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Ruminal Microbiota, Fermentation Process, Enteric Methane Emissions, and Animal Performance

Edited by
Ana Isabel Roca-Fernández and Magdalena Arévalo Turrubiarte

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Guest Editors

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About the Editors

Ana Isabel Roca-Fernández

Ana Isabel Roca-Fernández is an Assistant Professor of Animal Production in the Department of Anatomy, Animal Production and Veterinary Clinical Sciences at the Faculty of Veterinary Science, University of Santiago de Compostela (USC), Spain. She completed her PhD in Animal Production at the Agricultural Research Centre of Mabegondo (CIAM) and earned her international doctoral degree at the USC after completing a one-year PhD research period at TEAGASC-Moorepark (Ireland), AFBI-Hillsborough (North Ireland), Wageningen University (The Netherlands), INRA-Agrocampus Ouest (France), and DAIRYNZ (New Zealand), focusing on the sustainability of pasture-based milk production systems. During her two-year postdoctoral stays at INRA-Agrocampus Ouest (France) and USDA-ARS (USA), she conducted in vivo and in vitro experiments to mitigate greenhouse gas emissions from dairy cows by enhancing farm resiliency to climate change, working on different research projects such as MULTISWARD (FP7-244983) and Multifunctional Farms and Landscape to Enhance Ecosystem Services (8070-21000-008-00). Having been awarded the Extraordinary Doctoral Award, Roca-Fernández is a member of Research Group GI-1649, working on Agronomy and Animal Science. Her teaching responsibilities include lecturing on several subjects at undergraduate level and mentoring final degree, Master's, and PhD students. During her career she has published scientific papers related to the topic of this Special Issue in various journals.

Magdalena Arévalo Turrubiarte

Magdalena Arévalo Turrubiarte is a vHive Research Fellow at the University of Surrey, working within an innovation incubator affiliated with the School of Veterinary Medicine in Guildford, England, which is dedicated to animal health. She holds a Bachelor's degree in Veterinary Medicine and Zootechnics from the University of La Salle Bajío in León, Guanajuato, Mexico, and a Master of Science in Production and Animal Health, with a focus on ruminant metabolism and animal nutrition, from the National Autonomous University of Mexico (UNAM). Her doctoral research was conducted at INRAE–Agrocampus Ouest in Rennes, France, with a primary focus on mammary gland and stem cell phenotypes during milk production. She has also contributed to projects on tissue regeneration using mesenchymal stem cells derived from equines at the University of Turin's Veterinary School in Italy. Magdalena's work has primarily centered on animal production, and she has completed a postdoctoral fellowship in cardiac electrophysiology in racehorses. She has contributed to a chapter relating to dairy cattle and has authored published papers within the veterinary field that are focused on large animals.

Preface

This Special Issue, entitled "Ruminal Microbiota, Fermentation Process, Enteric Methane Emissions, and Animal Performance", ranges in scope from animal nutrition to animal production, including ruminal fermentation and its environmental influence on greenhouse gas emissions. The aim of this collection is to cover recent research findings relating to livestock microbiota, their role in digestion, and their impact on enteric methane emissions in ruminants. The purpose of this Special Issue is to contribute to scientific knowledge on the use of feed additives, by-products, or plant secondary metabolites for animal feeding to evaluate their potential in the reduction of ruminant methane emissions. The collection comprises thirteen contributions regarding different aspects of the topic mentioned above. Science-based results propose current solutions that can be implemented from a holistic One Health approach to focus on climate change from an animal–environmental perspective.

Ana Isabel Roca-Fernández and Magdalena Arévalo Turrubiarte
Guest Editors

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Editorial

Recent Research on Livestock Microbiota, Its Role in Digestion and Its Impact on Methane Emissions

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1. Introduction

The rumen is the largest compartment of the ruminant stomach and plays a central role in the digestive physiology of bovine, ovine, and caprine species. Within the rumen, a complex and diverse microbial ecosystem facilitates the fermentation of ingested feed, leading to the production of various gases—primarily methane and carbon dioxide. The composition and volume of these fermentation gases are mainly influenced by several factors such as dietary composition, microbial population dynamics, and the animal's overall health status, all which impact performance and production. To improve microbial fermentation efficiency and reduce gas emissions—particularly enteric methane emitted by ruminants—this Special Issue focuses on recent research related to "Ruminal Microbiota, Fermentation Process, Enteric Methane Emissions, and Animal Performance". Notably, the studies presented here examine the effects of dietary supplements, by-products, probiotic bacteria, feed additives, and specific feed ingredients on the modulation of ruminal microbiota composition. Only a few of the research papers published in this Special Issue will be highlighted here. For example, [1] demonstrated that a combination of essential oil blends and fumaric acid reduced methane gas emissions by up to 86% and increased propionate concentration by 9.5%, indicating significant shifts in the composition of the rumen microbiome. Similarly, [2] reported that using crop by-products could reduce gas emissions associated with forage production by approximately 26%. [3] investigated an acidogenic bacterial consortium derived from a brewery's waste for its potential as a probiotic. Their findings showed a reduction in methane-producing species in the rumen, along with increased daily weight gains and improved feed conversion rates in bovines fed high-forage diets, likely due to changes in the volatile fatty acid profile. Additionally, this Issue includes studies exploring various by-products, such as cashew nut shell extract and endosperm and mesocarp expellers from grugru palm, evaluating the rumen's microbial capacity to digest these byproducts. One study also identifies alterations in the ruminal microbiome, metabolome, and epithelial inflammatory response resulting from moderate feed restriction in Angus steers.

2. Conclusions

This Issue presents a variety of alternative feeding strategies involving additives, probiotics, and by-products—such as those mentioned above—that differ in composition. Their influence on the rumen microbiome, animal performance, and methane emissions has been assessed. The implementation of these recent scientific findings could significantly enhance feed efficiency and the sustainability of future ruminant production systems

worldwide. However, further research is needed to better understand rumen ecosystems and diet interactions in ruminants raised under different environmental conditions, with the goal of reducing their methane emissions. Studying the microbiome at different stages of animal growth may offer valuable insights into whether these feeding strategies can be applied from an early age to mitigate gas emissions. Moreover, metabolic studies on rumen bacteria, as well as genetic research, will help assess their impact on ruminant nutrition. Integrating these aspects represents a promising direction for improving livestock productivity, human health, and environmental sustainability from a holistic, One Health approach to addressing climate change.

Author Contributions: M.A.-T. Writing—Original Draft Preparation; A.I.R.-F. Writing—Review and Editing. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: We would like to extend our sincere gratitude to all the authors of the thirteen manuscripts featured in this Special Issue for their valuable contributions to the topic proposed by the Guest Editors, "Ruminal Microbiota, Fermentation Process, Enteric Methane Emissions, and Animal Performance". We highly appreciate their interest in publishing their research in this Issue. Special thanks are also due to the editorial staff of *Ruminants* for their ongoing administrative and technical support.

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Article

Rumen Degradation of Endosperm and Mesocarp Expellers from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. in Sheep Grazing Either Natural Pastures or *Brachiaria brizantha* cv. Marandu

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Simple Summary: Sheep producers in many countries of the world usually feed their animals with natural or improved pastures. In these situations, pastures alone usually do not cover animal needs, and supplementation is required. By-products from the oil industry are extensively used in animal feeding, and endosperm and mesocarp expellers from grugru palm may play an important role in animal feeding in the countries where it is distributed. To assess the protein value of an ingredient, the extent and rate of rumen degradation are central characteristics. However, associative effects with other components of the diet occur, and rumen degradation of individual ingredients should be assessed in the usual feeding conditions of an animal. On these grounds, the objective of this study was to provide information about rumen degradation of endosperm and mesocarp expellers from grugru palm in sheep grazing natural or cultivated monophytic pastures, and supplemented with a mixture of both expellers. The use of this mixture slows down the rate of degradation of the pastures, the effect of which is more intense in animals grazing natural swards. Supplementation with this mixture also increases the average daily gain of sheep, more substantially with multi-species natural pastures.

Abstract: Twenty-four ewes (eight fistulated in the rumen) were assigned to a 2 × 2 factorial design. The treatments included the pasture grazed (natural mixed swards—NMS or cultivated monophytic (*Brachiaria brizantha* cv. Marandu) swards—CMS) and the level of supplementation: without supplement or with a 1% supplement (dry matter (DM)/live weight). The supplement included one-third endosperm expeller (ENE) and two-thirds mesocarp expeller (ME) from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. In sacco rumen degradation of ENE, ME, NMS, and CMS was assessed in fistulated sheep, as were rumen fermentation variables. Average daily gain (ADG) was assessed in non-fistulated animals during a 7-week period. Potential degradability of the DM and crude protein of the expellers was not affected by the type of pasture grazed or the level of supplementation ($p > 0.05$), but the fractional rate of degradation (c) of DM was three times faster ($p < 0.01$) for ME than for ENE. The potential degradability of neutral detergent fiber was 34% higher ($p < 0.0001$) for ENE, with no differences in c ($p > 0.1$). Supplementation slowed down the c of the DM of the pastures, especially in animals grazing NMS (24% lower). Treatments affected rumen pH, concentration of volatile fatty acids, and proportion of valerate, in different ways. The use of the supplement increased ADG of sheep (six-fold in sheep grazing NMS and 40% in those grazing CMS). The use of a mixture of one-third ENE plus two-thirds ME as a protein supplement in sheep grazing either multi-species natural pastures or monophytic swards of *Brachiaria brizantha* cv. Marandu slows down the fractional rate of degradation of the pastures, the effect of which is more intense in animals grazing natural swards. This supplementation also increases average daily gain, more substantially with multi-species natural pastures.

Keywords: ruminants; extensive systems; oil industry; by-products; rumen function

1. Introduction

Grugru palm (*Acrocomia aculeata* (Jacq.) Lodd. ex Mart.) is widely distributed throughout the American continents, especially in Latin America, and has the potential to be introduced in Central Africa, Southern Asia, and Northern Australia as a bioenergetic source [1]. Although the predominant use of this species is in the production of bioenergy [2], it has been also reported for pharmaceuticals [3], medicine [4], human food [5], animal feed [6], and even in the masonry industry [7]. Oil extraction generates by-products such as endosperm (ENE) or mesocarp (ME) expellers, which can be efficiently used in animal feeding [6,8,9]. It is estimated that 20 t of fruits are produced per ha and year, from which ca. 0.9 t of ENE and 5.2 t of ME are obtained [10]. The processing of grugru palm fruits to obtain ENE and ME has been described by Loup [11]. Roughly, the fruits are stored for up to several months in containers to help keep them dry. During this period, some mechanical separation of the pericarp and the mesocarp occurs. After the storing–drying period, the pericarp is mechanically separated from the rest of the fruit, and then the mesocarp is pressed to obtain the mesocarp oil. The by-product of the oil extraction is known as ME. On its side, the endocarp is broken down and the endosperm is chemically separated with the aid of dihydrate kaolinite, which creates a density gradient. This way, the endosperm can be collected at the top of the tank after washing to eliminate the chemical. Then, it is dried down to a maximum of 9% dry matter, and ground and pressed to obtain the endosperm oil. The by-product of the oil extraction is known as ENE.

The published literature dealing with the chemical composition of both ENE and ME is very scarce [12], but data (n = 45 for ENE, and n = 25 for ME) from the Laboratory of Bromatology, Nutrition, and Animal Feeding of the Faculty of Veterinary Sciences, of the National University of Asunción, in Paraguay, indicate that ENE is a protein-rich by-product (346 $\pm$ 15.2 g crude protein/kg dry matter) with a moderate content in ether extract (98.7 $\pm$ 27.90 g/kg dry matter). On the other hand, ME is a low-protein (63.6 g/kg dry matter) by-product with a higher content in ether extract (146 $\pm$ 16.4 g/kg dry matter) than ENE. As a result, ENE could be used as a protein supplement and ME as an energy supplement. However, and due to the lack of published information on the digestion and performance of the animals consuming them, the criterion for its use by the producers is just their chemical composition.

The extent of protein degradation in the rumen is considered central to systems that have been proposed for the evaluation of protein requirements for ruminants [13]. These systems consider the microbial need for rumen-degradable N and also the host animal's need for amino acids derived either from microbial protein or from undegraded protein from the feed. The extent of degradation determines both the degradable part available for the rumen microbes and the undegraded protein that may be available for host animal enzymic digestion. Not only the extent of degradation but also the rate of degradation is extremely important [13].

When assessing rumen degradation of individual ingredients of a ration, it must be considered that it can be influenced by associative effects with the other components of the diet (e.g., forage vs. cereal grains [14]). Assessment of rumen degradation of any feedstuff should then be carried out in the usual feeding conditions of a determined animal. In this respect, sheep producers in the countries where grugru palm is more broadly distributed usually feed their animals with natural [15] or improved [16] pastures. Moreover, when by-products of *Acrocomia aculeta* (Jacq.) Lodd. ex Mart. are used, they are fed in the form of cake, a mixture of ENE and ME [6,8].

The main objective of the present paper was to provide information about rumen degradation of ENE and ME from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. in sheep grazing either natural pastures or cultivated *Brachiaria brizantha* cv. Marandu. The influence of supplementing the sheep with a mixture of ENE and ME on rumen degradation of ENE

and ME was also studied. The hypothesis is that the diet consumed by grazing sheep (type of pasture grazed, and supplementation or not with a mixture of one-third ENE and two-thirds ME) will affect rumen degradation of ENE and ME, rumen fermentation, and animal performance.

2. Materials and Methods

The trial was carried out at the sheep farm Condominio Stanley, in the Department of Caaguazú, San José de los Arroyos district, 122 km from Asunción, capital city of Paraguay (25°40′31.29″ S, 56°41′19.92″ W).

2.1. Experimental Design

Twenty-four non-pregnant, non-lactating Hampshire Down cross-bred grazing ewes (36 ± 3.8 kg (SEM) at the beginning of the experiment) were randomly assigned to four treatments (six ewes/treatment) in a 2 × 2 factorial arrangement. The factors were the type of pasture grazed (natural mixed swards (NMS) or cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu), and the level of supplementation (non-supplemented (NS) or supplemented (S) with a mixture of one-third ENE and two-thirds ME at a daily rate of 1% live weight (LW) on a dry matter basis). Weight homogeneity of the four animal groups was sought, and two animals assigned to each treatment were fistulated in the rumen 30 days before the adaptation period to the experimental diets. Four paddocks of 15,000 m^2 each were fenced, two of them with NMS and the other two with CMS. Within each type of pasture, one of the paddocks was supplemented, whereas the other one was not. Animals had access to automatic drinkers (one per paddock) during the whole experiment. The most important species of NMS were *Andropogon lateralis* Nees and *Axonopus compressus* (Sw.) P.Beauv. (*ca*. 14% cover each), *Sorghastrum agrostoides* (Speg.) Hitchc. (*ca*. 8% cover), *Paspalum notatum* Flüggé (*ca*. 7% cover), *Elyonurus latiflorus* (Nees ex Steud.) Hack and *Imperata brasiliensis* Trin. (*ca*. 3% cover each), *Paspalum devincenzii* Parodi (*ca*. 2% cover), *Paspalum guaraniticum* Parodi var. crovettoi and *Axonopus iridaceus* (Mez) Hitchc. & Chase ex Rojas (*ca*. 1.7% cover each), and *Paspalum maculosum* Trin. (*ca*. 1.3% cover). Other species present in the sward, even though at low percentages of cover, were *Mimosa* spp. and *Vernonia chamaedrys* Less. The supplement was offered at 17:00 in troughs installed in each paddock (one per paddock, shared by all animals in a paddock). Animals had access to the assigned pasture for 12 h each day (from 7:30 am till 7:30 pm), and then were housed in wooden barns, made exclusively for the trial, during the night. All the supplement provided was consumed by the animals, but no measurement of pasture intake could be made. In this respect, exclusion cages were not available at the farm to estimate pasture intake from agronomic measurements. Apart from this unavailability, this technique does not take into account the selection exerted by the animals on different species and plant parts, and this could be important in sheep grazing NMS. The *n*-alkanes technique was discarded due to its numerous odds (need for fecal recovery of the odd-chain alkanes present in the biomass consumed, for example). Calculation of pasture intake by subtracting the concentrate contribution from animal requirements was considered inaccurate. Weekly samples of pastures from the four paddocks were collected to assess the evolution of chemical composition. Both ME and ENE were obtained as described by Loup [10] and provided by Industrial Aceitera SACI (Capiatá, Paraguay). Samples of the two swards, the two expellers, and the supplement, were ground in a hammer mill fitted with a 1 mm sieve size, and analyzed at the Laboratory of Bromatology, Nutrition, and Animal Feeding, Faculty of Veterinary Sciences, National University of Asunción, Paraguay. The procedures of AOAC [17] were followed for dry matter (DM; ref. 934.01), organic matter (OM; ref. 942.05), crude protein (CP; ref. 976.05), and ether extract (EE; ref. 2003.05). Concentration of neutral detergent fiber (aNDFom) was analyzed as described [18], using α-amylase and sodium sulfite, and results were expressed exclusive of residual ashes. Acid detergent fiber (ADFom) and acid detergent lignin (Lignin (sa)) were analyzed as described by AOAC [17] (ref. 973.18), and Robertson and Van Soest [19], respectively.

Neutral detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were obtained as in [20]. The chemical composition of pastures, expellers, and supplement is given in Table 1.

Table 1. Nutrient composition (g/kg dry matter ± standard error of the mean) of natural mixed swards (NMS), cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu, mesocarp (ME), and endosperm (ENE) expellers of *Acrocomia aculeata* (Jacq.) Lodd. ex Mart., and supplement made of 1/3 ENE and 2/3 ME (n = 32 for NMS and CMS).

	NMS	CMS	ME	ENE	Supplement
Dry matter (g/kg)	n.d.	n.d.	914	940	925
OM	843 ± 17.3	834 ± 20.0	840	852	826
CP	51 ± 7.7	47 ± 7.9	83	344	226
NDF	711 ± 22.1	652 ± 24.1	586	305	422
NDIN	n.d.	n.d.	61	333	144
ADF	372 ± 17.0	304 ± 10.4	335	260	252
ADIN	26 ± 3.5	20 ± 4.8	26	93	66
ADL	55 ± 3.0	31 ± 4.5	153	103	105
EE	30 ± 5.6	31 ± 4.5	175	150	166
ME (Mjul/kg dry matter)	7.99	9.62	8.87	10.68.	10.87

OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; NIDN, neutral detergent insoluble nitrogen, ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; ADL, acid detergent lignin; EE, ether extract. ME, metabolizable energy estimated from ADF content according to the equation proposed by Mertens [21] (net energy for lactation (NE; kcal/kg dry matter) = (2.469–0.0351) · % acid detergent fiber; R^2 = 0.849; Metabolizable Energy = NE/0.61); n.d., not determined.

The care and management of animals were performed according to the Spanish Policy for Animal Protection RD 1201/05, which meets the EU Directive 86/609 on the protection of animals used for experimental and other scientific purposes, and the experimental protocol was approved by the Ethical Committee for Animal Research of the University of Zaragoza (PI48/20). The protocols applied were also covered by the Protection and Animal Welfare Act 4840/13, from the Government of Paraguay.

Non-fistulated sheep (four per treatment) were exclusively used for productive performance studies (average daily gain-ADG) during a 7-week period during the time of the year with more pasture productivity and quality. The ADG of fistulated animals was not included in order to avoid any influence of the surgery on that variable. Once a week, at 7:30 h, all ewes were weighed (with a precision of ±100 g) before taking them to the pasture, and ADG was estimated as the regression coefficient of individual LW on time.

2.2. Rumen Degradation of Pastures and Expellers

After the diet adaptation period, which lasted 12 days, NMS, CMS, ME, and ENE were incubated in polyester bags (10 × 16 cm; 48 μm pore size), in the rumen of the fistulated ewes, for 2, 4, 8, 16, 24, 48, and 72 h after first accession to the pastures. The pastures were incubated in the animals grazing them (n = 4,) whereas the expellers were incubated in all fistulated animals (n = 8). Rumen degradability of DM, crude protein (CP), and neutral detergent fiber (NDF) was assessed following the procedures described by Vanzant et al. [22]. About 5 g of the ground (2 mm) feedstuffs was incubated in duplicate bags per incubation time and ewe. Samples of pastures for incubation were taken from five 1 m^2 squares thrown randomly within each type of sward. The content inside each square was manually reaped at a height of 2–3 cm from the ground for NMS, and at 10 cm from the ground for CMS. The content of the five squares within each sward was pooled and dried in a forced-air oven at 60 °C for 48 h. Bags withdrawn from the rumen after each incubation time were rinsed with cool (*ca*. 15 °C) tap water and then frozen at −20 °C. Once all bags had been withdrawn, they were washed for 10 min in an automatic washing machine. Two additional bags per each feedstuff, containing non-incubated material, were

also washed to determine the soluble fraction. After washing, bags were dried at 65 °C for 48 h, and the residues inside were stored in hermetic plastic bags until analysis.

Analysis of CP and NDF in the bags' residues was performed at the Laboratory of Animal Nutrition, Faculty of Veterinary Sciences, University of Zaragoza, Spain, following the same procedures used for feedstuffs.

The disappearance of DM, CP, and NDF from polyester bags on time was fitted to the first-order kinetic equation described by Ørskov and McDonald [13]:

$$y = a + b\ (1 - e^{-ct}),$$

where 'y' represents degradation at a given time of incubation ('t'), 'a' the soluble fraction in the rumen, 'b' the non-soluble but potentially degradable fraction, and 'c' the fractional rate of degradation of fraction 'b'. Potential degradability is represented by the sum of 'a' and 'b'.

2.3. Rumen Fermentation

The next day after the assessment of rumen degradability, samples of rumen liquid were taken right before, and then at 4, 8, 12, 16, and 24 h after accession of the animals to the pasture. Fluid (about 200 mL) was removed using a plastic hose (40 cm long and 0.8 cm internal diameter) connected to a 50 mL syringe. Representative samples were taken moving the tube in all directions inside the rumen while sampling. Then, rumen pH was immediately measured, in triplicate, using a portable pH-meter HACH, model HQ40d. After pH recording, fluid was strained through four layers of sterile gauze, and aliquots were taken, in duplicate, for analysis of ammonia (10 mL of rumen fluid in 13 mL plastic tubes containing 0.2 mL of 50% sulfuric acid). Sampling was repeated one month later, just before the last weighing of the animals. In this second sampling, volatile fatty acids (VFA) were also analyzed by duplicate (4 mL of rumen liquid in 5 mL plastic tubes containing 1 mL deproteinizing solution: 5 mL H_3PO_4 and 0.5 g methyl valeric acid (as internal standard) in 250 mL milli Q water). All samples were frozen at −20 °C until analysis.

Ammonia nitrogen concentration in rumen samples was assessed by the colorimetric method described by Weatherburn [23] at the Laboratory of Water, Faculty of Exact and Natural Sciences, National University of Asunción, Paraguay. The analysis of VFA was carried out at the University of Zaragoza following the procedures described by Gimeno et al. [24].

2.4. Statistical Analysis

Analysis of the variance of all variables was carried out using the SAS Software (SAS Inst. Inc., Cary, NC, USA, v 9.2). Data of final LW and ADG were analyzed with PROC GLM, following the model

$$y = \mu + TS_i + SL_j + TSSL_{ij} + \varepsilon_{k(ij)},$$

where TS_i represents the effect of the type of sward grazed, SL_j the effect of the supplementation level, $TSSL_{ij}$ their interaction, and $\varepsilon_{k(ij)}$ the experimental error. Type of sward, supplementation level, and their interaction were included in the model as fixed factors, and animal as random. Values were corrected by covariance using the initial body weight as a covariate.

Potential degradability and fractional rate of degradation of DM, CP, and NDF of the two swards were also analyzed with PROC GLM using the same model as above. The only difference was that the random effect was the animal within the type of sward and supplementation level. For the degradability of the expellers, the model used was

$$y = \mu + TS_i + SL_j + TE_k + TSSL_{ij} + TSTE_{ik} + SLTE_{jk} + TSSLTE_{ijk} + \varepsilon_{k(ij)},$$

where TS_i represents the effect of the type of sward grazed, SL_j the effect of the supplementation level, TE_k the type of expeller incubated, $TSSL_{ij}$ the interaction between type of sward and supplementation level, $TSTE_{ik}$ the interaction between type of sward and type of expeller, $SLTE_{jk}$ the interaction between supplementation level and type of expeller, $TSSLTE_{ijk}$ the triple interaction between type of sward, level of supplementation and type of expeller, and $\varepsilon_{k(ij)}$ the experimental error. All factors were included as fixed, whereas the random effect was the animal within the type of sward and supplementation level.

Values of pH and ammonia concentration in the rumen liquid were analyzed as repeated measures with the MIXED procedure, following the model

$$y = \mu + TS_i + SL_j + D_k + H_{l(k)} + TSSL_{ij} + TSD_{ik} + TSH_{il(k)} + SLD_{jk} + SLH_{jl(k)} + TSSLD_{ijk} + TSSLH_{ijl(k)} + \varepsilon_{m(ijkl)},$$

where TS_i represents the effect of the type of sward grazed, SL_j the effect of the supplementation level, D_k the sampling day, $H_{l(k)}$ the sampling time within a day, $TSSL_{ij}$ the interaction between type of sward and supplementation level, TSD_{ik} the interaction between type of sward and day of sampling, $TSH_{il(k)}$ the interaction between type of sward and time of sampling within a day, SLD_{jk} the interaction between supplementation level and day of sampling, $SLH_{jl(k)}$ the interaction between supplementation level and time of sampling within a day, $TSSLD_{ijk}$ the triple interaction between type of sward, level of supplementation, and sampling day, $TSSLH_{ijl(k)}$ the triple interaction between type of sward, level of supplementation and time of sampling within a day, and $\varepsilon_{m(ijkl)}$ the experimental error. All effects were included as fixed factors, whereas the random factor was the animal within the type of sward and supplementation level. Sampling time within a day was used as a repeated measure.

The total concentration of VFA in the rumen, and molar proportions of acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate were also analyzed as repeated measures with the MIXED procedure. In this case, the model was

$$y = \mu + TS_i + SL_j + H_k + TSSL_{ij} + TSH_{ik} + SLH_{jk} + TSSLH_{ijk} + \varepsilon_{l(ijk)},$$

where TS_i represents the effect of the type of sward grazed, SL_j the effect of the supplementation level, H_k the sampling time, $TSSL_{ij}$ the interaction between type of sward and supplementation level, TSH_{ik} the interaction between type of sward and sampling time, SLH_{jk} the interaction between supplementation level and time of sampling, $TSSLH_{ijk}$ the triple interaction between type of sward, level of supplementation and time of sampling, and $\varepsilon_{l(ijk)}$ the experimental error. All effects were included as fixed factors, whereas the random factor was the animal within the type of sward and supplementation level. Sampling time was used as a repeated measure. For rumen fermentation variables, the variance–covariance structure was selected based on the lowest Akaike information criterion. Differences were considered significant if $p < 0.05$. A tendency was considered if $0.1 > p > 0.05$.

3. Results

3.1. Rumen Degradability of Pastures

Potential degradability of the DM and NDF of the two pastures was affected ($p < 0.05$) by the interaction between pasture type and supplementation level. For DM, this interaction was reflected in higher values for NS than S in sheep grazing NMS but not in those grazing CMS (Table 2). For NDF, the interaction reflected higher values for NS than S in sheep grazing NMS but the opposite in those grazing CMS. Sheep grazing CMS showed higher values than those grazing NMS for both DM and NDF regardless of the supplementation level. The potential degradability of CP was affected only by the type of pasture ($p < 0.0001$), with higher values for CMS (34.9% vs. 58.5%).

The fractional rate of degradation of DM was affected by the type of pasture ($p < 0.0001$; 0.030 h^{-1} vs. 0.040 h^{-1} for NMS and CMS, respectively) and level of supplementation ($p = 0.0030$; 0.038 h^{-1} vs. 0.032 h^{-1} for NS and S animals, respectively). The interaction

between both factors affected CP's fractional rate of degradation. This interaction was the result of higher values for S than for NS (2.4-fold) in animals grazing NMS but not in those grazing CMS. Also, the fractional rate of degradation of CP in NS animals was 31% lower for those grazing NMS, whereas for S animals the lower values (32% lower) were for those grazing CMS. The fractional rate of degradation of NDF was affected by pasture type ($p < 0.0001$; 0.025 h^{-1} vs. 0.040 h^{-1} for NMS and CMS, respectively) and level of supplementation ($p = 0.0172$; 0.034 h^{-1} vs. 0.031 h^{-1} for NS and S, respectively).

Table 2. Potential degradability (a + b; g/100 g dry matter), and fractional rate of degradation (c; h^{-1}), of dry matter (DM), crude protein (CP), and neutral detergent fiber (NDF) of natural mixed swards (NMS) or cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu, non-supplemented (NS) or supplemented (S) with 1 kg dry matter/100 kg live weight of a mixture of 1/3 of endosperm expeller and 2/3 of mesocarp expeller from Acrocomia aculeata (Jacq.) Lodd. ex Mart.

Pasture (P)		**NMS**		**CMS**			***p*-Value**		
Supplementation (S)		**NS**	**S**	**NS**	**S**	**SEM**	***p***	**S**	***p* × S**
DM	a + b	44.6 bA	39.9 aA	68.7 B	70.7 B	1.11	<0.0001	0.13	0.003
	c	0.034	0.026	0.041	0.038	0.0019	<0.0001	0.003	0.064
CP	a + b	32.7	37.1	54.8	62.3	4.60	<0.0001	0.10	0.65
	c	0.070 aA	0.168 bB	0.102 B	0.115 A	0.0133	0.32	0.0004	0.002
NDF	a + b	42.9 bA	33.9 aA	63.8 aB	68.1 bB	1.43	<0.0001	0.049	0.0002
	c	0.026	0.024	0.041	0.038	0.0013	<0.0001	0.017	0.60

SEM: standard error of the mean; a, b: different lower-case letters indicate differences between supplementation levels within a pasture type at $p < 0.05$; A, B: different upper-case letters indicate differences between types of pasture within a supplementation level at $p < 0.05$.

3.2. Rumen Degradability of Expellers

The potential degradability of DM and CP of both ENE and ME was not affected by the type of pasture or the level of supplementation ($p > 0.05$). There were also no differences between ENE and ME (Table 3). However, the fractional rate of degradation of DM was faster ($p = 0.007$) for ME (0.129 h^{-1}) than for ENE (0.044 h^{-1}). The potential degradability of NDF was higher ($p < 0.0001$) for ENE (72.5%) than for ME (54.4%) with no differences in fractional rate of degradation ($p > 0.1$). None of the interactions between pasture type, level of supplementation, and expeller type, were significant ($p = 0.20$).

Table 3. Potential degradability (a + b; g/100 g dry matter), and fractional rate of degradation (c; h^{-1}), of dry matter (DM), crude protein (CP), and neutral detergent fiber (NDF) of mesocarp (ME) and endosperm (ENE) expellers from Acrocomia aculeata (Jacq.) Lodd. ex Mart. incubated in the rumen of sheep grazing natural mixed swards (NMS) or cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu, non-supplemented (NS) or supplemented (S) with 1 kg dry matter/100 kg live weight of a mixture of 1/3 ENE and 2/3 ME.

Pasture (P)		**NMS**				**CMS**								
Supplementation (S)		**NS**		**S**		**NS**		**S**				***p*-Value**		
Expeller (E)		**ME**	**ENE**	**ME**	**ENE**	**ME**	**ENE**	**ME**	**ENE**	**RSD_1**	**RSD_2**	***p***	**S**	**E**
DM	a + b	81.0	81.5	79.2	69.7	78.9	82.9	80.6	82.1	2.94	7.32	0.20	0.21	0.72
	c	0.072	0.054	0.130	0.043	0.150	0.035	0.165	0.045	0.0049	0.0696	0.33	0.46	0.007
CP	a + b	83.9	87.7	81.3	63.3	76.4	84.7	79.6	89.1	9.45	15.76	0.39	0.21	0.82
	c	0.037	0.044	0.044	0.027	0.131	0.033	0.269	0.032	0.1291	0.1373	0.10	0.50	0.073
NDF	a + b	59.2	75.3	51.5	65.2	51.0	75.8	55.0	73.7	3.87	6.83	0.63	0.11	<0.0001
	c	0.049	0.079	0.117	0.059	0.123	0.055	0.081	0.068	0.0622	0.0552	0.77	0.81	0.20

RSD_1, residual standard deviation for comparison between pasture types, levels of supplementation, and their interaction; RSD_2, residual standard deviation for comparison between expellers and their interactions.

3.3. Animal Performance

Final LW was affected by the interaction between sward type and supplementation level ($p = 0.0334$), with no differences between supplementation levels for animals grazing

CMS, but higher values for supplemented vs. non-supplemented sheep grazing NMS (Table 4). No differences were found for the type of pasture grazed. A marked positive effect of supplementation on ADG ($p = 0.007$) was evident. Supplemented animals grazing NMS grew, on average, nearly 90 g/d more than non-supplemented animals (more than 6-fold), whereas the increment was less than 25 g/d in animals consuming CMS (40% higher in supplemented animals).

Table 4. Final live weight (FLW) and average daily gain (ADG) of sheep grazing natural mixed swards (NMS) or cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu, non-supplemented (NS) or supplemented (S) with 1 kg dry matter/100 kg live weight of a mixture of 1/3 of endosperm expeller and 2/3 of mesocarp expeller from Acrocomia aculeata (Jacq.) Lodd. ex Mart.

Pasture (P)	**NMS**		**CMS**		**SEM**	***p*-Value**		
Supplementation (S)	**NS**	**S**	**NS**	**S**		***p***	**S**	***p* × S**
FLW (kg)	37.5 a	41.7 b	39.0	39.8	0.69	0.54	0.005	0.033
ADG (g/d)	17	104	58	81	15.5	0.75	0.007	0.066

SEM: standard error of the mean; a, b: different letters indicate differences between supplementation levels within a type of pasture at $p < 0.05$.

3.4. Rumen Fermentation

Sheep consuming NMS showed higher (2.2% on average) rumen pH ($p = 0.028$) than those grazing CMS (Table 5), and supplementation tended ($p = 0.056$) to reduce rumen pH. Animals grazing CMS tended to have a higher concentration of VFA in the rumen than those grazing NMS ($p = 0.09$). The molar proportion of valerate was also 84% higher ($p = 0.0055$) in CMS animals. For the rest of the variables, there was no effect ($p > 0.1$) of either the type of pasture grazed or the level of supplementation. The interaction between these two factors was not significant ($p > 0.1$) in any case.

Table 5. Average daily rumen pH, daily average concentrations of volatile fatty acids (VFA; mmol/L) and ammonia (mg/100 mL), and daily average molar percentage (mmol/100 mmol) of the main VFA in the rumen fluid of sheep grazing natural mixed swards (NMS) or cultivated monophytic swards (CMS) of *Brachiaria brizantha* cv. Marandu, non-supplemented (NS) or supplemented (S) with 1 kg dry matter/100 kg live weight of a mixture of 1/3 of endosperm expeller and 2/3 of mesocarp expeller from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart.

Pasture (P)	**NMS**		**CMS**			***p*-value**		
Supplementation (S)	**NS**	**S**	**NS**	**S**	**SEM**	***p***	**S**	***p* × S**
pH	6.51	6.41	6.38	6.25	0.043	0.028	0.056	0.74
Total VFA	53.3	51.7	89.7	76.5	19.44	0.090	0.62	0.69
Ammonia	5.90	6.66	11.29	8.04	1.643	0.11	0.49	0.29
Acetate	56.9	57.9	52.4	53.5	3.62	0.16	0.70	0.98
Propionate	12.2	10.5	15.5	14.1	2.41	0.11	0.42	0.94
Butyrate	4.89	5.07	5.54	5.38	1.745	0.72	0.99	0.90
Isobutyrate	0.853	0.958	0.895	0.731	0.3431	0.72	0.91	0.61
Valerate	0.309	0.433	0.653	0.709	0.0804	0.0055	0.19	0.59
Isovalerate	0.850	1.111	1.008	0.833	0.4691	0.87	0.90	0.55

SEM: standard error of the mean.

The interaction between supplementation level and time of sampling affected the molar proportions of propionate ($p = 0.02$) and butyrate ($p = 0.032$). The proportion of propionate was higher in NS than in S animals at all times except at 4 h after accession to the pasture. The proportion of butyrate was higher in NS than in S animals at 8 and 12 h, but lower at 4, 16, and 24 h.

4. Discussion

According to their chemical composition (Table 1), both NMS and CMS can be classified as pastures of relatively poor quality. Their content in CP was below 70 g/kg DM, considered the threshold for an adequate activity of rumen microorganisms, and for maintaining an adequate intake [25]. In addition, the amount of structural carbohydrates was high, and typical of tropical grasses (Table 1).

With respect to the expellers from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart., both had a content of residual oil (EE in Table 1) higher than 15% because of the mechanical process of extraction. This makes them a suitable energetic supplement. In addition, ENE showed a high concentration in protein (34.4%), comparable to other expellers from oleaginous plants, which makes it also a suitable protein supplement. The mixture of one-third ENE and two-thirds ME showed a CP content similar to commercial concentrates for grazing sheep in Paraguay (*ca*. 14% on a dry matter basis). At a daily rate of 1% LW, the protein content of the mixture is sufficient to maintain grazing sheep with characteristics similar to those of the animals used in the present experiment, and even to allow moderate growth rates (50–100 g/day).

We are aware that the low number of experimental units (sheep) used in the present trial may compromise the veracity, repeatability, and reliability of our results. This low number was obliged by the on-farm tropical conditions of the experiment, as maintaining cannulated sheep alive in the wet tropical conditions of the study is especially difficult. However, many variables reached statistical significance and this indirectly indicates that the low number of animals was not a limitation in this study. Undoubtedly, future studies should be based on more powerful statistical designs.

4.1. Rumen Degradability

The lower potential degradability and fractional rate of degradation of NMS compared to CMS (Table 3) could be explained by the higher content in NDF, ADF, and ADL of the former (Table 1). To this respect, Van Soest [26] pointed out that rumen digestion of a determined ingredient or combination of them is negatively affected by its NDF content, and also by the degree of lignification of the ADF fraction. The potential degradability of DM of CMS was lower than that reported (79.4%) by Lopes et al. [27], but it must be taken into account that these authors worked with unsupplemented cattle instead of sheep, and that their pasture had a higher content in CP, which may have helped the rumen microorganisms to ferment more. Unfortunately, we have not been able to find published papers dealing with the rumen degradability of different pastures in sheep subjected to varying feeding conditions (e.g., forage-to-concentrate ratio). In our experiment, the potential degradability of the DM of CMS was not affected by the supplementation level, as previously stated by Oliveira et al. [28] in cattle. However, supplementation negatively affected the fractional rate of degradation of the DM of both NMS and CMS, probably due to the high EE level of the supplement. The fractional rate of degradation of the DM of CMS in our experiment was higher than that found by Lopes et al. [27] or Oliveira et al. [28] in non-supplemented cattle but huge variations may be found in pasture quality according to soil characteristics or climate conditions. Between-species differences (cattle vs. sheep) are also expected.

Regarding CP degradation of the pastures in our experiment, the potential degradability may be considered moderate. However, our values were lower than those reported by Lopes et al. [27] or Ibrahim et al. [29], both in cattle. Again, differences in animal species and feeding conditions might account for at least part of the dissimilarities. On the other hand, the fact that supplementation increased the fractional rate of degradation of CP in sheep grazing NMS but not in those grazing CMS is probably due to the lower ME content of the former (Table 1). We can speculate that bacteria in the rumen of NMS sheep had a less favorable environment, in terms of energy availability, than those in the rumen of CMS sheep. Then, the energy boost provided by the supplement would have had a more intense effect on sheep grazing NMS than on those grazing CMS. Lopes et al. [27] reported

3–4-fold lower values of fractional rate of degradation of CP in cattle grazing CMS than those reported in the present experiment, whereas Oliveira et al. [28] and Ibrahim et al. [29] had intermediate values. Between-species differences, together with diverse environmental and feeding conditions are surely responsible for the dissimilarities.

The potential degradability of NDF of the pastures in the present experiment was increased by supplementation in animals grazing CMS but was decreased in sheep grazing NMS (Table 2). Even though microbiome studies were not carried out in the present work, a likely explanation could be a change in bacterial populations, which could have been modified as a result of different factors, including the potential presence of bioactive compounds in the supplement (e.g., phenolic compounds, vitamin C, or β-carotene/vitamin A; Oliveira et al. [30]). The values of potential degradability of NDF of CMS presented in this work were similar to those reported by Lopes et al. [27] in cattle, and intermediate between those found by Oliveira et al. [28] and Ibrahim et al. [29], also in cattle. With respect to the fractional rate of degradation of NDF of CMS, our values were similar to those given by Ibrahim et al. [29] but higher than those found by Lopes et al. [27] or Oliveira et al. [28] in cattle.

It can be argued that the feeding behavior, metabolism, and physiology of cattle are different from those of sheep; hence, a comparison of our results to others obtained from the former species would be useless. Unfortunately, we were not able to find specific information in the literature dealing with the rumen degradability of *Brachiaria brizantha* cv. Marandu or the natural pastures used in the present work, in sheep subjected to varying feeding conditions (e.g., pasture-to-concentrate ratio). An alternative could have been to compare our results to those obtained in sheep fed different pastures, but in our opinion, the plant species has a greater impact on rumen degradation than the animal species consuming them.

On the other hand, it appears that even in grazing cattle, the feeding conditions (e.g., supplementation vs. non-supplementation) greatly affect the degradation of the pastures. The obvious conclusion then is that both potential degradability and fractional rate of degradation of grazed pastures should be assessed in each determined feeding system.

With respect to the grugru palm expellers, no information has been found in the literature with respect to their degradation in the rumen. Hence, the results of the present experiment will be compared to rumen degradation variables of other ingredients usually included in the concentrates given to grazing sheep in the area of our study. As an example, the average potential degradability of DM of ENE (78.9%) was lower than that reported by Sauvant et al. [31] for soybean meal (97%) or palm kernel meal (92%) but similar to that of sunflower meal (78%) or copra meal (83%). On the other hand, the average fractional rate of degradation of DM of ENE (0.044 h^{-1}) was lower than that reported by Sauvant et al. [31] for soybean meal (0.080 h^{-1}), sunflower meal (0.085 h^{-1}), or copra meal (0.150 h^{-1}), but similar to that of palm kernel meal (0.040 h^{-1}). Similar comparisons can be made for CP and NDF of ENE, or for DM, CP, and NDF of ME ([32,33]). Even in the case of CP of ENE, other authors [34] found comparable or higher values with soybean meal or faba bean, respectively. In summary, ENE has the advantages of sunflower meal in terms of protein content and its potential degradability, but has a slower degradation rate. As a result, this by-product could be used when slow-degraded carbohydrates are abundant in the diet or when the flow of undegraded protein to the intestines is desirable. Of course, the competitive price between ENE and other protein supplements should be considered. In the case of ME, its use as a unique supplement would be limited by its low protein content. Assuming additivity for potential degradability and fractional rate of degradation, the use of a mixture of one-third ENE plus two-thirds ME as a protein supplement seems useful in grazing sheep.

4.2. Effects of Grazed Pasture and Supplementation on Animal Performance

In the present work, individual intake of concentrate was not assessed and pasture intake was not measured so the only available traits of animal performance were final weight and ADG.

Final LW was increased with supplementation in animals grazing NMS but not in those consuming CMS, whereas ADG was enhanced more substantially in the first group (Table 2). As stated above, we can speculate that bacteria in the rumen of NMS sheep had a less favorable environment, in terms of energy availability, than those in the rumen of CMS sheep. Then, the energy boost provided by the supplement would have had a more intense effect on sheep grazing NMS than on those grazing CMS.

In sheep grazing natural pastures with different levels of supplementation (40.4% ground corn, 56.6% soybean meal, and 3% minerals), Dantas et al. [35] also found increases in LW when the animals were supplemented at 0%, 1%, and 1.5% LW. In the same way, Arias and Ocampos [15] recorded higher ADG in lambs grazing natural pastures supplemented with 250 g of commercial concentrate compared to non-supplemented animals.

With respect to CMS, Carvalho et al. [16] were not able to find differences in final LW between sheep grazing *Brachiaria brizantha* cv. Marandu with different types of supplementation (mineral mix, NaCl plus ground corn, mineral mix plus urea/ammonium sulfate (9:1), NaCl, ground corn plus soybean meal, or mineral mix plus urea/ammonium sulfate (9:1) plus NaCl plus soybean meal). Similarly, Oliveira et al. [36] did not observe differences in final LW between supplemented (with 180 g DM/day of supplements containing ground corn, wheat meal, and either cotton cake, soybean meal, or urea) or non-supplemented sheep grazing *Cynodon dactylon* (L.) Pers. Voltolini et al. [37] also failed to find differences in final LW between sheep grazing *Cenchrus ciliaris* L. supplemented at different levels (0%, 0.33%, 0.66%, and 1% LW, on a dry matter basis, of a mixture of ground corn, wheat bran, soybean meal, and urea) or non-supplemented. On the contrary, Almeida et al. [38] recorded higher final LW in supplemented (mixture of ground corn, soybean meal, Mesquite pod meal, wheat meal, sorghum meal, and urea) ewes grazing *Urochloa mosambicensis* (Hack.) Dandy compared to non-supplemented animals. It must be considered that the different trials were performed in different agronomic and climatic conditions, and with different animal species and stocking rates, but the positive effect of supplementation was much higher in terms of ADG and final LW in animals grazing multi-species pastures of low quality than in those consuming high-quality mono-species swards. This outcome was also obtained in the present experiment.

Regarding specific supplementation with *Acrocomia aculeata* (Jacq.) Lodd. ex Mart by-products, the available information is very scarce. Azevedo et al. [6] report the results of an experiment with confined male lambs receiving a complete diet (300 g sorghum silage and 700 g concentrate per kg DM) with different proportions of grugru palm cake (a mixture of unknown proportions of ENE and ME). The concentrate was formulated with corn, soybean meal, cottonseed hulls, and 0, 100, 200, or 300 g grugru palm cake/kg DM. These authors did not find an effect of including grugru palm in the concentrate on final LW or ADG, probably due to the high quality of the forage. In the present paper, supplementation with a mixture of one-third ENE and two-thirds ME, at a daily rate of 1% LW on a DM basis, enhanced ADG in sheep grazing NMS or CMS, more pronouncedly in the former. To the best of our knowledge, no information has been published regarding the animal performance of grazing sheep supplemented with grugru palm cake, and hence our results can be considered original.

In terms of economic profitability, sheep production based on pasture is advantageous due to lower production costs compared to systems that are more intensive. However, grazing systems are limited in terms of productive efficiency, and supplementation may increase the profitability of the farm by allowing faster rates of growth, as in the present work, and reducing the time to slaughter [39]. Nevertheless, the cost of the supplement must be considered as it could exceed the expected benefits. In our case, the cost of the supplement provided by Industrial Aceitera SACI was 135 EUR/t, whereas one kg of lamb

was paid at EUR 3.13 in the local market at the time of the trial. With these figures, the extra ADG of supplemented animals represented 0.282 and 0.078 EUR/animal and day for animals grazing NMS and CMS, respectively. As the cost of supplementation was the same (0.049 EUR/animal and day), this practice was economically profitable regardless of the type of pasture consumed.

4.3. Effects of Grazed Pasture and Supplementation on Rumen Environment

Rumen pHs were within the range of values considered normal for ruminants grazing tropical pastures, and the higher figures for animals consuming NMS vs. CMS ($p = 0.028$) were matched with a lower concentration ($p = 0.09$) of total VFA (Table 5). No publications have been found dealing with rumen fermentation variables in grazing ruminants supplemented with a mixture of one-third ENE and two-thirds ME. As a result, the discussion has been focused on the comparison of our results with those obtained in trials carried out with tropical pastures either supplemented or non-supplemented.

Muinga et al. [40] did not find differences in pH, VFA, or ammonia concentrations in the rumen of steers fed either *Cenchrus purpureus* (Scumach.) Morrone alone or supplemented with one or two kg of *Leucaena leucocephala* (Lam.) de Wit or with *Leucaena leucocephala* (Lam.) de Wit plus 1 kg maize bran per animal (on a dry matter basis). The range of VFA concentrations was 75–86 mmol/L, similar to the values obtained in the present work for CMS either supplemented or non-supplemented. In the paper by Muinga et al. [40], ammonia concentration ranged between 15.2 mg/100 mL and 25.2 mg/100 mL, and pH varied between 6.42 and 6.95, values slightly higher than those found in the present work. In our research, the average ammonia concentration was higher than 5 mg/100 mL, the threshold suggested by Satter and Slyter [41] for the correct functioning of rumen bacteria. Foster et al. [42] did not find differences in pH, ammonia, or VFA concentration, and molar proportions of the different VFA in the rumen of lambs fed *Paspalum notatum* Flüggé hay with or without supplementation with soybean meal (4.25% of the ration DM). On the other hand, Morais et al. [43], in a trial with cattle grazing *Brachiaria brizantha* cv. Marandu supplemented with a commercial compound feed at 0.5% LW, reported average ammonia concentrations of 13.12 mg/100 mL, higher than that obtained in the present work. Morais et al. [43] also found higher VFA concentrations than in the present work. Manella et al. [44] observed VFA concentration values between 55.9 mmol/L and 71.4 mmol/L in cattle grazing *Brachiaria brizantha* cv. Marandu and supplemented with either a protein concentrate or *Leucaena leucocephala* (Lam.) de Wit, with these values being lower than those reported in the present experiment. Also, ammonia concentrations reported by Manella et al. [44] were lower (between 1.7 mg/100 mL and 2.2 mg/100 mL) than those found in our trial. Carvalho et al. [16] measured the rumen pH of sheep grazing *Brachiaria brizantha* cv. Marandu and supplemented with various types of ingredients (minerals, energy, energy–protein, and protein) and also found no differences between treatments. These authors measured also ammonia concentration, which was higher in the protein- and protein–energy-supplemented animals. Their values for these treatments were also higher than those obtained in the present work.

In our work, supplementation of sheep grazing either NMS or CMS with a mixture of one-third ENE and two-thirds ME did not affect variables defining rumen fermentation. However, grazing CMS produced a numerically lower acetate/propionate ratio than grazing NMS (5.09 vs. 3.59), whereas supplementation increased that ratio (4.02 vs. 4.65 for non-supplemented and supplemented animals, respectively). The production of propionic incorporates 2H, competing as a sink with CH_4 [45] so, in the absence of an economic study, supplementation of sheep grazing either NMS or CMS with a mixture of one-third ENE and two-thirds ME does not seem to be a good strategy to reduce the emissions of methane from this type of production. The high-fat content of the supplement (166 g/kg DM) likely produced a shift in the microbiome [46].

5. Conclusions

Assuming additivity for the potential degradability and fractional rate of the degradation of ENE and ME, the use of a mixture of one-third ENE plus two-thirds ME as a protein supplement seems useful in sheep grazing either multi-species natural pastures or monophytic swards of *Brachiaria brizantha* cv. Marandu. Such supplementation slows down the fractional rate of degradation of the pastures, the effect of which is more intense in animals grazing natural swards. Due to the effects of the pasture grazed and level of supplementation, rumen degradation of grugru palm expellers should be carried out in each specific feeding schedule in grazing sheep.

Supplementation with a mixture of one-third ENE and two-thirds ME from *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. to sheep grazing either multi-species natural pastures or monophytic swards of *Brachiaria brizantha* cv. Marandu increases average daily gain, more substantially with multi-species natural pastures. Hence, this type of supplementation could be recommended especially in animals grazing low-quality pastures.

Grazing CMS seems to decrease the acetate/propionate ratio compared to grazing NMS, whereas supplementation with grugru palm by-products at 1 kg dry matter/100 kg live weight seems to increase that ratio. Supplementation of sheep grazing CMS with a mixture of one-third ENE and two-thirds ME, therefore, does not seem to be a good strategy for reducing the emissions of methane from this type of production.

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ruminants

MDPI

Article

Feed Restriction in Angus Steers Impacts Ruminal Bacteria, Its Metabolites, and Causes Epithelial Inflammation

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Simple Summary: Diet composition and intake level are important factors affecting microbial communities in the rumen. A low level of feed intake, common during the transition into lactation or periods of stress (e.g., transport, comingling), changes ruminal microbiome composition. The specific objectives of our study were to harvest ruminal digesta and epithelial biopsies from ruminally cannulated steers before, during, and after feed restriction to determine alterations in the relative abundance of 16 major ruminal bacteria, metabolite profiles, and mRNA abundance of inflammatory gene markers. Short-term feed restriction increased the abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124), highlighting their role in degrading rapidly fermentable carbohydrates to intermediates such as lactate and succinate. The significant decrease in the levels of most metabolites during feed restriction was likely due to both a lack of feed for microbes and changes in their digestive processes. Thus, short-term feed restriction affected the ruminal bacteria composition, lowered the levels of most ruminal metabolites, and caused inflammation.

Abstract: We identified alterations in the ruminal microbiome, metabolome, and epithelial inflammatory response due to moderate feed restriction (FR). Ruminal digesta and epithelial biopsies from seven ruminally cannulated Angus steers were initially collected during ad libitum access to feed (PRE). After a 10 day recovery, steers underwent a 3-day FR period (FRP) at 25% intake of PRE followed by a 15 day recovery (POST) phase with ad libitum access to feed. At the end of FRP and POST, ruminal digesta and epithelial biopsies were collected again for microbial DNA and tissue RNA extraction. RT-qPCR was applied for relative microbial abundance and RNA extraction. Metabolite profiling of digesta was performed via GC-MS. The abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124) was higher ($p < 0.05$) during FRP than PRE and POST, while *Lactobacillus* spp. *(C25)*, *Escherichia coli (EC42405)*, *Fibrobacter succinogenes*, and *Megaspheara elsdenii* abundances were lower in FRP than PRE ($p < 0.05$). The *TNF* and *TLR2* mRNA abundance was greater in FRP than PRE ($p < 0.05$). Among 15 detected amino acids, glutamine, isoleucine, lysine, phenylalanine, threonine, and valine were lower ($p < 0.05$) in FRP than PRE. Metabolite pathway analysis revealed alterations in amino acid, fatty acid, vitamin, and energy metabolism during FRP ($p < 0.05$). The mRNA of the proinflammatory genes *TNF* and *TLR2* in the epithelium peaked ($p < 0.05$) at FRP and remained higher at POST. Results indicated that a short FR influenced ruminal bacteria, reduced concentrations of most metabolites, and triggered an inflammatory response.

Keywords: beef cattle; immune response; microbiota; rumen function

1. Introduction

Diet composition, e.g., forage and concentrate level, and rate of intake are two well-known effectors of changes in the numbers and profiles of microbial communities in the rumen [1,2]. The lack of access to feed, as it may occur normally during the transition into lactation, or during periods of stress (e.g., transport, comingling) also can impact the physiology and metabolism in ruminants. Feed restriction (FR) in Holstein cows induces a dramatic reduction in milk production and changes milk composition. A comprehensive review of the available literature [3] concluded that FR increases milk fat content and somatic cell count while reducing milk protein and lactose content. Additionally, this review discussed the impact of FR on the endocrine system, noting decreased concentrations of insulin, IGF-1, and leptin [3], which resulted in heightened fat and muscle mobilization, as well as a decrease in ileal villus height and crypt depth [4,5].

Angus–Holstein calves exposed to restricted concentrate feeding had greater intestinal permeability, demonstrated by higher lactulose concentrations in the bloodstream [6]. Employing a 40% reduction in feed intake to mimic decreased voluntary daily dry matter consumption in dairy cows had adverse effects on the structure of the ileum and resulted in increased levels of circulating lipopolysaccharide (LPS)-binding protein and serum amyloid A [4]. As determined by the paracellular transport marker Cr-EDTA, it is noteworthy that even a 40% FR induced a "leaky gut" [4].

Short-term FR in beef heifers resulted in elevated plasma cortisol, haptoglobin, and ceruloplasmin levels, indicating an inflammatory response [7]. In beef cattle, a 5-day 25% reduction in feed intake led to decreased ruminal total volatile fatty acid and propionate concentrations, which were linked to a reduced total absorption rate of volatile fatty acids [8,9]. Depending on the extent of FR, research has suggested that the overall barrier function of the digestive tract may be compromised, potentially allowing harmful molecules like LPS to pass into the bloodstream [10]. Additionally, at a molecular level, FR at 0.4 times the maintenance energy requirements increased the abundance of leptin receptor mRNA in both the duodenum and liver of steers [11].

Feed restriction triggered alterations not only in metabolic processes and physiology but also in the ruminal microbiome. For example, in Nellore bulls, a period of FR decreased the abundance of *Prevotella ruminicola* species and *Succinovibrio* genus [12]. In pregnant ewes, the *Lentisphaerae* and *Elusimicrobia* phylum abundance and the genus abundance of *Papillibacter*, *Comamonas*, and unclassified *Neisseriaceae* were greater during FR [13]. These animals also had a lower abundance of *Suttonella*, *Desulfobulbus*, and *Howardella*. Along with reported changes at the microbial level due to diet, the application of NMR (nuclear magnetic resonance) spectroscopy nearly 15 years ago allowed the first evaluation of changes in ruminal metabolites in lactating dairy cows as a function of the incremental feeding of cereal grain [14]. A simultaneous evaluation of metabolites and major bacteria in the rumen during FR could provide valuable information. For example, it may allow the identification of metabolites that can have toxic or inflammatory effects on the ruminal epithelium, as reported in dairy cows fed high-grain diets at parturition [15].

We hypothesized that a short period of FR dysregulates the abundance of major ruminal bacteria and metabolite profiles in the rumen and triggers an upregulation of inflammation-related markers in the epithelium. The specific objectives were to harvest ruminal digesta and epithelial biopsies from ruminally cannulated steers before, during, and after FR in order to assess changes in the relative abundance of 16 major ruminal bacteria along with metabolite profiles via GC-MS, and the mRNA abundance of inflammatory gene markers.

2. Materials and Methods

2.1. Animal Handling, Experimental Design, and Sample Collection

The Institutional Animal Care and Use Committee (IACUC) at the University of Illinois approved the procedures (#19182). Seven cannulated Angus steers (663 $\pm$ 73 kg BW) from the University of Illinois Beef Unit herd were used. Previous studies focused

on the effect of nutrition on the ruminal environment (including mRNA abundance) have used 6 to 8 [16–18] or even 5 animals [19] and demonstrated that this range is appropriate in terms of statistical power. Furthermore, the use of a repeated sampling protocol over time, as we reported in a previous study of the ruminal epithelium in the periparturient period of dairy cows [20], enhances the statistical power because each animal serves as its own control. Thus, the choice of 7 steers to address our objectives was deemed appropriate. Steers were free of clinical disease and were fed a typical finishing diet composed of 40% corn silage, 15% modified wet distiller grain, and 35% dry rolled corn as a total mixed ration (TMR). The first sample collection occurred at day 5 during a 15-day period where steers had ad libitum access to feed in group-fed conditions. At the end of PRE, all steers underwent a 3-day FR period (FRP) during which they were fed at 25% of the PRE period. An additional 15-day period was used as a post-FR period (POST) during which steers had ad libitum access to feed similar to PRE. Ruminal digesta was collected at the beginning of the experiment (day 0) and at the end of FRP and POST (days 13 and 28). Steers were initially housed in a pen of 4.88 × 4.88 m in dimension and with an individual waterer. Individual intakes were not recorded while steers remained in this pen. The pen was constructed of 5.08 cm galvanized steel tubing, had slatted concrete floors covered by interlocking rubber matting, and was in a barn constructed of a wood frame with a ribbed metal roof and with siding on the north, west, and east sides. The south side of the barn was covered with polyvinyl chloride-coated 1.27 by 1.27 cm wire mesh bird screen and equipped with retractable curtains for wind protection. After the first sampling, steers were moved to a metabolism barn with tie stalls. Stalls (2.3 × 1.3 m) were equipped with individual feed bunks and nonsiphoning automatic water bowls. The barn was equipped with a heating, ventilation, and air-conditioning system, providing a controlled environment set at 18.3 °C. Steers remained in their individual stalls until the end of FRP. The amount of TMR fed to each steer during the FRP period was calculated based on the group-fed estimates of dry matter intake. There were no TMR refusals during the FRP period. During POST, the steers were returned to the original barn and remained as a group. Four layers of gauze were used to filter ruminal contents, and the mixed digesta sample was immediately stored in liquid nitrogen and subsequently at −80 °C until analysis. Ruminal papillae were biopsied from the ventral sac using surgical scissors after pulling the tissue out of the rumen via the cannula in a chute. Biopsy samples were immediately stored in liquid nitrogen and eventually at −80 °C until analyses. Steers did not show signs of discomfort during the procedure, and no analgesics were administered.

2.2. DNA Extraction and RT-qPCR

The microbial DNA from 500 µL of ruminal digesta was extracted using a commercial kit (QIAamp® PowerFecal® DNA Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, NC, USA), and the DNA was stored at −80 °C until analysis. The RT-qPCR protocol for bacterial abundance determination followed the exact steps as in one of the previous studies [21]. Details and primers are in the Supplementary File. The primer sequences were originally published in one of our previous studies [21].

The original standard was produced by mixing samples and subsequently applied to serial dilution to generate the standard curve. The relative 16S rRNA copy standard curve was calculated via the Ct value and log10 of the relative copies. The relative copy of the original standard was set to 100%. The slope and intercept were calculated from the relative copy standard curve for exponential amplification value (EAMP, amplicon doubling per cycle) calculation. The equation for determining relative 16S rRNA copies per run was:

$$\mathrm{E_{AMP}} = 10^{\left(-\frac{1}{slope}\right)};\ \text{Relative copies per run} = \mathrm{E_{AMP}}^{(intercept-Ct)}$$

To obtain the relative 16S rRNA copies per nanogram (ng) of DNA, the relative 16S rRNA copies per run were divided by the DNA amount (ng). Subsequently, the 16S rRNA copies per microliter (μL) of ruminal digesta were derived by multiplying the relative 16S rRNA copies per ng of DNA by the DNA concentration (ng/μL) of the ruminal digesta sample.

2.3. RNA Extraction, cDNA Synthesis, and RT-qPCR

Approximately 50 mg of tissue was homogenized with 1 mL Qiazol (Qiagen, Hilden, Germany), and RNA was extracted according to our published protocols (Supplemental File). The average RNA Quality Number was 9.48. The cDNA and RT-qPCR were performed according to our previous protocols (Supplemental File).

2.4. Metabolomics Analysis

Samples of ruminal digesta were delivered to the Metabolomics Unit of the High-Throughput DNA Sequencing and Genotyping Unit of the W. M. Keck Biotechnology Center at the University of Illinois, Urbana-Champaign, for metabolomics analysis via Gas chromatography–mass spectrometry (GC-MS). Details are in the Supplementary File. Pathway enrichment analysis using the 124 detected metabolites was performed via MetOrigin (https://metorigin.met-bioinformatics.cn/home/; accessed on 17 April 2023) [22]. The match index was calculated by the number of matched metabolites with significant differences divided by the number of metabolites in a specific pathway.

