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Tesis presentada por Rokia Temmar y dirigida por el Dr. **Sergio Calsamiglia** y Dra. **María Rodríguez**, del Departamento de Ciència Animal i dels Aliments de la Universitat Autònoma de Barcelona.

Bellaterra, 22 de diciembre de 2022

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CERTIFICO:

Que la memoria titulada "**Los efectos de los aditivos alimentarios, aceites esenciales o ácido guanidinoacético, sobre la fermentación microbiana del rumen bovino en estudios *in vitro*"**, presentada por **Rokia Temmar**, ha sido realizada bajo mi dirección y, considerándola finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos que correspondan, firmo el presente certificado en Bellaterra, 22 de diciembre de 2022.

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“Praise be to Allah, Lord of the Universe, the Almighty and Merciful, who has inspired us and filled us with blessings, we give him thanks;

To my mother and my father, who supported, guided, helped me, and surrounded me with all their love, their affection, their patience, and their understanding;

To my sisters, my brother, nieces and nephews for always being by my side;

In memory of my dear mother-in-law, may Allah have mercy on her;

To my husband Ali for his love, his patience, and for always being by my side;

To my father-in-law and my husband's brothers, Walid and Hamza, for their sympathy and kindness”.

I dedicate this work

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SHORT BIOGRAPHY

Rokia Temmar from Algiers (Algeria, 1984), graduated in Agricultural Engineering "Animal Production" at the Higher National Agronomic School (ENSA, Algiers, DZ) in 2009. She obtained a master in science journalism at the Higher National Journalism School (ENSJ, Algiers, AL) in 2014. After obtaining a competitive grant from the Spanish Ministry of Foreign Affairs (2015-17), she earned the double official M. Sc. degree in "Animal Nutrition" (2017) from the International Centre for Advanced Agronomic Mediterranean Studies (CIHEAM-IAMZ) and the University of Zaragoza (Zaragoza, ES). Thereafter, she was contracted as a Research Assistant at the Department of Animal and Food Sciences of the University Autonomous of Barcelona (UAB) in Bellaterra (ES), where she applied research and performed her Ph.D. in Animal Production (2017-22). During this period, she updated her knowledge with several expertise courses on ruminant nutrition, analytical methodology, statistical analyses, and languages. She also earned the official qualification of "Research staff user of animals for experimentation" (UAB - Generalitat de Catalunya). She presented their experimental results in international meetings (ADSA, EAAP), received a grant from the CowefficieNcy EU project for 3 months stay of research at the Department of Medical-Veterinary Sciences of the University of Parma (Parma, IT). The current Ph.D. thesis is a final requisite for obtaining her PhD with a mention as International Doctorate.

SCIENTIFIC PUBLICATIONS

Publications in International peer-reviewed journals (SCI)

Temmar, R., M. E. Rodríguez-Prado, G. Forgeard, C. Rougier, and S. Calsamiglia. 2021. Interactions among natural active ingredients to improve the efficiency of rumen fermentation in vitro. Animals 11:1205. https://doi.org/10.3390/ani11051205.

Simoni, M., **R. Temmar**, D. A. Bignamini, A. Foskolos, A. Sabbioni, M. Ablondi, A. Quarantelli, and F. Righi. 2020. Effects of the combination between selected phytochemicals and the carrier's silica and Tween 80 on dry matter and neutral detergent fiber digestibility of common feeds. Ital. J. Anim. Sci. 19:723–738. https://doi.org/10.1080/1828051X.2020.1787882.

International Conference symposia

Temmar, R., M. E. Rodríguez-Prado, A. Kihal, V. K. Inhuber, and S. Calsamiglia. 2022. Effects of guanidinoacetic acid in dairy or beef microbial conditions on rumen microbial fermentation and nutrient flow from a continuous culture system. ADSA

Annual Meeting. J. Dairy Sci. 105:366. https://www.adsa.org/Meetings/2022-Annual-Meeting/Abstracts.

Temmar, R., M. E. Rodríguez-Prado, A. Kihal, V. K. Inhuber, and S. Calsamiglia. 2022. Effects of guanidinoacetic acid on rumen microbial fermentation in continuous culture system. 73rd Annual Meeting EAAP. Book of abstracts. Porto, PT. https://doi.org/10.3920/978-90-8686-937-4.

Temmar, R., M. E. Rodriguez-Prado, G. Forgeard, C. Rougier, and S. Calsamiglia. 2020. Improving rumen microbial fermentation profile with natural active ingredients. ADSA Virtual Annual Meeting. J. Dairy Sci. 103:41–42. https://www.adsa.org/Meetings/2020-Annual-Meeting/Abstracts.

Temmar, R., M. E. Rodriguez-Prado, G. Forgeard, C. Rougier, and S. Calsamiglia. 2020. Exploring synergistic interaction between essential oils in vitro rumen microbial fermentation. ADSA Virtual Annual Meeting. J. Dairy Sci. 103:42. https://www.adsa.org/Meetings/2020-Annual-Meeting/Abstracts.

LIST OF ABBREVIATION

A:P	Acetate to propionate ratio
AA	Amino acid
AC	Active compound
ADF	Acid detergent fiber
ADG	Average daily gain
ADP	Adenosine diphosphate
AGAT	Arginine glycine amidino transferase
ATP	Adenosine triphosphate
BAC	Blend of anise and cassia oils
BCVFA	Branched-chain volatile fatty acids
BW	Body weight
CAGR	Compound annual growth rate
CCO	Corn cob
COR	Coriander oil
CP	Crude protein
CTR	Control
DIAT	Diatomaceous earth
DM	Dry matter
DMI	Dry matter intake
EMPS	Efficiency of microbial protein synthesis
EO	Essential oils
EOAC	Essential oil active compound
EU	European Union
Exp	Experiment
FDA	Food and drug administration
G:F	Gain to feed ratio
GAA	Guanidinoacetic acid
GAMT	Guanidinoacetate methyltransferase
GHG	Greenhouse gases
GIN	Ginger oil
HAP	Hyper-ammonia producing bacteria
LEM	Lemongrass oil
MON	Monensin
NDF	Neutral detergent fiber
OM	Organic matter
RAP	Rapeseed oil
SAM	S-adenosyl methionine
SIL	Silica-sunflower
TMR	Total mixed ration
VFA	Volatile fatty acids

ABSTRACT

The effects of feed additives, essential oils or guanidinoacetic acid, on rumen microbial fermentation in *in vitro* studies

Three studies were carried out in order to evaluate the effect of supplementing dietary additives, essential oils (EO) or guanidinoacetic acid (GAA), on rumen microbial fermentation to improve rumen fermentation efficiency in cattle using *in vitro* studies. In study 1, a total of 12 EO: anise star, cassia, geraniol, lemongrass (LEM), limonene, thyme, tea tree, coriander (COR), capsicum, black pepper, turmeric, and ginger (GIN), in Exp. 1 at three doses; and different combinations of LEM, COR and GIN oils in Exp. 2, were evaluated in 24-h *in vitro* batch microbial fermentation. All treatments, negative control and monensin (MON) as positive control were added to 1:1 rumen fluid:buffer containing a 50:50 forage: concentrate diet with an initial pH of 6.6. In Exp.1, data were analyzed with the MIXED procedures of SAS, and treatment means were compared to the control using Dunnett's multiple comparison test. Significance was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. In Exp. 2, data were analyzed according to the Simplex Centroid Design using R-Studio. In Exp.1, LEM tended to increase the propionate proportion and tended to decrease the acetate to propionate ratio. Anise star, COR, and thyme tended to increase butyrate proportion. Capsicum, COR, and thyme decreased NH_3-N concentration. In Exp. 2, a synergy was observed between LEM and COR that resulted in an increase in total VFA and propionate proportion, and a decrease in the acetate to propionate ratio. However, the addition of high doses of GIN to the LEM + COR mix had an antagonistic effect on the rumen fermentation profile.

In study 2, two 24-h *in vitro* studies were conducted to evaluate the effect of a blend of anise star and cassia oils (BAC) and the effect of adding LEM with four different carriers: silica-sunflower (SIL), rapeseed oil (RAP), diatomaceous earth (DIAT) and corn cob (CCO), on rumen microbial fermentation. In Exp. 1, treatments were: no additive as a negative control, monensin (MON) as a positive control, BAC, LEM, SIL, RAP, DIAT, CCO, LEM+SIL, LEM+RAP, LEM+DIAT and LEM+CCO. In Exp. 2, BAC and LEM were used to estimate total gas and methane production. All treatments were added to a 1:1 rumen fluid:buffer for Exp.1, and 1:4 rumen fluid:buffer for Exp.2, containing a 50:50 forage: concentrate diet with an initial pH of 6.6. In Exp.1, data were analyzed with the GLM procedures of SAS, and orthogonal contrasts analysis was used for comparison between control, EO and carriers. In Exp. 2, data were analyzed with the MIXED procedure of SAS, and differences between means were evaluated using Tukey's multiple comparison test. Significance was declared at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. In Exp. 1, BAC and LEM increased total VFA, and SIL, RAP and CCO tended to increase total VFA. Among the combinations of EO+carriers, only the combination LEM-RAP had an effect, decreasing ammonia-N concentration. In Exp. 2, BAC decreased total gas and methane production, and the ratio methane:total gas. In contrast, LEM increased total methane and methane:total gas ratio.

In study 3, eight dual flow continuous culture fermenters were used in 2 periods (7 days adaptation and 3 days sampling) to evaluate the effect of GAA on rumen microbial fermentation and nutrient degradation in dairy and beef cattle type diets. The study was a randomized block design. Treatments (n = 4) were arranged in a 2 × 2 factorial, with factors being the type of fermentation conditions: beef (pH between 5.5 and 6.5; diet 10:90 forage:concentrate, 16.3% CP and 17.6% NDF) or dairy (pH between 5.8 and 6.8; diet 50:50 forage:concentrate, 17.1% CP and 30.0% NDF); and GAA: 0 vs. 2 g/d. Temperature (38.5 ºC), liquid (0.10/h) and solid (0.05/h) dilution rates were kept constant. Diets (90 g/d DM) were fed in 3 portions/d. Effluent samples were collected from a

composite of the 3 sampling days, and bacteria were isolated on the last day of each period from fermenters for protein metabolism calculations. Fermenter samples were taken 3 h after the morning feeding of sampling days for microbiome analysis. Fermentation data were analyzed with the PROC MIXED of SAS and the microbiome diversity and composition with R-Studio. Significance was set at $P < 0.05$ and $0.05 < P < 0.10$ was considered a tendency. No differences were observed in true OM degradation. Degradation of NDF, the proportions of acetate and butyrate, the acetate to propionate ratio, NH_3-N concentration, the flow of total N and ammonia N, the efficiency of microbial protein synthesis, and alpha and beta diversity of microbial population were higher in dairy than in beef. Total VFA and the propionate proportion were higher in beef than in dairy. The GAA increased NH_3-N concentration and the flow of total and ammonia N. The microbial degradation of GAA was higher in dairy (69.8%) than in beef (6.30%) fermentation conditions.

In conclusion, careful selection, and combination of EO may result in useful mixtures with synergistic interactions to modulate rumen microbial fermentation profile. The use of GAA did not affect rumen microbial fermentation. However, the GAA degradation was higher in dairy than in beef, which require GAA protection from rumen microbial degradation in the case of dairy cows.

RESUMEN

Los efectos de los aditivos alimentarios, aceites esenciales o ácido guanidinoacético, sobre la fermentación microbiana del rumen en estudios *in vitro*

Se llevaron a cabo tres estudios para evaluar el efecto de la suplementación de aditivos dietéticos, aceites esenciales (EO) o ácido guanidinoacético (GAA), en la fermentación microbiana ruminal para mejorar la eficiencia de la fermentación ruminal en bovinos mediante estudios *in vitro*. El primer estudio está compuesto por dos Exp. En el Exp. 1, se utilizaron un total de 12 EO: estrella de anís, cassia, geraniol, limoncillo (LEM), limoneno, tomillo, árbol de té, cilantro (COR), pimienta negra, cúrcuma y jengibre (GIN), a tres dosis en Exp. 1; y en el Exp. 2 se utilizaron diferentes combinaciones de los EO LEM, COR y GIN. En ambos casos, se evaluaron como estudios *in vitro* de 24 h de fermentación microbiana. Todos los tratamientos se incubaron en una solución que contenía una proporción 1:1 líquido ruminal:tampón y se utilizó una dieta 50:50: forraje:concentrado con un pH inicial de 6.6. En el Exp.1 se analizaron los datos con el PROC MIXED de SAS y se compararon las medias de tratamiento con el control mediante la prueba de comparación múltiple de Dunnett. La significación se estableció en $P < 0.05$, y $0.05 < P < 0.10$ se consideró una tendencia. En el Exp. 2, los datos fueron analizados según el Simplex Centroid Design usando R-Studio. En el Exp.1, el LEM tendió a aumentar la proporción del propionato y a disminuir la proporción del acetato: propionato. La estrella de anís, el COR y el tomillo tendieron a aumentar la proporción de butirato. El capsicum, el COR y el tomillo disminuyeron la concentración de N-amoniacal. En el Exp. 2, se observó una sinergia entre LEM y COR que resultó en un aumento en la proporción total de VFA y propionato, y una disminución en la proporción de acetato:propionato. Sin embargo, la adición de altas dosis de GIN a la mezcla LEM + COR tuvo un efecto antagonista en el perfil de fermentación ruminal.

En el estudio 2, se realizaron dos Exp. *in vitro* de 24 horas para evaluar el efecto de una mezcla de aceites de anís estrellado y cassia (BAC) y el efecto de agregar LEM con cuatro portadores diferentes: sílice-girasol (SIL), aceite de colza (RAP), tierra de diatomeas (DIAT) y mazorca de maíz (CCO), sobre la fermentación microbiana ruminal. En el Exp. 1, los tratamientos fueron: control negativo (no aditivo), monensina (MON) como control positivo, BAC, LEM, SIL, RAP, DIAT, CCO, LEM+SIL, LEM+RAP, LEM+DIAT y LEM+CCO. En el Exp. 2, se utilizaron BAC y LEM para medir la producción total de gas y metano. Todos los tratamientos se incubaron en una solución que contenía una proporción 1:1 líquido ruminal:tampón para Exp.1, y de 1:4 para el Exp. 2. Se utilizó una dieta 50:50: forraje:concentrado, con un pH inicial de 6.6. En el Exp.1 se analizaron los datos con el PROC GLM de SAS y se realizaron contrastes ortogonales para la comparación entre control, EO y los portadores. En el Exp. 2 se analizaron los datos con el PROC MIXED de SAS y la comparación de medias se realizó mediante la prueba de comparación múltiple de Tukey. La significación se declaró en $P < 0.05$, y $0.05 < P < 0.10$ se consideró una tendencia. En el Exp. 1, BAC y LEM aumentaron el total de VFA, y SIL, RAP y CCO tendieron a aumentar el total de VFA. Entre las combinaciones de EO+portadores, solo la combinación LEM-RAP tuvo un efecto, disminuyendo la concentración de NH_3-N. En el Exp. 2, BAC disminuyó la producción total de gas y de metano, y la relación metano: gas total. En contraste, el LEM aumentó metano total y la relación metano: gas total.

En el estudio 3, se utilizaron ocho fermentadores de cultivo continuo de doble flujo en 2 períodos (7 días de adaptación y 3 días de muestreo) para evaluar el efecto de GAA en la fermentación microbiana ruminal y la degradación de nutrientes en dietas de tipo

vacuno lechero y de carne. El estudio fue un diseño de bloques al azar. Los tratamientos (n = 4) se organizaron en un factorial 2×2, siendo los factores las condiciones de fermentación: bovino de carne (pH entre 5.5 y 6.5; dieta 10:90 forraje:concentrado, 16.3% PC y 17.6% NDF) o bovino de leche (pH entre 5.8 y 6.8; dieta 50:50 forraje:concentrado, 17.1% CP y 30.0% NDF); y la adición de GAA: 0 vs. 2 g/d. La temperatura (38.5 ºC), la tasa de dilución de líquidos (0.10/h) y sólidos (0.05/h) se mantuvieron constantes. La dieta (90 g/d DM) se suministró en 3 porciones/d. Se recolectaron muestras compuesta del efluente de los 3 días de muestreo, y se aislaron bacterias el último día de cada período de cada fermentador para el cálculo del metabolismo proteico. Se colectaron además muestras de cada fermentador 3 h después de la alimentación matutina de los días de muestreo para el análisis de microbioma. Los datos de fermentación se analizaron con el PROC MIXED de SAS y la diversidad y composición del microbioma con R-Studio. La significación se estableció en $P < 0.05$ y $0.05 < P < 0.10$ se consideró una tendencia. No se observaron diferencias en la degradación verdadera de la OM. La degradación de NDF, las proporciones de acetato y butirato, la relación acetato: propionato, la concentración de NH_3-N, el flujo de N total y N amoniacal, la eficiencia de la síntesis de proteínas microbianas y la diversidad alfa y beta de la población microbiana fueron más altas en las condiciones de fermentación del bovino lechero que en bovino de carne. La proporción total de VFA y propionato fue mayor en bovino de carne que en bovino lechero. El GAA aumentó la concentración de NH_3-N y el flujo de N total y amoniaco. La degradación microbiana de GAA fue mayor en condiciones de fermentación bovino lechero (69.8%) que en de bovino de carne (6.30%).

