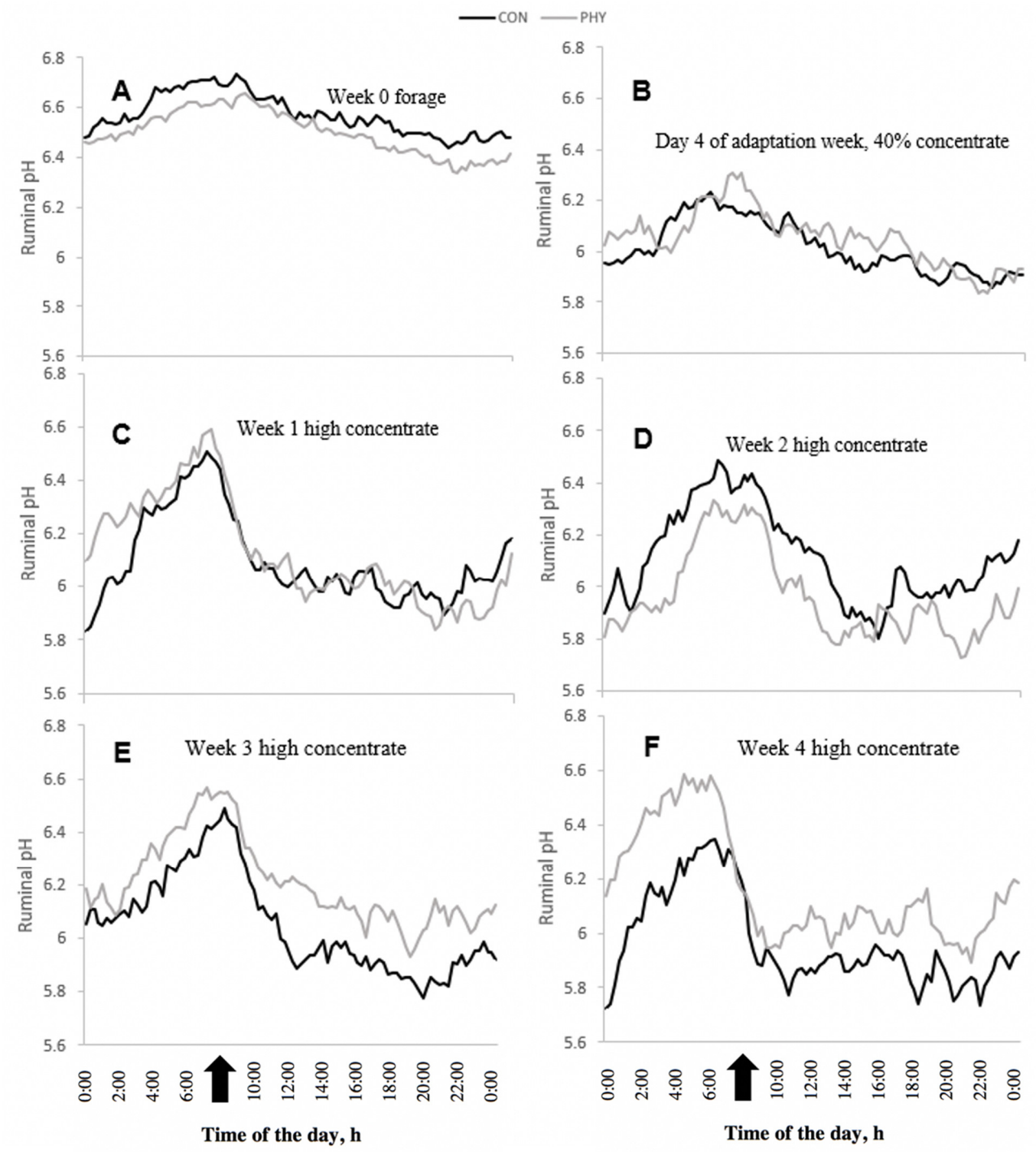

Figure 1. Diurnal variation of ruminal pH in various weeks in cows fed either forage-only or with 65% concentrate in a diet, without supplementation (CON) or supplemented (PHY) with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (Mentha arvensis) and cloves powder (Syzygium aromaticum). Time of feeding: 08:00 h (⬆). (A) Week 0 forage, SEM = 0.05. (B) Day 4 of adaptation week, 40% concentrate, SEM = 0.08. (C) Week 1 of high concentrate, SEM = 0.09. (D) Week 2 of high concentrate, SEM = 0.12. (E) Week 3 of high concentrate, SEM = 0.12. (F) Week 4 of high concentrate, SEM = 0.12. p-Values: Time < 0.01; Week < 0.01; Time × Week < 0.01. SEM: The largest standard error of the mean.

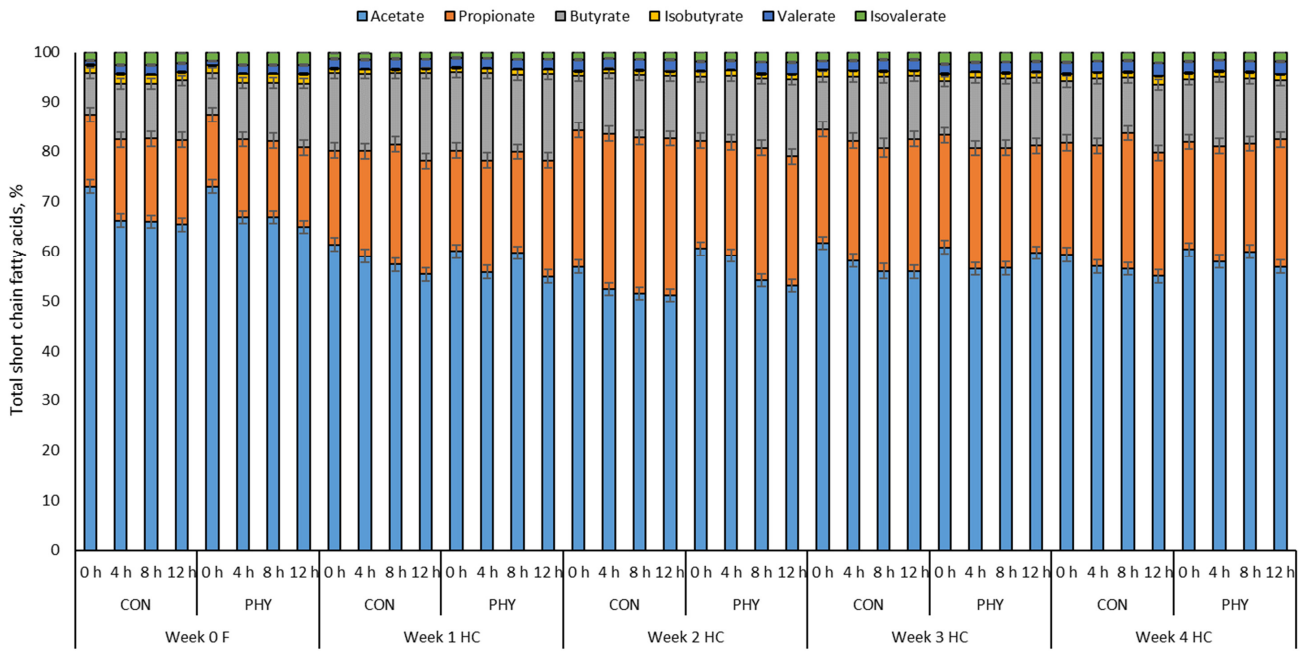

Figure 2. Variation of ruminal short chain fatty acid fermentation from 0 to 12 h post-feeding in cows fed either forage-only (F) or a high concentrate (HC), without supplementation (CON) or supplemented (PHY) with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*). *p*-Values: Acetate, Time < 0.01, Trt = 0.26, Diet < 0.01, Time × Trt × Diet × Week < 0.01; Propionate, Time < 0.01, Trt < 0.05, Diet < 0.01, Time × Trt × Diet × Week < 0.01; Butyrate, Time < 0.01, Trt = 0.23, Diet < 0.01, Time × Trt × Diet × Week < 0.01; Isobutyrate, Time = 0.36, Trt = 0.28, Diet < 0.01, Time × Trt × Diet × Week < 0.01; Valerate, Time < 0.01, Trt = 0.30, Diet < 0.01, Time × Trt × Diet × Week < 0.01; Isovalerate, Time = 0.45, Trt < 0.05, Diet < 0.01, Time × Trt × Diet × Week < 0.01.

In general, the fermentation and SCFA profile in the reticulum followed a pattern similar to that observed in the rumen (Figures S1 and S2). In the reticulum, PHY supplementation increased acetate, butyrate and isovalerate ($p < 0.05$) and decreased propionate ($p < 0.05$) compared to CON in wk 2 HC. Similarly, the A:P ratio increased with PHY compared to CON ($p < 0.05$) in wk 2 HC (Table S1). Influenced by time post-feeding ($p < 0.05$), acetate decreased consistently across weeks. However, propionate increased 12 h after feeding for both groups, and in wk 1 only for CON, whereas the proportion of butyrate increased after feeding in wk 0 and 3 for both CON and PHY, but this fatty acid displayed an increased post-feeding only in wk 2 for PHY (Figure S2).

3.3. Ruminal and Reticular Lactate and Ammonia

There was an increase in ruminal total lactate concentration ($p < 0.05$) when cows consumed the HC ration with average values of 0.17 mM and 1.10 for wk 0 and wk 4 HC, respectively (Table 3). An interaction between diet, week of feeding and PHY supplementation was observed for total and D-lactate concentration with a trend towards decreased total lactate ($p = 0.06$) and a reduction in D-lactate ($p < 0.05$) with PHY supplementation compared to CON in wk 3 HC. In addition, there was an interaction between diet, week of feeding and PHY supplementation for ruminal ammonia with this variable being greater for PHY cows in wk 2 HC ($p < 0.05$) compared to CON. Nevertheless, during wk 3 HC, PHY cows showed lower ruminal ammonia concentration ($p < 0.05$) compared to CON. Time of feeding did not influence total lactate throughout the experiment (Figure 3). However, in wk 3 HC at 0 h, PHY had a lower total lactate concentration ($p < 0.05$) and at 4 h after feeding PHY tended to reduce total lactate compared to CON ($p = 0.06$). Considering the

influence of time of feeding, the total ammonia concentration in the rumen increased with increasing time post-feeding with an exception in wk 2, 4 HC CON, and wk 3, 4 HC PHY.

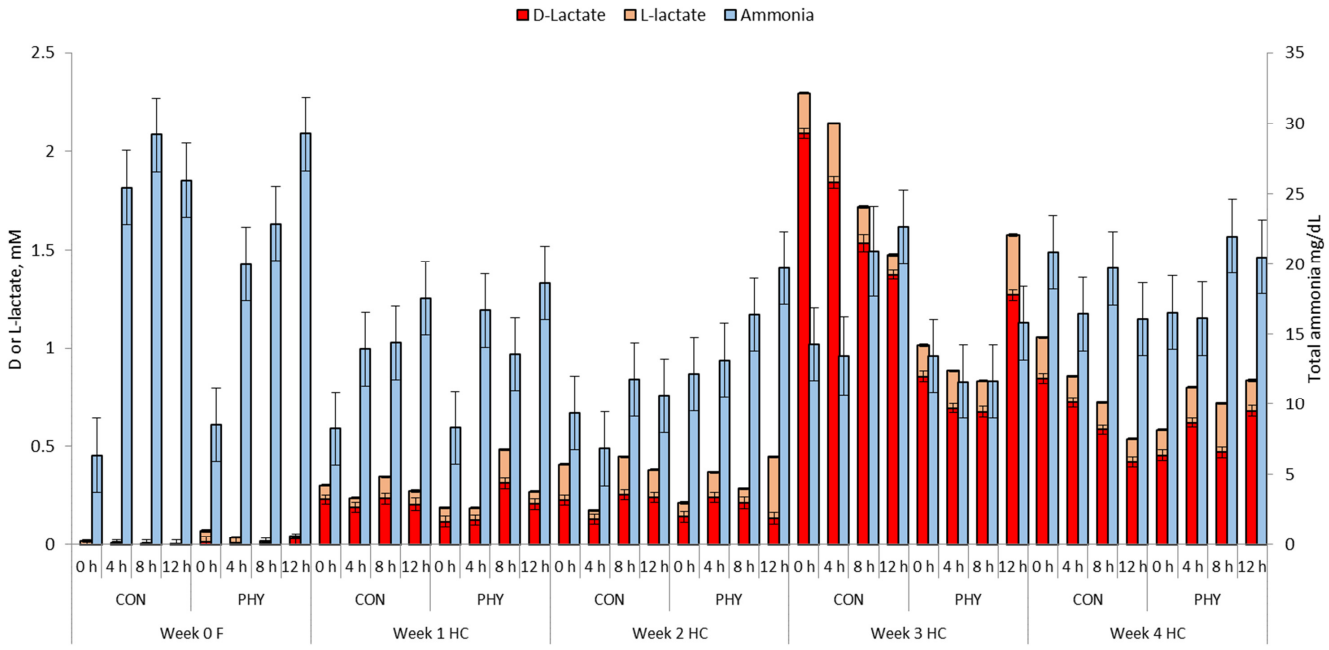

Figure 3. Variation of ruminal D-lactate, L-lactate (mM), and total ammonia concentration (mg/dL) from 0 to 12 h post-feeding in cows fed either all-forage (F) or a high concentrate (HC), without supplementation (CON) or supplemented (PHY) with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*). *p*-Values: D-lactate, Time = 0.99, Trt = 0.42, Diet < 0.01, Time × Trt × Diet × Week < 0.01; L-lactate, Time = 0.63, Trt = 0.60, Diet < 0.01, Time × Trt × Diet × Week < 0.05; Total ammonia, Time < 0.01, Trt = 0.90, Diet < 0.01, Time × Trt × Diet × Week < 0.01.

Total reticular lactate concentration showed a similar pattern to that observed in the rumen, with an overall increment from 0.32 mM (wk 0) to 1.16 mM (wk4). Similarly, PHY supplementation increased reticular ammonia concentration in wk 2 HC ($p < 0.05$), and during wk 3, HC decreased their reticular ammonia concentration compared to CON ($p = 0.08$) (Table S1). Total lactate concentration in reticulum was not influenced by time. However, that was not the case for L-lactate, which increased with time post-feeding, in wk 1 and 4 HC. Similarly, total ammonia increased after feeding, but not in wk 1, 2, 4 HC for CON and 3, 4 HC for PHY (Figure S3).

3.4. Systemic Inflammation and Liver Health Biomarkers

The SAA concentration increased almost 3-fold from forage feeding to HC feeding, whereas the Hp was not different (Table 4). Both APP were influenced by PHY supplementation after 2 weeks in feed. For example, PHY decreased the Hp blood concentration in wk 3 HC ($p < 0.05$) and tended to decrease it in wk 4 HC ($p = 0.08$), whereas PHY supplementation reduced SAA compared to CON in wk 3 HC ($p < 0.05$). The activity of liver enzymes AST, GLDH, and GGT were only influenced by changes in diet composition and increased with HC diet compared to wk 0 ($p < 0.05$); meanwhile, ALP did not change despite diet or supplementation (Table 4).

Table 4. Effect of supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) on liver enzymes and acute phase proteins in cows consuming a forage diet or a high concentrate diet [1].