2.5. Statistical Analysis

Data were analyzed using the MIXED procedure of SAS OnDemand for Academics (SAS Institute Inc., Cary, NC, USA; https://welcome.oda.sas.com/login). Experimentally, variations observed across periods were assumed to occur primarily due to the presence or absence of FR. The model included the fixed effect of the period (PRE, FRP, and POST) and the random effect of the steer. The bacterial abundance was normalized via log[p/(1 − p)], where *p* represents the relative abundance of bacterial species. The real-time quantitative PCR data were log2 transformed prior to statistical analysis. The final least square means (LSM) were back-transformed based on the original LSM generated in SAS (*OLSM*): 2^{OLSM}. The standard error of the mean (SEM) was transformed as $\left(2^{OLSM}\right) \times (\log 2) \times OLSM$. The separation of least squares means was performed via the PDIFF procedure with Tukey adjustment.

3. Results and Discussion

3.1. Abundance of Specific Bacteria

The relative abundance of *Clostridium* spp. (*C122*) and *Selenomonas ruminantium* was greater than 100‰ (Table 1). The relative abundance of *Lactobacillus* spp. (*C25*) and *Escherichia coli (EC42405)* were lower ($p < 0.05$) in FRP than POST. The relative abundance of *Fibrobacter succinogenes* and *Megaspheara elsdenii* was lower ($p < 0.05$) in FRP compared with PRE and tended to be lower in FRP ($p = 0.06$, $p = 0.08$) compared with POST. Although no significant differences in *Butyrivibrio fibrisolvens* and *Succinimonas amylolytica* relative abundance were detected between PRE and FRP, their relative abundance was greater ($p < 0.05$) in POST compared with FRP. The *Succinivibrio dextrinosolvens* relative abundance was ~60-fold greater ($p < 0.05$) in FRP than PRE and POST. Similar to *Sucinivibrio dextrinosolvens*, *Streptococcus bovis* and *Bifidobacteria* spp. (N124) relative abundance was greater ($p < 0.05$) in FRP compared with PRE and POST. The *Prevotella bryantii* relative abundance was greater ($p < 0.05$) in POST compared with FRP. In contrast, the *Eubacterium ruminantium* relative abundance in PRE tended ($p = 0.06$) to be lower than FRP, and its relative abundance in FRP was greater ($p < 0.05$) than in POST. The 16S rRNA, the ribosomal RNA in eukaryotes, was used as an indicator of total bacterial numbers in our study. Two universal bacterial primers were selected from our previous study to calculate the relative 16S rRNA copies

relative to the original standard. Both universal 1 and 2 16S rRNA copies (Figure 1) were greater ($p < 0.05$) in FRP than PRE and POST.

Table 1. Abundance (relative units) of major bacterial species in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).‰: per myriad (per ten thousand). Bacteria are arranged in descending order of relative abundance in PRE.

Species	Period			*p*-Value
	PRE	FRP	POST	
>100‰ in PRE				
Clostridium spp. *(C122)*	578.27 ^{b}	687.05 ^{ab}	989.57 ^{a}	0.03
Selenomonas ruminantium	140.65	89.01	119.34	0.10
<100‰, >0.1‰ in PRE				
Lactobacillus spp. *(C25)*	0.63 ^{a}	0.09 ^{b}	0.58 ^{a}	<0.01
Escherichia coli (EC42405)	1.43 ^{a}	0.19 ^{b}	1.14 ^{a}	<0.01
Fibrobacter succinogenes	0.79 ^{a}	0.29 ^{b}	0.72 ^{ab}	0.02
Megaspheara elsdenii	1.32 ^{a}	0.28 ^{b}	0.70 ^{ab}	<0.01
Butyrivibrio fibrisolvens	0.64 ^{a}	0.41 ^{a}	0.10 ^{b}	<0.01
Succinimonas amylolytica	0.25 ^{ab}	1.87 ^{a}	0.06 ^{b}	0.02
Bacteroides spp. *(BF25)*	1.39 ^{a}	0.46 ^{ab}	0.14 ^{b}	0.05
Succinivibrio dextrinosolvens	0.55 ^{b}	76.00 ^{a}	1.36 ^{b}	<0.01
Rumicoccus flavefaciens	1.80	0.63	0.87	0.31
Rumicoccus albus	0.31	0.05	0.21	0.17
<0.1‰ in PRE				
Streptococcus bovis	0.018 ^{b}	0.211 ^{a}	0.010 ^{b}	0.01
Bifidobacteria spp. (N124)	0.008 ^{b}	0.130 ^{a}	0.002 ^{b}	<0.01
Prevotella bryantii	0.035 ^{ab}	0.009 ^{b}	0.129 ^{a}	<0.01
Eubacterium ruminantium	3.29×10^{-6} ^{ab}	9.02×10^{-6} ^{a}	1.48×10^{-6} ^{b}	<0.01

^{a,b} Means in the same row with different superscripts differ ($p < 0.05$).

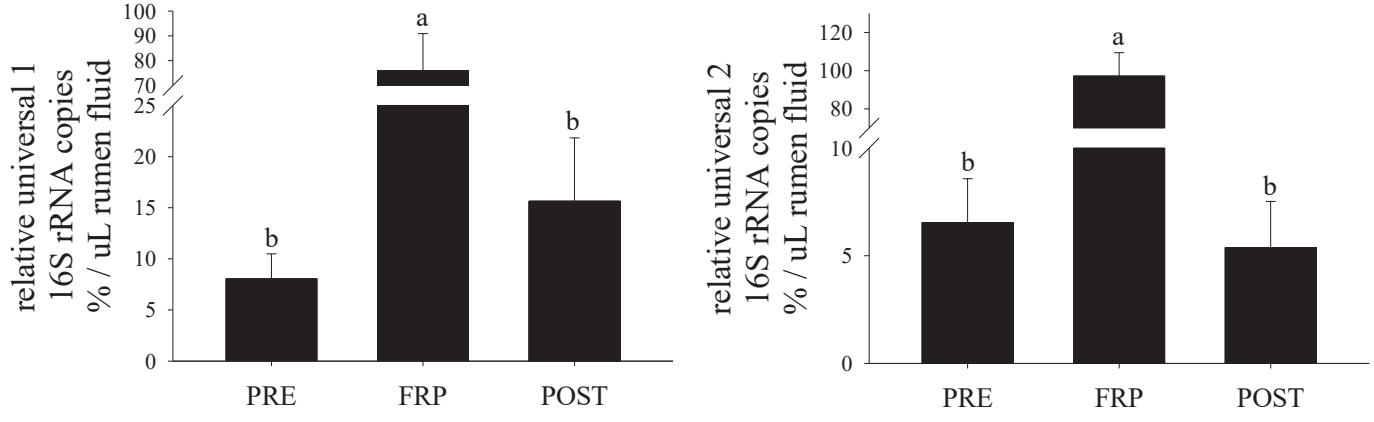

Figure 1. Relative universal 1 and 2 16S rRNA copies per μL of ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST). ^{a,b} Means differ ($p \leq 0.05$).

Feed restriction clearly dysregulated the profiles of bacteria in the rumen, but many appeared to recover following the FR period. Although ruminal pH was not measured in the present study, and it is known that FR often decreases pH [8,15], the recovery can be attributed, in part, to the increase in pH within the rumen after animals are allowed free access to feed [8]. Among those bacteria that experienced increases during FRP and a return to values similar to PRE, the increase in *Succinivibrio dextrinosolvens* during FR could have been related to its ability to utilize ammonia and urea [23] during the shortage of feed. The utilization of non-protein nitrogen such as ammonia and urea increased the nitrogen

sources for the ruminants. The increase in *Streptococcus bovis* and *Bifidobacteria* spp. (N124), both acid-resistant bacteria, during FR agrees with the notion that a lack of access to feed likely decreases ruminal pH.

Furthermore, this response also suggested that lactic acid production in the rumen increased during FRP. The decrease in *Lactobacillus* spp. *(C25)* and *Escherichia coli (EC42405)* abundance, both lactic acid producers, during FRP was surprising and potentially correlated with the decrease in *Megasphaera elsdenii* abundance, which utilizes lactic acid and prevents drastic drops in pH [24]. This response agreed to some extent with the lack of statistical changes in lactate concentration. It is well known that lactate accumulation induces ruminal acidosis and could be fatal because the D-isomer of lactate is a neurotoxin [25]. Research with cattle [26] and sheep [27] identified a connection between lactate production and utilization, feed conversion efficiency, and methane production. These studies suggest that high-producing animals, which exhibit lower methane emissions, tend to have a ruminal microbiota with increased activity of lactate-producing and lactate-utilizing bacteria, particularly *Megasphaera elsdenii* and *Coprococcus catus (Lachnospiraceae)*. The proposed explanation is that compared with fermentation by hydrogen-producing microorganisms, carbohydrate fermentation involving lactate cross-feeding produces less hydrogen, and consequently less methane. Instead, the carbon is primarily directed towards propionate, which enhances the energy available from the diet for the host [28]. Thus, the decrease in lactate-producing and lactate-utilizing bacteria (*Lactobacillus* spp. *(C25), Escherichia coli (EC42405)*, and *Megasphaera elsdenii*) potentially could increase the methane production and consequently reduce energy availability.

Despite the dysregulation in the abundance of these major bacterial species, not all the bacteria altered during FRP returned to values detected in PRE even after the 15-day of ad libitum access to feed. In fact, the nearly two-fold increase in *Clostridium* spp. *(C122)* abundance in POST relative to PRE was surprising given that it was the most abundant species in the present study. Although several species of *Clostridium* are considered pathogens that cause disease to animals, e.g., *Clostridium botulinum*, *Clostridium tetani*, and *Clostridium perfringens* [29–31], species such as *Clostridium butyricum* can metabolize acetate and lactate to butyrate under normal and stressed conditions [32]. *Clostridium botulinum* produces neurotoxins and can grow in the cow's gastrointestinal tract [33]. *Clostridium perfringens* causes enterotoxemia in dairy cows [34,35]. The fact that *Clostridium* spp. *(C122)* increased linearly in spite of the return to full feed for a period of 15 days also raises the possibility that they exert positive effects within the ruminal ecosystem [36]. Whether such effects are due to their cellular components and metabolites, such as butyrate and indolic compounds, is unclear [37]. What seems evident judging from non-ruminant studies is that metabolites such as these can play a probiotic role directly by interacting with gut cells [36].

The decrease in the abundance of *Fibrobacter succinogenes* during FRP was not unexpected as it is an important bacteria for fiber digestion [1]. Although the abundance of other fibrolytic bacteria (*Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*, *Rumicoccus flavefaciens*, *Rumicoccus albus*, *Prevotella bryantii*) did not differ between PRE and FRP, a decrease in cellulose digestion ability was reported during FR [38]. Thus, it appears that some fibrolytic bacteria are more susceptible to short periods of undersupply of nutrients.

Despite the fact that FR is often considered an unfavorable condition for the ruminal microbiota, i.e., due to a lack of nutrients, the increase in relative copies of 16S rRNA during FRP indicated a greater bacterial concentration in ruminal digesta. Whether there were differences in the numbers of live or dead bacterial cells could not be assessed. It is likely that the decrease in fermentable solid feed in the rumen during FR potentially caused bacteria to detach from the rumen solids. Consequently, while bacterial concentration appeared higher in ruminal digesta during FRP, it remains uncertain if this effect was a true reflection of the whole rumen. Furthermore, variations in bacterial composition may influence the number of 16S rRNA copies, as a single bacterial genome could contain several or even more than 10 copies of 16S rRNA [39]. Because of the substantial impact

of dominant bacteria and its composition on metabolite concentrations [40], we sought to investigate metabolite profiles in ruminal digesta.

3.2. Pro-Inflammatory mRNA Abundance

The mRNA abundance of tumor necrosis alpha (*TNF*, also known as *TNFA*) and toll-like receptor 2 (*TLR2*) in ruminal tissue was greater ($p < 0.05$) during FRP than PRE (Figure 2). Intermediate responses for *TNF* and *TLR2* were observed at POST relative to PRE and FRP.

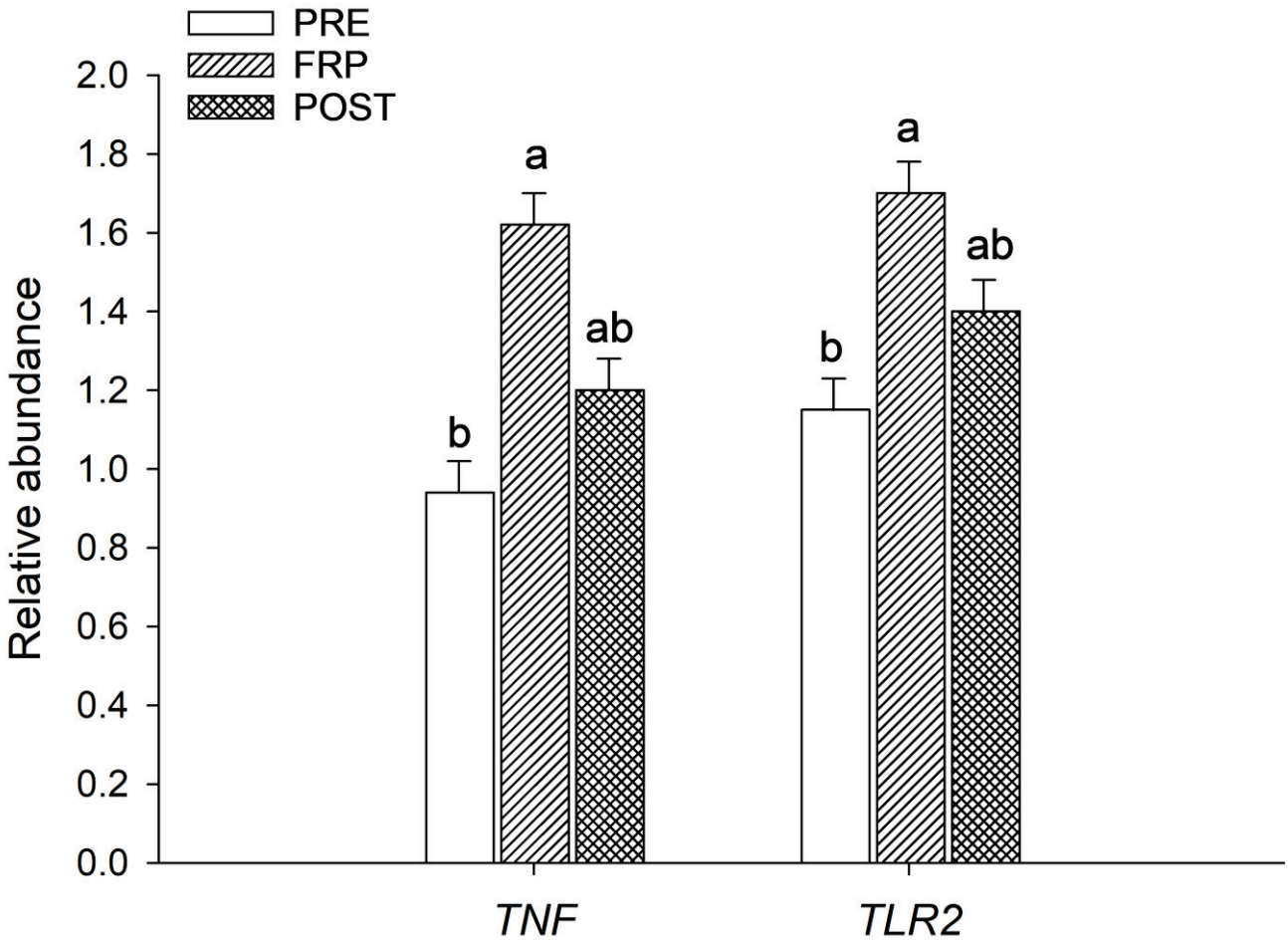

Figure 2. mRNA abundance (relative units) of the proinflammatory genes tumor necrosis factor (*TNF*) and toll-like receptor 2 (*TLR2*) in ruminal epithelium from beef steers during a pre-feed-restriction period (PRE), a 3-day 25% feed-restriction period (FRP), and a post-feed-restriction period (POST) ($n = 7$). ^{a,b} Means differ ($p \leq 0.05$).

The higher *TNF* and *TLR2* during FRP suggested the existence of an inflammatory state in the ruminal epithelium. In dairy heifers, a 24 h FR increased the plasma concentration of LPS-binding protein, which is considered a pro-inflammatory and gut permeability marker [7]. Although we did not assess gut permeability in the present study, if, in fact, the FRP caused the disruption of the ruminal barrier, it may have allowed for the passage of bacterial products capable of eliciting an inflammatory response. For example, the increased passage of bacterial-derived lipoteichoic acid and lipopolysaccharide across the gut epithelium could explain the upregulation of *TNF* and *TLR2* [41,42]. Furthermore, the alterations in bacterial species observed in this study and others that were not assessed could have resulted in the production of bioactive molecules that interacted with the ruminal epithelium and caused a pro-inflammatory response [15]. The sections below

discuss the possibility of some of the metabolites detected as potential triggers of immune responses in the ruminal epithelium.

3.3. Metabolites

A total of 124 metabolites were identified in ruminal fluid. Among these, 92 were consistently detected across all three periods (Tables 2–6). Additionally, 18 metabolites were exclusively found in the PRE and POST periods (Table 7), while 12 metabolites were uniquely identified in the PRE-FR period, including adenosine, b-sitosterol, docosanoic acid, gluconic acid, heptanoic acid, isomaltose, mannitol, mimosine, orotic acid, sebacic acid, tetracosanoic acid, and urocanic acid. Notably, alfa-Tocopherol was solely detected in the POST period, and tricarballylic acid was restricted to the FRP and POST periods. The detected metabolites were enriched in pathways outlined in Table 8. The predicted metabolic pathways were assumed to reflect major alterations in the microbial community.

Table 2. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value of metabolites associated with amino acids in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE, and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Amino acids					
Alanine	788 ^{a}	256 ^{b}	468 ^{b}	72.9	0.01
Aspartic acid	728	288	552	121	0.11
Beta-alanine	9.43 ^{a}	2.50 ^{b}	4.63 ^{b}	0.99	0.01
Glutamic acid	556 ^{a}	121 ^{b}	289 ^{b}	59.7	0.01
Glutamine	918 ^{a}	273 ^{b}	690 ^{a}	92.9	0.01
Glycine	504 ^{a}	117 ^{b}	314 ^{ab}	46.1	<0.01
Isoleucine	361 ^{a}	69.0 ^{b}	190 ^{ab}	55.9	0.03
Leucine	210	41.7	91.1	45.4	0.09
Lysine	1160 ^{a}	248 ^{b}	707 ^{ab}	132	0.01
Phenylalanine	157	48.7	108	30.3	0.11
Proline	122	19.4	84.6	39.3	0.25
Serine	314 ^{a}	73.4 ^{b}	178 ^{ab}	32.3	0.01
Threonine	394 ^{a}	89.6 ^{b}	189 ^{ab}	62.9	0.04
Tryptophan	8.50	1.73	3.03	2.25	0.16
Valine	362 ^{a}	80.4 ^{c}	226 ^{b}	28.1	<0.01
Amino acid-related metabolites					
Cadaverine	33.9 ^{a}	4.77 ^{b}	24.5 ^{a}	4.41	0.02
Fumaric acid	6.73 ^{a}	3.07 ^{b}	5.60 ^{a}	0.28	<0.01
N-Acetylglutamic acid	133 ^{a}	39.3 ^{c}	84.7 ^{b}	10.2	<0.01
Ornithine	127 ^{a}	15.0 ^{c}	49.3 ^{b}	5.01	<0.01
Putrescine	339 ^{a}	24.3 ^{b}	128 ^{ab}	68.2	0.04
γ-Aminobutyric acid	20.2 ^{a}	10.6 ^{b}	14.2 ^{ab}	1.41	0.02
1,3-diaminopropane	30.8 ^{a}	6.80 ^{b}	16.9 ^{b}	3.45	0.01
Glyoxylic acid	160	83.9	139	17.8	0.06
Succinic acid	109 ^{b}	128 ^{b}	205 ^{a}	14.4	0.01
p-hydroxyphenylacetic acid	10.9 ^{b}	12.9 ^{b}	22.8 ^{a}	1.83	0.01
Glyceric acid	70.2	42.6	45.2	31.3	0.80
Pyruvic acid	2.30	1.53	3.13	0.53	0.17
Spermidine	31.5	11.9	34.0	6.77	0.11
Urea	11.3	12.1	10.4	1.37	0.69
Amino acid derivatives					
N-methylalanine	11.3 ^{a}	1.43 ^{b}	13.2 ^{a}	1.87	0.01
L-methionine sulfoxide	18.4 ^{a}	3.27 ^{b}	9.97 ^{ab}	2.98	0.03
N-alpha-acetyl-l-lysine	86.0 ^{a}	28.5 ^{b}	50.8 ^{ab}	9.05	0.01
Pentanoic acid, 5-amino	26.0	10.8	12.1	5.65	0.19

^{a,b,c} Means in the same row with different superscripts differ ($p < 0.05$).

3.3.1. Amino Acids and Associated Metabolites

Among 15 detected amino acids (AAs), 11 (alanine, beta-alanine, glutamic acid, glutamine, glycine, isoleucine, lysine, phenylalanine, serine, threonine, and valine) were lower ($p < 0.05$) in FPR compared with PRE (Table 2). Similarly, aspartic acid and leucine concentrations were also lower ($p \leq 0.10$) in FRP compared with PRE. After FR, only glutamine and valine were returned to levels in PRE ($p < 0.05$), while glycine and lysine tended ($p \leq 0.10$) to increase. Among 27 predicted affected pathways (Table 8), 14 were related to AA metabolism or biosynthesis. Among these metabolites belonging to AA-related pathways, cadaverine, fumaric acid, n-acetylglutamic acid, and ornithine concentrations decreased ($p < 0.05$) in FRP compared with PRE and subsequently increased in POST. Similarly, putrescine, γ-aminobutyric acid, and 1,3-diaminopropane concentrations also decreased in FRP compared with PRE ($p < 0.05$) but did not recover in POST. The glyoxylic acid concentration also tended to be lower ($p < 0.10$) in FRP than PRE. Despite concentrations of succinic acid and p-hydroxyphenylacetic acid not changing in FRP, concentrations in POST were greater ($p < 0.05$) than in FRP. Regarding AA derivatives, the N-methylalanine, L-methionine sulfoxide, and N-alpha-acetyl-l-lysine concentrations were lower ($p < 0.05$) in FRP than PRE. N-methylalanine concentrations recovered ($p < 0.05$) during POST, while L-methionine sulfoxide and N-alpha-acetyl-l-lysine concentrations did not.

It is not surprising that concentrations of most AAs, their derivatives, and related metabolites were lower during FRP, indicating that fewer nitrogen sources were available to the animal and microbiome. The concentrations of several AAs and cadaverine in the rumen increased with a higher ratio of barley grain to barley silage in dairy cow diets [14,15]. Altering these dietary ratios resulted in elevated crude protein content [14], increasing AA and cadaverine concentrations. Cadaverine, originating from lysine, serves as an intermediate in various pathways and is involved in cell wall and siderophore biosynthesis [43]. Putrescine, another polyamine, is produced from arginine and ornithine or from the agmatinase pathway [44]. The production of gamma-aminobutyrate is derived from glutamate decarboxylation. In our study, the reduced concentrations of several AA-related metabolites (cadaverine, putrescine, and gamma-aminobutyrate) and AA derivatives (N-methylalanine, L-methionine sulfoxide, and N-alpha-acetyl-l-lysine) further demonstrated the close association between AA metabolism and protein intake. Thus, under a low dietary protein condition due to FR, microbes break down less protein into AAs and ammonia, a preferred nitrogen source [45].

3.3.2. Metabolites Associated with Ribose, Nitrogenous Bases, and Their Derivatives

Table 3 lists metabolites associated with ribose, nitrogenous bases, and their derivatives. The adenine, thymine, uracil, hypoxanthine, xanthine, 2-deoxy-D-ribose, erythronic acid, and ribose concentrations in FRP were greater ($p < 0.05$) than in PRE and POST. Similarly, the tyrosine and inosine concentrations were greater ($p < 0.05$) in FRP than PRE, while their concentrations were not significantly different in FRP and POST. Predicted alterations in metabolic pathways are discussed below.

The downregulation of metabolites associated with ribose, nitrogenous bases, and their derivatives indicated that FR dampened nucleoside and nucleotide metabolism. Purine and pyrimidine nucleotides serve as precursors for nucleic acids, exerting partial control over cell growth. In addition, they function as metabolic signals, energy providers, and essential components of coenzymes in various biological processes [46].

In the "purine metabolism" pathway, inosine, hypoxanthine, and xanthine are metabolites related to the degradation of guanine and adenine [47]. Ribose, purine, and pyrimidine are essential components of nucleotides [48], and erythronic acid is the product of ribose degradation. Under non-FR conditions, when compared with low-producing dairy cows, the downregulation of hypoxanthine, guanosine, and cytosine in ruminal digesta from high-producing dairy cows was associated with the overall downregulation of "purine production" and "pyrimidine metabolism" pathways [49]. Saleem et al. [15] proposed that a high-grain diet induced bacterial cell lysis, leading to greater levels of xanthine,

uracil, alanine, ornithine, and ethanolamine, all of which were suggestive of increased cell death. Such a response may partly account for the lower hypoxanthine concentration in high-producing cows. Thus, the fact that FR resulted in decreased concentrations of xanthine, uracil, alanine, ornithine, and ethanolamine in the present study suggested the possibility that during feed restriction, fewer microbial cells survived in the rumen, and most of the measured 16S rRNA copies originated from dead bacterial cells or from bacteria that entered a dormant state by forming endospores [50]. Further investigation is warranted to explore the viability of bacteria and the state of sporulation during FR.

Table 3. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites associated with ribose, nitrogenous bases, and their derivatives in ruminal digesta from beef steers (n = 7) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Adenine	3.87 [a]	0.93 [b]	3.30 [a]	0.49	0.02
Cytosine	5.93	10.3	7.30	2.43	0.48
Thymine	153 [a]	21.7 [b]	164 [a]	26.0	0.02
Tyrosine	217 [a]	50.0 [b]	130 [ab]	27.5	0.01
Uracil	772 [a]	165 [b]	672 [a]	29.2	<0.01
Inosine	108 [a]	28.1 [b]	35.2 [b]	9.69	0.01
Hypoxanthine	404 [a]	74.6 [b]	326 [a]	23.2	<0.01
Xanthine	464 [a]	89.5 [b]	377 [a]	26.1	<0.01
2-deoxy-D-ribose	289 [a]	14.2 [b]	185 [a]	37.9	0.01
Erythronic acid	22.0 [a]	2.43 [b]	22.5 [a]	4.40	0.03
Ribose	3535 [a]	748 [b]	3015 [a]	128	<0.01

[a,b] Means in the same row with different superscripts differ ($p < 0.05$).

3.3.3. Fatty Acids and Associated Metabolites

Concentrations of several fatty acids (FA) including tetradecanoic acid, pentadecanoic acid, palmitic acid, stearic acid, oleic acid C18:1 (11), oleic acid C18:1 (9), and heptadecanoic acid decreased ($p < 0.05$) in FRP compared with PRE (Table 4). However, only the concentrations of tetradecanoic acid, palmitic acid, and stearic acid returned to the levels in PRE ($p < 0.05$) during POST. The "biosynthesis of unsaturated fatty acids" and "fatty acid biosynthesis" pathways were predicted to be altered by FR.

Table 4. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for fatty acids and associated metabolites in ruminal digesta from beef steers (n = 7) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Fatty acids					
Tetradecanoic acid [1] (C14:0)	86.2 [a]	39.5 [b]	84.4 [a]	5.42	<0.01
Pentadecanoic acid (C15:0)	26.4 [a]	6.77 [b]	17.4 [ab]	2.61	0.01
Palmitic acid (C16:0)	1275 [a]	394 [b]	688 [b]	79.3	<0.01
Stearic acid (C18:0)	3816 [a]	597 [b]	1166 [b]	311	<0.01
Oleic acid C18:1 (11)	129 [a]	12.0 [b]	59.9 [ab]	24.3	0.05
Oleic acid C18:1 (9)	80.1 [a]	14.1 [b]	49.9 [ab]	8.25	<0.01
Heptadecanoic acid [2] (C17:0)	16.7 [a]	2.77 [b]	9.50 [ab]	2.36	0.02
Fatty acid-related metabolites					
Hexadecanol	15.6 [a]	4.77 [b]	20.7 [a]	1.66	<0.01
Octadecanol [3]	6.63	3.37	5.07	1.56	0.39
Tetracosanol	9.13 [a]	1.67 [b]	5.40 [ab]	1.20	0.01
Stigmastan-3-ol	24.7 [ab]	14.6 [b]	37.2 [a]	3.29	0.01

[a,b] Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as myristic acid; [2] also known as margaric acid; [3] also known as stearyl alcohol.

Unsurprisingly, the concentrations of FA were lower due to a lack of feed, which consequently downregulated the FA-related pathways in the microbiome. FA synthesis is highly energy-intensive, and it is crucial that the biophysical properties of the phospholipid membrane consistently maintain fluidity. Thus, the alterations in FA concentrations and related pathways potentially could alter the quantity and the composition of the FA in the phospholipids produced [51]. The "Biosynthesis of unsaturated fatty acids" and "Steroid biosynthesis" pathways were upregulated in ruminal digesta from high-producing compared with low-producing dairy cows [41], a response that was associated with greater dry matter intake in the former [52]. Many studies have already demonstrated that FR has a negative impact on production [3], and the observation in the present study of the downregulation of key metabolites in the "Biosynthesis of unsaturated fatty acids" and "Fatty acid biosynthesis" pathways induced by FR seems to underscore the importance of these pathways to the microbiota.

At high concentrations of unsaturated fatty acids (USFA), these are incorporated into bacterial cell membranes, disrupting membrane fluidity and function and causing toxicity [53,54]. To mitigate this effect, ruminal bacteria biohydrogenate USFA into saturated fatty acids (SFA), resulting in a higher concentration of SFA than USFA in the rumen [55]. Palmitic acid (C16:0) and stearic acid (C18:0) are major fatty acids of ruminal bacteria and protozoa [56]. Bacteria import and assimilate exogenous FA, with the FadL-FadD complex facilitating the uptake of long-chain FA for use as an energy source or for incorporation into complex lipids (e.g., phospholipids) [57]. It is likely that FR results in lower FA synthesis within cells as well as reduced FA uptake by the cells. Such effects likely depress microbial metabolism, aligning with predictions of alterations in the "Biosynthesis of unsaturated fatty acids" and "Fatty acid biosynthesis" pathways in the present study.

Hexadecanol, octadecanol, and tetracosanol are fatty alcohols with 16, 18, and 24 carbons, respectively. Some bacteria can produce fatty alcohols [58], and their production is increased by fatty acid starvation in *Escherichia coli* [59]. However, it is worth noting that long-chain fatty alcohols also exhibit antibacterial activity [60,61], although specific details regarding the types of fatty alcohols involved are not well-documented. Limited studies have addressed the relationship between long-chain fatty alcohols in the rumen. For example, ethanol, a short-chain fatty alcohol, has been shown to increase the digestion of cellulose, rumen fill, gas production in vitro, and the utilization of sulfate for AA and glutathione synthesis. However, it had little effect on the VFA concentrations in the rumen [62,63].

3.3.4. Metabolites Associated with Energy Sources

The glucose and N-acetyl glucosamine concentrations were lowest ($p < 0.05$) in FRP, and their concentrations in POST were lower than in FRP (Table 5). Similarly, the glycerol and glycerol-3-phosphate concentrations were lower ($p < 0.05$) in FRP than PRE and POST. The fructose concentration was greater ($p < 0.05$) in PRE than FRP and POST. The arabinose concentration in POST was greater ($p < 0.05$) than in FRP. The Arabitol concentration in FRP tended to be lower ($p < 0.10$) than in PRE and POST.

Starch is the most important storage form of energy in cereal grains fed to cattle, while cellulose is a crucial component of the cell wall in forages [64]. Glucose in the rumen is derived from the hydrolysis of starch and cellulose [65,66]. Cellulose and lignin content in feed can be accessed via the acid detergent fiber analysis [67], whereas neutral detergent fiber allows the measurement of cellulose, lignin, and hemicellulose [68]. The microbiota hydrolyzes cellulose into glucose and cellobiose [65,69]. Hemicellulose, another crucial polysaccharide in the cell wall, is hydrolyzed by bacteria into glucose, xylose, and arabinose, all of which serve as energy sources [65,70,71]. Fructan is also a significant non-fiber carbohydrate in ruminant feed, primarily composed of fructose [72] that is degraded by the ruminal microbiota [72–74], with the resulting fructose transported into bacteria for fermentation [75–77]. Monosaccharides in the rumen are typically products hydrolyzed from polysaccharides by the rumen microbiome [78]. Consequently, the lower

concentrations of most monosaccharides detected during FR may have been due to a lack of available polysaccharides from the feed for the ruminal microbiome and also the host.

Table 5. Relative concentrations (per 100 μL digesta), standard error of the mean (SEM), and *p*-value for metabolites associated with energy sources for microbes in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
Disaccharides					
Cellobiose	5050	884	1978	1129	0.09
Maltose	1444	220	411	318	0.07
Monoglycerides and derivatives					
Arabinose	142 [ab]	88.1 [b]	178 [a]	21.0	0.04
Arabitol	5.03	2.73	4.87	0.59	0.06
Fructose	90.0 [a]	4.83 [b]	30.1 [b]	7.66	<0.01
Glucose	13,810 [a]	1576 [c]	5872 [b]	323	<0.01
N-acetyl glucosamine	106 [a]	9.23 [c]	57.3 [b]	6.66	<0.01
Mannose	21.6	2.50	32.6	13.9	0.38
Other					
Glycerol	763 [a]	243 [b]	840 [a]	93.9	<0.01
Glycerol-3-phosphate	8.27 [a]	3.37 [b]	8.27 [a]	0.79	0.01

[a,b,c] Means in the same row with different superscripts differ ($p < 0.05$).

3.3.5. Other Metabolites

Regarding other metabolites, '2-quinolinecarboxylic acid, 4,8-dihydroxy', hydrocinnamic acid, malic acid, nicotinic acid, and O-phosphoethanolamine concentrations in FRP were lower ($p < 0.05$) than in PRE and POST (Table 6). The azelaic acid and inositol concentrations were greater ($p < 0.05$) in PRE than FRP. The inositol concentration tended to be greater ($p < 0.10$) in POST than FRP. Interestingly, the pyrrole-2-carboxylic acid concentration was lower ($p < 0.05$) in PRE than FRP and POST. The glycolic acid concentration was greater ($p < 0.05$) POST than FRP, and its concentration tended to be greater ($p < 0.10$) in PRE than FRP. A list of metabolites that were only present in PRE and POST is in Table 7. The eicosanoic acid and pantothenic acid concentrations were greater ($p < 0.05$) in PRE than POST.

The fact that pantothenic acid (known as vitamin B5) was only detectable in PRE and POST and nicotinic acid (known as niacin and vitamin B3) exhibited markedly low concentrations during FR seems to underscore a precarious condition during FRP that rendered the animals deficient (at least transiently) in these two important B vitamins [79]. The fact that ruminal microbiota are important sources of B vitamins to the host is well known [80,81]. However, just as for several metabolites, the dynamics among the B vitamin synthesis, degradation, passage out of the rumen, and possibly ruminal absorption occur simultaneously and render the interpretation of concentrations challenging. In general terms, it seems safe to assume that differences in the supply of readily fermentable carbohydrates and fiber can impact vitamin B production by the microbiota [82,83]. In the context of the present study, the fact that species such as *Streptococcus bovis* and *Megaspheara elsdenii* require pantothenic acid and likely use an excess synthesized by other bacteria [84,85] could account for the increase in abundance of the former and the inability to detect pantothenic acid during FRP. In the case of nicotinic acid, the fact that the intake and ruminal digestibility of starch in lactating dairy cows are highly positively correlated with its ruminal synthesis [84] likely explains the ~80% decrease in concentration during FRP.

Table 6. Relative concentrations (per 100 μL digesta), standard error of the mean (SEM), and *p*-value for metabolites in ruminal digesta from beef steers (n = 7) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	FRP	POST	SEM	*p*-Value
1,3-dihydroxyacetone	44.4	8.60	47.0	14.9	0.21
2-quinolinecarboxylic acid, 4,8-dihydroxy	4.40 [a]	1.07 [b]	3.40 [a]	0.36	<0.01
3-(3-hydroxyphenyl)propionic acid	12.53	4.00	17.1	5.83	0.34
3-hydroxybutanoic acid	4.10	1.67	1.27	0.72	0.08
Aminomalonic acid	47.0	18.6	34.4	8.60	0.14
Azelaic acid	241 [a]	96.0 [b]	149 [ab]	21.9	0.01
Benzeneacetic acid	210	210	130	29.1	0.18
Benzoic acid	9.17	7.63	7.00	1.74	0.68
Benzoic acid, 3-hydroxy	2.43	1.80	3.00	1.12	0.76
Dodecanedioic acid	15.4	10.1	16.1	5.32	0.70
Ethanolamine	106	67.2	77.4	18.4	0.23
Glutaric acid	8.10	8.17	13.5	1.87	0.14
Glycolic acid	32.6 [ab]	11.4 [b]	36.8 [a]	6.08	0.04
Hydrocinnamic acid	466 [a]	174 [b]	416 [a]	29.9	<0.01
Indole-3-acetic acid	1.87	1.70	2.47	0.36	0.36
Inositol	34.3 [a]	1.77 [b]	31.7 [ab]	8.34	0.04
Inositol-2-phosphate	8.93	1.83	8.70	2.17	0.10
Lactic acid	140	98.7	193	30.0	0.17
Lactose	5.30	0.33	3.90	1.27	0.08
Malic acid	170 [a]	26.9 [c]	108 [b]	12.7	<0.01
Nicotinic acid [1]	30.6 [a]	5.80 [b]	18.7 [a]	2.38	<0.01
O-phosphoethanolamine	42.9 [a]	9.57 [b]	47.0 [a]	5.22	<0.01
Propan-1,2-diol	20.7	18.1	33.4	5.88	0.23
Pyrrole-2-carboxylic acid	4.97 [b]	8.07 [a]	7.43 [ab]	0.67	0.04
Suberic acid	9.17 [a]	4.37 [b]	7.20 [a]	0.58	<0.01
Valeramide	3.47	3.10	3.03	0.80	0.88

[a,b,c] Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as niacin and vitamin B3.

The sole metabolite that increased during the FRP, pyrrole-2-carboxylic acid, is derived from fungi, plants, and bacteria [86]. Considering that FR decreased plant biomass in the rumen, the presence of pyrrole-2-carboxylic acid likely originated from fungi and bacteria. Although fungi constitute less than 10% of the total microbial biomass of the rumen in normal conditions, they produce a number of important biopolymer-degrading enzymes, e.g., xylanases, cellulases, chitinases, pectinases, and esterases [87]. Some evidence suggests that dietary fiber is the main driver of fungal growth in the rumen [88]. Thus, a period of FR, as in the present study, would be expected to reduce the fungal population. The sole study reporting concentrations of pyrrole-2-carboxylic acid in the rumen pertains to a comparison of ruminal microbiota and metabolome between lactating Montbéliarde × Holstein and Holstein cows, with the former having greater concentrations of this compound when fed the same corn silage-based diet [89]. Although the relative abundance of fungal species was not assessed, the genus *Succiniclasticum* sp. was the only statistically significant genus and was two-fold more abundant in the Montbéliarde × Holstein cows. The genus *Succiniclasticum* was first identified and characterized in 1995 [90] from the rumen of a dairy cow fed a silage-based diet. This organism had an optimal growth rate with succinate and did not produce propionate. It could be possible that in the present study, the marked increase in *Succinivibrio dextrinosolvens* abundance during FR not only explains the decrease in glucose concentration (Table 5) but also indirectly explains the increase in pyrrole-2-carboxylic acid. Because *Succinivibrio dextrinosolvens* produces succinate as a major endproduct [91], it is possible that *Succiniclasticum* sp. also thrived in those conditions. Dietary protein in the rumen also could alter pyrrole-2-carboxylic acid, as indicated by a study in which Tibetan sheep fed with 12.1% protein compared to 10.1% protein had greater ruminal concentrations of this molecule [92]. A positive correlation between Rikenellaceae_RC9_gut_group and pyrrole-2-carboxylic acid was also revealed in another study [92]. Further investigations are warranted to better understand the origin of pyrrole-2-carboxylic acid in the rumen and its relationship with the microbiota. This is particularly important given the potential physiological activities that this and chemically related carboxaldehydes can have in the body [86].

Table 7. Relative concentrations (per 100 µL digesta), standard error of the mean (SEM), and *p*-value for metabolites in ruminal digesta from beef steers ($n = 7$) before (PRE) and during a 3-day feed-restriction period (FRP) at 25% intake of PRE and after a 15-day recovery (POST).

Metabolite	PRE	POST	SEM	*p*-Value
1-Pentadecanol	2.91	4.54	2.09	0.64
2,8-Quinolinediol	18.5	18.6	2.09	0.96
2-Methylsuccinic acid	6.82	3.13	0.86	0.09
3-Hydroxyphenylacetic acid	42.2	33.0	5.25	0.27
3-Hydroxypyruvic acid	1.48	1.17	0.25	0.48
Adipic acid	9.75	8.23	2.23	0.68
Dodecanoic acid (C12:0)	11.0	10.9	1.03	0.93
Eicosanoic acid	25.0 [a]	6.18 [b]	2.39	0.02
Galactose	36.4	16.3	8.25	0.23
Glyceraldehyde	0.83	1.03	0.29	0.66
Glycylproline	7.32	5.42	0.92	0.28
Guanine	1.55	1.06	0.55	0.37
Linoleic acid	25.7	13.9	7.54	0.35
N-acetyl mannosamine	15.1	10.4	3.03	0.38
Panthotenic acid [1]	15.9 [a]	6.96 [b]	0.81	0.02
Pseudo uridine	18.5	10.9	2.21	0.12
Sedoheptulose	39.2	38.7	19.4	0.99
Sorbitol	5.92	8.56	1.28	0.28

[a,b] Means in the same row with different superscripts differ ($p < 0.05$). [1] Also known as vitamin B5.

Table 8. Match index, *p*-value, and matched metabolites of predicted pathways in ruminal digesta from steers that were affected during feed restriction. Metabolites in **bold** font had statistically significant differences. Metabolites in **green** were only present before (PRE) and after (POST) feed restriction. Metabolites in **blue** were only present before feed restriction (PRE). The match index was calculated with the number of significantly different matched metabolites divided by the number of metabolites in a specific pathway.

Pathway ID	Name	Match index	*p*-Value	Matched Metabolites
Pathways associated with amino acids				
KO00220	Arginine biosynthesis	21.74%	<0.0001	**Fumaric acid; L-Glutamic acid; Ornithine; L-Glutamine; N-Acetylglutamic acid; L-Aspartic acid**; Urea
KO00250	Alanine, aspartate, and glutamate metabolism	21.43%	<0.0001	**Gamma-Aminobutyric acid; Fumaric acid; L-Glutamic acid;** L-Alanine; Succinic acid; L-Glutamine; L-Aspartic acid; Pyruvic acid
KO00410	beta-Alanine metabolism	20.00%	<0.0001	**1,3-Diaminopropane; Beta-Alanine; Gamma-Aminobutyric acid; Pantothenic acid; Uracil**; L-Aspartic acid; Spermidine
KO00970	Aminoacyl-tRNA biosynthesis	17.31%	<0.0001	**Glycine; L-Glutamic acid; L-Tyrosine; L-Alanine; L-Threonine; L-Isoleucine; L-Lysine; L-Glutamine; L-Valine**; L-Phenylalanine; L-Proline; L-Aspartic acid; L-Leucine; L-Tryptophan
KO00470	D-Amino acid metabolism	16.07%	<0.0001	**Glycine; L-Glutamic acid; L-Alanine; L-Lysine; Ornithine; L-Glutamine; N-Acetylglutamic acid; Putrescine; Cadaverine**; L-Proline; L-Aspartic acid; Pyruvic acid
KO00480	Glutathione metabolism	15.63%	0.0001	**Glycine; L-Glutamic acid; Ornithine; Putrescine; Cadaverine**; Spermidine
KO00290	Valine, leucine, and isoleucine biosynthesis	13.04%	0.0039	**L-Threonine; L-Isoleucine; L-Valine**; Pyruvic acid; L-Leucine
KO00910	Nitrogen metabolism	10.53%	0.0287	**L-Glutamic acid; L-Glutamine**
KO00430	Taurine and hypotaurine metabolism	9.09%	0.0377	**L-Glutamic acid; L-Alanine;** Pyruvic acid
KO00260	Glycine, serine, and threonine metabolism	8.51%	0.0040	**1,3-Diaminopropane; Glycine; L-Threonine; Hydroxypyruvic acid;** Glyoxylic acid; Glyceric acid; L-Aspartic acid; Pyruvic acid; L-Tryptophan
KO00330	Arginine and proline metabolism	7.94%	0.0018	**1,3-Diaminopropane; Gamma-Aminobutyric acid; L-Glutamic acid; Ornithine; Putrescine;** Glyoxylic acid; L-Proline; Pyruvic acid; Urea; Spermidine
KO00350	Tyrosine metabolism	7.14%	0.0076	**p-Hydroxyphenylacetic acid; Fumaric acid; L-Tyrosine; Succinic acid;** Pyruvic acid
KO00360	Phenylalanine metabolism	6.98%	0.0207	**Fumaric acid;** L-Tyrosine; Succinic acid; L-Phenylalanine; Phenylacetic acid; Pyruvic acid
KO00310	Lysine degradation	5.77%	0.0361	**L-Lysine; Succinic acid; Cadaverine**
Pathways associated with nitrogenous bases and derivatives				
KO00230	Purine metabolism	9.30%	<0.0001	**Adenine; Adenosine; Glycine; Guanine; Hypoxanthine; Inosine; Xanthine; L-Glutamine;** Glyoxylic acid; Urea
KO00030	Pentose phosphate pathway	8.33%	0.0137	**D-Glucose; D-Ribose; Gluconic acid;** Glyceric acid; Pyruvic acid
KO00240	Pyrimidine metabolism	8.20%	0.0015	**Beta-Alanine; Orotic acid; Thymine; Uracil; L-Glutamine;** Urea
Pathways associated with fatty acids				
KO01040	Biosynthesis of unsaturated fatty acids	11.11%	0.0015	**Palmitic acid; Linoleic acid; Stearic acid; Arachidic acid**
KO00650	Butanoate metabolism	9.09%	0.0032	**Gamma-Aminobutyric acid; Fumaric acid; L-Glutamic acid; Succinic acid;** Pyruvic acid; 3-Hydroxybutyric acid
KO00061	Fatty acid biosynthesis	5.66%	0.0379	**Palmitic acid; Myristic acid (also called Tetradecanoic acid);** Dodecanoic acid
Pathways associated with energy sources				
KO00052	Galactose metabolism	10.87%	0.0004	**Glycerol; D-Galactose; myo-Inositol; Sorbitol; Isomaltose**
KO00020	Citrate cycle (TCA cycle)	10.00%	0.0316	**Fumaric acid; Succinic acid;** Pyruvic acid
KO00630	Glyoxylate and dicarboxylate metabolism	8.93%	0.0010	**Glycine; L-Glutamic acid; Succinic acid; L-Glutamine; Hydroxypyruvic acid;** Glyoxylic acid; Glyceric acid; Pyruvic acid
KO00520	Amino sugar and nucleotide sugar metabolism	4.50%	0.0192	**D-Xylose; D-Galactose; N-Acetyl-D-glucosamine; D-Fructose; N-Acetyl mannosamine**
Pathways associated with vitamins				
KO00770	Pantothenate and CoA biosynthesis	14.81%	0.0005	**Beta-Alanine; Pantothenic acid; Uracil; L-Valine;** L-Aspartic acid; Pyruvic acid
KO00760	Nicotinate and nicotinamide metabolism	7.84%	0.0054	**Gamma-Aminobutyric acid; Fumaric acid; Succinic acid; Nicotinic acid;** L-Aspartic acid; Pyruvic acid
Other predicted pathways				
KO00997	Biosynthesis of various other secondary metabolites	8.89%	0.0034	**L-Tyrosine; L-Alanine; L-Lysine; Ornithine;** L-Phenylalanine; L-Tryptophan

4. Conclusions

The short-term feed restriction clearly impacted a number of bacterial species studied, with the increased abundance of *Succinivibrio dextrinosolvens*, *Streptococcus bovis*, and *Bifidobacteria* spp. (N124) underscoring their unique niche in the degradation of rapidly-fermentable carbohydrates to intermediates such as lactate and succinate that could serve as substrates for other microorganisms. The marked decrease in concentrations during feed restriction for most metabolites detected was not surprising and likely reflected a combination of the reduction in substrates for the microorganisms and the alterations in the biochemical pathways of the microbiota. In that context, the dramatic decrease in vitamins B3 and B5 during feed restriction highlighted a potential transient deficiency in the supply of these vitamins to the animal. The origin of pyrrole-2-carboxylic acid in the rumen (the only metabolite with a marked increase during feed restriction), its relationship with the microbiota, and its physiological activities in the animal's tissues merit further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants4030028/s1, Supplemental methods: DNA microbe RT-qPCR; Metabolomic analysis; RNA extraction; RNA RT-qPCR; Relative mRNA abundance; Table S1: Species-specific primers for the quantification of target ruminal bacterial species; Table S2: GenBank accession number and sequence of primers for Bos taurus used to analyze gene expression; Table S3: Median Ct, Median ΔCt Slope, coefficient of determination of the standard curve (R^2), and efficiency of amplification.

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ruminants

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Article

Potential of Combined Yeast Culture and Enzymatically Hydrolysed Yeast to Improve In Vitro Dry Matter and Nutrient Degradability of Different Feedstuffs

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Simple Summary: Enzymatically hydrolysed yeast is a novel form of yeast culture with positive effects as prebiotics used to improve gastrointestinal health in ruminants. However, their effect on ruminal nutrient digestion is unclear. This study evaluated the effects of a combined yeast culture and enzymatically hydrolysed yeast on dry matter, fibre, and crude protein ruminal digestibility in vitro. Seven different substrates inclusive of legume and grass forages and commercial supplemental feedstuffs were incubated for 24 and 48 h, with or without enzymatically hydrolysed yeast, and again for 0, 2, 4, 8, 16, 24, and 48 h to determine crude protein degradability. Enzymatically hydrolysed yeast improved the fibre digestion of *Brachiaria arrecta* by 32%, while the dry matter and crude protein digestibility in soybean meal and *Glircidia sepium* were reduced by 16.2% and 38.5%, respectively, after 24 h of incubation. Therefore, enzymatically hydrolysed yeast has potential to improve ruminal fibre digestibility and modify the crude protein degradation of different substrates, which may contribute to the improved utilization of fibrous feedstuffs and efficiency in nitrogen and crude protein metabolism in ruminants.

Abstract: Live yeast cultures have been a popular additive in ruminant feeds to improve fermentation efficiency, rumen, and intestinal health. However, very little is known about inactive yeast culture and hydrolysable yeast cells on nutrient digestibility in ruminants. Therefore, this study was conducted to determine the effects of a combined yeast culture and enzymatically hydrolysed yeast (YC+EHY) on in vitro dry matter and nutrient digestibility. Seven chemically contrasting substrates, including the leaves and petiole of forage plants (*Trichanthera gigantea*, *Gliricidia sepium*, *Leucaena leucocephala*, and *Brachiaria arrecta*), agriculture by-products (soybean meal and rice hulls), and a commercial concentrate feed, were incubated in vitro with and without YC+EHY to determine dry matter (DM), crude protein (CP), neutral detergent fibre (NDF), and acid detergent fibre (ADF) digestibility after 24 and 48 h of incubation. A second experiment evaluated in vitro CP degradability by incubating substrates for 0, 2, 4, 8, 16, 24, and 48 h with and without YC+EHY. Incubation with YC+EHY reduced 24 h DM and CP digestibility in soybean meal and *G. sepium* by 16.2% and 38.5%, respectively. Conversely, the ADF digestibility of *B. arrecta* incubated with YC+EHY increased by 32%. In vitro ruminal DM and nutrient digestibility were unaffected by YC+EHY after 48 h of incubation. The rate of CP degradability in the commercial concentrate and rice hull inoculated with YC+EHY increased sharply between 16 and 24 h post-incubation and generally plateaued afterwards. Similarly, YC+EHY significantly increased CP degradability in *L. leucocephala* after 8 and 16 h of incubation. The 16 h CP degradation in *T. gigantea* without YC+EHY was significantly higher. It was therefore concluded that YC+EHY has potential to improve ruminal ADF digestibility and modify ruminal CP degradation dependent on the type of substrate.

Keywords: common feedstuffs; crude protein degradability; hydrolysable yeast; ruminal fibre digestibility; tropical forages

1. Introduction

Traditionally, dairy cows in tropical environments are managed in rotational grazing systems [1,2] and offered tree forages, agro-industrial by-products, or commercial concentrates to supplement graze pastures [3]. Tropical graze pastures vary in their nutritional profile and are generally poorly digested by ruminants. For example, the quality and nutritive value of tropical grasses vary with the stage of regrowth, species, and season [4]. Unlike temperate regions, dry matter digestibility and the rate of ruminal degradation of tropical grasses are lower at similar stages of regrowth [4]. Low digestibility limits the supply of energy and nutrients to the animal, which negatively affects productivity. While supplementing tropical grasses with inexpensive tree forages and agricultural by-products can improve digestibility, low availability limits their use by farmers. Further, some tree forages contain anti-nutritive compounds like tannins and saponins that negatively affect ruminal digestion directly or indirectly [5]. Therefore, commercial concentrates are the most common supplemental feedstuff used by dairy farmers in the Caribbean because they are readily available, conveniently packaged, easy to use, and have no antinutritional properties. However, the amount farmers use is limited because concentrate feeds are extremely expensive. This implies that other approaches might be necessary to improve digestive efficiency and feed utilization on dairy farms.

Several reports suggested that feed additives derived from live or inactive yeast cultures can improve digestibility in ruminants [6,7]. The current use of concentrate feeds offers the opportunity for the inclusion of additives into feeding systems for dairy cows, providing that the added benefits are clear and cost-effective. Some yeast cultures can produce useful compounds such as glucose during ruminal fermentation, which improved intake and nutrient digestibility in ruminants [8]. Similarly, other reports documented improvements in ruminal fermentation, the activity of cellulolytic microbes, and increase milk yield with the inclusion of yeast cultures [9,10]. As a result, yeast cultures, especially active yeast, have rapidly gained popularity.

However, very little is known about yeast cultures combined with enzymatically hydrolysable yeast (YC+EHY), a unique approach used to exploit the additional benefits of yeast cultures [11,12]. The production of enzymatically hydrolysed yeast involves the use of specific enzymes to produce redefined functional carbohydrates like mannooligosaccharides (MOS), D-mannose, and β-glucans from the cell wall of *Saccharomyces cerevisiae* [13,14]. These unique carbohydrates are popular components of prebiotics used in animal feed to improve gastrointestinal health [15]. Apart from a few studies that have demonstrated some benefits like methane reduction and reduced microbial colonization time in some substrates [16], increased dry matter intake, average daily gain, rumen fermentation profile, and microbial population from YC+EHY inclusion in feeds for ruminants [12,17], little is known about how YC+EHY affects nutrient digestion in the rumen. Therefore, the aim of this study was to determine the effects of a commercial YC+EHY on in vitro ruminal dry matter, NDF, ADF, and CP digestibility of contrasting feedstuffs commonly used to feed ruminants in the Caribbean. We tested the hypothesis that ruminal DM and nutrient digestibility will improve with the addition of YC+EHY.

2. Materials and Methods

2.1. Substrates

This study was performed with seven (7) contrasting feedstuffs, including three (3) high-protein tree forages (*Trichanthera gigantea*, *Gliricidia sepium*, and *Leucaena leucocephala*), one (1) grass forage (*Brachiaria arrecta*), two (2) agriculture by-products (soybean meal and rice hulls), and a commercial concentrate for lactating dairy cows. Four samples of each feedstuff were collected at the University of the West Indies Field Station or commercial dairy farms in Trinidad and Tobago over a 2-week period.

These feedstuffs are widely used to feed ruminant animals in tropical environments as either supplemental or basal feedstuffs. The grass forage was cut approximately 5 cm above ground level with a sharp knife. Rice hulls were collected from a local rice mill. Leaf

and petiole were harvested from mature *T. gigantea*, *G. sepium*, and *L. leucocephala* plants during the vegetative growth stage.

2.2. Analysis of Chemical Composition

The feedstuffs were dried to a constant weight in a force-draft (Gallenkamp, Model: OHG097.XX1.5, Manchester, M27 8WA, UK) oven set at 60 °C, followed by grinding in a hammer mill (Thomas Wiley Laboratory mill, model 4; Swedesboro, NJ, USA) to pass through a 2 mm sieve. Dried samples were completely ashed in a muffle furnace set a 550 °C for 8 h. Ash was estimated as the loss of organic matter. Complete dry matter (DM) was determined by oven-drying approximately 1.0 g of pre-dried samples at 105 °C for 24 h [18]. The concentration of crude protein (CP) was estimated from the analysis of total nitrogen (N) following the Kjeldahl method [18]. Crude protein was calculated as N × 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and lignin were determined sequentially. Both NDF and ADF analysis were conducted with the ANKOM^{2000} fibre analyser (model: A2000I, ANKOM Technology, Macedon, NY, USA) and expressed as ash-free. Sodium sulphite and amylase (α) were included in the NDF procedure. Lignin was determined by solubilizing cellulose with 72% sulphuric acid [19].

2.2.1. In Vitro Ruminal DM and Nutrient Digestibility

A Holstein-graded cow of approximately 500 kg body weight that was fitted with a rumen fistula was the donor of the inoculum. The care and management of this experimental animal were guided by the recommendations of the National Research Institute [20] for the management of terrestrial animals for research purposes. The cow was housed in a well-ventilated shed with concrete floor and a galvanized roof with free access to clean drinking water and mineral block. Feeding was performed once daily at approximately 0900 h with an ad libitum supply of freshly cut *B. arrecta* grass supplemented with approximately 750 g of commercial concentrate. Inoculum and buffer preparations were performed according to ANKOM Technology, method no. 3, for in vitro true digestibility using the ANKOM DAISY^{II} Incubator. The rumen digesta was manually extracted from multiple sites within the rumen before morning feeding. The liquid from the digesta was squeezed out and immediately filtered through four layers of cheesecloth into a pre-warmed thermos (39 °C) while being purged with CO_2. The rumen fluid and approximately 400 g of rumen digesta were transported to the lab for further processing. Microbes that adhere closely to feed particles were included in the inoculum by blending the 400 g of rumen digesta at a high speed for approximately 30 s. The buffered rumen inoculum was prepared by mixing ANKOM buffer with rumen fluid in a 4:1 ratio, achieving a final pH of 6.7 under constant purging with CO_2. Each ANKOM jar contained approximately 1600 mL of buffered rumen fluid. Approximately 0.5 g of each ground substrate was heat-sealed (model: 1915/1920, ANKOM Technology, Macedon, NY, USA) into ANKOM F57 filter bags. The sealed filter bags were placed in the ANKOM jars and incubated in the rotating Daisy^{II} digestion chamber (model: D200, ANKOM Technology, Macedon, NY, USA) at 39 °C. A 10 mL aliquot of YC+EHY was added to 2 of the 4 Daisy^{II} incubation jars. The aliquot was prepared by dissolving 3 g of YC+EHY in 200 mL of pre-warmed buffer [16]. Half the samples were incubated with and without the YC+EHY. Ankom filter bags with samples were incubated for 24 and 48 h intervals in buffered inoculum with or without combined YC+EHY. Twenty-five (25) samples and three (3) blanks were distributed evenly on either side of each jar.

At the end of the incubation, the filter bags were washed thoroughly with ice water to abruptly stop the microbial fermentation, followed by oven-drying at 60 °C to constant weight. Sample residues were analysed for DM, CP, NDF, and ADF, following the previously outlined methods.

2.2.2. In Vitro Ruminal CP Degradability

A second set of samples were prepared for in vitro ruminal CP degradability and incubated for 0, 2, 4, 8, 16, 24, and 48 h intervals following the procedure previously described with the Daisy^{II} incubator [3]. Degradability at 0 h was performed by rinsing samples in cold tap water, allowing them to air-dry, and then oven-drying them at 60 °C for 48 h. Two (2) blank samples were included in each incubation interval. The post-incubation sample residue was analysed for Kjeldahl N. [18] and converted to CP, as previously described.

2.3. Experimental Design and Statistical Analysis

The layout of this study was a 7 × 2 factorial arrangement (7 substrates × 2 treatments) in a completely randomized design with four replicates. Statistical analysis was performed with the Mintab 19 statistical software. Statistical significance was declared at $p < 0.05$ following an ANOVA by the general linear model:

$$Yij = \mu + Fi\ (i = 1 - 2) + Eij$$

where Yij = dependent variable (DM and nutrient degradability), μ = overall mean, Fi = effect of YC+EHY additive, and Eij = random error. Substrates incubated with or without YC+EHY were the main effects. Tukey's multiple comparison test separated the treatment means.

3. Results

3.1. Chemical Composition of Substrates

The substrates selected for this study (Table 1) had contrasting chemical compositions ($p < 0.001$). The ash, NDF, and ADF were highest in *B. arrecta*. The concentrations of CP were the highest in soybean meal and lowest in rice hull. Soybean meal also had the lowest fibre and lignin contents. *T. gigantea* had the lowest NDF and ADF contents among the forages.

Table 1. Dry matter and chemical composition of substrates.

Substrates	DM (g/kg)	Chemical Composition (g/kg DM)				
		Ash	CP	NDF	ADF	Lignin
Com. Concentrate	906 ^{a}	93.0 ^{de}	209 ^{b}	378 ^{d}	222 ^{d}	40.0 ^{de}
G. sepium	221 ^{c}	101 ^{cd}	204 ^{b}	694 ^{bc}	599 ^{b}	139 ^{c}
L. leucocephala	280 ^{b}	64.0 ^{f}	208 ^{b}	771 ^{ab}	644 ^{b}	293 ^{a}
Rice hull	898 ^{a}	126 ^{b}	67.9 ^{e}	687 ^{bc}	626 ^{b}	179 ^{b}
Soybean meal	890 ^{a}	75.0 ^{ef}	483 ^{a}	210 ^{e}	125 ^{e}	10.0 ^{e}
B. arrecta	158 ^{d}	115 ^{bc}	123 ^{d}	837 ^{a}	715 ^{a}	71.0 ^{d}
T. gigantea	160 ^{d}	242 ^{a}	153 ^{c}	628 ^{c}	542 ^{c}	174 ^{b}
SEM	3.70	3.50	1.30	9.90	11.5	5.40
Significance (*p*-value)	0.000	0.000	0.000	0.000	0.000	0.000

^{a,b,c,d,e,f} Means with a column that do not share a letter are significantly different. DM = dry matter; CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; SEM = standard error of the mean.

3.2. In Vitro Ruminal Dry Matter and Nutrient Digestibility

Incubation with combined YC+EHY had no effect ($p > 0.05$) on the 24 h in vitro ruminal dry matter and nutrient digestibility, except for DM in soybean meal, CP in *G. sepium*, and ADF in *B. arrecta* (Table 2). The addition of YC+EHY caused a reduction in 24 h DM digestibility in soybean meal by 16.2% and CP digestibility in *G. sepium* by 38.5%. On the other hand, the ADF digestibility in *B. arrecta* increased by approximately 32% when incubated with YC+EHY. The addition of YC+EHY did not affect the 48 h in vitro ruminal dry matter and nutrient digestibility (Table 3).