En conclusión, la selección cuidadosa y una adecuada combinación de EO puede resultar en mezclas útiles con interacciones sinérgicas para modular el perfil de fermentación microbiana ruminal. El uso de GAA no afectó la fermentación microbiana del rumen. Sin embargo, la degradación del GAA fue mayor en bovino leche que en bovino de carne, lo cual indica que la protección del GAA contra la degradación microbiana ruminal es requerida en el caso del bovino lechero.

RESUM

Els efectes dels additius alimentaris, olis essencials o àcid guanidinoacètic, sobre la fermentació microbiana del rumen en estudis *in vitro*

Es van dur a terme tres estudis per avaluar l'efecte de la suplementació d'additius dietètics, olis essencials (EO) o àcid guanidinoacètic (GAA), a la fermentació microbiana ruminal per millorar l'eficiència de la fermentació ruminal en bovins mitjançant estudis *in vitro*. A l'estudi 1, un total de 12 EO: estrella d'anís, càssia, geraniol, llimonet (LEM), limonè, farigola, arbre de te, coriandre (COR), pebrot negre, cúrcuma i gingebre (GIN), a Exp. 1 a tres dosis; i diferents combinacions d'olis LEM, COR i GIN a Exp. 2, es van avaluar durant 24 h de fermentació microbiana per lots in vitro. Tots els tractaments es van afegir a 1:1 líquid ruminal: tampó que contenia una dieta 50:50: farratge: concentrat amb un pH inicial de 6.6. En el Exp.1 es van analitzar les dades amb el procediment mixt de SAS i es van comparar les mitjanes de tractament amb el control mitjançant la prova de comparació múltiple de Dunnett. La significancia es va establir a $P < 0.05$, i $0.05 < P < 0.10$ es va considerar una tendència. En el Exp. 2, les dades van ser analitzades segons el Simplex Centroid Design utilitzant el R-Studio. En el Exp.1, el LEM va tendir a augmentar la proporció del propionat i ha disminuir la proporció de l'acetat al propionat. L'estrella d'anís, el COR i la farigola van tendir a augmentar la proporció de butirat. El capsicum, el COR i la farigola van disminuir la concentració d'amoníac-N. En el Exp. 2, es va observar una sinergia entre LEM i COR que va resultar en un augment en la proporció total de VFA i propionat, i una disminució en la proporció d'acetat a propionat. Tot i això, l'addició d'altes dosis de GIN a la barreja LEM + COR va tenir un efecte antagonista en el perfil de fermentació ruminal.

A l'estudi 2, es van realitzar dos Exp. *in vitro* de 24 hores per avaluar l'efecte d'una barreja d'olis d'anís estrellat i cassia (BAC) i l'efecte d'afegir LEM amb quatre portadors diferents: sílice- gira-sol (SIL), oli de colza (RAP), terra de diatomees (DIAT) i panotxa de blat de moro (CCO), sobre fermentació microbiana ruminal. En el Exp. 1, els tractaments van ser: control negatiu, monensina (MON) com a control positiu, BAC, LEM, SIL, RAP, DIAT, CCO, LEM+SIL, LEM+RAP, LEM+DIAT i LEM+CCO. En el Exp. 2, es va utilitzar BAC i LEM per estimar la producció total de gas i metà. Tots els tractaments es van afegir a 1:1 líquid ruminal:tampó per al Exp.1, i 1:4 líquid ruminal:tampó pel Exp.2, que contenia una dieta de 50:50: farratge: concentrat i amb un pH inicial de 6.6. Al Exp.1 es van analitzar les dades amb els procediments GLM de SAS i es va utilitzar l'anàlisi de contrastos ortogonals per la comparació entre control, EO i portadors. Al Exp. 2 es van analitzar les dades amb el procediment mixt de SAS i es van avaluar les diferències entre mitjanes mitjançant la prova de comparació múltiple de Tukey. La significancia es va declarar en $P < 0.05$, i $0.05 < P < 0.10$ es va considerar una tendència. En el Exp. 1, BAC i LEM van augmentar el total de VFA, i SIL, RAP i CCO van tendir a augmentar total de VFA. Entre les combinacions de EO + portadors, només la combinació LEM-RAP va tenir un efecte i va disminuir la concentració de NH3-N. En el Exp. 2, BAC va disminuir la producció total de gas i metà, i la relació metà/gas total. En contrast, LEM va augmentar el metà total i la relació metà/gas total.

A l'estudi 3, es van utilitzar vuit fermentadors de cultiu continu de doble flux en 2 períodes (7 dies d'adaptació i 3 dies de mostreig) per avaluar l'efecte de GAA a la fermentació microbiana ruminal i la degradació de nutrients en dietes de tipus lacti i boví de carn. L'estudi va ser un disseny de blocs aleatoris. Els tractaments (n = 4) es van organitzar en un factor 2×2, sent els factors el tipus de condicions de fermentació: boví de carn (pH entre 5.5 i 6.5; dieta 10:90 farratge:concentrat, 16.3% PC i 17.6% NDF) o boví de llet (pH entre 5.8 i 6.8; dieta 50:50 farratge:concentrat, 17.1% CP i 30.0% NDF);

i GAA: 0 vs. 2 g/d. Temperatura (38.5 ºC), velocitat de dilució de líquids (0.10/h) i sòlids (0.05/h). Les dietes (90 g/d DM) es van alimentar en 3 porcions/d. Es van recollir mostres d'efluents d'un compost dels 3 dies de mostreig, i es van aïllar bacteris el darrer dia de cada període a partir de fermentadors per al càlcul del metabolisme proteic. Les mostres de fermentador es van prendre 3 hores després de l'alimentació matinera dels dies de mostreig per a l'anàlisi de microbioma. Les dades de fermentació es van analitzar amb el procediment mixt de SAS, i la diversitat i composició del microbioma amb R-Studio. La significança es va establir a $P < 0.05$ i $0.05 < P < 0.10$ es va considerar una tendència. No es van observar diferències en la degradació verdadera de l'OM. La degradació de NDF, les proporcions d'acetat i butirat, la relació acetat a propionat, la concentració de NH_3-N, el flux de N total i amoníac N, l'eficiència de la síntesi de proteïnes microbianes i la diversitat alfa i beta de la població microbiana van ser més altes en boví lleter que en boví de carn. La proporció total de VFA i propionat va ser més gran en boví de carn que en boví lleter. El GAA va augmentar la concentració de NH_3-N i el flux de N total i amoníac. La degradació microbiana de GAA va ser més gran en condicions de fermentació boví lleter (69.8%) que en de boví de carn (6.30%).

En conclusió, la selecció acurada i la combinació d'EO pot resultar en barreges útils amb interaccions sinèrgiques per modular el perfil de fermentació microbiana ruminal. L'ús de GAA no va afectar la fermentació microbiana del rumen. No obstant això, la degradació del GAA va ser més gran en boví lleter que en boví de carn, cosa que requereix la protecció del GAA contra la degradació microbiana ruminal en el cas del boví lleter.

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Literature review

1.1. Introduction

Ruminal fermentation is a key process in ruminant nutrition. Due to the symbiotic relationship between the rumen microbiota and the host animal, ruminants can benefit from all types of plant material degraded by rumen bacteria (Van Soest, 1994). In addition, this microbiota can synthesize high-quality protein from dietary nitrogen. However, this fermentation process is accompanied by losses of energy in the form of methane and protein in the form of nitrogen. These losses constitute an economic loss and threat to the environment, hence the need to reduce these emissions through the modulation of ruminal fermentation towards the production of more propionate and less acetate, methane, and nitrogen emissions.

Several strategies have been used to improve the fermentation efficiency in ruminants, including the use of ionophore antibiotics, such as monensin, as feed additives, which have shown positive results by reducing the population of Gram-positive bacteria. (McGuffey et al., 2001; Duffield et al., 2008). However, the spread of antibiotic resistance from animals to humans and the growing public concern about its use, led to the ban on the use of antibiotics as growth promoters in the UE since January 2006 (Directive1831/2003/CEE; EC, 2003). Hence, the need to find alternatives to replace this kind of growth promoters.

Essential oils (EO) have been valued since ancient time and by different civilization for their therapeutic qualities in disease prevention and treatment, and for their flavors and taste in cooking (Hyldgaard et al., 2012). From the year 2000, EO regained great interest because they are natural products with high antimicrobial activity, and many *in vitro* screenings were set up to identify the individual effect of EO on rumen microbial fermentation, but results were variable and inconsistent among studies (Calsamiglia et al., 2007; Benchaar et al., 2008). These EO can be combined and, when a combination of EO occurre, the effect may be additive, synergistic, or antagonistic. A common problem in most feed additives for ruminants that are available in the market are based on combination of different EO or their main active compounds without considering whether there is compatibility among them. While synergistic or additive effects are beneficial,

the potential antagonistic effects may render the mix ineffective or negative (Fandiño et al., 2020).

Another growth promoter additive that was treated in this thesis is the guanidinoacetic acid (GAA) which is the direct precursor of creatine and its phosphorylated derivative phosphocreatine (Ostojic, 2015; 2017). The GAA was used for the first time for humans, then in chickens and pigs. Several studies have shown that GAA could improve growth performance, promote muscle development, and improve health status in monogastric when used as a feed additive (He et al., 2018; Liu et al., 2021). Recently, scientists have been interested in studying the effect of supplementing ruminant diets with GAA. Li et al. (2020) reported that the supplementation of the Angus bull diet with GAA improved rumen VFA production, microbial growth, and enzyme activity. However, some studies have reported that GAA can be 50 % degraded in the rumen (Speer et al., 2020).

The aim of using these feed additives is to improve rumen fermentation efficiency and reduce the environmental impact of methane and nitrogen emissions.

1.2. Ruminal fermentation

Dairy and beef cattle are mammals with 4 stomach compartments, namely rumen, reticulum, omasum, and abomasum. Of these digestive organs, the rumen, which is an anaerobic fermentation chamber, has the largest volume, accounting for more than 80% of the four stomach compartments (Van Soest, 1994). The rumen and reticulum are rich in microorganisms, mainly composed of bacteria, protozoa and fungi that perform the fermentation. Together, these compartments are responsible for breaking down ingested feed (Dryden, 2008). The omasum and abomasum have the function of recycling water and minerals, and of secreting digestive enzymes, respectively, while the digestion takes place in the abomasum and intestine. This configuration allows the ruminant to have a microbial pre-digestion of the feed in the rumen, facilitating the extensive use of the fiber present in the diet (Kamra, 2005).

The rumen is considered an ecosystem rich in microorganisms that live in symbiosis with the host (Figure 1.1) and contains one of the most diverse and dense populations of microorganisms known in nature. These microorganisms adapted to live in an anaerobic environment characterized by a pH between 5.5 and 7.0, and a temperature between 38 and 41ºC, degrade, through hydrolysis and fermentation processes, most components of

the diet. An overview of physical, chemical and microbial characteristics of the rumen is presented in Table 1.1 (Mackie et al., 2001).

Table 1.1. An overview of physical, chemical and microbiological characteristics of rumen ecosystem (Adapted from Mackie et al., 2001).

Physical properties	
Dry matter (%)	10 - 18
Osmolality	250 - 350 mOsmol/kg
pH	5.5 – 7.0 (Mean 6.4)
Redox potential	−350 to −400 mV
Temperature	38 - 41°C
Chemical properties	
Amino acids and oligopeptides	<1 mmol/L (present 2-3 h post feeding)
Ammonia	2 - 12 mmol/L
Dietary component (cellulose, hemicelluloses, pectin)	Always present
Endogenous (mucopolysaccharides)	Always present
Gas phase (%)	CO_2 65, CH_4 27, N_2 7, O_2 0.6, H_2 0.2
Growth factors	Good supply; branched-chain fatty acids, other unknown
Lignin	Always present
Minerals	High Na; generally good supply
Nonvolatile acids (mmol/l)	Lactate <10
Soluble carbohydrates	<1 mmol/L (present 2-3 h post feeding)
Trace elements/vitamins	Always present; good supply of vitamins B
Volatile fatty acids (mmol/l)	Acetate 60–90, propionate 15–30, butyrate 10–25, branched chain and higher 2–5
Microbial properties	
Anaerobic fungi	10^{3-5} /g (6 genera)
Bacteria	10^{10-11} /g (>200 species)
Bacteriophage	10^{7-9} /g particles/mL
Ciliate protozoa	10^{4-6} /g (25 genera)

1.3. Ruminal ecosystem

Ruminants rely on their rumen microorganisms to enzymatically break down and ferment ingested plant material to provide energy in the form of volatiles fatty acids (VFA), and protein supply to meet maintenance, growth and production (milk or meat) requirements (Van Soest, 1994; Greathead, 2003). Rumen microbes can be divided into

three main groups: bacteria, protozoa, and fungi. There is an extensive microbiology literature on the subject. For bacteria alone, there are at least between 300 and 400 phylotypes and more than 20 protozoa identified to date (Firkins and Yu, 2006; Edwards et al., 2004). The filtered rumen contents consisted of a microbial suspension containing about 10^{10} bacteria and 10^{6} protozoa per milliliter, but this is not uniform because many protozoa and bacteria are associated with the solid digesta. The average concentration of microorganisms in solid matrices is higher than in free suspensions up to an order of magnitude. Also, fungi are associated with the solid digesta (Hungate, 1988; Czerkawski, 1988).

- **Bacteria**

The group of bacteria in the rumen is very diverse, but the actual number of active bacteria depends on the animal species, the type and chemical composition of the fermented substrate, the frequency of feeding, and many other factors, known or unknown. The bacterial density in the rumen can exceed 10^{11} bacteria /g of rumen content (Mackie et al., 2001; Wright et al., 2011). They represent about half of the rumen biomass and a major part of rumen metabolic activity, which is inversely proportional to the size of the microbe. Typically, only 15-20 genera are present in an animal rumen at any given time, a number enough to perform important rumen functions. However, once a new ingredient is introduced into the diet, bacteria modify their metabolism to turn fermentation towards the utilization of the new ingredient (Ørskov, 1994).

The bacterial community in the rumen can be divided according to the fermentation substrate into bacteria that break down cellulose, hemicellulose, starch, sugars, intermediate acids, proteins, pectins or lipids, or according to the fermentation end-products into bacteria that produce VFA, methane, NH_3, …etc. Bacteria can also be classified according to their localization in the rumen into bacteria attached to feed particles, which constitute about 75% of bacteria and contribute to a large extent to the breakdown of food in the rumen. A second, more non-specific bacterial group associated with the liquid phase and is made up of bacteria that have been released from the particles and populations with high rates of division that subsist on soluble nutrients in the ruminal fluid. Finally, a third group of facultative anaerobic bacteria adhered to the ruminal epithelium. These bacteria associated with the ruminal epithelium quickly consume the oxygen that enters with feed and water and are specialized to degrade epithelial cells without actively intervening in the degradation of substrates. They also have great

protease and urease activity, through which they intervene in the recycling of urea (Cheng and Costerton, 1977).