Item [4]	Forage Diet Week 0		High Concentrate Week 1		High Concentrate Week 2		High Concentrate Week 3		High Concentrate Week 4			*p*-Values [3]		
	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM [2]	D	T	I
Hp, µg/mL	103.0	141.9	142.3	308.6	257.9	452.0	621.2 [a]	90.6 [b]	429.4 [x]	96.4 [y]	1.82	0.12	0.46	0.19
SAA, µg/mL	2.83	2.86	10.25	15.04	19.61	29.85	28.67 [a]	8.87 [b]	12.7	9.14	1.53	<0.01	0.60	0.23
ALP, U/L	7.70	7.10	7.43	8.10	7.59	8.33	8.49	8.20	6.78	7.33	1.10	0.50	0.73	0.27
AST, U/L	67.85	72.80	63.21	66.40	76.28	86.98	98.06	94.92	85.31	98.12	1.08	<0.01	0.32	<0.01
GLDH, U/L	4.80	6.01	5.56	5.22	9.94	10.87	13.26	12.17	10.86	11.67	1.20	<0.01	0.78	<0.01
GGT, U/L	21.21	20.99	21.56	20.89	25.64	25.11	30.78	25.64	29.26	29.59	1.09	<0.01	0.57	<0.01

[1] CON: A control diet containing no phytogenic product; PHY: supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*); [2] The largest standard error of the mean; [3] *p*-Values for the effect of diet (D), phytogenic treatment (T) and the diet × week × treatment interaction (I); [4] Values were transformed using the log function after checking for normal distribution. Hp: Haptoglobin, SAA: Serum Amyloid A, ALP: Alkaline Phosphatase, AST: Aspartate aminotransferase, GLDH: Glutamate Dehydrogenase, GGT: Gamma-Glutamyl Transferase; [a,b] Means with different superscripts indicate a significant difference ($p < 0.05$) between CON and PHY; [x,y] Means with different superscripts indicate a tendency for significant differences ($0.05 < p \leq 0.10$) between CON and PHY.

4. Discussion

In the present study, the HC diet contained an average of 28.5% starch and 18.0% peNDF > 8 mm, which, according to recent recommendations of Khorrami et al. [1], can be considered an acidogenic diet with a high risk of inducing SARA. Our main hypothesis stated that PHY supplementation will reduce the negative effects caused by SARA as the HC challenge is prolonged. In fact, we observed that from wk 3 HC, PHY started to modulate the pH and SARA indices, and this coincides with the decrease in D-lactate in the same week compared to CON. The risk of SARA has been largely characterized in the literature, with Zebeli et al. [5] suggesting a time threshold of 314 min per day with a pH below 5.8. This study also showed that a possible threshold of time when pH drops below 5.8 consistently during the 4 weeks of concentrate feeding could be 310 min per day, considering an average of the highest and lowest time below the SARA threshold and a 25% security margin. Kröger at al. [12] reported similar results, but with only two consecutive weeks of high concentrate after forage feeding and with a plant compound inclusion level of 3 g/cow/day. Other researchers [25] evaluating a mixture containing menthol also included levels from 3–6 g/cow/day. Phytogenic compounds such as menthol, levomenthol, β-linaloolm, anethole, hexadecanoic acid and ρ-menthane have been demonstrated to have similar effects and the potential for increasing rumen fluid pH in cows fed a 50:50 concentrate to forage ratio compared to a control diet [26]. Complementing this finding, Castillo-Lopez [13] reported that menthol tended to reduce the concentration of propionate in the rumen. This may have a positive effect on the consecutive weeks in terms of pH. For example, thymol, the main monoterpene phenol in thyme oil used in the PHY blend, contains p-cymene and γ-terpinene [27], which have demonstrated a positive influence in terms of modulating rumen pH, as well as menthol [28], the main component of essential oils of peppermint. Similarly, eugenol tended and increased ruminal pH in high grain diets [29,30], respectively; suggesting a positive effect on rumen microbial population [31,32]. Therefore, we speculate that the mechanisms by which these phytogenic compounds modulate ruminal fermentation may be through the action of the ruminal microbial community, particularly the acetate- and propionate-producing bacteria, which are largely responsible of fermentation and acid production in the rumen. However, the microbial community was not evaluated in the present study; thus, we are unsure which microbial taxa were affected.

In this study, CON cows experienced severe SARA, as indicated by increased time spent with a pH below 5.8, compared to PHY in wk 3 and 4 of HC. A plausible explanation for the higher mean ruminal pH and decreased SARA indices observed with PHY supplementation in wk 3 of HC feeding is a slower rate of starch degradability in the rumen [33].

This is supported by the lower D-lactate concentration indicating a decreased production of lactate. Our results suggest a positive impact of PHY in mitigating the detrimental effects of high concentrate feeding and decreasing the risk for SARA by rumen fermentation in high concentrate diets. Additionally, D-lactate increased from wk 1 and 2 compared to wk 3 by 2.5 and 2.3 fold for CON and PHY, respectively. The low increment of D-lactate in PHY further supports the modulation of rumen fermentation with supplementation. Our results coincide with Qumar et al. [20], as ruminal lactate displayed approximately a 10-fold increment in their study when cows transitioned to a high grain ration. Other authors have reported that cattle experiencing SARA showed concentrations of lactate of 2.29 mM [34] with high-yield lactating cows. In our experiment, ruminal lactate reached maximum values of 1.54 and 1.18 mM in wk 3 of HC for CON and PHY, respectively. To the best of our knowledge, there are no reports showing the impact of supplementing phytogenic compounds on lactate fermentation. However, discrepancies with our results, and those reported in the literature for lactate during SARA may be based on the method of detection, physiological stage of cows, and values below the detection limit. A possible explanation for the tendency to decrease DMI goes together with a more stable change in daily DMI as demonstrated by Stone [8] indicating that abrupt changes in intake can be reflected in stronger dietary SARA insults.

Our results showed that, in general, the fermentation pattern and SCFA profile between the reticulum and the rumen were similar. This reflects the close connection and constant exchange of digesta between compartments, with the difference that the first one is constantly buffered with saliva and carbon dioxide removal. Minor differences in lactate or ruminal SCFA fatty acid concentration noted between the rumen and the reticulum may be explained by differences in the physiology of both compartments. Differences in the water and saliva influx into the reticulum may distinguish it from the rumen and could influence the SCFA profile. High concentrate diets have also been shown to decrease ruminal acetate, isobutyrate and the acetate to propionate ratio, and to increase propionate and valerate in rumen fluid for two [11] or four consecutive weeks on high concentrate [35]. In addition, the accumulation of ruminal SCFA increases the risks for SARA. Thus, during the last few years, there has been a special interest in the use of essential oils, phytogenic additives and secondary plant compounds in ruminant diets to positively modulate rumen fermentation and to improve its efficiency [10]. Supplementation with phytogenic compounds has been shown to result in lower propionate concentrations in the rumen in a diet with a 50:50 concentrate to forage ratio [25]. In addition, Neubauer et al. [11] reported an increase in ruminal butyrate in high concentrate feeding after supplementing with phytogenic compounds in the TMR, and those results are in agreement with our findings in wk 2 of HC.

The variation in the proportions of individual SCFA in the rumen can be influenced by diet composition or the rate of production and utilization of those acids. For example, the reduction in acetate and the increase in propionate with time post-feeding found in the present experiment, especially in wk 4 of HC, may be explained by the increased availability of readily fermentable carbohydrates in the HC diet. Interestingly, we found that in wk 2 propionate was consistently lower for PHY compared to CON post-feeding, which indicates the regulation of activity of propionate-producing bacteria with PHY supplementation immediately after diet consumption and up to 12 h post-feeding. Other reports have found that eugenol did not modify SCFA fermentation when included in diets of lactating cows with a 47:53 concentrate to forage ratio mainly including corn and soybean meal with different inclusion levels (25–75 mg/kg DM) [36]. Differences in the effect of phytogenic compounds on ruminal fermentation and function may also be explained by differences in the composition or dosages used, as well as rumen microbial activity or absorption rate.

Ruminal ammonia concentration followed an opposite pattern to that observed for SCFA and lactate as reported by Sato [37]. Specifically, the decrease in ruminal pH was associated with a decrease in ammonia concentration. Lana et al. [38] demonstrated that

better assimilation of ammonia is not only influenced by increased microbial synthesis in high concentrate diets but also by the deamination rate. These results possibly reflect a more efficient microbial use of ammonia during high grain diet [39]. Another possible explanation may be related to changes in the abundance of hyper-ammonia producing bacteria closely involved in the generation of ammonia in the rumen due to phytogenic compounds [40], which would influence the concentration of ammonia, as observed in wk 2 and 3 of HC. However, it has been reported that feed additives did not show an effect on ammonia concentration after feeding a high concentrate diet (65% DM) for two consecutive weeks [11]. Discrepancies between our results and those from Neubauer et al. [11] may be explained by the shorter length of the HC challenge. In this study, the peak of total ammonia concentration was reached mostly 8 or 12 h after feeding similar than results reported by Sato [37] with rumen ammonia peak at 8 h after feeding in beef cattle.

The HC feeding and SARA was associated with increased inflammation markers, especially SAA. In this study, the SAA increased starting with wk 1 HC and this elevated APR was maintained throughout the trial for 4 weeks, being more pronounced in the CON group. However, preventive supplementation in 3 weeks demonstrated a decreased inflammation response (wk 3 and 4) compared to CON. Thus, PHY seemed to reduce the release and/or transfer of microbial toxins and biogenic amines to the bloodstream or to ease inflammation by increasing the hepatic clearance via bile [7,41], regulating the APR and inflammation in cattle, as reported by Yang et al. [42] and Oh et al. [43]. Rodrigues et al. [44] found that a mixture of condensed tannins fed to a group of lactating cows when fed a diet with 39% concentrate diet had a lower Hp concentration than the control group which is similar to our experiment during wk 3 and 4 HC. Interestingly, Yang et al. [42] reported a reduction in SAA when steers were supplemented with plant compounds similar to our results for wk 3 HC. Furthermore, liver enzymes increase when there is damage present in the tissue and, as a result, new organelles are produced to counteract inflammation. In the present study, the threshold values listed by Wille et al. [45] for activities of GLDH, and GGT (10.5 and 27 U/L, respectively) were exceeded from wk 3 of HC, suggesting a negative impact of high grain feeding on animal systemic health, and an impairment of liver function. The increase in the activity of GLDH and GGT after the change from a forage to HC diet coincided with results reported by Kröger et al. [46] once lactating cows were switched from a ration with 40:60 concentrate to forage ratio to one with 60% concentrate and may be explained by liver damage [47]. Furthermore, Lakhani et al. [48] reported similar results for ALP, but an increase in AST after the inclusion of a phytogenic feed additive in buffalos fed a 50:50 concentrate forage ratio. However, those results contrast findings from the present study, where systemic GGT did not change with PHY supplementation. Other reports have suggested that plant compounds can suppress oxidative stress and improve liver health status in different animal species [49]. Finally, our results may also suggest that animals in other physiological conditions such as non-lactating cows with a reduced DMI and nutrient demands show different results to the reports in the literature.

5. Conclusions

Overall, the study showed that a 65% concentrate diet successfully induced SARA and that supplementation with a phytogenic feed additive during the HC diet improved rumen SCFA profiles, and increased rumen pH after 2 weeks of high concentrate feeding. The improved rumen fermentation, reduced SARA indices and decreased inflammation response suggest a supplementation strategy of at least three weeks for PHY to contribute to reduce the negative effects of high concentrate feeding in dairy cattle. The PHY blend of L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) possibly influenced the microbial population by modulating rumen fermentation. Therefore, supplementation with 0.04% of the phytogenic compound mixed in the TMR can be recommended to lower the impact of high-concentrate feeding in dairy cows.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12091201/s1, Table S1: Effect of supplementation with a phytogenic feed additive on reticular short chain fatty acid profile, ammonia and lactate in cows consuming a forage diet or a high concentrate diet [1]; Figure S1: Boxplots illustrating the fermentation pattern and short chain fatty acid profile in the reticulum and rumen with time post-feeding according to diet and duration of high concentrate feeding in Holstein cows without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*); Figure S2: Variation of reticular short chain fatty acid fermentation from 0 to 12 h post-feeding in cows fed either all-forage (F) or a high concentrate (HC), without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*); Figure S3: Variation of reticular D-lactate, L-lactate (mM), and total ammonia concentration (mg/dL) from 0 to 12 h post-feeding in cows fed either all-forage (F) or a high concentrate (HC), without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*).

Author Contributions: Conceptualization, R.R.-C., E.C.-L., S.R., R.M.P., N.R. and Q.Z.; methodology, R.R.-C., E.C.-L., S.R., R.M.P. and Q.Z.; data curation R.R.-C. and E.C.-L.; writing—original draft preparation, R.R.-C. writing—review and editing R.R.-C., E.C.-L., S.R., R.M.P., N.R. and Q.Z.; project administration, E.C.-L. and R.M.P.; funding acquisition, Q.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development. BIOMIN Holding GmbH supports financially the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock. Open Access Funding by the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock.

Institutional Review Board Statement: The methods and protocols followed in this experiment were approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to the law for animal experiments 2012—TVG 2012 (protocol number: BMBWF-68.205/0003-V/3b/2019). This study was part of a larger experiment that evaluated other parameters that are presented in a companion paper.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the staff at the dairy farm of Vetmeduni Vienna for their help. The assistance of Anita Dockner, Sabine Leiner, and Manfred Hollmann in the sample analysis and support in the laboratory is very much appreciated. We are also thankful to Thomas Enzinger, Parisa Kheirandish and Claudia Lang for their help in sampling.

Conflicts of Interest: Nicole Reisinger is employed by BIOMIN Holding GmbH, a company that manufactures and trades feed additives. However, this circumstance did not influence the design of the study or bias the presentation and interpretation of results.

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4.3.Publication 3

Effect of duration of high-grain feeding on chewing, feeding behavior, and salivary composition in cows with or without a phytogenic feed supplement.

Rivera-Chacon, R.; Ricci, S.; Petri, R.M.; Haselmann, A; Reisinger, N.; Zebeli, Q.; Castillo-Lopez, E.

Animals (Basel). 2022; 12(15):2001

doi.org/10.3390/ani12152001

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Effect of Duration of High-Grain Feeding on Chewing, Feeding Behavior, and Salivary Composition in Cows with or without a Phytogenic Feed Supplement

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Simple Summary: This study evaluated the effects of the duration of high-grain feeding and a phytogenic feed supplement on the chewing, eating, and lying behavior as well as the salivation dynamics in dairy cows. A control group of cows with no supplementation was compared to a group receiving a phytogenic feed supplement. An increased duration of the high-grain diet increased meal size, but reduced rumination, the total chewing time, and the chewing index. Similarly, as the experiment progressed, the cows sorted against short feed particles. The results also showed that the duration on the high-grain diet increased the salivary pH; however, the salivary phosphate decreased at the start of high-grain feeding. Feed ensalivation also decreased after 4 weeks of consuming the high-grain diet. The supplemented cows sorted in favor of fiber-rich feed particles in week two and had greater salivary pH in week four on the high-grain diet. Our study showed that the duration of feeding exacerbates the negative impacts of high-grain diets in cows. However, supplementation with the feed additive mitigated some of these negative effects.