Table 2. Effect of YC+EHY on 24 h in vitro ruminal dry matter and nutrient digestibility.

Substrate	YC+EHY (+/−)	DM (g/kg)	24 h In Vitro Digestibility (g/kg DM)		
			CP	NDF	ADF
Com. Concentrate	(+)	571 ^{b}	109 ^{b}	129 ^{fg}	27.0 ^{fg}
	(−)	607 ^{b}	97.0 ^{b}	130 ^{fg}	25.0 ^{fg}
G. sepium	(+)	557 ^{b}	59.0 ^{c}	398 ^{ab}	315 ^{a}
	(−)	560 ^{b}	96.0 ^{b}	402 ^{a}	312 ^{a}
L. lecocephala	(+)	239 ^{ef}	59.0 ^{c}	302 ^{cd}	162 ^{cd}
	(−)	233 ^{ef}	54.0 ^{c}	291 ^{cd}	164 ^{cd}
Rice hull	(+)	215 ^{ef}	17.0 ^{ef}	120 ^{fg}	79.0 ^{ef}
	(−)	271 ^{de}	4.00 ^{f}	162 ^{ef}	103 ^{de}
Soybean meal	(+)	625 ^{b}	199 ^{a}	72 ^{g}	50.0 ^{g}
	(−)	746 ^{a}	217 ^{a}	101 ^{fg}	50.0 ^{efg}
B. arrecta	(+)	361 ^{c}	47.0 ^{cd}	315 ^{bc}	297 ^{a}
	(−)	344 ^{cd}	35.0 ^{cde}	275 ^{cd}	225 ^{b}
T. gigantea	(+)	239 ^{ef}	34.0 ^{cde}	271 ^{cd}	191 ^{bc}
	(−)	172 ^{f}	27.0 ^{def}	229 ^{de}	177 ^{bc}
SEM		14.6	4.40	14.9	9.80
Significance (*p*-value)					
Substrate		0.000	0.000	0.000	0.000
YC+EHY		0.043	0.728	0.797	0.666
Substrate × YC+EHY		0.000	0.000	0.135	0.001

^{a,b,c,d,e,f,g} Means within a column that do not share a letter are significantly different (effect of YC+EHY within substrate). DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; SEM = standard error of the mean; YC+EHY (+/−), substrates incubated with (+) or without (−) yeast culture and enzymatically hydrolysed yeast.

Table 3. Effect of YC+EHY on 48 h in vitro ruminal dry matter and nutrient digestibility.

Substrate	YC+EHY (+/−)	DM (g/kg)	48 h In Vitro Digestibility (g/kg DM)		
			CP	NDF	ADF
Com. Concentrate	(+)	719 ^{b}	157 ^{b}	201 ^{b}	71.0 ^{d}
	(−)	714 ^{b}	147 ^{b}	192 ^{b}	77.0 ^{d}
G. sepium	(+)	746 ^{b}	166 ^{b}	515 ^{a}	419 ^{a}
	(−)	739 ^{b}	162 ^{b}	516 ^{a}	408 ^{ab}
L. lecocephala	(+)	378 ^{d}	71.0 ^{c}	383 ^{a}	237 ^{c}
	(−)	378 ^{d}	63.0 ^{cd}	391 ^{a}	242 ^{c}
Rice hull	(+)	306 ^{d}	38.0 ^{d}	150 ^{b}	108 ^{d}
	(−)	326 ^{d}	34.0 ^{d}	167 ^{b}	126 ^{d}
Soybean meal	(+)	972 ^{a}	450 ^{a}	204 ^{b}	123 ^{d}
	(−)	970 ^{a}	446 ^{a}	201 ^{b}	112 ^{d}
B. arrecta	(+)	551 ^{c}	83.0 ^{c}	457 ^{a}	386 ^{ab}
	(−)	531 ^{c}	74.0 ^{c}	444 ^{a}	370 ^{ab}
T. gigantea	(+)	513 ^{c}	57.0 ^{cd}	412 ^{a}	327 ^{bc}
	(−)	539 ^{c}	62.0 ^{cd}	401 ^{a}	358 ^{ab}
SEM		19.6	5.9	24.9	15
Significance (*p*-value)					
Substrates		0.000	0.000	0.000	0.000
YC+EHY		0.872	0.171	0.936	0.740
Substrate × YC+EHY		0.954	0.914	0.999	0.827

^{a,b,c,d} Means within a column that do not share a letter are significantly different. DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre. SEM = standard error of the mean; YC+EHY (+/−), substrates incubated with (+) or without (−) yeast culture and enzymatically hydrolysed yeast.

3.3. In Vitro Ruminal Crude Protein Degradability

The in vitro ruminal CP degradability was unaffected by YC+EHY up to 16 h post-incubation in most substrates except *T. gigantea* and *L. leucocephala* (Figure 1). The rate of CP degradability in the commercial concentrate and rice hull increased sharply between 16 h and 24 h post-incubation and generally plateaued afterwards when inoculated with YC+EHY. Similarly, YC+EHY significantly increased CP degradability after 8 h in *L. leucocephala* and 24 h in *T. gigantea* and the commercial concentrate.

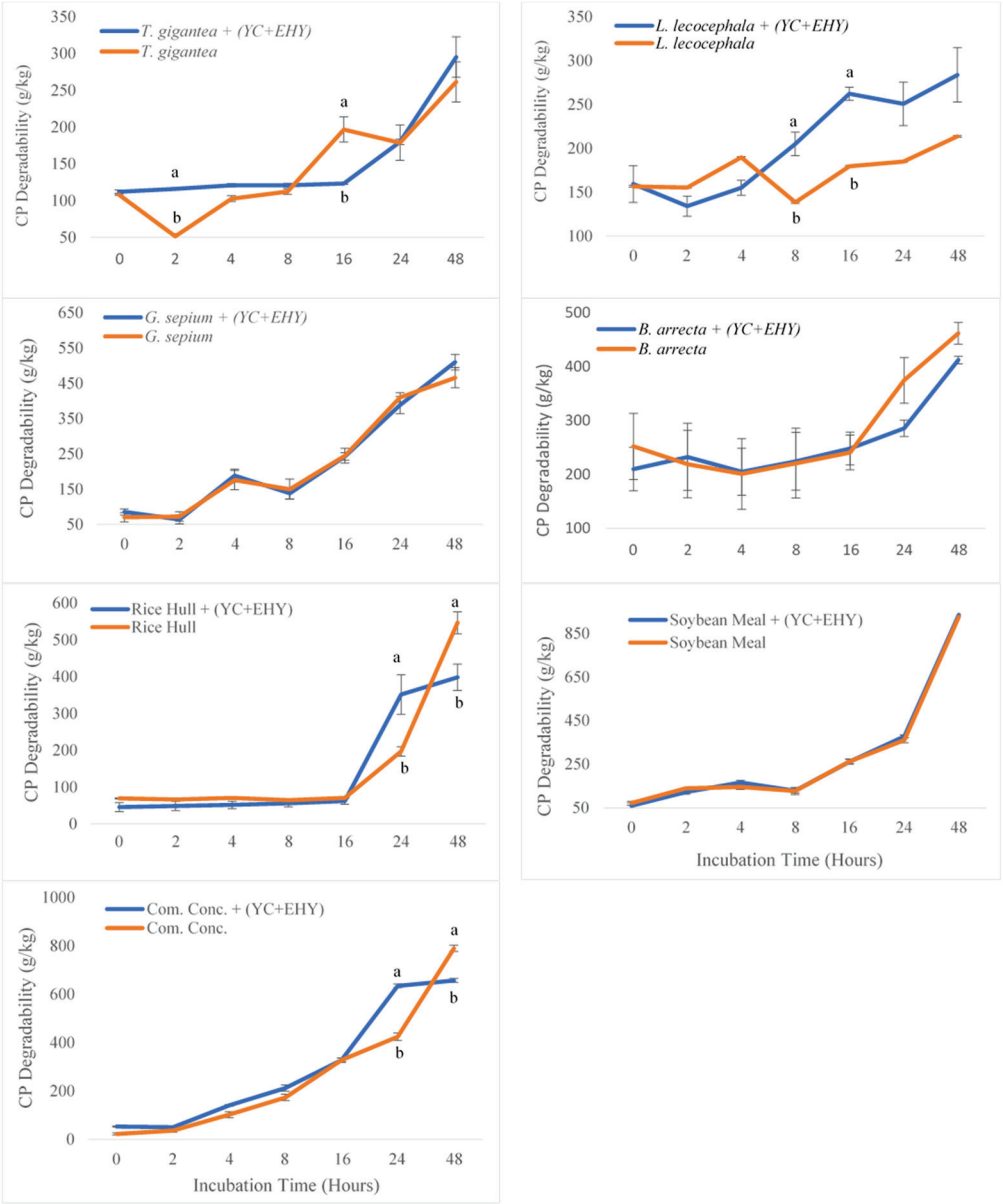

Figure 1. In vitro ruminal CP degradability substrates incubated with and without YC+EHY. [a,b] CP degradability at specific incubation times that do not share a letter is significantly different ($p < 0.05$).

4. Discussion

4.1. Chemical Composition of Substrates

The CP content of soybean meal in the present study is like other reports [21]. Soybean meal is the most popular protein feedstuff used in animal feeds [21,22]. It is also one of the most expensive feedstuffs [23]. Leguminous tree forages such as *G. sepium* and *L. leucocephala* had CP contents above 200 g/kg DM, making them potentially cheap

alternatives (partial or complete) to soybean meal [24,25]. However, high concentrations of fibre and lignin in *G. sepium* and *L. leucocephala* may reduce intake and digestibility [3]. Also, CP utilization can be limited by tannins and other phenolic compounds that are usually present in high concentrations in these browse forages [24,26] unless mitigating measures are adopted. The high fibre and lignin contents of the grass and tree forages in the present study are likely attributed to an advance stage of maturity [1]. Low-quality feedstuff like rice hull is mainly used as a source of roughage when the supply of grass forages is limited, e.g., in the dry season. With CP content below 80 g/kg DM, rice hull will supply less than the minimum amount of nitrogen needed to satisfy the rumen microbial requirements and maintain optimum rumen function [27]; therefore, careful supplementation with a good-quality N/protein source is required.

4.2. In Vitro Ruminal Dry Matter and Nutrient Degradability

The ruminal DM and nutrient digestibility post 48 h of incubation were unaffected by YC+EHY, while the 24 h ruminal digestibility of DM, CP, and ADF was influenced by YC+EHY in some substrates. This is an indication that the activity of YC+EHY may be short-term and is limited to the early periods post-feeding where rumen fermentation is highest. In the present study, a significant 16.2% reduction in the dry matter digestibility of soybean meal and a 38.5% reduction in CP digestibility in *G. sepium* were observed with the addition of combined YC+EHY post 24 h of incubation. On the other hand, ruminal ADF digestion in *B. arrecta* increased by approximately 32%. The significant reduction in DM digestibility in soybean meal without a significant concomitant reduction in the more resistant fractions like NDF and ADF was surprising, notwithstanding a nominal decline in NDF digestion. This was particularly unexpected since hydrolysable yeast is known to promote the development of cellulolytic bacterial population such as *Ruminococcus* spp., which increases fibre digestion [28]. This was most likely the reason for the significant increase in ADF digestion in *B. arrecta* forage incubated with YC+EHY in this study. However, some in situ studies reported that hydrolysable yeast did not affect fibre digestion [12,17]. Variations in fibre digestion are likely due to the availability of soluble nutrients and the concentration of fibre, especially the cellulose and spatial distribution of lignin in the cell wall of the substrate. Most studies where hydrolysable yeast did not affect fibre digestion used substrates with total fibre below 400 g/kg DM [12,17], like soybean meal and the commercial concentrate in the present study. It is important to note that *B. arrecta* forage had the highest fibre contents in the present study, suggesting the potential of YC+EHY to stimulate ruminal fibre digestion, particularly in substrates with extremely high fibre. The reduction in the 24 h CP digestibility in *G. sepium* incubated with YC+EHY is contrary to a previous report [12] where a significant increase in CP digestibility in beef cattle supplemented with hydrolysable yeast was observed. The presence of fermentable carbohydrates and highly soluble protein such as amino acids and nucleotides in YC+EHY could enhance ruminal CP digestibility [12]. Therefore, it is possible that the additional protein provided by YC+EHY in the present study could have caused more protein to be bound to tannin in *G. sepium*, resulting in a reduction in ruminal CP degradability. However, YC+EHY did not have a similar effect on *L. lecocephala*, a tannin-rich forage like *G. sepium*. *Leucaena lecocephala* is believed to have a higher tannin content and protein binding potential than *G. sepium* [29]. Therefore, the reason for the reduction in CP digestibility in *G. sepium* is not clear.

Few studies reported contrasting results regarding the influence of hydrolysable yeast on ruminal or total tract CP digestion in different livestock species, [12,30] but no previous work was found reporting the effects of combined YC+EHY on ruminal CP degradability at different incubation intervals. Yeast-based additives have gained popularity for modulating ruminal fermentation, but their mechanisms of action are not clearly understood [31]. This is especially the case with ruminal CP degradability. Many factors affect CP degradability in the rumen, like the relative concentrations of non-protein nitrogen and the physical and chemical characteristics of the protein that comprise the true protein fraction of the

feedstuff [32]. In experiment 2 of the present study, the ruminal CP degradability was unaffected by YC+EHY up to 16 h post-incubation in most substrates except *T. gigantea* and *L. leucocephala*. The decline observed in the 2 h and 24 h CP degradability in *T. gigantea* may be accidental. The limited effect of YC+EHY on the in vitro ruminal CP degradability within the initial 16 h of fermentation could be an indication that the supply of readily soluble N was adequate to satisfy the microbial requirements during the early stages of digestion and/or YC+EHY had little to no effect on the microbial colonization in some substrates. Interactions with phenolic compounds like tannins, saponins, and alkaloids known to be present in these browse forages can also be a possible cause [24,33]. The suppressive mechanism of YC+EHY on CP degradability in *L. leucocephala* post the 4 h incubation interval is not clear, but it can be important in increasing the supply of rumen undegradable protein to the small intestine. Low CP degradability in *T. gigantea* is suspected to be resulting from high-fibre-bound N, rendering most of the protein insoluble and inaccessible to rumen microbes [33]. However, significant increases in CP degradability after 8 h in *L. leucocephala* and 24 h in rice hull and the commercial concentrate could be pointing to the potential of YC+EHY to increase the ruminal degradation of the more slowly degrading protein fraction. Further, improved CP degradability in beef cattle was attributed to the additional supply of highly soluble protein by the hydrolysable yeast [12].

5. Conclusions

This study demonstrated that combined YC+EHY has the potential to improve ruminal ADF digestibility and modify ruminal CP degradation dependent on the type of substrate.

This can be an indication that supplementation with YC+EHY can improve the utilization of fibrous feedstuffs in particular and enhance the efficiency of nitrogen and crude protein metabolism in ruminants. Further studies are recommended to determine the nutritional mechanisms and mode of actions of YC+EHY. For example, it is worth knowing if and how YC+EHY modifies rumen microbial populations and if there are interactions with phenolic compounds in some browse forages.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The original data described in the study are openly available at https://hdl.handle.net/2139/56699 (17 May 2024).

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Conflicts of Interest: The authors have no competing interests related to this work to declare.

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ruminants

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Article

Annual Change in the Composition of Bulk Tank Milk Microbiota in Northern Kanagawa Prefecture, Japan

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Simple Summary: A cooling system called a bulk tank is used by farmers to temporarily store milk prior to shipment, and the milk stored in the tank is called bulk milk. Producing high-quality bulk milk leads to producing high-quality milk and dairy products. However, bulk milk is difficult to control because it is affected by the environment in which the cows are raised, the sanitary conditions of the milking equipment, and many other factors, and is composed of many types of bacteria. Additionally, few studies have examined the bacterial composition of bulk milk in Japan. Therefore, the objective of this study was to analyze the bacterial composition of bulk milk in Japanese farms through the year. We conducted interviews regarding the herds' general information, milking hygiene, and hygienic management of milking equipment. In addition, the bacterial composition of bulk milk was also examined using 16S rRNA gene full-length amplicon sequence analysis. The results suggest that the bacterial composition of bulk milk may not be constant throughout the year and may be susceptible to bacteria that are tolerant to low temperatures. This finding would be a basis for knowledge regarding the microbial control of bulk milk.

Abstract: Bulk tank milk microbiota (BTMM) is affected by various factors, including the characteristics of raw milk, microflora on teat surfaces, and the milking system. Clarifying the influence of these factors is important for producing high-quality dairy products. This longitudinal study describes the annual changes in BTMM at six dairy farms in northern Kanagawa Prefecture, Japan. Bulk tank milk samples were collected six times a year (in February, April, June, August, October, and December of 2022) to give a total of thirty-six samples. After bulk tank somatic cell counts (BTSCC) had been determined, we performed 16S rRNA gene amplicon sequence analysis to clarify the composition of the BTMM. Although no annual changes were observed in the BTSCC and alpha-diversity index, a significant difference in the beta-diversity index was observed between February and August ($p = 0.0315$). In February, the proportions of the psychrophilic genera *Listeria* and *Enterococcus* were significantly increased ($p < 0.05$). Similarly, in August, the proportion of commensal milk microbiota in the genera *Catenibacterium* and *Acetobacter* were significantly increased ($p < 0.05$). The results of this study suggest that the composition of BTMM in this region changed throughout the year, which may have been influenced by psychrophilic bacteria in winter.

Keywords: bulk tank milk microbiota; psychrophilic bacteria; 16S rRNA-based metagenomic analysis

1. Introduction

The production of high-quality milk and dairy products necessitates the use of high-quality raw milk. One of the crucial factors for evaluating the quality of raw milk is its microbial content, which also serves as a criterion for economic trade. Consequently, it is

important to have knowledge of, and to understand, the composition of bacterial flora in raw milk, along with the temporal changes and potential causes of these changes.

Microbial contamination of raw milk in bulk tanks, where milk from different individuals is pooled and stored until shipment, occurs not only via milk from infected individuals, but also from other sources of contamination, such as microflora on the teat surface and milking equipment [1]. As a result, the composition of the bulk tank milk microbiota (BTMM) is typically very diverse. In particular, mastitic udders are known to shed large numbers of pathogenic microorganisms into milk, and amplicon-based high-throughput sequencing analysis has shown that the composition of the BTMM can be frequently affected by bacterial taxa associated with mastitis (e.g., *Staphylococcus* and *Streptococcus*) [2]. However, milk from individuals without clinical or subclinical mastitis may also contain mastitis-causing pathogens. And other factors, such as farm management practices and seasonality, have also been shown to significantly influence the microbiota detected in milk [3]. Recently, it has been shown that the composition of BTMM varies not only among farms, but also by geographic region and sampling time [4]. In addition, studies on breeds other than Holsteins have also been reported, showing that the flora of Jersey cows changes depending on whether they are milked in cool (November) or hot (July) seasons [5].

Thus, BTMM is a complex group that includes numerous species from diverse sources, and what we learn about them will help us to produce high-quality milk. However, the causes of the diversity and compositional variations of BTMM in Japan have not been clarified.

The objective of this study was therefore to clarify the annual variation in the composition and diversity of the BTMM. The causes of this variation were examined over the course of a year by continuously observing bulk milk from dairy farms that employ the same feeding pattern and which are located in the same region. The analysis used metagenomic analysis, which does not require culturing and can detect difficult-to-culture bacteria.

2. Materials and Methods

2.1. Farm Selection and Milk Sample Collection

This study was conducted on six farms (A, B, C, D, E, and F) within the same region, located in northern Kanagawa Prefecture. Bulk tank milk from these farms was collected at bi-monthly intervals, six times in total (February, April, June, August, October, and December of 2022). Each farmer was interviewed to ascertain the number of dairy cattle, cattle breeds, feeding system, feeding pattern, origin of feed (i.e., self-supplied or purchased), milking type, milking frequency, milking equipment cleaning methods, bulk tank cleaning methods, elimination of abnormal milk by pre-milking, milking machine manufacturer, and bulk tank inspection frequency. Bulk tank milk was aseptically collected in 10 mL DNA-free tubes by a veterinarian, refrigerated immediately at 4 °C before noon, and brought to the laboratory for testing within 3 h. Bulk tanks used by farmers were kept at around 4 °C.

2.2. Weather Information

The 2022 meteorological data (temperature and rainfall) of six farms located in the same area were investigated using Japan Meteorological Agency data [6] and are shown in Figure 1. Average monthly temperatures ranged from 3.9 °C to 27.4 °C, with the lowest in January and the highest in August. Average monthly precipitation ranged from 19.5 mm to 254.5 mm and tended to be lower in winter (January, February, and December).

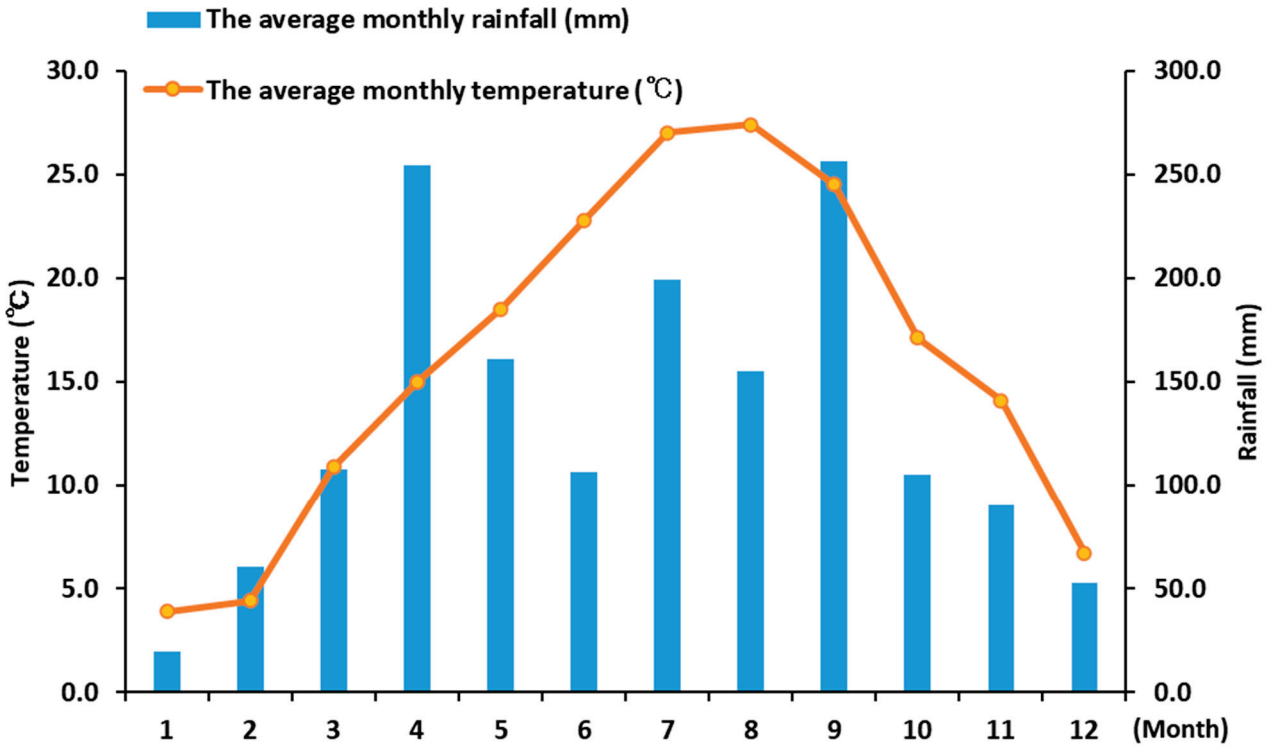


Figure 1. The temperature and precipitation for January to December 2022 in the subject area. The line graph shows the average monthly temperature, and the bar graph shows the average monthly rainfall.

2.3. Milk Test

The 10 mL DNA-free tube containing the sample was inverted and mixed well, and the sample was injected into a disposable cassette (DeLaval cassette 928658 80, International AB, Tumba, Sweden) containing small amounts of regents which, when mixed with the milk, react with the nuclei of the somatic cells. Then, a cassette with a DeLaval cell counter (DCC; DeLaval International AB, Tumba, Sweden) was set to measure the bulk tank milk somatic cell count (BTSCC) according to the manufacturer's instructions.

2.4. DNA Isolation and Pyrosequencing

DNA was extracted from bulk tank milk samples using an ISOSPIN Fecal DNA kit (NIPPON GENE, Tokyo, Japan). Briefly, bulk tank milk samples were homogenized for 10 min at 4350 rpm using a bead mill (Shakeman 6; Bio Medical Science, Tokyo, Japan). DNA was then extracted using a centrifugation-based column purification protocol. Extracted DNA was concentrated using a Quantus^{TM} Fluorometer (Promega Corporation, Madison, WI, USA) and purity was determined by measuring the absorbance ratios at 260/280 nm and 260/230 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Universal primers S-D-bact-1391-a-A-17 and S-D-bact-0008-c-S-20 [7] were used to amplify the V1–V9 region of 16S rRNA by PCR, which was performed using KAPA HiFi^{TM} HotStart ReadyMix (2×) (Nippon Genetics, Tokyo, Japan). The total reaction volume used for PCR was 20 µL, which consisted of KAPA HiFi^{TM} HotStart ReadyMix (10 µL), 0.25 µM of each primer, nuclease-free water (7 µL), *Haloarcula japonica* (2.8×10^3 molecules/µL), and genomic DNA (100 ng). *Haloarcula japonica* was spiked in uniformly as an internal control. The PCR was performed in a thermal cycler (ASTEC, Fukuoka, Japan) with the following settings: initial annealing step at 95 °C for 5 min, followed by 98 °C for 20 s, 69 °C for 15 s, and 72 °C for 60 s for 35 cycles, followed by a final extension step of 72 °C for 5 min. Subsequently, for library preparation, a second PCR was performed using a Rapid Barcoding Kit (SQK-RBK004: Oxford Nanopore Technologies, Oxford, UK) for DNA labelling, with incubation at 30 °C for 1 min and 80 °C for 1 min. All labeled PCR products were then purified using AMPure® XP beads (Beckman Coulter,

Brea, CA, USA), and DNA concentration was measured using a Quantus™ Fluorometer, with purity and quantity further assessed by NanoDrop spectrophotometry.

For the sequencing of library construction, labeled DNA (10 μL) was mixed with Rapid Adapter (1 μL) at room temperature for 5 min. The library mixture DNA (11 μL) was then mixed with sequencing buffer (34 μL), nuclease-free water (4.5 μL), and loading beads (25.5 μL), and sequencing was performed on a Spot-on Flow Cell R9 version (FLO-MIN106D; Oxford Nanopore Technologies) using a MinION™ Mk1C device (Oxford Nanopore Technologies). Data acquisition and FASTQ file creation were performed using MINKNOW software ver. 21.11.6 (Oxford Nanopore Technologies).

The resulting FASTQ files were trimmed and filtered with NanoFilt software ver. 2.8.0 [8]. Specifically, the minimum average read quality score targeted was less than 10, all sequences shorter than 500 nucleotides were removed, and the first 50 nucleotides of all reads were trimmed. After trimming and size selection, an average of 28,860 reads (4031–169,625) per sample were retained for bacterial identification. Each read was subjected to a minimap2 search against 5850 representative bacterial genome sequences stored in the Genome Sync database [9]. Taxonomic groups were determined based on the National Center for Biotechnology Information classification database [10]. Low-presence taxa (<0.01% of all reads) were excluded from the analysis.

2.5. Statistical Analyses

Annual changes in BTSCC and the alpha-diversity indexes (Richness, Simpson's, and Shannon's indexes) of the BTMM across the targeted farms were analyzed by the Friedman test, a nonparametric test. This analysis was performed using EZR plugin (ver. 1.61; Saitama Medical Center, Jichi Medical University) for the R statistical package (The R Foundation for Statistical Computing, Vienna, Austria) [11]. p values less than 0.05 were considered to indicate statistically significant differences. The composition of BTMM was compared monthly using non-parametric tests for similarity analysis (ANOSIM), which used the Bray–Curtis distance scale to measure compositional differences. p values less than 0.05 were considered to indicate statistically significant differences. This analysis was performed using the PAST 4.13 version software package [12]. Linear Discriminant Analysis Effect Size (LEfSe) analysis via the online Galaxy interface was performed to identify the specific bacterial species that showed significant differences in abundance in each month, as indicated by the ANOSIM test results [13].

3. Results

The total head of cattle on the farms in this study ranged from 20 to 51 (average 28.5 head), and all were Holstein Friesian (Table 1). The feeding mixture was component feeding, and all were fed using a tie-stall system, with the exception of Farm F, which used purchased feed. Farm F supplemented the feed with dent corn silage at 13 kg/day/head, but only in December. The milking type was an automatic milking system for all farmers, and the milking frequency was two times/day. Interviews also revealed that bulk tanks were automatically cleaned after each milk collection using a disinfectant, and that bulk tank cooling inspections were conducted once a year (Farm A, B, D, E, and F). The milking equipment cleaning methods, pre-milking, and milking equipment manufacturers are shown in Table 1. For Farm C, some items could not be investigated due to the ranchers' circumstances.

The BTSCC measurement results for each sample are shown in Table 2. BTSCC averaged 137,555 cells/mL overall, with the highest densities observed in December (average 184,833 cells/mL) and the lowest densities observed in February (average 117,833 cells/mL). The results of the Friedman test (Bonferroni correction) showed no significant differences between the months (Figure 2A). The alpha diversity indices (Richness (Figure 2B), Simpson's (Figure 2C), and Shannon's (Figure 2D)) showed no significant differences across all groups for any parameters, according to the Friedman test with Bonferroni correction.

Table 1. Characteristics of farms included in the study.

Farm	Number of Milking Cows	Milking Type and Frequency (/Day)	Elimination of Abnormal Milk by Pre-Milking	Milking Equipment Cleaning Methods	Bulk Tank Cleaning Methods	Milking Machine Manufacturer
A	21	Automatic 2	Yes	Alkaline cleaning (once a day) with acid cleaning (once every 3 days)	Automatic cleaning after each milk shipment	SAC
B	26	Automatic 2	No	Alkaline cleaning (once a day) with acid cleaning (once every 3 days)	Automatic cleaning after each milk shipment	DeLaval
C	30	Automatic 2	Unavailable	Unavailable	Automatic cleaning after each milk shipment	Unavailable
D	20	Automatic 2	Yes	Alkaline cleaning (once a day)	Automatic cleaning after each milk shipment	SAC
E	51	Automatic 2	Yes	Alkaline cleaning (once a day)	Automatic cleaning after each milk shipment	ORION
F	23	Automatic 2	Yes	Alkaline cleaning (once a day) with acid cleaning (once every 3 days)	Automatic cleaning after each milk shipment	DeLaval

Table 2. Bulk tank milk somatic cell count (mL^{-1}) measurement results for each sample.

Farm	February	April	June	August	October	December
A	102,000	172,000	143,000	196,000	194,000	329,000
B	72,000	86,000	118,000	80,000	46,000	72,000
C	141,000	103,000	194,000	191,000	47,000	100,000
D	175,000	184,000	123,000	147,000	254,000	216,000
E	152,000	107,000	170,000	171,000	181,000	331,000
F	65,000	76,000	77,000	23,000	53,000	61,000

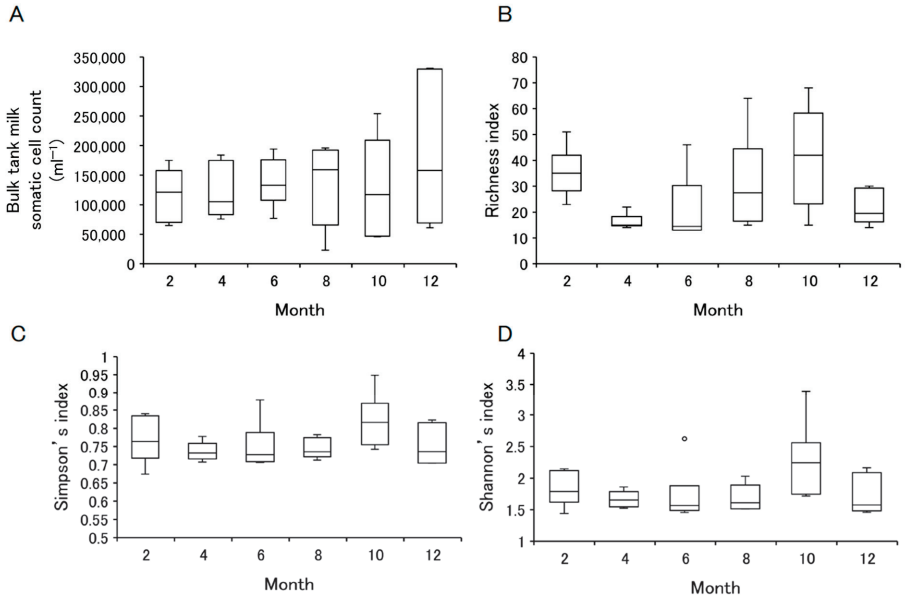


Figure 2. Annual trends in (**A**) bulk tank milk somatic cell count (BTSCC), and the alpha-diversity indices (**B**) richness, (**C**) Simpson's, and (**D**) Shannon's across the target farms. Bars represent means ± standard deviation. The Friedman test with Bonferroni correction showed no significant differences across any of the groups ($p < 0.05$).

A farmer-to-farmer comparison of the BTMM composition on the target farms is shown in Figure 3. The composition ratio of each farm was mainly composed of the phylum *Actinobacteria*, phylum *Firmicutes*, and phylum *Proteobacteria*, and there were no significant differences among farmers, although farmers B and E had a higher proportion of *Euryarchaeota* in February.

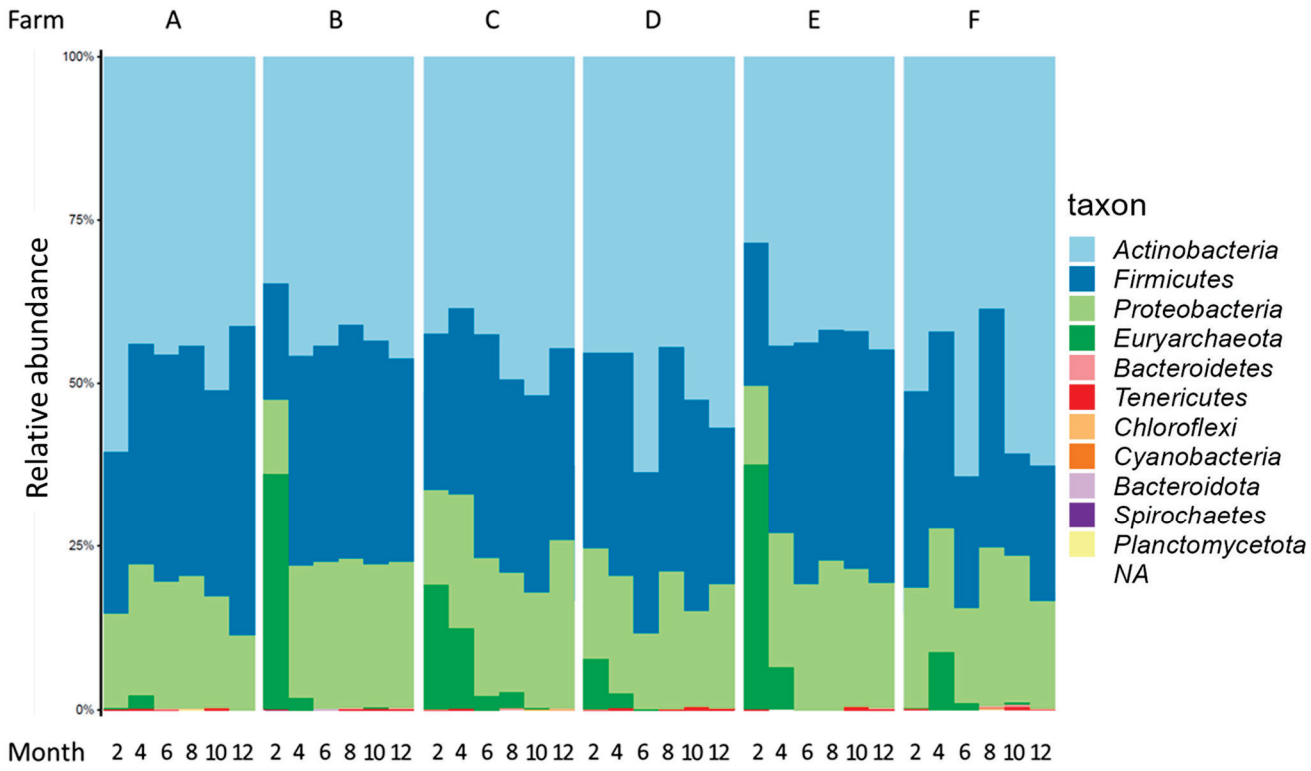


Figure 3. Monthly composition of bulk tank milk microbiota (BTMM) for each farm. The composition of BTMM in all months for all farms was mainly composed of the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, except in February in farms B and E.

Annual changes in the composition of BTMM on the target farms are shown in Figure 4 (A: 100% stacked bar graph, B: principal coordinate analysis). The BTMM consisted mainly of the phyla *Actinobacteria*, *Firmicutes*, and *Proteobacteria*, but some taxa in the phylum *Euryarchaeota* and other species were also identified. For example, various species were identified, including *Mycolicibacterium* spp. and *Kocuria* spp. in the phylum *Actinobacteria*, *Catenibacterium* spp. and *Acetanaerobacterium* spp. in the phylum *Firmicutes*, and *Acetobacter* spp. in the phylum *Proteobacteria*. According to the results of the ANOSIM test with Bonferroni correction, there was a significant difference ($p = 0.0315$) in BTMM composition between February and August (Figure 4A). Principal coordinate analysis, employing the Bray–Curtis distance to compare bacterial composition similarity across different months, showed that the BTMM composition varied among farmers in February; however, the composition was similar in August (Figure 4B).

The bar graph (Figure 5A) and cladogram (Figure 5B) show the bacterial species that varied significantly between February (red) and August (green), as indicated by the LEfSe analysis. The genera *Listeria* and *Enterococcus* accounted for significantly larger proportions of the BTMM in February, and *Catenibacterium* and *Acetobacter* in August.

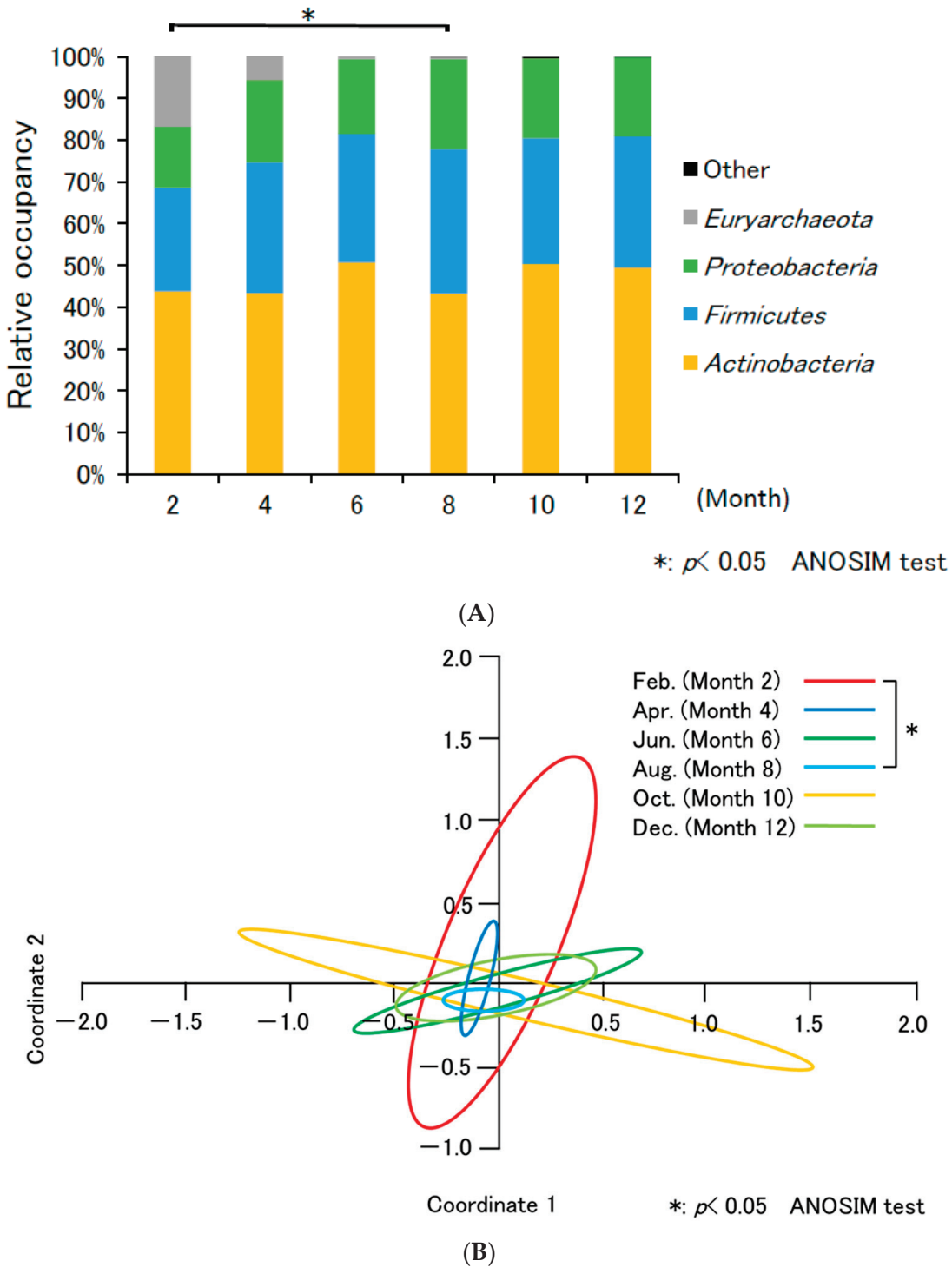
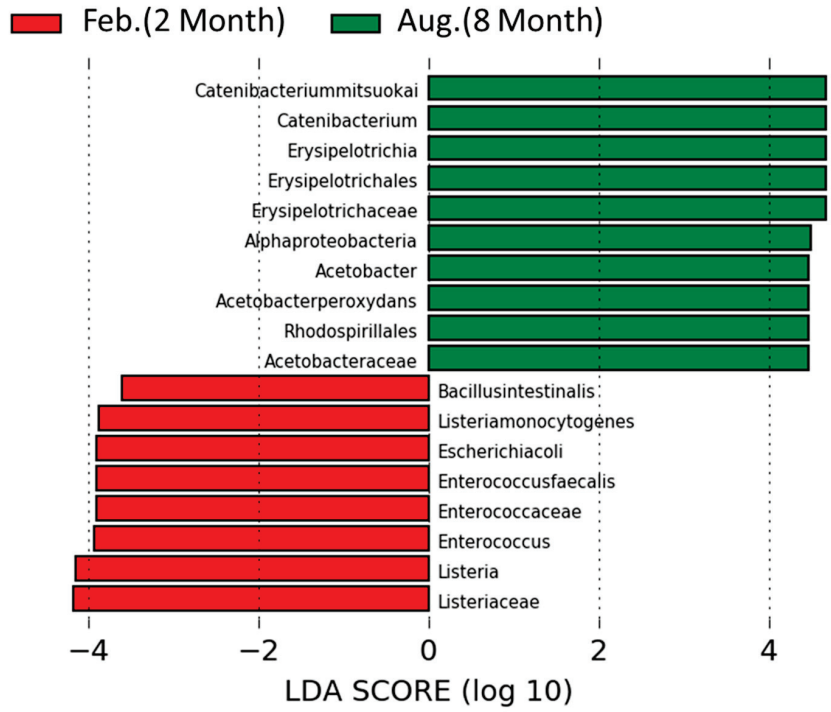
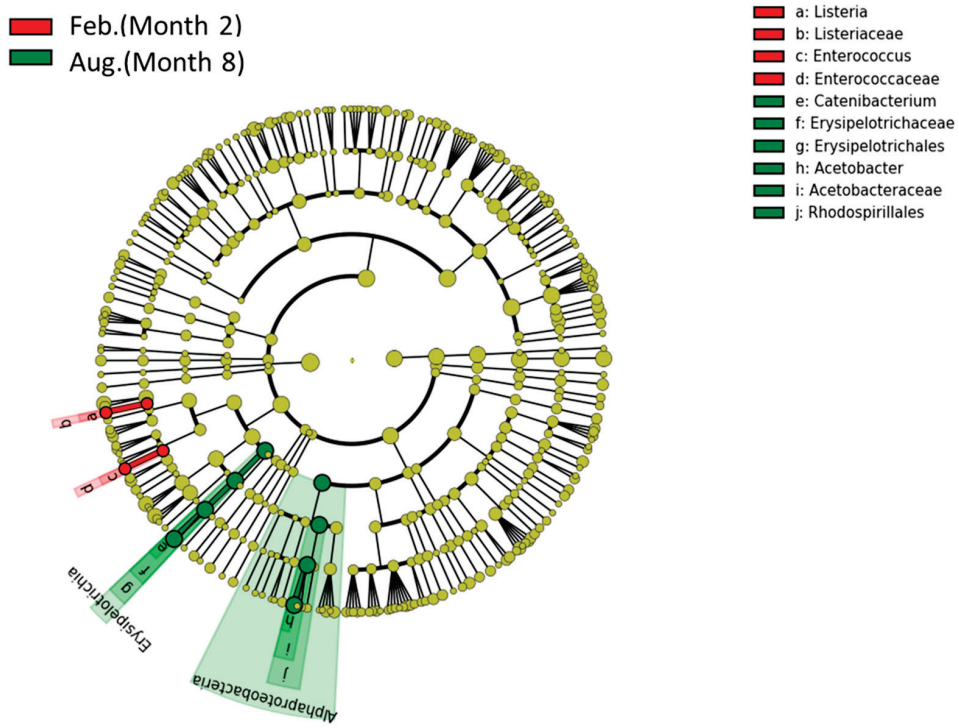


Figure 4. (**A**) Stacked bar graph showing annual changes in bulk tank milk microbiota (BTMM) composition, and (**B**) principal coordinate analysis based on Bray–Curtis distance. Asterisks indicate significant differences estimated by the analysis of similarities (ANOSIM) test with Bonferroni correction. The major phyla were Actinobacteria, Firmicutes, and Proteobacteria, in descending order of abundance, and the ANOSIM test showed a significant difference in the composition of the microbiota in February and August.



(**A**)



(**B**)

Figure 5. Differences in the abundance of bacterial taxa in the milk microbiota in February and August. The bacterial taxa that differed significantly between February and August are shown in red and green, respectively. (**A**) List of bacterial taxa detected by linear discriminant analysis (LDA) and linear discriminant analysis effect size (LEfSe) analysis. For the LEfSe analysis, a Kruskal–Wallis test alpha value of 0.05 and an LDA score of <2.0 ($p < 0.01$) were used as thresholds. The bacterial taxa in February and August had positive and negative LDA scores, respectively. (**B**) Taxonomic cladogram generated from the LEfSe analysis.

4. Discussion

No variation was observed in the average BTSCC on the target farms over the course of the year. Somatic cell counts in milk are used as an indicator of intramammary infection [14], and the reasons for variation in BTSCC have been attributed to the contamination of bulk milk with mastitis-causing bacteria [15], as well as herd size, season, and the timing of calving [16]. BTSCC is generally considered to be abnormal milk in excess of 300,000/mL [17], and some samples in this study exceeded that standard. However, because the milking techniques and hygiene and milk line and bulk tank cleaning methods of the target farmers in this study were sanitary, and because they eliminated abnormal milk by performing pre-milking (Table 1), it was unlikely that abnormal milk would enter the bulk milk. However, given that the herds in this study were the same size and had no seasonal breeding, and given that there were no inter-farm differences or annual variations in BTSCC, and in addition that the study was based on bulk milk that was actually shipped, we see no problem in using these samples for a comparative analysis of BTMM between farms.

The analysis of alpha-diversity indexes of the BTMM, including richness, Simpson's, and Shannon's indices, did not show any annual variation. However, the richness index, which represents the amount of bacterial species in the flora, exhibited an increasing trend, with greater variation observed after the summer. This trend was noted for both the richness index and the BTSCC, although the differences were not statistically significant. These results differed from those of a previous study [18], which reported a negative correlation between the richness index and BTSCC. The reason for this is that the study by Oliveira et al. [18] observed an increase in BTSCC with an increase in the percentage of *Streptococcus* spp. and *Staphylococcus* spp., whereas the present study did not observe an increase in the relative percentage of *Streptococcus* spp. or *Staphylococcus* spp. Moreover, Shannon's index, which is weighted by the percentage of rare species, tended to be higher compared to the other indices (i.e., Simpson's and Shannon's indices) that represent the evenness of bacterial flora. This suggests that the number of rare species constituting the flora increased after the summer season.

BTMM composition ratios were similar among farms, but the percentage of *Euryarchaeota* phylum was higher in February in farmers B and E. This was thought to be due to the higher percentage of archaea (*Haloarcula japonica*) readings added as an inner control, i.e., the relatively low amount of bacterial DNA in the samples. This may also have an effect on the extremely high percentage of *Euryarchaeota* in February compared to other months when compared together as a group by month.

The percentage of *Pseudomonas* spp. in the bulk milk of the farmers included in this study was small and did not fluctuate throughout the year. Since Davide et al. [2] pointed to the inadequate cleaning of milking equipment as the cause of high amounts of *Pseudomonas* spp. in bulk milk, it was thought that the cleaning of milking equipment was sufficient for the farmers in the study. On the other hand, *Streptococcus* spp. was found in several samples, and especially in those with BTSSC above 300,000/mL (December, Farm A and Farm E), at a higher rate (19.49%, 0.50%) than in the other samples (0.12–0.21%). Previous studies have attributed the increase in *Streptococcus* spp. to the contamination of milk from cows with clinical mastitis [2]. Although the cows in this study were clinically observed prior to milking and screened for mastitis milk by pre-milking to ensure that they did not have clinical-type mastitis before milking, the possibility could not be ruled out that milk abnormal enough to affect BTMM was introduced.

Listeira spp. were detected in the bulk milk of the farms included in this study, which is consistent with previous reports [19–21]. The origin of *Listeria* spp. has been linked to fecal contamination due to cattle being fed improperly stored silage [22]. However, the farmers in this study fed almost no silage to their cattle, and no significant increase in *Listeria* spp. was observed in bulk milk at the only instance of silage feeding (December of Farm F). The detection of *Listeria* spp. was concentrated in February, the month with the lowest average temperature, suggesting that the origin of *Listeria* spp. was related to

temperature rather than silage feed. We also believe that the detection of *Listeria* spp. is greatly influenced by temperature, as it has been reported [5] that BTMM in Jersey cattle differs between summer and winter and may be influenced by an increase or decrease in bacteria that is related to temperature.

All of the farms in this study fed their cattle purchased forage, and only on Farm F in December did they use their own feed (dent corn silage), but no bacterial species had a prominent increase in composition percentage in that month alone. The relationship between diet and milk microbiota composition was reported to be different for raw milk microbial communities of cows fed low-starch/high-fiber (LSHF) diets and high-starch/low-fiber (HSLF) diets [23], suggesting that BTMM may be influenced by the diet administered. However, these associations could not be determined because the starch concentration and fiber content of diets were not investigated in this study. Other studies have reported that BTMM varies by breed [24], but the dairy cows in this study were all Holsteins, and the results of this study were not considered to be affected by breed differences.

BTMM composition differed significantly between February and August. The results of the principal coordinate analysis across farms also showed that the BTMM composition in February was highly variable, whereas it was similar in August. It has previously been shown [2] that BTMM composition is influenced by mastitis-causing bacteria, such as *Streptococcus* spp. and *Staphylococcus* spp., in the short term, and by climate and feed in the long term (i.e., on an annual basis). Since this study was not conducted over a multi-year period, the effect of climate could not be determined. However, meteorological data show that monthly average temperatures were lowest in January and highest in August, and when combined with the fact that BTMM composition was significantly different in February and August, it is possible that temperature plays a significant role in the change in BTMM composition. Also, given the similarities in BTMM observed across farms in August, it seems likely that the farms may have been exposed to similar climatic conditions.

The difference in the composition of BTMM between February and August was due to the higher proportion of *Listeria* and *Enterococcus* spp. in February and *Catenibacterium* and *Acetobacter* spp. in August, respectively. Since *Listeria* spp. and *Enterococcus* spp., both of which were abundant in February, are psychrophilic [25], it is possible that some herds were affected by these bacteria due to cold conditions, which was one of the reasons for the variation observed in the composition of BTMM among farms. On the other hand, *Catenibacterium* spp. and *Acetobacter* spp., which accounted for a large proportion of the microfloral community in August, were reported to be indigenous flora in normal milk [26]. The small variation in BTMM composition among herds in August suggests that there were no significant differences across farms in terms of bacterial exposure during the summer months in this study. These results suggest that although there were no differences among herds in terms of environmental exposure to bacteria in August, some herds may have been exposed to bacteria that were tolerant to the low temperatures in February.

This study has several potential limitations. First, this study examined the relative composition of microbiota in bulk tank milk, and did not consider the absolute number of bacteria in milk. Second, the results of BTMM composition reflect not only the udder flora of each herd, but also the results of the bacterial contamination of bulk tanks. However, the bacterial flora on teat surfaces, milking equipment, and bulk tank hygiene were not examined in this study. Consequently, the origin of the bacteria affecting the composition of the BTMM could not be determined. Third, we could not obtain descriptions of animals, such as average parity, calving date, body weight, body condition score, feeding system by farm (feed ingredients and nutritional value), and average total dry matter intake, due to personal information. Therefore, it was not possible to examine in detail the effects of feed, individual cow differences, and other factors on the BTMM composition ratio.

5. Conclusions

The results of this study suggest that the BTMM composition across the herds examined in this study was not constant throughout the year, and that exposure to psychrophilic

bacteria in the winter was a factor in the difference. The results also suggest that temperature is likely to affect the BTMM composition ratio.

Author Contributions: R.I. and Y.S. (Yasunori Shinozuka) conceived of the study, participated in the design and coordination of the study, analyzed and interpreted data, and wrote the manuscript. K.K. and Y.S. (Yuko Shimizu) collected samples on the farm, prepared samples, and analyzed them in the laboratory. K.K. provided all data from the farm, including health status and nutrition. Y.S. (Yuko Shimizu) participated in the sequence alignment and drafting of the manuscript. T.K. and K.K. supervised the entire study. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was a non-interventional study that focused on bulk tank milk before shipment, so ethical approval from Azabu University was not required.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this study will be made available by the authors on request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Communication

Chemical Composition and In Vitro Nutritive Evaluation of Pomegranate and Artichoke Fractions as Ruminant Feed

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Abstract: The aim of this work was to assess the chemical composition and in vitro ruminal fermentation of samples (n = 3) of pomegranate (peels (PPs) and seeds (PSs)) and artichoke (hearts (AHs) and stems (ASs)) wastes. Dried orange pulp (DOP) and tomato pomace (TP) were used as reference feeds. All wastes had low dry matter (DM; lower than 33.0 and 12.0% for pomegranate and artichoke, respectively). The DM of pomegranate fractions was rich in sugars (>42.0%) and contained low protein (<8.0%) and neutral detergent fiber (NDF; <27.0%), whereas that of both artichoke fractions had high protein (>18.0%) and NDF (>36.0%) and low sugars content (<9.2%). Pomegranate seeds were more rapidly and extensively fermented in vitro than PPs, but both were less degradable and contained less metabolizable energy (ME) than DOP (7.43, 11.0 and 12.5 MJ ME/kg DM, respectively). Although AHs were more rapidly fermented and produced more volatile fatty acids (VFAs) than ASs, both had lower ME content than TP (9.50, 7.25 and 12.5 MJ ME/kg DM). The analyzed wastes had lower ME content than other by-products, but they were extensively fermented by ruminal microorganisms and could be used as ruminant feeds.

Keywords: pomegranate wastes; artichoke wastes; in vitro; rumen fermentation; energy content; gas production

1. Introduction

Food waste has a substantial environmental and economic impact and represents an inefficient use of global resources, and thus it is essential to reduce the amount of wasted food. Fruits and vegetables produce the largest share of food waste, considering the waste generated during cultivation, harvesting, processing, distribution, and consumption. Current estimates indicate that in the EU, as much as 41.4 and 45.7% of fruit and vegetable production, respectively, is lost or wasted throughout the entire food supply chain, and these figures can rise to over 55% in other regions worldwide [1]. Furthermore, fruits and vegetables account for 76% of the total food waste generated during primary production, especially at the postharvest stage, when between 37 and 55% of the total harvest can be discarded [2] due to the high-quality standards imposed by consumers. Moreover, food waste can also be extremely polluting and its handling is usually challenging [3].

Pomegranate (*Punica granatum* L.) fruits are consumed worldwide, and its production is estimated to be greater than 3.8×10^6 t per year [4]. Pomegranate processing into juice or other products generates a large quantity of waste products, mainly peels, which can represent as much as 50% of the fruit [5]. Previous studies have evaluated the effect of including pomegranate waste products in ruminant diets on animal performance and product quality [5–8], and although no effect has been observed on milk production or growth performance, the antioxidant status of cow's and ewes' milk and kid's meat has been increased, with a healthier fatty acid profile. However, information on their nutritive value and ruminal fermentation is more limited [8].

Artichoke (*Cynara scolymus* L.) is another crop widely consumed worldwide, with a production of approximately 0.7×10^6 t per year [9]. However, only a small proportion of

the crop is edible and waste products (leaves, external bracts, and stems) may represent approximately 80% of the harvested biomass [10]. Previous studies have evaluated the characteristics of ensiled artichoke waste [11,12] and its effect on milk quality in both dairy sheep, observing a reduction in fat and total free fatty acids and an increase in total free amino acids in cheese [13], and dairy goats, observing a healthier lipid and mineral profile for human consumption in milk [14]. However, information on characteristics and ruminal fermentation of artichoke wastes is still scarce. Therefore, the objective of this study was to evaluate the chemical composition, in vitro ruminal fermentation, and energy content of samples of pomegranate and artichoke fruits and their fractions.

2. Materials and Methods

All the experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid (Appro-val number PROEX 212.2/22). Animal care and ruminal sampling followed the Spanish regulations for experimental animal protection.

2.1. Pomegranate and Artichoke Samples

Three pomegranate (*Punica granatum* L.) and artichoke (*Cynara scolymus* L.) samples were obtained from local markets in3 different weeks during the autumn season. Each sample of pomegranate consisted of 10 fruits that were weighed and manually separated into peels (albedo, rind, and membrane) and seeds (aril and seeds). Each artichoke sample consisted of 15 pieces, which were weighed and divided into hearts and external parts (outer leaves and stems). Both fractions of each sample (3 samples for both pomegranate and artichoke) were independently weighed and dried at 40 °C in an air-forced oven until constant weight to determine the dry matter (DM) content. In addition, one sample of each dried orange pulp (DOP) and tomato pomace was evaluated in the study to be used as reference feeds for pomegranate and artichoke samples, respectively, as both by-products are widely used in ruminant feeding in practice and have similarities in the chemical composition with the samples studied. The DOP sample was commercially available, whereas tomato pulp was obtained from a tomato processing industry. After drying, all samples were ground to pass a 1 mm screen using an automatic centrifugal mill (Retsch ZM 200, Haan, Germany) to carry out chemical composition analysis and in vitro ruminal incubations.

2.2. Animals, Feeding and Ruminal Fluid

Four adult Lacaune sheep (64.3 $\pm$ 2.11 kg body weight; 3 years old), with a permanent rumen cannula, were used as rumen fluid donors for in vitro incubations. The sheep were fed a diet based on grass hay and concentrate in a 2:1 proportion, which contained 114 g of crude protein (CP), 365 g of neutral detergent fiber (NDF), and 160 g of acid detergent fiber (ADF) per kg DM. The diet was administered daily in two equal portions at 9:00 and 18:00 at a restricted level (45 g dry matter (DM)/kg body weight $^{0.75}$) and animals had free access to freshwater and were individually housed in floor pens.

About 400 g of ruminal content was manually obtained from each sheep through the rumen cannula before the morning feeding (9:00 h) using barbeque tongs with shovels. The ruminal content was filtered through four layers of cheesecloth and the obtained fluid was immediately transported to the laboratory (less than 15 min after collection) into thermal flasks (one for the fluid from each donor sheep) for conducting the in vitro incubations.

2.3. In Vitro Incubations: Experimental Design and Sampling

Two similar in vitro trials were carried out to determine gas production kinetics and fermentation parameters of the fractions of pomegranate and artichoke and of the reference feeds. The incubations were conducted as described by De Evan et al. [15] in two consecutive weeks—gas production kinetics first and fermentation parameters second. Briefly, 200 mg of DM of each sample (3 samples per each studied fraction and the two

reference feeds) was carefully weighed into 60mL glass vials. The ruminal fluid of each sheep was independently mixed with a pre-warmed (39 °C) culture medium [16] in a 1:4 ratio which was modified by excluding the trypticase and replacing the $(NH_4)HCO_3$ with $NaHCO_3$ to obtain a N-free medium. Each vial was filled with 20 mL of the mixture using a Watson-Marlow 520UIP31 peristaltic pump (Watson-Marlow Fluid Technology Group, Cornwall, UK) under CO_2 flushing. The vials were sealed with rubber stoppers and incubated at 39 °C. In addition, vials without substrates (blanks; two per inoculum) were included to correct for the endogenous gas production. This procedure was followed to obtain 4 different replicates (one vial per sheep inoculum) per incubated sample.

In the first in vitro trial the vials were incubated for 120 h and the amount of gas produced in each vial was measured at different time intervals (3, 6, 9, 12, 15, 22, 26, 31, 36, 48, 58, 72, 96, 120 and 144 h after incubation) using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a plastic syringe. The second in vitro trial was performed in a different week as described before and lasted for 24 h. After this time, the gas production was measured, the content of the vials was homogenized by handshaking, and the pH was determined with a pHmeter Crison GPL 21 (Crison Instruments, Barcelona, Spain). Finally, 3 mL of each vial content was mixed with 3 mL of 0.5 M HCl and frozen at −20 °C until the volatile fatty acid (VFA) and NH_3-N concentrations analyses.

In addition, the potential DM degradability (PDMD) was estimated using an Ankom Daisy^{II} incubator (Ankom Technology Corp., Fairport, NY, USA), at the same time as the incubation was performed to measure the gas production kinetics. Three hundred mg of each feed was weighed into filter bags (Ankom Corp #57; 25 μm pore size; Ankom Technology Corp., Fairport, NY, USA) in triplicate, and the bags were incubated at 39 °C in a 1:4 mixture of ruminal fluid (mixture of all sheep) and the culture medium described previously [16]. After 144 h, the bags were washed with cold water, dried at 60 °C for 48 h, and weighed to calculate the PDDM. This value was used to estimate the DM effective degradability (DMED) as described later.