- **Protozoa**

Rumen ciliate protozoa are larger organisms than bacteria and represent only a small fraction, about 10^6 cells / mL, but in terms of mass, protozoa are almost equal to that of bacteria present in the rumen, representing about 50% of the viable biomass in the rumen (Mackie et al., 2001; Wright et al., 2011). Cellulolytic and hemicellulolytic protozoa play an important role in the degradation of carbohydrates and proteins. They have a positive role in the degradation of starch (but less rapidly than that of bacteria). Other protozoa can consume lactic acid and limiting the risk of acidosis. Some types of protozoa can scavenge oxygen in such a way that they have a stabilizing effect on anaerobiosis. Most of them degrade proteins very efficiently and release amino acids and NH_3. Ciliated protozoa produce large amounts of hydrogen, which is a substrate for methanogens. Ciliated species are predators of other protozoa, prokaryotes and fungi. Indeed, a single protozoan cell can swallow up to several thousand bacteria in an hour, so they play a very important role in the stability of the rumen microbial population (Williams et al., 2020). Also, ciliates can protect readily fermentable carbohydrates (sugars and starches) from sugar/starch-utilizing bacteria to ensure a constant energy and protein supply to the animal in the form of short-chain volatile fatty acids. Therefore, organic acids are not produced in abundance immediately after feeding animals and these sugars are released slowly during the day. The species of ciliate protozoa commonly observed in a rumen are *Dasytricha ruminantium, Entodinium caudatum, E. exiguum, Epidinium caudatum* and *E. Bicaudatum* (Kamra, 2005).

- **Methanogens**

Methanogens are one of the main representatives of the archaea group. In the rumen, the number of methanogens is about 10^9/g of rumen content (Mackie et al., 2001; Wright et al., 2011). Previously, methanogens were assigned to bacteria, but were later classified as a separate archaeal domain due to special cell wall structures and 16S rRNA sequences (Janssen and Kirs, 2008; Frey et al., 2010), Although bacteria and archaea have the same ancestors, methanogens establish themselves in the gastrointestinal tract within 1-3 days after birth and reach their peak within 3 weeks (Skillman et al., 2004). Methanogens are a unique group of microorganisms that produce methane as the end-product of their

metabolism, but they depend on the H_2 and CO_2 produced from the fermentation of monosaccharide by rumen microorganism to produce methane (Mackie et al., 2013). In a process called syntrophic hydrogen transfer, methanogens receive hydrogen and use it to reduce carbon dioxide to methane (Krause et al., 2013; Lan and Yang, 2019). The function of the methanogens as hydrogen sink in the rumen is very important, because the accumulation of hydrogen slows down the fermentation process and its efficiency due to an accumulation of reducing equivalents (Boadi et al., 2002). Therefore, methanogens maintain steady-state fermentation in the rumen (Garrity et al., 2007; Jin et al., 2014). However, the methane produced by ruminants represent a loss of energy for the animal and has a negative impact on the environment, because it is considered a greenhouse gas. Therefore, many research projects have focused on finding strategies that can mitigate methane production in the rumen without affecting rumen fermentation efficiency (Hook et al., 2010; Patra, 2012; Henderson et al., 2015).

- **Fungi**

The number of rumen anaerobic fungi is about 10^6/g of rumen content and represent 5-8 % of total rumen biomass (Mackie et al., 2001; Wright et al., 2011). They grow slowly within 8-10 days after birth. Its appearance in the rumen was not identified until much later because they were confounded with and classified as flagellated protozoa. Rumen fungi were first discovered in 1975 due to the presence of chitin in their cell walls (Orpin, 1975). They play a role in fiber degradation as they fully hydrolyze the non-lignified tissues of plants and partially degrade the lignified tissues of plants (Akin and Benner, 1988). One study showed that alfalfa and bermudagrass stems incubated with fungi were weakened by 55% and 57%, respectively, while the bacteria alone only weakened them by 42% and 29%, respectively (Akin et al., 1990). So far, only 6 genera of rumen anaerobic fungi have been identified, namely *Neocallimastix*, *Piromyces* (formerly *Piromonas*), *Caecomyces* (formerly known as *Sphaeromonas*), *Orpinomyces*, *Anaeromyces* (formerly *Ruminomyces*), and *Cyllamyces* (Orpin, 1977; Gold et al., 1988).

- **Bacteriophages**

Bacteriophages are another important component of the rumen microbiome and are present at more than 10^9 particles/mL (Mackie et al., 2001; Wright et al., 2011). These phages contribute to bacterial mass turnover in the rumen which may be considered not useful for the animal host (Klieve and Swain, 1993). The specificity of phages for specific

rumen bacteria can be exploited by lysis to remove undesirable rumen bacteria and control methanogen population and other community members in the rumen (Klieve et al., 1999; Bach et al., 2003). McAllister and Newbold (2008) reported siphophages capable of infecting the methanogens *Methanobacterium, Methanobrevibacter* and *Methanococcus spp*. Despite lack of knowledge, ruminant phages and their enzymes represents an interesting opportunity to control methanogenic populations and can be used as strategy to reduce methane production.

1.4. Microorganism-host relationship in the rumen

Microbial populations interact in the ecosystem of the rumen to maximize the efficiency of feed fermentation (Van Soest, 1994). There are two types of microorganisms: those that ferment feeds and those that produce fermentation products from fermented feeds (Dai et al., 2015). The second population has the essential function of eliminating the final product of the first fermentation group and providing the factors necessary for their growth. Therefore, many end-products that were not detected in mixed cultures of rumen microorganisms were found in pure cultures. An example of these relationships is branched-chain volatile fatty acids (BCVFA), which are produced by amylolytic species and are essential for cellulolytic species for the synthesis of amino acids or to produce long-chain fatty acids (Van Soest, 1994).

The relationship between the rumen microbiota and the host animal is called a symbiotic relationship (Figure 1.1). This is a sustainable biological relationship that benefits 2 or more organisms belonging to different species (here rumen microbiota and the host animal; Bladen et al., 1961). This relationship can be illustrated by the degradation of the plant cell wall (Scharen et al., 2018). After ingesting these cell walls, fungi and bacteria attach to their surfaces (Bryant, 1973). Fungi first act mechanically through the development of rhizomes in the plant cell wall. The attachment of bacteria and fungi to the fibers allows access of bacterial enzyme secretions to the plant cell wall. Rumination (approximately 12 hours per day) is a physiological phenomenon whereby the host animal regurgitates previously rapidly ingested feed, then chews and finally swallows the contents. Chewing makes it possible for microorganisms to open new attack fronts in plant tissues and stimulate the flow of saliva. The smaller the particle size, the greater the bacterial colonization. On the other hand, protozoa ingest tiny plant particles to digest them. The fermentation produces VFA, NH_3, and gases such as methane and

carbon dioxide, which are exhaled by ruminants. The VFA are absorbed by animals at the level of rumen epithelial cells, avoiding a drop in rumen pH that would be detrimental to further microbial degradation (Jouany, 1994; Lobo and Faciola, 2021).

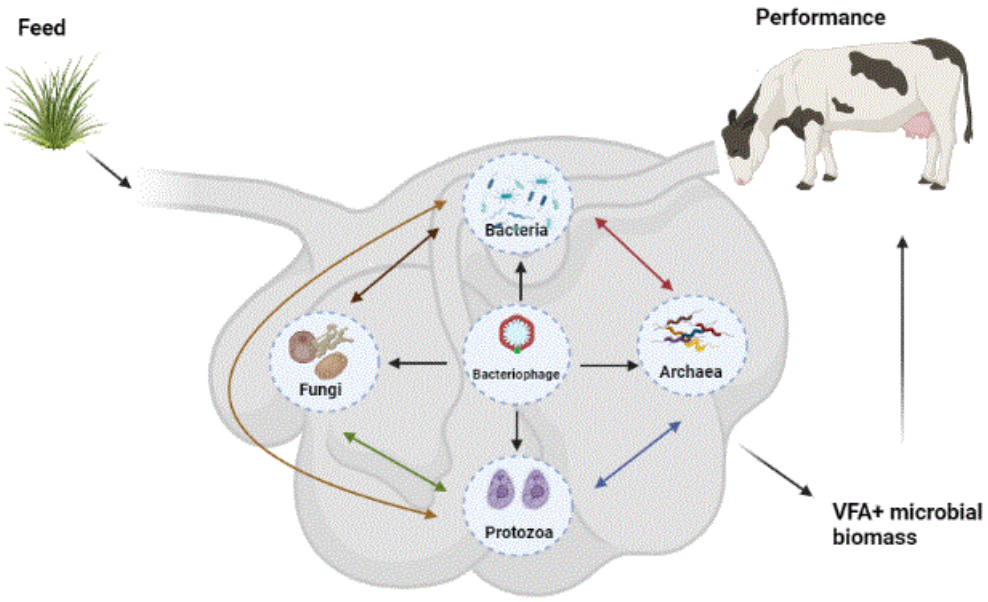

Figure 1.1. Relationship between rumen microbial ecosystem and, between microorganism and the host.

1.5. Modulation of rumen microbial fermentation

Due to the symbiotic relationship between the host animal and the large microbial population in the rumen, the more undigestible parts of the plant (cellulose, hemicellulose, and lignin) can be degraded. This process (Figure 1.2) results in the production of short chain VFA, the main source of energy for ruminants, and microbial protein (Greathead, 2003). However, the efficiency of diet utilization in the rumen is not totally complete. The fermentation of carbohydrates and amino acids in the rumen is always accompanied by a loss of energy and nitrogen. Of the digestible energy consumed by cows, 2-12% is lost as methane (Johnson and Johnson 1995; Henderson et al., 2015), and 65-80% of the ingested N is excreted in feces and urine (Tamminga, 1992). Hence the need to modulate rumen microbial fermentation.

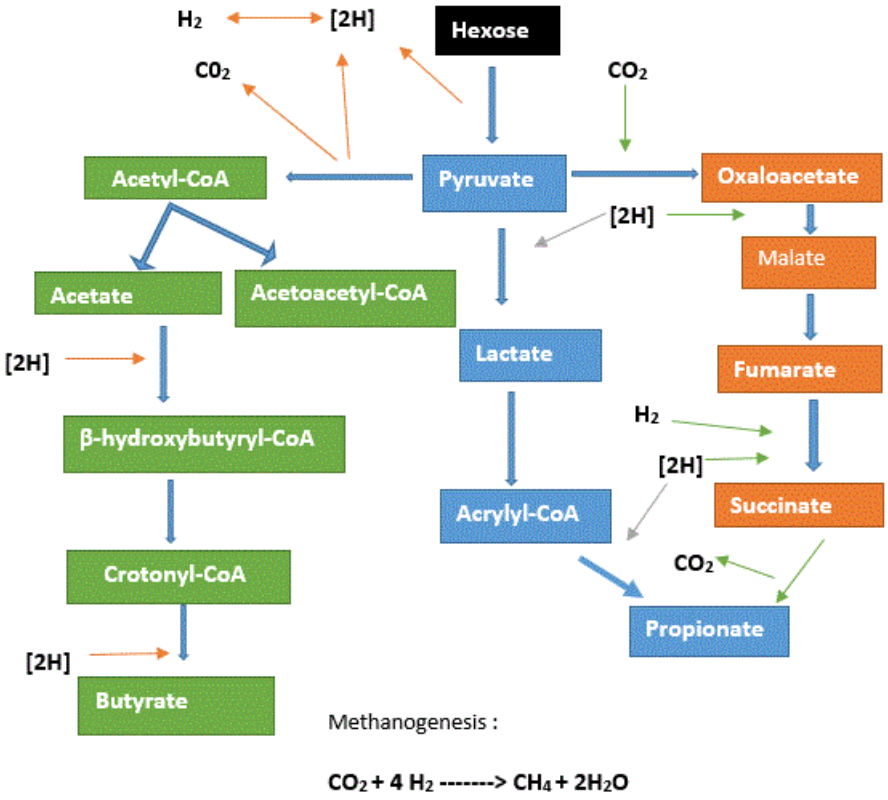

Figure 1.2. Rumen fermentation pathways.

1.6. Feed additives as modulator of rumen fermentation

Feed additives are non-nutritive compounds supplemented to ruminant diets to improve nutrient utilization by the animal (Adesogan, 2009). They function in several ways: they regulate rumen pH, reduce lactic acid accumulation, increase the degradability of rumen organic matter and dietary fiber, and reduce the risk of metabolic disease (Newbold and Rode, 2006). Feed additives can be used to modulate rumen microbial fermentation and improve ruminal energy and nitrogen utilization, by reducing methanogenesis and lowering the acetate:propionate ratio without reducing total VFA (Hristov et al., 2013). Also, feed additives enhance rumen fermentation by reducing proteolysis, peptide hydrolysis and amino acid deamination, thereby reducing the production and losses of NH_3 to the environment (Alexander et al., 2008; Adesogan, 2009). The primary goal of using dietary feed additives is to improve feed efficiency, animal performance and reduce the environmental impact of methane and nitrogen emissions. Finally, feed additive should be cost effective and approved by legal authorities.

1.6.1. Ionophores

Ionophores such as Rumensin (monensin sodium), Bovatec (lasalocid sodium), and Salinomycin, are feed additives known for their antimicrobial properties (Yang et al., 2007). However, monensin, which is a polyether carboxylic ionophore produced by a naturally occurring strain of *Streptomyces cinnamonensis* (Haney and Hoehn, 1967) and is administered orally to cattle as the sodium salt, is the most common ionophore used to improve feed efficiency (Duffield et al., 2008). Monensin is highly lipophilic and can alter the ion exchange gradient of Gram-positive bacteria membranes and reducing its population density compared to Gram-negative bacteria. Gram-negative bacteria have a complex cell wall, which Gram-positive bacteria don't have. This cell wall has a lipopolysaccharide layer consisting of protein channels (porins) with a size which doesn't allow monensin to pass through and infiltrate Gram-negative bacteria (McGuffey et al., 2001). In fact, these antibiotics act by modifying the microbial population and rumen fermentation patterns via a mechanism of ion transfer through the membranes (McGuffey et al., 2001; Duffield et al., 2008). The bacterial cells try to correct this ion imbalance, causing a loss of energy which can later lead to the death of bacteria (Russell and Strobel, 1989). The fermentation end-products of Gram-positive bacteria include acetate, H^+, CH_4 and NH_3 (Odongo et al., 2007). Monensin supplementation improves rumen function and animal performance by reducing the production of these metabolites, resulting in an increase in propionate molar proportion, and a decrease in butyrate molar proportion and acetate to propionate ratio (NRC, 2001). Also, the fermentation end products of Gram-negative bacteria are mainly propionate and succinate and are accompanied by less methane production (Odongo et al., 2007). These effects increase energy retained in VFA (NRC, 2001). Additionally, monensin may alter nutrient profiles to promote animal growth and performance (NRC, 2001).

The use of antibiotics as growth promoters in animal feed has shown positive results in the dairy and beef industries (McGuffey et al., 2001; Duffield et al., 2008). However, with the emergence of antibiotic-resistant bacteria and the growing consumer awareness of the risk of transmission of antibiotic residues to humans through animal products, including milk and meat (Barton, 2000), the use of antibiotics as growth promoters in animal feeds has been banned in the European Union since January 2006 (Directive1831/2003/CEE; EC, 2003). Since that, scientists have become interested in evaluating other alternatives to control specific microbial populations to modulate rumen

fermentation (Calsamiglia et al., 2007). Among several other rumen-modifying products, essential oils (EO) have aroused great interest over the past two decades and still today, for their virtues and benefits known since antiquity. Many studies on EO and their active compound (EOAC) have been conducted in *in vitro* batch culture system and provided evidence that EO and EOAC have the potential to enhance energy and/or nitrogen utilization by modifying rumen microbial fermentation profile (Calsamiglia et al., 2007; Giannenas et al., 2013).

1.6.2. Essential oils

1.6.2.1. Definition of essential oils

Since ancient time and by different civilizations, EO have been used for different purposes from culinary to health purposes, but also in perfumes and cosmetics industries. The term EO derives from the drug Quinta Essentia, named by Paracelsus von Hohenheim of Switzerland, reformer of medicine in the 16th century (Guenther, 1948; Brenner 1993). The term "essential" means taste or odor and refers to the property of these organic substances to provide typical odors and flavors of many plants (Calsamiglia et al., 2007). There are various definitions for EO given by different studies or organizations, among others:

- European Pharmacopoeia (2008): "essential oils are an odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating".
- The International Organization for Standardization (ISO 9235, 2013): "The essential oil is the product obtained from a natural raw material of plant origin by means of either distillation or mechanical processes from the epicarp of citrus fruits, or by dry distillation. The essential oil is then separated from the aqueous phase by physical methods".

Essential oils are declared as GRAS (Generally recognized as safe for human and animal consumption; FDA, 2004). These complex compounds are produced as secondary metabolites from aromatic plants, spices, and herbal plants (Bakkali et al., 2008; Voon, 2012). They can be produced by different parts of plant including flowers, roots, bark,

leaves, seeds, peel, fruits, wood and the whole plants (Hart et al., 2008; Hyldgaard et al., 2012) to provide protection to the plant against external invasion such as pathogenic microorganisms, and may also have important ecological functions (Greathead, 2003; Calsamiglia et al., 2007).