Abstract: Switching diets from forage to a high-grain (HG) diet increases the risk of rumen fermentation disorders in cattle. However, the effects of the duration of the HG feeding, after the diet switch, on animal behavior and health have received considerably less attention. This experiment primarily aimed to assess the effects of the duration of an HG diet on the chewing, eating, and lying behavior and salivation dynamics in a control group (CON) and a group of cows receiving a phytogenic feed supplement (TRT) at 0.04% (DM basis), which included L-menthol, thymol, eugenol, mint oil, and cloves powder. The experiment was a crossover design with nine non-lactating cows, and two experimental periods with an intermediate washout of four weeks. In each period, the cows were first fed a forage diet for a week to collect baseline measurements representing week 0; then, the diet was switched over a week to HG (65% concentrate), which was fed for four continuous weeks (week 1, week 2, week 3, and week 4 on an HG diet, respectively). The cows were divided in two groups of four and five animals and were randomly allocated to CON or TRT. The data analysis revealed that at the start of the HG feeding, the dry matter intake and the cows' number of lying bouts increased, but the eating time, rumination time, and meal frequency decreased, resulting in a greater eating rate. We also found that an advanced duration on an HG diet further decreased the rumination time, total chewing time, chewing index, and sorting in favor of short feed particles, with the lowest values in week 4. The feed bolus size increased but feed the ensalivation decreased in week 4 compared to week 0. The dietary switch increased salivary lysozyme activity, and the advanced duration on the

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Citation: Rivera-Chacon, R.; Ricci, S.; Petri, R.M.; Haselmann, A.; Reisinger, N.; Zebeli, Q.; Castillo-Lopez, E. Effect of Duration of High-Grain Feeding on Chewing, Feeding Behavior, and Salivary Composition in Cows with or without a Phytogenic Feed Supplement. *Animals* **2022**, *12*, 2001. https://doi.org/10.3390/ani12152001

Received: 22 June 2022
Accepted: 5 August 2022
Published: 8 August 2022

HG diet increased salivary pH, but salivary phosphate decreased in weeks 1 and 2 on the HG diet. Supplementation with TRT increased sorting in favor of physically effective NDF (peNDF) in week 2 and increased salivary pH in week 4 on an HG diet. Overall, the negative effects of the HG diet in cattle are more pronounced during the initial stage of the HG feeding. However, several detrimental effects were exacerbated with the cows' advanced duration on feed, with host adaptive changes still observed after 3 and 4 weeks following the diet switch. The TRT mitigated some of the negative effects through the temporal improvement of the salivary properties and the intake of peNDF, which are known to modulate rumen fermentation.

Keywords: dairy cow; rumination; saliva; feed sorting; phytogenic feed additive

1. Introduction

Feeding cattle high-grain (HG) diets is commonly implemented in the dairy and beef cattle production industry to enhance the energy intake for milk or accelerate daily gains, respectively. Grains are rich in starch and less voluminous and are thereby a better source of metabolizable energy than forages for cattle diets in many parts of the world. However, feeding large amounts of grain is known to influence chewing behavior, which could affect animal health due to the increased risk of gut disorders. More specifically, HG diets have been reported to impair rumination and total chewing times, which are essential physiologic processes in ruminants [1–4] often used as indicators of cattle welfare and health [5]. Additionally, the lying behavior of cattle is an important indicator of their comfort and welfare [6]. For instance, Haley et al. [7] demonstrated that lying time is closely related to comfort and health changes. Cattle ruminate more while lying down than when standing. However, feeding them HG diets may change the lying behavior of cows [2], with longer lying times and shorter rumination times usually reflecting distress and discomfort [8].

The feed-sorting behavior in cattle may also be affected by a change in dietary composition [9–11]. For example, cows are known to select diet fractions during eating, sorting for shorter particles in the ration (concentrate) and refusing longer particles (forages) [12–14]. Another report by Greter and DeVries [15] demonstrated that cows fed with a 54% grain diet sorted against long particles and tended to sort in favor of short particles. Nonetheless, this feeding behavior may contribute to further impaired chewing activity and salivation due to the reduced intake of dietary physically effective NDF (peNDF) and could consequently affect animal health or gut function. Specifically, peNDF in the diet is important because it stimulates chewing activity, greater salivary buffer secretion, and the regulation of ruminal pH [16]. Therefore, diet composition plays an important role in feed sorting, with the time invested in eating and ruminating positively correlated with the intake of peNDF [17]. In this regard, diets with more fiber are associated with more meals per day and reduced eating rates [18], which positively modulates rumen fermentation.

The essential physiologic role of chewing in cattle is based on its contribution to stimulating salivary secretion [19,20]. Salivary buffers help stabilize the ruminal pH [21] because salivary buffers such as bicarbonate and phosphate represent important components for ruminal proton removal in the rumen [22]. Furthermore, salivary secretions contain different proteins such as mucins, lysozymes, and immunoglobulins [23], which contribute to health and gut function [24]. Therefore, an increase in mastication and salivary flow can enhance the rumen acid–base balance and ultimately improve health [5,25]. In this context, phytogenic compounds, such as thymol and thyme oil, have shown a potential to modulate the salivary secretions in cattle. Similarly, menthol has been reported to stimulate chewing and increase salivation in non-ruminants [26,27].

The dietary shift to HG feeds is known as the time with the greatest risk for cattle health due to the major adaptive changes occurring in the host during this interval. However, there is limited research on the effects of the duration of the HG feeding challenge after the

dietary change on salivary secretions [28] as well as the chewing activity and lying behavior. In addition, there is a paucity of information regarding the effect of the supplementation of feed with phytogenic compounds on salivary composition and production [29], chewing activity, or the eating behavior of cows. Thus, there is a need to fill or strengthen these research gaps in the scientific literature. Therefore, the aims of this study were to evaluate the effect of the duration of an HG feeding challenge on the chewing activity, eating and lying behavior, and salivary composition and production in dairy cows without or with phytogenic feed supplementation. Our hypothesis stated that the advanced duration of the HG challenge would exacerbate the negative effects on rumination, the eating and lying behavior, and the salivary production and composition. We also hypothesized that the phytogenic supplementation would alleviate the decrease in the chewing activity as well as improve the feed sorting and salivary properties.

2. Materials and Methods

2.1. Animals, Experimental Design, Treatments and Management

This study was part of a larger experiment; details on animal management, feeding, as well as the results regarding ruminal fermentation have been published in a companion paper [30]. The animal protocol was approved by the institutional ethics and animal welfare committee of University of Veterinary Medicine Vienna and the Austrian national authority (protocol number: BMBWF- 68.205/0003-V/3b/2019).

Briefly, we used nine rumen-cannulated, individually-fed, dry Holstein cows in a cross-over experimental design. Cows were blocked in 2 groups of 4 and 5 animals and assigned to a control diet (CON) or a diet with 0.04% (DM basis) of a phytogenic feed additive based on a blend of mint oil (*Mentha arvensis*), cloves powder (*Syzygium aromaticum*) and thymol, including L-menthol and eugenol (TRT; Digestarom®, DSM GmbH). The inclusion rate of the phytogenic supplement was defined based on previous studies.. Details of this product have also been reported in the companion paper [30]. Each experimental period consisted of 6 weeks. During the first week of each period, cows were fed a solely forage diet including 45% grass silage, 45% corn silage, and 10% grass hay (DM basis). This week of forage feeding was used to collect baseline measurements representing week 0. In the following week, an HG feeding challenge was induced through an increment in the proportion of concentrate in the total mixed rations (TMR, 10% daily increments, DM basis). After the dietary change, the HG ration contained 26.25% grass silage, 8.75% corn silage, and 65% of a pelleted concentrate based on barley and triticale ground grains (DM basis; Supplementary Table S1), and this HG ration was fed for 4 consecutive weeks (week 1, week 2, week 3, and week 4 on the HG, respectively). Figure 1 depicts the overall experimental outline for each of the 2 periods. Between these experimental periods there was a washout interval, which lasted 4 weeks.

During the experiment, cows were housed in a free-stall barn with deep litter cubicles (2.6 × 1.25 m, straw litter) and free-choice mineral blocks. Before the initiation of the study, cows were randomly allocated to the feed bins, so that each cow was trained using an ear tag transponder to allow access to only one feed bin throughout the experiment. Therefore, individual feed intake was continuously recorded since each feed bin was equipped with an electronic scale (Insentec B.V., Marknesse, The Netherlands). This feeding approach enabled the collection of data related to eating behavior and feed sorting for each cow. Additionally, there was no feed bunk competition due to space limitations or determined by the social hierarchy because each cow had her own feed bin. Therefore, each cow was used as an experimental unit. Diets were automatically mixed every day (Trioliet Triomatic T15, Oldenzaal, The Netherlands), and were offered in the individual feed bins to each cow at 0800 h. When needed, the amount of water added to the diet was adjusted during mixing to target 46% DM in the TMR. Cows were fed targeting 10% of feed refusals.

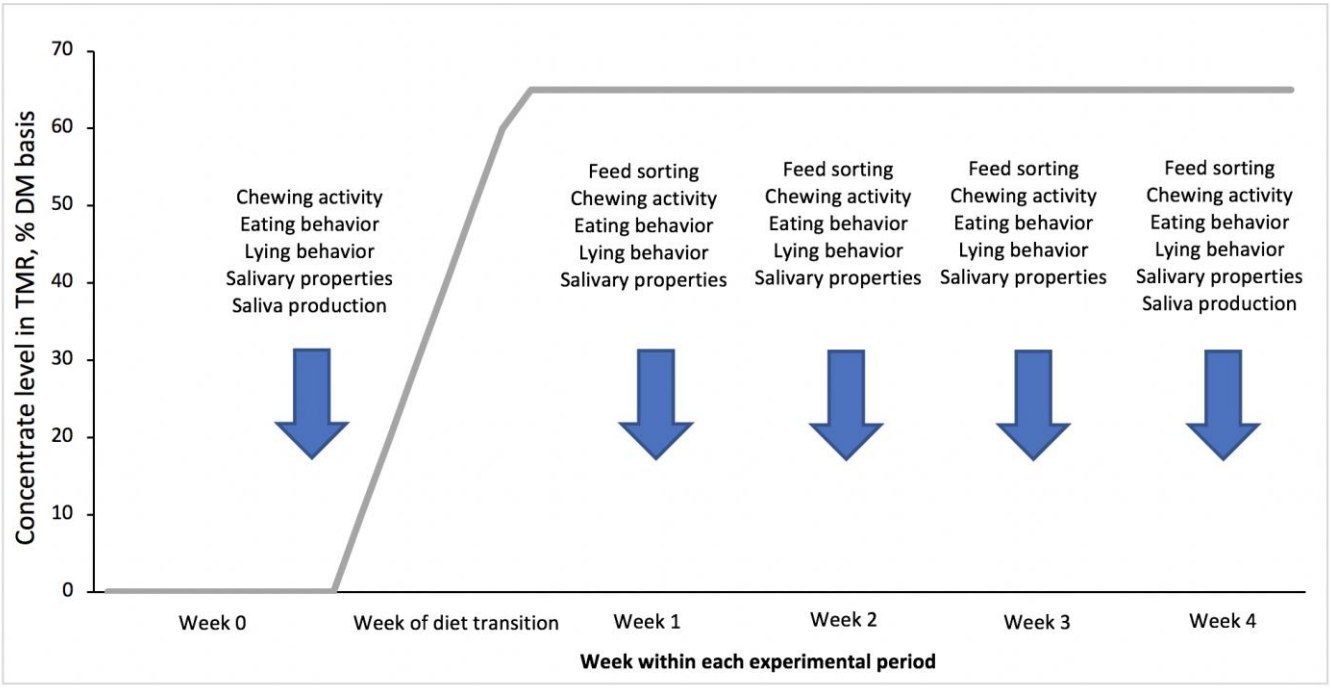

Figure 1. General outline of each of the 2 experimental periods illustrating the dietary adaptation to (HG) feeding and the duration of the HG feeding challenge. The blue arrows indicate the time points for major samplings as well as measurements performed.

2.2. Collection of Feed Samples and Analyses for Chemical Components

Dry matter concentration of the TMR was determined every day by drying samples at 100 ◦ C for 24 h. Using these data, DM content of the TMR was adj usted if needed by changing the amount of water added to the diet during mixing. Individual feed samples were collected at the beginning and at the end of each experimental period, while TMR samples were collected once a week for chemical composition. Chemical composition of each TMR sample was evaluated, and then values were averaged by chemical component across sampling weeks. Details on the laboratory analyses as well as the method used for the evaluation of particle size distribution of the rations have been reported in the companion paper [30].

2.3. Evaluation of Chewing, Feed Sorting and Eating Behavior of Cows

Evaluation of chewing activity was conducted weekly (Figure 1), with the first evaluation performed in week 0, and the following conducted in each of the 4 weeks of the HG feeding regimen. This analysis included eating time, ruminating time, number of chews per minute and per feed bolus, chewing index, drinking time, and drinking gulps. These parameters were evaluated following Kröger et al. [31]. To do so, noseband pressure sensors (RumiWatch System, ITIN + HOCH GmbH, Fütterungstechnik, Liestal, Switzerland) were used for 3 consecutive full-days each week in all cows simultaneously. The evaluation of chewing activity with these systems is based on the detection of changes in pressure, which are monitored by the sensors when cows ruminate or eat, and according to the animal's head position. Halters were placed on the cows for adaptation 12 h before the initiation of data collection for chewing activity. After measurements were completed, the recorded raw data were transferred using the interface software RumiWatch Manager (version 2.2.0.0; Itin and Hoch GmbH) and processed with the evaluation software RumiWatch Converter (Version 0.7.3.2). Graphs outlining diurnal variations of rumination and eating time were constructed for a detailed description of a full-day period within each experimental week.

Feed sorting behavior was evaluated for each cow once a week (Figure 1), starting from the first week of the HG feeding regimen using the methodology described by

Haselmann et al. [32] and Stauder et al. [3]. Specifically, particle size distribution of TMR offered and in the feed refusals collected in the following day were measured. Samples of TMR were collected; on the following day, refusal samples were collected from each feed bin in the morning before the new feed was offered. Feed sorting for each cow was expressed through the change in particle size distribution (as-fed basis) of the provided TMR in relation to the refusals. According to Leonardi and Armentano [12], feed selection of each particle size was calculated as the percentage of the actual as-fed intake from the predicted as-fed intake, expressed as the selection index. Predicted intake of a specific particle size was estimated as the product of as-fed intake and the proportion of this specific fraction in the offered TMR.

Eating behavior was evaluated for each cow weekly (Figure 1). The variables evaluated included valid visits, visit duration, visit size, meal frequency, meal duration, meal size, and eating rate. A valid visit was defined when a cow stayed at the feeder for 4.5 min and consumed at least 200 g of DM. Additionally, when the time interval between the end of one visit and the start of the next one was shorter than 29.5 min, these visits were considered as part of a single meal. This time interval was calculated based on the methods described by Tolkamp et al. [33] and DeVries [34]. Eating rate was estimated following the protocol of Beauchemin et al. [35].

2.4. Evaluation of Lying Behavior

Lying behavior was measured in one of the 2 experimental periods. These measurements were collected during the same 3 days used for evaluation of chewing activity and was performed in all cows using data loggers (HOBO Pendant G Acceleration Data Logger). For this purpose, loggers were placed on the external side of the hind left leg using a self-adherent bandage (UKAL cohesive flexible bandage, France). Prior to the attachment, each logger was fixed to a silicon mat to avoid chafing. The recording interval was set at 30 s. After 3 days of data collection, the loggers were removed, and raw data were downloaded using the software HOBOware PRO. Lying data were processed using the Ledgerwood's algorithms for lying/standing bouts and laterality [36]. Lying behavior variables were calculated on a daily basis and included: standing time, total lying time, lying time on the left side, lying time on the right side, total lying bouts, lying bouts to the left, and lying bouts to the right.

In addition, the data of rumination activity collected in the corresponding experimental period were combined with the lying behavior. To perform these calculations, both parameters were matched in 10 min intervals. This enabled the calculation of rumination times while standing or lying. For these calculations, we considered a minimum time of 9.5 min to assume that cows were lying for the complete 10-min interval.

2.5. Saliva Collection and Evaluation of Salivary Characteristics, and Measurement of Saliva Production

Saliva samples were collected orally once a week (Figure 1) to evaluate salivary physico-chemical characteristics, with the first collection conducted in week 0, and the following collections performed in each of the 4 weeks of the HG feeding regimen. Details of saliva samplings have been described by Castillo-Lopez et al. [28] and Ricci et al. [37]. Briefly, saliva collections were conducted with a vacuum pump before the morning feeding. Then, aliquot samples were frozen immediately at −20 °C. At the end of the experiment, samples were thawed, and pH was measured using a portable pH meter (Mettler-Toledo, AG; Analytical CH; Schwerzenbach, Switzerland). Additionally, other salivary physico-chemical characteristics including buffer capacity, bicarbonate, phosphate, mucins, lysozyme activity, and total proteins were measured at the end of the experiment as previously described by Castillo-Lopez et al. [28].