2.4. Analyses of Chemical Composition

All chemical fractions were analyzed in duplicate. The DM (ID 934.01), ash (ID 942.05), and ether extract (EE; ID 920.39) were analyzed following the procedures of AOAC [17]. Other chemical analysis and analysis of NH_3-N and volatile fatty acids (VFA) concentrations in the vial's content were performed as described by De Evan et al. [15].

2.5. Calculations and Statistical Analysis

Gas production data were fitted to the model Gas = A $(1 - e^{(-c\,(t - lag))})$ using the Proc NLIN of the SAS [18]. In this model, A is the asymptotic or potential gas production, *c* is the fractional gas production rate, *lag* is the time until gas production begins, and t is the gas measurement time. In addition, the average gas production rate (AGPR) was defined as the gas production rate in the period from the incubation start to the time taken to reach half of the A value, and it was calculated as: AGPR = A *c*/[2 (ln2 + *c lag*)]. The DMED was estimated as: DMED = [(PDMD × *c*)/(*c* + *k*p)] $e^{(-kp \times lag)}$ for a *k*p (rumen passage rate) of 0.042 per h. The metabolizable energy (ME) content of the samples was estimated from the amount of gas produced at 24 h of incubation (G24; mL per 300 mg of DM incubated) and the content in CP and EE (expressed as g/kg DM) using the following equation [19]: ME = 2.43 + 0.1206 × G24 + 0.0069 × CP + 0.0187 × EE.

All statistical analyses were performed with the SAS package [18]. Data on chemical composition of pomegranate and artichoke fractions were analyzed independently for each by-product as a one-way analysis of variance, with the by-product fraction being the main effect. Gas production and fermentation parameters were analyzed using the PROC MIXED of SAS using the following statistical model, in which the effect of the by-product fraction was considered fixed and that of the inoculum was considered random: Y ij = μ + Ti + Yj + eij, where Y ij = observation; μ = overall mean for each parameter; Ti = effect of

by-product fraction; Yj = effect of inoculum; and eij = random error. Values of $p < 0.05$ were considered statistically significant, and those < 0.10 were considered trends.

3. Results and Discussion

3.1. Chemical Composition of Pomegranate and Artichoke Wastes

The chemical composition of the pomegranate and artichoke fractions and of the reference feeds is shown in Table 1. On average, the pomegranate fruits contained 36.5 and 63.5% of peels and seeds (fresh matter basis). The DM content of the pomegranate was greater ($p = 0.027$) in peels than in seeds, although it was low in both fractions. Both pomegranate fractions were characterized by low CP and EE content (<7.2% and 1.6% of DM, respectively), but had high total sugars levels (>46% of DM). Peels were more fibrous than seeds, having more than twice the amount of NDF, ADF and lignin than seeds ($p \leq 0.003$), whereas CP and sugars content were significantly lower ($p \leq 0.008$) in peels than seeds. The NDICP was a low proportion of total CP in seeds, but it was greater ($p < 0.001$) in peels, reaching an average 28.1% of total CP. A sample of DOP was selected as a reference feed due to its similar low CP and high sugars content. The chemical composition of the DOP sample was in the range previously reported in the feed tables [20–22].

Table 1. Chemical composition (g/100 g dry matter unless otherwise stated) of pomegranate and artichoke fractions (n = 3) and of a sample of each dried orange pulp (DOP) and tomato pomace used as reference feeds [1].

	Pomegranate				Artichoke					
Item	**Peels**	**Seeds**	**SEM [1]**	***p* =**	**DOP**	**Hearts**	**Stems**	**SEM [1]**	***p* =**	**Tomato Pomace**
Dry matter (g/100 g)	32.4	20.0	2.57	0.027	91.1	11.9	7.02	0.386	0.001	26.0
Ash	3.59	2.47	0.152	0.007	3.11	9.70	9.05	0.252	0.140	3.70
Crude protein (CP)	3.80	7.17	0.474	0.008	5.84	24.0	18.6	0.65	0.004	17.3
Ether extract	1.60	1.55	0.186	0.872	4.90	2.31	1.55	0.231	0.083	10.7
Total sugars	42.6	75.3	1.4	<0.001	46.5	9.09	6.90	0.858	0.145	12.3
Neutral detergent fiber (NDF)	26.7	12.8	0.90	<0.001	16.3	36.2	51.2	2.05	0.007	54.1
Acid detergent fiber	18.6	8.64	1.10	0.003	9.73	23.7	34.8	1.52	0.007	40.8
Lignin	6.80	3.90	1.255	0.178	0.81	7.45	6.57	0.872	0.514	21.7
NDICP (% CP) [2]	28.1	4.97	1.49	<0.001	5.11	26.5	24.2	3.18	0.629	12.8
Lignin (% NDF)	25.1	30.2	4.25	0.444	4.97	20.4	12.9	2.01	0.056	40.1

[1] SEM: standard error of the mean (n = 3); [2] NDICP: neutral detergent insoluble crude protein expressed as g/100 g crude protein.

The type of pomegranate by-product analyzed in different studies varies highly, and therefore some differences in the chemical composition can be expected. In agreement with our results, others [23,24] found greater CP content in seeds than in peels, but the content of NDF and ADF was greater in the seeds, which contrasts with our results. In previous studies [23–26], CP content of pomegranate peels varied from 2.5 to 8.4% (DM basis), whereas NDF, ADF and EE content ranged from 20.6 to 31.6, from 11.7 to 21.2, and from 0.40 to 5.25% of DM, respectively, and our samples were within the ranges previously reported for CP, NDF and ADF, although greater EE content was observed.

The nutrient content (DM basis) of pomegranate seeds after extracting the juice reported by others [23,24] was variable for CP (11–15%), NDF (43–68%), ADF (31–49%), ash (0.7–2.8%) and EE (0.6–10%). We observed lower content of CP, NDF and ADF in the seeds compared to these studies, but ash and EE contents were in the range described. These differences could be explained by the high concentration of sugars in the seeds of our study (75% of DM), which would cause a reduction in the concentration of other nutrients. In general, differences in chemical composition of pomegranate by-products can be justified

by variations in production and growing conditions and in pomegranate varieties, but also by differences in processing.

Artichoke hearts and stems represented an average 46.5 and 53.6% (fresh matter basis) of the whole vegetable, respectively, and both fractions contained low DM (Table 1). The DM of both artichoke fractions were characterized by high CP and NDF content, although about 25% of the total CP was linked to NDF, thus reducing its availability for ruminants. In addition, both fractions had low EE and total sugars content. Artichoke hearts had greater CP and lower NDF and ADF than stems ($p \leq 0.007$), but there were no differences between fractions in ash, total sugars and lignin content. As previously reported [11,12,15,27–29], the CP, NDF, ADF, lignin and EE content of artichoke stems (DM basis) can range from 10 to 18%, 43 to 57%, 30 to 43%, 4.3 to 10%, and 0.8 to 5.5%, respectively, and the composition of the stems analyzed in our study is generally within the range of values reported. The variations observed in the chemical composition of artichoke by-products among studies may be largely due to the proportion of each fraction.

Tomato pomace was selected as a reference feed for comparison with artichoke samples due to its similar CP and NDF content, although tomato pomace contained more EE and total sugars than both artichoke fractions. The chemical composition of the tomato pomace sample used in our study agrees well with the values reported previously [20,21].

3.2. In Vitro Fermentation of Pomegranate Wastes

Both pomegranate fractions and the DOP used as a reference were fermented in vitro with ruminal fluid from sheep to assess the gas production kinetics (Table 2). Compared to peels, pomegranate seeds had greater ($p < 0.001$) values of A, *c*, *Lag* and AGPR, indicating greater fermentation of pomegranate seeds compared to peels. Both DMED and ME content were greater ($p < 0.001$) for the seeds, which agrees well with the greater content in sugars and lower NDF content of this fraction compared with peels (Table 1).

Table 2. Parameters of gas production kinetics of pomegranate (n = 3) and artichoke fractions (n = 3) and of a sample of each dried orange pulp and tomato pomace used as reference feeds [1].

Gas Production Parameters						
Sample	**A (mL/g)**	***c*** **(%/h)**	***Lag*** **(h)**	**AGPR (mL/h)**	**DMED (%)**	**ME [2] (MJ/kg DM)**
Pomegranate peels	147	4.23	0.053	4.49	36.0	7.43
Pomegranate seeds	244	7.83	1.788	11.4	53.2	11.0
SEM [3]	1.6	0.171	0.1422	0.127	0.58	0.044
p =	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Reference feed						
Dried orange pulp	360	8.61	0.306	20.7	61.7	12.5
Sample						
Artichoke hearts	188	3.71	2.63	4.40	33.2	9.50
Artichoke stems	206	2.78	3.37	3.61	23.1	7.25
SEM	1.9	0.061	0.193	0.071	0.64	0.119
p =	<0.001	<0.001	0.014	<0.001	<0.001	<0.001
Reference feed						
Tomato pomace	199	7.28	2.33	8.28	31.5	11.1

[1] A: potential gas production; *c*: fractional rate of gas production; *Lag*: is the time needed to start gas production; AGPR: average gas production rate; DMED: dry matter effective degradability estimated for a rumen particulate outflow of 0.042 per h; [2] ME: metabolizable energy was calculated from gas production at 24 h as well as chemical composition as proposed by Menke and Steingass [19]; [3] SEM: standard error of the mean (n = 12; 3 samples × 4 replicates).

Mirzaei-Aghsaghali et al. [23] observed greater in vitro gas production for pomegranate peels compared with seeds in 96 h in vitro incubations with ruminal fluid from steers, but the seeds were obtained after extracting the juice and had lower content in non-structural carbohydrates than the peels. In contrast, Delavar et al. [24] reported similar gas production

patterns for both peels and seeds. The gas production values observed in the current study for both pomegranate fractions were greater than others reported previously [24,30,31], which can be partly due to the fact that our samples had not previously been used for juice extraction. In addition, the source of ruminal fluid and the experimental methodology can also influence in vitro gas production.

Both pomegranate fractions were less fermented than DOP despite all samples having high total sugars and low NDF content. However, pomegranate NDF was more lignified than the NDF of DOP (Table 1) and lignin is one of the main factors that reduce ruminal fiber degradation. The ME content of the DOP used as a reference (12.5 MJ ME/kg DM) was similar to the values previously reported in the feed tables (12.2, 11.5 and 12.1 MJ ME/kg DM) for [20–22], respectively, indicating that this sample could be considered representative of this by-product. The ME of pomegranate peels and seeds was 61.9 and 91.7% of that of DOP, respectively. As DOP can replace cereals in the diet of ruminants [32], pomegranate by-products, and especially those including whole seeds, can be used as an energy source for ruminants.

Compared with peels, pomegranate seeds showed greater ($p < 0.001$) gas and total VFA production after 24 h of incubation (Table 3), and consequently lower pH ($p < 0.001$) as the total VFA production and ruminal pH are usually negatively correlated. The molar proportion of acetate was greater ($p < 0.001$) in pomegranate peels than in the seeds, whereas the propionate proportion was lower ($p < 0.001$), resulting in a greater ($p < 0.001$) acetate/propionate ratio for the peels fraction. The observed differences in the VFA profile are consistent with the lower sugars and greater NDF content of peels compared to seeds and is in accordance with the results of Kara [25], who studied the in vitro fermentation of pomegranate peels using ruminal fluid from goats as the inoculum.

Table 3. Fermentation parameters of pomegranate (n = 3) and artichoke fractions (n = 3) and of a sample of each dried orange pulp and tomato pomace used as reference feeds after 24 h of incubation with ruminal fluid from sheep [1].

				Molar Proportions (mol/100 mol)					
Sample	**Gas (mL/g DM)**	**pH**	**Total VFA (μmol/g DM)**	**Acetate (Ac)**	**Propionate (Pr)**	**Butyrate (Bt)**	**Minor VFA**	**Ac/Pr (mol/mol)**	**NH_3-N (mg/L)**
Pomegranate peels	96.3	6.76	4.48	57.5	28.6	11.8	2.13	2.02	61.6
Pomegranate seeds	210	6.46	8.46	52.3	34.7	10.4	2.61	1.52	79.2
SEM [2]	1.60	0.010	0.092	0.43	0.49	0.56	0.160	0.040	1.95
p =	<0.001	<0.001	<0.001	<0.001	<0.001	0.105	0.048	<0.001	<0.001
Reference feed									
Dried orange pulp	235	6.44	8.90	64.8	21.0	10.9	3.30	3.09	87.8
Sample									
Artichoke hearts	109	6.72	6.57	62.0	25.7	7.57	4.64	2.42	182
Artichoke stems	97.7	6.79	6.15	64.5	24.1	6.88	4.57	2.68	162
SEM [2]	1.70	0.037	0.810	0.27	0.30	0.103	0.066	0.039	2.5
p =	0.025	0.217	0.003	<0.001	<0.001	<0.001	0.482	<0.001	<0.001
Reference feed									
Tomato pomace	135	6.80	6.28	66.0	23.2	7.32	3.48	2.84	162

[1] VFA: volatile fatty acids; minor VFA: calculated as the sum of isobutyrate, isovalerate, and valerate; and [2] SEM: standard error of the mean (n = 12; 3 samples × 4 replicates).

The greater NH_3-N concentrations ($p < 0.001$) observed for the seeds agrees well with the greater CP content and the lower NDICP proportion of this fraction compared with the peels (Table 1), which would result in a greater amount of N available to be used by rumen microorganisms. The fermentation pattern of the DOP used as a reference (Table 3) was quite similar to that of pomegranate seeds, although fermentation of DOP resulted in a greater acetate/propionate ratio, probably due to differences in the chemical composition. In addition, DOP showed greater NH_3-N content than both pomegranate fractions despite having intermediate CP content, which could be due to greater CP degradability in the DOP as indicated by the lower amount of NDICP (Table 1).

3.3. In Vitro Fermentation of Artichoke Wastes

The parameters of gas production of artichoke fractions and the sample of tomato pomace used as a reference are shown in Table 2. Potential gas production (A) of artichoke stems was 9.6% greater ($p < 0.001$) than that of artichoke hearts. Although the NDF content of the stems was significantly greater (51.2 vs. 36.2% of DM), the lower lignification of the stems' NDF (12.9% of lignin in the NDF) compared with the hearts' (20.4%) can help to explain these results. Despite the lower potential gas production, the hearts fermented more rapidly than the stems, resulting in significantly greater ($p < 0.001$) values of c, AGPR, DMED and ME, and lower ($p = 0.014$) *Lag* values. Others [29,30] reported greater gas production than in our study for artichoke stems and bracts, respectively, after in vitro incubation with ruminal fluid from sheep for 96 h. Similarly, greater in vitro DM degradability for ensiled artichoke waste (69%) [12], for fresh artichoke (50.6%) [33], and for artichoke stems (65.6%) [30] and bracts (63.4%) [29] have been observed in other studies. These results reflect the great variability in the values that can be found in the literature, which can be attributed to the variability in the composition of by-products, but also to the variable methodologies used to determine DM degradability.

Both artichoke fractions were less rapid and extensively fermented than the tomato pomace used as a reference feed, which was likely due to the greater content in easily fermented fractions in the tomato pomace [3]. Tomato pomace has been classified as a medium-quality fibrous ingredient [3], and its ME content reported in the feed tables was between 9.5 and 11.2 MJ /kg DM [20,21] which is in good agreement with the value observed in our study (11.1 MJ/kg DM). The ME content of artichoke hearts and stems was 85.6 and 65.3% of that of tomato pomace, respectively, indicating that they have lower nutritive value for ruminants than this by-product.

Artichoke hearts produced greater ($p \leq 0.025$) gas and total VFA than artichoke stems in 24 h of in vitro ruminal incubation (Table 3), which is in agreement with the greater gas production rates (*c* and AGPR) and DMED values previously observed for hearts. The greater ($p < 0.001$) acetate proportions and lower ($p < 0.001$) proportions of propionate observed for stems are consistent with the greater NDF content of this fraction compared with artichoke hearts (Table 1), which is also reflected in the greater acetate/propionate ratio ($p < 0.001$) observed for the stems. Madrid et al. [33] analyzed the in vitro fermentation of both fresh and boiled whole artichoke using ruminal fluid from goats and observed greater proportions of acetate and lower proportions of propionate than in the present study, but it should be taken into account that VFA production was measured after 72 h incubation and acetate proportion usually increases and that of propionate decreases as incubation time progresses [34]. Compared with artichoke hearts, the stems had lower ($p < 0.001$) NH_3-N concentration, which could be attributed to their lower CP content. Compared with the sample of tomato pomace used as a reference, both artichoke fractions produced lower amounts of gas, but similar amounts of total VFA. However, the estimated ME content of tomato pomace was greater than that for artichoke stems, which could be due to the greater EE concentration of tomato pomace.

4. Conclusions

Discarded pomegranates had low dry matter content, but the dry matter was rich in sugars and contained low protein and fiber. Pomegranate peels were more fibrous than seeds and were less degraded in the rumen. The metabolizable energy content of pomegranate seeds was similar to that of dried orange pulp and therefore it could be a high-energy feed for ruminants. However, pomegranate peels had lower energy content and therefore the energy content of discarded pomegranates would be influenced by the proportion of each fraction. Discarded artichokes had very low dry matter content, but the dry matter was rich in protein and medium-lignified fiber. The estimated metabolizable energy content of artichoke hearts and stems indicates that they have lower nutritive value for ruminants than tomato pomace. Both wastes could be used in ruminant feeding, but

given their low dry matter content, effective low-cost storage methods are needed for their preservation.

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Effect of Tannin Inclusion on the Enhancement of Rumen Undegradable Protein of Different Protein Sources

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Abstract: Tannins can be utilized to increase rumen undegradable protein (RUP) by their capacity to form complexes with diverse nutrients present in the feed. In that regard, high-performance ruminants demand elevated RUP levels. The objective of this study was to evaluate the effects of incorporating varying levels of tannin into three protein sources (cottonseed, peanut, and soybean meals) on ruminal kinetic parameters, ruminal fermentation, and intestinal digestibility. Thus, three in situ experiments were conducted to investigate the ruminal degradation kinetics, where Fraction A represents the soluble portion, Fraction B relates to the portion potentially degraded in the rumen, and kd denotes the degradation rate of Fraction B, for both dry matter (DM) and crude protein (CP) in the rumen. Additionally, the study assessed dry matter effective degradability (ED), rumen undegradable protein (RUP), and intestinal digestibility (ID). These experiments utilized three cannulated animals for the in situ incubations. Regarding cottonseed meal in terms of DM degradation kinetics, tannin inclusion had a quadratic effect on fraction A ($p < 0.01$), B ($p = 0.10$, trend), kd ($p = 0.03$), and ED ($p < 0.01$). Fraction A of CP had a cubic effect ($p = 0.03$), being greater for the control compared with the other treatments. The inclusion of tannin linearly increased RUP ($p < 0.01$). The RUP proportion increased 29, 33, and 45% when 20, 40, and 60 g/kg tannin were used, respectively, compared to the control. For peanut meal, the A fraction of protein and RUP responded quadratically as tannin was included in peanut meal ($p < 0.01$). However, tannin levels did not affect fraction B of protein and ID. Regarding soybean meal, fractions A and B of DM and ED had cubic effects ($p < 0.01$), being greater for the control compared with the other treatments, and responded quadratically as tannin increased. Also, tannin inclusion had a cubic effect on fractions A and B of protein, RUP, and ID ($p < 0.01$). The cubic behavior showed greater B fraction and ID and lower A fraction and RUP for the control compared other treatments ($p < 0.01$). Tannins offer a promising avenue for elevating RUP levels in diets featuring cottonseed and peanut meals. Nevertheless, no advantages were observed when treating soybean meal with tannin.

Keywords: beef cattle; ruminal degradation; protein degradation; tannins

1. Introduction

Ruminant animals still exhibit significantly lower feed efficiency compared to poultry and swine, indicating the pressing need for further advancements in this area [1–3]. Furthermore, protein is the costliest component among the feed constituents [4]. Hence, there is a need for research endeavors to optimize the utilization of this nutrient, which in the case of ruminants is classified into rumen-degradable protein (RDP) and rumen-undegradable

protein (RUP) [5]. The RDP undergoes conversion into amino acids by rumen microorganisms, thereby serving as the primary source of metabolizable protein for ruminants [4]. However, microbial protein alone is insufficient to meet the requirements of high-producing animals [6]. Thus, high-producing animals require a greater RUP contribution to fulfill their metabolizable protein needs.

Soybean, peanut, and cottonseed meals are the main protein sources used in feedlot cattle production in Brazil [7]. Although widely utilized, a significant portion of these feeds' protein is fermented in the rumen [8,9]. Thus, researchers have sought processing techniques (physical and chemical) to enhance protein protection against ruminal fermentation and improve protein utilization [10]. Several processing methods have been developed to manipulate the ruminal degradation of protein sources with the objective to increase feedstuff RUP, including autoclaving [11], microwave treatment [12,13], toasting or irradiation [14], heating [15,16], malic acid [16], xylose [17], and tannins [18,19]. Among these processing techniques, the addition of condensed tannins is noteworthy. Condensed tannins, recognized for their anti-nutritional properties, can form stable complexes with protein when added at low concentrations, resulting in increased resistance to rumen microbial degradation [20,21]. Thus, studies have shown that the addition of condensed tannins, at low concentrations, can increase RUP [22]. However, the optimal levels of tannin utilization for protein protection are not yet well-established. It is important to note that this protein protection should not interfere with intestinal protein digestibility, should not cause harm to animals, and should not adversely affect the ruminal microbial population [23].

There are uncertainties regarding the use of tannins to increase RUP, such as the lack of specific dosages and their effects on different feed ingredients, such as peanut and cottonseed meals. Furthermore, there are limited studies on the effectiveness of increasing RUP supply on ruminal fermentation. In this regard, the use of in vitro techniques allows for controlled studies and provides relevant information on ruminal fermentation of feedstuffs [24,25]. Therefore, this study aimed to evaluate different levels of tannin inclusion in three protein sources (cottonseed, peanut, and soybean meals) on ruminal kinetic parameters, ruminal fermentation, and intestinal digestibility. Our hypothesis is that tannin treatment can effectively protect the protein in these feed ingredients against ruminal fermentation, leading to an increase in RUP and improving nutrient utilization efficiency.

2. Materials and Methods

2.1. Location and Ethical Approval

The experiments took place at the Beef Cattle Research Center, located at the Institute of Animal Science in Sertãozinho, São Paulo, Brazil. This study was conducted in full compliance with the guidelines set forth by the Animal Use Ethics Committee of the Institute of Animal Science. The committee approved the protocol for animal care and handling under the reference number 249-19.

2.2. Experimental Designs and Chemical Analysis

Three experiments were performed for individual feed evaluation: cottonseed (Exp.1), peanut (Exp.2), and soybean (Exp.3) meals. Within each experiment, feed were submitted to four levels of tannin (85% condensed and 15% hydrolyzed from *Acacia mearnsii*, Tanac SA, Montenegro, RS, Brazil) including: 0, 20, 40, and 60 g/kg (DM basis). Furthermore, a commercial soybean-based product was also evaluated as positive control (SoyPass®, Nutron Cargill, São Paulo, SP, Brazil) in Exp.3.

For in situ trials, three Nellore bulls (BW of 397 ± 51 kg, 24 months old, and a body condition score of 3.5), cannulated in the rumen, were used for ingredients incubation. The experimental design included a randomized complete block (animal). For each ingredient, simultaneously in each animal, bags were incubated in the rumen for 0, 2, 4, 8, 12, 24, or 48 h. Filter bags were incubated for each treatment in duplicate in each animal and timepoint, totaling 42 observations per treatment. In the in vitro trials, a setup comprising four 4-L digestion vessels (TE-150, Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil) was employed. These vessels were equipped with a gentle rotational mechanism and a temperature controller and used in a 24 h fermentation batch. Thus, the experimental design included a randomized complete block (vessel), with a total of 26 filter bags (experimental units) incubated for each treatment.

The ingredients used in these studies underwent grinding using a 2 mm screen (Wiley mill; Thomson Scientific Inc., Philadelphia, PA, USA) for all incubations and analyses. Subsequently, the samples were subject to chemical analyses, including dry matter (DM; method G-003/1), ash (method M-001/1), crude protein (method N-001/1), and ether extract (method G-005/1), following the procedures outlined in Detmann et al. [26]. The organic matter (OM) content was calculated as the difference between DM and ash. To determine neutral detergent fiber, the samples were treated with alpha thermo-stable amylase, with the omission of sodium sulfite, in accordance with the method by Van Soest et al. [27] and adapted for the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA). Detailed information on the chemical compositions of the experimental ingredients can be found in Table 1. The tannin treatments were prepared as follows: 600 g of each sample was weighed using a digital scale (Mettler Toledo, model ME2002E, Polaris Parkway, Columbus, OH, USA) and placed in metal molds. Then, the tannin was weighed and homogenized with each sample. After homogenization, distilled water (in a 1:2 ratio) was added to the mixture, which was left to stand for 6 h. After resting, the sample was transferred to a ventilated oven at 60 °C for 72 h.

Table 1. Chemical composition of protein sources.

Composition	Cottonseed Meal	Peanut Meal	Soybean Meal
Dry matter, g/kg	910	915	910
Crude protein, g/kg DM	543	628	524
Ether extract, g/kg DM	16.4	12.8	19.4
Neutral detergent fiber, g/kg DM	174	164	157
Ash, g/kg DM	63.5	53.9	62.3

2.3. In Situ Procedures and Calculations

The animals were situated in an enclosed barn and placed in individual tie stalls. They were provided with a diet consisting of 40% forage and 60% concentrate, which included a mixture of 60% corn silage, 24.9% dry ground corn, 13% soybean meal, 0.2% urea, and 1.9% mineral supplement. Bulls were adapted to this diet for 14 d before the commencement of the study, and they had continuous access to water. Each ingredient was weighed and then inserted into Nylon bags (Sefar Nitex, Switzerland, Fairport, NY, USA) with a porosity of 50 μm and a surface area of 400 cm^2. These bags were subsequently placed inside each animal for incubation, ensuring a bag surface area to mass ratio of 15 mg/cm^2. Samples were incubated in the rumen by attaching the bags to a steel chain with a weight at the end to allow for continual immersion within ruminal contents. Bags were placed into the rumen in the reverse order of incubation hours so that all bags were removed at the same time for washing.

After removal, the bags were immersed in an ice-cold saline solution for 15 min to halt microbial activity and detach bacteria from the feed fraction. Subsequently, the bags underwent a thorough washing in a washing machine using cold running tap water until the rinsing water became clear. Bags designated as "0 h" were not subjected to rumen incubation but were rinsed alongside the incubated bags. Following this, the bags were dried in an oven at 55 °C for 72 h. Upon completion of the drying process, each bag was individually weighed. The residues from each diet were carefully extracted from the bags and placed into labeled plastic bags to create a sample for each diet corresponding to each animal and incubation time. These residual samples from different time points in the bags were utilized to estimate the parameters of ruminal degradation.

The DM and CP degradation profiles were estimated asymptotic function [28]:

$$Yt = A + B \times (1 - e - (kd \times t)),$$

where Yt is the fraction degraded in time 't', g/kg; A is the water-soluble fraction, g/kg; B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate of fraction b, h^{-1}; and t is time, h.

The effective degradability (ED, g/kg) of DM was calculated using the model [29]:

$$ED = A + [B \times kd/(kd + kp) \times e\text{-}kpt),$$

where A is the water-soluble fraction, g/kg; B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate of fraction b, h − 1; t is time, h; and kp is the rumen passage rate (k) of 0.074 h^{-1}, obtained from the equation developed for concentrates [30].

The rumen undegradable protein (RUP) content in ingredients was calculated as:

$$RUP = B \times [kp/(kp + kd)],$$

where B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate, h^{-1}; kp = passage rate, h^{-1}.

2.4. Intestinal Digestibility Procedures and Calculations

In the context of in vitro trials, a setup was utilized that included four digestion vessels, each with a capacity of 4 L (TE-150, Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil). These vessels were equipped with a controlled system to ensure slow rotation and maintained temperature, enabling a 24 h fermentation batch. To assess the intestinal digestion of RUP, a three-step in vitro procedure was employed [31,32]. In brief, 0.335 g (on a dry matter basis) from the 12 h timepoint of the in situ incubation for each ingredient was weighed and placed into R520 bags (Ankom Technology, Macedon, NY, USA). These bags were then sequentially incubated with constant rotation at 39 °C using a TE-150 incubator (Tecnal Equipamentos Científicos, Piracicaba, SP, Brazil). The incubation process involved the use of pepsin solution (P-7000, Sigma, St. Louis, MO, USA) for 1 h, followed by pancreatin solution (P-7545, Sigma, St Louis, MO, USA) for 24 h. Upon completion of the incubation period, the bags were rinsed with tap water until the effluent water became clear. Subsequently, the samples were dried in an oven at 60 °C for 48 h. The residues remaining in the bags were then analyzed to determine the dry matter (DM) and nitrogen (N) content.

2.5. Statistical Analysis

The DM and CP fractions, ED, RUP, and ID were first determined for each replication and compared using a completely randomized model design. The parameters were compared through a regression analysis, with differences considered statistically significant at

a level of $p \leq 0.05$ and trending when $0.05 < p \leq 0.10$. Thus, the levels of tannin inclusion were examined for linear, quadratic, and cubic responses using the following model:

$$Y_{ijk} = B_0 + B_1 X_i + B_2 X_i^2 + P_j + A_k + e_{ijk}$$

where Y_{ijk} represents the response variable obtained from the *i*th level of tannin in the diet of the *j*th incubation and the *k*th experimental unit. The indices *i*, *j*, and *k* denote the levels of inclusion of tannin, the random factor of incubation, and the random factor of bottle, respectively. The regression parameters of the model are denoted as B_0, B_1, and B_2. The X_i represents the effect of the *i*th level of the fixed quantitative factor (inclusion of tannin), P_j represents the effect of the level of the random factor incubation, A_k represents the effect of the level of the random factor, and e^{ijkl} represents the residual error, assumed to follow a normal distribution (0, s^2). All analyses were run using the PROC GLIMMIX of SAS (SAS on Demand, online version).

3. Results

3.1. Cottonseed Meal

Ruminal degradation parameters of DM and CP, as well as protein ID can be found in Table 2 and Figure 1. Regarding DM degradation kinetics, tannin inclusion had linear and quadratic effects on fraction A ($P_{\text{Lin.}} < 0.01$; $P_{\text{Quad.}} < 0.01$), B ($P_{\text{Quad.}} = 0.10$, trend), kd ($P_{\text{Lin.}} = 0.10$, trend; $P_{\text{Quad.}} = 0.03$), and ED ($P_{\text{Quad.}} < 0.01$). Fraction A of CP had linear and cubic effects ($P_{\text{Lin.}} < 0.01$; $P_{\text{Cub.}} = 0.03$), being greater for the control compared with the other treatments and responded quadratically as tannin increased. Tannin levels did not affect Fraction B of CP ($P_{\text{Lin.}} = 0.72$; $P_{\text{Quad.}} = 0.77$; $P_{\text{Cub.}} = 0.87$). The inclusion of tannin linearly increased RUP ($p < 0.01$). Compared to the control (0 g/kg tannin), the RUP proportion increased 29, 33, and 45% when 20, 40, and 60 g/kg tannin were used, respectively. Furthermore, ID had a cubic effect ($p < 0.01$), being greater for the control compared with the other treatments and responded quadratically as tannin increased.

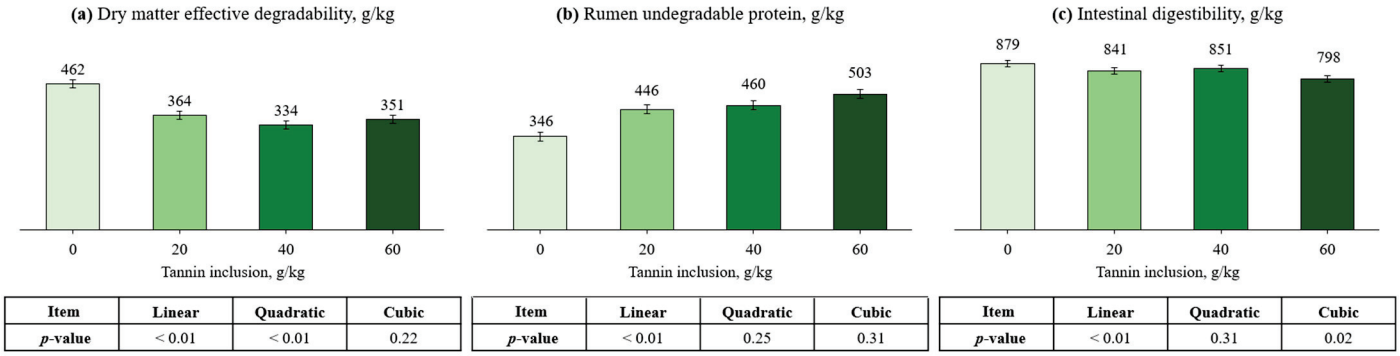
Table 2. Effects of tannin inclusion on rumen degradation parameters of cottonseed meal.

Item [1]	Tannin Inclusion, g/kg				SEM [2]	*p*-Value		
	0	20	40	60		Linear	Quadratic	Cubic
Dry matter								
A, g/kg	194	113	104	112	34.7	<0.01	<0.01	0.13
B, g/kg	595	629	644	599	7.93	0.82	0.10	0.71
kd, h^{-1}	0.059	0.049	0.041	0.051	0.002	0.10	0.03	0.45
Crude protein								
A, g/kg	345	315	331	293	5.76	<0.01	0.71	0.03
B, g/kg	581	572	561	569	10.4	0.72	0.77	0.87
kd, h^{-1}	0.084	0.060	0.046	0.044	0.001	<0.01	0.08	0.92

[1] A, water-soluble fraction; B, potentially degradable water-insoluble fraction; kd, degradation rate of fraction B; [2] SEM, standard error of the mean.

3.2. Peanut Meal

The effects of tannin treatment in peanut meal can be found in Table 3 and Figure 2. For DM kinetics, tannin inclusion had a quadratic effect on fraction A ($p < 0.01$). Furthermore, fraction B had a cubic effect ($p < 0.01$), being lower for the control compared with the other treatments and responded quadratically as tannin increased. On the other hand, tannin levels did not affect kd ($P_{\text{Lin.}} = 0.56$; $P_{\text{Quad.}} = 0.38$; $P_{\text{Cub.}} = 0.15$). Nevertheless, the inclusion of tannin linearly decreased ED ($p = 0.02$). Regarding CP degradation kinetics, tannin levels did not affect fraction B ($P_{\text{Lin.}} = 0.08$; $P_{\text{Quad.}} = 0.08$; $P_{\text{Cub.}} = 0.29$) and ID ($P_{\text{Lin.}} = 0.37$; $P_{\text{Quad.}} = 0.70$; $P_{\text{Cub.}} = 0.84$). The A fraction ($p < 0.01$) and RUP ($p = 0.05$) responded quadratically as tannin was included in peanut meal.



Item	Linear	Quadratic	Cubic
p-value	< 0.01	< 0.01	0.22

Item	Linear	Quadratic	Cubic
p-value	< 0.01	0.25	0.31

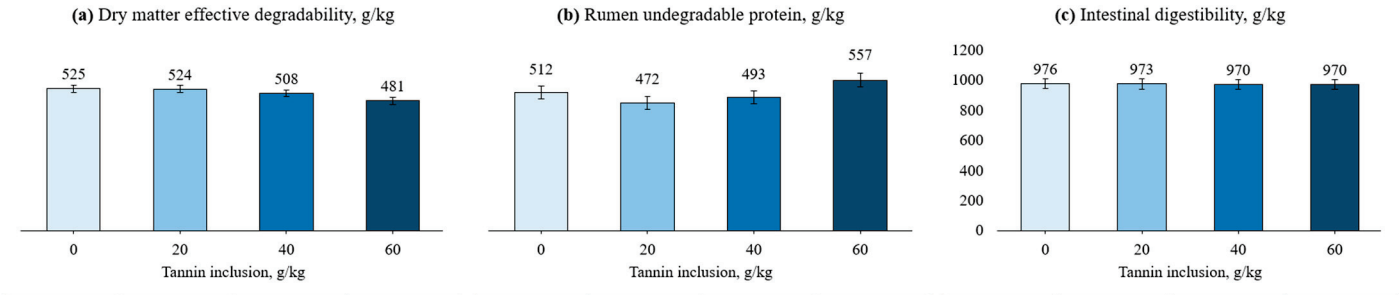
Item	Linear	Quadratic	Cubic
p-value	< 0.01	0.31	0.02

Figure 1. Effects of tannin inclusion on dry matter effective degradability ((**a**), ED), rumen undegradable protein ((**b**), RUP), and intestinal digestibility (**c**), of cottonseed meal. ED = A + [B × kd/(kd + kp) × e-kt] [29]; RUP = B × [kp/(kp + kd)], where A is the water-soluble fraction, g/kg; B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate of fraction b, h^{-1}; t is time, h; and kp is the rumen passage rate of 0.074 h^{-1} [30].

Table 3. Effects of tannin inclusion on rumen degradation parameters of peanut meal.

Item [1]	Tannin Inclusion, g/kg				SEM [2]	*p*-Value		
	0	20	40	60		Linear	Quadratic	Cubic
Dry matter								
A, g/kg	250	228	212	231	398	0.04	<0.01	0.40
B, g/kg	757	832	749	791	23.5	0.86	0.48	<0.01
kd, h^{-1}	0.043	0.040	0.047	0.036	0.003	0.56	0.38	0.15
Crude protein								
A, g/kg	195	219	226	128	3.50	<0.01	<0.01	0.07
B, g/kg	829	772	838	885	13.9	0.08	0.08	0.29
kd, h^{-1}	0.041	0.048	0.037	0.040	0.001	0.61	0.72	0.25

[1] A, water-soluble fraction; B, potentially degradable water-insoluble fraction; kd, degradation rate of fraction B; [2] SEM, standard error of the mean.



Item	Linear	Quadratic	Cubic
p-value	0.02	0.47	0.70

Item	Linear	Quadratic	Cubic
p-value	0.17	0.05	0.86

Item	Linear	Quadratic	Cubic
p-value	0.37	0.70	0.84

Figure 2. Effects of tannin treatment on dry matter effective degradability ((**a**), ED), rumen undegradable protein ((**b**), RUP), and intestinal digestibility (**c**), of peanut meal. ED = A + [B × kd/(kd + kp) × e-kt] [29]; RUP = B × [kp/(kp + kd)], where A is the water-soluble fraction, g/kg; B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate of fraction b, h^{-1}; t is time, h; and kp is the rumen passage rate of 0.074 h^{-1} [30].

3.3. Soybean Meal

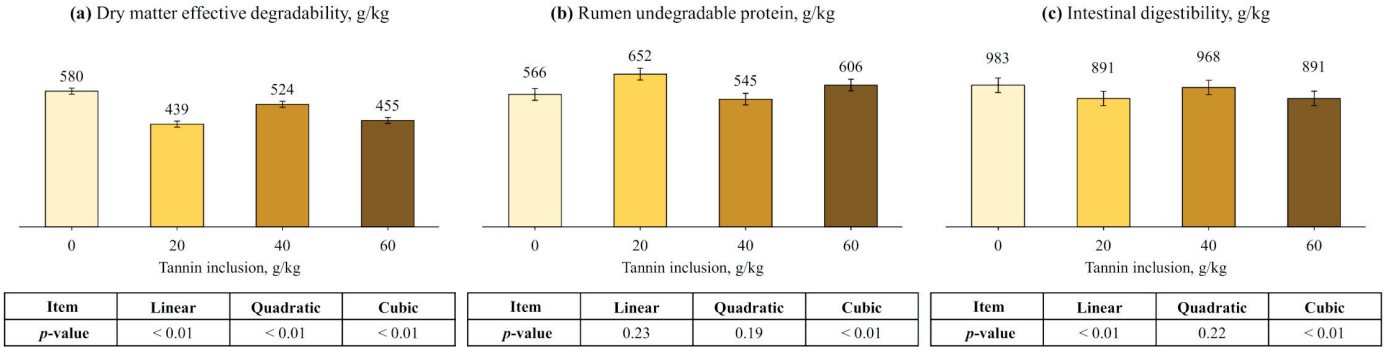
Regarding DM degradation kinetics, fractions A and B, and ED had cubic effects ($p < 0.01$), being greater for control compared with the other treatments and responded quadratically as tannin increased (Table 4 and Figure 3). However, tannin levels did not affect kd ($P_{Lin.} = 0.68$; $P_{Quad.} = 0.41$; $P_{Cub.} = 0.34$). For CP kinetics, tannin inclusion had a quadratic effect on fraction kd, heating the greatest value at 60 g/kg ($p < 0.01$). Also, tannin

inclusion had a cubic effect on fractions A and B, RUP, and ID ($p < 0.01$). The cubic behavior showed greater B fraction and ID, and lower A fraction and RUP for control, compared other treatments. Then, tannin inclusion had a quadratic effect on these parameters.

Table 4. Effects of tannin inclusion on rumen degradation parameters of soybean meal.

Item [1]	Tannin Inclusion, g/kg				SEM [2]	*p*-Value		
	0	20	40	60		Linear	Quadratic	Cubic
Dry matter								
A, g/kg	287	182	208	183	3.19	<0.01	<0.01	<0.01
B, g/kg	731	698	785	667	6.43	0.32	0.08	<0.01
kd, h^{-1}	0.049	0.042	0.049	0.050	0.002	0.68	0.41	0.34
Crude protein								
A, g/kg	128	147	197	117	5.02	0.70	<0.01	<0.01
B, g/kg	912	811	926	903	17.4	<0.01	<0.01	<0.01
kd, h^{-1}	0.038	0.025	0.030	0.062	0.002	<0.01	<0.01	0.71

[1] A, water-soluble fraction; B, potentially degradable water-insoluble fraction; kd, degradation rate of fraction B; [2] SEM, standard error of the mean.



Item	Linear	Quadratic	Cubic
p-value	< 0.01	< 0.01	< 0.01

Item	Linear	Quadratic	Cubic
p-value	0.23	0.19	< 0.01

Item	Linear	Quadratic	Cubic
p-value	< 0.01	0.22	< 0.01

Figure 3. Effects of tannin treatment on dry matter effective degradability ((**a**), ED), rumen undegradable protein ((**b**), RUP), and intestinal digestibility (**c**), of soybean meal. ED = A + [B × kd/(kd + kp) × e-kt] [29]; RUP = B × [kp/(kp + kd)], where A is the water-soluble fraction, g/kg; B is the potentially degradable water-insoluble fraction, g/kg; kd is the degradation rate of fraction b, h^{-1}; t is time, h; and kp is the rumen passage rate of 0.074 h^{-1} [30].

4. Discussion

The rise of the rumen undegradable protein (RUP) content of a diet is essential to fulfilling the requirements of high-performance animals [6]. In that regard, tannins can be utilized to increase RUP by their capacity to form complexes with diverse nutrients present in the feed [21,33]. However, to our knowledge, the optimal levels of tannin inclusion on protecting the amino acids of common protein sources (cottonseed, peanut, and soybean meals) from ruminal fermentation remain unclear. Regarding ruminal kinetic parameters, fraction A is soluble and readily available for ruminal degradation, fraction B is potentially degraded in the rumen within a specific timeframe, and kd is the degradation rate of fraction B [34,35]. Thus, we hypothesized that the treatment of these feed with tannin could modify the ruminal kinetic parameters of DM and CP. Indeed, tannin inclusion reduced the soluble fraction of DM for all tested feed, reaching lower values at 40 g/kg for cottonseed and peanut meals and 60 g/kg for soybean meal. Interestingly, the tannin effect was higher on protein soluble fraction, where 60 g/kg was the treatment with lower values for all feeds. The explanation for these results lies in the fact that tannin treatment can facilitate the creation of complex compounds with low solubility in the rumen [36]. Moreover, hydrogen bond interactions between tannin and protein could be the reason for the higher effect on protein soluble fraction [37,38]. The inclusion of tannins has also resulted in a reduction in the percentage of protein degradation in the rumen over time for both cottonseed and

soybean meals, at 60 and 20 g/kg, respectively. This reduction in Kd indicates a shorter duration for the protein ruminal fermentation; this, in turn, can facilitate the enhancement of amino acid flow and absorption in the animal's intestine [23,39,40]. Thus, our results suggest that tannin treatment changed ruminal nutrient kinetics by protecting the feed from ruminal fermentation.

The use of reduced concentrations of tannins in the diet can be an alternative for optimizing protein supply to ruminants [41]. This is attributed to the capacity of tannins to bind with proteins and form tannin–protein complexes. This alteration in fermentation kinetics promotes greater ruminal escape and consequently increases RUP levels [42]. Thus, the changes in the ruminal kinetics caused by tannin inclusion were reflected in the reduction in ED and an increase in RUP levels, as expected. Regarding ED, caution must be exercised when including tannins as their usage may lead to toxic effects on ruminal microorganisms [43]. This could result in a reduction in fiber degradation and organic matter digestibility [43,44]. The utilization of condensed tannins could lead to a reduction in substrate degradation by hindering the attachment of microbes to feed particles and by forming bonds with nutrients and microbial enzymes [37]. Furthermore, there is a possibility of tannin complexation with other compounds, such as starch [45]. The interaction between tannins and starch is considered pH-independent but can be influenced by factors like tannin solubility [45]. This could explain the results observed for peanut meal (a feed with higher starch content). In this case, there was a linear decrease in digestibility and a quadratic effect on RUP. Specifically, there was a reduction in digestibility for the first two tannin levels, followed by an increase in the 60 g/kg treatment. Thus, it appears that the highest tannin level offers the most favorable conditions for improving protein–tannin complexation in both cottonseed and peanut meals.

The increase in RUP implies a decrease in the quantity of substrates accessible for microbial fermentation within the ruminal environment [46,47]. However, it is important to emphasize that this protection should occur only at the ruminal level, leaving it susceptible to digestion and absorption in the other compartments of the animal's gastrointestinal tract [48]. Thus, we hypothesized that tannin treatment could increase the RUP content of the tested feeds without impairing the intestinal digestibility of protein. Indeed, the lack of differences observed for the peanut meal treatments indicates that protein–tannin complexes were sufficiently robust to shield the protein from rumen fermentation but not strong enough to prevent their breakdown after passing through the rumen. The complexes formed for feed protection between protein and tannin should be reversible when exposed to pH values lower than the ruminal pH, such as those found in the abomasum and small intestine [21,49]. On the other hand, the cubic effect observed for the protein intestinal digestibility of cottonseed and soybean meals suggests that higher levels of tannin inclusion (60 g/kg) could also have a detrimental impact on post-rumen protein utilization.

Therefore, our results lead us to the conclusion that tannin treatment may enhance the RUP content of cottonseed and peanut meals. However, caution must be exercised regarding the levels of tannin inclusion as an excess of tannins could potentially impact the availability of amino acids for intestinal absorption, as observed with soybean meal. For this feed, the alteration in ruminal kinetics has also impacted the RUP, but the reductions in intestinal digestibility did not lead to an improvement in protein utilization. In fact, the control treatment exhibited 1.01%, 2.91%, and 3.36% more RUP being digested in the intestine compared to the increasing levels of tannin inclusion. Considering that the reactivity of tannins with proteins can be influenced by factors such as the chemical structure, molecular weight of tannins, and amino acid content of proteins [40,45,50], it appears that soybean meal was less affected by these treatments. Some studies observed that doses of up to 20 g/kg of dry matter (DM) resulted in reduced protein digestibility when Jersey steers were fed diets based on soybean meal [51]. According to these authors, this effect may be attributed to several factors, including the binding effect of tannins with proteins, the lack of dissociation of a portion of the tannin–protein complex in the abomasum, potential inactivation of intestinal enzymes, or re-binding to proteins.

In summary, the amount of RUP digested in the intestine was as follows: for cottonseed meal: 0 g/kg tannin inclusion (304 g/kg), 20 g/kg tannin inclusion (375 g/kg), 40 g/kg tannin inclusion (392 g/kg), and 60 g/kg tannin inclusion (401 g/kg); for peanut meal: 0 g/kg tannin inclusion (500 g/kg), 20 g/kg tannin inclusion (459 g/kg), 40 g/kg tannin inclusion (478 g/kg), and 60 g/kg tannin inclusion (540 g/kg). Thus, our results suggest the potential inclusion of tannins for each feed. Regarding both cottonseed and peanut meals, we highlight the inclusion of 60 g/kg of tannins, which resulted in 31.9% and 8.14% more RUP being digested in the intestine compared to the non-processed treatment, respectively. Subsequent actions will involve conducting studies to assess the rumen fermentation parameters of these ingredients.

5. Conclusions

Tannins present a promising alternative for enhancing RUP levels in diets containing cottonseed and peanut meals. However, the effectiveness of tannins is contingent upon their dosage. Therefore, the most favorable outcomes under these experimental conditions were observed with a tannin inclusion level of 60 g/kg for both feeds. This dosage indicates effective protection of protein from ruminal microbial degradation without compromising its absorption at the intestinal level. This suggests that tannins can serve as a viable alternative for processing these feedstuffs. However, no benefits were observed from treating soybean meal with tannin. Concerning the treated cottonseed and peanut meals, it is advisable to conduct additional research to assess the impact of these ingredients on ruminal fermentation parameters and animal performance.

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Institutional Review Board Statement: This study was carried out in strict accordance with the recommendations of the Animal Use Ethics Committee of the Instituto de Zootecnia. Animal care and handling protocols were approved by this committee (Protocol Number: 249-19).

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Essential Oil Blends with or without Fumaric Acid Influenced In Vitro Rumen Fermentation, Greenhouse Gas Emission, and Volatile Fatty Acids Production of a Total Mixed Ration

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Abstract: The growing interest in improving rumen fermentation and mitigating methane emissions necessitates the use of essential oil blends (EOB) and fumaric acid (FA). This study evaluated the synergistic effect of four EOB with or without FA supplementation on in vitro dry matter digestibility, greenhouse gas emission, and total volatile fatty acid production using inoculum from three rumen-cannulated Black Angus beef cows. The study was arranged in a 4 × 2 + 1 factorial design to evaluate the effects of the four EOB and two FA levels on a total mixed ration (TMR). The EOB dosage was 100 µL while FA was added at 3% of total mixed ration. The EOB × FA interaction ($p < 0.05$) influenced the dry matter, neutral detergent fiber, and hemicellulose degradabilities. All the EOB and FA (EFA) treatments decreased ($p < 0.001$) the dry matter degradability compared to the control (TMR substrate only). The EFA4 treatment reduced the neutral detergent fiber and hemicellulose degradabilities compared to the control. The ruminal pH was influenced ($p < 0.001$) by both the EOB and FA inclusion, and the EOB × FA interaction was significant. The microbial mass was higher ($p < 0.001$) in the EFA1, EFA4, and EOB4 compared to the control and the EOB3 treatments. The EFA1 and EOB1 produced less ($p < 0.001$) gas than the control by 29.1 and 32.1%, respectively. Compared with the control, the EFA1 and EOB1 treatments decreased ($p < 0.001$) methane gas by 90.8% and 86.4%, respectively, while the carbon dioxide was reduced ($p = 0.004$) by 65.7 and 57.9%, respectively. The EOB × FA interaction was significant ($p < 0.001$) for the total and individual volatile fatty acid concentrations. The inclusion of FA increased the propionate concentration by 9.5% and decreased ($p = 0.02$) the acetate concentration by 4%. In summary, the synergistic effect of the EOB and FA offers an effective way to reduce greenhouse gas emission and enhance total volatile fatty acids.

Keywords: essential oils; fumarate; ruminants; methane; batch culture

1. Introduction

Ruminants could utilize high fibrous feed resources by relying on the rumen microbiome to extract the nutrients. Through the fermentation processes, plant lignocellulosic materials are degraded by anaerobic digestion to yield volatile fatty acids (VFA) and other methanogenic products such as hydrogen, carbon dioxide (CO_2), hydrogen sulfide (trace amount), and acetic acid [1]. Thus, ruminants have been implicated to contribute 14.5% of anthropogenic greenhouse gas (GHG) emissions toward global warming and climate change [2]. Of greater interest are methane (CH_4) and CO_2, because methanogens can reduce the CO_2 generated during anerobic digestion to CH_4 through the hydrogenotrophic methanogenesis pathway. Besides, CH_4 gas accounts for up to 12% of dietary gross energy, and its global warming potential surpasses that of CO_2 by over 28 times [1,3]. In view of

this, reducing the GHG emissions from ruminant animals without compromising their production performance is a high-priority challenge that must be addressed. Nutritional interventions for reducing the enteric CH_4 and CO_2 emission include adding dietary lipids or various feed additives to the ration [4]. Essential oils (EO) are a natural source of plant secondary metabolites and bioactive compounds, with several antimicrobial properties that hold potential to modify rumen microbiome including bacteria, protozoa, and fungi [5,6]. They influence ruminal metabolic activity, reduce methane emission, and inversely increase the molar proportion of propionate [7]. Since EO vary in their chemical structure and bioactive constituents, combining two or more single EO to form a unique EO blend (EOB), or a combination of EO with other anti-methanogenic agents, is presumed to be effective in mitigating the methane emission from ruminants [8]. The combinations of various EOB were found to modify the rumen fermentation processes in vitro [9], in vivo [10], or both [5] with promising results. Blanch et al. [5] reported that 300 mg/L of essential oil blend (Next Enhance®; NE300) containing cinnamaldehyde and garlic oils, reduced the total gas, CH_4 emission, and VFA profile and increased the propionate proportion.

Fumaric acid (FA) is a key metabolic intermediate of the propionate–succinate pathway, which is recognized to enhance ruminal propionate production by scavenging the hydrogen available for methanogens, thereby offering a potent means to reduce CH_4 emissions [4]. Previous studies [3,11] have reported a reduction in CH_4 production, total VFA, and acetate-to-propionate ratio, while propionate increased with FA supplementation. Based on the prospects of EOB and FA, it was hypothesized that combinations of EO and FA would synergistically improve feed digestion and reduce GHG emission. Furthermore, there is a paucity of available literature on the synergy of EOB containing a mixture of four or more individual EO and FA on in vitro rumen fermentation. Lin et al. [7] reported that the addition of FA and a mixture of essential oils (clove, oregano, cinnamon, and lemon) or their bioactive substances, decreased ammonia nitrogen concentration, total VFA content, acetate-to-propionate ratio, increased propionate proportion, and inhibited the growth of methanogens and protozoa. In the present study, twelve EO with different bioactive substances were mixed to form four unique EOB based on a previous study from our lab, which showed that certain EO consistently reduced GHG without a significant negative effect on nutrient digestibility [9]. The study hypothesis was that the synergistic effects of EOB with or without FA supplementation would positively influence the ruminal fermentation profile and improve the nutrient degradation. Hence, the study was conducted to investigate the effects of EOB, with or without FA, on CH_4 and CO_2 production, fermentation characteristics, gas production, and VFA concentrations.

2. Materials and Methods

2.1. Study Ethical Approval

The study protocol for using essential oil blends and cannulated cows as a rumen liquid donor was approved by the Institutional Animal Care and Use Committee (LA22-0019), North Carolina A&T State University, Greensboro. The cannulated cows were maintained according to the University Farm standards.

2.2. Test Ingredients

A total of twelve individual, commercially available EO were used. Four different EOBs were formulated as follows: EOB1 [garlic, lemongrass, cumin, lavender, and nutmeg; 4:2:2:1:1]; EOB2 [anise, clove, oregano, cedarwood, and ginger; 4:2:2:1:1]; EOB3 [clove, oregano, peppermint, and eucalyptus; 3:3:2:2]; and EOB4 [clove, anise, peppermint, and oregano; 4:3:2:1]. The proportion of each essential oil used in the blends was based on a previous study from our laboratory [9]. Fumaric acid (99+%) purchased from Thermo Fisher Scientific, Branchburg, NJ, USA was used in this study. Samples of corn silage, alfalfa hay, and concentrate were obtained from the North Carolina A&T State University Farm. Samples were oven-dried (Isotemp Oven, Thermo Fisher Scientific, Allentown, PA, USA) and milled through a 1-mm screen (Cutting Mill SM100, Retsch GmbH, Haan, Germany).

A total mixed ration (TMR) formulated consisted of 60% corn silage, 20% alfalfa hay, and 20% concentrate on a dry matter (DM) basis was used as the substrate for the in vitro batch culture study. Three rumen-cannulated Black Angus beef cows (1506 ± 70 kg) were used as rumen inoculum donors. They were maintained on pasture, and fed with grass hay and a mineral mixture as supplement.

2.3. Chemical Analysis

The chemical composition of each ingredient (corn silage, alfalfa hay, and concentrate) and TMR formulated were determined using standard procedures [12]. The dry matter (DM, #930.15) was determined by oven drying at 55 °C to constant weight (Thermo Scientific Heratherm OGS100, Thermo Electron LED GmbH, Langenselbold, Germany). Nitrogen (N, #954.01) was quantified using an organic elemental analyzer (2400 CHNS, PerkinElmer, Waltham, MA, USA) and crude protein (CP) was calculated as N × 6.25. Ether extract (EE; #920.39) was determined using the ANKOM XT15 (ANKOM, Macedon, NY, USA) extractor. The ash content (CA, #942.05) was determined by combustion of samples in a muffle furnace at 550 °C (Lindberg Blue M, Thermo Fisher Scientific, Pittsburgh, PA, USA). The organic matter (OM) was determined by subtracting the weight of the ash after ignition and reported in percentage. The neutral detergent fiber (NDF) was determined [13] with modifications for using thermo-stable α-amylase and sodium sulfite; and the acid detergent fiber (ADF; #973.18) content was analyzed using automated the ANKOM fiber analyzer (F57 Fiber Filter Bags, 200 Fiber Analyzer, ANKOM Technology). To obtain ADL, the cellulose was removed from the ADF by soaking with concentrated H_2SO_4 based on the ANKOM Technologies analytical methods. The chemical composition of the ingredients and substrate are presented in Table 1 below.

Table 1. Chemical composition (% dry matter) of ingredients and total mixed ration *.

	Corn Silage	Alfalfa Hay	Concentrate	Total Mixed Ration
Dry Matter	37.0	82.8	89.6	67.8
Organic matter	96.5	91.0	83.3	93.0
Crude Protein	6.72	17.0	16.6	13.6
Crude Fat	4.63	3.26	8.62	4.89
Ash	3.52	9.03	16.7	7.03
NDF	58.9	49.7	74.4	61.9
ADF	14.4	9.35	15.2	11.7
ADL	10.4	18.2	10.4	13.7

* *n* = 10 replicates; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; ADL, Acid detergent lignin.

2.4. In Vitro Batch Culture Incubation

The in vitro batch culture assay followed the standard procedures from our lab [9]. The study was arranged as a 4 × 2 + 1 factorial design to evaluate the effects of the four EOB and two FA levels (with or without). The nine treatments were designated as follows: EOB1 with FA (EFA1); EOB2 with FA (EFA2); EOB3 with FA (EFA3); EOB4 with FA (EFA4); EOB1 without FA (EOB1); EOB2 without FA (EOB2); EOB3 without FA (EOB3); EOB4 without FA (EOB4); and control (TMR substrate only). The EOB dosage was 100 µL while the FA was added at 3% of the TMR. The ruminal contents were obtained from the various rumen regions of three ruminally cannulated Black Angus beef cows. It was strained through four layers of cheesecloth into an insulated thermos and transported immediately to the Ruminant Nutrition Laboratory. The buffer was prepared according to McDougall's recipe and maintained in a water bath at 39 °C until dispensed into 100 mL serum bottles. Samples (500 ± 50 mg) of the substrate were weighed into ANKOM filter bags (ANKOM Technology Corp., Macedon, NY, USA) and placed in the serum bottle for dry matter and fiber degradation, while some samples were weighed directly into the serum bottles for estimation of the microbial mass and the efficiency of microbial production. Six bottles with no substrate were also included as blank. The rumen fluid was mixed with the buffer

solution in a proportion of 1:3 (vol/vol) at 39 °C under continuous flushing with CO_2. Sixty milliliters of buffered medium were added anaerobically to each serum bottle. The bottles were sealed immediately with a 14 mm butyl rubber stopper plus aluminum crimp cap and incubated at 39 °C for 24 h on an orbital shaker at a speed of 125 rpm. The incubation was repeated on a different date to make two runs, with nine replicates per run.

2.5. Sampling and Analyses of Gas Production and Digestibility

After 6 and 24 h of incubation, all the culture serum bottles were measured by inserting a 22 mm gauge needle attached to a manometer (VWR International, Randor, PA, USA) to determine the gas pressure, which was later used to estimate the total gas production. The concentrations of methane, ammonia, carbon dioxide, and hydrogen sulfide gases were determined using a portable gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA) [9]. Thereafter, samples of the ruminal liquid contained in each bottle were collected to determine the pH values (Fisherbrand™ FE150 pH benchtop meter, Fisher Scientific, Waltham, MA, USA). Then, the ANKOM bags were removed from the bottles, rinsed, and dried in an oven [55 °C; 48 h] to determine the apparent DM degradability. The NDF, ADF, and ADL content of the residues in the fiber bags were also determined. The in vitro apparent degradable dry matter (IVADDM) was derived from the microbial mass data, and the in vitro true degradable dry matter (IVTDDM) was estimated after 24 h of incubation.

2.6. Estimation of Microbial Mass

The microbial mass was determined according to the protocol described [9,14]. The contents from the serum bottles (n = 3 per treatment per run) were transferred into pre-weighed centrifuge tubes (Thermo Fisher Scientific, Rochester, NY, USA) after they had been incubated for 24 h. As a correction factor, blanks (bottles without substrate during incubation) were also included in the process. The samples were centrifuged at 15,000$\times$ g for 15 min at 4 °C. Afterward, the supernatant was decanted, and the centrifuged substrate was kept in a freezer for 24 h. The frozen samples were freeze dried for 72 h using a freeze dryer (L-200, BÜCHI Lyovapor, New Castle, DE, USA). The tubes were then reweighed, and the microbial mass was calculated as: Feed (DM) incubated − [pellet (DM) − blank pellet (DM)]/Feed (DM) incubated.

2.7. Ammonia Nitrogen Determination

The ammonia nitrogen (NH_3-N) contents in the ruminal liquid were determined by the Kjeldahl method. After 24 h of incubation, 25 mL of ruminal liquid from each bottle was collected into 5 mL diluted H_2SO_4 (72%) and stored in −20 °C prior to analysis. The samples were thawed, added to 50 mL of NaOH (32%), and distilled into a 25 mL boric acid indicator using a BÜCHI Distillation Unit (K-355, BÜCHI Lyovapor, New Castle, DE, USA). The distillate was then titrated against diluted HCl (0.1N) until it changed back to its original color.

2.8. Volatile Fatty Acid Analysis

After 24 h of incubation, samples of the ruminal liquid (15 mL) were collected from six bottles per treatment, preserved with 3 mL of 25% (wt/wt) metaphosphoric solution, and immediately frozen at −20 °C for the VFA determination. The VFA profile in the ruminal liquid was quantified by using gas chromatography (Agilent 7890B GC system/5977B GC-MSD/7693 autosampler, Agilent Technologies, Santa Clara, CA, USA) with a capillary column (Zebron ZB-FFP, Phenomenex Inc., Torrance, CA, USA) as previously described [14]. A metaphosphoric acid and crotonic acid (trans-2-butenoic acid) mixture was used as the internal standard, while acetic, propionic, butyric, isobutyric, valeric, and isovaleric acid were used as quantitative external standards [13,15].