1.6.2.2. Chemical composition and classification of essential oils

Essential oils are natural mixtures made up of about 20 to 60 active compounds (AC). These AC are characterized by their low molecular weight and their different concentrations, but only two or three AC are in the majority in the mixture, from 20 to 70%, the rest being present in traces (Bakkali et al., 2008). In general, the AC with the higher concentration is responsible for the biological effects of EO.

The chemical nature of essential oils determines their effectiveness. Essential oils with phenols or aldehydes such as carvacrol, eugenol or thymol as their main AC have very potent antibacterial activity (Benchaar et al., 2008). Whereas EO containing terpene alcohols, ketones or esters such as beta-myrcene, alpha-geranone or geranyl acetate, or EO containing terpene hydrocarbons, have weaker antimicrobial activity (Bassole and Juliani, 2012).

The AC of EO may be classified in two main groups, terpenes and phenylpropanes, which have different precursors and are synthesized by distinct metabolic pathways (Chao et al., 2000; Calsamiglia et al., 2007) as shown in Figure 1.3.

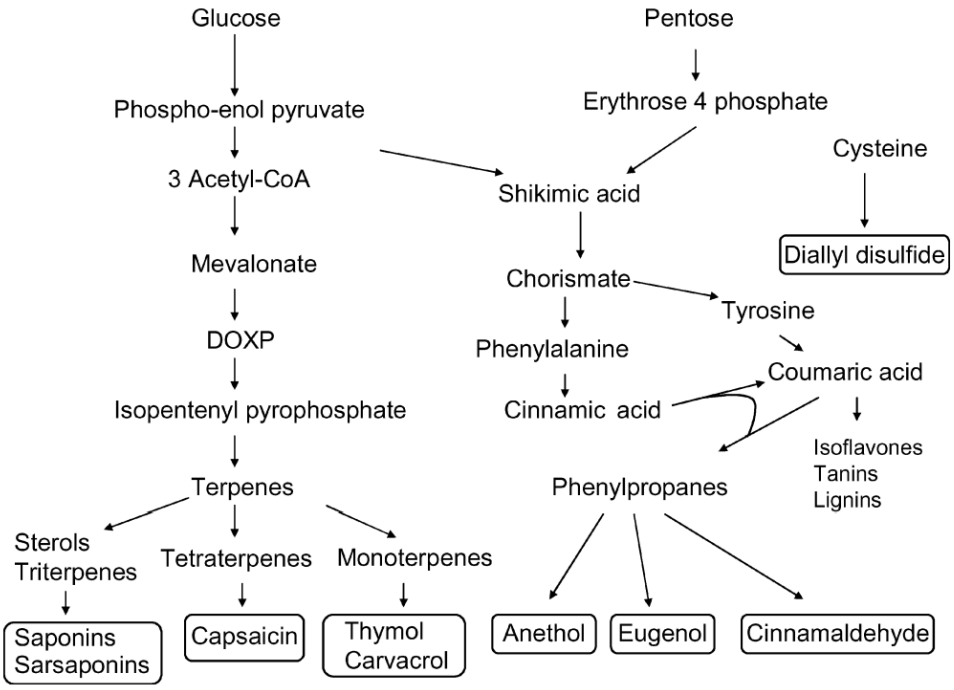

Figure 1.3. Metabolic pathways of the biosynthesis of the main plant extract active compounds (Calsamiglia et al., 2007).

- **Terpenes**

Terpenes are the most common class derived from the secondary metabolism of plants. More than 15,000 are known and present a very high chemical variability and complexity which makes it difficult to assemble them under the same chemical properties. Terpenes are made from the union of 5 carbons isoprene unit which contain an isopentenoid structure and are lipophilic in most cases (Gershenzon and Croteau, 1991).

The main terpenes are the monoterpenes (C10), consisting of two isoprene units and represents 90% of essential oils, followed by the sesquiterpenes (C15), consisting of three isoprene units. The rest, hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) are less abundant than the mono and the sesquiterpenes (Bakkali et al., 2008). Terpenes have several functional groups, mainly carbures, alcohols, aldehydes, ketones, ethers and esters. When a terpene contains an oxygen atom, is called a terpenoid (Bakkali et al., 2008).

- **Phenylpropanes**

Even though phenylpropanoids are less abundant than terpenes, some EO may contain abundant or significant proportions of these components (Sangwan et al., 2001). Phenylpropane are composed of a chain of three carbons linked to an aromatic ring of six carbons. Phenylalanine (an aromatic AA) is the precursor of the synthesis of phenylpropanoids through the shikimate pathway. This pathway is functional only in microorganisms and plants and represents the biosynthetic pathway towards aromatic amino acids (Sangwan et al., 2001; Calsamiglia et al., 2007).

1.6.2.3. Mechanism of action of essential oils

Essential oils have different properties, including the antioxidant effect by the neutralization of free radicals or decomposition of peroxides (Burt, 2004). Basil, cloves and thyme have strong antioxidant properties (Aruoma, 1998). Essential oils also have anti-inflammatory activity against allergens by inhibiting histamine release (Maruyama et al., 2005), or as a chemo protectant against cancer. Compounds obtained from garlic and turmeric play an important role in cancer prevention and cure (Edris, 2007). In addition, EO can have cytotoxicity, allelopathic, repellent and insecticidal properties. However, in the present study, only the antimicrobial activity of EO will be studied.

The antimicrobial activities of EO is complex and still misunderstood. Due to the large number of different groups of chemical compounds present in the EO, their antimicrobial activities cannot be limited to a one specific mechanism but rather to several targets in the cell (Burt, 2004). As demonstrated in Figure 1.4, EO can affect rumen microbes in different sites and ways (Skandamis et al., 2001; Carson et al., 2002). The hydrophobicity of EO allows them to spread into the lipids of the bacterial cell membrane and mitochondria, thus disrupting their structures and making them more permeable which leads to a subsequent loss of cellular components (Sikkema et al., 1994; Griffin et al., 1999). Then, EO can penetrate inside the cell and acidification can occur, which blocks the production of cellular energy (ATP) by leakage of ions, collapse of proton pumps, protein translocation and depression of the membrane potential (Benchaar et al., 2008).

Another hypothesis that explains the mechanism of action of EO is their capacity to coagulate the cytoplasm and harm proteins, lipids, membranes and cell walls, which can

lead to macromolecule loss followed by cell lysis and death (Cox et al., 2000; Lambert et al., 2001; Saad et al., 2013). However, in many cases, bacteria can escape the bactericidal effect of EO and can survive using its ion pumps to compensate the lack of balance but grow slowly because all their energy is diverted to this function (Calsamiglia et al., 2007). All these effects cited above are more effective with Gram-positive bacteria compared to Gram-negative bacteria, and this is because of the presence of a hydrophilic cell wall around the Gram-negative bacteria, and therefore, not allowing to penetrate lipophilic substances as EO (Chao et al., 2000; Calsamiglia et al., 2007). However, the external membrane of Gram-negative bacteria is not completely impermeable, and some EO can penetrated. Benchaar et al. (2008) reported that EO that have low molecular weight such as thymol and carvacrol can penetrate the cell membrane of Gram-negative bacteria (Cox et al., 2001a; Calsamiglia et al., 2007). These low-molecular weight compounds can penetrate the outer cell wall and interact with the lipid bilayer of cells. (Griffin et al., 1999; Dorman and Deans, 2000; Calsamiglia et al., 2007). Patra (2011) suggested that EO influence microbial population pattern, and can inhibit also hyper ammonia producing bacteria, which is involved in amino acid deamination, and reduced ammonia concentration in the rumen.

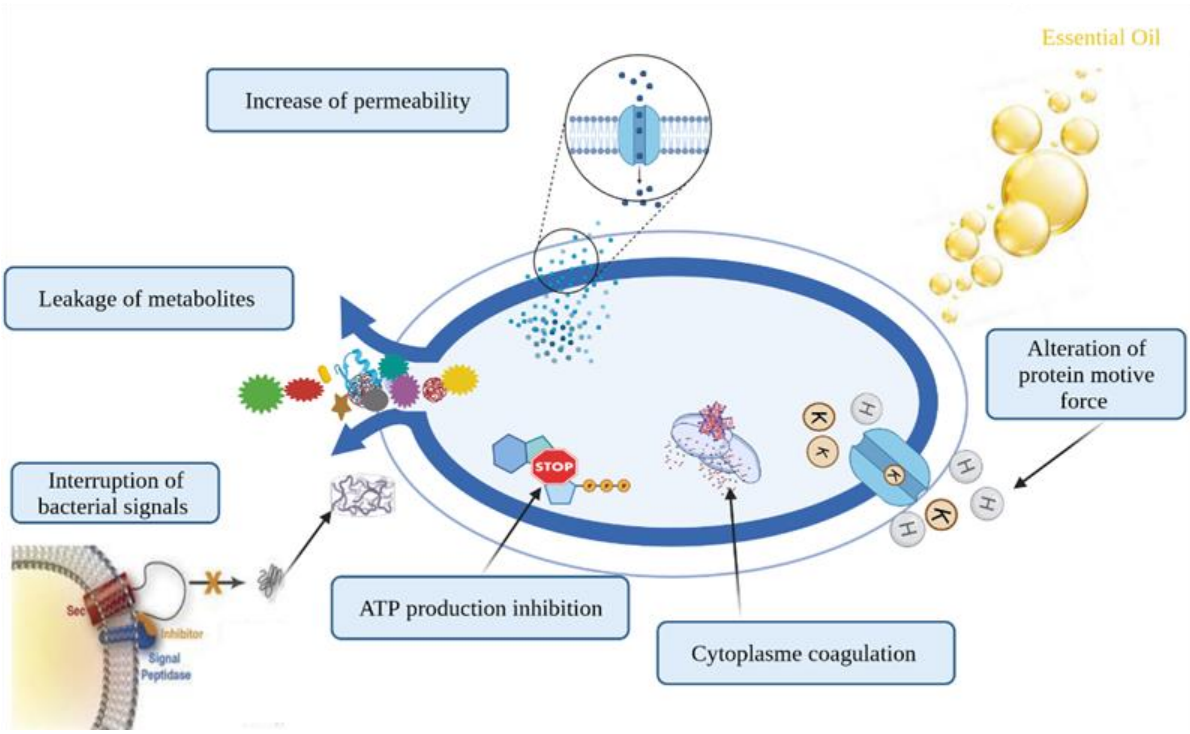

Figure 1.4. Mechanisms of action of essential oils on bacterial cell.

- **Specificity of EO activity against bacteria**

There is a large literature available on the mechanism of action of EO and their main AC (Calsamiglia et al., 2007; Hyldgaard et al., 2012). But an extensive description is beyond the scope of this thesis, and the most frequently reported mechanism of action of EO and their AC are summarized in Table 1.2.

Essential oils and their AC possess a wide range of actions against a broad spectrum of bacteria, including those that are resistant to antibiotics (Fisher and Philips, 2009). In general, the antimicrobial activity of EO is attributed to their chemical proprieties, hydrophobicity and to their chemical structure (Ultee et al., 1999; Benchaar et al., 2009). As mentioned above, EO are powerful antibacterial against Gram-positive and, sometimes, against Gram-negative such as *Aeromonas hydrophila* and *Campylobacter jejuni,* two Gram-negative bacteria that have been showen to be particularly sensitive to EO activity (Oussalah et al., 2007). The EO with phenol as main active compounds, such as clove and cinnamon oils, are strongly anti-microbial towards a large spectrum of micro-organisms (Hili et al., 1997; Helander et al., 1998), followed by aldehydes and alcohols (Dorman and Deans, 2000; Bruni et al., 2004; Sacchetti et al., 2005). In fact, aldehydes such as cinnamaldehyde have strong antibacterial properties due to the electronegativity created by the arrangement of aldehydes conjugated to carbon-carbon double bonds. This electronegativity allows cinnamaldehyde to inhibit electron transfer and enzyme activity (Dorman and Deans, 2000). Eugenol, a main active component in both cinnamon and clove oils, is also antimicrobial due to its ability to disrupt the cell membrane, causing cell lysis (Di Pasqua et al., 2007), as well as inhibiting several microbial enzymes by binding to them (Burt, 2004). All these modes of microbial inhibition have led the interest of using these plant compounds as natural rumen modifiers (Calsamiglia et al., 2007).

Table 1.2. Mechanism of action of some essential oils (Langeveld et al., 2013).

Essential oils	Mode of action	Reference
Oregano	Reduction in lipase and coagulase activity, enzyme inhibition.	Carneiro de Barros et al., 2009
Carvacrol	Membrane disruption, inhibition of ATPase activity, membrane destabilization, leakage of cell ions, fluidization of membrane lipids, reduction of proton motive force.	Ultee et al., 2002 ; Gill & Holley, 2006a, b; Di Pasqua et al., 2007
Thymol	Membrane disruption with potential intracellular targets, citrate metabolic pathway disruption.	Trombetta et al., 2005 ; Di Pasqua et al., 2007, 2010
p-Cymene	Membrane disruption.	Ultee et al., 2002
Cinnamaldehyde	Membrane disruption by inhibiting ATPase activity.	Gill & Holley, 2004, Gill & Holley, 2006a, b.
Cinnamic acid	Membrane disruption.	Hemaiswarya & Doble, 2010 ; Chen et al., 2011
Eugenol	Membrane disruption by inhibiting ATPase activity, possible efflux pump blocker, reduction of several virulence factors at sub inhibitory concentrations.	Gill & Holley, 2006a, b; Di Pasqua et al., 2007; Hemaiswarya & Doble, 2009; Qiu et al., 2010; Bolla et al., 2011
Tea tree	Inhibition of membrane-located metabolic events leading to inhibition of respiration and increased membrane permeability.	Cox et al., 2001b
γ-Terpinene	Membrane disruption.	Oyedemi et al., 2009

1.6.2.4. Effect of essential oils on rumen microbial fermentation

Results of 24-*in vitro* batch culture studies, that evaluated the effect of EO and/or EOAC on dairy rumen microbial fermentation are summarized in Table 1.3.

- **Volatile fatty acids**

Volatile fatty acids are the main end-product of rumen microbial fermentation, providing up to 80% of the metabolizable energy consumed by ruminants (Owens and Goetsch, 1988). There are formed by one to seven carbon atoms, exist as straight or branched chain compounds, including formic acid, acetic acid, propionic acid, butyric

acid, isobutyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid, hexanoic acid, and heptanoic acid (Bergman, 1990). Acetic, propionic, and butyric acids are the major forms of VFA, mainly produced by the fermentation of plant materials such as cellulose, fiber, starch, and sugars (Bergman, 1990). The aim of using EO in ruminant studies, is to shift ruminal fermentation profile towards more propionate and less acetate and acetate to propionate ratio, without affecting total VFA. These effects result in more efficient fermentation and less emission of methane (Calsamiglia et al., 2007). Many studies have shown that some EO and their AC can accomplish this target, although the results on total and individual VFA are heterogeneous and inconsistent between studies (Busquet et al., 2005a; Chaves et al., 2008; Patra and Saxena, 2010). Benchaar et al. (2007a) reported an increase in the concentration of total VFA when an EO mixture (750 mg/d) was added to a dairy cow diet based on alfalfa silage. However, this same EO mixture (750 mg/d) decreased total VFA when added to a dairy cow ration based on corn silage. These results suggested that the effect of EO and their AC may be affected by the type of fermented substrate (Calsamiglia et al., 2007). The effect of EO and EOAC on total and individual VFA can be different according to the dose used. Busquet et al. (2006; Table 1.3) tested a range of EO and EOAC *in vitro* at increasing doses (0, 3, 30, 300 and 3000 mg/L), and reported that at higher doses (300 and 3000 mg/L) the majority of EO and EOAC decreased total VFA concentration, and acetate and propionate molar proportion. Cade, capsicum, ginger, and yucca did not affect total and individual VFA at different doses (Busquet et al., 2006). Also, Castillejos et al. (2006) tested different doses of EO and EOAC (0, 5, 50, 500, 5000 mg/L; Table 3) and observed that the higher the dose of EO and EOAC the lower the concentration of total VFA. In contrast, Benchaar et al. (2007b; Table 1.3) also tested a range of EO, among others thymol, eugenol, cinnamaldehyde at doses ranging from 200 to 800 mg/L but did not observed effect on total and individual VFA. The decrease of total VFA observed in the study of Busquet et al. (2006) and Castillejos et al. (2006) mainly at higher doses of EO is not beneficial to the animal, because VFA are the main energy source of ruminants, and this reduction indicates a loss of feed energy from the fermentation of structural carbohydrates (Benchaar et al., 2009; Szumacher-Strabel and Cieslak, 2012). Essential oils can also be affected by ruminal pH. In the study conducted by Cardozo et al. (2005), six EO at different doses (0, 0.3, 3, 30, and 300 mg/L) were tested at two different pH (7.0 and 5.5) and observed that decreasing the pH from 7.0 to 5.5 resulted in a decrease in total VFA concentration, acetate

proportion, branched chain VFA, acetate: propionate ratio and ammonia N, and in an increase in the proportion of propionate.