The measurement of salivary production and evaluation of feed boli characteristics were conducted twice during each experimental period, in week 0 and week 4. The protocols for collection of feed boli and sample analyses as well as calculations are also reported in

detail in Castillo-Lopez et al. [28]. The evaluated parameters related to salivary production included feed boli size, saliva content in feed boli, salivation rate (g saliva flow/min), and feed ensalivation (g saliva/g feed DM).

2.6. Statistical Analysis

Statistical analyses were performed using the Proc Mixed of SAS (version 9.4; SAS Institute, Cary, NC, USA). Data were checked for outliers and normality, if the normality assumption was not met, transformations were performed as described in Rivera-Chacon et al. [30]. The statistical model included the fixed effects of the experimental period, duration of the HG feeding regimen in weeks, TRT supplementation, and the interaction between duration of the HG feeding regimen × phytogenic supplementation. The cow was included as random effect in the model. In addition, data from the same cow at different times were analyzed as repeated measures, with a first order variance-covariance structure matrix. Each cow was considered as the experimental unit. The multiple comparisons of means were performed using the PDIFF option. The transformed data were back-transformed after the analysis of variance. Additionally, we conducted Pearson correlation analyses using Proc corr of SAS between rumen fermentation variables (ruminal pH and short chain fatty acids) vs. rumination time, total chewing time, salivary properties (bicarbonate content, phosphate content, buffer capacity, and pH), feed insalivation, and feed sorting. From a preliminary statistical power analysis that we conducted [29] according to Stroup [38] and Kononoff and Hanford [39], using similar variables as those evaluated in this study, we observed that a minimum of n = 4 is required to obtain a statistical power of 0.82 with $\alpha = 0.05$, an acceptable level.

We report the results as LSM as well as the largest standard error of the mean (SEM). Statistical significance was declared when $p \leq 0.05$, and statistical tendencies are discussed if $0.05 < p \leq 0.10$.

3. Results

3.1. Dietary Characteristics

During the week of the diet change, the dietary composition drastically shifted from 50.4 to 30.9% NDF. Additionally, there was also an abrupt reduction in fibrous long feed particles and peNDF. At the same time, there was an increase in the dietary starch content (Supplementary Table S1). Thus, this diet shift represented an adequate experimental approach to induce an HG feeding challenge on the animals and to evaluate their host adaptive responses with respect to advanced days of feeding.

3.2. Chewing Activity and Eating Behavior

The rumination time was strongly impaired by the duration of the HG feeding and decreased from 348 to 245 min/day from week 1 to week 4 of the HG feeding regimen, independent of the TRT. Similarly, the total chewing time (minutes of chewing per day) was highly influenced by the duration of the HG feeding regimen with a pronounced reduction observed in week 4 on an HG diet ($p < 0.05$). The chewing index (total chewing time/kg DMI) was markedly decreased at the start of the high-grain challenge, and the values were maintained at low levels throughout the entire HG feeding period. In addition, the eating time showed a decreasing pattern with the lowest value found in week 4 of the HG feeding (145 min per day), independent of the TRT. Nonetheless, drinking gulps and drinking time followed a different pattern, which increased as the HG diet was implemented ($p < 0.05$) and both variables reached the highest values in week 4 on the HG (Table 1).

The dry matter intake (DMI) was greater for week 1 on an HG diet compared to week 0, with estimates of 8.0 and 13.0 kg for week 0 and week 1 on the HG, respectively. The greatest DMI was reached in week 3 on the HG, with an average of 14.1 kg. Furthermore, eating and feed bunk visits were mostly affected immediately at the start of the HG challenge. For instance, the visit duration (min) and meal frequency (#/day) decreased, whereas the visit size (kg DMI per visit) increased from week 0 to week 1 of the HG feeding regimen. In

addition, the meal size (kg of DM) and eating rate increased immediately from the start of the HG feeding. The advanced duration on the HG diet increased the meal size compared to week 1 on the HG. We also found that supplementation with TRT tended to reduce the meal size in week 2 of the HG feeding regimen (Table 2).

Table 1. Effect of duration on a high-grain diet on chewing activity of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

Item	Week 0		Week 1		Week 2		Week 3		Week 4		SEM [2]	*p*-Values [3]		
	CON	TRT	CON	TRT	CON	TRT	CON	TRT	CON	TRT		DUR	TRT	I
Ruminating time, min/d	296	374	353	344	363	301	304	267	273	218	40.8	0.09	0.64	0.22
Eating time, min/d	186	183	156	178	147	157	150	157	146	145	13.3	<0.01	0.63	0.58
Ruminating, chews/min	63.4	63.5	60.7	62.3	61.4	62.3	61.3	63.0	60.9	61.8	0.78	<0.01	0.22	0.53
Ruminating, chews/bolus	47.4	47.0	48.2	49.3	49.4	50.1	50.8	52.7	48.2	50.2	1.52	<0.05	0.45	0.87
Total chewing time, min/d	534	546	490	524	500	464	451	431	426	362	40.2	<0.01	0.67	0.65
Drinking time, min/d *	4.87	5.45	7.51	6.84	8.74	10.28	10.52	8.85	11.63	13.09	1.26	<0.01	0.91	0.66
Drinking gulps, #/day *	68.5	74.5	96.6	97.9	117.8	138.9	148.9	122.0	149.8	188.3	1.25	<0.01	0.85	0.57
Chewing index, chewing time/kg DMI *	63.6	73.7	34.7	32.1	34.6	32.7	32	29.5	32.0 ^{x}	27.0 ^{y}	1.07	<0.01	0.36	0.07

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*), (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of duration of high-grain feeding (DUR), phytogenic feed supplement (TRT), and the interaction of duration on high-grain × supplementation (I). * Due to lack of normal distribution, values were first log transformed prior to statistical analysis. $^{x, y}$ Within corresponding week, means with different superscripts indicate a tendency for a difference ($0.05 < p \leq 0.10$) between CON and TRT.

Table 2. Effect of duration on a high-grain diet on DMI and eating behavior of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

	Week 0		Week 1		Week 2		Week 3		Week 4			*p*-Values [3]		
Item	CON	TRT	CON	TRT	CON	TRT	CON	TRT	CON	TRT	SEM [2]	DUR	TRT	I
DMI, kg	8.57	7.35	13.5	13.7	13.8	12.6	14.6	13.6	14.1	13.3	0.64	<0.01	0.11	0.69
Valid visits [4], #/day	11.9 ^{x}	10.3 ^{y}	10.9	11.4	10.1	10.5	10.5	10.9	9. 8	9.8	0.71	0.22	0.92	0.43
Visit [4] duration, min	10.2	9.9	9.0	9.2	9.4	9.4	8.6	9.5	8.4	9.0	0.49	<0.01	0.59	0.56
Visit [4] size, kg DMI	0.57	0.55	1.02	0.97	1.07	0.97	1.34 ^{x}	1.06 ^{y}	1.04	1.08	0.11	<0.01	0.43	0.51
Meal [5] frequency, #/day	6.2 ^{x}	5.5^{y}	4.9	5.4	4.9	5.1	5.1	5.0	4.7	4.7	0.29	<0.01	0.98	0.29
Meal [5] duration, min	27.9	28.5	28.1	29.0	28.3	27.3	28.3	27.4	29.7	28.1	0.96	0.45	0.70	0.32
Meal [5] size, kg DMI	1.29	1.28	2.82	2.7	2.87 ^{x}	2.48 ^{y}	2.9	2.69	3.13	2.95	0.15	<0.01	0.28	0.54
Eating rate, g DM/min	46.09	47.09	101.8	94.6	100.2	91.3	103.7	98.8	105	105.6	5.33	<0.01	0.56	0.34

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*) (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of duration of high-grain feeding (DUR), phytogenic feed supplement (TRT), and the interaction of duration on high-grain × supplementation (I). [4] A valid visit was defined when a cow stays in the feeder for at least 4.5 min and consuming at least 200 g of DM. [5] A meal was defined as the sum of close visits initiated in less than 29.5 min interval after the end of previous visit. Meal duration calculated as sum of visits + intervals between visits within average meal. $^{x, y}$ Within corresponding week, means with different superscripts indicate a tendency for a difference ($0.05 < p \leq 0.10$) between CON and TRT.

From week 1 of the HG feeding regimen, there was a change in the pattern of the time spent eating throughout the day. Specifically, in week 0 (Figure 2A), there were multiple peaks for eating time distributed during the first 13 h after feed delivery. However, from week 1 of the HG feeding regimen onwards (Figure 2B–E), the eating time decreased, and the predominant peak of the eating time was generally observed shortly the after delivery of the diet in the feed bins early in the morning.

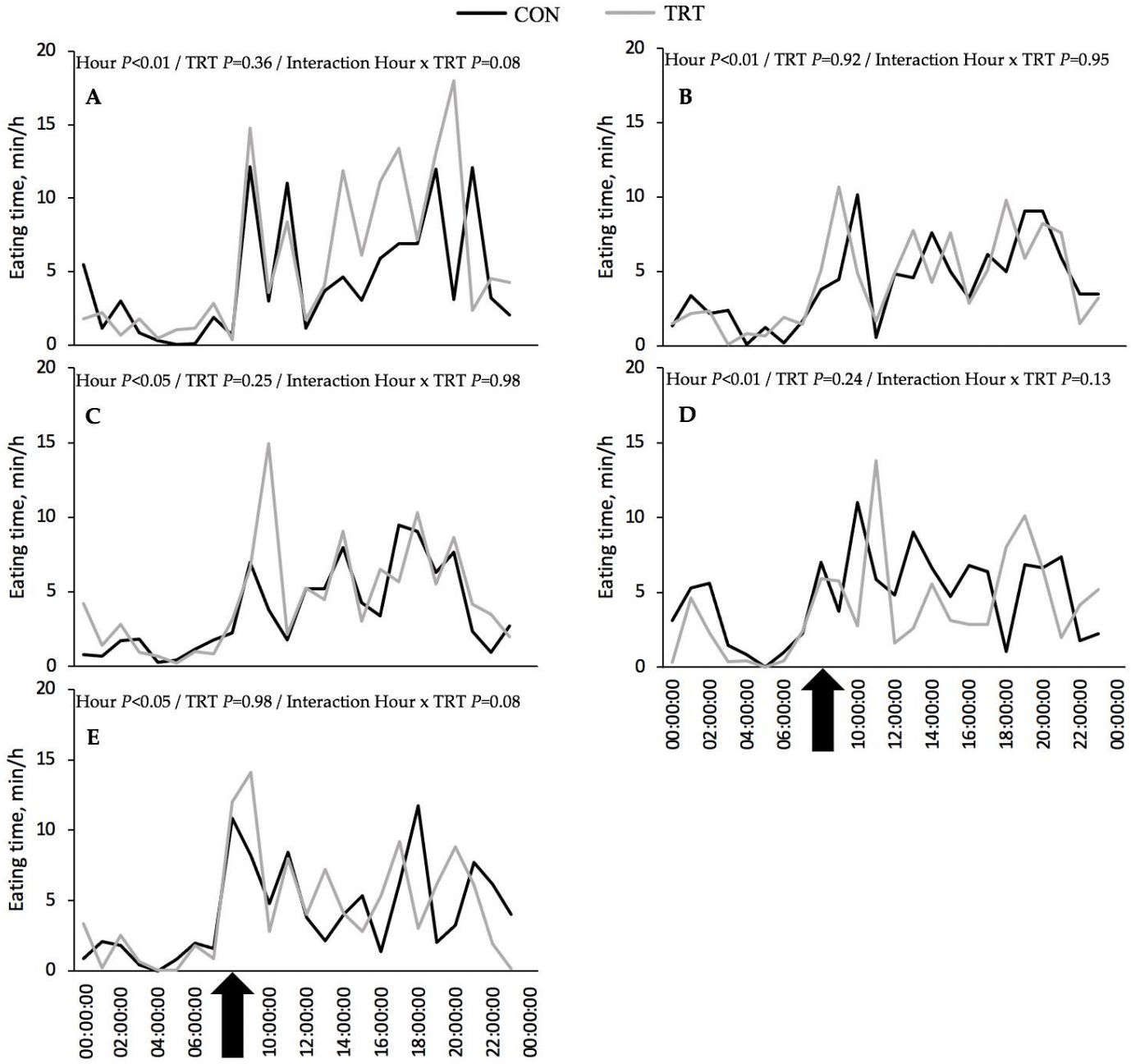

Figure 2. Diurnal variation of eating time according to duration on a high-grain diet in a control group of Holstein cows (CON) or a group receiving a phytogenic feed supplement (TRT) based on L-menthol, thymol, eugenol, mint oil, and cloves powder (0.04%, DM basis). (A)—Week 0; (B)—Week 1 on high-grain; (C)—Week 2 on high-grain; (D)—Week 3 on high-grain; (E)—Week 4 on high-grain. The black arrows indicate the time of feeding.

3.3. Feed Sorting Behavior

The feed-sorting behavior analysis showed that in week 3 on the HG diet, sorting for medium size feed particles decreased ($p = 0.01$) independent of the TRT. In addition, sorting for short size feed particles tended ($p = 0.08$) to decrease due to the advanced duration of the HG diet. With regard to feed supplementation, during week 2 of the HG feeding regimen, the TRT group sorted in favor of larger particles of feed, while the CON cows followed the opposite pattern. However, in weeks 3 and 4 on the HG diet, both groups sorted in favor of larger feed particles. In addition, in week 2 on the HG diet, TRT cows showed a greater preference for peNDF than CON ($p < 0.05$; Table 3).

Table 3. Effect of duration on a high-grain diet on feed sorting behavior of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

	Week 1		Week 2		Week 3		Week 4			*p*-Values [3]		
Particle Fraction [4]	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**SEM [2]**	**DUR**	**TRT**	**I**
Long	92.0	101	81.5 [b]	107 [a]	106	102	108	103	7.68	0.36	0.35	0.11
Medium	108	112	116	114	103	107	106	110	3.06	0.01	0.37	0.66
Short	88.0	72.7	73.6	58.3	68.4	67.0	66.4	60.0	8.39	0.08	0.25	0.68
Fine	92.5	71.4	63.8	70.0	81.6	67.3	83.3	65.7	12.29	0.55	0.15	0.57
peNDF [5] $_{>8\ mm}$	106	105	101 [b]	114 [a]	104	107	106	106	2.56	0.80	0.07	0.02
peNDF $_{>1.18\ mm}$	98.3	99.0	93.3 [b]	101 [a]	97.6	100	97.5	96.1	1.51	0.44	0.04	<0.01

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*) (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of duration of high-grain feeding (DUR), phytogenic feed supplement (TRT), and the interaction of duration on high-grain × supplementation (I). [4] Measured according to Kononoff et al. [40]. Values lower than 100 indicate decreased preference (sorting against the corresponding particle fraction); values greater than 100 indicate increased preference (sorting in favor of the corresponding particle fraction). [5] Physically effective NDF. [a, b] Within corresponding week, means with different superscripts indicate a significant difference ($p < 0.05$) between CON and TRT.

3.4. Lying Behavior and Rumination According to Animal Position

There was a tendency for the total standing time to decrease at the start of the HG feeding ($p = 0.10$), and the total lying time tended to increase ($p = 0.10$), but with no effect from the TRT supplementation ($p = 0.93$). The total lying bouts and lying bouts to the left or right side were greater from week 1 on the HG diet onwards compared to week 0, with these variables being greater ($p < 0.05$) for TRT in week 2 of the HG diet compared to the CON cows. There was a tendency for a greater time spent lying on the left side for the TRT compared with CON cows ($p = 0.10$) in week 1 of the HG diet (Table 4).