2.9. Statistical Analysis

Data generated on nutrient degradability, greenhouse gas production, fermentation parameters, and volatile fatty acids concentration were analyzed using the General Linear Model in a 4 × 2 + 1 factorial arrangement (SAS 9.4 version; SAS Institute Inc., Cary, NC, USA). The treatments were 4 EOB with or without fumaric acid. The main effects of EOB and FA treatments and their interactions were examined. Significant means were separated at $p \leq 0.05$ using the Duncan multiple range test.

Data were analyzed using the model below:

$$Yijk = \mu + EOBi + FAj + (EOB \times FA)ij + eijk$$

where Yijk is the dependent variable, μ is the overall mean, EOBi is the essential oil blend effect, FAk is the fumaric acid effect, (EOB × FA)ij is the interaction effect between the essential oil blend and fumaric acid, and eijk is the residual error.

3. Results

3.1. Dry Matter and Fiber Fractions Degradability

The interaction effects of the EOB and FA significantly ($p < 0.01$) influenced dry matter degradability (DMD), NDF degradability (NDFD), and hemicellulose degradability (HEMD; Table 2). All the EOB, either with or without FA, decreased ($p < 0.001$) DMD compared to the control. Significantly lower ($p < 0.05$) DMD, NDFD, and HEMD values were observed in the EFA4 treatment. The inclusion of FA reduced ($p = 0.028$) DMD by 6.2% while NDFD, ADFD, ADLD, HEMD, and cellulose degradability were not affected.

Table 2. Effects of essential oil blends with or without fumaric acid on dry matter and fiber fractions degradability of the total mixed ration.

Treatments	DMD (%)	NDFD (%)	ADFD (%)	ADLD (%)	HEMD (%)	CELD (%)
Control	52.99 ^{a}	70.72 ^{a}	56.03	19.78	14.70 ^{a}	36.25
EFA1	39.47 ^{bc}	67.72 ^{ab}	55.17	17.74	12.61 ^{ab}	37.37
EFA2	40.35 ^{bc}	67.37 ^{ab}	54.40	17.80	12.97 ^{ab}	36.60
EFA3	38.22 ^{bc}	68.19 ^{ab}	55.94	19.26	12.25 ^{ab}	36.68
EFA4	36.80 ^{c}	66.23 ^{b}	56.30	17.97	9.94 ^{b}	38.33
EOB1	38.89 ^{bc}	68.46 ^{ab}	56.96	19.09	11.50 ^{ab}	37.87
EOB2	42.57 ^{bc}	69.53 ^{ab}	55.71	19.29	13.82 ^{ab}	36.41
EOB3	44.03 ^{b}	70.66 ^{a}	56.06	17.46	14.60 ^{a}	38.60
EOB4	39.51 ^{bc}	66.62 ^{ab}	55.38	20.37	11.24 ^{ab}	35.01
SEM	0.769	0.357	0.203	0.380	0.352	0.401
p value						
EOB	0.1370	0.0353	0.3582	0.9066	0.0152	0.7103
FA	0.0280	0.0543	0.1845	0.3142	0.2561	0.7603
EOB × FA	<0.001	0.0085	0.1539	0.6575	0.0116	0.5303

DMD, Dry matter degradability; NDFD, Neutral detergent fiber degradability; ADFD, Acid detergent fiber degradability; ADL, Acid detergent lignin degradability; HEMD, Hemicellulose degradability; CELD, Cellulose degradability; SEM, Standard error of means; $^{a–c}$ Means with different superscripts within the same column differ, $p < 0.05$.

3.2. In Vitro Digestibility and Fermentation Parameters

The interaction of the EOB and FA influenced ($p < 0.001$) the pH, undegraded DM, IVADDM, IVTDDM, partitioning factor (PF), and microbial mass (Table 3). The pH, which ranged from 6.61 to 6.69, was influenced ($p < 0.001$) by both the EOB and FA inclusion. Undegraded DM values were significantly ($p < 0.001$) higher in the treatments tested than in the control but were similar among the EOB and EFA treatments. Treatments EFA1, EFA4, and EOB4 decreased the IVADDM ($p < 0.001$) by almost 33% compared to control. All the EOB decreased ($p < 0.001$) the IVTDDM compared to the control. The EOB1 and EOB4 treatments had higher ($p < 0.001$) PF compared with EFA4. Higher ($p < 0.001$)

microbial mass was noted for the EFA1, EFA4, and EOB4 compared to the control and EOB3 treatments. The NH_3-N concentration was significantly influenced ($p < 0.001$) by the FA addition and the EOB × FA interaction. All the EOBs and EFAs decreased ($p < 0.001$) NH_3-N compared to the control. Meanwhile, EFA2 resulted in a 26% decrease in the NH_3-N content in relation to the control. Also, FA inclusion reduced ($p < 0.001$) the ruminal NH_3-N content by nearly by 31%.

Table 3. Effects of essential oil blends with or without fumaric acid on in vitro digestibility and fermentation parameters of the total mixed ration.

Treatments	pH	Undegraded DM	IVADDM	IVTDDM	PF	Mmass (g/kg DM)	NH_3-N (mg/dL)
Control	6.64 ^{b}	0.159 ^{b}	0.592 ^{a}	0.693 ^{a}	2.57 ^{ab}	0.065 ^{cd}	16.14 ^{a}
EFA1	6.61 ^{d}	0.184 ^{a}	0.391 ^{d}	0.636 ^{b}	2.74 ^{ab}	0.124 ^{a}	12.21 ^{ef}
EFA2	6.64 ^{b}	0.182 ^{a}	0.450 ^{bcd}	0.644 ^{b}	2.62 ^{ab}	0.099 ^{abc}	11.88 ^{f}
EFA3	6.62 ^{cd}	0.189 ^{a}	0.523 ^{ab}	0.628 ^{b}	2.76 ^{ab}	0.054 ^{d}	12.61 ^{def}
EFA4	6.64 ^{bc}	0.186 ^{a}	0.393 ^{d}	0.631 ^{b}	2.29 ^{b}	0.120 ^{a}	13.73 ^{bcd}
EOB1	6.64 ^{bc}	0.181 ^{a}	0.423 ^{cd}	0.644 ^{b}	2.99 ^{a}	0.113 ^{ab}	14.01 ^{bc}
EOB2	6.69 ^{a}	0.183 ^{a}	0.494 ^{bc}	0.640 ^{b}	2.85 ^{ab}	0.074 ^{bcd}	14.06 ^{bc}
EOB3	6.62 ^{bcd}	0.178 ^{a}	0.498 ^{bc}	0.647 ^{b}	2.65 ^{ab}	0.076 ^{bcd}	14.85 ^{b}
EOB4	6.69 ^{a}	0.183 ^{a}	0.399 ^{d}	0.636 ^{b}	2.96 ^{a}	0.120 ^{a}	13.39 ^{cde}
SEM	0.004	0.0014	0.0106	0.0029	0.034	0.0045	0.217
p value							
EOB	<0.001	0.8969	<0.001	0.3819	0.3646	<0.001	0.5107
FA	0.0001	0.0901	0.4328	0.0601	0.0363	0.7162	<0.001
EOB × FA	<0.001	<0.001	<0.001	<0.001	0.0305	<0.001	<0.001

IVADDM, In vitro apparent degradable dry matter; IVTDDM, In vitro true degradable dry matter; PF, Partitioning factor; Mmass, Microbial mass; SEM, Standard error of means; $^{a–f}$ Means with different superscripts within the same column differ, $p < 0.05$.

3.3. Gas Production and GHG Emissions

The effects of the EOBs and FA on the total gas production, methane, carbon dioxide, ammonia (NH_3), and hydrogen sulfide (H_2S) are presented in Table 4. The interaction of the EOB and FA produced a significant ($p < 0.001$) effect on total gas production, CH_4, CO_2, NH_3, and H_2S concentrations. The EFA1 and EOB1 treatments produced less ($p < 0.001$) gas than the control by 29.1% and 32.1%, respectively. The EOB with or without FA significantly ($p < 0.001$) reduced the CH_4 and CO_2 gases. Compared with control, the EFA1 and EOB1 treatments decreased ($p < 0.001$) the CH_4 gas by nearly 90.8% and 86.4%, respectively, while the CO_2 was reduced ($p = 0.004$) by approximately 65.7% and 57.9%, respectively. Both the EFA4 and EOB4 treatments had lower ($p < 0.001$) NH_3 gas compared with the other treatments, whereas the EOB4 produced the least ($p < 0.001$) H_2S gas. Among the EOB group, the EOB3 had the highest ($p < 0.001$) gas volume, while the EOB1 had the least gas volume, both at the 6 and 24 h post incubations. The inclusion of the FA e increased ($p < 0.001$) the gas production only at 6 h. EOB1 significantly ($p < 0.001$) reduced thCH_4 by 79–85% and CO_2 by 36–53% compared to other blends. However, the EOB4 had lower NH_3 (60–68%) and H_2S (79–92%) concentrations when compared to the other EO blends.

3.4. Volatile Fatty Acids Production

The effects of the EOB and FA on the total and molar proportions of the volatile fatty acid production are shown in Table 5. The interactions between the EOB and FA were significant ($p < 0.001$) for the total volatile fatty acid (TVFA), acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and acetate-to-propionate ratio (APR). The EFA1 increased ($p < 0.001$) the butyrate concentration by 80.7% compared to the control. The isobutyrate concentration was reduced ($p < 0.001$) in all the EOB with or without FA when compared with the control. The EFA1 increased ($p < 0.001$) the valerate concentration compared to the rest of treatments. EFA4 increased the isovalerate concentration, whereas the EFA1 had the lowest value. The EOB2 and EOB4 increased ($p < 0.001$) the APR by 38.6 and 28.6%, respectively, compared to the control, while the EFA1 and EOB1 reduced it by

18.9 and 17.1%. Also, FA inclusion decreased (p = 0.02) acetate concentration by 4% and increased propionate concentration by 9.5%.

Table 4. Effects of essential oil blends with or without fumaric acid on total gas volume and greenhouse gases production of the total mixed ration.

Treatments	Gas Volume (mL/g DM)	Methane (mg/g DM)	Carbon Dioxide (mg/g DM)	Ammonia (mmol/g DM)	Hydrogen Sulphide (mg/g DM)
Control	181 ^{a}	7.69 ^{a}	37.3 ^{a}	61.7 ^{ab}	213 ^{bc}
EFA1	128 ^{d}	0.71 ^{d}	12.9 ^{b}	75.5 ^{a}	511 ^{a}
EFA2	158 ^{abc}	5.18 ^{ab}	27.2 ^{ab}	73.1 ^{a}	202 ^{bc}
EFA3	160 ^{ab}	5.36 ^{ab}	29.5 ^{ab}	58.3 ^{ab}	179 ^{bc}
EFA4	147 ^{bcd}	4.42 ^{abc}	24.7 ^{ab}	26.6 ^{b}	43.5 ^{bc}
EOB1	123 ^{d}	1.05 ^{cd}	15.7 ^{b}	90.1 ^{a}	505 ^{a}
EOB2	148 ^{bcd}	5.10 ^{ab}	25.3 ^{ab}	80.2 ^{a}	217 ^{b}
EOB3	158 ^{abc}	6.29 ^{ab}	31.0 ^{ab}	72.1 ^{a}	217 ^{b}
EOB4	133 ^{cd}	3.95 ^{bcd}	20.3 ^{ab}	25.7 ^{b}	38.3 ^{c}
SEM	2.3	0.379	1.62	3.86	24.85
p value					
EOB	<0.001	<0.001	0.003	<0.001	<0.001
FA	0.077	0.808	0.883	0.316	0.857
EOB × FA	<0.001	<0.001	0.004	<0.001	<0.001

SEM, Standard error of means; $^{a–d}$ Means with different superscripts within the same column differ, $p < 0.05$.

Table 5. Effects of essential oil blends with or without fumaric acid on total and molar proportion of VFA production of the total mixed ration.

Treatments	TVFA (mM)	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	APR
Control	77.90 ^{a}	0.678 ^{bc}	0.201 ^{bc}	0.104 ^{e}	0.0049 ^{a}	0.011 ^{b}	0.0021 ^{ab}	3.39 ^{d}
EFA1	59.49 ^{d}	0.579 ^{f}	0.211 ^{ab}	0.188 ^{a}	0.0031 ^{c}	0.017 ^{a}	0.0014 ^{e}	2.75 ^{e}
EFA2	62.28 ^{cd}	0.657 ^{cd}	0.188 ^{cd}	0.138 ^{c}	0.0041 ^{b}	0.011 ^{b}	0.0019 ^{bc}	3.50 ^{cd}
EFA3	72.52 ^{ab}	0.666 ^{bcd}	0.198 ^{bc}	0.120 ^{d}	0.0039 ^{b}	0.010 ^{b}	0.0018 ^{bc}	3.36 ^{d}
EFA4	59.83 ^{cd}	0.652 ^{d}	0.166 ^{ef}	0.166 ^{b}	0.0039 ^{b}	0.011 ^{b}	0.0022 ^{a}	3.94 ^{b}
EOB1	59.46 ^{d}	0.606 ^{e}	0.216 ^{a}	0.161 ^{b}	0.0037 ^{b}	0.011 ^{b}	0.0017 ^{d}	2.81 ^{e}
EOB2	69.44 ^{abc}	0.708 ^{a}	0.151 ^{f}	0.126 ^{cd}	0.0038 ^{b}	0.009 ^{b}	0.0017 ^{cd}	4.70 ^{a}
EOB3	66.94 ^{bcd}	0.683 ^{b}	0.177 ^{de}	0.123 ^{d}	0.0041 ^{b}	0.010 ^{b}	0.0019 ^{bcd}	3.86 ^{bc}
EOB4	58.75 ^{d}	0.662 ^{bcd}	0.152 ^{f}	0.169 ^{b}	0.0039 ^{b}	0.011 ^{b}	0.0020 ^{bc}	4.36 ^{a}
SEM	1.101	0.0054	0.0033	0.0038	0.00007	0.0004	0.00004	0.089
p value								
EOB	<0.001	<0.001	<0.001	<0.001	<0.001	0.0019	<0.001	<0.001
FA	0.9547	0.0210	0.0199	0.2520	0.1967	0.0580	0.7181	0.0047
EOB × FA	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

TVFA, Total volatile fatty acids; APR, Acetate Propionate Ratio; SEM, Standard error of means; $^{a–f}$ Means with different superscripts within the same column differ, $p < 0.05$.

4. Discussion

4.1. Dry Matter and Fiber Fractions Degradability

The decrease in the DMD as a result of EOB (with or without FA) inclusion would adversely affect the actual amounts of various nutrients accessible to the animals. Also, the decrease in the NDFD and HEMD would be correlated with the lower TDN and, consequently, the dietary energy content. Some authors reported that the EOs and EOBs had negative effects on DM and fiber digestibility [16,17], which has been attributed to the phenolic and non-phenolic compounds that intrude and disintegrate into the cell membrane of the rumen fibrolytic bacteria and protozoa, inhibiting their metabolic activities. Metwally et al. [10] reported significantly lower in situ rumen dry matter degradability of a TMR with the EO blend (Crina® Ruminant) but the affected individual ingredients (grass silage,

maize silage, soybean meal, rapeseed meal, and wheat grain) differently. The absence of an effect on the ADFD and ADLD with the inclusion of EOBs with or without FA in this current study agrees with a previous report by [9], who observed no effect of EOBs on the ADFD and ADLD of high-forage and high-concentrate diets.

4.2. In Vitro Digestibility and Fermentation Parameters

Ruminal fluid pH is a critical index to evaluate microbial fermentation activity, because most ruminal cellulolytic bacteria are pH sensitive [18]. In this study, the rumen pH (6.61–6.69) was influenced by both the EOB inclusion and the EOB $\times$ FA interaction. The reduced pH was linked to a decrease in the gas production, DM disappearance, and total VFA concentration [19]. Expectedly, pH was reduced by 0.03 unit with the FA inclusion without compromising the TVFA concentration. This contradicts the assumption that a reduction in the rumen pH could result in VFA accumulation, thereby limiting microbial fermentation [18]. The undegraded DM and IVTDDM values were significantly lower than the control but are similar among the EOB and EFA treatments. The chemical structures of the essential oils, the synergy of various bioactive substances in the EOB, the inclusion dosage, and the diet type are key factors responsible for the variations in in vitro apparent and true dry matter digestibility. The lack of effect on IVTDDM among the EOB groups agrees with the findings of [20] who reported that anise, clove, ginger, and oregano administered individually at varying doses had no effect on IVTDDM.

The PF points out the relationship between the feed degradability, the gas production, and the efficiency of microbial biomass synthesis [9,20]. In this study, the EOB1 and EOB4 had higher PF compared with the EFA4 treatment, despite no variation in undegraded DM and IVTDDM values among all the treated groups. This implies that lower amounts of degraded DM and OM were needed to generate 1 mL of gas, and that more volatile fatty acids and microbial biomass would be generated per unit of degraded substrate during the fermentation process. The microbial mass is a crucial outcome of ruminal fermentation and plays a vital role in the microbial protein synthesis. The higher microbial mass observed in the EFA1, though similar to the EFA4 and EOB4, could be attributed to the lower gas produced and the inhibition of methanogens [19].

4.3. Gas Production and GHG Emissions

Methane is linked to dietary energy loss, a decrease in the production efficiency of ruminant animals, and an environmental hazard. It is formed by reducing CO_2 with hydrogen, which is produced by different ruminal microbes during feed digestion [17]. The effectiveness of EOB as rumen microbiome modifier to improve feed efficiency and reduce methane emission has been reported [8,9]. In this study, the EFA1 and EOB1 reduced the total gas production gas by 29.1% and 32.1%, the CH_4 gas by approximately 90.8% and 86.4%, and the CO_2 gas by nearly 65.7% and 57.9%, respectively. This study corroborates the fact that the EOB vary in their abilities to impact ruminal fermentation and inhibit GHG emission, particularly CH_4 production [9]. Combining multiple EOs with different bioactive compounds can have synergistic effects on rumen fermentation and methane reduction. Each EO may target specific microbial populations or metabolic pathways in the rumen, contributing to a more comprehensive methane-mitigation strategy. In addition, the effectiveness of specific EO combinations may vary depending on factors such as the type and proportion of the EOs used, the diet composition, and the animal species [5,7,9]. In this study, a higher proportion of garlic (40%) in EOB1 could be responsible for the most potent effect in reducing the CH_4 and CO_2. Patra and Yu [8] noted that garlic oil has significant amounts of organosulfur compounds, including alliin and allicin, diallyl sulphide, diallyl disulphide (DADS), and allyl mercaptan, with which it exerts anti-methanogenic effect more on methanogenic archaea than on rumen bacterial population. Reduced CH_4 emissions could be attributed to a decrease in the ruminal protozoa population, thereby resulting in better nutrient utilization efficiency. A higher protozoa population increases ruminal protein degradation and reduces dietary protein and energy utilization efficiency. Molho-

Ortiz et al. [19] reported that the inclusion of garlic, cinnamon, eucalyptus, and rosemary essential oils decreased methane production compared to their aqueous extracts in a basal diet. Moreover, the addition of FA to EOB1 further enhanced the antimicrobial activity of this unique blend to inhibit methanogenesis. Also, fumaric acid is a key intermediate in the succinate–propionate pathway and propionic acid precursor, which absorbs the H_2 sink to reduce CH_4 from ruminal fermentation [7,11]. This suggests that the FA could have diverted the H_2 toward the propionate pathway. In support, Baraz et al. [21] reported that the combination of disodium fumarate and thyme essential oil decreased gas and methane production in vitro compared with their single use. Bayaru et al. [11] reported that fumaric acid supplementation reduced methane production by 23.0% in Holstein steers. The observed reduction in total gas produced during enteric fermentation and GHG emission could be attributed to the reduction in the rumen microbial population, particularly hyperammonia-producing bacteria, by the EOB and FA. In agreement, Lin et al. [7] reported a significant decrease in microbial populations of protozoa, methanogens, *Fibrobacter succinogenes*, and *Butyrivibrio fibrisolvens* in Hu sheep fed a combination of monosodium fumarate and EOB containing eugenol, carvacrol, citral and cinnamaldehyde. Furthermore, the efficacy of EOB and FA on GHG reduction is diet dependent and is more pronounced on low quality than high quality diets. Li et al. [3] reported that fumaric acid supplementation exerted a greater CH_4-decreasing effect on Xinong Saanen dairy goats fed low forage: concentrate particle size diet (31.72%) compared with high forage: the concentrate particle size diet (17.9%). Bayaru et al. [11] also noted that fumaric acid reduced carbon dioxide production by 20.5%.

Ammonia production is related to feed protein digestion and deamination of amino acids by mostly proteolytic bacteria and a group of hyper-ammonia-producing bacteria [17]. In the current study, the EFA4 and EOB4 significantly reduced NH_3 gas compared with other treatments, whereas the EOB4 produced the least H_2S gas concentrations (79–92% lower) when compared to the other EOBs. These observations confirm that different EOBs could exert varying inhibitory effects on Gram-positive rumen bacteria, including ammonia hyper-producing bacteria, and protozoa. The reduction in ammonia concentration with EOB without or with FA inclusion is consistent with some previous reports [8,9,22]. In addition, Lin et al. [23] reported that a monosodium fumarate and essential oils combination reduced ammonia nitrogen in the rumen of Hu sheep.

Gas production depicts the accessibility of degradable carbohydrates, particularly cellulose, for enteric fermentation, and is positively correlated to VFA production [19]. In the present study, EFA1 and EOB1 reduced the total gas production (29.1% and 32.1%, respectively) and the total VFA by approximately 23.7% compared to the control. Our results are consistent with the study by [9], who reported that EOB reduced total gas production when compared with the control, and that variation exists in cumulative gas production among the EOBs groups.

4.4. Volatile Fatty Acid Production

Since VFA are the primary source of metabolizable energy for ruminants, strategies to increase their production from the diet would be beneficial to the animal. Phytochemicals including natural essential oils have been reported to alter the rumen microbiota, thus changing the end-products of ruminal fermentation such as volatile fatty acids [6]. In this study, the higher propionate molar proportion obtained in the EOB1 without or with FA inclusion (EFA1) suggests that such EOB could enhance the energy availability in beef cattle. This further implies that the EOB1 modifies the rumen microbiome by promoting the relative abundance of bacteria that are positively correlated with propionate concentration. Meanwhile, the inclusion of FA further increased propionate concentration by 9.5% and decreased the acetate contents by 4%. In the rumen, fumarate works as an alternative hydrogen acceptor and a metabolic precursor of propionate and succinate through decarboxylation and reduction reactions, respectively [11,22]. Additionally, Baraz et al. [21] reported that the simultaneous use of disodium fumarate and thyme essential oil

decreased gas production and molar proportions of acetate and butyrate, while propionate was increased. Lin et al. [23] observed that 200 mg/L of essential oil active components with or without fumarate reduced the gas production by 13.60 to 17.10%. Blanch et al. [5] observed a decrease in the total VFA production when testing a commercial EO blend (Next Enhance® 300) containing cinnamaldehyde and garlic oils at 400 mg/L. In addition, it has also been reported that a blend of disodium fumarate and thyme essential oil caused a significant increase in the molar proportion of propionate, and a decrease in acetate, butyrate, and acetate-to-propionate ratio [21]. Busquet et al. [24] demonstrated that garlic oil addition at 300 mg/L increased the proportion of propionate and butyrate and reduced acetate proportion. Bayaru et al. [11] reported a higher propionic acid, a decrease in butyric acid and isovaleric acid, and no significant change in acetic acid following FA supplementation alone. The EFA1 treatment increased the butyrate concentration by 80.7% compared to untreated TMR, suggesting better gut health. Increased butyrate concentration might also indicate that the predominant butyrate-utilizing bacteria might have been inhibited by the synergy of the EOB1 and fumarate. Furthermore, the accumulation of hydrogen gas when methane gas is suppressed could inhibit the growth of butyrate-utilizing bacteria [8]. Higher propionate and butyrate concentrations could also enhance the rumen structure and functions, thus contributing to nutrient absorption, gut wellness, and better health benefits to the cattle. Remling et al. [4] noted that propionate and butyrate also stimulate the growth of rumen papillae, thereby increasing the absorption surface for ruminants. Branched-chain VFA (BCVFA), such as isobutyrate, isovalerate, valerate, and 2-methylbutyric acid, are by-products of amino acid deamination in the rumen which are utilized by ruminal microbes as a source of carbon skeleton to synthesize branched-chain amino acids [18]. Higher valerate and isovalerate concentration in EFA1 and EFA4, respectively, indicates that such treatments would enhance cellulolytic bacteria population and fiber digestibility in the rumen.

5. Conclusions

The present results show that a synergy of EOBs and FA offers an effective way to reduce methane and carbon dioxide production. Both EFA1 and EOB1 had the greatest effect in reducing methane and carbon dioxide gases. The inclusion of EFA1 and EOB1 increased propionate concentration and decreased the acetate-to-propionate ratio. The varied effects of EOB with or without FA on nutrient digestibility, fermentation characteristics, microbial mass, and total VFA production implied modification of the rumen microbiome. Therefore, future studies to improve dry matter digestibility using a unique recombination of EOBs with FA or other additives would be required. Additionally, future studies will consider the inclusion levels of the EOB and FA to reduce their effect on dry matter digestibility.

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ruminants

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Article

Characterization of an Acidogenic Bacterial Consortium as Probiotic and Its Effect on Rumen Fermentation In Vitro and In Vivo

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Abstract: Probiotics are live microorganisms that promote host health through microbiota balance and immune modulation. We assessed an acidogenic bacterial consortium (ABC) with promising probiotic properties, focusing on its resilience during transit through the digestive tract in ruminants and determining its optimal in vitro dosage. The ABC exhibited antibiotic resistance, thrived at pH levels between 5 and 7 for 24 and 48 h, and showed a 77% survival rate in artificial gastric juice. Moreover, it not only endured bile salt exposure but also multiplied. The ABC exhibited 10.74% of coaggregation capabilities against *E. coli*. Optimal dosage determination revealed that 4×10^8 was the ideal concentration, as higher doses did not yield significant differences in dry matter digestion. In the in vivo trial with Limousin Heifers, the ABC led to enhanced total volatile fatty acid (VFA) production, increased daily weight gains, and improved feed conversion rates compared to the control group. These findings underscore the potential of the ABC as a probiotic to boost animal productivity and overall health.

Keywords: acidogenic bacterial consortium; probiotic properties; rumen fermentation; productive variables

1. Introduction

Ruminant nutritionists have developed many feed supplementation methods, such as antibiotic growth promoters, to enhance production by limiting the effects of pathogenic infections on ruminant productivity [1]. Maintaining a good balance in the rumen microbiota confers the correct growth for the host animal and greater production of animal food products. The rumen is an efficient animal–microbe mutualism system that benefits both participants [2], where rumen microbiota provide the main energy source for ruminants, the end-products of fermentation, volatile fatty acids (VFAs, mainly acetate, propionate, and butyrate), with the propionate being the only gluconeogenic VFA used by the host animal.

For decades, antibiotics have played a dual role in animal production as therapeutic agents and growth-promoting additives, with the aim of increasing productivity. However, the widespread use of antibiotics has led to severe concerns over microbial resistance and the exacerbation of residual effects in food intended for human consumption [3]. Consequently, several governments, including those in the European Union, the United

States, and Mexico, have significantly reduced or even banned the use of antibiotics as growth promoters.

Antibiotic resistance is a major public health issue that must be prevented and controlled, emphasizing the importance of research-driven initiatives to develop effective alternatives for use in animal production [4,5]. The introduction of alternative additives, such as plant-based products, prebiotics, and probiotics, was necessary, which are utilized in the prophylaxis and treatment of gastrointestinal diseases.

Probiotics are microorganisms that confer health benefits on their host animal when given in adequate doses due to their nutritional, immunological, bacteriostatic, and bactericidal effects [6–8], and they serve as a viable alternative for the prevention and treatment of certain calf pathologies, as well as enhancing their productivity [9]. Several studies reported the benefits of the oral administration of probiotics to ruminants, such as regulating and balancing the gut microbiota, reducing diarrhea, protecting against infections and diseases, and promoting the development and growth of animals [10].

Probiotics or directly fed microorganisms (DFM) can be (1) monostrain, containing one strain of one species, (2) multistrain, containing more than one strain of the same species or closely related species, for example, *Lactobacillus acidophilus* and *Lactobacillus casei*, and (3) multispecies, containing strains of different species belonging to one or preferably more genera [11]. The latter are known as bacterial consortia, which are a set of various species of microorganisms that interact in synchrony for mutual benefit within a community and are characterized by communication between themselves either through substance exchange or molecular signals, division of different tasks, and performing multi-step functions [12–14].

The probiotics demonstrated the ability to improve the rumen environment by consuming oxygen in the organ, modifying the ruminal environment, benefiting the growth of strictly anaerobic microbes such as cellulolytic bacteria, enhancing fiber digestibility, modifying the pH, and competing for adhesion sites on the rumen wall and intestinal mucosa. Furthermore, they have exhibited the capacity to regulate the profiles of VFA production, particularly propionate, which is the primary gluconeogenic VFA; this capability arises from specific bacteria present in bacterial consortia that can compete with methanogenic bacteria for hydrogen. This competition leads to increased volatile fatty acids and reduced methane production by efficient cow microbiota, aligning with a greater energy efficiency [13,14], and the production of antagonistic compounds such as bacteriocins A and B that affect the development of *E. coli* and *Salmonella* spp. [15–18].

However, the beneficial response to probiotic supplementation in ruminants needs to be more consistent, which is dependent on the microbial strain selected, combination of strains, dose, time, and frequency of supplementation. Thus, it is advisable to source potential target host probiotic strains from the target host intestine to recognize the nature of the microbiota of the target host. Furthermore, these microorganisms must be well identified and evaluated for their potential probiotic activities, such as tolerance to temperature and pH, gastric digestibility conditions, and antibiotic resistance, to be considered Generally Recognized as Safe (GRAS, FDA) [19]. In our previous work [20], we evaluated the effect of oral administration of an acidogenic bacterial consortium (ABC) on changes in VFA proportions in ruminant sheep, resulting in the capacity to alter ruminal fermentation and increasing the production of short-chain fatty acids (SCFAs) [20,21].

Therefore, this study aimed to characterize an ABC as a potential probiotic, evaluating different in vitro probiotic tests (susceptibility to antibiotics, tolerance to temperature and pH, antagonist effects, and simulated gastric and intestine conditions), as well as its in vivo effects on VFA production, changes in rumen microbiota, and productivity parameters such as daily weight gain and blood glucose levels in Limousin heifers.

2. Materials and Methods

In this section, we provide an overview of the materials and methods employed in our study to assess the safety and efficacy of our new probiotic candidate. The primary objective of our research is to determine whether this probiotic meets the stringent criteria

for GRAS status, ensuring its safety for consumption. Also, we conducted in vivo trials involving Limousine heifers to assess the effects of our probiotic on productive parameters within the livestock industry, to evaluate the probiotic's influence on factors such as weight gain, feed efficiency, and ruminal microbial populations. Our methodology is designed to evaluate the ABC safety profile, probiotic properties, and potential benefits.

2.1. Broth and Agar Nutrient Medium Preparation

A highly acidogenic bacterial consortium (ABC) obtained from a brewery's waste was used [21]. It was genetically characterized, and the results are shown in Table 1. For their maintenance and preservation, the microorganisms were grown at 37 °C under anaerobic conditions in a nutrient medium (NM) containing (per liter) glucose, 2 g; yeast extract, 1 g; tryptone, 2 g; KH_2PO_4, 1 g; K_2HPO_4, 1.66 g; $(NH_4)_2SO_4$, 0.5 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $MgSO_4$, 0.1 g; $NaHCO_3$, 3.5 g; L-cysteine, 0.5 g; vitamin solution, 1.25%; and mineral solution, 1.25%. The medium was gassed with CO_2, and pH was adjusted to 6.5 with HCl, before autoclaving for 15 min at 121 °C [21].

Table 1. Microorganisms present in the acidogenic bacterial consortium (ABC).

Phylum	Family
Actinobacteria	*Propionibacteriaceae* *Nocardioidaceae*
Bacteroidetes	*Rikenellaceae* *Prophyromonadaceae*
Chloriflexi	*Anaerolinaeceae* *Caldilineacea*
Firmicutes	*Ruminococcaceae* *Clostridiaceae* *Veillonellaceae* *Peptococcaceae* *Enterococcaceae*
Nitrospira	*Nitrospiraceae*
Proteobacteria	*Enterobacteriaceae* *Syntrophobacteraceae* *Syntrophaceae* *Methyloccystaceae* *Desulfovibrionaceae* *Vibrionaceae*
Spirochaetes	*Spirochaetaceae*

The mineral solution was composed of (g/L) $FeCl_2$-$4H_2O$, 2 g; $CoCl_2$-$6H_2O$, 0.05 g; EDTA, 0.5 g; $MnCl_2$-$4H_2O$, 0.5 g; $NiCl_2$-$6H_2O$, 0. 05 g; Na_2SeO_3, 0.1 g; $AlCl_3$-$6H_2O$, 0.05 g; H_3BO_3, 0.05 g; $ZnCl_2$, 0.05 g; $(NH_4)_6Mo_7O_{24}$-$4H_2O$, 0.05 g; and $CuCl_2$-$2H_2O$, 0.05 g.

The vitamin solution was composed of (mg/L) biotin, 2; folic acid, 2; pyridoxine-HCl, 10; riboflavin, 5; thiamine, 5; nicotinic acid, 5; pantothenic acid, 5; vitamin B_{12}, 0.1; p-aminobenzoic acid, 5; and lipoic acid, 5.

Both solutions were added once the autoclaving process was completed. To prepare the inoculum, 49 mL of this NM solution was transferred to a 50 mL conical tube, subsequently, 0.5 mL of the ABC glycerol stock culture was inoculated into the tube, and the inoculated NM solution was then incubated anaerobically at 37 °C. The bacterial growth curve and generation time were measured every 2 h until an OD 600 nm of 0.5–0.6 was reached.

For the agar plates, 23 g of agar (A1296, Sigma-Aldrich, Taufkirchen, Germany) was placed in the NM and sterilized. After this, like the culture broth, the vitamin and mineral solution were added and poured into sterile Petri dishes before it solidified, ensuring an adequate amount (approximately 12 mL) to cover the entire surface of the plate.

To create and maintain anaerobic conditions in the culture media, GasPak™ EZ Anaerobe Systems (BD, Sparks, MD, USA) were utilized.

2.2. Acidogenic Bacterial Consortia Characterization and Culture Conditions

The ABC obtained from a brewery's waste was used [20]. It was genetically characterized, and the microbiota compositions are shown in Table 1. For their maintenance and preservation, the microorganisms were grown at 37 °C under anaerobic conditions in a nutrient medium (NM) broth.

The bacterial growth curve and generation time were measured every 2 h until an OD 600 nm of 0.5–0.6 was reached.

For the agar plates, 23 g of agar (A1296, Sigma-Aldrich, Taufkirchen, Germany) was placed in the NM and sterilized. After this, like the culture broth, the vitamin and mineral solutions were added. Then, the growing ABC was added and poured into sterile agar Petri dishes before it solidified, ensuring an adequate amount (approximately 12 mL) to cover the entire surface of the plate.

To prepare agar NM and maintain incubation in anaerobic conditions in the culture media, GasPak™ EZ Anaerobe Systems (BD, Sparks, MD, USA) were utilized at 37 °C for 24 h.

Generation Time of the Bacterial Consortium

Generation time refers to the time required for a cell to divide or a population to double. To determine the generation time of the consortium, the Ratkowsky equation [22] was used:

$$TG = \ln 2 / \mu$$

where TG = generation time and μ = the growth rate value, which is determined by ln of T/t, where T = final biomass time and t = initial biomass time. The generation time of ABC was determined based on bacterial growth from a culture with a 1:10 ratio over 12 h in MN at 37 °C. From this culture, the required amount was taken to inoculate a new culture to achieve an OD 600 nm of 0.1. Measurements were taken every hour using the UV-Vis spectrophotometer HP/Agilent 8453 (SpectraLab Scientific Inc., Markham, ON, Canada) until reaching the maximum logarithmic phase.

2.3. Probiotic Tests

2.3.1. Susceptibility to Antibiotics

To assess antibiotic susceptibility, the ABC was subjected to an antibiogram test which comprises a profile or pattern of results from antibiotic susceptibility testing specific to a bacterial strain or species, offering insights into the susceptibility or resistance of bacteria to various antibiotics. It is desirable for a probiotic not to present antibiotic resistance.

One hundred microliters of ABC culture (OD 600 nm = 0.5–0.6) was seeded on NM agar plates. Once the ABC was seeded, it was left to dry for 3–5 min, and then, the following multidiscs with antibiotics were added and incubated anaerobically at 37 °C for 24 h: Gram (−): AK Amikacin (30 μg), AM Ampicillin (10 μg), CB Carbenicillin (100 μg), CL Chloramphenicol (30 μg), NET Netilmicin (30 μg), NF Nitrofurantoin (300 μg), and NOF Norfloxacin (10 μg). Gram (+): DC Dicloxacillin (1 μg), CLM Clindamicin (30 μg), E Erythromycin (15 μg), PE Penicillin (10 U), VA Vancomycin (30 μg), and TE Tetracycline (30 μg). Gram (−) and (+): AM Ampicillin (30 and 10 μg, respectively), CF Cephalothin (30 μg), CFX Cefotaxime (30 μg), CPF Ciprofloxacin (5 μg), GE Gentamicin (10 μg), and SXT Sulfamethoxazole/Trimethoprim (25 μg) (MULTIBAC-ID, Investigación Diagnostica, Iztapala, CDMX, MEX). The diameter of the inhibition zone (mm) was measured, and the results were expressed as resistant (R), intermediate (I), and susceptible (S) according to the manufacturer's instructions.

2.3.2. Determination of Tetracycline Resistance Genes Tet M, Tet K, and Tet W

To verify antimicrobial safety and determine whether tetracycline resistance was found in genomic or plasmid DNA, PCR tests for the resistance genes Tet M (F: GTGGACAAAG-GTACAACGAG; R: CGGTAAAGTTCGTCACACAC), Tet K (F: TTATGGTGGTTGTAGC-TAGAAA; R: AAAGGGTTAGAAACTCTTGAAA), and Tet W (F: GAGAGCCTGCTATAT-GCCAGC; R: GGGCGTATCCACAATGTTAAC) were performed. PCR amplification for the Tet M, Tet K, and Tet W genes was carried out from genomic and plasmid DNA previously extracted from the ABC. The PCR conditions were 95 °C—5 min; 95 °C—30 s, 64 °C—30 s, 72 °C—30 s; 30 cycles and a final alignment of 72 °C—7 min.

2.3.3. Resistance to pH

One hundred microliters of ABC culture (OD 600 nm = 0.5–0.6) was inoculated in NM broth at different pH values of 2, 3, 4, 5, 5.5, 6, 7, and a control at pH = 6.5 was used. Then, it was incubated anaerobically at 37 °C for 24 and 48 h. After incubation, six 10-fold serial dilutions (v/v, ratio) were made starting at 1:1 $\times$ 10^1 (v/v, ratio). One hundred microliters of the dilution was taken and plated on NM agar and grown anaerobically at 37 °C for 24 h. Results were expressed as survival in percentage of CFU/mL.

2.3.4. Gastric Juice Resistance Test

Five 15 mL tubes were inoculated with 1 mL of ABC in 10 mL of artificial gastric juice (2 g NaCl and 3.2 g pepsin/L at pH 2.5); they were incubated at 37 °C for 30, 60, 90, and 120 min, extracting one tube at each time. From each tube extracted, 100 µL of the sample was taken, seeded in plates with NM agar, and incubated at 37 °C for 24 h; then, colonies were counted. Resistance was estimated by comparing the viable cell counts with and without artificial gastric juice, using the formula

$$R = (CFC / gj) / (CFU / wogj) \times 100$$

where R is the resistance to gastric juice, CFU is the number of colony forming units, gj is milliliters of medium with gastric juice, and wogj is the milliliters of medium without gastric juice.

2.3.5. Resistance to Bile Salts

One hundred microliters of ABC culture (OD 600 nm = 0.5–0.6) were inoculated in NM broth at pH 6.5 with or without 0.3% ox-bile salt and grown anaerobically at 37 °C. The growth curve of ABC culture was monitored by measuring OD 600 nm every 2 h up to 6 h. The culture was then diluted by 10-fold serial dilutions to 1:1 $\times$ 10^7 (v/v, ratio). One hundred microliters of the dilution was taken and plated on NM agar and incubated anaerobically at 37 °C for 24 h. The survival rate was expressed as the percentage of CFU/mL.

2.3.6. Coaggregation Capacity

Coaggregation capacity plays a crucial role in elucidating how pathogens adhere to host tissues, serving as a pivotal factor in the initiation of infections. In the context of probiotic tests, it also provides valuable insights into guiding antimicrobial strategies. This knowledge offers potential avenues to disrupt detrimental bacterial interactions, ultimately contributing to the promotion of health and well-being.

For the evaluation of coaggregation capacity, ABC was grown in NM until a value of 0.5 at OD 600 nm was achieved. An aliquot of 750 µL of the ABC was mixed with the same volume of a suspension of the coaggregation partner *E. coli* (INCQS 00219, origin ATCC 8739) and then vortexed for 30 s. The OD value of 600 nm was determined at time 0 (baseline, just after mixing the suspensions) and after 60 min of incubation at 37 °C. Coaggregation was measured using the equation

$$\% \text{ coaggregation} = [(OD0 - OD60) / OD0] \times 100,$$

where

OD0 refers to the initial OD value determined at time 0 and OD60 refers to the OD value of the supernatant after incubation for 60 min.

2.3.7. Tolerance to Temperature

One hundred microliters of ABC culture (OD 600 nm = 0.5–0.6) was inoculated in NM broth and incubated anaerobically at 30, 37, and 45 °C, with OD 600 nm measured at 24 and 48 h. Survival results were expressed as the percentage of CFU/mL.

2.3.8. Antagonistic Activity

Bacterial antagonism was evaluated using the disc diffusion method [23] with modifications, employing two pathogenic strains (*S. typhimurium* ATCC 19028 and *E. coli* ATCC 11229, donated by the Facultad de Medicina, Universidad Autónoma de Quéretaro). The ABC cultures were incubated anaerobically in NM broth and the pathogenic strains in LB broth at 37 °C overnight. One hundred microliters of pathogens were spread on Mueller–Hinton agar plates. ABC cultures were centrifuged at 3000 rpm for 5 min, and after that, ABC cultures supernatants were added to sterile paper discs and placed on the Mueller–Hinton agar plates. After incubation at 37 °C for 24 h, the growth inhibition zone (mm) was measured. The antagonistic activity was considered positive when the average growth inhibition zone diameter was $\geq$10 mm.

2.3.9. Adhesion to Mucus

Sheep small intestinal mucin was prepared according to the methods described by Vigil et al. [24] and used to assess the adhesion of ABC. Briefly, ABC was labeled by incubation with 250 µL of 4,6-dichlorotriazinyl aminofluorescein (DTAF) in 500 µL of PBS at 60 °C for 2 h and washed three times by resuspension in PBS and collected by centrifugation at 10,000× *g* 10 min at 4 °C. Ten µL of labeled ABC was mixed with 200 µL of ovine mucin and incubated at 37 °C for 2 h, washed three times with PBS and diluted 1:1000 and 1:100,000, and fixed with paraformaldehyde (PFA 4%) on microscope slides. Adherence was expressed as CFU/mL. Viable bacteria adhered to the mucus were observed under a microscope. ImageJ was used to quantitate viable bacteria. For microscopic analysis, the MosaiX module for the APOTOME system with the 40×/1.30 DIC (UV) VIS-IR M27 Plan-Apochromat oil immersion objective was used to obtain a full mosaic image (1 mm^2). Three individual image stacks were collected and assembled using the MosaiX system (Carl Zeiss, Jena, Germany) for each histological slide. ImageJ64 and FIJI were used for the analysis of the 2D images. Quantitation of particles was performed using Yen's image thresholding method [25].

2.4. In Vitro Disappearance of Dry Matter and VFA Production

To assess the ABC's capacity to modify volatile fatty acid profiles in vitro and to evaluate its potential as a fiber digestibility enhancer, we conducted in vitro experiments to measure the dry matter disappearance and VFA production. These analysis results provide information about the possible effects of the ABC administration in vivo.

To measure the volatile fatty acid (VFA) production and the in vitro dry matter disappearance (IVDMD) by the ABC in rumen liquid, a pre-inoculum was prepared with the ABC in NM broth and allowed to grow for 8 h under anaerobic conditions at 37 °C. Subsequently, an inoculum was prepared by transferring a volume of 1 mL of the pre-inoculum to a 50 mL tube with 49 mL of NM broth. The inoculum was incubated at the same temperature and anaerobic conditions as the pre-inoculum until an OD 600 nm of 0.5, corresponding to the exponential growth phase of the ABC, was reached. Once the desired OD was reached, the inoculum was centrifuged at 5000 rpm for 15 min to separate a cell pellet from the liquid culture medium. The cell pellet was later added to the Daisy jars, in different doses (4×10^8; 4×10^{10}; 4×10^{12}) to find the optimal dose; this optimal dose

shows the minimum amount of CFU/mL in which ABC improves fiber digestibility at the rumen level. The results of this test were used in the in vivo test.

The rumen fluid was obtained from Brown Swiss bulls (using the same bull for each sampling to minimize individual variation). the extraction procedure consisted of introducing a probe into the rumen through the cannula, filtered with clean gauze and collected in a clean container with an airtight lid, and gassed with CO_2. It was kept at 39 °C during transfer to the laboratory, where upon arrival, it was mixed with artificial saliva buffer solution [21].

The IVDMD technique at 24 and 48 h was performed following the protocol recommended by the manufacturer of the Daisy II incubator (ANKOM Technology, Fairport, NY, USA) using FN-57 bags with a pore size of 25 μm.

The substrate for the ruminal microorganisms was the same diet consumed by the Brown Swiss bulls from which ruminal liquid was obtained, and this was previously ground to 2 mm before being added to the bags.

The bags were previously washed with acetone for five min and completely air-dried, then weighed individually, and 0.5 g of ground feed was placed inside each bag. They were heat-sealed and stored at room temperature. In each of the 4 jars in the Daisy II incubator, we placed 10 FN-57 bags. Subsequently, we added artificial saliva buffer to the jars, which was prepared by combining buffer A and buffer B in a 5:1 ratio. Buffer solution A comprised the following per liter: K_2HPO_4, 10 g; $MgSO_4$-$7H_2O$, 0.5 g; NaCl, 0.5 g; $CaCl_2.2H_2O$, 0.1 g; urea, 0.5 g. Buffer solution B contained the following per liter: Na_2CO_3, 15 g; Na_2S-$9H_2O$, 1 g. The resulting mixture was adjusted to achieve a final pH of 6.8 and maintained at a temperature of 39 °C until it was incorporated into 400 mL of rumen fluid [21]. Additionally, 5 mL of the ABC culture (OD 600 nm = 0.5–0.6) was introduced into the mixture.

The nylon bags with the substrate (diet) were recovered. They were washed with running water. After washing, the bags were dried in an oven at 60 °C for 12 h and were weighed. The following formula was used to calculate the percentage of IVDMD:

$$\% \text{ Disappearance} = 100 - (P3 - (P1 \times C)) \times 100 \text{ / } P2$$

where

P1 = Original weight of the bag;

P2 = Weight of the sample;

P3 = Final weight of the bag (after in vitro fermentations);

C = Correction factor (final weight of the blank bag/original weight of the blank bag).

For the quantification of volatile fatty acids (VFAs; acetate, propionate, and butyrate) at three distinct time points (0, 24, and 48 h), two samples from each jar were taken and placed in separate 15 mL conical tubes, with approximately 10 mL of ruminal fluid per tube for VFA extraction, and each collected sample was read in duplicate in the gas chromatograph.

The sample preparation for gas chromatograph was as follows: 1.5 mL of the ruminal fluid collected was centrifuged at 3500× *g* for 10 min at 4 °C to sediment the cell pellet, then 1200 μL of supernatant was recovered in a clean Eppendorf tube, and 240 μL of 25% metaphosphoric acid was added to obtain a 5:1 ratio. The tubes were incubated on ice for 30 min to sediment the proteins and then centrifuged at 12,000× *g* for 15 min at 4 °C. The supernatants were finally filtered with glass fiber membranes and stored in 1:5 dilution at −20 °C until analysis.

An Agilent 7890B gas chromatograph was used. The chromatography conditions were as follows: 170 °C oven, 190 °C injector, 210 °C detector, a column with a flow rate of 2.5 mL/min, and an FID (Flame Ionization Detector).

2.5. Effect of the Addition of Fresh ABC to Limousin Heifers

The in vivo experiment was carried out at the Centro de Enseñanza, Investigación y Extensión en Producción Animal en el Altiplano (CEIEPAA-UNAM), located in Tequisquiapan, Querétaro.

The protocol was approved by CICUAE-UNAM (CICUAE.DC-2019/4.2, UNAM). Fourteen Limousin heifers (n = 7), weighing approximately 200 kg at the beginning of the experiment and with an age of 9 months, were used. These heifers had a BCS of 3–3.5. The animals were housed in individual pens and fed a diet based on forage (alfalfa dry) and concentrate Nucalf 2, NUTEC® (1.5 kg/animal/day).

Two treatments were tested: the ABC (4×10^8 CFU) treatment, which was offered daily to the animals mixed with 50 g of ground corn, and the control treatment with 50 g of ground corn without probiotics. The animals were assigned in pairs to either treatment using a pair-feeding statistical design. Every 28 days (on 2 consecutive days), the animals were weighed, and blood samples were collected to measure blood glucose levels, while ruminal fluid samples were obtained to assess whether ABC alters the microbiota composition and VFA quantification.

For the growth of the ABC, the reagents described in the in vitro experiment were used in the process. Subsequently, an inoculum was prepared by transferring a volume of 1 mL of the pre-inoculum to a 50 mL Erlenmeyer flask with 49 mL of NM broth. The inoculum was incubated at the same temperature and anaerobic conditions as the pre-inoculum until an OD 600 nm = 0.5, corresponding to the exponential growth phase of the microbial consortium, was reached. Once the desired OD was reached, the inoculum was centrifuged at 5000 rpm for 20 min to separate the cell pellet from the liquid culture medium.

Blood was taken from the jugular vein. Following the aseptic procedures for obtaining the sample, it was collected in vacutainer tubes without anticoagulant; the blood sample was centrifuged to obtain the serum and from this, the concentration of glucose per animal was determined [26], measuring the absorbance in a spectrophotometer at 540 nm.

Rumen fluid was obtained using the oral probing technique [27]. Two samples were collected per animal, these were placed in 15 mL conical tubes, in an approximate amount of 10 mL of ruminal fluid per tube. One of the samples was used for DNA extraction and subsequent analysis of the microbial species present in the rumen, and the other for VFA quantification using the same procedure described in Section 2.4.

2.5.1. DNA Extraction and Microbiota rRNA 16s Sequencing

To verify if the ABC modified the microbial population in the rumen samples present in our study, genomic DNA was used to determine the bacterial diversity. DNA extraction was performed according to the RBB+C method [28]. DNA concentration was measured with a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA), and DNA integrity was verified by agarose gel electrophoresis (1%). The purified DNA was subject to partial 16S DNA gene sequencing to confirm bacteria identity. Sequencing of the rumen microbiome was performed by Research and Testing Laboratory (RTL) Genomics (Lubbock, TX, USA) with Illumina Myseq sequencer. Illumina produces FASTQ files with a phred offset of +33. FASTQ files contain all the raw sequence data generated by the sequencer; they may contain information regarding the primer for amplicon sequencing. To allow for more run flexibility, RTL genomics used a 2-step PCR process for Illumina sequencing that uses universal adapters and sequences the forward and reverse primers. FASTQ files generated by Illumina sequencer come in 2 forms depending on the method used: paired or single end. In this case, we used pair-end sequence.

Results were expressed as relative abundance (%). Data quality control and analyses were mostly performed using the USEARCH V.11 pipeline. The FASTQ forward and reverse files were merged into a single FASTQ file per sample, quality control and processing included removing adapters and cutting the sequences to length-based filtering of 400 bp (reads smaller than 200 bp were excluded from the analysis). The resulting read files were then aligned to RDP V.16 to define operational taxonomic units (OTUs) for taxonomic assignment; the OTUs table was generated at 97% identity. The Uclust method was used to cluster the reads into OTUs [29].

2.5.2. Relative Quantification of *Lactobacillus* spp. and *Propionibacterium* spp.

In order to verify the microbial population in ABC obtained from 16S sequencing, qPCR was performed. DNA extraction was performed according to the RBB+C method [22]. A total of 50 ng of DNA measured in a Nanodrop 1000 spectrophotometer were analyzed in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used were universal primers: BACT1369 F: CGGTGAATACGTTCYCGG; PROK1492 R: GGWTACCTTGTTACGACTT [23]; Lactobacillus F: AGCAGTAGGGAATCTTCCA; Lactobacillus R: CACCGCTACACATGGAG [24]; Propionibacterium F: ACGGGAGGCAGAGTGGG; and Propionibacterium R: TCTCCTACDMKCYCTTTAC. Conditions of PCR: 40 cycles; 95 °C 10 min, 95 °C 10 s, 56 °C 15 s, 72 °C 15 s. Melting curve: 56 °C 1 min, increasing temperature up to 95 °C. Samples were analyzed in duplicate. Relative abundance of the DNA target was calculated using the Bacchetti de Gregoris et al. [25] method.

2.6. Statistical Analysis

Data were obtained in triplicate (antibiotic susceptibility, bile salts resistance, resistance to pH, temperature tolerance, antagonistic activity, and relative quantification of *Lactobacillus* spp. and *Propionibacterium* spp.) or duplicate (in vitro adhesion assay, disappearance of dry matter, and VFA production in vitro) and expressed as standard error of the mean (SEM); differences between samples were analyzed by one-way analysis of variance (ANOVA) and Tukey's Kramer tests, and the differences with $p < 0.05$ were considered significant. Data were analyzed using the SAS version 9.3 software (SAS Institute, Cary, NC, USA).

For the evaluation of the effect of the probiotic on the productive variables, as well as the quantification of blood glucose and VFA production, a pair feeding analysis was performed thus resulting in 7 pairs, and subsequently, a Student's *t*-test for paired samples was used for data analysis.

$$t = \frac{\mathrm{XD} - \mu 0}{\mathrm{SD} / \sqrt{\mathrm{n}}}$$

For this equation, the difference D between all the pairs has to be calculated:
XD is the mean of the differences;
SD is the standard deviation of the differences;
$\mu 0$ is the constant different from 0;
n is the sample size.

Taxonomic assignment was performed using the RDP V.16 reference database with 97% identity. Data were analyzed in the R platform (3.6) with the Phyloseq library.

3. Results

3.1. Growth Rate

According to the ABC growth curve and the analysis of the equation according to [30], the ABC generation time (GT) was determined to be 8.8 h, calculated based on the equation $GT = \ln 2/\mu$ [22]. The growth curve is shown in Figure 1.

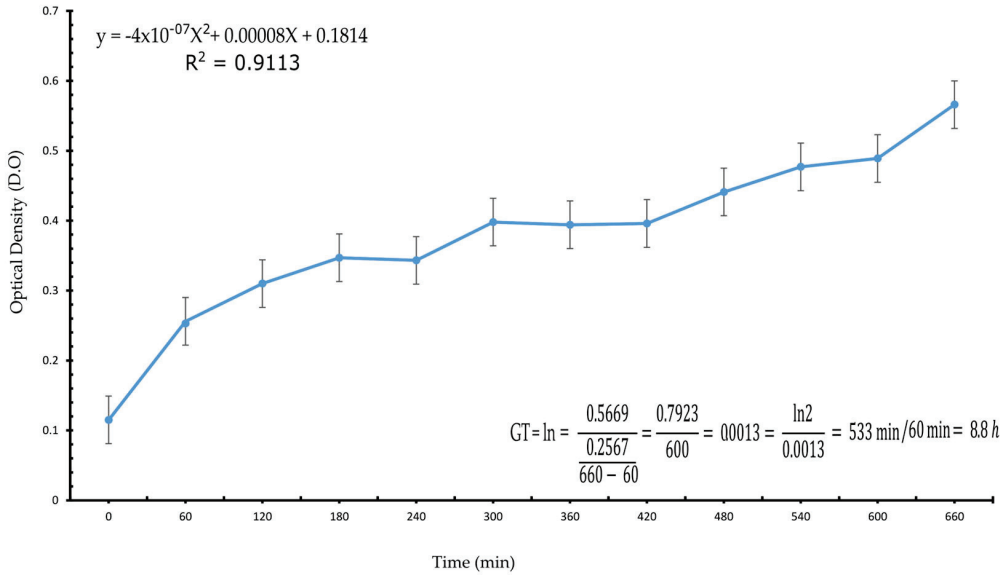

Figure 1. Growth curve of ABC in NM broth. The bars represent the SEM. GT = generation time.

3.2. Probiotic Tests

3.2.1. Susceptibility to Antibiotics

The results of the antibiograms are presented in Table 2. The ABC recorded susceptibility to the antibiotic's sulfamethoxazole + trimethoprim, vancomycin, ampicillin (commonly used for digestive disorders), and cephalothin; however, the ABC recorded resistance against gentamicin and tetracycline.

Table 2. Antibiograms for Gram-positive and Gram-negative bacteria tested in the ABC.

Gram-Positive	ABC	Gram-Negative	ABC
SXT (25 µg)	Susceptible	AM (10 µg)	Susceptible
PE (10 u)	Resistant	AK (30 µg)	Susceptible
VA (30 µg)	Susceptible	GE (10 µg)	Susceptible
TE (30 µg)	Resistant	CF (30 µg)	Susceptible
AM (10 µg)	Susceptible	NET (30 µg)	Susceptible
CPF (5 µg)	Intermediate	CL (30 µg)	Intermediate
GEN (10 µg)	Resistant	SXT (25 µg)	Intermediate
		NF (300 µg)	Susceptible

SXT = sulfamethoxazole + trimethroprim, PE = penicillin, VA = vancomycin, TE = Tetracycline, AM = ampicillin, CPF = ciprofloxacin, GEN = gentamicin, AK = amikacin, GE = gentamicin, CF = cephalotin, NET = netilmicin, CL = chloranmphenicol, NF = nitrofurantoin, ABC = acidogenic bacterial consortium.

3.2.2. Tetracycline Resistance Genes Tet R Tet M and Tet W

Genomic and plasmid DNA extraction of ABC was performed for PCR and determined where tetracycline resistance was present.

Tet R and Tet W genes were not found in ABC, and in the case of the Tet M gene, it was found in the genomic DNA.

3.2.3. Resistance to pH

Table 3 shows the number of CFUs from the resistance test of the ABC at different pH for 24 and 48 h. For 24 and 48 h at pH 6.5 and 7, there is a higher CFU growth, in contrast to acidic pH 2 and 3, which did not record growth at 48 h. No differences were found in the number of CFUs at pH 5.5 at 48 h and pH 6 at 24 h (2.6×10^{10} and 1.9×10^{10}, respectively), and similarly, there was no difference in CFUs at pH 5.5 and 6 incubated for 48 h compared to CFUs at pH 7 incubated for 24 h (2.6×10^{10}, 3.0×10^{10} and 3.5×10^{10}, respectively). The highest number of CFUs is observed when the ABC is incubated at pH 6.5 and 7 for 24 and 48 h (3.7×10^{10}, 4.1×10^{10}, 3.5×10^{10}, and 4.0×10^{10}, respectively).

Table 3. pH resistance of ABC at 24 and 48 h.

pH	2.0	3.0	4.0	5.0	5.5	6.0	6.5	7	SEM
24 h	6.6×10^{7} a,+	3.3×10^{8} a,+	5.0×10^{9} a,b,+	6.0×10^{9} a,b,+	8.7×10^{9} b,+	1.9×10^{10} c,+	3.7×10^{10} e,+	3.5×10^{10} d,e,+	2.7×10^{8}
48 h	0 a,+	0 a,+	3.8×10^{9} a,b,+	9.8×10^{9} b,+	2.6×10^{10} c,d,*	3.0×10^{10} d,*	4.1×10^{10} e,+	4.0×10^{10} e,+	2.7×10^{8}

[a–e] Different superscripts in the same row indicate significant differences ($p < 0.0001$, $n = 3$). +,* Different superscripts in the same column indicate significant differences between time points ($p < 0.0001$, $n = 3$). ABC = acidogenic bacterial consortium, SEM = standard error of the mean.

3.2.4. Gastric Juice Resistance Test

The pH value in the abomasum varies from 2 to 4 depending on the diet. Therefore, ABC was challenged at pH 2, 3, and 4 for 2 h which is the average time the digesta remains in the abomasum. Table 4 shows the results of the CFU after the ABC was incubated at different pH. At pH 4, the highest number of CFUs was observed (5.0×10^{5}) compared to pH 2 and 3 (3.6×10^{4} and 9.0×10^{4}, respectively). Table 5 shows the survival results of the ABC. The ABC has a survival of 100% at 60 min of incubation and 76.7% at 150 min of incubation, meaning that ABC is able to withstand the acidity and the presence of pepsin.

Table 4. CFUs of ABC at 2 h incubated in NM at different pH.

	2.0	3.0	4.0
CFUs	3.0×10^{7} a	9.0×10^{7} a	5.0×10^{8} b
SEM	782,209.60	782,209.60	782,209.60

[a,b] Different superscripts in the same row indicate significant differences ($p < 0.01$, $n = 3$). CFUs = colony forming units, ABC = acidogenic bacterial consortium, SEM = standard error of the mean.

Table 5. Survival rate (%) of ABC on artificial gastric juice.

Bacteria	30 min	60 min	90 min	120 min	150 min
ABC	25.0	100.0	83.3	65.0	76.7

ABC = acidogenic bacterial consortium.

3.2.5. Resistance to Bile Salts

Table 6 shows the results of the challenge to bile salts. In the ABC, no differences were found between the control bacteria (grown in NM) and the treatment (NM enriched with bile salts). The ABC was found to be resistant to bile salts.

Table 6. CFUs of ABC incubated for 6 h in NM and bile salts.

Bacteria	Incubation Broth	CFUs	SEM
ABC	NM	1.6×10^{6}	842,120.3
ABC	NM + Bile salts	1.4×10^{6}	842,120.3

($p > 0.05$, $n = 3$) CFUs = colony forming units, ABC = acidogenic bacterial consortium, SEM = standard error of the mean, NM = nutrient medium.

3.2.6. Tolerance to Temperature

Figure 2 shows the results of tolerance to the different temperatures at 24 and 28 h. At 24 h, a higher DO was observed compared to at 48 h. Growth was better at 30 °C, then 37 °C, and finally, at 45 °C ($p < 0.05$).