In general, the addition of higher doses of EO and EOAC to diet may decreased VFA production. However, at low doses EO had no effect on VFA. Also, pH and diet type influence the effect of EO. Careful selection of EO doses, pH and diet are needed to have a positive effect on the production of VFA.

- **Ammonia-N concentration**

Ammonia is considered one of the end products of rumen fermentation and at the same time serves as a substrate for microbial growth. Ammonia production in the rumen is mainly produced from the fermentation of dietary protein. The non-protein nitrogen sources like urea, which are rapidly converted to ammonia in the rumen, contributes with a very small amount (Russell et al., 1991). Ammonia production rates in the rumen often exceed ammonia utilization by utilizing species and large amounts can be accumulated. Excess ammonia is absorbed across the rumen wall and converted to urea in the liver. Some of the urea is recycled back to the rumen via saliva, but most of it is excreted in the urine (Nolan, l975). Two different groups of organisms are responsible of the production of ammonia in the rumen: species present in large numbers but with low deamination activity, and those present in low numbers but with very high deamination activity. The latter are the hyper- ammonia producing bacteria species (HAP). The main HAP species are *Clostridium aminophilum, Clostridium sp. strain R, Clostridium sticklandii, Peptostreptococcus sp. strain C and Peptostrteptococcus anaerobius* (Russell and Strobel, 1988; Chen and Russell, 1989; Paster et al., 1993). The HAP bacteria are considered to deaminate more than 25% of the dietary protein (Krause and Russell, 1996).

Deamination of amino acids in the rumen, which results in the loss of NH_3 through the rumen wall, is one of the main causes of ineffective nitrogen retention by ruminants and environmental pollution (Benchaar et al., 2008). Hence, the need to modulate rumen microbial fermentation to reduce NH_3 production and, as a result, improve the efficiency of rumen dietary protein utilization and reduce contamination (Rychlik and Russell, 2000). Essential oils and their AC can affect ammonia production in different ways. Newbold (1997) reported that EO can decrease ammonia concentration in the rumen by the reduction in the number of protozoa, because they play an important role in protein degradation (Jouany, 1996). Some studies suggested that EO and EOAC can inhibit

protein degradation to ammonia by two mechanisms. First, by reducing proteolysis which avoid the degradation of protein to peptides (Newblod et al., 2004). Second, by the direct inhibition of HAP and their deaminase activities (Wallace et al., 2004). McIntoch et al. (2003) used a blend of EO (CRINA® ruminants, that contains thymol, eugenol, vanillin, and limonene on an organic carrier) for 48-hour *in vitro* study to investigate the effect of feeding 1g/d of this blend to cannulated cows on protein metabolism, and reported that the main effect of EO was on deamination stage and inhibited only *C. sticklandii* and *P. anaerobius* while, the other HAP species tested, *Clostridium aminophilum*, was not affected. A similar conclusion was reached by Newbold et al. (2004) when reported a decrease of 25% in amino acid deamination after 24 h of incubation in the ruminal fluid of sheep fed 100 mg/d of the same blend of EO. However, Benchaar et al. (2007a) reported that the addition of 0.75 g/d of a blend of EO CRINA® did not affect ammonia concentration in the rumen. Essential oils can also improve N metabolism by inhibiting protein degradation. Ferme et al. (2004), in a continuous culture study, reported the capacity of garlic oil to modify microbial population profile by reducing the population of *Prevotella ruminantium* and *P. bryantii* mainly responsible for protein degradation and AA deamination in the rumen. Also, Busquet et al. (2005b) reported that clove bud decreased proteolysis and peptidolysis without affecting deamination.

- **Enteric methane production**

Methane is considered a powerful greenhouse gas (GHG). Within the livestock sector, cattle (beef and dairy) have the highest total GHG emissions and more than 40% comes from enteric CH_4 fermentation (Gerber et al., 2013). Enteric CH_4 is produced from anaerobic fermentation of feeds, which takes place mainly in the rumen with a minor contribution from the hindgut. No single microbial species is responsible for complete degradation of substrates in the rumen. Instead, a complex succession of organisms takes part in the cooperative catabolism of substrates and the production of gases, CO_2 and H_2, as fermentation end-products (Buddle et al., 2011). Methanogens bacteria use the H_2 produced during the fermentation process to reduce CO_2 to CH_4 (Hungate, 1967). In fact, the purpose of methane production in the rumen is to prevent hydrogen accumulation (Bodas et al., 2012), because the accumulation of a large amount of H_2 in the rumen reduces microbial activity and reduces fermentation rates, which slows the efficiency of rumen fermentation and the conversion of feed into VFA (McAllister and Newbold, 2008). However, methane production represents an energy loss, which can vary from 5%

to 15% of total energy intake, depending on intake and dietary composition, which determine the diversity, size, and activity of the microbial population in the rumen (Johnson and Johnson, 1995). By modulating rumen microbial fermentation with EO and EOAC, we expect to improve animal feed efficiency and reduce environmental impact of GHG. Essential oils can inhibit methane production by different ways; directly by reducing methanogen population because EO have antimicrobial activities against a wide spectrum of microbes (Kamra et al., 2012). Indirectly, EO can reduce protozoa number and consequently reduce methanogens because of the symbiotique relationship between protozoa and methanogens (Kamra et al., 2012) which contribute significantly (about 37%) to methane production (Szumacher-Strabel and Cieślak, 2010). Also, EO may decrease feed digestion and decrease methane production, because of the positive correlation between feed degradation and methane production (Kamra et al., 2012). Evans and Martin (2000) tested different doses of thymol (50, 100, 200 and 400 mg/L) and reported that at high dose (400 mg/L) thymol strongly decreased methane concentration *in vitro*. Also, Chaves et al. (2008; Table 1.3) reported that using EO and EOAC (20, 100 and 250 mg/L) *in vitro* decreased methane production, but also decreased propionate proportions and increased the A:P ratio. Benchaar and Greathead (2011) investigated the potential of EO to reduce enteric methane emissions in ruminants. They concluded that *in vitro* studies with EO from thyme, oregano, cinnamon, garlic, horseradish, rhubarb, and frangula reduced CH_4 production in a dose-dependent manner. Busquet et al. (2005c) also observed a 69 to 74% reduction in CH_4 production when diallyl disulphide and garlic oil were added to an *in vitro* fermentation study at 300 mg/L, respectively, suggesting that a direct inhibition of rumen methanogenic archaea occurred. Wang et al. (2009) showed *in vivo* that feeding a mixture of EO based on oregano (0.25 g/d) to sheep for 15 days decreased methane production. However, Beauchemin and McGinn (2006) found no effect on methane production from feeding a commercial blend of EO to beef cattle (1 and 2 g/d; CRINA® ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK).

From the examples cited above, we conclude that methane production can be inhibited at different doses by EO and EOAC, but this decrease in many cases is accompanied by a decrease in total VFA concentrations and feed digestion. Many EO used *in vitro* affected methane production, but they can have negative effects on rumen efficiency and palatability *in vivo*. Thus, *in vivo* studies are needed to determine the

effectiveness of these compounds. The challenge is to identify EO that selectively inhibit rumen methanogenesis at realistic feeding rates without negative effects on rumen microbial fermentation profile, feed digestion and animal productivity.

Table 1. 3. Results of 24-h *in vitro* studies on the effect of EO and EOAC on dairy rumen microbial fermentation and methane production.

Treatment	Dose (mg/L)	Inicial pH	Diet	TVFA (Mol/100mol)	Acetate %	Propionate %	Butyrate %	A:P	N-NH_3 (mg/100mL)	CH_4	Reference
Geraniol	300-900	7	60:40	↓ (300, 600, 900)	↓ (600, 900)	↓ (300, 600)	↑ (300, 600)	↑ (300, 600) ↓ (900)	NM	↓ (300, 600, 900)	Joch et al. (2017)
Camphene				↓ (600, 900)	↑ (600, 900)	↓ (900)	↑ (900)	↑ (600, 900)		NE	
Eugenol	844-1067	NM	70:30	↓ (1067)	NE	↓ (1067)	↑ (1067)	↑ (1067)	NM	↓ (1067)	Joch et al. (2016)
Carvacrol				↓ (976)		NE	NE	NE		↓ (976)	
Citral				↓ (888)	↑ (888)	↓ (888)	↑ (888)	↑ (888)		↓ (888)	
Limonene				NE	NE	NE	NE	NE		↓ (844)	
1,4-Cineole				NE	NE	NE	NE	NE		↓ (887)	
γ-Terpinene				NE	NE	NE	↑ (850)	NE		NE	
p-Cymene				↓ (860)		↓ (860)	↑ (860)	↑ (860)		NE	
Linalool				↓ (870)		↓ (870)	↑ (870)	NE		↓ (870)	
Bornyl acetate				NE		NE	NE	NE		↓ (986)	
α-Pinene				NE	NE	NE	NE	NE		↓ (858)	
β-Pinene				↓ (866)	NE	NE	NE	NE		↓ (866)	
Cinnamon	50-1600	NM	50:50	NE	NE	NE	NM	NM	↓ (100-1600)	↑ (200-1600)	Nanon et al. (2015) *
Clove				NE	NE	NE			↓ (50-1600)	↑ (100,150, 200)	
Garlic				↑ (200)	NE	NE			↓ (200-1600)	↑ (200-1600)	
Lemongrass				↑ (200)	NE	NE			↓ (200-1600)	↑ (50, 200)	
Ginger				NE	NE	NE			↓ (200-1600)	↑ (150-1600)	

↓ the corresponded dose of EO decreases the studied variable; ↑ the corresponded dose of EO increases the studied variable; NE no effect; NM not mentioned.
*Study was done with rumen fluid from beef heifers.

Table 1. 3. (Continued).

Treatment	Dose (mg/L)	Inicial pH	Diet	TVFA (Mol/100mol)	Acetate %	Propionate %	Butyrate %	A:P	N-NH_3 (mg/100mL)	CH_4	Reference
Anethol	3-3000	7	50:50	↓ (300, 3000)	↓ (30, 300, 3000)	↓ (3000)	↑ (300, 3000)	NM	NE	NM	Busquet et al. (2006)
Anise oil				↓ (3000)	↓ (300, 3000)	↓ (3000)	↑ (300, 3000)		NE		
Benzyl salicylate				↓ (3000)	↓ (300, 3000)	↑ (300, 3000)	↑ (300, 3000)		NE		
Cade oil				NE	NE	NE	↑ (3000)		NE		
Capsicum oil				NE	NE	NE	NE		↓ (3000)		
Carvacrol				↓ (300, 3000)	↓ (300)	↓ (300),	↑ (300)		↓ (3000)		
Carvone				↓ (3000)	↓ (3000)	↓ (3000)	↑ (3000)		↓ (3000)		
Cinnamaldehyde				↓ (3000)	NE	↑ (3000)	NE		↓ (300, 3000)		
Cinnamon oil				↓ (3000)	↑ (3000)	NE	NE		↓ (3000)		
Clove bud oil				↓ (300, 3000)	↓ (300)	NE	↑ (300)		↓ (3000)		
Dillweed oil				NE	NE	↓ (3000)	↑ (3000)		NE		
Eugenol				↓ (3000)	NE	↑ (3000)	↑ (300)		↓ (3000)		
Fenugreek				NE	NE	↑ (3000)	NE		↓ (300, 3000)		
Garlic oil				↓ (3000)	↓ (300, 3000)	↑ (300)	↑ (3000)		NE		
Ginger oil				NE	NE	NE	NE		↑ (300)		
Oregano oil				↓ (300, 3000)	NE	↑ (3000)	↑ (300)		↑ (300)		
Tea tree oil				↓ (3000)	↓ (3000)	↓ (3000)	↑ (3000)		NE		
Yucca				NE	NE	NE	NE		NE		

↓ the corresponded dose of EO decreases the studied variable; ↑ the corresponded dose of EO increases the studied variable; NE no effect; NM not mentioned.

Table 1. 3. (Continued).

Treatment	Dose (mg/L)	Inicial pH	Diet	TVFA (mol/100mol)	Acetate %	Propionate %	Butyrate %	A:P	N-NH3 (mg/100mL)	CH4	Reference
limonene	5-5000	7	60:40	↓ (50,500, 5000)	↓ (5000)	NE	NE	↓ (5000)	↓ (500)	NM	Castillejos et al. (2006)
Guaiacol				↓ (5, 50, 5000)	↓ (5,50,500, 5000)	NE	NE	N. E	↓ (5-5000)		
Vanillin				↓ (5000)	↓ (500, 5000)	↑ (5000)	NE	↑ (5000)	↓ (5000)		
Thymol				↓ (500, 5000)	↑ (500)	↓ (500) ↑ (5000)	NE	↑ (500)	↓ (500,5000)		
Eugenol				↓ (5000)	↓ (5)	↓ (500)	NE	↓ (5)	↓ (50,500, 5000)		
Clove leaf	200-800	5.52-5.58	51:49	NE	NE	NE	NE	NE	NE	NM	Benchaar et al. (2007b)
Cinnamon leaf				NE	NE	NE	NE	NE	NE		
Carvacrol				NE	NE	NE	NE	NE	NE		
Thymol				NE	NE	NE	NE	NE	NE		
Eugenol				NE	NE	NE	NE	↑ (800)	NE		
Oregano				NE	NE	NE	NE	NE	NE		
Thyme				NE	NE	NE	NE	NE	NE		
Sweet orange				NE	NE	NE	NE	NE	NE		
Cinnamaldehyde				NE	NE	NE	NE	NE	NE		
Anethol	20-250	6.5	51:49	NE	NE	NE	NE	NE	↑ (20)	NE	Chaves et al. (2008)
Cinnamon leaf				NE	NE	↓ (250)	↑ (250)	↑ (250)	NE	↓ (250)	
Garlic				NE	NE	↓ (100, 250)	↑ (100, 250)	↑ (100, 250)	↑ (100)	↓ (100, 250)	
Juniper berry				NE	NE	NE	NE	NE	↑ (20)	↓ (20)	
p-cymene				NE	NE	NE	NE	NE	↑ (20)	↓ (20)	

↓ the corresponded dose of EO decreases the studied variable; ↑ the corresponded dose of EO increases the studied variable; NE no effect; NM not mentioned.

1.6.2.5. Additivity, synergism, and antagonism relationship between essential oils

The chemical structure of EO and their active compounds, their interactions, and their individual proportions are key factors in determining the antimicrobial activity of EO (Dorman and Deans, 2000; Marino et al., 2001; Delaquis et al., 2002). Interactions between one or more EO can be additive, synergistic or antagonistic. An effect is considered additive when the combined effect equals the sum of the individual effects (Burt, 2004). When the combination is greater than the sum of its parts, there is a synergistic effect (Davidson and Parish, 1989). However, these interactions don't always have a positive effect. Antagonist effects may occur when one or more compounds are less effective when used together than when used alone (Davidson and Parish, 1989). Blending two or more EO creates a mixture that is different from the individual ingredients and may be more powerful without increasing the dose. Some studies have demonstrated that whole EO have a greater antibacterial activity than the mixtures of their major components, suggesting that minor components may be critical to the synergistic activity, although antagonistic and additive effects have also been observed (Gill et al., 2002; Mourey and Canillac, 2002).

Bassolé and Juliani (2012) reviewed the antimicrobial activity of combined EO and/or their main AC. In Table 1.3 are summarized some examples of the antimicrobial activity of mixes of EO. The addition of phenolic monoterpenes and phenylpropanoids, which are strong antimicrobial, to other components increase the bioactivities of these mixtures. For example, the combination of thymol with carvacrol, and both EO with eugenol were synergistically active against *E. coli* strains (Bassolé and Juliani, 2012). In other studies, the combination of thymol and carvacrol resulted in additive effect against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Lambert et al., 2001; Table 1.3) or antagonistic effect against *S. aureus, Bacillus cereus* and *E. coli* (Gallucci et al., 2009). The combination of carvacrol with eugenol was synergistically active against *E. coli* strains (Pei et al., 2009), but was antagonist against *S. aureus, B. cereus* and *E coli* in another study (Gallucci et al., 2009). In general, compounds with similar structures exhibit an additive rather than a synergistic effect (Bassolé and Juliani, 2012). Also, in many cases, additive and synergistic effects can occur when phenolic and alcoholic compounds are mixed (Lambert et al., 2001; de Azeredo et al., 2011; Bajpai et al., 2012). In contrast, antagonistic effects have been attributed to the interaction between non-oxygenated and oxygenated monoterpene hydrocarbons (Hammer et al., 1999; Goñi et

al., 2009). These differences between mixed EO may vary depending on their chemical composition, geographic origin, harvesting or extraction methods, which are the main factors responsible for varying levels of their activity against Gram-positive and Gram-negative bacteria. These differences confirm that EO and their main AC need to be tested before deciding the composition of the mix.