Table 4. Effect of duration on a high-grain diet on lying behavior of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

	Week 0		Week 1		Week 2		Week 3		Week 4			*p*-Values [3]		
Item	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**SEM [2]**	**DUR**	**TRT**	**I**
Standing time, h/d	10.8	11.0	10.3	9.2	10.1	10.0	9.7	10.0	10.2	10.6	0.77	0.10	0.93	0.53
Total lying time, h/d	13.2	13.0	13.7	14.8	13.9	14.0	14.3	14.0	13.8	13.5	0.77	0.10	0.93	0.53
Lying time, right side, h/d	6.4	6.4	6.8	6.8	6.4	7.3	5.8 [y]	6.8 [x]	6.4	6.4	0.47	0.27	0.38	0.32
Lying time, left side, h/d	6.8	6.8	6.8 [y]	8.0 [x]	7.2	6.9	8.2	7.4	7.5	7.2	0.61	0.07	0.95	0.11
Total lying bouts *, #/d	13.6	12.5	16.8	15.2	14.7 [b]	18.5 [a]	15.6	15.0	14.3	12.0	1.13	<0.01	0.74	0.14
Lying bouts, right side, #/d	6.3	6.7	9.0	8.7	7.2 [b]	12.4 [a]	7.6	8.6	7.2	5.8	1.56	<0.05	0.37	0.10
Lying bouts, left side, #/d	6.2	6.7	8.7	8.5	7.2 [b]	11.6 [a]	8.6	8.7	7.4	7.6	1.05	<0.05	0.13	0.06
Rumination standing, min/d [+]	113	110	74.2	74.7	58.2	57.3	40.1	52.9	48.0	50.6	2.17	0.08	0.82	0.44
Rumination lying right, min/d [+]	124	98.4	83.1	64.1	65.9	49.4	46.4	44.7	55.0	42.6	2.22	<0.01	0.58	0.97
Rumination lying left, min/d	153 [y]	266 [x]	101 [b]	255 [a]	127	126	119	74.7	69.1	85.0	3.13	<0.05	0.21	0.11

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*), (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of duration of high-grain feeding (DUR), phytogenic feed supplement (TRT), and the interaction of duration on high-grain × supplementation (I). [a, b] Within corresponding week, means with different superscript indicate a significant difference ($p < 0.05$) between CON and TRT. [x, y] Within corresponding week, means with different superscripts indicate a tendency for significant difference ($0.05 < p \leq 0.10$) between CON and TRT. * Due to lack of normal distribution, values were first log-transformed prior to statistical analysis. [+] Due to lack of normal distribution, values were first root square-transformed prior to statistical analysis.

The rumination time while standing and while lying on the right or left decreased consistently (by 56, 63, and 60%, respectively) from week 0 and throughout the 4 weeks on the HG diet ($p < 0.05$; Table 4). In general, rumination times while lying down on the left side were greater than rumination time on the right side. In addition, the supplementation increased the rumination time while lying on the left side in week 1 on the HG diet ($p < 0.05$) compared to the CON.

3.5. Feed Bolus Ensalivation and Salivary Physico-Chemical Properties

Feed bolus size (as-is or on a DM basis) was greater in week 4 of the HG feeding challenge ($p < 0.05$) compared to week 0. The total amount of saliva in the feed bolus and the flow of saliva did not show a diet effect ($p = 0.97$), but the feed ensalivation (g saliva/g feed bolus) was highly influenced by the diet, with a strong reduction of 51% observed when the HG diet was fed ($p < 0.01$; Table 5). Supplementation with TRT did not change the feed bolus or saliva flow ($p \geq 0.13$). The Pearson correlation analyses showed a positive correlation between the ruminal pH and feed ensalivation (r = 0.54; $p < 0.01$), a negative correlation between the ruminal total short chain fatty acids and feed ensalivation (r = −0.53; $p < 0.01$), and a positive correlation between the ruminal acetate to propionate ratio and feed ensalivation (r = 0.62; $p < 0.01$).

Table 5. Effect of a dietary shift from only forage to a high-grain diet on feed bolus size and salivation of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

	Week 0		Week 4			*p*-Values [3]		
Item	**CON**	**TRT**	**CON**	**TRT**	**SEM [2]**	**DI**	**TRT**	**I**
Feed bolus size (as is), g	239	245	302	283	22.6	<0.05	0.76	0.55
Feed bolus size (DM), g	31.6	29.8	70.5	64.8	5.7	<0.01	0.49	0.72
Saliva in bolus, g	139	150	143	146	10.9	0.97	0.51	0.71
Feed ensalivation, g saliva/g feed	5.31	6.32	2.74	3.00	0.48	<0.01	0.13	0.59
Saliva flow, g/min	69.7	75.0	71.8	73.3	4.49	0.97	0.51	0.71

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*) (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of diet (DI), phytogenic feed supplement (TRT), and the interaction of diet × supplementation (I).

Several salivary physico-chemical properties changed due to the diet shift or the duration of the HG feeding regimen (Table 6). For example, we observed an increase in salivary pH with the increased duration on the HG diet, with the greatest values observed in weeks 3 and 4 on the HG diet. Compared to the CON, the TRT supplementation increased salivary pH ($p < 0.05$) in week 4 on the HG diet. In addition, the salivary lysozyme activity increased from the start of the HG feeding ($p < 0.05$), reaching the highest values in weeks 3 and 4 on the HG diet, an average increase of around 45% compared to week 0. On the other hand, we found a reduction in the salivary phosphate concentration during weeks 1 and 2 of the HG feeding regimen, but this variable increased in weeks 3 and 4 on the HG diet. In addition, TRT supplementation tended to increase the salivary buffer capacity ($p = 0.09$) in week 3 on the HG diet compared to the CON.

The concentrations of salivary bicarbonate, total mucins, and total proteins were not affected by the change in diet or the duration of the HG feeding. However, considering the reduction in feed bolus ensalivation, there was an overall reduction in the supply of these salivary components per gram of feed bolus.

Table 6. Effect of duration on a high-grain diet on salivary physico-chemical properties of non-lactating Holstein cows not supplemented or receiving a phytogenic feed supplement [1].

	Week 0		Week 1		Week 2		Week 3		Week 4			*p*-Values [3]		
Item	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**CON**	**TRT**	**SEM [2]**	**DUR**	**TRT**	**I**
Salivary pH	8.86	8.88	8.96	8.87	8.94	8.98	9.02	9.04	8.86 [b]	9.02 [a]	0.05	<0.05	0.59	0.11
Buffer capacity, decamol HCl/L/ΔpH	0.013	0.014	0.014	0.013	0.013	0.014	0.014 [y]	0.015 [x]	0.014	0.014	0.001	0.64	0.18	0.26
Bicarbonate, mM	67.7	69.6	73.6	68.5	67.8	70.0	73.1	71.8	73.4	74.1	3.68	0.20	0.93	0.64
Phosphate, mM	11.7	12.2	9.95	10.8	10.4	10.9	13.1	13.8	12.7	12.2	0.89	<0.01	0.64	0.88
Mucin, mg/mL	1.75	1.30	1.30	1.48	1.41	1.83	1.19	1.23	1.10	1.16	0.25	0.15	0.84	0.40
Lysozyme activity, U/mL/min *	24.9	26.4	45.1	34.6	39.6	46.2	42.8	49.7	48.1	44.9	1.20	<0.01	0.96	0.63
Total protein, μg/mL	445	442	404	390	529	486	405	381	452	404	55.0	0.56	0.13	0.99

[1] CON: A control ration; TRT: inclusion of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*), and cloves powder (*Syzygium aromaticum*) (0.04%, DM basis). [2] The largest standard error of the mean. [3] *p*-values for the effect of duration of high-grain feeding (DUR), phytogenic feed supplement (TRT), and the interaction of duration on high-grain × supplementation (I). * Due to lack of normal distribution, values were first log transformed prior to statistical analysis. [a, b] Within corresponding week, means with different superscripts indicate a significant difference ($p < 0.05$) between CON and TRT. [x, y] Within corresponding week, means with different superscripts indicate a tendency for a difference ($0.05 < p \leq 0.10$) between CON and TRT.

4. Discussion

This study aimed to evaluate the effect of the duration of an HG feeding challenge on chewing, eating, and lying behaviors as well as the salivary production and composition in cows with or without a phytogenic feed supplement. In agreement with our hypothesis, there was a reduction in the rumination time not only at the start of the HG feeding regimen, but also due to advanced duration on the HG diet. Consequently, the reduction in the rumination time likely contributed to the lower feed ensalivation observed after 4 weeks of the HG feeding regimen. Furthermore, the total chewing time decreased because of the duration on the HG diet. Chewing activity is essential for adequate rumen function because it stimulates salivation and normal rumen fermentation [16]. It has also been reported that a greater chewing time improves feed digestion by exposing nutrients and increasing feed surface area, thereby facilitating the activity of microbial enzymes [41]. Therefore, our findings clearly indicate that the duration on an HG diet exacerbates the negative effects on the chewing activity in cattle. Our observations for rumination time were lower than the values reported by Ben Meir et al. [42] for lactating cows consuming diets with similar levels of concentrate. These contrasting findings may be because of the higher DMI intake in lactating cows used by Ben Meir et al. [42], with intakes twice as high as the dry cows utilized in our study, resulting in more ruminal digesta available for rumination. Although there are limited data regarding the effects of phytogenic supplementation on rumination, the research conducted by our group showed that a blend of essential oils increased the rumination time during the first 2 weeks of an HG feeding challenge compared to a control TMR [43]. In another experiment, Castillo-Lopez et al. [29] demonstrated in a short-term trial that thymol supplementation tended to increase chews/min. However, in the present study, this effect was not observed, suggesting that thymol may exert only a temporary stimulating effect on chewing activity.

Our findings also indicate that the DMI increased from the first week of the HG feeding regimen. These results support reports from Dann et al. [44] for diets containing similar levels of starch. The increase in the DMI might have occurred because of the small particle size of the HG diet, which allows for a greater feed intake due to the decreased gut fill. In addition, the greater feed consumption may be explained by the improved feed acceptability with the inclusion of concentrate in the diets. Our findings showed a greater meal size and eating rate due to the advanced duration on the HG diet, which is a factor that increases the risk of ruminal acidosis, because this results in the accumulation of volatile fatty acids in the rumen. The latter observations explain the greater feed boli measured after 4 weeks on the HG. In general, the number of meals per day observed

in this study were lower compared to previous studies in lactating cows [18,33,42]. This may be because the nutrient utilization and energy metabolism are slower in dry cows compared to lactating animals. With regard to the effect of the TRT on the DMI, reports suggest that individual phytogenic compounds may influence feed intake [29]. In this trial, the TRT tended to reduce meal sizes in week 2 on the HG diet compared to the CON. This may be explained by the increased intake of long feed particles and peNDF for the TRT in that week, which contributed to gut fill. The increased preference for long feed particles possibly was due to olfactory and gustatory stimulation of TRT. This is a beneficial effect because of the role of fibrous feed ingredients in the regulation of ruminal pH, particularly when there is a need to modulate fermentation due to a low ruminal pH, as reported in the companion paper [30]. Furthermore, the findings from the correlation analyses in this study agree with our expectations and showed that a greater amount of saliva per gram of the feed that cows consume contributes to an increased ruminal pH by neutralizing the acids produced in the rumen. The positive association between the feed ensalivation and the ratio of acetate to propionate agrees with the simultaneous change in the feed ensalivation and acetate production due to a change in the proportion of the concentrate in the diets. For example, forage-based rations are associated with greater feed ensalivation and an increased acetate production.

The majority of the studies on feed sorting behavior have evaluated feed management or the fiber of the diet [3,25,45], but the influence of the duration of the HG challenge or phytogenic supplementation on feed sorting remains yet to be elucidated. Interestingly, the present study showed that the advanced duration on the HG diet decreased the cows' preference for the short feed particles. This may reflect a response of the cows to counteract the negative effects of low fiber diets on rumen pH, because short feed particles are rich in readily fermentable starch that increase ruminal fermentation and acidification.

Our hypothesis also stated that the lying time would increase with the duration of the HG feeding regimen. Several experiments have demonstrated that there is a close relationship between the standing and lying time and laminitis in cows. For example, reduced lying times and abnormal standing times seem to be indicators of the development of laminitis [46]. In this study, we found that there was an increase in the number of lying bouts on either the right or left side from the start of the HG feeding regimen, and these increased values were maintained throughout the HG feeding challenge. Greater lying bouts may reflect animal discomfort and may be due to the effect of the acidogenic diets that lead to damage in the lamina of the foot [47]. Furthermore, Fukasawa et al. [48] reported similar results, describing a tendency to increase lying bouts when implementing high concentrate feeding compared to forage feeding. The higher nutritive value of the diet has been suggested to influence lying time or lying bouts as well, with increasing lying times associated with a greater body condition score [6]. In this study, there was an increase in the body weight of cows (69 kg), which could have contributed to the greater lying bouts throughout the HG feeding.

Our results show that laterality of the lying behavior followed a similar pattern as reports from Tucker et al. [49], with a fairly even distribution between the left or right lying times. Previous research has demonstrated that cows preferably ruminate while lying down [17,50], which coincides with our findings. It is possible that as the cows experienced increased discomfort as a consequence of intensive rumen fermentation, they preferably ruminated while lying on the left side. These results are supported by pioneering findings by Bailey and Balch [51] and Albright [52], who suggested that lying on the left side is a strategic position for cows to increase rumination efficiency, because this position may facilitate the regurgitation process of the digesta due to the improved alignment of the esophagus with the rumen contents. In addition, the TRT group increased its propensity to lie on the left compared to CON, especially in the second week on the HG diet. Nonetheless, the exact association between the TRT supplementation and the lying side of cows remains to be elucidated.

Another aim of this study was to evaluate the changes in salivary composition and dynamics. In agreement with our hypothesis and with previous studies [28,35], the change to an HG diet had a negative impact on feed ensalivation. Beauchemin et al. [35] also suggested that eating time may influence salivary secretion in cows, which supports our findings showing that a lower eating time was associated with a lower feed ensalivation in week 4 on the HG diet. Moreover, salivary secretion is not influenced by rumination alone. For instance, it has been demonstrated that when the eating rate increases, there is a reduction in the daily salivation of cows [35]. This supports our findings showing that when the cows consumed the HG rations at a faster pace, the feed ensalivation was lower. With regard to the effect of phytogenic compounds on salivation, the increased salivation rate via individual phytogenic compounds previously reported [37] in a short-term trial was not confirmed in the present study. These contrasting findings may be because the stimulus for the salivation flow of individual phytogenic compounds could decrease when combined with other compounds. It is also possible that differences in the effects of these compounds are related to their distinct modes of action, with some substances being active in the oral cavity [53], while others influence salivary secretion through the olfactory stimulation of the nervous system [54,55].

Salivary physico-chemical properties play important roles in gut function and health. In this trial, we observed that salivary lysozyme activity increased at the start of the HG feeding regimen. The salivary lysozyme is known to act as an antimicrobial bioactive component [56,57]. Thus, this observation may reflect a host response to counteract a potential outgrowth of pathogens due to the drastic diet shift [57]. Additionally, we found that salivary pH increased with the advanced time on the HG diet, which may be a host response for ruminal pH regulation, given the role of saliva for proton removal and ruminal pH balance. On the other hand, we found a reduction in salivary phosphate during the first 2 weeks on the HG, an observation that is highly relevant because of the role of this salivary buffer in the regulation of ruminal pH. The latter effect may exacerbate the reduction in ruminal pH commonly observed when cattle are switched from forage to HG rations. Another finding was the tendency for TRT to increase the salivary buffer capacity in the third week of the HG feeding regimen, and to increase the salivary pH in the fourth week compared to CON; these changes may have contributed to the greater ruminal pH for TRT reported in those weeks in the companion paper [30]. However, there was no increment in bicarbonate or phosphate due to TRT. These findings indicate that the salivary pH or buffer capacity are also influenced by factors other than salivary major buffers [19,51]. Although the mechanism by which TRT supplementation influences buffer capacity or pH is not clear at the moment, TRT possibly influenced the profile of specific proteins in saliva [58] increasing salivary pH, a topic that deserves further investigation. Another potential explanation to the change in the salivary buffer capacity and pH is the hydration status, as reported in other studies [59], indicating that hydration status affects these salivary variables in other animal species.