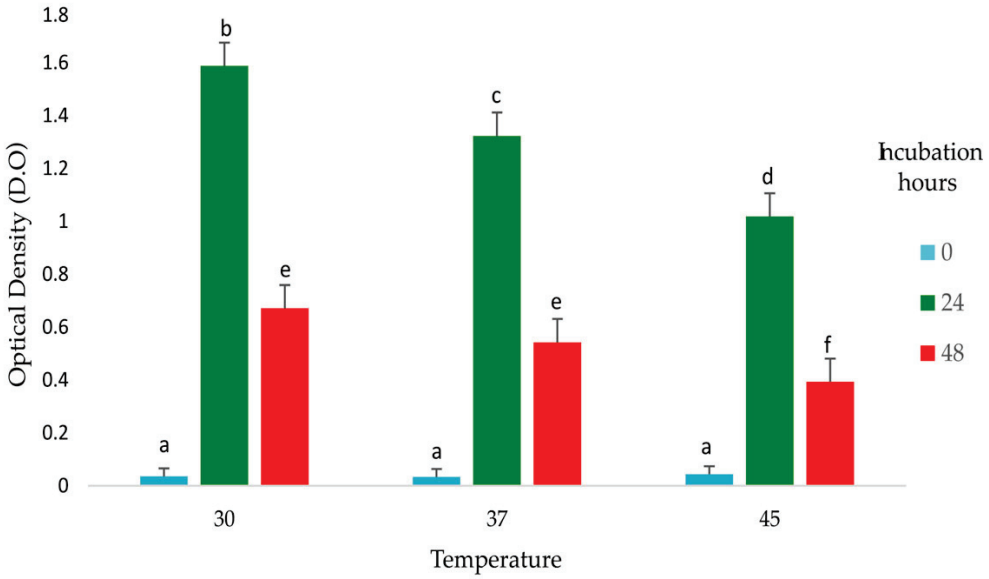

Figure 2. Temperature (°C) resistance of ABC at different hours of incubation. Different letters indicate significant differences. ($p < 0.05$, $n = 3$).

3.2.7. Coaggregation Capacity

The ABC demonstrated a coaggregation capacity of 10.74% against *E. coli* during the 60 min test.

3.2.8. Antagonistic Activity

ABC showed positive inhibition against *E. coli* because they had a zone of inhibition of 10.5 ± 0.5 mm. No inhibition was observed for *S. typhimurium* (8 ± 1.5 mm).

3.2.9. Adhesion to Mucus

Figure 3 shows the results of the adhesion to mucins, was much higher for the ABC compared to *P. acidipropionici* counts. In contrast, no viable bacteria were found in the negative control. These results suggest the potential for PCB adherence and colonization of the intestinal tract.

3.3. Disappearance of Dry Matter and VFA Production In Vitro

Table 7 shows the results of IVDMD. As can be seen at 24 h, there was no difference between treatments, but at 48 h, there was a better DMD in the treatments in which the ABC was added (51.73%, 51.43%, and 51.97% for treatments 4×10^8, 4×10^{10} and 4×10^{12}, respectively); however, no difference was found between them.

Table 7. Dry matter disappearance (%) at 24 and 48 h.

Time (h)	Control	ABC 4×10^8	ABC 4×10^{10}	ABC 4×10^{12}	SEM
24	45.43 $^{a,+}$	44.32 $^{a,+}$	43.38 $^{a,+}$	45.17 $^{a,+}$	0.91
48	48.86 ^{b,*}	51.73 ^{c,*}	51.43 ^{c,*}	51.97 ^{c,*}	0.91

^{a,b,c} Different superscripts in the same row indicate significant differences ($p < 0.01$, $n = 5$). +,* Different superscripts in the same column indicate significant differences between time points ($p < 0.0001$, $n = 3$). ABC = acidogenic bacterial consortium; SEM = standard error of the mean.

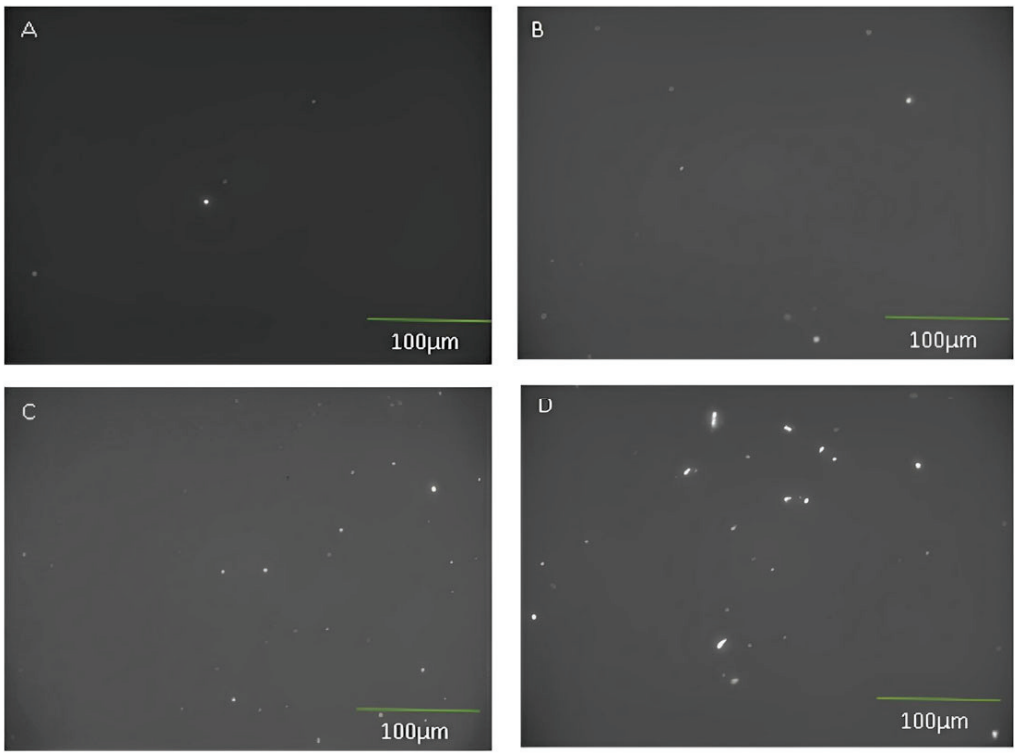

Figure 3. Adherence to mucins of ovine gut. (**A**) Negative control, (**B**) positive control *P. acidipropionici* + mucins, (**C**) bacterial consortium without mucins, (**D**) bacterial consortium + mucins.

The dose of 4×10^8 was selected for further experiments.

Table 8 shows the effect of ABC addition on VFA production in vitro. Increases of 72.7, 74.8, and 48.3% were observed with the addition of the ABC for acetate, propionate, and butyrate, respectively. For total VFAs, the increase was 72.7% ($p < 0.05$).

Table 8. Effect of the addition of the ABC to the rumen fluid on the VFA production in 24 h of in vitro fermentation.

	Control		ABC	
mMol/L	**Mean**	**SEM**	**Mean**	**SEM**
Acetate	23.8 [a]	5.7	41.6 [b]	4.7
Propionate	8.9 [a]	1.3	13.2 [b]	1.09
Butirate	2.8 [a]	1.0	6.5 [b]	0.86
Total VFAs	35.5	-	61.3	-
Acetate: Propionate ratio	2.7:1 [a]	0.11	3.2:1 [b]	0.09

[a,b] Different superscripts between rows indicate statistical differences ($p < 0.05$, $n = 3$). ABC = acidogenic bacterial consortium; SEM = standard error of the mean; VFAs = volatile fatty acids.

3.4. Effect of the Addition of Fresh ABC to Limousin Heifers

The animals were weighed on day 1 and then every 28 days for two consecutive days, to record their growth and development. When analyzing the data, a difference ($p \leq 0.001$) between treatments can be observed (Table 9), showing an improvement in the weight gain of up to 10 kg more in the animals that were supplemented with the ABC.

Table 9. Effect of ABC on body weight (IW, FW), dry matter intake (DMI), daily weight gain (DWG), and feed conversion (FC) of Limousin heifers.

Productive Parameters (kg)	Control	SEM	ABC	SEM
IW	274.9 [a]	11.6	283.0 [b]	14.3
FW	348.5 [a]	7.1	371.8 [b]	5.5
DWG	0.87 [a]	0.04	1.06 [b]	0.03
DMI	11.9 [a]	1.7	11.1 [b]	0.97
FC	11.4 [a]	2.18	9.22 [b]	1.80

[a,b] Different superscripts between columns indicate statistical differences ($p \leq 0.001$, $n = 7$). IW = initial weight, FW = final weight, DMI = dry matter intake, DWG = daily weight gain, FC = feed conversion, ABC = acidogenic bacterial consortium, SEM = standard error of the mean.

This was also demonstrated in the daily weight gain (DWG) (Table 9) of the animals where the difference between treatments ($p < 0.001$) was greater for the animals that received the ABC, with a DWG of 200 g more than the control group, which may demonstrate that the ABC has a beneficial effect on the animals' DWG.

Table 9 shows the effect of the ABC on the dry matter intake (DMI), daily weight gain (DWG), and feed conversion (FC) of Limousin heifers ($p < 0.001$).

3.4.1. Serum Glucose Determination

Glucose quantification was performed to determine the effect that increased VFA could have on circulating blood glucose. When analyzing the data by paired means, this was significant ($p = 0.05$) for the ABC group. Statistical analysis of each sample showed that the ABC group at day 85 had a significant increase in glucose, 90.52 (SD = 2.6) vs. 81.69 (SEM = 3.02) for the ABC and control groups, respectively (Table 10).

Table 10. Effect of ABC addition on blood glucose.

Days	Control (mg/dL)	SEM	ABC (mg/dL)	SEM
Day 1	79.07	4.14	80.46	4.43
Day 29	78.27	4.14	79.05	4.43
Day 57	84.48	4.14	88.86	4.78
Day 85	81.69 [b]	3.02	90.52 [a]	2.62

[a,b] Different superscripts between rows indicate statistical differences ($p < 0.001$, $n = 7$). ABC = acidogenic bacterial consortium, SEM = standard error of the mean.

3.4.2. ABC Effects on VFA Production

Table 11 shows the effect of probiotic addition on the molar concentrations of VFA in the rumen fluid. The concentration of acetate produced an increase of 14.5%, and there was an increase of 19% for propionate ($p < 0.05$). Butyrate was not different ($p > 0.05$). The total VFA difference was 15.4% ($p < 0.05$).

Table 11. Effect of ABC consortium addition on feed over VFA production (mmol/L) at the rumen level.

	Control	SEM	ABC	SEM
Acetate	94.87 [a]	71.35	108.46 [b]	65.62
Propionate	17.60 [a]	10.1	20.98 [b]	9.01
Butyrate	7.98 [a]	14.9	9.62 [a]	15.8
Total VFAs	120.45		139.06	

[a,b] Different superscripts between rows indicate statistical differences ($p \leq 0.05$, $n = 7$). ABC = acidogenic bacterial consortium, SEM = standard error of the mean, VFAs = Volatile fatty acids.

3.4.3. ABC Effects on Ruminal Microbiota

The possible changes in bacterial and archaeal populations in the rumen liquid after ABC administration are shown in Figures 4 and 5. No different changes in the bacterial

population at the species level were found between the ABC treatment and the control group (Figure 4). However, upon analysis of Archaea species, a decrease in methane-producing species was found (Figure 5, $p < 0.05$).

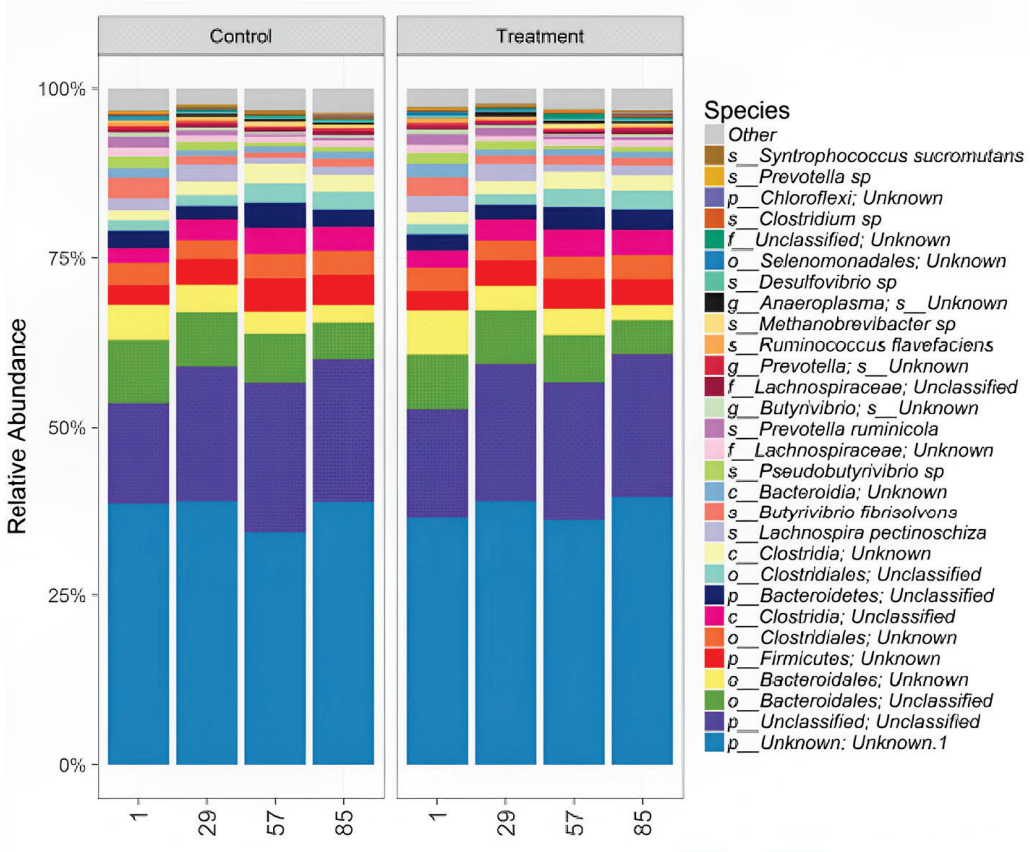

Figure 4. Effect of ABC on the relative abundance of bacterial species inhabiting the rumen of Limousin heifers.

3.4.4. Relative Quantification of *Lactobacillus* spp. and *Propionibacterium* spp.

The relative abundance of *Lactobacillus* spp. in the ABC was 0.091%, whereas the relative abundance of *Propionibacterium* spp. was 0.013%.

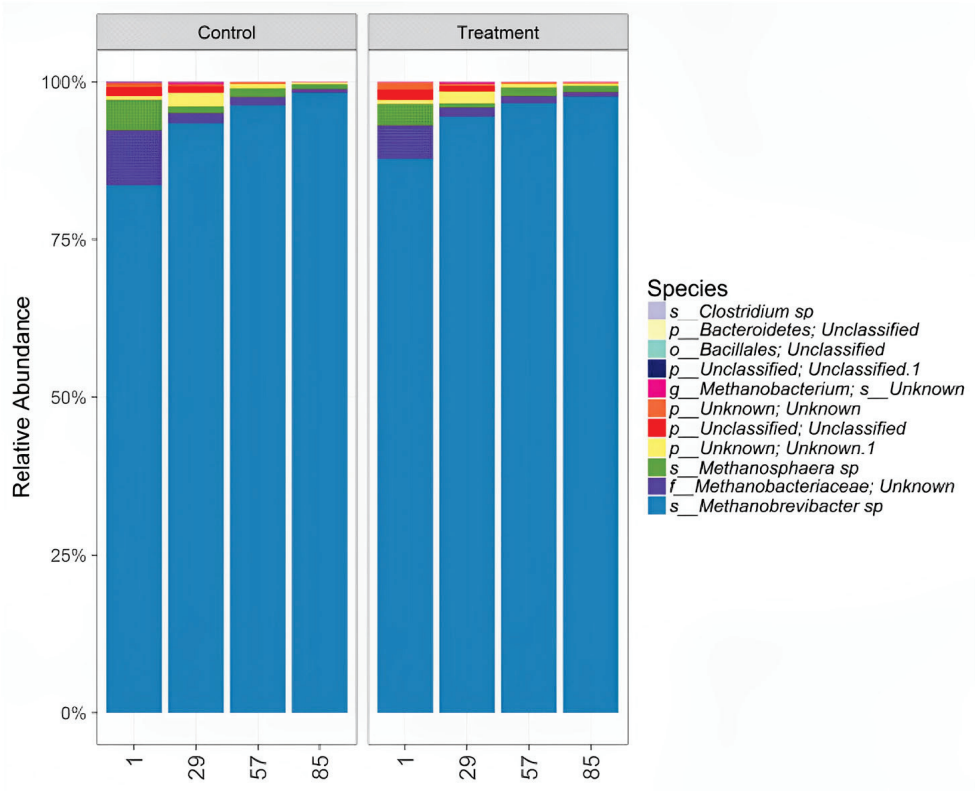

Figure 5. Effect of ABC on the relative abundance of Archaea species inhabiting the rumen of Limousin heifers.

4. Discussion

4.1. Probiotic Tests

The use of probiotics is subject to various laws to ensure their safety for both human and animal consumption. In the USA, microorganisms used for consumer purposes must have Generally Regarded as Safe (GRAS) status, regulated by the Food and Drug Administration (FDA). In Europe, the European Food Safety Authority (EFSA) introduced the term Qualified Presumption of Safety (QPS). The EFSA considers that bacterial strains carrying antibiotic resistance related to acquired genetic determinants have a high potential for horizontal spread and should not be incorporated into food. Otherwise, it considers that bacterial strains possessing antibiotic resistance by intrinsic or chromosomal mutation have a minimal or low potential for horizontal resistance spread and can generally be incorporated into food [31,32].

Resistance to gentamicin, erythromycin, and tetracycline was reported within the ABC; the bacterial genera present were *Lactobacillus* spp., *Enterococcus* spp. and *Bacillus* spp. Albuquerque et al. [33] and Argyri et al. [34], who worked with different strains of *Lactobacillus* spp., found intrinsic resistance to gentamicin, erythromycin, tetracycline, and kanamycin. Baccouri et al. [35], working with *Enterococcus faecalis* OB14, reported resistance to gentamicin and erythromycin. *Enterococcus* spp. resistance to gentamicin has been described as intrinsic, non-transmissible, and widely distributed [36,37]. This erythromycin resistance in *Lactobacillus* spp. has been associated with a single mutation in the 23S rRNA gene [38]. An important point and one to be taken with caution is the observed tetracycline resistance; some studies have described the tetracycline resistance genes tet (M), tet (L), tet (K), and tet (S) as chromosomal in lactic acid bacteria [38,39], while others have shown tet (M) as localized on plasmids [40], which can commonly move between bacteria. For the case of this ABC, the only gene of resistance to tetracycline was the Tet M, which is in the genomic DNA and does not represent a risk factor to transmit this resistance according to what has been established by the EFSA; when resistance is found in the chromosomal part

of the consortium, there is a minimal factor for the spread of horizontal resistance, and it can be incorporated into food [31,32].

Probiotics exert their beneficial effects on the host by modifying the composition and activity of the intestinal microbiota [41]. To achieve this, the bacterial strains used as probiotics must survive challenges in the host's digestive tract (DT) [42] including variations in pH throughout the ruminant DT compartments (reticulum–rumen, omasum, abomasum, small intestine, and colon), as well as resistance to bile in the intestine [43]. Our ABC exhibits robust survivability, particularly at low pH levels, with optimal survival at pH 6 and above, and it also demonstrates resistance to bile salts.

The pH is probably the most important factor affecting the microbial population and its activities. The rumen fermentation products, particularly acetate, propionate, lactate, and CH_4, are strongly affected by ruminal pH, mediated largely by the effect of pH on microbes [44]. The sensitivity of ruminal bacteria to pH is dictated by the pH gradient across their cell membranes and their ability to regulate intracellular pH. These physiolo gical pH ranges in the rumen and other parts of the DT are influenced by dietary factors. For instance, Kern et al. [45] measured the pH of the digestive tract of cattle fed with hay at a daily rate of 2% of body weight for 30 d. The DT pH values were 6.9, 3.1, 7.3, 7.0, and 7.2 for the rumen, abomasum, ileum, cecum, and terminal colon, respectively. Wheeler et al. [46] collected pH data from cattle fed with concentrates at 48.5 and 59.6% of the ration, reporting pH of 5.38 to 6.58, 2.27 to 4.16, 5.75 to 6.78, and 5.0 to 6.8 for the rumen, abomasum, ileum, and colon, respectively.

Ruminal pH greatly influences microbial activity, with the magnitude and duration of pH levels being key factors. The ABC thrived optimally at pH 6.5 and 7, both at 24 and 48 h, as shown by CFU counts (Table 3), similar to those reported by Maldonado et al. [47] who worked with different strains of *Lactobacillus* spp. obtained from the rumen of Bradford and Brangus cattle and found that *Lactobacillus* spp. tolerated acid pH (3.0) differently by the tested strains. Only *Lactobacillus fermentum* were able to grow at pH 3.0, which agrees with our results obtained for pH 2.0, 3.0, and 4.0 even though at 24 h there were still CFUs. The intolerance of *Enterococcus* spp. and *Weissella* spp. to highly acidic conditions is consistent with their inability to adapt to acid stress, as previously reported [48]. However, physiologically, ruminal pH does not reach values as low as 2.0, 3.0, 4.0, and 5.0 (without presenting any lethal pathology).

The ABC used in this work exhibited significant survival across a simulated gastrointestinal tract. Since probiotics should survive in the stomach (in this case the abomasum) where gastric juice is present, tolerance to acid pH is an important selection criterion for probiotics, with the pH of the abomasum being in the range of 2 to 4 depending on the type of diet [45,46] and a residence time of about 2 h.

Normally, this low pH causes strong decreases in bacterial counts as reported by Albuquerque et al. [33], who worked with several strains of *Lactobacillus* spp. Only one strain in *Lactobacillus fermentum* 296 was able to survive after 2 and 3 h at pH of 2.0, which agrees with our results, even though at pH of 4.0, there was a significantly higher amount of CFUs. Although there is survival of the ABC at this pH for 2 h, in vivo in the abomasum, probiotics are not necessarily challenged at pH values as low as 2.0 and 3.0 because the abomasal environment can be buffered by food components.

Bile comprises a complex mixture of components, encompassing proteins, ions, pigments, cholesterol, and diverse bile salts, which have demonstrated protective properties against pathogenic bacteria. The precise mechanisms underlying how bile triggers cell death remain poorly understood, and it remains uncertain whether cell death arises from membrane damage or from damage at the DNA-level, or both [49].

The ABC is resistant to bile salts at 0.3% and our result is like that reported by Sánchez et al. [50], who observed resistance to bile salts in four strains of *Lactobacillus* spp. isolated from calves. A factor that may favor the survival of these microorganisms in vivo in the duodenum is the presence of feed, since the bacteria may not be exposed to bile salts, and thus toxicity on the membranes could be avoided.

One of the relevant properties of some probiotics is the ability to coaggregate with certain pathogens and, consequently, prevent their access to the mucous membranes. We demonstrated that the bacterial consortium presents a 10.74% coaggregation capacity against *Escherichia coli*. This result is similar to those mentioned by Albuquerque et al. [33], who studied nine strains of *Lactobacillus* spp. as potential probiotics; they reported that the coaggregation capacity of their strains ranged between 2.4 and 41.3%. It is postulated that the coaggregation capacity depends on the bacterial species involved, being specific among strains and affected by the environmental conditions in which this interaction takes place. The pH and salt concentration of the medium modify this characteristic, probably because of the influence on the surface charges of the bacteria; thus, low pH and high electrolyte concentration favor it [51].

Another important characteristic of a probiotic is its ability to promote bacterial colonization of the intestinal mucosa [52]. In general, we observed good adhesion of ABC to mucus; in Video S1, we could observe the CFUs of the acidogenic bacterial consortium (ABC) adhered to mucins. Cueto-Vigil et al. [24] showed LAB had high adhesion to mucus, while López and Espinoza [53] found that *Lactobacillus plantarum* can adhere to and colonize intestinal cells in vitro. The ability of probiotics to reduce pathogenic microorganisms in the digestive tract may be due to the production of bacteriocin and exclusion due to competition by adherence to the intestinal epithelium. Indeed, some strains of *Lactobacillus* spp. and *Bifidobacterium* spp. have hydrophobic surface proteins that promote non-specific adhesion to animal cells, cover receptor binding sites, and prevent pathogenic microorganisms from binding to intestinal epithelium [53,54].

Among the benefits of various probiotics is the ability to improve the digestibility of feedstuffs and it is thought that, consequently, animal performance is improved. As can be seen in Table 7, dry matter disappearance was not affected by the treatments at 24 h. This was also reported by Ghorbani et al. [55] who worked with two probiotic bacterial strains and their combination (*Propionibacterium* P15 1×10^9 CFU/g and *Propionibacterium* P15 plus *Enterococcus faecium* 1×10^9 CFU/g) with a dosage of 10 g/animal/d in cannulated steers; they found no differences in DM digestion (in situ) on any of the substrates used. These findings have also been reported with other probiotic bacterial strains by authors such as Cagle et al. [56], with *Saccharomyces cerevisae*. Newbold et al. [57], also with *Saccharomyces cerevisae*, did not observe that degradation was significantly affected by treatment. Souza et al. [16], working with *Bacillus subtilis* spores, and Qiao et al. [58], also with a *Bacillus subtilis*, found no differences in dry matter digestibility.

Concerning VFA production, the ABC increases it by almost 15–19% in acetate and propionate, respectively. The use of probiotics in ruminants is recommended to improve intestinal health and productive variables in young animals. Different products have been used to manipulate ruminal fermentation [59]; our ABC has been shown to increase VFA production both in vitro and in vivo (Tables 8 and 11), which makes it a product with great potential for use in both dairy and beef cattle.

It is necessary then to clarify that the disappearance of dry matter does not have to be paired with VFA production, and we could speculate that the increase in VFA is due to a greater efficiency in the utilization of energy substrates available to ruminal bacteria, which is a possible mechanism of action of the ABC. It could also be due to less wastage of substrates or less availability of nitrogen, since a forage was used as a substrate for the bacteria and since this was limited, the bacteria had more carbohydrates available for conversion to VFAs.

4.2. Effect of the Addition of Fresh ABC to Limousin Heifers

Comparing the results obtained in this work with the use of the probiotic where the feeding of the animals was based on forages, we can see the efficacy of its use on weight gain, where there was an increase in DWG (1 kg daily), going from an average of 283 kg to 371 kg of weight in 84 days. If this growth trend is maintained, 400 kg calves could be obtained in 112 days.

Krehbiel et al. [41] reported that probiotic supplementation to fattening cattle can produce an increase of approximately 2.5 to 5% in DWG and 2% in feed efficiency. Arantzamendi et al. [60] described the use of *Bacillus toyoi* (Toyocerin®), in three different farm units and locations, with beef cattle, showing improvements for DWG of 10% and 6.2% for the final gain. Dick et al. [61] and Vyas et al. [62], where *Propionibacterium* spp. were tested as probiotics in steers, observed no change in DWG between the control and treated groups, nor was a reduction in DMI observed.

Triphati [63] tested three different yeast strains *Kluyveromyces marximanus*, *Saccharomyces cerevisiae*, *Saccharomyces uvarum*, and a combination of the three in a 1:1:1 ratio. A difference was observed in the weight gain of the animals supplemented with *Saccharomyces cerevisiae* and with the yeast mixture, where weight gains were 21% and 16%, respectively.

Alvarez [64] used a mixture of *Lactobacillus acidophilus*, *Bacillus thermophilum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*, and found no effect on DWG. These data agree with those reported by Krehbiel et al. [41], who concluded that the response in dry matter intake in animals consuming probiotics has been inconsistent and is dependent on factors such as climate, sanitary condition of the farm, and the health of the animals.

When observing the molar concentrations (Table 11) for the total VFA produced, it is evident that with the addition of the ABC, there is an increase in the concentration of VFAs. The concentration of acetate produced had an increase of 15%, and 19% for propionate. Butyrate was not different. The capacity shown by the ABC to modify the rumen environment causing an increase in VFAs (Tables 8 and 11) was notorious even with the use of forages, since diets with high forage content usually produce a lower amount of these; however, the acidogenic potential of the ABC could not be determined. The addition of concentrate or grain to the substrate may cause the expected impact on the propionic fraction, increasing the molar ratio of propionate at the expense of acetate causing a better acetate: propionate ratio [65].

Most of the work completed to study the effect of microbial additives on productive performance in ruminants has been carried out with growing and fattening animals or in production (dairy) [66].

Most studies report an increase in propionate and a decrease in the acetate: propionate ratio with supplementation of *Propionibacterium acidipropionici* [67–69] or propionate [70–72] in a variety of ruminant diets, suggesting that it is a useful tool to increase the glycogenic potential of the diet. Other researchers have reported no differences in total VFAs, but acetate was either decreased [67,69] or not altered [68] by supplementation with *Propionibacterium acidipropionici.* In the present study, an increase in total VFAs was observed for animals in the treated group, but there was no change in the acetate: propionate ratio.

Several studies have reported a successful establishment of products from one of the most important lactate-utilizing species in the rumen, *Megasphaera elsdenii* in sheep and cattle, where an increase in ruminal fermentation was seen to result in increased production of VFAs and helped to regulate ruminal pH preventing acidosis, although its mechanism of action is not fully elucidated [73,74].

These data on bacterial probiotics are consistent with the performance shown by the ABC, where it exerts an effect on ruminal fermentation, increasing weight gain and increasing VFAs of the animals that were supplemented with the consortium.

5. Conclusions

The evaluated ABC exhibits significant potential for use as a Direct-Fed Microbial (DFM) or probiotic due to several key attributes. Firstly, it demonstrates innocuousness, lacking antibiotic resistance and antibiotic resistance plasmids, it displays remarkable survivability under diverse digestive tract conditions encountered in ruminants. This includes tolerance to varying pH levels in the reticulum and rumen (ranging from 5.5 to 7.0), as well as robust survival in the abomasum, even in acidic environments. Furthermore, it exhibits resilience, with a 76.7% survival rate in the presence of HCl, pepsin, and pH 2.0, along with resistance to bile salts. Additionally, the ABC displays the ability to coaggregate

with *Escherichia coli* (10.74%) and enhances in vitro dry matter digestion (IVDMD) at 48 h (51.73%).

The ABC contributes to improved total volatile fatty acid (VFA) production both in vitro and in vivo, resulting in enhanced daily weight gain (DWG) and feed conversion in Limousin beef cattle. These attributes collectively position it as a promising candidate for probiotic use, especially in animals consuming high-forage diets.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants3040028/s1, Video S1: Colony-forming units (CFUs) of the acidogenic bacterial consortium (ABC) adhered to mucins. Staining with (5-(4,6-Dichlorotriazinyl) Aminofluorescein) (DTAF identified by color green, Thermo Fisher Scientific, USA) for the bacterial membrane and HOECHST-33342 (Blue, Thermo Fisher Scientific, Waltham, MA, USA) for DNA. The microscopy was performed with CONFOCAL-780LSM (Zeiss) 63X. The visualization was performed with AMIRA software (Thermo Fisher Scientific, Waltham, MA USA) in the CAVE VR System (automatic virtual environment, University of Illinois, Chicago, IL, USA).

Author Contributions: Conceptualization, O.M. and A.S.; methodology, D.C.-C., C.R.-R. and A.P.-T.; software, C.R.-R.; validation, C.R.-R. and L.G.-D.; formal analysis, C.R.-R. and D.C.-C.; investigation, O.M., C.R.-R. and L.G.-D.; resources, A.S.; data curation, C.R.-R. and O.M.; writing—original draft preparation, C.R.-R., O.M. and L.G.-D.; writing—review and editing, O.M., L.G.-D. and A.S.; visualization, C.L.-F.; supervision, A.S.; project administration, L.G.-D.; funding acquisition, A.S. and O.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by Comité Institucional para el Cuidado y Uso de Animales de Estudio CICUAE-UNAM (CICUAE.DC-2019/4.2, UNAM).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available upon request. Please contact the authors at ofemora66@unam.mx for access to the data.

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Conflicts of Interest: The authors declare no conflict of interest.

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ruminants

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Effect of Vegetable Oils or Glycerol on the In Vitro Ruminal Production of Greenhouse Gases

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Abstract: The objective of this research was to evaluate the ruminal fermentation parameters and in vitro Greenhouse gas (GHG) production derived from the fermentation of a balanced sheep diet with the addition of vegetable oils (canola, corn, safflower, and sunflower) or glycerol at different proportions (0, 20, and 40 g/kg of dry matter, DM). The fermentations showed that the highest G_{max} was obtained with the addition of 40 g/kg of sunflower oil and 20 g/kg of glycerol with values of 180.97 and 179.95 mL/g DM, respectively. The treatment with 40 g/kg DM of corn oil showed the lowest values in CH_4 production (7.15 mL/g DM when compared to the control) and it seemed to be a potential feeding strategy for reducing GHG emissions without affecting gas production. However, the N-NH_3 content for this treatment in both doses (1.90 and 1.88 mg/dL) indicated that some toxicity for the animal could be expected.

Keywords: fermentation process; methane production (CH_4); enteric emissions; feed ingredients; ruminant diets

1. Introduction

Greenhouse gases (GHG) such as CO_2, CH_4, and N_2O absorb infrared radiation and consequently impact global warming [1]. The enteric production of CH_4 by livestock is considered an important source of GHG emissions from the agricultural sector, with ruminant livestock being responsible for the annual global production of approximately 80 million tons of CH_4, which represents nearly 33% of anthropogenic CH_4 emissions and is implicated as a driver of global climate change [2–4]; furthermore, the livestock sector is responsible for GHG emissions of 5.6–7.5 $GtCO_{2e}$/year [5].

Methane has a 100 year global warming potential that is twenty-five times greater than the equivalent amount of CO_2, whereas enteric methane is an end product of the microbial fermentation of food generated within the gastrointestinal tract, particularly in the rumen [6]. Ruminal degradation of food also produces short-chain fatty acids (in particular acetic, propionic, and butyric acids), as well as CO_2 and H_2, which are converted into CH_4 by methanogenic bacteria. It is estimated that 6–18% of the gross energy intake is wasted as ruminal methane [7]; for this reason, the formation of CH_4 also represents a loss of energy from the animal's diet. Therefore, the development of mitigation strategies to reduce CH_4 emissions from ruminants is currently the subject of scientific and public

interest [3]. Studies indicate that an improvement in the quality of diet will reduce the emissions of CH_4 [8]. However, several factors influence ruminal production, such as the consumption of dry matter, lipids supply, a fiber-free carbohydrate diet, ingredient digestibility, and forage/concentrate ratios [7]. Some research indicates that the addition of vegetable oils improves the mechanisms involved in the antimethanogenic effects in the rumen [6], since lipids have been recognized as effective dietary additives to reduce enteric methane production [9]. Corn, canola, sunflower, and safflower oils have different profiles of polyunsaturated fatty acids. Canola oil is rich in monounsaturated oleic acid and has a relatively high proportion of polyunsaturated linoleic acid (omega-6) and a low proportion of alpha-linolenic acid (omega-3). Sunflower oil has a higher proportion of linoleic acid and a low proportion of oleic acid. Corn oil has a high proportion of linoleic acid and a low proportion of oleic acid. Safflower oil has a relatively high proportion of linoleic acid and a low proportion of oleic acid, similar to sunflower oil [9].

The polyunsaturated fatty acids present in vegetable oils have a toxic effect on ciliated protozoa, which are the rumen microorganisms involved in the production of H_2 (the main substrate for the enteric production of CH_4); hence, a reduction in the number of ruminal protozoa is generally associated with a decrease in CH_4 production due to a reduction in H_2 availability in the rumen [10].

On the other hand, glycerol or crude glycerin is a by-product of biodiesel, which can be used as a feed ingredient in ruminant diets without affecting the performance of beef cattle in fattening diets [11]. Additionally, it could inhibit the degradation of fats by bacteria and promote the ruminal passage of the total lipid content, thus providing higher proportions of beneficial unsaturated fats. Moreover, an excess of glycerol can be absorbed by both the ruminal and intestinal mucosa, as it is a direct gluconeogenic source for the ruminant [12]. The gross energy of glycerin varies from 13 to 25 MJ/kg, depending on its composition [13].

The final products of ruminal fermentation depend on the ingredients of the diet, which are volatile fatty acids (VFA) mainly composed of acetate, propionate, and butyrate with a lower acetate:propionate (A/P) ratio for concentrates compared to fibrous feeds. VFA supply an important part of the energy and carbon requirements of the ruminants and are largely absorbed through the rumen wall [3,7]. The higher amount of propionate formed during ruminal fermentation allows H_2 capture, thus restricting the formation of CO_2, which does not favor methanogenic bacteria. The production of acetate and butyrate releases CO_2 and H_2, which are precursors of CH_4 in the ruminal environment [7]. Any glycerol that is not absorbed through the rumen epithelium increases the concentration of propionate, butyrate, and valerate in the rumen whilst decreasing acetate [4].

Ruminant nutrition studies are aimed at establishing diets that maximize microbial protein production in the rumen since they reduce the need to supplement animal feed with non-degradable protein sources in the rumen. From an ecological point of view, the increase in carbon fixation in microbial biomass reduces carbon losses in the form of CO_2 and CH_4 [14]. N-NH_3 in the rumen is one of the most important variables that determine ruminal proper functioning [15], since it is essential for bacterial multiplication. An adequate amount of N-NH_3 in the ruminal fluid is needed by the bacteria, mainly for the synthesis of their body proteins [16]. The availability of fermentable carbohydrates in a ruminal environment and maintaining optimal levels of N-NH_3 in the ruminal medium during most of the day optimize the use of energy for microbial growth and increase metabolizable proteins [17–19]. It has been stated that the maximum microbial efficiency occurs when the concentration of ruminal N-NH_3 is between 5 and 8 mg/100 mL since the synthesis of protein in the rumen reaches a maximum at such a range [20].

Livestock production must be considered by the global scientific community when addressing the challenge of climate change [5]. Limiting the increase in emissions from the livestock sector is certainly a challenge. However, there are opportunities to simultaneously increase productivity and decrease the intensity of emissions in such a manner that the economy and livelihoods in rural areas will not be hampered [21]. Therefore, the study

of the intake in ruminants is becoming increasingly important in the search to improve the understanding of the digestive processes that occur in the previously mentioned multi-compartment system.

The current challenge is to increase the productivity of ruminants without negatively affecting the environment [2], which is why, in the present work, the effect of vegetable oils (canola, corn, safflower, and sunflower) or glycerol was evaluated (0, 20 and 40 g/kg DM) in an extruded diet based on the consumption of alfalfa for sheep by considering its response on the ruminal fermentation parameters (N-NH_3 concentration and VFA production), the accumulated gas production, and the gaseous fraction using in vitro techniques.

2. Materials and Methods

The data obtained from in vitro gas production kinetics, methane, and CO_2 production, as well as ruminal fermentation parameters were analyzed using a completely randomized design with doses [20 and 40 g/kg DM] and additives [corn, canola, safflower, sunflower, and glycerol] as factors. Each treatment was randomly selected and subjected to each analysis as a triplicate. The treatments consisted of a diet composed of 500 g/kg of a previously designed extrudate [22], 300 g/kg of corn, 190 g/kg of alfalfa, and 10 g/kg of minerals, to which were added 0, 20, and 40 g/kg of vegetable oils (canola, corn, safflower, and sunflower) in DM basis or crude glycerin. After extrusion processing, treatments were dried in a forced-air oven at 55 °C for 48 h and crushed in a Wiley-TP4274E70024 mill (Thomas Scientific, Morelia, Michoacán, México) with a 1 mm sieve looking for uniformity in particle size.

Three Suffolk breed sheep (80.5 ± 3.7 kg body weight) provided with a ruminal cannula were used as ruminal fluid donors. The sheep were housed in individual pens and fed twice a day (9:00 and 16:00 h) with a maintenance diet based on corn silage. Whole rumen contents were collected before the morning feeding and strained through a polyester monofilament fabric (250 μm mesh aperture) to remove solids. Inocula were obtained by mixing equal parts of rumen fluid from all animals, and 10 mL were immediately inoculated into 120 mL vials containing 30 mL of an anaerobic buffer solution kept at 39 °C [23]. The vials were incubated anaerobically at 39 °C for up to 24 h. The vials without substrate were used as controls.

The laboratory analyses were carried out within the hour of recollection according to [24], where the buffered mineral solution was prepared and kept at 39 °C under continuous CO_2 gassing as an anaerobic medium. Gas production was determined according to the methodology described by [25].

One gram of sample was added to each flask with 120 mL of ruminal fluid and a nutrient solution mixture in a 1:2 v/v ratio [26]; three replicates per treatment (thirty in total) and three fermentation flasks were used as blanks (containing ruminal fluid, nutrient solution, and one gram of the compound diet) and were incubated using a Daysi^{II} incubator (ANKOM Technology, Macedon, NY, USA) for 96 h.

In vitro accumulated gas pressure was measured automatically for 96 h using a piece of automatic gas measurement equipment (ANKOM RF Gas Production System) equipped with a pressure transducer connected to each cylinder that transmits the gas cumulative pressure values by radiofrequency to a computer, and readings were taken at 0, 0.5, 1, 2, 3, 6, 12, 24, 36, 48, 72, and 96 h after inoculation. The obtained gas production profiles were adjusted to the Gompertz sigmoidal equation of three parameters [27]:

$$PG = Gmax\, e^{-Ae^{-kt}} \quad (1)$$

where PG = gas production (mL/g DM), $Gmax$ = maximum gas production (mL/g DM), A = lag or adaptation phase (h), t = time (h), and k = gas production rate (h^{-1}).

The gas samples of the fermenters were obtained from the headspace at 12 h to calculate the gas fraction (CO_2 and CH_4) using the Biogas 5000 equipment (Landtec, Dexter, MI, USA); the liquid samples were centrifugated at 2500 rpm for 5 min and filtered for the

analysis of N-NH_3 and VFA [28], and frozen immediately afterward. VFA were analyzed on a 6890 N gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector and an HP-Innowax polyethylene glycol capillary column (30 m × 0.32 mm × 0.15 µm, J&W Scientific, Folsom, CA, USA). The oven temperature was set from 80 °C (held for 1 min) to 120 °C at a rate of 20 °C/min to 205 °C at a rate of 10 °C/min and held for 2 min. Hydrogen was the carrier gas at a constant flow rate of 40 mL/min and 1 µL of the sample was injected in no split mode. The peaks were identified by comparing retention times with VFA standards. The results were expressed as mmol of each VFA per 100 mmol of total VFA detected.

The ruminal environment was evaluated with N-NH_3, of which the samples were analyzed using a Genesys 10S VIS UV visible spectrophotometer (Thermo Scientific™, Madison, WI, USA) at a wavelength of 630 nm [28].

The data obtained from animal performance were analyzed using a completely randomized design with a generalized linear model. Experimental data were adjusted to quadratic models and regression coefficients were obtained. Statistical significances of the regression terms were examined by variance analyses (ANOVA) for the three parameters of the Gompertz sigmoidal equation ($p < 0.05$).

The gas production of fermentations, composition of the generated gas, N-NH_3, and VFA production were analyzed using the MIXED procedure of the statistical package SAS (SAS Institute, Inc., Cary, NC, USA), and the comparison of means ($p < 0.05$) and variance were performed using least-squares differences.

3. Results and Discussion

The formulation of the diet consisted of 12 g 100 g^{-1} soybean meal, 15 g 100 g^{-1} dried distillers' grains with solubles (30% protein), 7 g 100 g^{-1} molasses, 30 g 100 g^{-1} nixtamalized corn (NC), and 27:9 g of cottonseed meal (*Gossypium hirsutum* L.): g of NC 100 g^{-1}. The chemical composition of the evaluated diet is shown in Table 1.

Table 1. Proximal chemical analysis of balanced optimal diet for sheep [22].

Sample	Moisture [%]	Total Dry Matter [%]	g 100 g^{-1}				
			Crude Protein	Crude Fat	Crude Fiber	Nitrogen Free Extract	Ashes
Optimal food	8.66	91.34	27.25	4.24	12.21	46.95	9.35
Requirement *	-	90	14.5	3	10	52	7.5

* Nutritional requirements for sheep less than 1 year old [29].

Total gas production after inoculation of all treatments is shown in Figure 1. These results might be attributed to an increment of amylolytic bacteria since some oils are toxic to cellulolytic bacteria and protozoa [30]. The differences among treatments could be the result of the type and amount of oils [9].

The fermentation kinetics of feed can be determined by the gas production and by the storage of short-chain volatile fatty acids [12]; therefore, to carry out the ruminal degradation of the substrate, it must undergo a colonization time or lag time that allows the enzymes to reach the substrate (lag or adaptation phase, A). In this research, the inclusion of different oils at different levels showed no significant differences ($p > 0.05$) for any treatment (Table 2), which could indicate that the gas production kinetics from the in vitro fermentation of vegetable oils or glycerol might not be affected by the colonization of microorganisms in the rumen [12]. Regarding the gas production profiles, an increase was obtained for all treatments compared to the control. The highest gas productions obtained were 180.97 and 179.95 mL/g DM for sunflower (at a rate of 40 g/kg DM) and glycerol (at a rate of 20 g/kg DM), respectively, thus representing a 40% increment compared to the control. For the glycerol treatment, the results obtained might indicate the presence of non-structural carbohydrates associated with glycerin, which are rapidly metabolized (between

4 and 6 h), thus providing greater synchronism with fast nitrogen sources degradation and, consequently, increased gas production [12]. Ref. [7] evaluated ruminal fermentation with added glycerin that showed a rapid fermentation during the first 12 h of incubation. However, in this research, in terms of the gas production rate (k), similar values with the control were obtained, except for the safflower (20 g/kg DM) and glycerol (20 and 40 g/kg DM) treatments, which resulted in a decrement of 38% compared to the control (0.11 h^{-1}), indicating a longer time to achieve maximum gas production. Safflower oil is rich in polyunsaturated fatty acids and is a rich source of linoleic acid (0.76 of the total fatty acids) [31] with availability in the carbon chain that could be saturated with H_2.

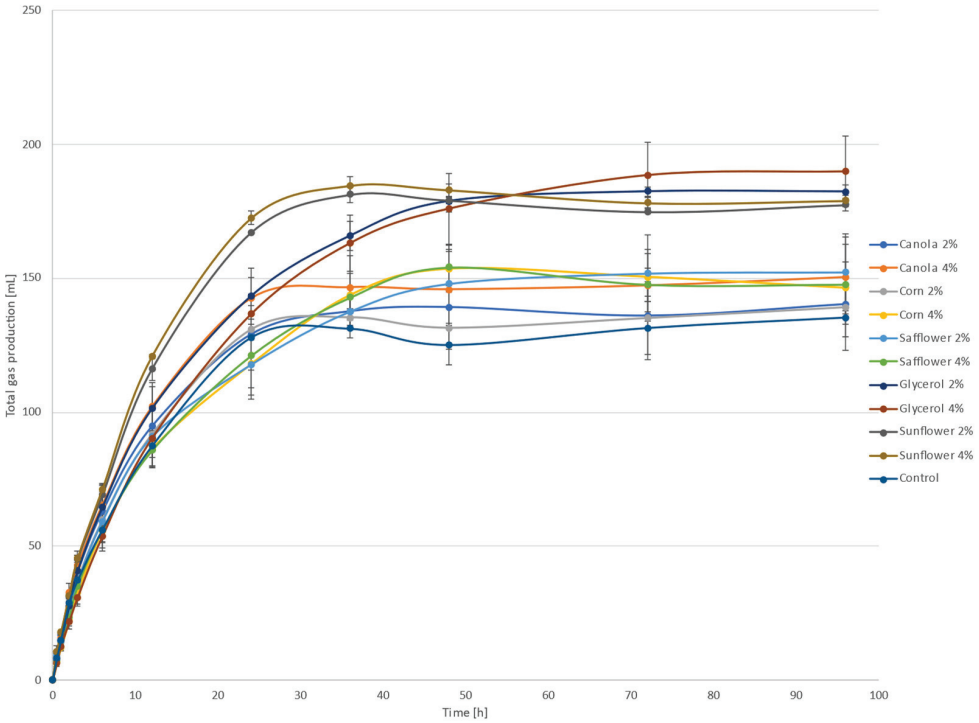

Figure 1. Total gas production after inoculation.

Table 2. Parameters of the Gompertz model.

Treatment	Control	Canola		Corn		Safflower		Sunflower		Glycerol		EE
Dose	0%	2%	4%	2%	4%	2%	4%	2%	4%	2%	4%	
A	2.50 ^{a}	2.50 ^{a}	2.49 ^{a}	2.48 ^{a}	2.57 ^{a}	2.47 ^{a}	2.51 ^{a}	2.61 ^{a}	2.65 ^{a}	2.51 ^{a}	2.67 ^{a}	0.030
Gmax	128.85 ^{d}	132.55 ^{d}	140.45 ^{c}	139.20 ^{c}	152.30 ^{b}	157.05 ^{b}	151.45 ^{b}	177.80 ^{a}	180.97 ^{a}	179.95 ^{a}	173.20 ^{a}	2.133
k	0.18 ^{a}	0.19 ^{a}	0.17 ^{a}	0.16 ^{a}	0.13 ^{a}	0.11 ^{b}	0.14 ^{a}	0.16 ^{a}	0.17 ^{a}	0.11 ^{b}	0.11 ^{b}	0.007

EE: Standard error of the mean for Dose; $^{a–d}$: Equal letters indicate no statistically significant difference ($p > 0.05$); *A*: lag or adaptation phase (h); *Gmax*: maximum gas production (mL); *k*: gas production rate (h^{-1}).

Table 3 indicates that the linear coefficients of the dose and all the vegetable oils and the glycerol had a negative significant effect ($p < 0.05$) on the lag phase, except for glycerol and sunflower. Regarding the maximum gas production and gas production rate, all the linear coefficients and their interactions with the dose showed a significant effect ($p < 0.05$). Such effects could also be attributed to the different profiles of the polyunsaturated fatty acids of the used oils and glycerol [6,9]. Canola and corn oil showed a negative significant effect ($p < 0.05$) on maximum gas production, which could be attributed to changes in the microbial community of rumen fluid and digesta associated to the total VFA concentration, the molar proportions of acetate, isobutyrate, butyrate, and total protozoa [32].

Table 3. Regression coefficients of responses of gas production parameters.

	Intercept	Dose	Canola	Corn	Safflower	Glycerol	Sunflower	Dose*Canola	Dose*Corn	Dose*Safflower	Dose*Glycerol	Dose*Sunflower	R^2
A	2.539	**0.026**	**−0.038**	**−0.017**	**−0.049**	**0.052**	**0.092**	−0.029	0.016	−0.007	0.051	−0.005	0.728
Gmax	153.551	0.985	**−17.051**	**−7.801**	**0.699**	**23.024**	**25.832**	**2.965**	**5.565**	**−3.785**	**−4.3597**	**0.599**	0.974
k	0.152	0.0001	**0.031**	**−0.006**	**−0.029**	**−0.039**	**0.012**	**−0.009**	**−0.012**	**0.016**	**0.001**	**0.003**	0.875

Bold values indicate significant effect $p < 0.05$. *A*: lag or adaptation phase (h); *Gmax*: maximum gas production (mL); *k*: gas production rate (h^{-1}).

The production of CH_4 and CO_2, as well as the CH_4/CO_2 ratio (Table 4), yielded 13.09 mL (50% increment, compared to the control) and 73.70 mL (34% increment, compared to the control) of CH_4 and CO_2, respectively, as maximum values with the 40 g/kg DM sunflower treatment. However, CH_4/CO_2 ratio presents values with significant differences for all treatments, showing the highest values for the safflower treatment 20 g/kg DM (0.190), representing an increment of 20% compared to the control. Additionally, it was observed that the treatments with added corn oil in both doses presented the lowest CH_4 production, indicating decreases of 7 and 18% for 20 and 40 g/kg DM treatments, respectively, compared to the control. A lower H_2 production per unit of fermented feed resulting in a lower CH_4 formation might have occurred, which is associated with an increase in the amount of propionate among the final fermentation products in the rumen [3].

Table 4. Gas fraction resulting from the in vitro gas production kinetics (mL).

Treatment	Control	Canola		Corn		Safflower		Sunflower		Glycerol		EE
Dose	0%	2%	4%	2%	4%	2%	4%	2%	4%	2%	4%	
CH_4	8.73 ^{b}	10.18 ^{b}	9.08 ^{b}	8.19 ^{b}	7.15 ^{c}	10.89 ^{b}	8.88 ^{b}	11.74 ^{b}	13.09 ^{a}	10.61 ^{b}	9.65 ^{b}	0.357
CO_2	55.01 ^{c}	62.04 ^{b}	56.40 ^{b}	55.64 ^{b}	49.96 ^{c}	57.35 ^{b}	53.68 ^{c}	73.38 ^{a}	73.70 ^{a}	61.18 ^{b}	55.59 ^{b}	2.101
CH_4/CO_2 ratio	0.1587 ^{i}	0.1641 ^{f}	0.1609 ^{g}	0.1472 ^{j}	0.1431 ^{k}	0.1900 ^{a}	0.1655 ^{e}	0.159 ^{h}	0.1777 ^{b}	0.1734 ^{d}	0.1737 ^{c}	0.00003

EE: Standard error of the mean for Dose; $^{a–k}$: Equal letters indicate no statistically significant difference ($p > 0.05$).

According to [7,9], increasing the propionate amount and decreasing the A/P ratio results in lower H_2 production in the rumen and, therefore, lower CH_4 production due to the net consumption of H_2 in propionate synthesis, possibly improving the efficiency of the energy use of feed.

The VFA obtained values are presented in Table 5. The highest value of acetic acid was registered for the sunflower treatment of 40 g/kg DM with an increase of 41% compared to the control, with an acetate yield as the end product of the fermentation that exceeded propionate production. However, the obtained results indicate that for both glycerol concentrations, acetate and butyrate values increased compared to the control, while propionate concentrations decreased, which is consistent with an increase in CH_4 production [33]. In the case of propionic acid, all treatments resulted in values below the control. Yet, the highest values correspond to the treatment of corn 40 g/kg DM (18.35%) and safflower 20 g/kg DM (17.18%); consequently, from the obtained values of acetic and propionic acid, the A/P ratio showed the lowest values for both corn 40 g/kg DM (3.41%) and safflower 20 g/kg DM (3.61%) treatments without significant differences ($p > 0.05$) to the control. The 40 g/kg DM corn treatment presented the lowest values both in the A/P ratio and in CH_4/CO_2 ratio (in this case, obtaining the lowest value even below the control). On the other hand, the 20 g/kg DM safflower treatment presented the second lowest value in the A/P ratio, but also showed the highest amount of CH_4 per mL of CO_2 produced. Regarding butyric acid, no significant differences ($p > 0.05$) were found among treatments.

Table 5. Volatile fatty acid concentrations (%).

Treatment	Control	Canola		Corn		Safflower		Sunflower		Glycerol		EE
Dose	0%	2%	4%	2%	4%	2%	4%	2%	4%	2%	4%	
Acetic	55.59 ^{c}	73.82 ^{a}	72.24 ^{a}	70.95 ^{a}	62.10 ^{b}	61.38 ^{b}	72.77 ^{a}	73.45 ^{a}	78.29 ^{a}	70.13 ^{a}	74.90 ^{a}	2.100
Propionic	18.99 ^{a}	12.61 ^{b}	13.86 ^{b}	14.71 ^{b}	18.35 ^{a}	17.18 ^{a}	12.87 ^{b}	12.58 ^{b}	10.75 ^{b}	13.99 ^{b}	12.57 ^{b}	0.896
Butyric	6.01 ^{a}	7.22 ^{a}	7.12 ^{a}	6.85 ^{a}	8.80 ^{a}	9.42 ^{a}	7.59 ^{a}	7.36 ^{a}	6.13 ^{a}	8.34 ^{a}	7.02 ^{a}	0.615
TVFA (mM)	70.49 ^{b}	75.70 ^{b}	69.34 ^{b}	44.59 ^{c}	33.54 ^{d}	29.42 ^{d}	76.45 ^{b}	78.80 ^{b}	103.40 ^{a}	65.28 ^{b}	77.45 ^{b}	2.625
A/P ratio	2.95 ^{c}	6.10 ^{b}	5.38 ^{b}	5.34 ^{b}	3.41 ^{c}	3.61 ^{c}	5.87 ^{b}	6.27 ^{b}	7.83 ^{a}	5.17 ^{b}	6.28 ^{b}	0.568

EE: Standard error of the mean for Dose; TVFA: Total volatile fatty acids; $^{a–d}$: Equal letters indicate no statistically significant difference ($p > 0.05$).

The optimal N-NH_3 concentrations of 5.06 and 5.15 mg/dL (Table 6) were obtained with the 20 g/kg DM safflower and 40 g/kg DM sunflower treatments, respectively. In contrast, 20 g/kg DM (1.90 mg/dL) and 40 g/kg DM (1.88 mg/dL) corn oil treatments showed lower concentrations than those registered for the control treatment. N-NH_3 maximum concentration was generally reached approximately two hours after the protein intake provided by feeding [20]. Samples were analyzed 12 h after fermentation. In in vitro experimentation, N-NH_3 concentration is an indicator of protein degradability because there is no nitrogen uptake or recycling compared to those obtained using in vivo ruminal media [29], as indicated by [13]. Something similar could have occurred with the obtained N-NH_3 values from the other treatments since the protein had been mostly degraded by the time the analysis sample was collected. The authors of [30] obtained values ranging from 10.8 to 13.8 mg/dL of ammonia nitrogen in ruminal fermentations with organic oils added at three levels to their evaluated diets: 2% fish oil; 2% fish oil and 1.5% soybean oil; and 2% fish oil and 3% soybean oil, which were higher than the levels and results from this research.

Table 6. Ammoniacal nitrogen concentrations (mg/dL).

Treatment	Control	Canola		Corn		Safflower		Sunflower		Glycerol		EE
Dose	0%	2%	4%	2%	4%	2%	4%	2%	4%	2%	4%	
Concentration	3.34 ^{c}	3.74 ^{b}	2.97 ^{c}	1.90 ^{d}	1.88 ^{d}	5.06 ^{a}	4.18 ^{b}	3.89 ^{b}	5.15 ^{a}	4.11 ^{b}	4.15 ^{b}	0.152

EE: Standard error of the mean for Dose; $^{a–d}$: Equal letters indicate no statistically significant difference ($p > 0.05$).

4. Conclusions

In this research, an effective ruminal fermentation process was presented with an adaptation phase without significant differences among the treatments tested herein, and with an increase in gas production which was favorable for all treatments compared to the control. The obtained values were consistent with the gaseous fraction observed due to GHG production and showed a decrease of 7 and 9.5% in the methane ratio for each mL of CO_2 for the treatments with 20 g/kg and 40 g/kg of added corn oil, respectively. The treatment with corn oil showed the lowest value in CH_4 production, which could represent a potential feeding strategy for reducing GHG emissions without affecting gas production.

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Article

Cactus Pear Silage to Mitigate the Effects of an Intermittent Water Supply for Feedlot Lambs: Intake, Digestibility, Water Balance and Growth Performance

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Abstract: The aim of this study was to evaluate the intake, digestibility, water balance and growth performance of lambs receiving diets containing cactus silage under an intermittent water supply. Thirty-six male, uncastrated Santa Inês lambs with an initial weight of 19.8 ± 2.1 kg and age of 6 months were distributed in a 3 × 3 factorial arrangement, with three proportions of cactus pear in the diets (0 (control diet containing Tifton hay), 21% and 42% of dry matter) and three periods of intermittent water supply (0, 24 and 48 h), with four repetitions. Lambs that received diets non-isonitrogenous with cactus silage showed higher intakes of dry matter ($p < 0.001$), total digestible nutrients ($p < 0.001$), water excretion via faeces ($p < 0.001$) and water balance ($p < 0.001$). Lambs that received diets with cactus silage showed higher digestibility of total carbohydrates, non-fibre carbohydrates ($p = 0.005$), water intake via food ($p < 0.001$), total water intake ($p < 0.001$), water excretion via urine ($p < 0.001$) and water balance ($p < 0.05$), when compared to the control diet. Lambs that received diets with cactus silage promoted growth performance ($p = 0.001$). When using 42% forage cactus silage in place of Tifton hay and water offered at 48 h intervals, intake, digestibility, and performance of feedlot lambs were improved.

Keywords: animal production; succulent feed; water restriction

1. Introduction

The semi-arid region of the Brazilian northeast is one of the world's most densely populated dryland regions [1]. Its outstanding characteristics are high temperatures (annual average of 28 °C [2]), low rainfall (annual average of less than 800 mm [3]), relative air humidity around 55% [2], evaporative demand greater than 2000 mm/year, Thornthwaite aridity index ≤ 0.50, annual water deficit ≥ 60% and periodic droughts, which result in a water deficit for most of the year [3]. These factors directly influence the vegetation, its economy, and the feeding of animals and humans [4].

Due to food limitations and the lower nutritional value of available pastures, the dry season poses a serious challenge to animal production in semi-arid regions. Due to

its adaptation to the region's soil and climatic conditions, high water content, potential for biomass production (241.75 t/ha green matter and 12.46 t/ha dry matter [DM]) and nutritional value (as a source of energy, non-fibre carbohydrate), the spineless cactus is a substitute compared to traditional forage sources and common food found in these areas [5,6].

It has excellent palatability, high metabolizable energy (11.38 MJ/kg DM; [7]), high digestibility (690–780 g/kg) [8], and a high water content (109 g/kg DM [9]), contributing to the supply of dietary water for the animal. However, the low content of DM (109 g/kg DM [9]), crude protein (44.6 g/kg DM [9]), neutral detergent fibre (260.3 g/kg DM [10]) and acid detergent fibre (146 g/kg DM [9]) impair its supply as the sole source of water to animals.

According to [6], in a study on the performance of lambs fed spineless cactus silage associated with forages adapted to the semi-arid environment, the diets resulted in an average weight gain of 0.268 kg/day, with greater body weight gain for the animals receiving spineless cactus silage (15.2 kg, approximately 0.293 kg/day) and spineless cactus + gliricidia silage (15.1 kg, approximately 0.303 kg/day) diets, due to the higher DM intake that these diets provided. The values found are above that established by [11] (200 g/day). When using forage palm silage as a ratio to evaluate the performance of lambs, Bendaou et al. [12] observed that animals fed with silage gained 195 g per day of weight, in comparison with those fed with a conventional diet with a weight gain of 255 g per day.

In addition, water supports the maintenance of homeostasis and is connected to all metabolic activities. IBGE [13] estimates that there are 13.5 million sheep in the semi-arid area of Brazil alone. These animals would need 41.1 million litres of water per day if they drank about 3 L per animal each day [14]. This number may be considerably higher if we consider the water content of the feed that these animals consume, the water used to produce the feed, and the water necessary to clean the cages and other equipment. So, the amount of water needed for animal husbandry is significant and should be used intelligently in order to maximize the efficiency of its abstraction and usage, which will have a positive impact on the environment.

A scarcity of water for animal consumption has the consequences of reducing growth, well-being and health, and increasing stress, generating negative impacts on productive and economic factors. According to [14], in sheep, water restriction can lead to skin retraction, dry eyes, weight loss, low food intake, dry faeces and reduced urine excretion.

This is important because during the dry season, the lack of water severely limits livestock production, and herds frequently need to travel several kilometres to reach a water source. In such cases, an intermittent water supply can be used as a strategy to mitigate the effects of water scarcity [15].