Table 1.3. The antimicrobial interactions of certain combined essential oil components against Gram-positive and Gram-negative bacteria (Adapted from Bassolé & Juliani, 2012).

Reference	EO Components	Effect	Targeted bacteria
Lambert et al. (2001)	Thymol / Carvacrol	Additive	*Staphylococcus Aureus, Pseudomonas Aeruginosa*
Pei et al. (2009)	Thymol / Carvacrol Cinnamaldehyde/ Thymol	Synergism Synergism	*Escherichia Coli* *Escherichia Coli*
Bassole et al. (2010)	Carvacrol / Linalool Eugenol / Linalool Eugenol / Menthol	Synergism	*Listeria monocytogenes, Enterobacter aerogenes, E. coli, P. aeruginosa*
Gutierrez et al. (2008)	*O. vulgare/O. basilicum*	Additive	*B. Cereus, E. Coli, P. Aeruginosa*
Fu et al. (2007)	*S. aromaticum/ R. officinalis*	Antagonism	*Aspergillus niger*
Gallucci et al. (2009)	Carvacrol / Myrcene	Antagonism	*S. aureus, B. cereus, E coli*

The mechanism of action of the antimicrobial activities of individual EO or their active compounds is widely studied compared with the mechanism of action of combined EO or their active compounds. However, there are some recognized mechanisms of antimicrobial interaction that would produce synergy. The proposed hypothesis to understand the mechanism of action of combined EO were tested mainly with thymol, carvacrol, cinnamaldehyde and eugenol. Pei et al. (2009) suggested that the synergistic effects between carvacrol and eugenol or between thymol and eugenol is due to the fact that carvacrol and thymol disrupt the outer membrane of *E. coli*, facilitating the entry of eugenol into the cytoplasm and its binding with proteins. Also, Pei et al. (2009) observed that the synergistic effect between eugenol and cinnamaldehyde might be due to the

interaction of these components with different bacterial proteins or enzymes. Zhou et al. (2007) reported that thymol or carvacrol expand the permeability of the bacterial cytoplasmic membrane allowing cinnamaldehyde to be more easily transported into the cell resulting in synergistic effect. Also, thymol or carvacrol could increase the number, size, or duration of existence of the pores created by the binding of cinnamaldehyde to proteins in the cell membrane, so that a synergistic effect can occur when these two components are used in combination. Fei et al. (2011) reported that the combination of basil with oregano resulted in synergistic effect against *Escherichia coli*. Also, the combination of basil with bergamot or a combination of oregano with bergamot resulted in synergistic effect against *S. aureus* and *B. subtilis* respectively. The combination of oregano with perilla act synergistically and damage the integrity of the cell membrane of *S. cerevisiae*.

All EO synergy-antagonism studies have been carried out mainly in the cosmetic and food industries. However, since scientists became interested in the use of EO and their AC as an additive for animal feed, several products based on combination of different EO or their active molecules appeared on the market (i.e., CRINA-Ruminants®, DSM, Switzerland; AGOLIN®, Agolin Sa, Switzerland, XTRACT®, Pancosma, Switzerland) but without evidence of additivity or synergy. Belanche et al. (2020) in a meta-analysis on the effect of AGOLIN® (Agolin Sa, Biere, Switzerland; mainly containing: coriander seed oil, eugenol, geranyl acetate and geraniol) reported that short *in vitro* studies showed small and inconsistent effect on rumen fermentation and methane emission. However, *in vivo* studies (more than 4 weeks of feeding 1g/d of AGOLIN® to dairy cow) increased milk yield, fat and protein corrected milk and feed efficiency, and decreased methane production without changes in rumen fermentation, milk composition and feed intake. Blanch et al. (2016) studied the effect of another blend of EO (Next Enhance® 300, NE300, containing cinnamaldehyde and garlic oil encapsulated product) at different doses (200, 300 and 400 mg/L) *in vitro* and *in vivo*. They reported that this blend, *in vitro* at 300 mg/L decreased methane production, acetate proportion, and ammonia-N concentration, and increased propionate proportion without affecting total VFA production. *In vivo*, NE300 increased milk yield in multiparous lactating dairy cows after 15 of adaptation.

Another blend of EO that was widely studied is CRINA® (CRINA-Ruminants®, AKZO Nobel Surface Chemistry Ltd, Hertfordshire, UK; mainly containing: thymol,

eugenol, vanillin, and limonene on an organic carrier). Newbold et al. (2004) reported that apart from the decrease (about 25%) in amino acid deamination measured *in situ*, CRINA® had no major influence on other aspects of ruminal fermentation. Castillejos et al. (2005; 2007) also tested the same product, CRINA®, and reported an increase in total VFA concentration and acetate proportion. However, Castillejos et al. (2005; 2007) did not observe any effect of CRINA® on N metabolism. McIntosh et al. (2003) reported that some species of hyper ammonia- producing bacteria (HAP) were sensitive to the addition of CRINA® and deamination was reduced.

All these examples of blend of EO cited above among are commercialized but, surprisingly, the evidence for their possible additivity, synergistic or antagonistic effects is non-existent. Few studies have been specifically designed to prove additivity, synergistic or antagonistic effects of EO on rumen microbial fermentation. Recently, Fandiño et al. (2020) used different combinations of six EO (tea tree, oregano, clove bud, thyme, rosemary, and sage) at four doses (10, 50, 200 and 400 mg/L) to explore possible additive or synergy-antagonism effects of these EO combinations under feedlot beef-type ruminal microbial fermentation conditions *in vitro*. Fandiño et al. (2020) observed an antagonistic interaction on rumen microbial fermentation when clove was mixed with thyme and oregano oils. Fandiño et al. (2020) emphasized the importance of designing specific studies to identify the relationship between EO before mixing them to avoid any combination with possible antagonistic effects on rumen microbial fermentation and dairy production.

1.6.3. Guanidinoacetic acid

1.6.3.1. An overview of creatine

Creatine (*α*-*N*-methylguanidino acetic acid) is a nitrogenous organic amino acid playing essential roles in energy metabolism. This compound serves to provide a constant supply of high-energy phosphate bonds by allowing the rapid resynthesize of adenosine triphosphate (ATP) and the maintenance of cellular ATP levels in cells. Tissues such as muscles and the brain, generally, have very high energy requirements and contain the highest concentrations of creatine (Wyss and Kaddurah-Daouk, 2000; da Silva et al., 2009). Creatine can be obtained from dietary sources or synthesized endogenously from three essential amino acids (methionine, glycine and arginine) in the liver, kidneys and pancreas (Baracho et al., 2015). Then, creatine is transferred via the blood by specific

creatine transporters to body tissues that have high energy requirements (Kley et al., 2013). Total creatine includes free creatine plus phosphocreatine (Wyss and Kaddurah-Daouk, 2000). Around 95% of creatine (60% phosphocreatine and 40% free creatine) is found in skeletal muscle, with only 5% of creatine located in the brain, kidney and testes (Bemben and Lamont, 2005). The body needs creatine permanently for the growth and development of muscle mass, mainly in growing animals where the requirement for creatine is higher compared to adult animals. The degradation of creatine in the body can occur through an irreversible non-enzymatic reaction, and the by-product formed is creatinine which is excreted in urine (Riesberg et al. 2016). This loss of creatine in the form of creatinine should be continuously replaced by the combination of diet and endogenous synthesis of creatine.

- **Creatine synthesis**

Creatine synthesis requires the presence of two enzymes: arginine glycine amidino transferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Cells pick up creatine through a specific transporter, SLC6A8. Mitochondrial or cytosolic creatine kinases convert creatine to its high-energy homologue phosphocreatine (Figure 1.5; Béard and Braissant, 2010). Phosphocreatine dephosphorylation yields energy, and ADP is converted to ATP. Phosphocreatine, which is critical for the maintenance of energy in cells is generated by the transfer of a phosphate group from ATP to creatine via creatine kinase in a reversible reaction resulting in the release of ADP (Guimaraes-Ferreira, 2014). A high ratio of ATP to ADP in the cell when the ATP consumption rate is low leads to phosphocreatine synthesis through transfer of a phosphoryl group from ATP to creatine via creatine kinase (Wyss and Kaddurah-Daouk, 2000). The ATP can be generated through three main ways in skeletal muscle to maintain the appropriate ATP/ADP ratio in the cytosol: creatine, glycolysis in the cytosol, and oxidative phosphorylation in the mitochondria (Vander Heiden et al., 2009). Creatinine is the end-product of phosphocreatine and creatine metabolism through an irreversible nonenzymatic reaction, and it is eliminated by the kidneys and passed out in the urine (Brosnan and Brosnan, 2016).

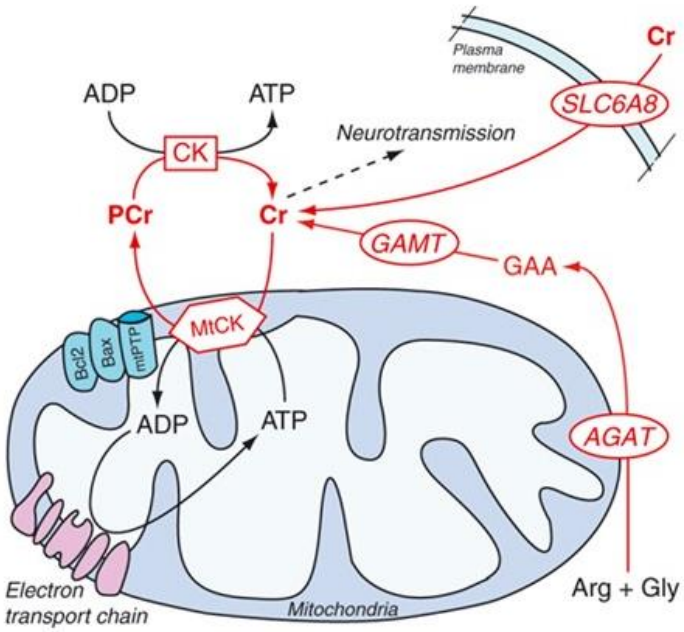

Figure 1.5. Metabolic pathway of creatine (Béard and Braissant, 2010).

1.6.3.2. An overview of guanidinoacetic acid

Guanidinoacetic acid (GAA) is a nitrogenous organic acid which can be obtained from dietary sources or mainly synthesized endogenously in the kidney from glycine and arginine (Ostojic, 2015). It was first identified in humans about 80 years ago (Weber, 1934) and has been used therapeutically, mainly for cardiac and poliomyelitis diseases, since the 1950s (Fallis and lam, 1952).

Commercial GAA is usually white or off-white crystalline powder with no irritating odor. It is synthesized from chemical raw materials, and contained 96% minimum of GAA, 1% maximum of starch and 1% maximum of water. The commercial product based on GAA can be processed into premixes, mineral mixes, and compound feeds. Its technical properties guarantee homogeneous mixability and stability against demixing, and in term of thermostability, GAA is more stable in aqueous solutions (Khajali et al., 2020), and economically less expensive (about 40%) than creatine (Ostojic, 2017). For those reasons, supplementation with GAA is preferred over creatine. The chemical

formula is $C_3H7N_3O_2$ (molecular weight = 117.11 g/mol), and the structure of the molecule is shown in Figure 1.6.

Figure 1.6. Chemical sturture of guanidiniacetic acids molecule.

From the regulatory point of view, the Food and Drug Administration (FDA, 2016) allowed the use of GAA and declared it as a safe substance that spares arginine and serves as a precursor of creatine in broiler chicken and turkey drinking water and feeds. Also, the European Commission allowed its use as feed additive for growing chickens and pigs (Regulation (EU) 2016/1768; EC, 2016).

1.6.3.3. Metabolic pathway of guanidinoacetic acid

The ultimate goal of GAA supplementation is to enhance creatine synthesis which is a form of energy storage in muscle. In the body of the vertebrate, the synthesis of creatine according to the de novo synthesis, follows two main steps (Figure 1.5 and 1.7).

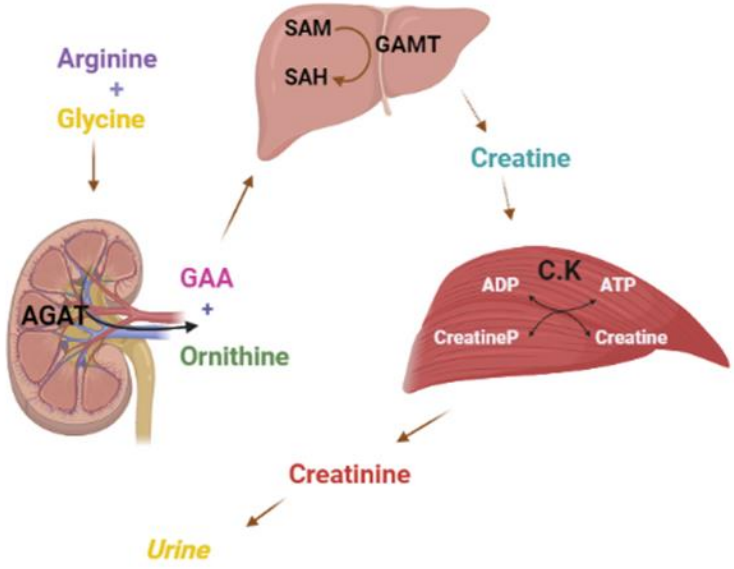

Figure 1.7. Metabolic pathway of guanidinoacetic acid.

First, the formation of GAA and ornithine, when glycine obtains a guanidine group from the amino acid L-arginine in a biochemical reaction catalyzed by L-arginine:glycine amidino transferase (AGAT, generic gene name GATM). This initial reaction occurs mainly in the kidney. In the second step, that takes place in the liver, GAA is methylated by S-adenosyl methionine (SAM) to creatine. The enzyme that catalyzes this reaction is guanidinoacetate N-methyltransferase (GAMT; Wyss and Kaddurh-Daouk 2000).

The formation of GAA by L-arginne and glycine is regulated by a negative feedback mechanism involving the effect of serum creatine and ornithine concentrations on AGAT activity. However, dietary supplementation with GAA bypasses this rate-regulating mechanism. Therefore, increasing dietary intake of arginine and glycine does not increase creatine synthesis or its concentration in muscle tissue above such regulatory levels (Khajali et al., 2020).

1.6.3.4. Physiological effect of guanidinoacetic acid supplementation

Recently, GAA has generated interest as dietary additive because of its beneficial physiological functions, among others:

- GAA acts as an immediate precursor of creatine (Ostojic et al., 2013).
- Its role as a neuromodulator and stimulator of hormone secretion, mainly insulin being more powerful than arginine or creatine in increasing insulin secretion (Ostojic, 2014).
- The addition of GAA to the diet avoids the endogenous synthesis of GAA which spare arginine for other physiological functions, including protein anabolism, cellular signaling, and hormonal release (Dilger et al., 2013).
- The GAA may activate the gamma amino butyric acid (GABA) receptors in the brain and peripheral tissues and affect neuronal excitability, muscle tone and/or brain development. (Neu et al., 2002).
- The GAA can also be used as a substitute for creatine in creatine deficient situations, but its ability to replenish cellular energy appears to be inferior to that of creatine.