5. Conclusions

The shift to HG feeding regimens has been perceived as the time with the greatest risk for cattle health because of the major adaptative changes that occur during this period. Our findings show that the negative impacts of an HG challenge are pronounced immediately after the dietary change. However, several effects were worsened by the duration on the HG diet, with host adaptive changes still observed after 3- and 4-weeks following diet change. More specifically, the advanced duration on the HG diet further decreased rumination and total chewing times. In addition, we found that the cows displayed a decreased preference for short feed particles, which might have been a response to modulate ruminal fermentation. The diet change increased the cows' lying bouts, and these values were maintained throughout the HG feeding challenge, which may reflect the animals' discomfort. Additionally, we found that the duration of the HG feeding regimen increased salivary pH, which was likely a response to counteract the reduced feed

insalivation. Furthermore, the HG rations reduced feed bolus ensalivation in week 4 and salivary phosphate in weeks 1 and 2 on the HG diet. The positive effects of TRT included an increase in salivary pH in week 4 on the HG diet and increased the cows' preference for fibrous feed particles in week 2 on the HG diet.

Given the effects of diet on animal behavior and salivation dynamics, further research is needed to counteract the negative effects of the HG diets not only during dietary change but also once the cattle have adapted to the HG rations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani12152001/s1, Table S1: Ingredients, chemical composition, particle size fractions, and physically effective fiber of the diets fed to cows during the study.

Author Contributions: Conceptualization, R.R.-C., E.C.-L., S.R., R.M.P., N.R. and Q.Z.; data curation, R.R.-C., E.C.-L. and A.H.; Investigation, R.R.-C., S.R. and E.C.-L.; writing—original draft preparation, R.R.-C.; writing—review and editing, R.R.-C., E.C.-L., S.R., R.M.P., A.H., N.R. and Q.Z; project administration, E.C.-L. and R.M.P.; funding acquisition, Q.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been supported by Austrian Federal Ministry for Digital and Economic Affairs and the National Foundation for Research, Technology and Development. BIOMIN Holding GmbH supports financially the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock. Open Access Funding by the Christian Doppler Laboratory for Innovative Gut Health Concepts of Livestock.

Institutional Review Board Statement: The methods and protocols followed in this experiment were approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna and the Austrian national authority according to the law for animal experiments 2012—TVG 2012 (protocol number: BMBWF- 68.205/0003-V/3b/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within this article or in the Supplementary Material.

Acknowledgments: Thanks to the personnel of the veterinary farm of the University of Veterinary Medicine Vienna for the assistance received. Thanks to Anita Dockner, Sabine Leiner, Arife Sener-Aydemir, Silvia Nöbauer, Manfred Hollmann, Thomas Enzinger, Parisa Kheirandish, Elmar Draxler, and Claudia Lang for their help. We thank Matthias Wenter (Südtiroler Rinderzuchtverband) for his support in data analysis of lying behavior.

Conflicts of Interest: Nicole Reisinger is employed by BIOMIN Holding GmbH, a company that manufactures and trades feed additives. Nonetheless, this fact did not interfere in the design, analysis, or interpretation of the data.

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5. GENERAL DISCUSSION

This thesis primarily aimed to investigate the responses of salivation dynamics and salivary composition of cows under SARA, and whether these responses can be modulated by supplementation of single phytogenic compounds. For this, the first part of the thesis assessed the rather short-term effects, which were evaluated in a high-grain diet using nine non-lactating rumen-cannulated cows after the animals have eaten 2.5 kg of DM (feed + treatment) for up to 4 hours. The diet consisted of 65% concentrate and 35% forage (DM basis). Each of the nine single phytogenic compounds were evaluated at a 1X and 10X increase fold concentration.

For the second part of this thesis, the main aim was to look into animal response in terms of rumen fermentation parameters, inflammation, salivation, chewing and lying behavior of cows under a long-term SARA challenge, and to verify if such parameters can be modulated by a PAS. Therefore, nine non-lactating rumen-cannulated cows were divided in two groups (5 and 4 animals each) and were used in a change-over experimental design. First the cows received a TMR with forage (grass, corn silage and hay) for one week to be used as a baseline, and then high grain adaptation was conducted through a daily increment of 10% concentrate diet until reaching 65% concentrate diet and 35% forage (DM basis) on the last day of the adaptation week and were fed the HC diet for 4 more consecutive weeks. During the first day of the baseline diet, the cows started receiving either the PAS or a control (CON) dosed in the rumen through the cannula. Feed, water, and mineral blocks were provided *ad libitum*. Cows had access to a feed bunk with an electronic scale and daily feed intake was recorded automatically. Each run lasted 41 days, which included one week of baseline, one week for diet adaptation and 4 weeks of HC diet (SARA challenge), with a washout period between runs of 4 weeks in which the cows received an only forage-based diet.

Summarizing, these experiments demonstrated an effect on saliva both in the short and in the long term, most importantly in terms of saliva composition after inclusion of different phytogenic compounds. An effect of the PAS was evident from the third week of HG feeding, as confirmed by both the ruminal fermentation parameters (local effect) and the inflammation and tissue damage proteins (systemic effect). However, we observed reduced salivation, which could relate to smaller meal size. Taken all together, the results suggest a positive effect of the PAS in the long term, confirming our hypothesis and providing interesting insights for future research.

Data from this research have been published as three peer-reviewed papers in two scientific journals. The first publication from experiment 1 in *Frontiers in Physiology* (Ricci et al., 2021). The second and third publications from experiment 2 in *Animals* (Rivera-Chacon et al., 2022a; Rivera-Chacon et al., 2022b).

5.1. Salivary changes in cows fed an acidogenic diet supplemented with phytogenic compounds

Salivation and saliva properties are highly important for ruminants, especially under SARA conditions. First, a drop of salivation flow could be seen as a cause of SARA (Beauchemin, 1991; Beauchemin and Penner, 2009; Hossain, 2020), and secondly, changes in the salivary properties due to SARA are believed to impact rumen health and digestion processes (Giger-Reverdin, 2018; Castillo-Lopez et al., 2020a). Yet, there has been limited research during the last two decades in salivary dynamics and composition and how salivary variables are affected by changes in fiber proportion in dairy cows' diets. The same is true regarding the effects of phytogenic compounds on saliva. Pioneering research stated that saliva secretion declined when cattle were fed forage compared with ground or pelleted diets (Wilson and Tribe, 1963; Weston and Hogan, 1967). A few decades later Maekawa et al. (2002) proved that saliva secretion decreased as concentrate in

the diet increases, and our findings confirmed these results. It is important to highlight that saliva consists of a complex mixture of organic compounds, proteins, and ions together with microorganisms, food and cellular debris (Pedersen et al., 2002). Our study demonstrated that salivary composition can be influenced by diet or feed intake, disagreeing with older studies (Balch, 1958; Bailey 1961), which had postulated that salivation rate was higher for only concentrates compared with forage and secretion and phosphate increased with concentrates compared to only hay, respectively. On the other hand, other studies support our findings in relation with saliva pH and increasing amounts of saliva secretion while eating (McDougall, 1948). In the experiment evaluating the effect of individual phytogenic compounds, the fact that the same response was registered for 5 different compounds seem to support the physiological defense mechanism theory that decreased saliva pH based on acid proline rich proteins (Burrit et al., 1987; Sadashivappa Pateel et al., 2022). Capsaicin and garlic oil seemed to have beneficial results in terms of saliva buffer capacity, buffer concentrations, and salivation dynamics, while menthol and mint oil tended to increase ensalivation. Taken all together, these effects might be beneficial for the cow's health, especially during SARA because they may counteract the fast SCFA build up in the rumen neutralizing the pH. Furthermore, the fact that different phytogenic compounds provided different beneficial effects supports the idea that using a blend of substances could enhance the positive effects on the salivation and on the rumen health.

There are different salivation values reported in the literature depending on diet composition (Keesman et al., 2016). For instance, Chibisa et al. (2016) reported lower ensalivation of feed (2.22 ml/g DM) in beef cattle and a diet with higher DM concentration. Our results for salivation rate are also lower in comparison with Maekawa et al. (2002) that worked with lactating cows and

higher DM intakes and reported values of 210 ml/min. The differences between studies might be due to not only the diet composition, but also the breed as well as the lactation stage.

For the second experiment there was only an effect of length of HC feeding, which reduced feed ensalivation after 4 weeks. There was no effect of PAS in regards to saliva dynamics. Additionally, the correlation analysis proved that greater saliva per gram of feed ingested generated positive effects in terms of ruminal pH, which could explain the greater mean ruminal pH for PAS in week 4. It is also relevant to point out that salivation seems to be influenced by the number of meals a day, as reported by Carter et al. (1990). The results from the second experiment seem to agree with this study, considering that cows had greater number of meals during the week of forage feeding, reaching the lowest number of meals during week 4 of HC feeding. Furthermore, this could be linked with the time when cows experienced SARA, because the lowest number of meals coincided with the lowest ensalivation and the lowest pH. Salivary phosphate decreased during the first 2 weeks of intensive concentrate feeding, which may explain why ruminal pH reached the lowest minimum values in week 2. Interestingly, PAS tended to increase salivary buffer capacity in week 3 and increased saliva pH in week 4 of concentrate feeding. A possible explanation could be that the CON group showed a tendency to greater meal size and visit size for weeks 2 and 3, respectively, compared with PAS. Particularly, CON cows tended to have lower salivary buffer capacity on week 3, which can explain lower mean and minimum ruminal pH, since these cows were somehow increasing the intake of concentrate per meal. Furthermore, increase in lysozyme activity with duration of high concentrate feeding may have been due to the changes in oral microbiome as a defense mechanism, considering that this salivary component can have bactericidal properties (Kaplan and Baum, 1993; Oliver and Wells, 2015; Gilmutdinov et al.,

2020). These changes could be also in response to SARA, since recent research has demonstrated alterations in salivary microbiome in cows experiencing subacute acidosis (Yang et al., 2022).

The differences between our findings from the long-term experiment and those observed in the short-term experiment could be due not only to the organoleptic properties of the substances tested, but also to the meal size. In fact, in the first experiment, the amount of ingested feed was controlled during the first 4 hours after feeding; however, the second experiment the animals had *ad libitum* access to the feeders.

Both experiments allowed us to affirm that salivary composition can be modulated with phytogenic compounds to a point to potentially reduce risk of SARA in cows fed HC diets for longer periods of time (4 weeks). Additionally, length of SARA challenge proved to modify physico-chemical salivary composition increasing pH, phosphate concentration and lysozyme activity. This confirms the hypothesis that changes in saliva composition due to PAS can impact rumen fermentation and inflammation parameters.

5.2. Ruminal pH and fermentation in cows with a phytogenic additive supplementation during SARA

If we consider the definition of subacute ruminal acidosis as at least 314 minutes per day with ruminal pH below 5.8 (Zebeli et al., 2008), both our experiments successfully induced SARA with a short dietary transition of one week to a 65% concentrate diet. Despite the recorded drops, ruminal pH during week 3 with PAS started showing increasing values, possibly due to decreasing D-lactate concentration. Similar results including increased mean ruminal pH in cows fed a 50:50 forage/concentrate ratio diet with the supplementation of menthol, levomenthol, β-linalool, anethole, hexadecanoic acid and ρ-menthane were reported by Kholif et al. (2020), and these findings can be linked to a reduction of propionate in the rumen (Kholif et al., 2021). Another plant

compound included in our PAS is menthol, that according to Castillo-Lopez et al. (2021b) tended to reduce propionate concentration in the rumen with a concomitant increase in acetate. This modulation on rumen fermentation was observed in our findings during week 2 and could be the reason why rumen pH increased during the following weeks. This indicates that the prolonged supplementation with menthol could prevent pH drops in animals fed acidogenic diets. Furthermore, thymol, that was also part of the PAS used in our experiment, contains ρ-cymene and γ-terpinene (Pinto et al., 2020) which exert ruminal pH modulation in a similar way as menthol (Patra et al., 2019b). Literature has also demonstrated that eugenol tended to increase mean ruminal pH in experiments with HC diets (Benchaar et al., 2007; 2008) because of positive shifts in rumen microbial population (Calsamiglia et al., 2007; Patra, 2011). Therefore, it can be speculated that the changes in ruminal pH occurred as a consequence of changes in ruminal microbial community in our experiment. In fact, results from one companion experiment demonstrated the capability of ruminal microbial niche to adapt during a prolonged SARA challenge (Ricci, 2022) which could relate with how rumen pH with PAS increased on the last 2 weeks of the experiment.

The CON group of cows experienced severe SARA as demonstrated by a greater time with pH below 5.8 in comparison with PAS in weeks 3 and 4 of HC feeding. This could be interpreted as a slower starch degradability rate in the rumen from week 3 onwards with PAS, similar to the results reported by Newbold et al. (2004) using essential oils. Results for D-lactate in our experiment coincide with previous reports (Qumar et al., 2016) where a tenth-fold increase occurred when diet was changed from forage to high-grain. However, it is necessary to point out the limited research reporting ruminal lactate concentration with PAS, which indicates that comparing our results to previous studies may be challenging.

The results of this thesis proved that there does not seem to be differences in the rumen and reticular fermentation pattern. In terms of rumen fermentation, different experiment lengths demonstrated a decrease in ruminal acetate, isobutyrate and acetate/propionate ratio whereas increased propionate and valerate feeding cows high-grain diets for two or four consecutive weeks, respectively (Neubauer et al., 2018; Pourazad, et al., 2016). It is common knowledge that the accumulation of SCFA in the rumen increases the risk of SARA, which justifies the on-going research using PAS in cattle nutrition, especially seeking for potential to modulate ruminal fermentation (Lillehoj et al., 2018). Reports from Neubauer et al. (2018) with similar concentrate inclusion using a phytogenic blend were similar to our findings for week 2 of HC feeding, with increasing butyrate values. This result has a positive implication in terms of rumen health (Miguel et al., 2019) and could imply positive outcomes in term of avoiding milk fat depression (Steele et al., 2011b) or decreasing proton release from fermentation per unit of organic matter, as demonstrated by Owens and Goetsch (1988). This experiment also demonstrated an increase in acetate on week 2 of HC: this has potential positive implications if we consider the *de novo* milk fat synthesis (Kajikawa et al., 1990), although both experiments presented in this thesis did not work with lactating cows. Additionally, proliferation of butyrate producing bacteria is a sign of a healthy gut (Clemente et al. 2012; Geirnaert et al. 2017) and would reflect positive effects contrasting SARA impacts, as demonstrated in the last 2 weeks of the experiment. Other literature also suggests that work with phytogenic compounds should consider a longer adaptation beyond two to three weeks (Joch et al., 2019), this may have positive implications on rumen fermentation and pH in a longer-term experiment.