Studies evaluating the effects of forage-cactus-based silages on intake, digestibility, water balance and performance have already been reported in different parts of the world, such as Zimbabwe, per [16]. However, this information with small ruminants is still incipient in the semi-arid region of Brazil; it is limited and generally considers the use of cactus pear silage to mitigate the effects of an intermittent water supply [15,17,18]. We hypothesized that cactus pear silage reduces water intake by lambs, meeting the water demand of the animals without affecting live weight gain.

The aim of this study was to evaluate the intake, digestibility, water balance and growth performance of lambs growing receiving diets containing cactus silage under an intermittent water supply.

2. Materials and Methods

2.1. Description of the Study Site

The experiment was conducted at the experimental Caatinga biome field of the Animal Metabolism Unit, Embrapa Semi-arid, located in Petrolina, state of Pernambuco, Brazil. The municipality is at 376 m altitude, at the geographical coordinates of 9°23′35″ S latitude and 40°30′27″ W longitude. The climate is BSwh' semi-arid, with summer rainfall [19]. The

mean annual rainfall is 570 mm, relative humidity is 36.73% and average annual maximum and minimum temperatures are 32.22 °C and 20.90 °C, respectively.

The present study was submitted and approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Bahia (Opinion no. 0005/2016).

2.2. Animals, Experimental Design and Diets

Thirty-six crossbred, growing, intact male Santa Inês lambs from the same herd and from a single delivery (6 months of age and 19.8 ± 2.1 kg body weight) were housed in individual pens (1.2 × 1.0 m) equipped with feeding and drinking fountains for the diet and water supply. The experiment lasted 84 days, including 10 days of adaptation. At the beginning of the adaptation period, animals were identified, weighed, treated against endo- and ectoparasites through the application of an oral solution (200 µg/kg body weight; Ivomec, Merial, Campinas, Brazil) and randomly assigned to the pens previously identified according to the treatment.

Treatments were arranged in a 3 × 3 factorial design comprising three levels of cactus silage replacing Tifton hay in the diet (0, 21% and 42% on DM basis) and three intervals for supplying water (0, 24, and 48 h), with four replications. The effect of variables isolated was evaluated when the interaction was not significant. Water was supplied intermittently according to the respective treatments: T1 = no water restriction (daily water supply), T2 = 24 h of restriction and then water supply for 24 h and T3 = 48 h of restriction and then water supply for 24 h. On the water supply days, fresh water was provided ad libitum in the morning (at 09:00 h).

Experimental diets consisted of forage cactus silage, Tifton hay and concentrate based on corn meal, soybean meal, wheat bran and mineral supplements (Table 1).

Table 1. Chemical composition and fermentative characteristics of the ingredients used in experimental diets.

Items (in g/kg Dry Matter)	Ground Corn	Soybean Meal	Wheat Bran	Tifton Hay	Cactus Silage
Dry matter [a]	872	886	867	887	74
Organic matter	980	929	949	937	831
Ether extract	48	27	25	16	26
Crude protein	104	529	198	56	83
NDFap	388	213	420	614	428
Acid detergent fibre	345	128	125	404	279
Total carbohydrates	827	395	726	865	721
Non-fibre carbohydrates	439	182	307	251	293
Cellulose	25	124	85	340	224
Hemicellulose	354	85	294	210	149
Acid detergente lignin	09	04	40	63	55
Total digestible nutrients	937.5	832.4	742.8	604.9	659.91
pH	-	-	-	-	4.95
Water-soluble carbohydrates	-	-	-	-	15.06
N-NH3 (%NM)	-	-	-	-	2.72
Buffer capacity	-	-	-	-	14.23

[a] in g/kg fresh matter; NDFap—neutral detergent fibre corrected to ash and protein.

Silage comprised Mexican Elephant Ear cactus (*Opuntia stricta* Haw) forage harvested at 24 months after regrowth. The material was chopped with a stationary forage harvester (PP-35, Pinheiro máquinas, Itapira, São Paulo, Brazil) to an average particle size of approximately 2.0 cm and stored in 200 L plastic-drum silos (89 cm × 59 cm × 59 cm) with a removable lid sealed with a metal ring. The silage was used after a minimum period of 60 d after its confection. The diets were formulated as non-isonitrogenous, so the crude protein contents were not similar; however, they were formulated according to the recommendations of [11] for estimated weight gains of 150 g/day. The roughage: concentrate ratio was 60:40, and cactus silage was used to replace three proportions of hay in the diet (0, 21% and 42% DM) (Table 2).

Table 2. Chemical composition of the experimental diets.

Items (% Dry Matter)	Forage Cactus Silage Levels		
	0%	**21%**	**42%**
Ground corn	28	23	18
Soybean meal	8	10	12
Wheat bran	3	6	9
Tifton hay	60	39	18
Forage cactus silage	-	21	42
Mineral supplement [a]	1	1	1
	Chemical composition (in g/kg DM)		
Dry matter [b]	858	697	537
Organic matter	936	916	895
Crude protein	118	130	143
Ether extract	29	28	27
NDFap	491	445	399
Acid detergent fibre	259	237	214
Total carbohydrates	788	756	724
Non-fibre carbohydrates	297	311	325
Cellulose	220	198	176
Hemicellulose	231	208	185
Acid detergent lignin	39	39	37
Total digestible nutrients	660	661	663

[a] on a fresh matter basis; NDFap—neutral detergent fibre corrected to ash and protein. [b] Guaranteed levels provided by the manufacturer (per kg in active elements): calcium—120 g (min.); phosphorus—87 g (min.); sodium—147 g (min.); sulphur—18 g (min.); copper—590 mg (min.); cobalt—40 mg (min.); chromium—20 mg (min.); iron—1800 mg (min.); iodine—80 mg (min.); manganese—1300 mg (min.); selenium—15 mg (min.); zinc—3800 mg (min.); molybdenum—10 mg (min.); fluorine—870 mg (max.); phosphorus (P) solubility in 2% citric acid—95% (min.).

Diets were provided twice a day, at 09:00 h and 15:00 h. The amount of feed offered was calculated according to the intake on the previous day. Amounts of feed offered and refused were weighed daily to calculate and adjust intake, allowing at least 10% leftovers in the trough. Weekly, samples of the offered food and refusals were individually collected per animal and stored at −20 °C for later laboratory analysis.

2.3. Intake and Digestibility of Nutrients

Daily DM intake was obtained from the difference between the total DM of the distributed feed and the total DM present in the refusals. Nutrient intake was determined as the difference between the total nutrients present in the ingested feed and the total nutrients present in the leftovers, on a total-DM basis.

A digestibility test was performed across 15 days in the final third of the experimental period: 10 days for adaptation followed by 5 days for data collection. For this, animals were distributed in metabolic crates provided with feeding arranged in a covered area. The faeces of each animal were collected using collection bags, which were fixed to the animals two days before the sampling period. Bags were weighed and emptied twice daily, and a sub-sample of 10% of the total amount was collected to form a composite sample for each treatment, which was stored at −20 °C. Urine was collected and weighed once daily in plastic buckets. The urine was then filtered, and 10 mL aliquots were collected and immediately diluted in 40 mL 0.03 N sulfuric acid [20].

2.4. Assessment of Water Intake

Water intake (WI) was evaluated daily. Water was supplied in plastic buckets (5 L) and weighed before being supplied and again 24 h later. This variable was estimated using buckets randomly placed around the experimental shed, with the same amount of water available for each treatment, being determined by the weight difference over 24 h. Water lost by evaporation was also considered in the calculation of water intake. Water balance

was evaluated according to [21]. The production of metabolic water (faeces and urine) was estimated from the chemical analysis of the diets and calculated by multiplying the consumption of carbohydrates, protein and digestible ether extract by the factors 0.60, 0.42 and 1.10, respectively.

Water balance (WB) was evaluated using the following equations [20]:

$$\text{Total water intake (TWI; kg/day)} = \text{water intake (corrected for evaporation)} + \text{water from the diet} \quad (1)$$

$$\text{Total water excretion (kg/day)} = \text{water excreted via urine (WEU)} + \text{water excreted via faeces (WEF)} \quad (2)$$

$$\text{Water balance} = \text{total water intake} - \text{total water excretion} \quad (3)$$

2.5. Growth Performance

Animals were weighed at the beginning and end of experimental period and after a 12 h period of solid food deprivation (with access to water) to obtain the initial body weight (IBW), final body weight (FBW), total weight gain (TWG), average daily gain (ADG) and feed conversion (FC). The following equations were used:

$$\text{TWG (kg)} = \text{FBW} - \text{IBW} \quad (4)$$

$$\text{ADG (g/day)} = \text{TWG/confinement days} \quad (5)$$

$$\text{FC} = \text{dry matter intake/ADG} \quad (6)$$

2.6. Laboratory Analysis

Samples of ingredients, diets, refusals and faeces were pre-dried in a forced-air oven at 55 °C for 72 h and ground to 1 mm particles in a knife mill (Wiley Mill, Marconi, MA-580, Piracicaba, Brazil). All chemical analyses were performed using the procedures described by [22] for DM (DM; Method 967.03), mineral matter (MM; Method 942.05), crude protein (CP; Method 981.10) and ether extract (EE; Method 920.29). Neutral detergent fibre corrected for ash and protein (using heat-stable alpha-amylase without sodium sulphite) NDFap [23,24] and acid detergent fibre (ADF) were determined as described by [25] and lignin was determined by treating the ADF residue with 72% sulfuric acid [26]. Hemicellulose (HEM) was calculated by the following equation:

$$\text{HEM} = \text{NDF} - \text{ADF} \quad (7)$$

Total carbohydrates (TC) were estimated according to the equation proposed by [27], as follows:

$$\text{TC (g/kg)} = 1000 - (\text{CP} + \text{EE} + \text{MM}) \quad (8)$$

where

CP = crude protein;

EE = ether extract;

MM = mineral matter.

Non-fibre carbohydrate (NFC) contents were calculated as proposed by [28]:

$$\text{NFC (g/kg)} = \text{TC} - \text{NDFap} \quad (9)$$

The apparent digestibility coefficient of nutrients was calculated as described by [29]:

$$\text{ADC} = \{[\text{Nutrients ingested (kg)} - \text{nutrients excreted in the faeces (kg)}]/\text{nutrients ingested (kg)}\} \times 100 \quad (10)$$

Total digestible nutrients (TDN) were estimated on the basis of the data on apparent digestibility and calculated according to [27]:

$$\text{TDN} = \text{DP} + \text{DNDF} + (\text{DEE} \times 2.25) + \text{DNFC} \quad (11)$$

where

DP = digestible protein;

DNDF = digestible neutral detergent fibre;

DEE = digestible ether extract;

DNFC = digestible non-fibre carbohydrates.

2.7. Statistical Analysis

Treatments were arranged in a distributed 3 × 3 factorial arrangement, with three proportions of cactus pear in the diets (0 (control diet containing Tifton hay), 21% and 42% of dry matter) and three periods of intermittent water supply (0, 24 and 48 h), with four repetitions. The effect of variables isolated was evaluated when the interaction was not significant.

Data were tested by Shapiro–Wilk and Levene's tests to check the normality of the residuals and homogeneity of the variances, respectively; once the assumptions were met, they were tested by ANOVA, and means were compared by Tukey's test, as well as the interactions between them, with a statistical probability of up to 5% ($p < 0.05$) considered as significant using the Statistical Analysis System version 9.4 (SAS Institute, Inc. Cary, NC, USA) software.

The following mathematical model was used:

$$\text{Yijk} = \mu + \alpha i + \beta j + (\alpha\beta)ij + k + eijk \quad (12)$$

where Y is the observed value of variable ijk that refers to the k-th repetition of the combination of the i-th level of factor A with the j-th level of factor B; μ is the mean of all experimental units for the variable; αi is the effect of the levels of forage cactus silage (i = 0, 21% and 42%) at the observed value Yijk; βj is the effect of the intermittent water supply (j = 0, 24 h and 48 h) at the observed value Yijk; αβij is the effect of the interaction between the levels of forage cactus silage and intermittent water supply; k is the block effect on the observation Yijk; and eijk is the error associated with the observation of Yijk.

3. Results

There was no significant effect of an intermittent water supply (drinking fountain), nor was there a significant effect of the interaction between water supply and cactus silage on the DM and nutritional fraction intake ($p > 0.05$; Table 3). The lambs that received diets with 21% and 42% cactus silage showed higher intakes of DM, MO, CP, NDFap, TC, NFC and TDN ($p < 0.05$) when compared to the lambs that received the control diet (Table 3). Lambs that received the diet with 21% cactus silage showed the highest EE intake ($p < 0.001$; Table 3).

Table 3. Daily intake of nutritional components and apparent digestibility of nutrients in lambs fed forage cactus silage under an intermittent water supply.

Itens	Cactus Silage (%)			Intermittent Water Supply (h)			SEM	*p* Value		
	0	21	42	0	24	48		CS	IW	CS × IW
				Intake (g/day)						
Dry matter	712.8 b	967.4 a	996.1 a	894.7	888.1	893.5	28.70	<0.001	0.991	0.954
Organic matter	617.9 c	1813.8 b	2339.9 a	1635	1485	1640	4.48	<0.001	0.493	0.963
Crude protein	94.9 b	145.0 a	153.3 a	129.2	131.0	132.9	5.49	<0.001	0.915	0.828
Ether extract	24.4 b	32.6 a	25.9 b	29.1	25.9	27.9	1.00	<0.001	0.333	0.749
NDFap	331.9 b	394.9 a	390.5 a	371.5	375.2	370.6	9.38	0.020	0.977	0.976
Total carbohydrate	546.9 b	703.3 a	712.0 a	657.1	652.9	652.3	18.90	<0.001	0.991	0.970
Non-fibre carbohydrates	234.3 b	325.5 a	314.6 a	298.1	282.4	293.9	9.77	<0.001	0.694	0.862
Total digestible nutrientes	470.4 b	642.7 a	635.3 a	596.9	577.7	573.8	21.21	<0.001	0.835	0.954
				Digestibility (g/kg)						
Dry matter	632.5	666.5	681.6	674.0	658.7	646.8	1.25	0.139	0.563	0.282
Organic matter	649.9	682.6	698.7	690.5	674.7	666.2	1.20	0.128	0.582	0.282
Crude protein	711.7	698.2	728.3	708.7	723.2	706.4	1.04	0.383	0.696	0.102
NDFap	598.0	613.7	621.5	627.1	609.3	596.8	8.50	0.730	0.602	0.245
Total carbohydrate	629.6 b	673.7 a	695.2 a	682.1	660.2	656.2	7.80	0.049	0.556	0.359
Non-fibre carbohydrates	704.5 b	761.7 a	779.7 a	765.2	735.5	745.2	1.14	0.005	0.389	0.737

Means followed by different letters differ by Tukey's test at the 5% probability level for the following effects: CS—cactus silage; IW—intermittent water supply; CS × IW—interaction effect for cactus silage and intermittent water supply; NDFap—neutral detergent fibre corrected to ash and protein; SEM—standard error of the mean; *p*-value—probability value.

There was no significant effect of an intermittent water supply, nor was there a significant effect of the interaction between water supply and cactus silage on the apparent digestibility of nutrients ($p > 0.05$; Table 3). Lambs that received diets with 21% and 42% cactus silage showed higher digestibility of TC ($p = 0.049$) and NFC ($p = 0.005$) in relation to the lambs that received the control diet (Table 3).

There was neither an effect of an intermittent water supply nor an effect of the interaction between water supply and cactus silage on water intake, water excretion and water balance ($p > 0.05$; Table 4). The control diet promoted higher water intake via drinker ($p < 0.001$; Table 4). Lambs that received the diet with 42% cactus silage showed higher total water intake and WEU ($p < 0.001$) in relation to the animals fed diets with 21% cactus silage and the control diet (Table 4). Diets with 21% and 42% cactus silage promoted higher WIF, WEF and WB ($p < 0.001$) when compared to the control diet (Table 4).

Table 4. Water balance of lambs fed forage cactus silage under an intermittent water supply.

Itens (g/Day)	Cactus Silage (%)			Intermittent Water Supply (h)			SEM	*p* Value		
	0	21	42	0	24	48		CS	IW	CS × IW
				Intake (g/day)						
Water intake via drinker	1403.7 a	711.0 b	156.0 c	837.1	857.8	530.3	109.71	<0.001	0.065	0.716
Water intake via food	120.2 b	2273.5 a	3432.8 a	2062.8	1903.2	2009.2	239.49	<0.001	0.145	0.586
Total water intake	1523.9 c	2984.5 b	3588.8 a	2899.9	2760.9	2539.5	173.12	<0.001	0.305	0.462
Water excretion via faeces	331.4 b	687.2 a	646.3 a	588.8	545.9	548.1	36.90	<0.001	0.733	0.358
Water excretion via urine	255.5 c	630.8 b	1090.3 a	787.1	551.6	661.6	72.43	<0.001	0.066	0.736
Water balance	937.0 b	1666.5 a	1852.1 a	1523.9	1663.5	1328.8	96.33	<0.001	0.224	0.767

Means followed by different letters differ by Tukey's test at the 5% probability level for the following effects: CS—forage cactus silage; IW—intermittent water supply; CS × IW—interaction effect for cactus silage and intermittent water supply; SEM—standard error of the mean; *p*-value—probability value.

Animals fed a diet with 42% cactus silage showed higher FBW in relation to the animals receiving the control diet ($p = 0.002$). A higher proportion of cactus silage in the diets promoted higher TWG ($p = 0.001$) and ADG ($p = 0.001$). Animals that received the control diet presented higher FC ($p = 0.028$) than animals fed diets with 42% cactus silage (Table 5). Animals that were given water every 48 h presented higher TWG ($p = 0.032$) and ADG ($p = 0.032$). Lower FC was found for animals that received drinking water every 48 h ($p = 0.007$) (Table 5).

Table 5. Growth performance of lambs fed cactus silage under an intermittent water supply.

Itens (g/Day)	Cactus Silage (%)			Intermittent Water Supply (h)			SEM	*p* Value		
	0	21	42	0	24	48		CS	IW	CS × IW
				Intake (g/day)						
Initial body weight (kg)	19.3	20.5	19.8	19.9	20.2	19.3	1.26	0.143	0.326	0.373
Final body weight (kg)	29.8 b	34.1 ab	35.1 a	32.8	32.0	34.2	2.57	0.0018	0.306	0.979
Total weight gain (kg)	10.5 b	13.6 a	15.4 a	12.8 ab	11.8 b	14.8 a	1.83	0.001	0.032	0.609
Average daily gain (g)	142.0 b	184.0 a	208.0 a	173.4 ab	159.1 b	200.8 a	24.75	0.001	0.032	0.609
Feed conversion (kg DMI/kg ADG)	5.0 a	4.4 b	4.1 b	4.7 a	4.9 a	3.9 b	0.49	0.028	0.007	0.081

Means followed by different letters differ by Tukey's test at the 5% probability level for the following effects: CS—cactus silage; IW—intermittent water supply; CS × IW—interaction effect for cactus silage and intermittent water supply; DMI—dry matter intake; ADG—average daily gain; SEM—standard error of the mean; *p*-value—probability value.

4. Discussion

4.1. Intake, Digestibility and Growth Performance

The use of forage cactus silage reduces water intake by lambs, meeting the animals' water demands without affecting growth performance. Thus, the use of forage cactus silage meets the demand for water in periods of water scarcity, attenuating the reduction in feed intake that would culminate in weight reduction due to the loss of body mass and water [30,31].

The average DMI observed was higher than the requirement recommended by the NRC [11], which is 780 g/animal/day for lambs at the age and weight range used in the present study, with gains of 150 g/day. The results of DMI observed for treatments with inclusion levels of forage cactus silage (21% and 42%) were also higher (967.4 and 894.7 g/kg DM, respectively) than the requirement recommended by the NRC [11]. Forage cactus silage, compared to diets containing Tifton hay, may provide higher DMI due to the high rate of DM degradability due to high concentrations of non-fibre carbohydrates, which may explain the results obtained in this study.

Cordova-Torres et al. [32] evaluated the effect of water deprivation (without water and ad libitum) and increasing levels of forage cactus (30%, 50% and 70% in replacement of Tifton hay) in the diets of growing lambs on DMI and obtained values lower than those of the present study when lambs were subjected to water stress (804 g/kg DM) and increasing levels of forage cactus (803 g/kg DM).

Thus, it is evident that there were no limitations on DMI, indicating that the use of cactus silages in place of Tifton hay in diets for small ruminants showed desirable fermentation properties and high acceptability by the animals, which favoured the increase in ADG and feed conversion (Table 5), in addition to providing water supply via food (Table 4), allowing animals to not reduce food intake when receiving water in amounts below their requirements.

The use of cactus silage to replace Tifton hay may have been one of the factors responsible for the highest intake of CP in animals fed 21% or 42% cactus silage in the diets, since the cactus silage presented in its composition a higher content of CP (8.3% DM) than Tifton hay (5.6% DM) (Table 2), which provided the animals with a crude protein intake above that recommended by the NRC [11], which is 117 g/day for animals in this category. Forage cactus, when well managed and fertilized, can provide a greater supply of nitrogen, as well as other bulky foods, which explains the high CP percentages in its composition. It should also be taken into account the fact that the diets are not isonitrogenous, having different levels of crude protein, which possibly increased the consumption of this ingredient.

Ether extract intake was higher in animals fed a diet with a composition of 21% cactus silage than in the other two levels tested, with higher consumption values than those reported by the NRC [11] (30 g/kg DM). Adequate energy intake levels for young lambs

are necessary for animals to develop and fulfil their potential [33]. This fact may explain the highest final weight values of the animals in the treatment with 21% forage cactus silage.

The decreasing levels of NDFap in the diets, as well as the decrease in the percentages of Tifton hay (Table 2), allowed higher intake of NDFap and NFC by lambs fed diets containing cactus silage. Forage cactus has a low content of NDFap, which is associated with a high content of soluble carbohydrates that increase the intake of NFC by lambs [17], corroborating the findings of the present study. According to [34], forage cactus can be considered a good source of non-fibre carbohydrates. Because of their rapid degradation, these nutrients improve the digestive flow through the gastrointestinal tract, increasing the intake of nutrients.

The absence of significant differences in DMI and nutrient digestibility in lambs under intermittent water supply in the present study can be seen as a positive fact, as it suggests that an intermittent water supply within 48 h can be used for lambs in feedlot, as a way to save water, without influencing the intake of DM and nutrient digestibility in these animals. These results can be evidenced by research carried out by [17] and [31] using an intermittent water supply (ad libitum and 24 and 48 h water restrictions) for lambs and goats, respectively. In a study evaluating the effect of water restriction on the growth performance of lambs fed replacement levels (30%, 50% and 70%) of Tifton hay for forage cactus under water restriction, [32] also did not observe an effect on animal performance. However, research carried out by [15] observed a reduction in DM consumption in lambs subjected to water restrictions of 24, 48 and 72 h.

With the use of forage cactus as silage, there was a change in the composition of the diet, mainly with regard to the proportions of non-fibre carbohydrates. These results may be related to low concentrations of ADF and ADL (Table 2) and a higher concentration of NFC in cactus silage in relation to Tifton hay, which probably increased ruminal degradation and nutrient digestion.

The increase in the proportion of non-fibre carbohydrates possibly provided better conditions in the rumen, since non-fibre carbohydrates are easily degraded, increasing the energy supply and improving the energy: protein ratio, which favours microbial growth and, therefore, digestion [35,36]. Thus, the reduced NFC digestibility for Tifton hay is related to the high content of non-fibre carbohydrates present in forage cactus, which after rapid fermentation in the rumen, promote a sharp decline in rumen pH, an increase in the rate of passage and, consequently, reduction in cellulolytic activity [37].

4.2. Water Balance

Animals that were given diets containing cactus silage had less need to seek water from the drinking fountain, as they ingested more water via food. This is due to the low DM content present in cactus silage (73.90 g/kg fresh matter; Table 1) and, consequently, the high moisture content in its composition, which demonstrates the efficiency of this forage in supplying water and its ability to significantly assist in animal watering in arid and semi-arid regions, where water can be a limiting factor in animal production.

Since the highest water intake was found in animals fed cactus silage, it was to be expected that there would also be greater excretion of water via faeces and urine in these animals, which in fact occurred. Water excretion via the faeces of animals that received cactus silage was more than twice that observed in animals that did not receive this food. This is justified by the highest water content of diets containing cactus silage. According to [11], the amount of water contained in ruminant faeces can be influenced by the water content of the diet; more humid diets and those with a higher mineral content generally result in a higher faecal water content.

As with the excretion of water via faeces, the excretion of water via urine increased with the use of cactus silage in the diet, showing an increasing behaviour as the proportion of cactus silage in the diet increased. Lambs fed diets containing 42% cactus silage excreted the largest amount of water, on average 1090.30 g/day. The authors of [38] reported that small ruminants fed diets containing forage cactus lowered their water intake via the

drinking fountain and excreted large volumes of urine, as compensatory mechanisms in the regulation of the total volume of water circulating in the body.

For animals to have good productive performance, it is necessary that the water balance in these animals is positive and stable, thus guaranteeing a water balance between their body fluids [30]. Lambs fed diets containing 21% and 42% cactus silage showed an average water balance of 1759.3 g/day water. The greater values of water balance for animals that ingested cactus silage in their diet emphasize the efficiency in the use of drinking fountain water and water contained in their food by small ruminants. Thus, it can be inferred that the water balance observed for both the cactus silage and the intermittent water supply was suitable.

5. Conclusions

Lambs' productive performance is improved when cactus silage substitutes up to 42% of DM of Tifton hay in non-isonitrogenous diets. On the other hand, an intermittent supply of water in periods of up to 48 h does not impair the performance of lambs under feedlot conditions.

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Can Associative Effects Affect In Vitro Digestibility Estimates Using Artificial Fermenters?

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Abstract: We aimed to test the associative effects among forages, and between forage and concentrates on the in vitro digestibility of dry matter and neutral detergent fibre using an artificial ruminal fermentation system. The study consisted of two assays, in which associative effects were evaluated among three forages, sugarcane, maize silage, and Tifton 85 hay under two incubation conditions (single feed or all feeds together in a jar), and the associative effects between sugarcane and soybean meal and/or ground maize. For the first assay, sugarcane digestibility increased ($p < 0.02$), whereas the maize silage digestibility decreased ($p < 0.01$) when forages were incubated together in the same jar. Tifton hay digestibility was not altered ($p \geq 0.57$) by the incubation condition. In the second assay, the sugarcane digestibility was depressed ($p < 0.05$) when the forage was incubated along with maize grain. For both assays, the pattern of repeatability for digestibility estimates presented an influence of the incubation condition. We concluded that the incubation of different feeds together in the same jar using artificial fermenters causes associative effects among them. These effects can influence the estimates of in vitro dry matter and fibre digestibility and alter their repeatability.

Keywords: concentrates; feed analysis; forages; in vitro systems; ruminal digestion

1. Introduction

The method for evaluating in vitro digestibility of ruminants' feeds suggested by Ti-lley and Terry [1] was based on the incubation of forage samples in individual tubes containing ruminal inoculum and buffer solution. However, the utilisation of artificial fermenters has increased in recent decades, as laboratorial analyses are considered to be simpler, faster, less expensive, and have greater operational capacity [2–4].

The first artificial fermenter model was developed by a North American company (DaisyII, Ankom Technology Corporation Fairport, New York, NY, USA) and was introduced to the public in 1994 [3]. However, nowadays, different commercial brands of this equipment can be found on the market. Overall, the equipment possesses four jars, where samples are kept in contact with the inoculum under controlled temperature and rotation. Particularly, the greater operational capacity of artificial fermenters occurs because several samples can be evaluated simultaneously [5] and the utilisation of filter bags could minimize errors caused by material transfer during filtration procedures [6]. On the other hand, the simultaneous evaluation of feeds with different chemical characteristics could cause associative effects among samples incubated in the same jar, influencing microbial populations and activity, and, consequently, altering feed digestibility estimates [7–9].

In vitro digestibility estimates of feeds can be used according to two main objectives: prediction of in vivo digestibility [10] and comparative evaluation among feeds [5,11]. In this sense, the occurrence of associative effects among feeds incubated together in a jar could affect both objectives by biasing the estimates, and by altering the ranking of feeds in relation to digestibility characteristics. However, studies performed to evaluate the

occurrence of associative effects amongst feeds using in vitro artificial fermenters are still scarce regarding tropical feeds.

Therefore, our objective was to study the occurrence of associative effects among forages, and between forage and concentrates on in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) using an artificial ruminal fermentation system.

2. Materials and Methods

The experiment was carried out at the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. The Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (protocol number 029/2019) approved all animal care and handling procedures applied in this work.

2.1. Incubations and Measurements

The experiment was performed in two assays. In the first, the occurrence of associative effects among forages with different chemical characteristics (sugarcane, maize silage, and Tifton 85 hay) was evaluated. In the second assay, the occurrence of associative effects between one forage (sugarcane) and energy and/or protein concentrate feeds (maize grain and, or soybean meal) was evaluated.

Primary samples of fresh sugarcane (*Saccharum officinarum* L.), maize silage (*Zea mays* L.), Tifton 85 hay (*Cynodon* sp. Rich.), maize grain, and soybean meal were obtained in Viçosa, Minas Gerais, Brazil. The high-moisture forages were oven-dried (55 °C/72 h). After that, all materials were processed in a knife mill (model 3; Arthur H. Thomas, Philadelphia, PA, USA) to pass through a 1 mm screen sieve and were analysed with regard to the contents of dry matter (DM, dried 105 °C/16 h; method G-003/1), nitrogen (N, Kjeldahl procedure; method N-001/2), and neutral detergent fibre (NDF, method F-002/1) according to the standard analytical procedures of the Instituto Nacional de Ciência e Tecnologia de Ciência Animal ([12], Table 1). The NDF contents were evaluated using a heat-stable α-amylase (Termamyl 2X; Novozymes, Araucária, Paraná, Brazil), omitting sodium sulphite, and were expressed including residual ash and protein. The crude protein (CP) content was expressed as N × 6.25.

Table 1. Average contents of crude protein (CP) and neutral detergent fibre (NDF) in the different study materials.

	CP	**NDF**
Feeds	**g/kg Dry Matter**	
Sugarcane	30.2	586
Maize silage	79.3	562
Tifton 85 hay	75.9	797
Soybean meal	515	233
Maize grain	84.0	198

In the first assay, the IVDMD and IVNDFD were evaluated with the forages incubated either in different jars or all of them simultaneously in the same jar. In the second assay, the digestibility of fresh sugarcane was evaluated by incubating only the forage in a jar or the forage along with maize grain, soybean meal, or maize grain plus soybean meal.

The digestibility assays were performed in a TE-150 artificial fermenter (Tecnal Equipamentos Científicos, Piracicaba, São Paulo, Brazil) and filter bags which were made of non-woven textile (100 g/m^2, [13]). The filter bags were 4 × 4.5 cm and were previously washed, dried, and weighed as described by Camacho et al. [4] to obtain the tare weight.

The incubations procedures lasted eight days, with each assay lasting four consecutive days, with two consecutive 48 h incubation runs [1]. In each assay, each incubation run included the evaluation of four fermentation jars containing 30 filter bags with approximately 500 mg test portions per filter bag (in a DM basis). All filter bags were heat-sealed.

In the first assay, the filter bags were distributed to the fermentation jars in each run as follows: one jar for each individual forage (n = 30 for each jar), and one jar for all forages simultaneously (n = 10 for each forage). In the second assay, the filter bags were distributed as follows: one jar for sugarcane (n = 30 for sugarcane), one jar for sugarcane and maize grain (n = 15 for each feed), one jar for sugarcane and soybean meal (n = 15 for each feed), and one jar for sugarcane, maize grain, and soybean meal (n = 10 for each feed). Each jar contained two blank filter bags. It must be noticed that the artificial fermenter possessed four jars (3200 mL).

McDougall's buffer solution [14] was used for all incubations ($NaHCO_3$, 9.80 g/L; anhydrous Na_2HPO_4, 3.71 g/L; KCl, 0.57 g/L; NaCl, 0.47 g/L; $MgSO_4.5H_2O$, 0.12; $CaCl_2.2H_2O$, 0.05 g/L). Urea was not added to the buffer solution in order to avoid favouring the microbial utilisation of the low-quality feeds [11,15], and to allow a clearer visualization of associative effects. The solution was prepared 24 h prior to each incubation run and kept in an acclimatized room (39 °C). Prior to each incubation, the pH of the buffer solution was adjusted to 6.8 by bubbling CO_2 into the solution, as described by Camacho et al. [4].

The ruminal inoculum was obtained from a rumen-fistulated Nelore heifer weighing 350 kg. The basal diet of the animal consisted of fresh sugarcane and a commercial concentrate containing 220 g CP/kg as fed. The commercial concentrate was composed of maize grain, soybean meal, wheat bran, urea, and minerals. The forage-to-concentrate ratio of the diet was 80:20 on a DM basis. The animal was fed ad libitum twice daily at 0800 and 1600 h, allowing approximately 100 g/kg in orts (as feed). The animal also had ad libitum access to fresh water and a complete mineral mixture, and was adapted to the basal diet for 14 days prior to the inoculum collections [16].

Ruminal inoculum (liquid and solid digesta) was collected at several points in the cranial and caudal rumen mat shortly before the beginning of incubation. Ruminal inoculum was stored in preheated (39 °C) thermal bottles and then mixed for a few seconds in a blender to homogenize liquid and solid phases [4]. The fluid was then filtered through four layers of cheesecloth. The steps from rumen inoculum collection to incubation onset were conducted within 20 min in an acclimatized room (39 °C). In each jar, 400 mL of ruminal inoculum and 1600 mL of McDougall's buffer solution were added (1:4 vol/vol). Carbon dioxide was flushed into the headspace of each jar, which was closed and placed into the preheated (39 °C) artificial fermenter.

After 48 h of incubation, the filter bags were washed with hot distilled water (90 °C) until the water became clear, and bags were gently pressed to remove gases. Then, to estimate the apparently undigested DM residue, filter bags were oven-dried (55 °C/24 h and 105 °C/16 h, sequentially), placed in a desiccator, and weighed.

For the IVNDFD evaluations, filter bags containing the incubation residues were placed into polypropylene screw-capped flasks (120 mL; autoclavable universal collection vial, Bioplast 2605, Porto Alegre, Rio Grande do Sul, Brazil) with 80 mL of neutral detergent solution and 500 μL of a heat-stable α-amylase (Termamyl 2X, Novozymes, Araucária, Paraná, Brazil). Flasks containing the filter bags were closed and autoclaved for 1 h at 105 °C [17]. After that, the filter bags were washed with hot distilled water (90 °C) and acetone. The drying and weighing procedures were performed as previously described.

The IVDMD and IVNDFD were calculated as

$$D = \frac{M - (R - B)}{M} \times 1000 \quad (1)$$

where D is the IVDMD or IVNDFD (g/kg); M is the incubated mass of DM or NDF (g); R is the undigested residue of DM or NDF (g); and B is the DM or NDF residue in blank filter bags (g).

To evaluate the pH and concentration of ammonia nitrogen (NH_3-N) in the medium (i.e., the mixture of rumen inoculum and buffer solution), aliquots of the fluid were taken from each jar within each incubation run, filtered through a triple layer of cheesecloth, and transferred to 50 mL conical polypropylene tubes with screw caps. The pH was immediately measured using a digital potentiometer (TEC-3P-MP; Tecnal, Piracicaba, São

Paulo, Brazil). Then, 1 mL of a H_2SO_4 solution (9 mol/L) was added to each tube, and all tubes were kept at 4 °C until NH_3-N analysis. Ammonia nitrogen was estimated by indophenol-catalysed colorimetric reactions (method N-006/1, [12]).

2.2. Statistical Analysis

For the first assay, the evaluation of the associative effects among forages was analysed according to the model:

$$Y_{ijkl} = \mu + F_i + C_j + FC_{ij} + R_k + \varepsilon_{ijkl} \quad (2)$$

where Y_{ijkl} is the IVDMD or IVNDFD measured on test portion l of forage i under incubation condition j in run k; μ is the general constant; F_i is the fixed effect of forage i; C_j is the fixed effect of the incubation condition j (i.e., single feed or all feeds together in a jar); FC_{ij} is the fixed effect of interaction between forage i and incubation condition j; R_k is the random effect of incubation run k; and ε_{ijkl} is the random error assumed to be NIID (0, ${\sigma^2}_\varepsilon$).

For the second assay, the evaluation of associative effects among forage and concentrate feeds followed the model:

$$Y_{ijk} = \mu + C_i + R_j + \varepsilon_{ijk} \quad (3)$$

where Y_{ijk} is the IVDMD or IVNDFD measured on test portion k under incubation condition i in run j; μ is the general constant; C_i is the fixed effect of the incubation condition i; R_j is the random effect of incubation run j; and ε_{ijk} is the random error assumed to be NIID (0, ${\sigma^2}_\varepsilon$).

All statistical evaluations were performed using the MIXED procedure of SAS 9.4. The components of variance were estimated according to the restricted maximum likelihood method. Significant results were declared at $p < 0.05$.

Initially, we performed an outlier evaluation on the overall dataset. An outlier was identified when its restricted likelihood distance was greater than 1.0. After the outlier elimination, analyses of variance were performed again to interpret the significance of the effects. For the model (2), when a significant interaction was found, we studied the effect of incubation condition nested within each forage using the SLICE statement of the MIXED procedure. When a significant effect of incubation condition was found in model (3), comparisons were performed using the Bonferroni's test.

After performing the analyses based on the full models (Equations (2) and (3)), a second set of analyses of variance was carried out. In this case, we evaluated the data obtained for each forage/incubation condition to quantify their specific residual variances. The following model was used:

$$Y_{ij} = \mu + R_i + \varepsilon_{ij} \quad (4)$$

where Y_{ij} is the IVDMD or IVNDFD measured on test portion j in incubation run i; μ is the general constant; R_i is the random effect of incubation run i; and ε_{ij} is the random error assumed to be NIID (0, ${\sigma^2}_\varepsilon$).

From the results obtained in model (4), the repeatability was calculated as

$$r = \frac{\sqrt{\hat{o}_\varepsilon^2}}{\bar{Y}} \times 100 \quad (5)$$

where r is the repeatability (%); $\hat{o}_\varepsilon^2$ is the residual variance [(g/kg)^2]; and $\bar{Y}$ is the average IVDMD or IVNDFD (g/kg).

The initial and final values of pH and NH_3-N concentration were evaluated following what was previously described, but considering each jar as a subject. Evaluation times (i.e., initial and final) were considered as repeated measures. Due to the restricted number of replicates ($n = 2$ for each assay), the (co)variance matrix structure was modelled according to a variance component structure (VC), and the degrees of freedom were estimated using

the Satterthwaite's approximation. Comparison among incubation conditions followed the previously stated methods.

3. Results

We did not detect variation between incubation runs ($p > 0.05$) for the response variables evaluated here. For the first assay, there was an interaction between forage and incubation condition ($p < 0.01$) for both IVDMD and IVNDFD. Sugarcane digestibility increased ($p < 0.02$), whereas maize silage digestibility decreased ($p < 0.01$) when the forages were incubated together in a same jar. Tifton hay digestibility was not altered ($p \geq 0.57$) by the incubation condition (Table 2).

Table 2. Average and standard error of the mean of in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) in several forages according to incubation conditions.

Forage	Incubation Condition [1]		
	Single Feed	**All Feeds**	***p*-Value**
	IVDMD (g/kg)		
Sugarcane	574 ± 20.2 (60)	600 ± 21.3 (20)	0.007
Maize silage	666 ± 20.2 (59)	625 ± 21.3 (20)	<0.001
Tifton 85 hay	552 ± 20.3 (55)	557 ± 21.3 (20)	0.599
	IVNDFD (g/kg)		
Sugarcane	347 ± 21.4 (58)	377 ± 23.1 (20)	0.019
Maize silage	503 ± 21.3 (60)	442 ± 23.1 (20)	<0.001
Tifton 85 hay	508 ± 21.4 (55)	501 ± 23.1 (20)	0.577

[1] Single feed, each feed located in different bags from a separated jar; All feeds, all the feeds located in different bags from the same jar. The number in parentheses represents the number of replicates.

In the second assay, the sugarcane IVDMD and IVNDFD did not change ($p > 0.05$) when the forage was incubated together with soybean meal or soybean meal plus maize (Table 3). However, its digestibility was depressed ($p < 0.05$) when the forage was incubated along with maize grain.

Table 3. Average and standard error of the mean of in vitro digestibility of dry matter (IVDMD, g/kg) and neutral detergent fibre (IVNDFD, g/kg) in sugarcane incubated alone or along with different concentrate feeds.

Incubation Condition	IVDMD [1]	IVNDFD [1]
Sugarcane + maize + soybean meal	660 ± 21.6 [a] (16)	422 ± 31.5 [a] (18)
Sugarcane + soybean meal	651 ± 21.2 [a] (29)	418 ± 31.0 [a] (29)
Sugarcane + maize	615 ± 21.2 [b] (29)	370 ± 31.1 [b] (29)
Sugarcane	641 ± 21.0 [a] (56)	407 ± 30.6 [a] (57)
p-value	<0.001	<0.001

[1] Means in column followed by different letters differ at $p < 0.05$. The number in parentheses represents the number of replicates for the forage feed.

The evaluation of associative effects among forages showed that the repeatabilities for digestibility of sugarcane and maize silage were improved when the incubation was performed with all feeds in the same jar (Figure 1). Though, the opposite pattern was verified for Tifton 85 hay, whose best repeatability occurred when this forage was incubated alone in the jar.

Regarding the associative effects between forage and concentrates, we did not observe variations in the repeatability for sugarcane IVDMD regardless of the incubation condition. However, repeatability for sugarcane IVNDFD was better when forage was incubated along with soybean meal (Figure 2). The incubation of forage alone or the addition of maize to the same incubation jar compromised the repeatability, which worsened when maize and soybean meal were added together to the incubation jar.

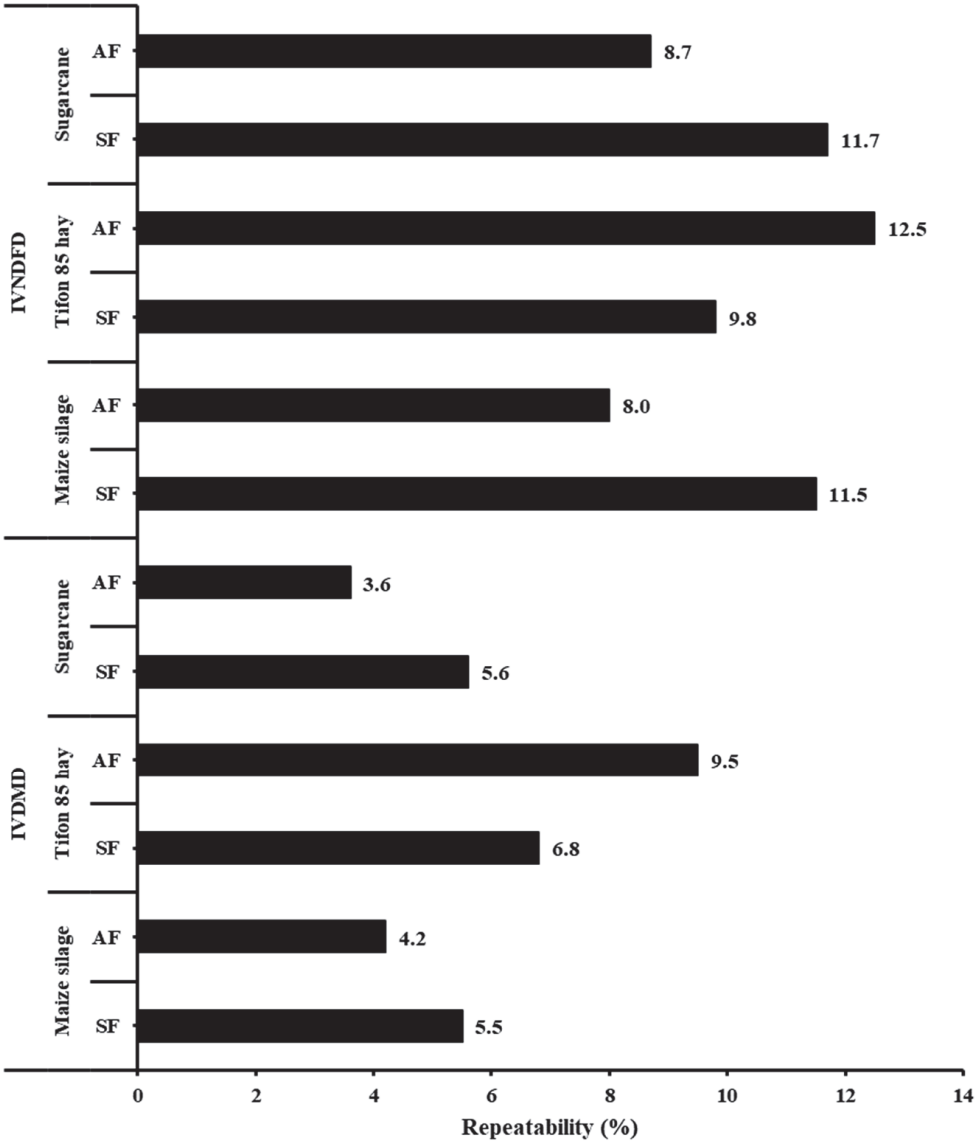

Figure 1. Repeatabilities for in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) according to incubation conditions in assay 1 [SF, single feed (each feed in a separated jar); AF, all feeds (all the feeds in the same jar)] for the three forages under study.

In the first assay, pH and NH_3-N concentration in the incubation jars were not affected by either incubation condition ($p \geq 0.30$), or by the interaction between incubation condition and time ($p \geq 0.35$). However, both variables differ according to the time of evaluation ($p < 0.01$). On average, the pH decreased (6.99 vs. 6.73) and NH_3-N concentration increased (0.51 vs. 4.94 mg/dL) from the beginning to the end of incubation period (Table 4). Regarding the evaluation of associative effects between forage and concentrates, both pH and NH_3-N concentration showed an interaction between incubation condition and time ($p < 0.01$). Slicing of this effect showed no differences between incubations conditions for the initial pH ($p > 0.90$) and NH_3-N ($p > 0.98$), whose average values were 6.91 and 2.16 mg/dL, respectively. The final pH was higher ($p < 0.05$) for the incubation of sugarcane along with soybean meal, when compared to the incubation of forage alone or along with maize. Incubating all feeds together caused an intermediate final pH. The final NH_3-N concentration followed the same pattern observed for the final pH.

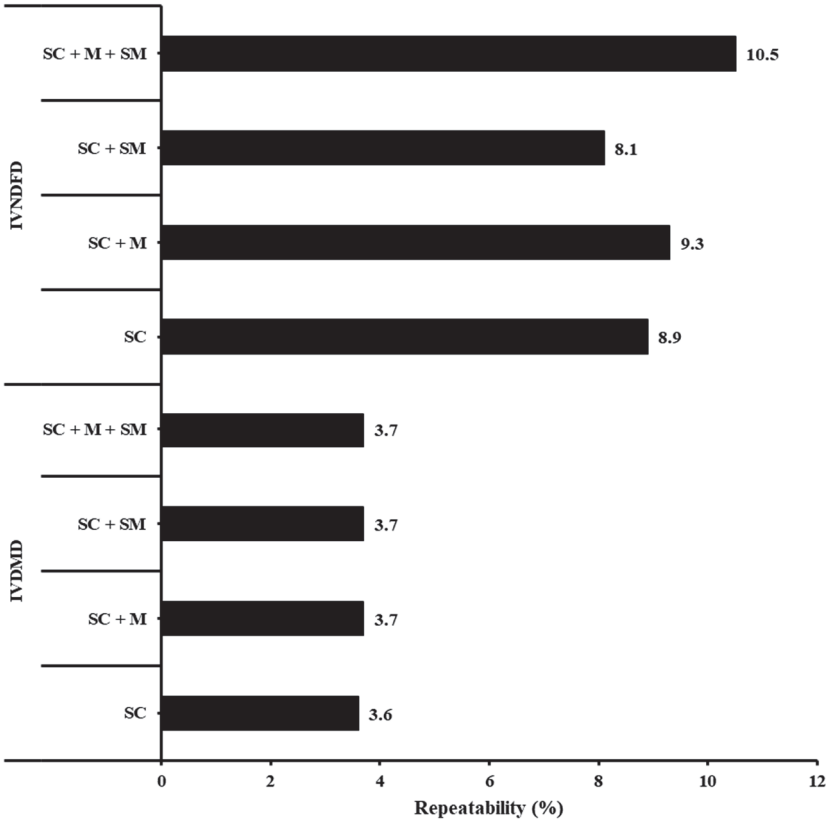

Figure 2. Repeatabilities for in vitro digestibility of dry matter (IVDMD) and neutral detergent fibre (IVNDFD) according to incubation conditions in assay 2 (SC, sugarcane; SC + M, sugarcane plus maize; SC + SM, sugarcane plus soybean meal; SC + M + SM, sugarcane plus maize plus soybean meal).

Table 4. Average descriptive values of pH and ammonia nitrogen (NH_3-N) concentration under different incubation conditions.

	Ph [1]		**NH_3-N (mg/dL)** [1]	
Incubation Condition	**Initial**	**Final**	**Initial**	**Final**
Forages (Assay 1)				
Sugarcane	7.01	6.76	0.47	5.73
Maize silage	6.99	6.77	0.62	6.27
Tifton 85 hay	7.00	6.72	0.29	4.03
All forages together	6.99	6.70	0.66	3.72
p-value				
Incubation condition (IC)	0.764		0.305	
Time (T)	<0.001		<0.001	
IC × T	0.809		0.350	
SEM	0.040		0.762	
Forage plus concentrates (Assay 2)				
Sugarcane + soybean meal + maize	6.91	6.69 ^{ab}	1.90	8.12 ^{ab}
Sugarcane + soybean meal	6.92	6.86 ^{a}	2.04	13.59 ^{a}
Sugarcane + maize	6.93	6.61 ^{b}	2.54	4.05 ^{b}
Sugarcane	6.90	6.63 ^{b}	2.17	3.69 ^{b}
p-value				
Incubation condition (IC)	0.009		0.039	
Time (T)	<0.001		0.001	
IC × T	0.012		0.028	
SEM	0.037		1.429	

[1] Means in column followed by different letters differ at $p < 0.05$.

4. Discussion

Associative effects occur when the digestibility of a mixture of feeds is different from the weighted sum of individual feed digestibilities [18]. In this sense, we understand that the results obtained in our work provide evidence for the occurrence of associative effects between forages, and also between forages and concentrates in terms of DM and fibre digestibility. In agreement with our results, several researchers have pointed out differences in digestibility characteristics when feeds are evaluated separately or together [5,7,19,20].

The effective ruminal digestibility of a feed is an integration between its potential digestibility (an inherent characteristic of the feed) and the digestion environment [21], and its expression depends on the intensity of microbial activity on the substrate [22]. The environment itself encompasses all major factors that can affect the activity of microbial enzyme systems on substrates, such as pH, minerals, nitrogen compounds (i.e., ammonia and peptides), branched-chain fatty acids, etc. [23]. Even though a part of the environment is defined by the own animal (e.g., buffer releasing and N recycling), the feed itself is a potential supplier of substrates for microbial growth, as well as influencing the rumen physicochemical characteristics. Consequently, the rumen environment conditions are interrelated with feed characteristics. When a mixture of feeds is fed into the rumen, most environmental conditions would be determined through the mutual interactions among the components of different feeds [21]. For the case of closed in vitro systems with a single donor animal, such as the one evaluated here, the environmental characteristics among the different incubation conditions would be almost exclusively affected by the feeds.

There are several causes for the occurrence of associative effects among feeds, which can manifest in either a positive or negative way [18]. Evidence of a positive associative effect was verified through sugarcane digestibility in the first assay, whose digestibility was increased by incubation along with the other forages. Overall, sugarcane is a forage with a low content of nitrogen compounds. Hence, under restriction of nitrogenous substrates (i.e., ammonia, amino acids, or small peptides), the microbial fibre degradation is compromised [24] and, consequently, digestibility decreases. When sugarcane was incubated along with forages with higher CP content, an improvement in the nitrogen availability for microbial growth possibly occurred, and its IVDMD and IVNDFD were increased. An improvement in ammonia concentration has not been detected in the first assay. However, it is likely that improvements in the availability of other forms of nitrogenous compounds (e.g., small peptides) were responsible for the better microbial degradation of sugarcane when incubated along with better quality forages. It is noteworthy that these forages had a higher CP content when compared to sugarcane.

On the other hand, the results from the first assay also showed that positive and negative associative effects can be simultaneously observed. Despite the improvement in sugarcane digestibility, the joint incubation of forages decreased the maize silage digestibility. Likely, the joint incubation increased the carbohydrate diversity in the medium. Under this condition, some microbial species (e.g., *Butyrivibrio fibrisolvens Bryant* and *Streptococcus gallolyticus* Orla-Jensen) may change their priority regarding the carbohydrate source used for energy metabolism and growth [25]. This priority shift could decrease the degradation of some carbohydrate sources that would previously be considered as a primary source for microbial growth. Moreover, the inclusion of fast-fermenting carbohydrates to the incubation medium might favour the growth of certain microbial groups with a greater competitive capacity for substrates [26]. Both statements could be associated with the high sucrose content in sugarcane, which seemed to negatively affect the microbial utilisation of maize silage. According to Huhtanen [18], the decrease in fibre digestibility tends to be more pronounced in higher quality forages.

Negative associative effects were also observed in the second assay, when sugarcane was incubated along with maize grain. This inhibited digestion of sugarcane emphasised the occurrence of the carbohydrate effect, which is characterised by a decrease in the cellulolytic activity when fast-fermenting carbohydrates (e.g., starch) are added to the medium. Its causes seem to involve an increased competition among microbial species for

essential nutrients and other compounds necessary for microbial growth. Species with a faster growth rate, such as those that degrade starch, outcompete the fibrolytic microorganisms, which have a slower growth rate, and imply a decreased fibre digestion [26–28]. The carbohydrate effect can be minimized or overcome when the availability of essential substrates in the medium increases, mainly in terms of amount and chemical profile of nitrogenous compounds [28]. This statement explains why the sugarcane digestibility was not compromised when soybean meal was incubated in the same jar, with or without the presence of maize grain, as soybean meal was able to improve the nitrogen availability in the medium.

On the other hand, the forages that showed an evident associative effect in the first assay (i.e., maize silage and sugarcane) also displayed an improved repeatability. Repeatability is a measure of the ability of the method to generate similar results for multiple preparations of the same sample considering the same intra-laboratorial conditions. This pattern highlights that despite the altered digestibility, there was greater homogeneity in microbial activity on the test portions when the feeds were incubated together in the same jar. However, even though Tifton hay has not shown evident associative effects on digestibility, its repeatability worsened when it was incubated together with the other forages. This differentiated pattern seems to indicate that associative effects may affect feed digestibility trough in different ways, which might include loss of precision in some cases. In a logical way, it can be understood that feed itself is an important factor that affects the incubation medium [21]. Thus, the associative effect may incur different changes in digestibility estimates depending on the combination of feeds incubated together in the same jar.

We should highlight that the associative effects evaluated herein are characteristic of artificial fermenters based on the Daisy incubator system, where several samples are incubated together in the same jar. Hence, these effects would not be observed in the classical method proposed by Tilley and Terry [1], in which each test portion is incubated in an individual test tube. According to Wilman and Adesogan [7], this particular associative effect in artificial fermenters could be associated with the escape of soluble material from the samples, which may influence the overall microbial population in the incubation medium. This statement agrees with the results obtained in our work.

In vitro digestibility estimates of feeds can be used according to two different objectives: prediction of in vivo digestibility and comparative evaluation among feeds [11]. For the first case, the occurrence of associative effects observed here warns us that the likelihood of the estimates obtained through artificial fermenters could be improved with the incubation of all feeds that compose the diet, following the proportion planned for in vivo use. In this case, it would be expected that the in vitro environment could present similar interactions among feeds compared to the in vivo ruminal digestion. However, simultaneous in vivo and in vitro evaluations are necessary to provide adequate support for this statement. Moreover, the mutual influence among the forages evaluated here shows that any evaluation aiming at discriminating feeds can be compromised if the incubation of those feeds is performed together in the same jar. However, more studies with a larger number of feeds are suggested to enhance our understanding about the influence of associative effects on a procedure to rank or discriminate feeds.

Additionally, researchers and technicians have used both forms of incubation worldwide (i.e., individually incubated feeds and collectively incubated feeds). Inadvertently, many of them have considered both techniques as similar. We showed that they are not similar. Consequently, most researchers and technicians may not be aware that interactive effects among feeds can affect the in vitro estimates of feed digestibility. Our results showed that they should be aware and should use this information in the interpretation of the laboratory test results and/or to adjust more adequate analysis protocols.

5. Conclusions

The incubation of different feeds together in the same jar using artificial fermenters causes associative effects among them. These effects can influence the estimates of in vitro dry matter and fibre digestibility, and change their repeatability pattern.

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Institutional Review Board Statement: The animal care and handling procedures were approved by the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (protocol number 029/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data generated during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Article

Effects of Cashew Nut Shell Extract on Ruminal Fermentation and Nutrient Digestibility under Continuous Culture

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Abstract: The overall objective of this study was to determine the dose response to four levels of cashew nut shell extract in a granulated form (CNSE, containing 59% anacardic acid and 18% cardol) on culture pH, rumen fermentation metabolites, and apparent nutrient digestibility in continuous culture fermenters. The study was conducted as a generalized randomized complete block design with four treatments and four replications per treatment. The four treatments were randomly assigned to eight fermenters for two incubation runs of 10 d. Treatments consisted of (1) Control (CO, no CNSE), (2) Control plus 100 ppm of CNSE, (3) Control plus 200 ppm of CNSE, and (4) Control plus 300 ppm of CNSE. Fermenters were fed 52 g/d (DM basis) of a total mixed ration (TMR; 17.0% crude protein (CP), 29.7% NDF, and 29.9% starch), divided between two feedings at 0800 and 2000 h. The apparent digestibility of dry matter (DM), organic matter (OM), and neutral detergent fiber (NDF) were not affected by CNSE supplementation. Similarly, CNSE had no effect on culture pH, total volatile fatty acids (VFA) or individual VFA molar proportions. These results suggest that at the dosages evaluated in this study, CNSE has no impact on the rumen fermentation profile and the apparent nutrient digestibility under continuous culture conditions.

Keywords: anacardic acid; continuous culture; digestibility; fermentation; feed additive

1. Introduction

Diet supplementation with some feed additives (e.g., antibiotics), can provide a competitive advantage for specific ruminal microbial populations, thus improving the overall feed utilization efficiency by changing fermentation patterns in the rumen [1,2]. However, there is growing concern about the usage of antibiotics in food animal production due to potential antibiotic resistance and drug residues. As a result, many countries are considering restrictions on the use of antibiotics in feed, and the European Union has banned the use of antibiotics as growth promoters in feed (Regulation 1831/2003/EC). Therefore, researchers are becoming increasingly interested in using natural alternatives such as tannins, essential oils, cashew nut shell extract, and other organic compounds as a way to replace antibiotics to lessen worries about human health risks [3,4].

Cashew nut shell liquid (CNSL) is a by-product of the processing of cashew nuts and is used in various industrial applications [5]. Furthermore, the phenolic compounds found in CNSL, such as anacardic acid, cardanol, and cardol, have been shown to have antimicrobial properties [6]. In particular, anacardic acids have been shown to inhibit gram-positive rumen bacteria [7]. Watanabe et al. [6] reported a decrease in active hydrogen and formate producers (e.g., *Ruminococcus flavefaciens*, *Eubacterium ruminantium*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens*) but increased propionate and succinate producing bacteria species (e.g., *Succinivibrio dextrinosolvens* and *Megasphaera elsdenii*).]. Furthermore, CNSL inhibited CH_4 formation and increased propionate production in a dose-dependent manner under in vitro conditions [6]. The reduction in methanogenesis has been attributed to a

reduction in hydrogen and formate, which are the most important electron donors used by methanogenic bacteria [8]. Although the effect of CNSL on CH_4 formation was also observed in vivo [9], the authors also reported a reduction on total VFA production when the diet fed to non-lactating cows was supplemented with CNSL. Interestingly, technical CNSL (cashew nut shell extract that contains cardanol and cardol as the main active ingredients, but no anacardic acids) had no effect on methane emission in lactating dairy cows but tended to increase NDF digestibility [10]. The variable effects of cashew nut shell byproducts on nutrient digestibility reported in the literature are probably associated with the type and amounts of phenolic compounds found in the additive, as a result of the extraction method, and the dose fed to the animals. Thus, CNSE fed at a level that does not compromise animal performance might be used as a dietary strategy to shift rumen fermentation to improve the efficiency of feed energy utilization in cows. However, there are only a few dose-response studies investigating the effects of CNSE on nutrient digestibility and rumen metabolism [6].

Based on these studies, we hypothesized that incorporating incremental levels of CNSE would not affect nutrient digestibility, but it will shift the production of VFA towards propionate. Thus, the objective of this study was to determine the dose response to four levels of CNSL in a granulated form (cashew nut shell extract, CNSE) on culture pH, rumen fermentation metabolites, and apparent nutrient digestibility in continuous culture fermenters.

2. Materials and Methods

2.1. Experimental Design and Treatments

The study was conducted as a generalized randomized complete block design with four treatments fed to continuous culture fermenters. Treatments consisted of (1) Control (CO, no CNSE), (2) Control plus 100 ppm of CNSE (L), (3) Control plus 200 ppm of CNSE (M), and (4) Control plus 300 ppm of CNSE (H). The granulated CNSE (Agri-Bio Business Department, Idemitsu Kosan Co., Ltd., Tokyo, Japan) contained 50% CNSE (59% anacardic acid and 18% cardol) and was further diluted by premixing it with corn grain before dietary treatment mixing. The content of CNSE in each dietary treatment was not measured to verify that CNSE was added to each fermenter at the intended level. However, in a recent study comparing two ionophore sources, premixes prepared with the same methodology averaged 105% of the expected ionophore content [11]. The CO diet was formulated to meet NRC (2001) requirements for a multiparous cow with 150 DIM, 40 kg/d of milk production, and 26.6 kg/d of predicted DMI (Table 1). The CNSE added to the CO in stepwise increments was equivalent to 2.5, 5.0, and 7.5 g/d per cow with a DMI of 26.6 kg/d for L, M, and H treatments, respectively. For each incubation run, treatments were randomly assigned to 1 of 8 fermenters and run for a 10-d period with 7 d for adaptation and 3 d for sample collection. Fuentes et al. [12] suggested a minimum of 5 d of adaptation to stabilize the microbial population within the cultures. We conducted two incubation runs (block), resulting in a total of 4 replicates per treatment. A total of 52.0 g of diet DM was fed to each fermenter daily in 2 equal portions at 0800 and 2000 h. Dietary treatments and treated corn were mixed using a commercial mixer (Commercial Series 8, KitchenAid, Benton Harbor, MI, USA).

Table 1. Ingredient composition of control diet.