1.6.3.5. Effect of guanidinoacetic acid supplementation in monogastric

Guanidinoacetic acid has been used in monogastric diets as a direct precursor of creatine that could spare arginine and allow this amino acid to further become available

for biosynthesis of growth-promoting polyamines including putrescine, spermidine and spermine in broiler chicken (Michiels et al., 2012; Dilger et al., 2013; Faraji et al., 2018). Creatine and creatine phosphate are essential for rapid growth and development of muscle tissue. Several studies have demonstrated the positive effects that GAA can have in improving growth performance in monogastric animals, promoting muscle development, and improving animal health. He et al. (2019) conducted two experiments, one in adult broilers and the second in one-day broilers, in which, a corn–soybean meal-basal diet was supplemented with 0, 600, 800, 1,000 or 1,200 mg GAA/kg of feed for 42 days. Results showed that dietary GAA inclusion increased average daily gain (ADG) and improved gain-to-feed (G:F) ratio from 1 to 42 days, with no effect on average daily feed intake. Also, GAA supplementation increased creatine concentration in plasma and kidney in broilers. For one-day broilers, dietary inclusion of 600 mg GAA/kg of feed significantly increased ADG and the G:F ratio. He et al. (2019) suggested that 600 mg GAA/kg of feed is the minimum dose for improving performance in broilers. Another study of supplementing broiler diet with two levels of GAA (0.6 g and 6.0 g GAA per kg of feed) from day 1 to 35 (starter and grower period), showed that 6 g GAA/kg of feed increased plasma and urinary levels of creatine and creatinine. Plasma homocysteine was also increased due to the increased demand for methyl groups to convert GAA to creatine. Additionally, there were increases in urinary excretion of GAA (3.37 to 102.62 mg/kg$^{0.75}$ of BW daily), creatine (1.07 to 29.43 mg/kg $^{0.75}$ of BW daily), and creatinine (3.40 to 66.97 mg/kg $^{0.75}$ of BW daily) by increasing levels of supplemental GAA from 0 to 6.0 g GAA/kg of feed, respectively, suggesting that the renal excretion is the main way to eliminate the excess of GAA and its metabolites, creatine and creatinine, in chicken (Tossenberger et al., 2016). In addition, dietary supplementation of GAA could prevent the negative effect of heat stress on growth performance, mortality rate, antioxidant status, and gut morphology in broiler chickens (Amiri et al., 2019).

Murakami et al. (2014) used GAA with meat-type quails, and observed that GAA, creatine, and creatinine were increased in eggs with increasing GAA levels. This demonstrated that GAA supplementation increased the transport of GAA from the bloodstream to the liver to be converted into creatine to enhance the transfer of creatine into the egg.

In growing-finishing pigs, He et al. (2018) conducted two experiments. In the first Exp. pigs were fed a basal corn-soybean meal diet supplemented with 0, 300, 600, 900,

and 1,200 mg GAA /kg of feed for 98 days. The G:F ratio increased with increasing dietary GAA supplementation, and the optimal amount of dietary GAA was 300 mg/kg of feed throughout the trial period to maximize G:F ratio, hot carcass weight, carcass length and lean percentage, that tended to increase with increasing dietary GAA content. Serum GAA and hepatic creatine tended to increase with dietary GAA by day 98. Furthermore, at day 98, serum ATP, muscle ATP and adenosine monophosphate increased with increasing GAA supplementation.

In the second Exp. conducted by He et al. (2018), pigs were also fed corn soy meal - basal diets supplemented with 0, 150, 300, 600, and 1,200 mg GAA /kg of feed for 35 days, and observed increases in final body weight, ADG, and G:F ratio with increasing dietary GAA. Maximal ADG and final body weight were observed with 300 mg GAA/kg of feed. Results showed that dietary GAA increased tissue creatine and ATP loads in pigs, resulting in improved growth performance. He et al. (2018) recommended a dose of 300 mg GAA /kg of feed to maximize the growth performance of growing-finishing pigs. Another study by Liu et al. (2015) showed that feeding GAA increased creatine concentration in longissimus dorsi and could improve meat quality in growing-finishing pigs. However, no effect of GAA supplementation was reported on ADG, feed intake or feed conversion in piglets (Teixeira et al., 2017).

1.6.3.6. Effect of guanidinoacetic acid supplementation in ruminants

Recently, scientists have been interested in studying the effect of supplementing ruminant diets with GAA given its positive effects observed in monogastric. Zhang et al. (2022) investigated the effects of dietary supplementation with different amounts of GAA (0, 500, 1000 and 1500 mg/kg DM) in the feed using 24 3-month-old male lambs. They reported that as the GAA level increased, the ATP content in quadriceps muscle linearly increased, and phosphocreatine levels and ADP levels in the longissimus dorsi muscle also increased linearly. The author recommended a dose of 500 and1000 mg/kg DM GAA to supplement lamb diets.

A conversion of GAA to creatine needs a methyl group, so, a methyl group deficiency may occur as a result of GAA supplementation. Ardalan et al. (2020) studied the metabolic responses of ruminally cannulated Holstein heifers to post-ruminal infusion of GAA (0, 10, 20, 30, and 40 g/d) with or without methionine (0 or 12 g/d of L-Met) as a

methyl group donor, to avoid methionine deficiency. They observed that GAA supplementation increased creatine supply to cattle and spared arginine, but without any effect on methionine.

Another study on the bioavailability of GAA was conducted by Speer et al. (2020) in which seven ruminally cannulated Holstein steers were ruminally or abomasally infused continuously with 10 or 20 g/d GAA. They observed that plasma creatine concentrations increased linearly with GAA infusion to the abomasum and tended to increase linearly when GAA was infused ruminally. Urinary creatine concentrations increased linearly with increasing amounts of GAA infused in the abomasum and the rumen. Plasma creatinine concentrations were not affected by GAA infusion to the abomasum or rumen. Urinary creatinine concentration decreased when GAA was infused abomasally. They also reported that the ruminal escape of GAA based on plasma creatine and urinary creatine concentrations were 47% and 49%, respectively, suggesting that the ruminally infused GAA was about 50% degraded in the rumen.

Li et al. (2020) used fourty-eight Angus bulls to study the effect of increasing GAA doses (0.3, 0.6 and 0.9 g/kg of DM) on rumen fermentation profile and microbial population, and observed that increasing GAA supplementation improved microbial growth, especially *R. flavifaciens*, *B. fibrisolvens*, *P. ruminicola* and *Rb. Amylophilus*, and increased the activities of cellobiases, pectinases and proteases. This increase in microbial growth resulted in higher nutrient degradation, higher total VFA and propionate proportion, and lower acetate proportion, acetate to propionate ratio and ammonia-N concentration. Also, Liu et al. (2021) used forty-four Angus bulls to study the effect of GAA (0 or 0.6 g/kg DM) with or without betaine as a methyl group donor (0 or 0.6 g/kg DM) for 80 days. They reported that the supplementation of GAA and betaine together increased ADG, BW and feed efficiency. This improvement in ADG and BW may be due to the improvement in intestinal morphology (Amiri et al., 2019) leading to increased DMI, nutrient digestibility, and blood creatine concentrations. The supplementation of GAA without betaine increased the population of total bacteria, *R. albus*, *B. fibrisolvens* and *Rb. amylophilus* and increased the activities of xylanases, cellobiases, proteases and α-amylases in the rumen which resulted in higher total VFA and ammonia-N concentration without affecting individual VFA or the acetate to propionate ratio.

As shown above, numerous studies have demonstrated the beneficial effects of GAA in promoting muscle development and muscle growth. Compared with creatine and

arginine, it has the advantages of good stability, low cost and better growth-promoting effect. However, the research on the effect of GAA on ruminants is limited, and the determination of effective doses in cattle, its rumen degradability, effects on the rumen and whether GAA is toxic, have not been fully explored. Therefore, more detailed studies are required.

CHAPTER 2

Objectives

CHAPTER 2

Objectives

The general objective of this thesis is to explore the effects of feed additives on improving rumen fermentation efficiency using essential oils or guanidinoacetic acid.

The specific objectives were:

- To explore additive, synergistic or antagonistic effects among essential oils to increase propionate and decrease acetate concentrations, and the acetate to propionate ratio.
- To study the effect of blend of essential oils (anise star and cassia) and lemongrass oil with or without carriers on rumen microbial fermentation.
- To evaluate the effect of adding guanidinoacetic acid to beef and dairy rumen fermentation conditions on rumen microbial fermentation and microbiome, and to determine its microbial degradability.

CHAPTER 3

Synergy-antagonism among essential oils

CHAPTER 3

Interactions among natural active ingredients to improve the efficiency of rumen fermentation *in vitro*[1]

3.1. Abstract

A total of 12 EO: anise star, cassia, geraniol, lemongrass (LEM), limonene, thyme, tea tree, coriander (COR), capsicum, black pepper, turmeric and ginger (GIN), in Exp. 1 at three doses; and different combinations of LEM, COR and GIN oils in Exp. 2, were evaluated in 24-h *in vitro* batch microbial fermentation. All treatments were added to 1:1 rumen fluid:buffer containing a 50:50 forage: concentrate diet with an initial pH of 6.6. In Exp.1, data were analyzed with the MIXED procedures of SAS, and treatment means were compared to the control using Dunnett's multiple comparison test. Significance was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. In Exp. 2, data were analyzed according to the Simplex Centroid Design using R-Studio. In Exp.1, LEM tended to increase the propionate proportion and tended to decrease the acetate to propionate ratio. Anise star, COR, and thyme tended to increase butyrate proportion. Capsicum, COR, and thyme decreased ammonia-N concentration. In Exp. 2, a synergy was observed between LEM and COR that resulted in an increase in total volatile fatty acids and propionate proportion, and a decrease in the acetate to propionate ratio. However, the addition of high doses of GIN to the LEM + COR mix had an antagonistic effect on the rumen fermentation profile.

[1]This article was published in: Temmar, R., M. E. Rodríguez-Prado, G. Forgeard, C. Rougier, and S. Calsamiglia. **2021**. Interactions among natural active ingredients to improve the efficiency of rumen fermentation *in vitro*. *Animals* 11:1205. https://doi.org/10.3390/ani11051205.

3.2. Introduction

Ruminal microbial fermentation can be modulated with the use of additives, resulting in improved efficiency of energy and protein utilization (Benchaar et al., 2008). Shifting ruminal fermentation profile toward more propionate and less acetate is more efficient and reduces the emission of methane (Busquet et al., 2005c). For example, the addition of monensin increased propionate proportion, and decreased acetate and butyrate proportions, and therefore increase the efficiency of converting feed energy into energy in the acid end-products which are available for absorption (Richardson et al., 1976; Tedeschi et al., 2003; Hristov et al., 2013). However, since the ban on the use of antibiotics as growth promoters in animal feeds in the European Union in January 2006 (EC, 2003) the interest has focused on natural alternatives.

Essential oils (EO) are the aromatic volatile fraction of plant secondary metabolites generally recognized as safe for human and animal consumption (Calsamiglia et al., 2007). Essential oils are characterized by their active compounds included in two main chemical groups: terpenoids and phenylpropanoids (Dorman and Deans, 2000; Calsamiglia et al., 2007). Numerous studies have demonstrated the ability of EO and their main active components to shift rumen microbial fermentation profile (Castillejos et al., 2006; Rodríguez-Prado et al., 2012; Foskolos et al., 2020). Many plant extract additives in the market are based on combinations of several EO. However, evidence of their possible additivity, synergistic or antagonistic effects is very limited, and only Fandiño et al. (2020) recently reported a negative interaction of clove bud when mixed with thyme or oregano oils on ruminal microbial fermentation. Tserennadmid et al. (2011) observed a synergy between α-pinene and limonene, two monoterpenes. Bassolé et al. (2010) observed a synergy between the monoterpene linalool and the phenolic compound eugenol. In contrast, Gallucci et al. (2009) observed an antagonistic effect between thymol and carvacrol, two phenolic compounds. However, all these studies on synergistic-antagonistic effects among EO have been conducted in the field of cosmetics and food processing (Burt, 2004; Bassolé and Juliani, 2012).

Only few studies have been specifically designed to prove additivity, synergistic or antagonistic effects of EO on rumen microbial fermentation. Recently, Fandiño et al. (2020) addressed this issue in high concentrate feedlot-type fermentation conditions and found an antagonistic effect when clove bud oil was mixed with tea-tree, thyme and (or)

oregano oils. Our hypothesis was that the combination of EO at doses that would not have an effect when supplemented alone would have additive or synergistic effects when supplemented together. The objective of the present study is to explore additive, synergistic or antagonistic effects among EO to increase propionate and decrease acetate concentrations and reduce the acetate to propionate ratio.

3.3. Materials and Methods

An animal care and use statement was not needed in this study since the rumen fluid was obtained from an abattoir.

3.3.1. Experiment 1: Individual essential oils

3.3.1.1. Experimental procedures

A group of 12 EO at three different doses (Table 3.1) were selected based on previous studies (Chao et al., 2000; Hristov et al., 2008; Langeveld et al., 2013), the Rumen Up project (https://www.abdn.ac.uk/research/rumen-up/ accessed on 18 September 2018), commercial availability, reasonable price and legal status in the EU. All EO were supplied by TECHNA France Nutrition (Couëron, Fr).

Table 3.1. Essential oils, their main active component and different doses used in Exp. 1.

Product	Active Compound and Purity (%)	Dose* (mg/L)		
		Low	Medium	High
Monensin	Monensin, 93	10		
Anise star	Trans-Anethol, 99	80	300	750
Black pepper	Piperine, 95	0.4	3	7.5
Capsicum	Capsaicin, 6	0.4	3	7.5
Cassia	Cinnamaldehyde, 75	80	300	750
Coriander	Linalool, 65	40	150	375
Geraniol	Geraniol, 84	80	300	750
Ginger	Gingerols, 63.2	10	40	150
Lemongrass	Citral, 75	80	300	750
Limonene	Limonene, 93	80	300	750
Tea tree	Terpinen-4-ol, 38.9	40	150	375
Thyme	Thymol, 47	80	300	750
Turmeric	Curcumin, 40	10	40	150

*The dose of each EO was calculated according to the % of purity of the main active compound.

The experiment was conducted in *in vitro* batch fermentation conditions (Tilley and Terry, 1963). Rumen fluid was collected at a slaughterhouse from four dairy cows in each period, filtered through four layers of cheesecloth, mixed and transported to the laboratory in thermos with no headspace. The rumen fluid was pooled and added to a phosphate-bicarbonate buffer (McDougall, 1948) in a 1:1 proportion, purged with N_2, and adjusted to an initial pH of 6.6. The diet used was a 50:50 forage to concentrate dairy cow diet with 17.9% crude protein, 30% neutral detergent fibre, and 21% acid detergent fibre, dry matter (DM) basis, and consisted (DM basis) of alfalfa hay (50.3%), ground barley grain (19.1%), ground corn grain (19.1%), soybean meal (10.9%), and a vitamin-mineral dairy cow premix (0.6%). The diet was designed to meet or exceed nutrient recommendations for a Holstein cow (650 kg) producing 30 kg of milk (NRC, 2001).

Incubations were conducted in 110-mL polypropylene tubes containing 50 mL of culture fluid with 0.5 g of the diet cited above ground through a 1-mm screen (Cyclotec CT 293, Foss, Barcelona, Spain). Treatments included a negative control without additive (CTR), monensin as a positive control (MON; Sigma-Aldrich Chemical, St. Louis, MO, USA), a blank without additive nor diet, and each of the 12 EO with the diet at three doses: low, medium, and high (Table 3.1). All essential oils were dissolved in ethanol in different proportions to reach the appropriate dose, and a total of 0.2 mL was added to the culture fluid in each tube. The equivalent amount of ethanol (0.2 mL) was added to the control and the blank. Each treatment was used in triplicate at each dose, and fermentations were replicated in two independent periods. Anaerobiosis was ensured by the addition of N_2 before sealing tubes with rubber stoppers. Incubations were conducted at 39 °C in a thermoregulated water bath.

3.3.1.2. Sample collection and chemical analyses

After 24 h of fermentation, final pH was measured with a pH meter (sensION+, HACH Company, Barcelona, Spain), and samples were collected for VFA (volatile fatty acids) and ammonia-N concentration analyses. Samples for VFA were analyzed by liquid chromatography. For that, 100 µL of sample was added to 50 µL of 2N HCl and shaken with a multivortex shaker for 5 s. Then, 1 mL of chloroform was added and shaken for 1 min again and centrifuged at 15,000 × g for 5 min at 4 °C. The

organic phase was collected and placed in a 2-mL Eppendorf containing 0.5 mL of 1M NaOH. Samples were shaken again for 3 min and centrifuged at 15,000 × g for 5 min at 4 °C. The aqueous phase (0.4 mL) was collected, 50 µL of 2N HCl were added, shaken with a multivortex shaker for 5 min and centrifugated at 14,500 × g during 5 min at 4 °C. The supernatant was placed in chromatographic glass vials before being injected for analysis. The HPLC system (HPLC 1100 series, Walldbroom, Germany) was composed of a quaternary pump, an automated injector, a column (Agilent, Sta. Clara, CA, USA) oven, and a UV detector. Branch-chained VFA were calculated as the sum of isobutyric and isovaleric acids. Ammonia-N concentration was analyzed as described by Chaney and Marbach (1962), where 4 mL sample of fermentation fluid were acidified with 4 mL of 0.2N HCl and frozen. After thawing, samples were centrifuged at 15,000 × g for 15 min at 7 °C, and the supernatant was analyzed by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK).