5.3. Inflammation and liver tissue damage in cows fed an acidogenic diet with phytogenic additive supplementation

Different inflammation markers were assessed in experiment 2. Evidently, the acidogenic diet is associated with increased concentrations of inflammation markers. In our experiment, SAA showed an increase from the first week of HC until week 3 in the animals that did not receive PAS. A possible explanation may include PAS effects on reducing the translocation of toxins or biogenic amines from the rumen/gut level to the bloodstream. Another potential alternative to reduce inflammation would be increasing the hepatic clearance of toxins via bile. Estimation of cholesterol levels to relate it with endotoxin stimulation has been performed in previous studies (Ametaj et al. 2005 and 2010), but was unfortunately not possible in our experiment. Effects of the PAS on cholesterol levels could imply a positive effect on toxin clearance and could justify the lower levels of SAA measured (Jafari et al., 2006; Humer et al., 2018). Another explanation might be the regulation of cell-growth and apoptosis in response to the PAS, which in turn could regulate the acute phase response in cattle. The reduction on SAA in the blood is a positive sign after PAS, showing that there is also less endotoxin in the rumen and may be due to reducing apoptosis, and therefore a reduced translocation able to reach the bloodstream as reported by Yang et al. (2010) and Oh et al. (2017) with cinnamon and capsicum, respectively. Additionally, Yang et al. (2010) reported lower SAA in steers fed plant compounds coinciding with our findings during week 3 of HC feeding. Haptoglobin is released during the acute phase response binding with hemoglobin to prevent iron utilization (Wassell, 2000), and Hp increase is commonly used as inflammation marker in cows with SARA (Gozho et al., 2006). A different experiment evaluating a diet with 39% concentrate in lactating cows supplemented with a mixture of phytogenic compounds demonstrated a decreased Hp concentration compared with the control group (Rodrigues et al. 2019), this agrees with our findings in the last two weeks of the SARA challenge.

The concentration of liver enzymes usually increases with tissue damage. Wille et al., 2010 indicated threshold values of 10.5 and 27 U/L for Glutamate Dehydrogenase (GLDH) and Gamma-glutamyl transferase (GGT), respectively. These values were overcome from the third week of HC feeding onwards, possibly reflecting impairment of liver function by the acidogenic diet. Different phytogenic compounds have demonstrated to improve liver health and reduce oxidative stress (Upadhaya and Kim, 2017). Although the phytogenic compound used in our experiment did not affect the liver parameters evaluated, it alleviated the negative effect of HC diets as shown by the inflammation markers, demonstrating the positive influence on animal health from the third week of HC feeding. Therefore, the use the PAS for at least three weeks may contribute to decrease systemic inflammation parameters in cows experiencing SARA. It is important to state that most of the research with phytogenic compounds has been conducted with mixtures of different kinds or concentrations, and there is limited literature citing individual components or additives to explain the exact mechanism of action.

5.4. Behavior of cows fed an acidogenic diet with phytogenic additive supplementation

Rumination time in the second experiment decreased, as it was influenced by low fiber level of the diet as well as duration of the HC challenge. Similarly, total chewing time (eating + rumination) decreased as weeks of HC feeding progressed. Literature has proved that chewing is an essential activity for adequate rumen function due to salivation stimulation and normal rumen fermentation (Mertens, 1997). Furthermore, ruminants spend less eating and ruminating time when fed pelleted or ground diets (Freer and Campling, 1963; Weston and Hogan, 1967). Additionally, chewing during rumination seems to have more relevance for saliva production than during eating (Bailey and Balch, 1961a; Ulyatt et al., 1984), which could explain why it was not possible to find differences in feed ensalivation while eating between PAS and CON. Considering the differences

in rumination time compared toother studies with similar concentrate levels, the lower rumination time reported in this thesis can be explained by the fact that our cows were not in high nutritive demand and therefore did not show greater DMI as a high-yield lactating cows. Even though to our knowledge, there is limited information due to the lack of studies relating the use of PAS with rumination, a different experiment demonstrated that a blend of essential oils increased rumination time during the first 2 weeks of HC feeding (Kröger et al., 2017). Castillo-Lopez et al. (2020) found that thymol tended to increase chews per minute in a short-term trial; however, this was not observed in our findings and may be due to the temporary effect of this plant compound when acting alone. Furthermore, both aforementioned experiments evaluated a phytogenic additive for shorter time compared to the long-term experiment presented in this thesis. Utilizing a PAS continuously for a period of 4 weeks seemed not to impact the rumination and chewing activity directly, although it showed an effect on salivation.

A TMR with high concentrate level is more palatable than an only forage one. Thus, we can state that a greater eating rate because of greater meal sizes when cows are fed HC diets contributes to increase the risk of SARA. Therefore, reduced meal size will implicate a reduction of acidosis risk, as demonstrated in week 3 of HC feeding in our study and previously reported by Beauchemin and Penner (2009).

There is a knowledge gap on feed sorting behavior of cows fed a PAS. Some research mostly focused on limited feed bunk area or differentiating multiparous from primiparous cows (DeVries and von Keyserlingk, 2006; DeVries et al., 2007). This thesis demonstrated that cows decreased their preference for shorter feed particles influenced by the length of the HC challenge: this could be an animal response to counteract the effects of a low fiber diet on rumen fermentation and low pH.

Lying behavior in dairy cattle has been studied mostly in relationship with infrastructure design, limited lying areas, or evaluating different feeding schedules. In the second experiment presented in this thesis, the focus was on assessing animal behavior with normal housing conditions and especially in relation to diet and PAS. We found that there was an increase in number of lying bouts from week 1 of HC compared to the forage week. Greater lying bouts are associated with animal discomfort and could be due to the damage in the lamina of the foot (Bergsten, 2003). Furthermore, in the second experiment the cows gained on average 69 Kg, contributing to greater lying bouts with HC feeding. Nevertheless, other researchers suggest that cows lying down more than 11 h a day are more productive than cows that lie down less time (Lovarelli, et al., 2020b); these values are lower than the ones recorded in the experiment, which could be explained by the fact that it included dry cows. Furthermore, we need to consider that the experiment presented in this thesis was conducted during the summer, and seasonality can influence lying behavior as demonstrated by Lovarelli et al. (2020a), with cows reducing lying times during summer and increasing lying time during winter.

In terms of laterality, cows seem to adapt to the effects of SARA by week 1 of HC: lying down to the left more than to the right while ruminating could be a way to reduce the negative impacts of acidosis and allow a more efficient rumination performance, as suggested by Balch (1955) and Albright (1987). Additionally, the increase in total lying bouts during week 2 of HC may be a result of the exacerbation on animal behavior of cows during the week cows started experiencing SARA.

5.5. Synopsis and outlook

The first aim of the research presented in this thesis was to study the implications of a short and prolonged acidogenic diet with 9 PHY and one PAS on saliva, rumen health, inflammation, and animal behavior. A second aim was to elucidate whether phytogenic additives can counteract the negative impacts of high-concentrate diets on dairy cows. We proved that different plant compounds can positively influence saliva secretion and physico-chemical composition. In the second experiment, it was possible to affirm that there is a positive correlation between greater saliva per gram of feed ingested and greater mean ruminal pH. A next step would include evaluation of saliva components in high-yield lactating cows and perhaps include capsaicin as one ingredient in future PAS formulations. In terms of saliva dynamics, it would have been desirable to evaluate saliva dynamics more times during the prolonged HC feeding to see if there were changes during the time the cows were eating the acidogenic diet and to estimate salivation while ruminating. Additionally, rumen fermentation demonstrated that phytogenic compounds can modulate rumen pH from the third week of HC. The phytogenic mixture included in experiment 2 seemed to influence rumen fermentation, therefore it would be of great interest to try to assess if these changes are reflected into the microbial community as well. Taking into account that lactating cows show their true potential genetic up to the peak of lactation, it will be interesting to evaluate prolonged challenges considering the cows nutrition requirements up to 90 days and assess if acute-phase proteins are positively influenced by phytogenic additive also in these conditions. In addition, animal behavior and phytogenic additives should be explored further. It was revealing to find that PAS reduced meal size with positive implications in rumen fermentation. The next steps could include assessing how animals change their behavior (eating, lying and ruminating combined) on a sudden or smoother transition towards the acidogenic diet, considering that in practical terms cows receive a prepartum diet 21-24 days before parturition to transition

immediately to a higher energy demand diet on day 1 of lactation. Finally, the results from this study may help link and understand better how PAS can reduce SARA incidence in dairy cattle.

6. REFERENCES (INTRODUCTION AND GENERAL DISCUSSION)

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7. SUPPLEMENTARY MATERIAL

7.1. Supplementary Material for Manuscript 1

Supplementation with phytogenic compounds modulates salivation and salivary physico-chemical composition in cattle fed a high-concentrate diet

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Supplementary Material

Supplementary Tables

Table S1. Ingredients, chemical composition, and physically effective NDF (peNDF) of the total mixed ration (TMR) fed to the cows, which was used to prepare the controlled meal.

Ingredients	**% of DM**
Grass silage	26.25
Corn silage	8.75
Rolled wheat grain	26.00
Pelleted concentrate mixture[1]	39.00
	% of DM (unless otherwise stated)
TMR chemical composition	
DM, % as fresh	46.4 ± 0.84
Crude protein (CP)	16.5 ± 1.41
Neutral detergent fiber (NDF)	32.4 ± 1.33
Acid detergent fiber (ADF)	19.7 ± 1.21
Starch	32.8 ± 1.75
Ether extract	3.0 ± 0.13
Non-fiber carbohydrates	41.9 ± 1.72
Ash	6.2 ± 0.10
peNDF[2] >8 mm	12.9 ± 0.6

[1]The pelleted concentrate mixture contained: triticale (3.75%), wheat (11.25%), rapeseed meal (34.0%), bakery by-product (45.0%), molasses (3.0%), mineral-vitamin premix for dairy cattle (2.0%), and limestone (1.0%).

[2]Physically effective NDF.

Table S2. Substances tested, scientific name of the plant source and relative dosages. Each substance (in powder) was mixed in 2.5 kg (dry matter basis) of feed.

Substance	Scientific name of plant source	1x dosage (mg/kg)	10x dosage (mg/kg)
Angelica root	*Angelica archangelica* L.	6.6	66
Capsaicin	*Capsicum* sp.	10	100
Garlic oil	*Allium sativum* L.	0.3	3
Gentian root	*Gentiana lutea* L.	6.6	66
Ginger	*Zingiber officinale*	40	400
L-menthol	*Mentha arvensis* L.	6.7	67
Mint oil	*Mentha arvensis* L.	15.3	153
Thyme oil	*Thymus vulgaris* L. and *Thymus zygis* L.	9.4	94
Thymol	Chemical synthesis	5	50

Table S3. Effect of supplementation with angelica root on salivary physico-chemical properties, salivation and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	CON	LOW	HIGH	SEM[4]	Linear	Quadratic
pH	8.81	8.73	8.76	0.067	0.61	0.52
Bicarbonate, mM	77.09	90.00	80.75	8.918	0.64	0.17
Phosphate, mM	10.54	9.95	11.67	1.117	0.42	0.35
Total proteins, µg/mL	312.8	370.0	283.0	42.47	0.58	0.19
Buffer capacity, mol of HCl/L/ΔpH	0.014	0.015	0.014	0.0009	0.99	0.60
Osmolality, mOsm/kg	254.6	241.9	249.7	3.18	0.05	<0.01
Lysozyme activity, U/mL/min	38.58	30.11	36.89	8.306	0.85	0.39
Mucins, mg/mL	1.55	1.37	1.44	0.318	0.62	0.56
Stimulated saliva[3]						
pH	6.81	6.61	6.69	0.069	0.17	0.09
Phosphate, mM	12.60	12.99	13.58	1.182	0.48	0.94
Buffer capacity, mol of HCl/L/ΔpH	0.038	0.033	0.035	0.0033	0.32	0.30
Osmolality, mOsmol/kg	386.0	326.8	393.6	64.10	0.82	0.07
Saliva dynamics						
Salivation rate, g/min	74.32	94.99	81.48	10.477	0.55	0.17
Ensalivation, g/g DM feed	4.18	4.34	4.25	0.356	0.86	0.75
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.883	1.198	1.015	0.123	0.33	0.08
Bolus size (as is), g	223.54	280.56	249.79	31.226	0.47	0.23
Bolus size (DM), g	37.52	46.47	43.18	6.745	0.47	0.44

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 6.6 ppm of angelica root in 2.5 kg (DM) of TMR; HIGH: 66 ppm of angelica root in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

Table S4. Effect of supplementation with gentian root on salivary physico-chemical properties, salivation and feed bolus dynamics of non-lactating Holstein dairy cows.

	Treatment[1]				*P*-value	
Unstimulated saliva[2]	CON	LOW	HIGH	SEM[4]	Linear	Quadratic
pH	8.87	8.80	8.70	0.052	0.03	0.84
Bicarbonate, mM	74.53	88.67	85.58	9.142	0.33	0.40
Phosphate, mM	9.21	9.39	9.30	1.580	0.96	0.93
Total proteins, µg/mL	315.8	226.1	163.4	82.65	0.10	0.84
Buffer capacity, mol of HCl/L/ΔpH	0.013	0.013	0.014	0.0011	0.93	0.82
Osmolality, mOsm/kg	250.1	246.4	249.6	4.38	0.88	0.26
Lysozyme activity, U/mL/min	41.64	41.12	32.18	9.078	0.40	0.69
Mucins, mg/mL	1.73	1.61	2.00	0.824	0.84	0.79
Stimulated saliva[3]						
pH	6.85	6.62	6.69	0.095	0.06	0.02
Phosphate, mM	11.41	14.10	10.81	1.969	0.70	0.02
Buffer capacity, mol of HCl/L/ΔpH	0.039	0.084	0.045	0.0270	0.82	0.05
Osmolality, mOsmol/kg	436.0	455.4	428.4	40.97	0.81	0.36
Saliva dynamics						
Salivation rate, g/min	75.16	85.00	90.24	7.338	0.08	0.74
Ensalivation, g/g DM feed	4.54	4.51	4.14	0.733	0.53	0.76
Ensalivation, l/kg DM feed/kg $LW^{0.75}$	0.062	0.060	0.051	0.014	0.42	0.75
Bolus size (as is), g	231.20	258.35	288.78	31.27	0.08	0.95
Bolus size (DM), g	36.80	43.18	48.80	8.347	0.11	0.95

[1]CON: a control diet prepared by combining 50 g of the wheat silica carrier and 2.5 kg (DM) of TMR; LOW: 6.6 ppm of gentian root in 2.5 kg (DM) of TMR; HIGH: 66 ppm of gentian root in 2.5 kg (DM) of TMR.
[2]Saliva samples collected from the mouth after treatment intake; data from samples collected before treatment were used as covariates.
[3]Saliva samples collected at cardia, by collecting and straining saliva from ingested feed boli.
[4]The largest standard error of the mean.

7.2.Supplementary Material for Manuscript 2

Supplementing a phytogenic feed additive modulates the risk of subacute rumen acidosis, rumen fermentation and systemic inflammation in cattle fed acidogenic diets.

Table S1. Effect of supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*) on reticular short chain fatty acid profile, ammonia and lactate in cows consuming a forage diet or a high concentrate diet[1].