Item	Control Diet (CO)
Ingredient, % of DM	
Corn silage [1]	37.0
Alfalfa hay [2]	15.0
Ground corn	23.7
Solvent soybean meal	9.5
Expeller soybean meal [3]	4.0
Soyhulls	8.0
Energy Booster 100	1.0
Mineral premix [4]	1.8

[1] Chemical composition (DM basis) was 7.2% CP, 38.6% NDF, and 30.2% starch. [2] Chemical composition (DM basis) was 26.5% CP, 30.3% NDF, and 3.1% starch. [3] SoyPlus, West Central Coop. (Ralston, IA). [4] Premix composition: guaranteed minimum concentration (as-fed basis): Ca 17.9%, Zn 7.2%, Mn 5.8%, Cu 1.5%, Fe 1.2%, I 1440 ppm, Co 768 ppm, and Se 288 ppm.

2.2. Continuous Culture Conditions

The care and handling of animals used for collecting rumen contents and in situ incubations were conducted as outlined in the guidelines of the Clemson University Committee on Animal Use (AUP2019-074). On day 0 of each incubation run, rumen fluid and solids were collected from two ruminally fistulated lactating dairy cows that were fed a diet containing 44% corn silage, 4.1% barley silage, and 51.9% concentrate mix (DM basis).

Within 20 min of collection, large particles were removed from the whole ruminal contents by filtration through 2 layers of cheesecloth, and the filtrate containing the microbial population was transferred immediately to the laboratory in a sealed container. With constant stirring, the filtered ruminal inoculum was diluted 1:1 with the buffer [13] and then added to completely fill (approximately 800 mL) each dual-flow fermenter that was modified in construction and operation from the design described by Teather and Sauer [14]. The main modifications were a reconfigured overflow sidearm that was angled downward at approximately 45° to facilitate emptying, a faster stirring rate (45 rpm) that still allowed stratification of particles into an upper mat, a middle liquid layer of small feed particles, a lower layer of dense particles, and a higher feeding rate [15,16]. The buffer solution [13] was delivered continuously using a peristaltic pump to achieve a 0.10/h fractional dilution rate. Each morning, the buffer solution was prepared and adjusted using 6 N NaOH or 3 N HCL to maintain the buffer's pH levels. All fermenters received the same buffer solution, thus treatment effects of CNSE had the opportunity to alter pH. Anaerobic conditions were maintained by purging the cultures with CO_2 at a rate of 20 mL/min and were checked nightly to verify consistency. The temperature of the fermenters was held at 39 °C by a circulating water bath. The culture pH was monitored daily by taking pH readings just before each feeding (Symphony H10P, VWR, Radnor, PA, USA).

2.3. Sample Collection and Analysis

On d 10 of each incubation run, a 5 mL sample of mixed culture contents was collected at 0 (before feeding), 2, 4, 6, 8, 10, and 12 h after feeding into polycarbonate centrifuge tubes containing 1 mL of 25% (*wt*/*v*) metaphosphoric acid and frozen at −20 °C until further analysis. The culture pH was determined immediately after sample collection using a calibrated portable pH meter (Symphony H10P, VWR, Radnor, PA, USA). The culture contents were thoroughly mixed (100 rpm) during the sampling collection and pH measurements to ensure proper mixing. On d 8, 9, and 10 of each incubation run, overflow from each fermenter was collected in 2-L Erlenmeyer flasks submerged in an ice bath and containing 10 mL of H_2SO_4 (50% solution) to prevent further microbial activity. The volume of the overflow flask was measured twice daily (before each feeding). After recording the total volume, a 20% sample of the overflow was collected and immediately frozen at a −20 °C. Frozen overflow samples were later thawed and composited by fermenter and incubation run, and a subsample was dried for 48 h at 55 °C and ground through a

1-mm screen using a Wiley Mill (Arthur H. Thomas Co., Philadelphia, PA, USA). A second subsample was centrifuged at 40,000× *g* for 20 min at 4 °C. The supernatant was carefully removed using 3 mL plastic transfer pipettes, and the remaining sample was immediately dried for 48 h at 100 °C. The resulting dry matter (DM) concentration was used to estimate daily overflow mass.

Ground feed and overflow samples were dried at 105 °C for 24 h to determine analytical DM. Ash concentration was determined after combusting samples in a furnace for 3 h at 600 °C (Method 942.05) [17]. Neutral detergent fiber (aNDFom) and ADFom concentrations were determined using an Ankom200 Fiber Analyzer (Ankom Technology, Faiport, NY, USA) and corrected for ash concentration. Sodium sulfite and α-amylase (Sigma no. A3306: Sigma Chemical Co., St. Louis, MO, USA) were included for NDF analysis [18]. For each ground feed and culture overflow sample, a subsample was submitted to Cumberland Valley Analytical Services (Waynesboro, PA, USA) to determine the concentrations of N (Method 990.03, AOAC) [19] and starch [20]. The feeds and diets' crude protein concentration was calculated as a percentage of N × 6.25. Samples of mixed culture contents were thawed and centrifuged at 40,000× *g* for 20 min at 4 °C. After centrifugation, the supernatant was filtered, diluted with 0.5 mL distilled H_2O, and combined with 100 µL of internal standard (86 µmol of 2 ethylbutyric acid/mL), in a 2 mL GC vial. Samples were injected into a Hewlett-Packard 6980 gas chromatograph (San Jose, CA, USA) fitted with a custom packed column for VFA-flame-ionization detection.

2.4. Statistical Analysis

Data were analyzed with the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) as a generalized randomized complete block design. For apparent digestibility data, the statistical model included the fixed effect of treatment, the random effect of incubation run (block), the random effect of fermenter, and the residual error. The above model was expanded to include the fixed effect of time as a repeated measure, and the interaction between treatment and time, to determine treatment effects on pH, TVFA and VFA molar proportions. The first-order autoregressive covariance structure was used to fit a time series-type covariance structure in which the correlation declines as a function of time. Pre-planned orthogonal contrasts were used to test linear and quadratic effects of treatments (CNSE level). Significant differences and tendencies to differ were declared at $p < 0.05$ and $p \leq 0.10$, respectively.

3. Results and Discussion

As polyphenols were the main contributions to the granulated CNSE, as expected, their increasing concentration in the diet had a limited effect on dietary composition (Table 2). Thus, dietary OM, CP, NDF, and starch content were similar between dietary treatments and averaged 93.9, 17.0, 29.3, and 29.5% (DM basis), respectively.

Table 2. Chemical composition of dietary treatment containing different levels of CNSE fed to continuous culture fermenters.

Item	CNSE (ppm)			
	0	**100**	**200**	**300**
DM, %	92.1	92.4	92.3	91.6
OM, % DM	94.2	93.6	94.1	93.8
CP, % DM	16.7	17.2	17.2	16.9
NDF, % DM	29.2	30.0	28.9	28.9
ADF, % DM	19.1	18.5	19.7	18.5
Starch, % DM	29.9	29.7	29.1	29.4

3.1. Culture Fermentation Measurements

Table 3 presents the least square mean on the effects of CNSE supplementation on culture pH, total VFA concentration and molar proportion of VFAs, and acetate-to-propionate

ratio. Averaged culture pH values were not different between CO and the increasing levels of CNSE (5.76 ± 0.18). As expected, there was a significant ($p < 0.01$) effect of pH over time and the pattern of change was similar between all treatments (Figure 1). The culture pH pre-feeding (time 0) was 5.79; it declined to a nadir on time 6 (5.40) and reached the highest pH at time 12 (6.03). These results are consistent with an in vivo [8] that observed no effect of CNSE on rumen pH. Watanabe et al. [6] observed a lower, albeit negligible, pH value (6.45) on the diets supplemented with different levels of CNSE compared with the control (6.59) under semicontinuous culture conditions.

Table 3. Effect of different levels of CNSE supplementation on continuous culture fermenters metabolites.

Item	CNSE (ppm)				SEM [2]	*p*-Value [1]	
	0	100	200	300		L	Q
pH	5.78	5.72	5.75	5.80	0.20	0.51	0.47
Total VFA, mM	76.8	70.5	72.3	71.2	10.9	0.46	0.51
VFA, mol/100 mol							
Acetate	37.5	39.2	35.8	38.1	1.57	0.96	0.51
Propionate	27.8	26.6	28.6	28.5	8.12	0.67	0.92
Butyrate	17.8	17.6	18.4	16.7	1.81	0.61	0.57
Isovalerate	0.82	1.12	1.09	0.82	0.44	0.82	0.26
Valerate	8.70	8.71	9.30	8.52	1.56	0.93	0.58
A:P [3]	1.24	1.29	1.17	1.21	1.29	0.64	0.78

[1] Probability of a linear (L) or quadratic (Q) effect of CNSE level in the diet. [2] Standard error of the mean (highest when uneven samples). [3] Acetate-to-propionate ratio.

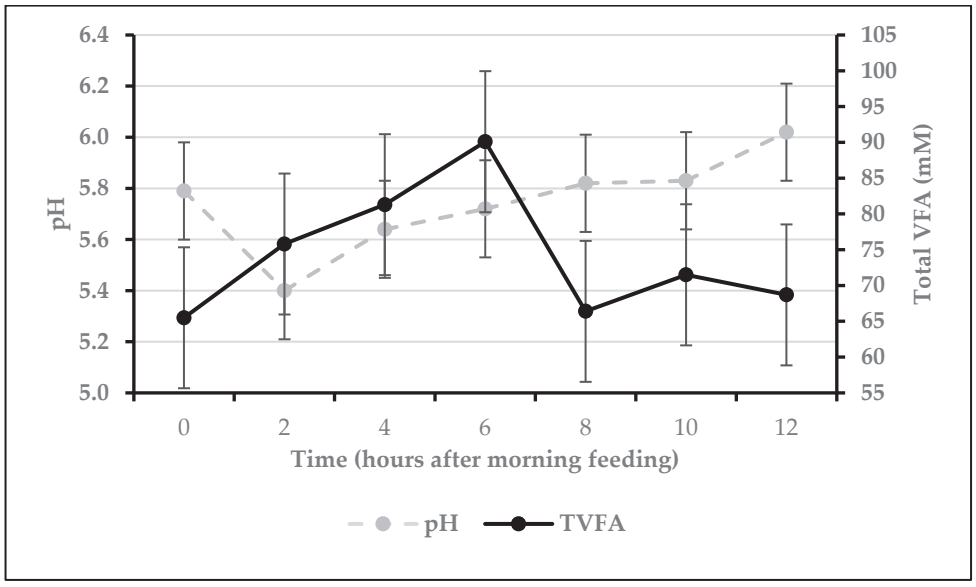

Figure 1. Changes in pH and total VFA of fermenter contents of all treatments (combined) before (0 h) and every two hours after the a.m. feeding on d 10. Bars indicate SEM.

Increasing levels of CNSE in the diet had no effect on total VFA concentration and ranged between 70.5 to 76.8 mM across all treatments. These numbers were similar to those previously reported studies on continuous cultures. For instance, total VFA concentrations were 77.1 mM [15] and 62.9 mM [16] in continuous cultures with no additives. Watanabe et al. [6] observed a small increase in total VFA concentration when CNSL was supplemented to the diet. Conversely, Shinkai et al. [9] reported a significant reduction in VFA concentration in two in vivo studies. Under the condition of this study, CNSE supplementation had no effect on the molar proportions of acetate (37.7 ± 2.35), propionate (27.8 ± 0.94), butyrate (17.6 ± 2.48), isovalerate (0.96 ± 0.18), valerate (8.81 ± 0.59), or A:P

ratio. Results reported in the literature on the effect of CNSE on the VFA profile are inconsistent. For example, both batch culture and continuous culture trials have found a consistent shift in VFA molar proportions towards lower acetate and higher propionate content when CNSL or CNSE were added to the diet [6,21]. Furthermore, similar changes on the molar proportions of acetate and propionate have been reported in an in vivo study [9]. However, when the same product as the one evaluated in this study was fed at 2.5 and 5.0 g/d per day to dairy cows during the transition period, the authors reported no treatment effect on the VFA profile [22]. Also, Sarmikasoglou et al. [23] observed no changes in VFA production or in the molar proportions of acetate and propionate when CNSE (the same product as in the current study) was fed at two different levels (100 or 200 ppm) and under continuous culture conditions.

When taken together, data from this study and from Sarmikasoglou et al. [23] and Goetz et al. [22] suggest that when CNSE with 59% anacardic acid and 18% cardol is fed at a level between 100 and 300 ppm, there is no shift in VFA molar proportion towards propionate. The inconsistent results between studies using CNSL or CNSE on propionate production highlights the importance that the phenolic composition and dose level of CNSE in the diet probably have on the rumen microbial population.

3.2. Apparent Digestibility of Nutrients

Table 4 summarizes the effects of CNSE supplementation on apparent digestibility. Under the condition of this study, apparent digestibility of DM (54.7% ± 7.6), OM (63.8% ± 5.9), NDF (58.8% ± 8.2), and starch (97.2% ± 1.3) were similar among the treatments. Average digestibility values were similar or higher to those previously reported in continuous cultures studies fed dairy diets [24–26]. Sarmikasoglou et al. [23] observed no effect on nutrient digestibility under continuous culture conditions. Shinkai et al. [9] reported a decrease in DM and OM digestibility in one of two feeding trials, when approximately 22 g/d per cow of CNSL (pellet form) was fed to dry cows. This level of supplementation was 4.6 times higher than the highest dose used in the current trial. Furthermore, Shinkai et al. [9] hypothesized that the discrepancy in DM and OM digestibility between the two trials was that a CNSL pellet with greater rumen diffusion was fed in the trial and digestibility was reduced by CNSL supplementation. In their recent study, Goetz et al. [27] observed no effects of CNSE supplementation on DM, OM, NDF, ADF, and starch digestibility. Branco et al. [10], reported that technical CNSL, rich in cardanol and cardol, had no effect on total-tract apparent digestibility of nutrients, except for a tendency to increase digestibility of NDF digestibility. Taken together, data from the current and previous in vitro and in vivo studies suggest that regardless of the level of supplementation, CNSE has little impact on apparent nutrient digestibility.

Table 4. Effect of different levels of CNSE supplementation on nutrient apparent digestibility from continuous cultures.

Item	CNSE (ppm)				SEM [2]	*p*-Value [1]	
	0	100	200	300		L	Q
DM, %	54.2	59.2	53.4	56.2	4.27	0.57	0.57
OM, %	63.3	66.2	62.6	65.5	3.33	0.74	0.85
NDF, %	57.8	62.1	55.7	59.9	4.72	0.71	0.71
Starch, %	97.8	97.5	96.8	97.4	0.65	0.47	0.15

[1] Probability of a linear (L) or quadratic (Q) effect of CNSE level in the diet. [2] Standard error of the mean (highest when uneven samples).

4. Conclusions

In this study, CNSE (containing 59% anacardic acid and 18% cardol) was added at incremental levels to continuous cultures of mixed ruminal microorganisms to determine how they affected the volatile fatty acid profile and apparent nutrient digestibility. Although CNSE supplementation did not influence the overall nutrient digestibility, the

results of this study do not support our hypothesis that increasing levels of CNSE in the diet will promote the production of propionate. Future studies should evaluate CNSE supplementation at a level not evaluated in this study and with a different diet composition before considering CNSE as an additive for ruminants.

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ruminants

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Article

The Lipidome of the Gastrointestinal Tract in Lactating Holstein Cows

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Abstract: The lipidome is a key determinant of structural and functional characteristics of tissues, contributing to optimal gut function and efficiency of nutrient use in the gastrointestinal tract (GIT). Our objective was to study lipidomic profiles in different sections of the GIT in lactating dairy cows and to link them with biological functions. We studied the lipid species in ruminal papillae and epithelium from duodenum, jejunum, and ileum harvested after slaughter from five lactating Holstein cows. Extracted lipids were identified by LC/MS/MS and analyzed via Lipidsearch, Metaboanalyst 5.0, and lipid ontology (LION). Of 1259 lipid species identified across the GIT, 387, 565, 193, and 86 were neutral lipids, phospholipids, sphingolipids, and derivatized lipids, respectively. Among the 1223 lipid species common to the GIT, a PLS-DA analysis revealed similar profiles for jejunum and ileum and discriminated them from rumen and duodenum. The content of 12 out of 28 lipid classes differed ($p < 0.05$) among GIT sections. The average fatty acid chain length in lipid species spanned from 9 to 37 carbons, and the average degree of unsaturation ranged from 0 to 6. The term 'membrane component' from LION analysis differed markedly between the rumen and the small intestine. Future studies will help better understand what factors (function or cellular component) in a given section of the GIT are related to the different lipid species. This is the first description of the lipidome profiles across sections of the GIT in lactating dairy cows. The unique lipidome profiles uncovered distinct structural and functional properties across the bovine GIT, which may impact the efficiency of nutrient use.

Keywords: lipid profiles; nutrition; rumen; small intestine; bovine

1. Introduction

Lipids play a crucial role in an animal's body and are the essential components of the membrane systems, including the cell membrane [1], endoplasmic reticulum (ER) [2], Golgi apparatus (GA), mitochondrial membrane, and vesicles [3]. The double layer of the cell membrane separates the cell contents from the extracellular matrix and plays a role in molecular transport. The different lipid species associated with the ER, GA, and mitochondria support the biological processes of living cells [4]. Phospholipids, the major components of the cell membrane system, form the lipid bilayer in the cell membrane system [5].

Within the enterocytes, a phospholipid monolayer decorated by proteins encapsulates the neutral lipids forming the lipid droplet (LD) in intestinal enterocytes [6] and adipose tissue [7]. The neutral lipids mainly include cholesteryl ester (ChE), triacylglycerol (TG), diglyceride (DG), and monoglyceride (MG). The absorbed dietary lipids from the intestinal lumen are used to synthesize chylomicrons and transport them into the lymph system and then the blood [6]. The fatty acids (FA) released from plasma lipoproteins are transported into cells for esterification into TG or for beta-oxidation to provide energy [8,9].

Various lipids can act as signaling molecules that regulate the metabolism of cells [4]. For instance, sphingolipids have important roles in membrane and lipoprotein structure

and act as second messengers for differentiation factors, cytokines, growth factors, and a growing list of agonists [10]. Sphingolipids play a role in cellular processes such as cell movement, membrane homeostasis, nutrient transport, endocytosis, and protein synthesis. Ceramide (Cer), sphingosine, and sphingosine-1-phosphate, subclasses of sphingolipids, regulate Gq/IP3/Ca2+ and G12/13/Rho/ROCK signaling pathways [11]. Acylcarnitines (AcCa) transport long-chain fatty acids (LCFA), and plasmalogens exert antioxidant functions [12]. Clearly, to better appreciate the potential role of the various lipid species found in the GIT in the context of function requires a high-throughput approach. Lipidomics is a powerful technology for studying all lipid species with mass spectrometry (MS).

The present study aimed to generate the first lipidome profiles of different sections of the bovine GIT and to link them with biological functions. The use of lactating and high-producing Holstein dairy cows provided an ideal opportunity to generate fundamental knowledge of the unique lipidome features of the rumen and the small intestine. These sections of the GIT have unique functions in the context of nutrient digestion and absorption. The lipidome approach would allow an initial exploration of the potential functions conferred by the lipid species detected in sections of the GIT. Our general hypothesis was that different sections of GIT have unique lipid profiles that confer unique functions.

2. Materials and Methods

2.1. Animal Handling and Experimental Design

The Institutional Animal Care and Use Committee (IACUC) at the University of Illinois approved the slaughter of the cows via captive bolt (#19161). Five healthy non-pregnant mid-lactation multiparous Holstein cows (3.40 $\pm$ 0.74 parities; 2.99 $\pm$ 0.09 body condition score; 759 $\pm$ 17 kg body weight) that were housed comingled with other cows in the University of Illinois Dairy Unit herd in free stalls with sand bedding and milked twice per day were selected. These cows were free of clinical disease, were culled due to failure to become pregnant, and were fed a typical total mixed ration composed of corn silage/alfalfa hay with water provided ad libitum. They averaged 136 $\pm$ 3 days in milk and 37 $\pm$ 6 kg milk/d before slaughter. The diet fed contained 17.4% crude protein, 1.74 Mcal/kg net energy for lactation, and 358 g/d methionine in the metabolizable protein (Supplementary Materials File Table S1) and was delivered to the feed bunk at 06:00 and 17:30 h daily. The day of sacrifice (06:00 h), cows were given 300 mg Xylazine intramuscularly to sedate (Rompun®, 100 mg/mL, Dechra, Kansas City, KS, USA), loaded two at a time into a livestock trailer (EBY Maverick LS livestock trailer, EBY, Seymour, IN, USA), and transported 0.8 km from the University of Illinois Dairy Unit to the Veterinary Diagnostics Laboratory, University of Illinois College of Veterinary Medicine, Urbana-Champaign. Cows became recumbent within 10 min of injection and were then euthanized with a penetrating captive bolt and removed from the trailer. Cows were then exsanguinated and within 10 min the body cavity was opened to gain access to the gut tissues.

2.2. Sample Collection

Tissue samples were harvested within 20 min from sacrifice. The ruminal papillae from the ventral sac of the rumen were harvested using surgical scissors. The small intestine was cut from the rumen and placed on a necropsy table where duodenal tissue was collected approximately 25 cm distal from the pyloric sphincter; jejunum was collected approximately 1 m proximal to the ileocecal junction, and the ileum approximately 18 cm proximal to the ileocecal junction [13]. Twenty-five cm segments from the duodenum, jejunum, and ileum were cut into pieces measuring approximately 10 cm $\times$ 20 cm and washed with phosphate-buffered saline to avoid food and microbial contamination. Then, a sterile scalpel blade was used to scrape the epithelium. Samples were collected into cryovials and immediately frozen in liquid nitrogen. Afterward, the tissues were transported to the laboratory and stored at -80 °C.

2.3. Lipid Extraction and Lipidomics

Lipids were extracted according to a previous protocol [14]. Briefly, approximately 30 mg of tissue was homogenized with 150 µL cold LC-MS grade methanol and 450 µL Methyl-tert-butyl-ether (MTBE). Three-hundred µL 25% cold LC-MS grade methanol was added to the samples and vortexed. After centrifugation at 14,000× g for 10 min, the supernatant was transferred to a fresh 10 mL glass tube and dried under a nitrogen gas stream. Lastly, the dried samples were redissolved in 250 µL acetonitrile/isopropanol (v/v, 7:3). Samples were then delivered to the Roy J. Carver Biotechnology Center, University of Illinois (Urbana) and used for Lipid profiling analysis by LC/MS/MS and Lipidsearch (Thermo-Fisher Sci, Waltham, MA, USA) [15].

2.4. Functional Mapping

After removing lipid species that could not be mapped against the lipid ontology (LION) [16], signal counts for individual molecular species were input for data formatting (replace 0 and/or NAs and the sample normalized by the sum of each sample) and enrichment in Ranking mode. A total of 1065 out of 1070 (99.53%) identifiers were matched to LION. The LION term and coordinate lipid species were mapped and reported in Supplementary File Table S2.

2.5. Statistical Analysis

The raw data were normalized by signal counts (to internal standard signals and sample weight). Lipid classifications and distribution bar plots were drawn with Sigma Plot (version 14.0; Systat Software Inc., San Jose, CA, USA). The total lipid content for each section of the GIT was calculated as the sum of signal counts for individual molecular species [15]. The average chain length and average unsaturation of FA were calculated taking into account all the carbons, and the degree of total unsaturation for each lipid species was divided by the chain number for each species and rounded to an integer number. Then, the signal counts of lipid species were summed for each term of average chain length, average unsaturation, and LION. The sum of signal counts for each term was then log2 transformed. The resulting data were analyzed using PROC MIXED in SAS OnDemand for Academics (SAS Institute Inc., Cary, NC, USA) https://welcome.oda.sas.com/login (accessed on 13 February 2022). The model tested the fixed effect of GIT section and statistical differences were determined using pairwise comparisons. SAS results were read in Matlab to extract the least squares means, standard errors of the means, and p-value. Heatmaps were drawn in R pheatmap with the (1.0.12) package. Log2 transformed lipid species data in all sections of the GIT were analyzed using Metaboanalyst 5.0 (https://www.metaboanalyst.ca/docs/Format.xhtml) [17]. By default, missing values were replaced with 1/5 of the minimum positive values of their corresponding variables, and Partial Least Squares Discriminant Analysis (PLS-DA) for the 2D scores plot was performed.

3. Results and Discussion

3.1. Overall Lipid Classification and Distribution

The untargeted lipidomic analyses detected 1259 lipid species in total, with 387 neutral lipids, 565 phospholipids, 193 sphingolipids, 86 derivatized lipids (biotinylation, diazomethane), and 28 fatty acyl and other lipids (Figure 1A). Among all the lipid species, the most abundant group was phospholipid, with 12 classes. Next were neutral lipids and sphingolipids, with five and four classes (Supplementary File Table S3).

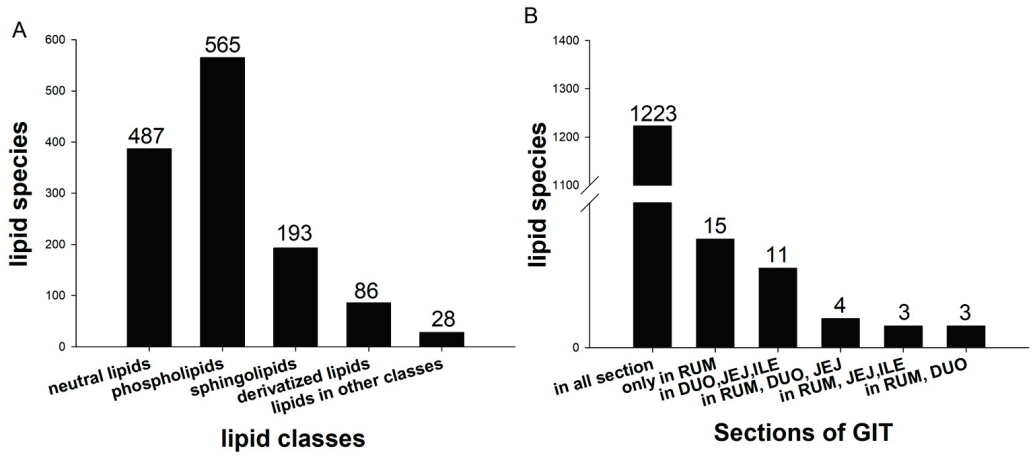

Figure 1. Classification (**A**) and distribution (**B**) of a total of 1259 lipid species identified across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows.

There were 1223 lipids in the rumen, duodenum, jejunum, and ileum. Fifteen lipids only exist in the rumen, which includes one neutral lipid, six phospholipids, two sphingolipids, and six derivatized lipids. Eleven lipid species only existed in the small intestine; four lipid species existed in rumen, duodenum, and jejunum, but not ileum; three lipid species existed in rumen, jejunum, ileum, but not duodenum; three lipid species only existed in rumen and duodenum (Figure 1B, Supplementary Materials Tables S4 and S5).

In the PLS-DA, the rumen and duodenum were separated from the ileum and jejunum (Figure 2). Lipid species with top variable importance in projection (VIP) scores were phosphatidylglycerol (34:2), triglyceride (20:4_20:4_20:4), phosphatidylglycerol (20:5_20:4), acyl carnitine (22:2), and phosphatidylcholine (38:0 e) (Supplementary File Table S6). The general analysis showed that lipid species' composition was more similar in jejunum and ileum, while the rumen and duodenum tended to have unique profiles.

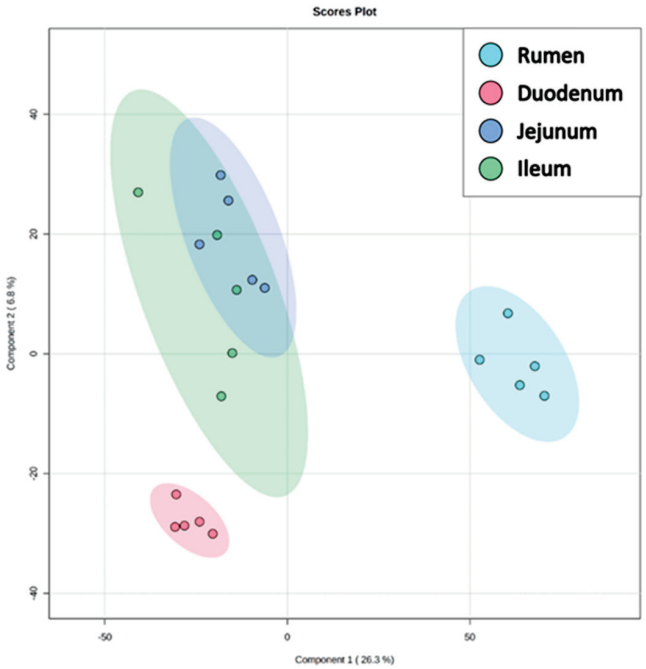

Figure 2. Partial Least Squares Discriminant Analysis (PLS-DA) of common lipid species identified across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows.

In a previous study, lipidomic data from blood revealed differences in phosphatidylglycerol (PG), phosphatidylcholine (PC), sphingomyelin (SM), and TG between cow plasma and calf serum. Compared with colostrum, the cow plasma or calf serum had a greater percentage of PC, phosphatidylinositol (PI), and SM, but a lower percentage of PG [18]. Dynamic changes in plasma lipidomic were also observed in the transition to lactation, where both C 36:6 and PC 32:3 were identified as potential biomarkers for the increase in lipolysis, ketogenesis, and hepatic lipid deposition after parturition [19].

Although few lipid composition data of the rumen and intestine in dairy cows are published, the lipid composition of the intestine was reported for rats, pigs, and rabbits. The rat Golgi membrane of the small intestine contains 26.9% PC, 16.1% cholesterol, 13.2% phosphatidylethanolamine (PE), 12.0% FA, and 10.4% SM (*w*/*w*) of total lipids. Other lipids include ChE, TG, PI, phosphatidylserine (PS), lysophosphatidylcholine (LPC), and cardiolipin (CL); the detected carbon numbers of FA ranged from 14–20 [20]. In the jejunal brush broader membrane of the rat, the main types of lipids were phospholipids, neutral glycolipids, cholesterol, TG, FA, gangliosides, MG, bile acids, and the phospholipids LPC, SM, PC, PS, PI, PE, CL, and lecithin [21,22]. The lipid composition of small intestinal brush broader membrane can be altered by age [21], glucocorticoids [23], fasting, and diabetes [22]. Rat jejunal and ileal microvillus membrane phospholipid contained ~23–31% PE, ~18–31% PC, ~16–30% PS, ~10–22% SM, ~3–4%LPC, and ~2–7% PI % of total lipid phosphorous [24]. Compared with another similar study that only measured PE, PC, PS, SM, and LPC of isolated microvilli membranes [25], the content of total phospholipids was slightly different. The 14-, 16-, 18-, and 20-carbon FA were detected in rat jejunal and ileal microvilli membranes [24]. Cholesterol, ChE, phospholipids, TG, cerebrosides, and gangliosides were detected in the intestinal mucus of rats, and their fractions could be altered by chronic ethanol feeding [26].

In the basolateral plasma membrane of pig intestinal mucosal cells, the main lipid classes were phospholipids and cholesterol, and the phospholipids include ~45% PC, ~31% PE, ~9% PS, ~7% SM, and ~6% PI *w*/*w* of total phospholipid phosphorus; the major carbon numbers of detected FA in PC and PE were 14–24 [27]. The pig intestinal brush border membrane contains ~33–40% PC, ~37–41% PE, ~8–12% PS, ~7–8% SM, ~4–5% PI, ~1–2% LPC, and ~1% of total phospholipids as diphosphatidylglycerol. The FA with 14-, 16-, 18-, 20-, 22-, 24-carbons were detected in these phospholipids [28]. In the jejunal and ileal mucosa of piglets, phospholipids contain ~46–51 mol PC, ~32–41 mol PE, ~9–11 mol SM, ~0.9–2 mol PS, ~0.3–4 mol LPC, and ~0.2–4 mol PI of 100 mol total phospholipids, and they were altered by malnutrition [29].

The rabbit jejunal and ileal basolateral membrane contains cholesterol and phospholipids containing ~33–60% PE, ~13–22% PC, ~12–15% PS, ~12–23% PI, and ~8–17% SM [30]. The rabbit small intestinal brush border membrane contained ~0.5 mg total lipid/mg protein, ~50 μg cholesterol/mg protein, and ~8 μg lipid phosphorous/mg protein; the phospholipids contained phosphatidic acid (PA), PE, ethanolamine plasmalogen, alkylacylgly cerophosphocthanolamine, PS, lysophosphatidylethanolamine (LPE), PC, alkyl and alkenylglycerophosphocholine, LPC, PI, and SM; the neutral lipids contained cholesterol, ChE, DG, TG, and FA [31].

In previous studies, specific parts of the membrane were isolated from intestinal cells, and the different lipid compositions were studied. The cell membrane mainly consists of cholesterol and phospholipids [21,31], and the main phospholipid classes were PE, PC, PS, PI, and SM. Clearly, the use of more robust techniques such as LC/MS/MS to analyze the lipidome in the present study allowed us to detect more lipid species and their profiles in the major sections of the GIT. One limitation of the present study is that we could not separate the brush border membrane from the basolateral membrane of intestinal cells. Thus, future research could help ascertain if these membranes have unique lipid profiles and how those confer unique physiological functions to the tissue.

3.2. Lipid Content Profiles

The lipid content across the GIT is reported in Table 1. Across 28 lipid classes, the lipid content of 12 lipid classes was significantly different among the 4 sections of GIT, with lipid content of 14 lipid classes in the rumen being significantly different compared with the small intestine. The content of CL, monolysocardiolipin (MLCL), LPC, dimethyl phosphatidylethanolamine, lysodimethylphosphatidylethanolamine, AcCa, and cyclic PA in the rumen was lower than in the small intestine ($p < 0.05$). In contrast, the lipid content of PE, DG, cholesterol ester, Cer, ceramide phosphate, ceramide phosphoethanolamines, methyl phosphatidylcholine (MePC), and coenzyme in the rumen was greater than in the small intestine ($p < 0.05$). ChE content was lower in duodenum and jejunum ($p < 0.05$). The zymosterol ester (ZyE) was the greatest in the duodenum ($p < 0.05$). Dimethyl phosphatidylethanolamine content was greater in the duodenum and jejunum ($p < 0.05$). Cardiolipins (CL), localized and synthesized in the inner mitochondrial membrane [32], contains four fatty acyl chains and a lipid dimer consisting of two phosphatidyl residues bridged by glycerol [33,34]. Most of the published studies of CL are related to the high density of proteins in the inner mitochondrial membrane. The mitochondria lacking CL failed to generate ATP during stressful conditions and destabilized the respiratory super complexes [35]. The CL stabilizes the tertiary structure of proteins, such as the ADP/ATP carrier, helps support the proton conduction of protein complexes, and increases super complex association and the arrangement of the protein complex [34]. Monolysocardio-lipin (MLCL) is generated during the degradation of CL through the action of phospholipases [34]. The fact that MLCL and CL content was greater in the small intestine than in the rumen ($p < 0.05$) suggested that mitochondria in the small intestine have a greater amount of inner membrane, likely because absorptive and metabolic processes in this section of the GIT require higher levels of energy consumption [36]. The PE content was greater in the rumen than the intestine, the LPC content was greater in the small intestine than the rumen ($p < 0.05$), and the PC content tended to be greater in the small intestine ($p = 0.06$). The PC content was markedly greater, ranging from 52–60%, followed by TG at 15–21% (Table 1). Of the published data available in bovine, similar analysis to ours revealed that in milk, the relative proportion of TG was ~4-to-10-fold greater than other lipid classes, including PC, DG, and Cer [37,38]. Furthermore, the milk lipidomic profiling was altered by subclinical intramammary infection and dietary supplementation of citrus peel extract and Eucommia ulmoides leaves [37–39]. Additional research might be warranted to explore how disease and nutrition could alter the GIT lipidome and the functional outcomes.

The primary structural lipid class in eukaryotic membranes are the glycerophospholipids, including PC (over 50% of phospholipids), PE (around 20% of phospholipids), PS, PI, and PA. The distributions of phospholipids are different in the plasma membrane, ER, mitochondria, GA, and endosomes [4]. The ER is the main site of lipid synthesis, including phospholipids, cholesterol, and Cer; the GA synthesizes sphingolipid and produces SM, glucosylceramide (GlcCer), lactosylceramide (LacCer), and higher-order GSLs, and these lipids are primarily transported to the plasma membrane [4]. LPC, LPE, Lysophosphatidylglycerol (LPG), and Lysophosphatidylinositol (LPI) result from the hydrolysis of PC, PE, PG, and PI. These molecules can serve as signal mediators by attaching to specific receptors and changing various cellular functions and metabolism [40–42]. The PC and PE are hydrolyzed to lysophospholipids and glycerophosphoryl bases before absorption in the rat [43]. Thus, the differences we detected for PE, LPC, and PC, for example, among sections of the GIT, indicated that unique epithelial membrane composition likely is associated with a unique function.

Table 1. Relative abundance, SEM, and *p*-value of common lipid classes across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows.

Class Group	Class Name	Rumen%	Duodenum%	Jejunum%	Ileum%	SEM	*p*-Value	DIJ * vs. Rumen
Phospholipids	Cardiolipin	0.118	0.154	0.151	0.152	0.000150	0.20	0.04
	Monolysocardiolipin	0.022 ^{b}	0.089 ^{a}	0.072 ^{a}	0.078 ^{a}	0.000194	0.01	<0.01
	Phosphatidylcholine	52.4	54.0	60.2	58.2	0.024640	0.07	0.06
	Phosphatidylethanolamine	6.009	4.180	5.112	5.170	0.004848	0.08	0.04
	Phosphatidylethanol	0.003	0.002	0.002	0.003	0.000004	0.18	0.32
	Phosphatidylserine	1.591	1.502	1.388	1.410	0.001011	0.49	0.20
	Phosphatidylglycerol	0.102	0.088	0.104	0.112	0.000150	0.42	0.95
	Phosphatidylinositol	0.059	0.067	0.067	0.077	0.000129	0.77	0.42
	Lysophosphatidylcholine	0.562 ^{b}	1.953 ^{a}	1.578 ^{a}	1.703 ^{a}	0.004832	0.05	0.01
	Lysophosphatidylethanolamine	0.060	0.041	0.045	0.058	0.000159	0.55	0.34
	Lysophosphatidylglycerol	0.001	0.001	0.001	0.001	0.000003	0.37	0.11
	Lysophosphatidylinositol	<0.001	<0.001	0.001	<0.001	0.000002	0.41	0.43
Neutral lipids	Monoglyceride	0.074	0.065	0.058	0.093	0.000100	0.10	0.85
	Diglyceride	1.026 ^{a}	0.684 ^{b}	0.645 ^{b}	0.707 ^{b}	0.000684	<0.01	<0.01
	Triglyceride	20.6	20.6	15.9	16.4	0.031940	0.59	0.43
	Cholesterol Ester	0.072 ^{a}	0.012 ^{c}	0.023 ^{bc}	0.028 ^{b}	0.000050	<0.01	<0.01
	Zymosterol Ester	0.016 ^{b}	0.091 ^{a}	0.021 ^{b}	0.012 ^{b}	0.000115	<0.01	0.07
Sphingolipids	Ceramides	1.037 ^{a}	0.612 ^{b}	0.500 ^{b}	0.533 ^{b}	0.000581	<0.01	<0.01
	Ceramides phosphate	0.003 ^{a}	<0.001 ^{b}	<0.001 ^{b}	<0.001 ^{b}	0.000003	<0.01	<0.01
	Ceramide phosphoethanolamines	0.001	<0.001	0.001	0.001	0.000002	0.17	0.03
	Sphingomyelin	9.348	9.501	8.615	10.210	0.013140	0.86	0.95
Derivatized lipids (biotinylation, diazomethane)	Bis-methyl phosphatidic acid	2.457	2.169	1.924	1.614	0.002693	0.16	0.08
	Dimethyl phosphatidylethanolamine	0.086 ^{b}	0.147 ^{a}	0.150 ^{a}	0.113 ^{b}	0.000113	<0.01	<0.01
	Lysodimethylphosphatidylethanolamine	0.003 ^{b}	0.013 ^{a}	0.010 ^{a}	0.010 ^{a}	0.000028	0.02	<0.01
	Methyl phosphatidylcholine	3.807 ^{a}	3.580 ^{ab}	3.104 ^{bc}	2.886 ^{c}	0.001793	0.01	0.01
Fatty acyl and other lipids	Acyl Carnitine	0.032 ^{c}	0.094 ^{a}	0.052 ^{bc}	0.082 ^{ab}	0.000143	0.02	0.01
	Coenzyme	0.492 ^{a}	0.344 ^{b}	0.282 ^{b}	0.349 ^{b}	0.000338	<0.01	<0.01
	Cyclic phosphatidic acid	0.0001	0.0011	0.0016	0.0014	0.000005	0.07	0.01

* DIJ = combination of duodenum, jejunum, and ileum, ^{a,b,c} Means on the same row differ ($p < 0.05$).

The dietary ChE is hydrolyzed to cholesterol and FA by cholesterol esterase [44]. The absorbed cholesterol is esterified by acetyl-CoA cholesterol-acetyltransferase within the intestinal cells [45]. Cholesterol is also a component of cell membranes, and its level may change the permeability of cellular membranes [46]. There are no data demonstrating that cholesterol or ChE can be absorbed from the rumen during digestion. Traditionally, the rumen is only considered to play a role in the absorption of volatile fatty acids [47]. Thus, it is unclear if the greater overall ChE content in the rumen, compared to the small intestine, is associated with a specific function.

Previous studies revealed that high-grain diets alter the abundance of genes associated with cholesterol homeostasis in ruminal papillae of dairy cows [46], suggesting that increased cellular cholesterol may be a sign of inflammation and increased cell proliferation [46,48,49]. In plants, vitamin D is synthesized from cholesterol and zymosterol [50]. Zymosterol is also an intermediate of ergosterol, cholesterol, campesterol, and stigmasterol in fungi and bacteria [51]. Thus, we infer that feed and microbial cells contribute zymosterol to the digesta available for digestion and absorption. Although the content of cholesterol increases and ZyE decreases gradually from duodenum to jejunum to ileum, it is unknown at which section of the GIT zymosterol is digested or absorbed. Based on available knowledge, it can be inferred that ZyE, along with cholesterol, FA, and MG, are absorbed in the jejunum. The fact that ZyE concentrations were lower in the jejunum may suggest this molecule is metabolized rapidly within the enterocytes or transported into the circulation. The greater DG in the rumen, compared to the small intestine, coupled with the lack of difference in MG and TG content across sections of the GIT suggested that DG may have specific functions in the rumen.

Ceramide (Cer) de novo synthesis occurs in the ER of the gut and uses sphingoid bases, palmitoyl-CoA, and serine. The complex sphingolipids, including SM, glucosylceramides, and gangliosides, are produced by the GA from Cer [52]. In humans and rats, Cer and/or its metabolites regulate proliferation, differentiation, and apoptosis in epidermal keratinocytes and contribute to innate immune function [53,54]. Cer also plays a crucial role in numerous physiologic and pathologic processes in the gut [52], and the accumulation of Cer in the intestine led to inflammation and cell death in mice [55]. The different Cer and Cer concentrations between the rumen and intestine could be associated with different cellular processes in each section.

Carnitine is a molecule that helps transport LCFA from the cytosol to the mitochondria for oxidation. The small intestine had a greater content of AcCa compared to the rumen, indicating a higher reliance in these sections of the GIT on LCFA transport and metabolism [56]. Although this is a novel outcome of the present study, it is important to recognize that the intestinal epithelial cells have other energy sources, such as glutamate, glutamine, glucose [57], and butyrate [58].

3.3. Chain Length and Unsaturation of Fatty Acids

The average chain length of FA in each lipid class is depicted in Figure 3; details are in the Supplementary File Table S7. Among phospholipids, PC had the highest signal counts among species with mainly 15 to 21 carbons in chain length (Figure 3A). Next was PE, with an average of mainly 17 to 19 carbons, and PS, with an average of mainly 18 to 20 carbons. The chain length of FA ranged from 9 to 22 carbons for the phospholipids. Among neutral lipids, the majority of TG signal counts ranged from FA with 15 to 20 carbons (Figure 3B). The FA chain length of neutral lipids ranged from 9 to 37 carbons. The ChE and ZyE had longer FA with up to an average of 30 and 37 carbons. The SM had the highest signal counts, averaging 17 to 22 carbons (Figure 3C). Next was the Cer with 17 to 22 carbons. Bis-methyl phosphatidic acid (BisMePA) signal counts were highest with 18 to 20 carbons, and MePC signal counts were highest with 16 to 18 carbons. The average FA chain length for all the lipids we measured was mainly from 13 to 23 carbons, and ChE and ZyE included very long chain fatty acids ($\geq$22 carbon).

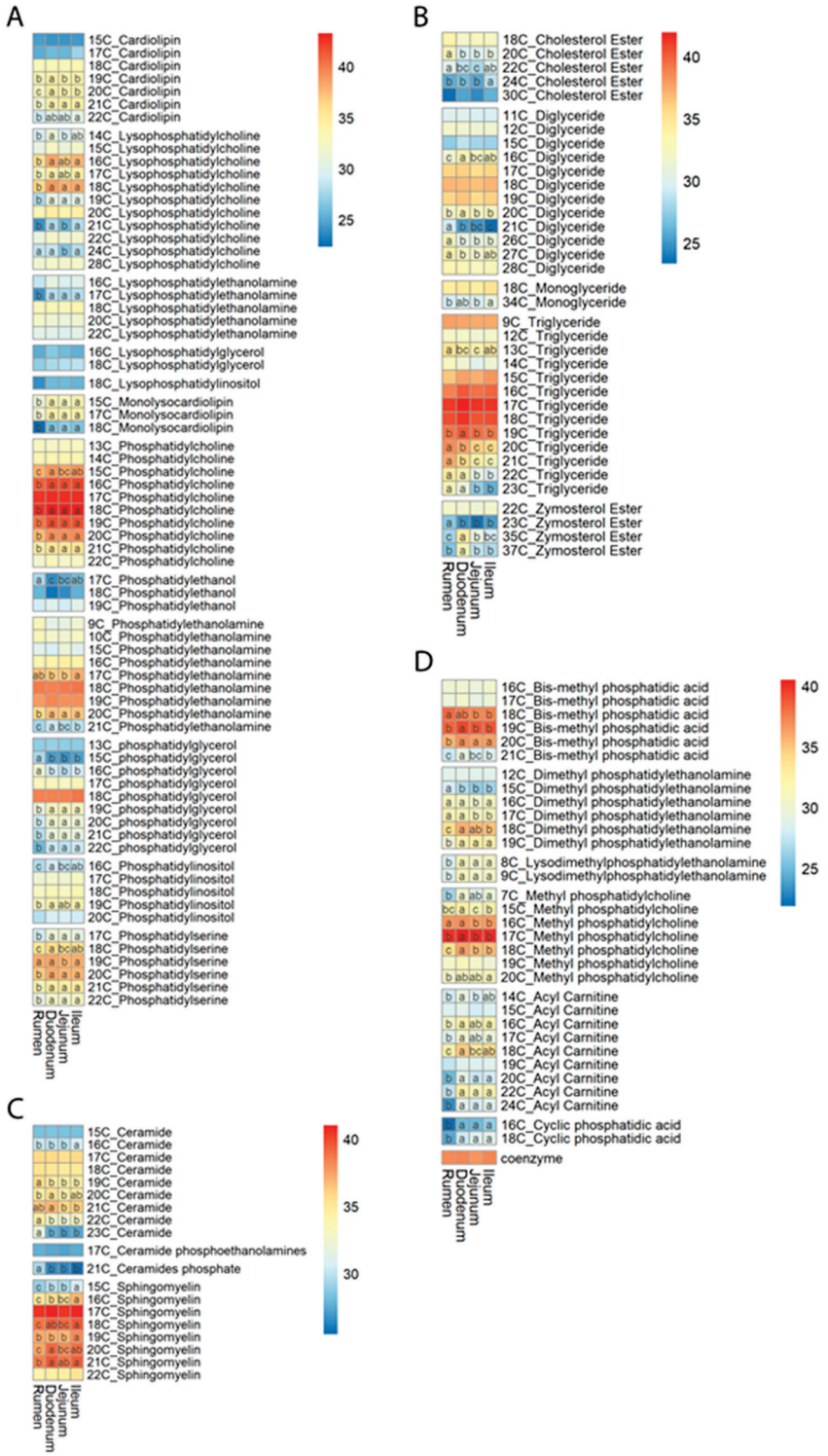

Figure 3. Signal counts of average fatty acid chain length in each lipid class across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows ((**A**): phospholipids, (**B**) neutral lipids, (**C**) sphingolipids, (**D**) derivatized lipids, fatty acyl, and other lipids). The letter C and a number denote the average fatty acid chain length. Red denotes high signal counts, yellow median signal counts, and blue low signal counts in each lipid class. ^{a,b,c} Means with on the same row differ ($p < 0.05$).

The major average degree of FA unsaturation ranged from 0 to 4 double bonds (Figure 4). However, species such as LPC, LPE, PC, PG, ChE, and MePC had up to five

double bonds. The ZyE even contained FA with 6 degrees of unsaturation, and its lowest degree of unsaturation was 3 (Figure 3D). The signal counts of CL and MLCL had an average degree of FA unsaturation of 1, 2, and 3. The signal counts of DG, TG, and SM had an average degree of FA unsaturation of 0, 1, and 2.

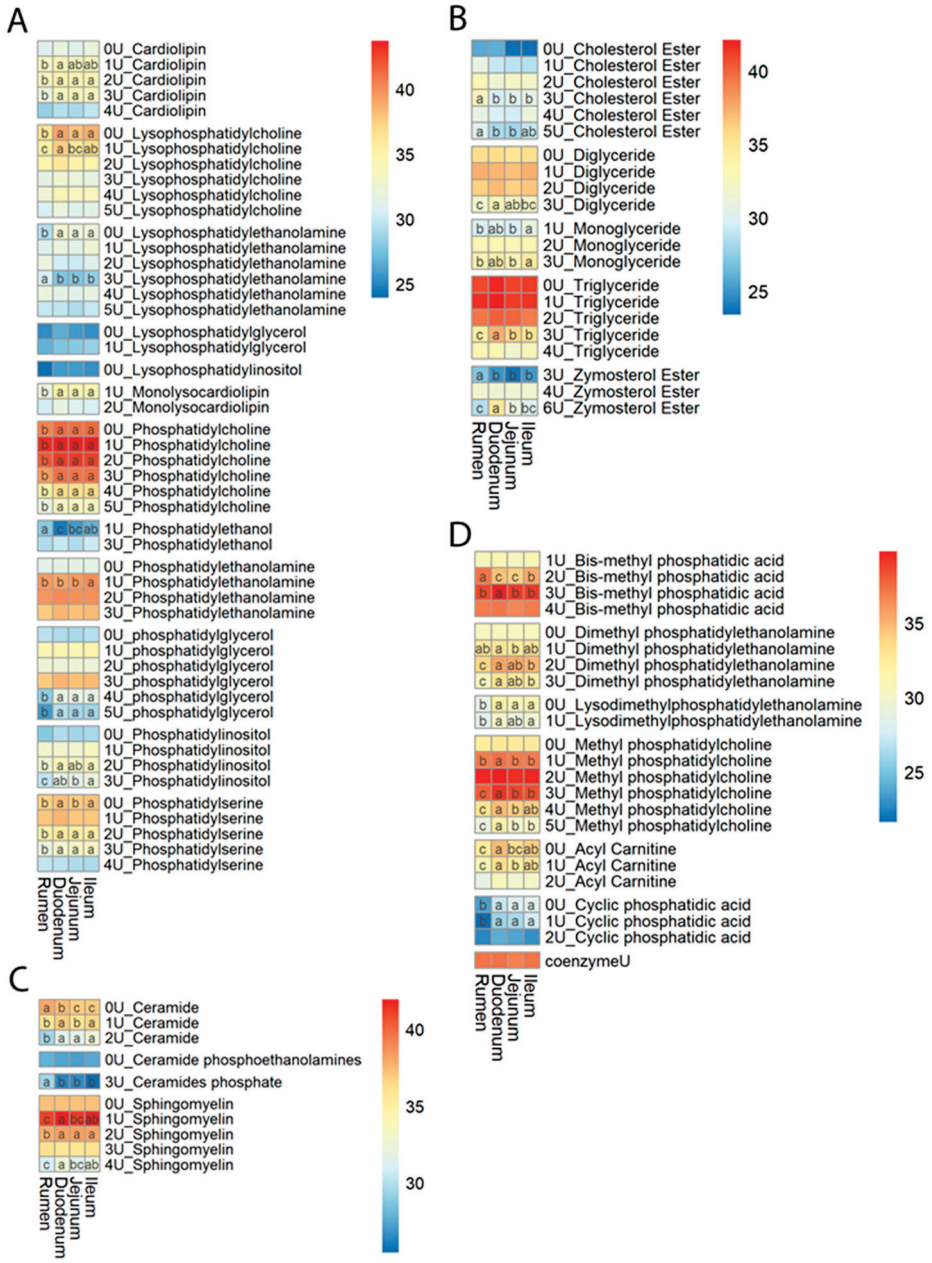

Figure 4. Signal counts of the average degree of unsaturation in each lipid class across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows ((**A**) phospholipids, (**B**) neutral lipids, (**C**) sphingolipids, (**D**) derivatized lipids, fatty acyl, and other lipids). The letter U and a number denote the average degree of unsaturation. Red denotes high signal counts, yellow median signal counts, and blue low signal counts for each lipid class. ^{a,b,c} Means with on the same row differ ($p < 0.05$).

The ruminal bacteria lipolyses esterified lipids in the diet, including TG, galactolipids, and phospholipids, to FA and the glycerol backbone [59]. The forage and concentrate fractions of dairy cow diets usually contain high amounts of C16:0, C18:0, C18:1, and C18:2 FA [60–62]. To avoid the antimicrobial effects of unsaturated FA (UFA), the microorganisms hydrogenate UFA to trans-FA intermediates and saturated fatty acid (SFA), such as

18:0 [59]. Endogenous (De novo synthesis) lipids of microbial origin and the exogenous FA contribute to the total lipid content of bacterial dry mass in the rumen ranging from 10–15% [59]. The ruminal microbial lipids contain higher concentrations of SFA, especially C16:0 (18–23 g/100 g FA) and C18:0 FA (36–52 g/100 g FA), and USFA concentrations are low. The concentration of C18:1 and C18:2 range from 9–14 and 1.8–3.3 g/100 g FA. [61]. The lipidomic data showed the total FA chain length and degree of freedom in each lipid species. Each lipid species had one or more than one FA, and we do not know all the FA chain length and degree of freedom for each FA, making it difficult to compare with previous diet and microbiome FA composition. In the future, more lipidomic data from the diet and ruminal digesta could potentially help a comparison with the present data and help assess potential functional relevance for the GIT.

The shortage of the way of presenting chain length and unsaturation in Figures 3 and 4 is that FA chain length and degree of freedom were calculated by the sum dividing the number of FA and these numbers were rounded, and the possible chain length and unsaturation were recorded in the Supplementary File Table S7. However, we can still have a general idea about their chain length and unsaturation. Among these lipid species, most FA have 16–20 carbons, and ChE and ZyE were mainly attached to very long chain FA (more than 22 carbon). It is unknown, to our knowledge, if the FA absorbed from the lumen participates in the synthesis of ChE and ZyE in the GIT.

3.4. Lipid Ontology

Figure 5 depicts the lipid ontology in each term within LION. In the function category, the sum signal counts of lipid storage and lipid-mediated signaling were not significantly different (Figure 5A). The sum of signal counts of membrane components in the rumen was lower than the small intestine ($p < 0.05$). The sum signal counts of ER, endosome, lysosome, and GA in the rumen were lower than in the intestine ($p < 0.05$) (Figure 5B). The sum of signal counts in very low transition temperature, low transition temperature, average transition temperature, and high transition temperature in the small intestine were greater compared with the rumen (Figure 5C).

The lack of exact concentration of lipids based on the signal counts and the difference in the number of lipid species associated with each term precludes a direct comparison of LION terms in the same section of the GIT. However, we can compare differences for the same term across the various sections of the GIT. Based on the lipid species, LION helped us predict the functions, cell components, and cell characteristics. The DG can either be a signaling molecule or a membrane component in the LION classification, but the LION cannot separate specific functions. The greater sum of signal counts for membrane components and ER, endosome, lysosome, and GA in the intestine predicted that the intestine has a more complex membrane system, potentially due to its unique function in the absorption of nutrients. A more in-depth morphological analysis in the future could help ascertain this prediction.

Lipid phase transitions involve interconversions between various polymorphic (different solid structures formed by lipids) and mesomorphic (the intermediate phase between liquid and crystal) lipid phases. External variables such as temperature, water content, pressure, aqueous phase composition, and the chemical structure of lipids affect lipid self-assembly in different phases [63,64]. With the same FA, the PC transition temperature is lower than PE, and the shorter FA and the more UFA, the lower the transition temperature [63,64]. In the transition temperature category, the LION analysis included 299 lipid species among PC, PS, PG, PE, and SM. Details are provided in Supplementary File Table S8. Within the intestine, the duodenum had greater lipid signal counts associated with the very low transition temperature term.

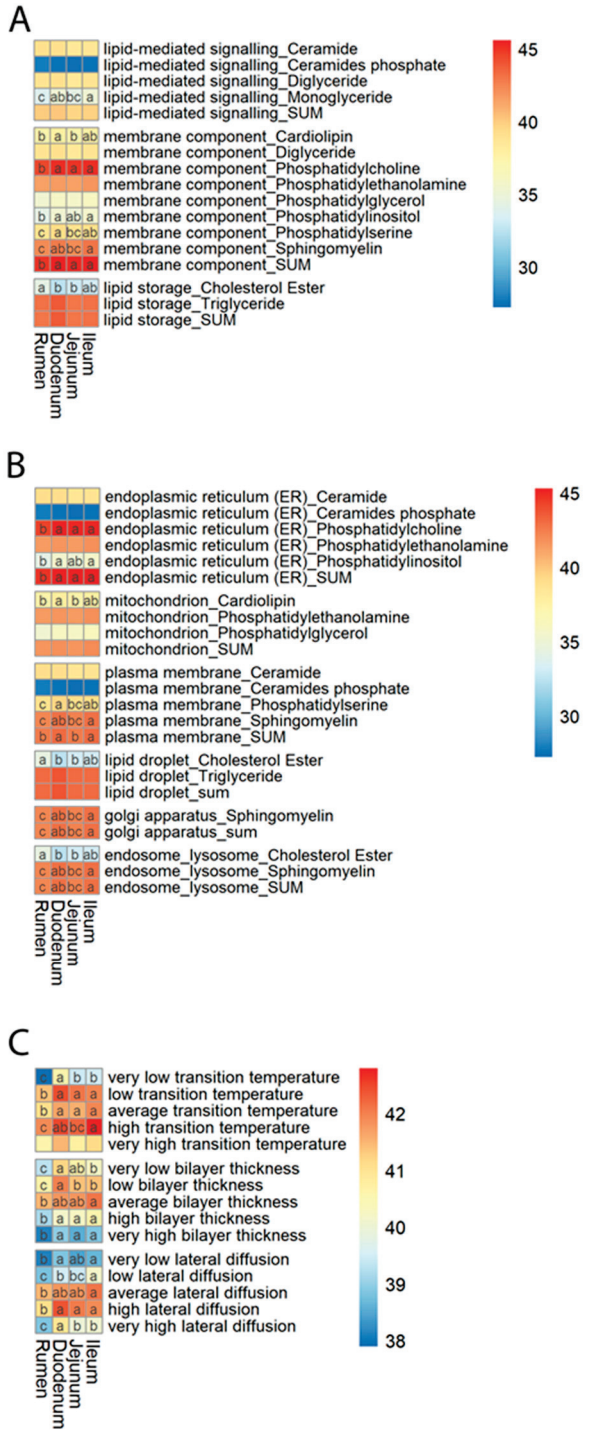

Figure 5. Signal counts of LION function terms in different LION term categories across the ruminal papillae and epithelium from duodenum, jejunum, and ileum of 5 lactating Holstein cows ((**A**) function, (**B**) cellular component, (**C**) transition temperature, bilayer thickness, and lateral diffusion.). Red denotes high signal counts, yellow median signal counts, and blue low signal counts in each lipid class. ^{a,b,c} Means with on the same row differ ($p < 0.05$).

The sum of signal counts in the rumen for bilayer thickness were lower than in the intestine in each LION term associated with bilayer thickness. In the bilayer thickness category, the LION analysis included 213 lipid species among PC, PS, PG, and PE, and its classification is mainly based on the head group of lipids, FA chain length, and unsaturation. Within the intestine, the duodenum tended to have greater sum of signal counts of very

low and low bilayer thickness terms. There were no significant differences among average, high, and very high bilayer thickness, which predicted that the duodenum had a thinner bilayer thickness compared with the jejunum and ileum.

Lateral diffusion is a key parameter in evaluating membrane fluidity of the lipid bilayer membrane and the interaction between the bilayer lipid membrane and solid substrates [65]. In the lateral diffusion category, the LION analysis included 219 lipid species among PC, PS, PG, and PE. Similar to the bilayer thickness category, the sum of signal counts for each term in this category in the rumen was lower than in the intestine. In the very high lateral diffusion category, the duodenum had the highest sum of signal counts, followed by the jejunum and ileum, and the signal counts of the rumen were very low. This indicated that the small intestine had more lipid species related to lateral diffusion. The sum of signal counts of low lateral diffusion in the ileum were highest, and there was no significant difference in very low, average, and high lateral diffusion among the intestinal segments. The sum of signal counts of very high lateral diffusion in duodenum compared with jejunum and ileum predicted that the ileum had less lateral diffusion than the duodenum and jejunum. The same evaluation indicated that the duodenum had higher lateral diffusion than jejunum and ileum.

4. Conclusions

For the first time, the lipid profiles across sections of the GIT have been determined using LC/MS/MS. We focused on these four sections because the rumen is the most important compartment in the forestomach, and the intestine is the major section where nutrient absorption occurs. Most of the lipid species that could be detected exist in all four sections of the lactating dairy cow GIT. The lipid composition of jejunum and ileum are very similar and differ markedly from the rumen and duodenum. Close to 50% of the lipid groups detected had a different profile across the four sections of the GIT. The average chain length of FA in the lipids detected ranged from 9 to 37 carbons, and the average degree of unsaturation ranged from 0 to 6. LION analysis predicted a more complex membrane system in the intestine than in the rumen, the duodenum had a thinner bilayer thickness among the small intestinal sections, and lateral diffusion ability was higher in the duodenum and lower in the ileum. A limitation of the present study was that the lipidomic data pertains to the whole tissue, disregarding the specific contribution of organelles. It was not feasible to ascertain if the differences in lipid profiles are partly associated with dietary or microbial sources. Due to the lack of published data, the functions and distribution of certain lipids in cells of the rumen could not be discerned. Future studies will have to be performed to better understand what factors (function or cellular component) in a given section of the GIT are related to the different lipid species.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ruminants3010007/s1: Table S1: ingredient composition of the diet; Table S2: LION terms and lipid identifiers; Table S3: lipid groups and classes; Table S4: Lipid species only exited only in rumen; Table S5: Lipid species that were in 2–3 sections of GIT; Table S6: variable importance point (VIP) score of PLS-DA; Table S7: average fatty acid chain and degree of unsaturation; Table S8: LION function.

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