	Forage diet Week 0		High concentrate Week 1		High concentrate Week 2		High concentrate Week 3		High concentrate Week 4			*P*-values		
Item	CON	PHY	CON	PHY	CON	PHY	CON	PHY	CON	PHY	SEM[2]	D	T	I
Total SCFA concentration, mM	74.1	77.2	105	96.2	107	101	109^{x}	94.0^{y}	108	109	3.36	<0.01	0.04	0.09
% of total SCFA														
Acetate	66.4	66.6	58.1	57.1	52.5^{b}	55.8^{a}	58.3	58.0	58.0	58.4	0.75	<0.01	0.56	<0.01
Propionate	16.2	15.8	20.8	21.8	29.6^{a}	23.8^{b}	23.5	22.8	23.5	23.2	0.71	<0.01	0.12	<0.01
Butyrate	10.4	10.6	15.6	16.1	12.0^{b}	14.0^{a}	12.7	13.0	12.7	12.7	0.53	<0.01	0.21	0.06
Isobutyrate	1.89	1.94	0.95	1.03	0.88	1.08	1.15	1.23	1.14	1.07	0.06	<0.01	0.40	<0.01
Isovalerate	2.40	2.53	1.40	1.36	1.54^{b}	1.98^{a}	1.66^{y}	1.97^{x}	1.62	1.57	0.11	<0.01	0.31	<0.01
Valerate	1.72	1.75	2.09	2.14	2.43	2.36	2.03	2.04	2.11	2.28	0.07	<0.01	0.55	0.63
Ratio of acetate to propionate	4.07	4.21	2.78	2.62	1.76^{b}	2.33^{a}	2.47	2.53	2.45	2.51	0.10	<0.01	0.19	<0.01
Ammonia, mg/dL	19.11	20.01	12.52	13.91	10.67^{b}	16.30^{a}	19.57^{a}	15.48^{b}	16.03	18.45	1.33	<0.01	0.15	<0.01
Lactate[4]														
D-lactate, mM	0.186	0.195	0.640	0.650	0.633	0.723	0.872	0.893	0.699	0.795	0.0015	<0.01	0.25	<0.05
L-lactate, mM	0.123	0.099	0.313	0.310	0.249	0.289	0.290	0.406	0.348	0.390	0.0007	<0.01	0.27	<0.01
Total lactate, mM	0.330	0.311	0.972	0.967	0.863	1.020	1.194	1.309	1.102	1.219	0.0020	<0.01	0.27	<0.01

[1]CON: A control diet containing no phytogenic product; PHY: supplementation with a phytogenic feed additive based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*).

[2]The largest standard error of the mean.

[3]*P*-values for the effect of diet (D), phytogenic treatment (T) and the diet × week × treatment interaction (I).

[4]Values were transformed using the root square function after checking for normal distribution, and were transformed back after the analysis.

[a,b]Means with different superscripts indicate a significant difference ($P < 0.05$) between CON and PHY.

[x,y]Means with different superscripts indicate a tendency for significant difference ($0.05 < P \leq 0.10$) between CON and PHY.

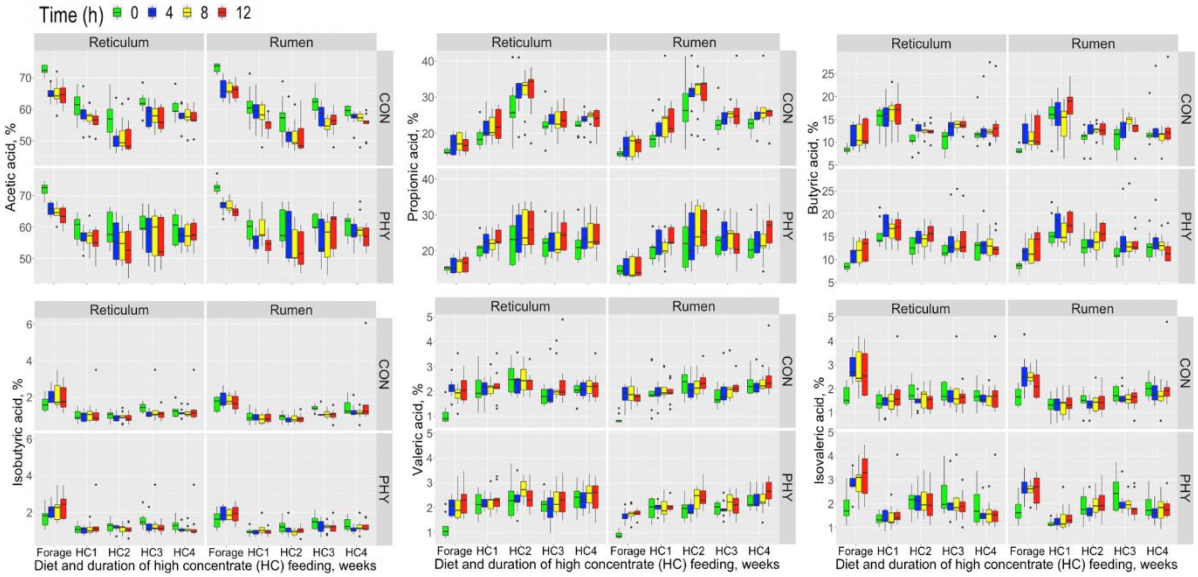

Figure S1. Boxplots illustrating the fermentation pattern and short chain fatty acid profile in the reticulum and rumen with time post-feeding according to diet and duration of high concentrate feeding in Holstein cows without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*).

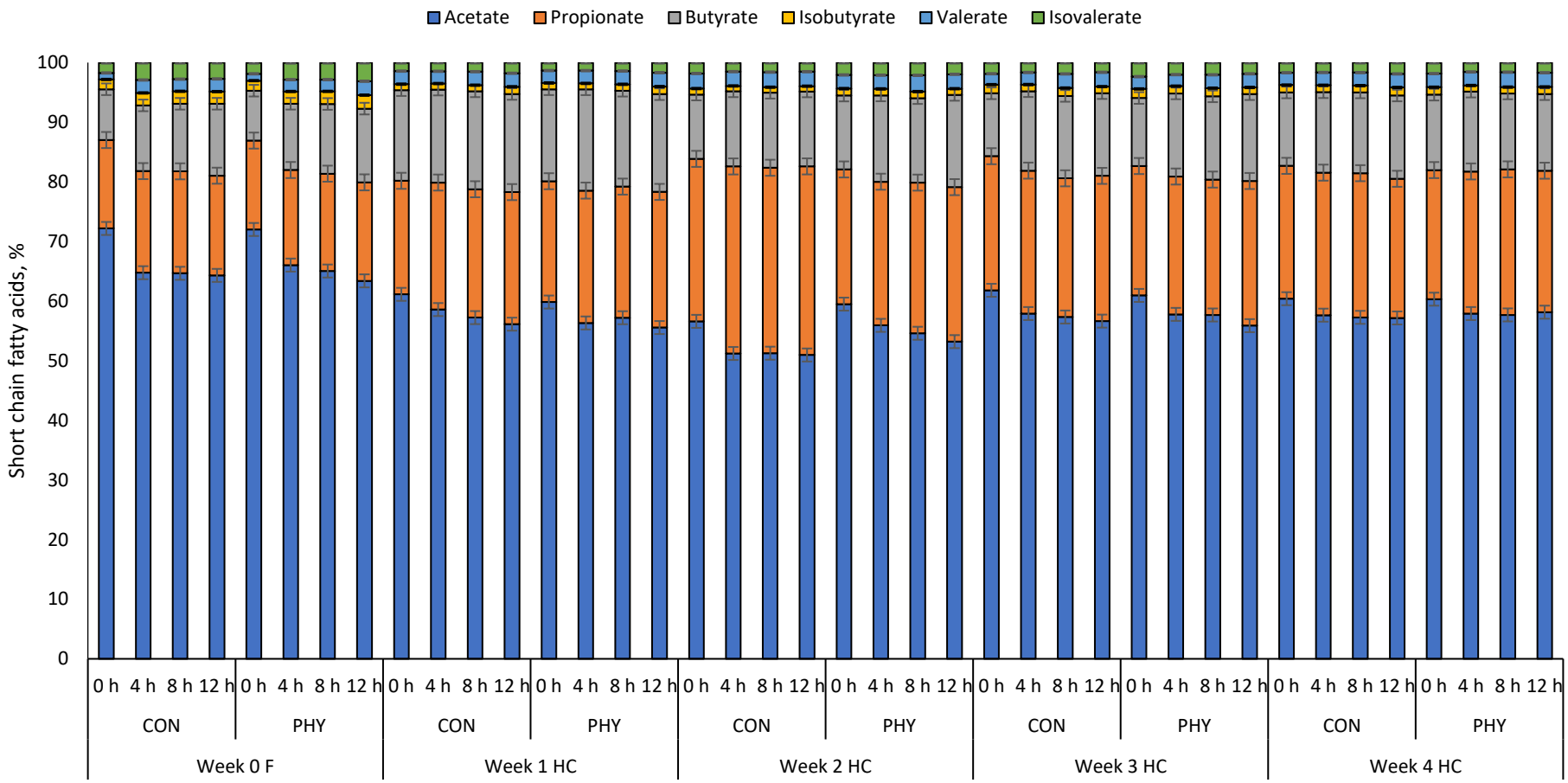

Figure S2. Variation of reticular short chain fatty acid fermentation from 0 to 12 h post-feeding in cows fed either all-forage (F) or a high concentrate (HC), without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*). *P*-values: Acetate, Time < 0.01, Trt = 0.54, Diet < 0.01, Time×Trt×Diet×Week < 0.01; Propionate, Time < 0.01, Trt = 0.17, Diet < 0.01, Time×Trt×Diet×Week < 0.01; Butyrate, Time < 0.01, Trt = 0.50, Diet < 0.01, Time×Trt×Diet×Week < 0.01; Isobutyrate, Time = 0.42, Trt = 0.67, Diet < 0.01, Time×Trt×Diet×Week < 0.01; Valerate, Time < 0.01, Trt = 0.64, Diet < 0.01, Time×Trt×Diet×Week < 0.01; Isovalerate, Time < 0.05, Trt = 0.29, Diet < 0.01, Time×Trt×Diet×Week < 0.01

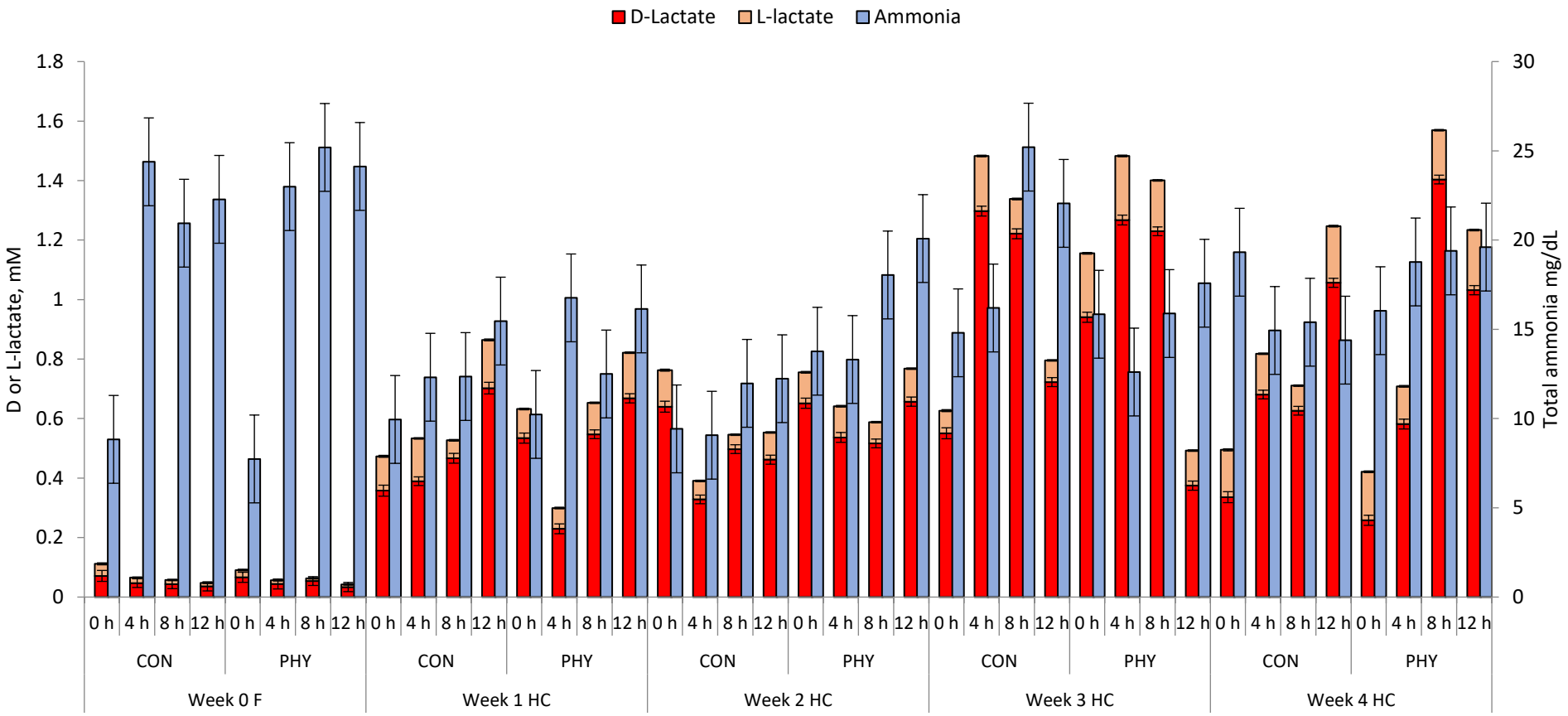

Figure S3. Variation of reticular D-lactate, L-lactate (mM), and total ammonia concentration (mg/dL) from 0 to 12 h post-feeding in cows fed either all-forage (F) or a high concentrate (HC), without supplementation (CON) or supplemented with a phytogenic feed additive (PHY) based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*). P-values: D-lactate, Time = 0.25, Trt = 0.48, Diet < 0.01, Time×Trt×Diet×Week < 0.01; L-lactate, Time = 0.16, Trt = 0.38, Diet < 0.01, Time×Trt×Diet×Week < 0.05; Total ammonia, Time < 0.01, Trt = 0.15, Diet < 0.01, Time×Trt×Diet×Week < 0.01

7.3.Supplementary Material for Manuscript 3

Effect of duration of high-grain feeding on chewing, feeding behavior, and salivary composition in cows with or without a phytogenic feed supplement.

Table S1. Ingredients, chemical composition, particle size fractions and physically effective fiber of the diets fed to cows during the study.

Item	Diet and treatment, % DM (unless otherwise stated)		
	Forage diet*	High-grain diet CON	High-grain diet TRT
Ingredients			
Grass silage	75	26.25	26.25
Corn silage	15	8.75	8.75
Grass hay	10	0	0
Control concentrate[1]	0	65	0
Treatment concentrate[2]	0	0	65
TMR chemical composition			
DM, % as fresh	32.4	45.1	44.0
Crude protein, %	17.2	19.6	19.3
Neutral detergent fiber, %	50.4	30.2	31.6
Acid detergent fiber), %	36.6	19.9	20.0
Starch, %	4.2	28.9	28.0
Ether extract, %	2.9	3.2	3.2
Non-fiber carbohydrates, %	18.4	39.5	39.0
Ash, %	11.0	6.8	6.7
Particle fraction (% retained)[3]			
Long	86.7	27.8	29.2
Medium	5.54	29.3	29.7
Short	7.30	20.3	18.8
Fine	0.50	1.4	1.1
Physical effectiveness factor	0.92	0.6	0.6
Physically effective NDF> 8 mm	47.5	17.3	18.6

[1]CON: The control concentrate mixture contained: barley grain (30.22%), triticale grain (18.1%), bakery by-product (23.08%), rapeseed meal (24.0%), molasses (3.0%), mineral-vitamin premix for dairy cattle (0.8%), limestone (0.5%), and salt (0.3%).

[2]TRT: The phytogenic concentrate mixture contained: barley grain (30.22%), triticale grain (18.04%), bakery by-product (23.08%), rapeseed meal (24.0%), molasses (3.0%), mineral-vitamin premix for dairy cattle (0.8%), limestone (0.5%), salt (0.03%). Formulated to provide 0.04% in the TMR of a phytogenic feed supplement based on L-menthol, thymol, eugenol, mint oil (*Mentha arvensis*) and cloves powder (*Syzygium aromaticum*), (TRT, Digestarom®, DSM Austria GmbH).

*This diet was common for both CON and TRT cows; during the week of baseline forage feeding, the mineral and vitamin premix without (CON) or with the phytogenic feed supplement (TRT) was introduced into the rumen through the ruminal cannula before the morning feeding to corresponding cows.

[3]Particle fractions determined with the Penn State Particle Separator with a 19-mm screen (long), 8-mm screen (medium), 1.18-mm screen (short), and a pan (fine), (Kononoff et al. 2003).