3.3.1.3. Statistical analyses

The effect of different doses within each treatment compared with control was analysed as a randomized block design using the MIXED procedure of SAS (v.9.4 SAS Institute, Inc., Cary, NC, USA). The dose was the fixed effect, and the period (block) was the random effect. Treatment means were compared to the control using the Dunnett's multiple comparison test (Miller, 1981), and were declared significant at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. All means are reported as least squares means.

3.3.2. Experiment 2: Mixtures of essential oils

3.3.2.1. Experimental procedures

Lemongrass (LEM), coriander (COR), and ginger (GIN) oils were chosen from the first experiment and used in different proportions according to the Simplex Centroid Design for experiments with mixtures (Cornell, 1990; Figure 3.1). The three points at the vertices of the triangle correspond to the pure essential oils. The midpoints of the sides of the triangle correspond to 1:1 mixture of two essential oils. The centre point of the triangle represents the three-component mixture in equal portions. Axial mixtures using different proportions were also investigated. These ten mixtures with different proportions of each component in the mix were prepared at a

total of 75 mg/L for a low dose and a total of 150 mg/L for a high dose (Table 3.2). A control (CTR; diet without EO) and a blank (rumen fluid and buffer without feed nor EO) were included within each run. All EO were diluted in ethanol, and the control and the blank were also dosed with the equivalent amount of ethanol (0.2 mL).

Fermentation conditions were the same as described in experiment 1. The incubations were conducted in triplicate and in two independent periods. At 24 h of fermentation, the final pH and samples for VFA and ammonia-N concentrations were collected and analysed as described in the first experiment.

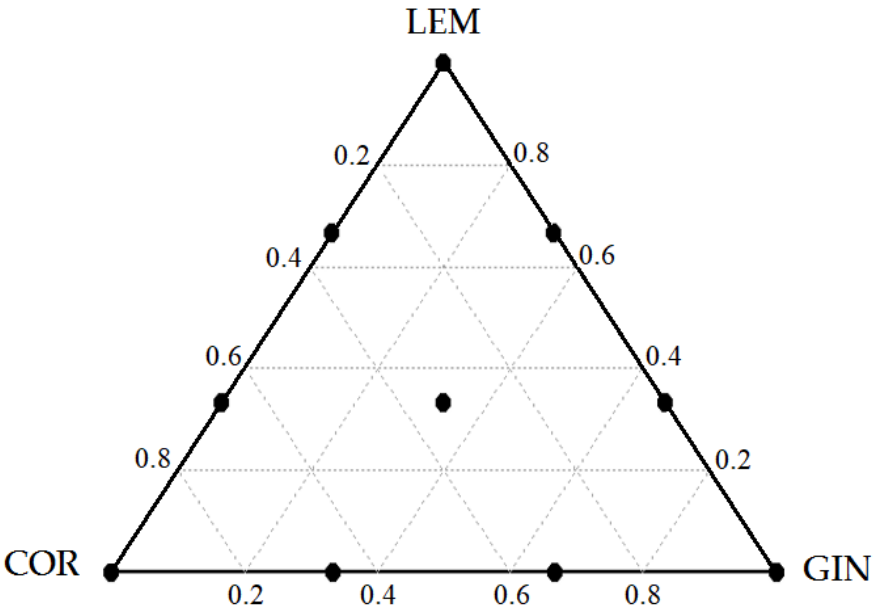

Figure 3.1. Simplex Centroid Design used to optimize the mixtures. The points correspond to the proportions of individual essential oils: lemongrass = LEM, coriander = COR, and ginger = GIN used in different combinations in this study.

Table 3.2. Determination of different proportions, combinations and doses of LEM, COR, and GIN used in experiment 2 according to the Simplex Centroid Design.

Treatments	Composition[1]	%			Low dose (mg/L)			High dose (mg/L)		
		LEM	COR	GIN	lem	cor	gin	LEM	COR	GIN
T1	LEM	1.00			75.0			150.0		
T2	COR		1.00			75.0			150.0	
T3	GIN			1.00			75.0			150.0
T4	LEM+COR	0.50	0.50		37.5	37.5		75.0	75.0	
T5	LEM+GIN	0.50		0.50	37.5		37.5	75.0		75.0
T6	COR+GIN		0.50	0.50		37.5	37.5		75.0	75.0
T7	LEM+cor+gin	0.50	0.25	0.25	37.5	18.8	18.8	75.0	37.5	37.5
T8	lem+COR+gin	0.25	0.50	0.25	18.8	37.5	18.8	37.5	75.0	37.5
T9	lem+cor+GIN	0.25	0.25	0.50	18.8	18.8	37.5	37.5	37.5	75.0
T10	LEM+COR+GIN	0.33	0.33	0.33	25.0	25.0	25.0	50.0	50.0	50.0

[1]The treatments (LEM=lemongrass, COR=coriander, GIN=ginger) are written in upper case when they are the major EO in the mixture and in lower case when they are the minor EO in the mixture.

3.3.2.2. Statistical analyses

The statistical analysis was performed in two steps. The effect of dose (high and low) of each mixture of EO was analyzed using the MIXED procedure of SAS with the fixed effects of dose, treatment and their interaction, and the random effect of period. Treatments were compared with control using the Dunnett test. Results are reported as least squares means and significance was set at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. The second analysis was for the Simplex Centroid Design using the R software from the R foundation for statistical computing (v. 4.0.2, 2020, https://www.R-project.org/ accessed on 2 February 2021). The analysis of such design is a multiple regression analysis without intercept (Cornell, 1990). The regression model included the three EO and their interactions as described below:

$$Y = a_{lem} \times LEM + a_{cor} \times COR + a_{GIN} \times GIN + b_{lem/cor} \times LEM \times COR + b_{lem/GIN} \times LEM \times GIN + b_{cor/GIN} \times COR \times GIN + c_{lem/cor/GIN} \times LEM \times COR \times GIN + \varepsilon.$$

where Y was the outcome (total VFA, A:P ratio, etc.); a, b, and c were the regression coefficient associated with corresponding term; LEM, COR, and GIN were the values of

the factor for the low and high doses (LEM + COR + GIN = 150 mg/L); and " was the residual error. Then, the sum of squares of the prediction error (PRESS) was calculated. For each fitted value, PRESS is obtained by deleting the i_{th} observation from the data set, estimating the regression equation from the remaining n-1 observations, then using the fitted regression function to obtain the predicted value for the i_{th} observation. The predictive ability of the model was assessed with PRESS. In general, the smaller the PRESS value, the better the model's predictive ability. The models adjusted in the second analysis was used to represent a contour plot. The contour plot provides a 2-dimensional view where all points that have the same response are connected by a line of constant responses.

3.4. Results

3.4.1. Individual essential oils

In general, the majority of EO screened in this experiment and monensin did not modify the rumen fermentation profile (Supplementary Table 9.1–9.7 in Chapter 9). The average concentration for total VFA was 74.0 ± 7.13. mM, for acetate proportion was 51.5 ± 1.92 mol/100 mol, for propionate proportion was 17.38 ± 1.58 mol/100 mol, for butyrate proportion was 20.0 ± 2.72 mol/100 mol, and for the A:P ratio was 3.07 ± 0.43. Total VFA concentration tended to increase only with the low dose of thyme oil. None of the doses of EO tested affected the acetate molar proportion. Only lemongrass oil at low dose tended to increase propionate proportion and tended to decrease the A:P ratio. Thyme oil at medium dose increased butyrate molar proportion. Coriander oil at low and high doses, and anise star oil at high dose tended to increase butyrate molar proportion. No effect of EO was observed on branch-chained VFA molar proportion. The effect of EO on ammonia-N is reported in Table 3.3. Capsicum, coriander, and thyme oils decreased ammonia-N concentration at low, medium, and high doses. Limonene tended to decrease ammonia-N concentration at medium and high doses. Cassia oil also tended to decrease ammonia-N concentration at high dose. However, geraniol increased ammonia-N concentration at low, medium, and high doses, and cassia oil tended to increase ammonia-N concentration at low and medium doses.

Table 3.3. Effect of essential oils on ammonia-N concentration (mg of N/100 mL) compared with control in *in vitro* rumen microbial fermentation of a 50:50 forage: concentrate diet.

Treatment	CTR	Dose[1]			SEM[2]	*P-V*alue
		Low	Medium	High		
Monensin	28.1	28.2			1.31	0.80
Anise star	28.1	29.2	28.5	27.5	2.60	0.56
Black peper	28.1	26.4	26.7	26.3	0.96	0.48
Capsicum	28.1	25.2*	24.3*	25.5*	1.56	0.02
Cassia	28.1	29.4+	30.8+	24.8+	1.74	0.09
Coriander	28.1	24.3*	23.2*	23.4*	0.99	0.01
Geraniol	28.1	33.1*	33.8*	27.8	1.06	0.03
Ginger	28.1	29.7	31.0	29.9	1.50	0.14
Lemongrass	28.1	32.5	31.9	31.1	1.68	0.21
Limonene	28.1	28.9	27.6+	25.7+	2.20	0.07
Tea tree	28.1	25.9	27.7	28.8	1.75	0.12
Thyme	28.1	26.4*	25.9*	17.2*	1.69	0.01
Turmeric	28.1	28.4	26.5	28.0	1.10	0.63

[1]Doses for each EO are reported in Table 3.1; [2]SEM: standard error of the mean.
*Means within a row differ from control ($P < 0.05$), + Means within a row differ from control ($P < 0.10$).

3.4.2. Mixtures of essential oils

Results of the analysis of variance which consisted of comparing the different treatments versus the control in the case of the low dose, showed no difference between the treatments and the control (data not shown). Therefore, the Simplex Centroid Design analysis was not applied on low dose treatments and will not be discussed further.

Results from the analysis of variance for the high dose used in this study of the control treatment and the Simplex Centroid Design treatments for total and individual VFA and ammonia-N are presented in Table 3.4, Table 3.5 represents the coefficient estimates and statistics values when using the interaction model of the Simplex Centroid Design treatments. Contour plot for total VFA and A:P ratio is presented in Figure 3.2a, b, respectively.

The sum of the squares of the prediction error (PRESS) was low for all the outcomes (Table 3.5), which reflects the fit of the model used in this analysis. This precision of prediction indicates that the model correctly represented the response surface. Also, the

high value of the R2 for all the outcomes (average $R^2 = 0.98$) indicates that the model was adequate for the analysis of the response surface.

Within the Simplex Centroid Design treatments, the three EO used individually increased total VFA, where the highest value was observed in COR followed by LEM and GIN. The significant coefficient of total VFA (Table 3.5) for the mix containing equal proportion of LEM and COR indicated that the two EO were synergistic in increasing total VFA concentration. This synergism is also illustrated in the left part of Figure 3.2a where the maximum of total VFA was observed when the mixture is formed by LEM and COR in a range of 65–90% with a maximum of 90% for the COR and completed with 10% of LEM. Figure 3.2a also shows that increasing the proportion of GIN in the mixture decreased total VFA concentration, and, therefore, has an antagonistic effect when mixed with LEM and COR.

Table 3.4. Effect of mixtures of lemongrass (LEM), coriander (COR) and ginger (GIN) oils according to the simplex centroid design on total and individual VFA, and ammonia-N concentration.

Treatment	Dose (mg/L)			TVFA[1]	Acetate	Propionate	Butyrate	A:P[2]	N-NH3
	LEM	COR	GIN	(m*M*)	(%)	(%)	(%)		mg/100 mL
T1	150			109.0*	52.4*	19.0*	24.6*	2.77+	18.1
T2		150		114.0*	54.1*	18.1	24.1*	3.02	17.9*
T3			150	107.0*	53.7*	18.7	23.9*	2.88+	18.7
T4	75	75		108.0*	50.2*	19.4*	24.8*	2.70+	18.7
T5	75		75	93.0	55.1*	18.4	21.8*	3.02	1–*
T6		75	75	91.0	56.8	18.2	21.9*	2.97+	18.6
T7	75	38	38	100.0*	54.8	17.6	18.0	3.06	20.4*
T8	38	75	38	97.0	57.9	17.5	17.6	2.83+	20.0
T9	38	38	75	104.0*	57.3	18.5	24.5*	2.83+	19.8*
T10	50	50	50	96.0	58.3	17.5	18.8	2.98+	18.8
Control	0	0	0	88.8	57.6	17.9	17.9	3.24	18.5
P–*V*alue				< 0.01	< 0.01	< 0.01	< 0.01	0.080	0.03
SEM				0.84	0.21	0.12	0.15	0.100	0.44

[1] Total volatile fatty acids.
[2] The acetate to propionate ratio.
*Means within a column differ from control ($P < 0.05$), [+] Means within a column differ from control ($P < 0.10$).

The three EO used individually decreased acetate, tended to decrease the A:P ratio, and increased propionate and butyrate molar proportions (Table 3.5). The mix containing equal proportions of LEM and COR resulted in the lowest A:P ratio and increased the propionate molar proportion (Table 3.5), suggesting that LEM and COR were synergistic in decreasing the A:P ratio and increasing propionate molar proportion. Figure 3.2b shows that the lowest A:P ratio was observed when the mixture is formed by LEM and COR in a range of 55–80% with a maximum of 80% for the COR and completed with 20% of LEM. In contrast, the addition of high doses of GIN increased the A:P ratio suggesting an antagonistic interaction of GIN versus LEM+COR. There were no synergies among the three EO on ammonia-N concentration. The lower value of ammonia-N was observed with COR used alone (Table 3.5).

Table 3.5. Results of the interaction model applied to the simplex centroid design mixing lemongrass (LEM), coriander (COR) and ginger (Gin) oils on total and individual VFA and ammonia-N concentration. For each term of the model, the regression coefficients (Coef), their standard error (SEM) and the associated p–Value are presented. The sum of squares of the prediction error is also shown (PRESS).

Treatment	TVFA[1] (m*M*)			Acetate%			Propionate%			Butyrate%			A:P[2]			NNH3[3]		
	Coef	**SEM**	***P***	**Coef**	**SEM**	***P***	**Coef**	**SEM**	***P***	**Coef**	**SEM**	***P***	**Coef**	**SEM**	***P***	**Coef**	**SEM**	***P***
LEM	111	4.54	<0.01	53.6	1.66	<0.01	18.9	1.62	<0.01	25.1	1.12	<0.01	2.74	0.31	<0.01	18.5	4.62	0.02
COR	114	4.54	<0.01	51.3	1.66	<0.01	18.8	1.62	<0.01	24.6	1.12	<0.01	3.04	0.31	<0.01	17.7	4.62	0.02
GIN	105	4.54	<0.01	55.0	1.66	<0.01	17.2	1.62	<0.01	23.3	1.12	<0.01	3.03	0.31	<0.01	18.6	4.62	0.02
LEM+COR	120	3.62	0.04	51.0	2.15	0.12	19.1	7.92	0.03	23.8	5.49	0.75	2.70	1.51	0.02	16.8	2.26	0.95
LEM+GIN	93	3.62	0.10	55.0	1.15	0.30	17.5	7.92	0.96	22.5	5.49	0.07	3.06	1.51	0.67	21.8	2.26	0.95
COR+GIN	97	3.62	0.14	54.1	2.15	0.78	17.0	7.92	0.91	23.0	5.49	0.20	2.83	1.51	0.87	22.3	2.26	0.89
LEM+COR+GIN	90	1.33	0.48	53.4	4.59	0.61	18.4	4.46	0.57	23.0	3.09	0.67	3.88	1.53	0.66	23.4	1.28	0.05
Statistical value																		
Linear	<0.01			<0.01			<0.01			<0.01			<0.01			<0.01		
Cuadratic	<0.01			<0.01			<0.01			<0.01			<0.01			<0.01		
RSD	1.21			2.35			2.29			1.58			0.44			6.54		
R^2	0.99			0.94			0.99			1.00			0.99			1.00		
Adjusted R^2	0.99			1.00			0.98			1.00			0.98			0.89		
Predicted R^2	0.97			1.00			0.97			0.99			0.96			0.80		
PRESS	0.07			0.07			0.03			1.23			0.03			0.09		

[1] Total volatile fatty acids.
[2] The acetate to propionate ratio.
[3] NNH_3 (mg/100 mL).

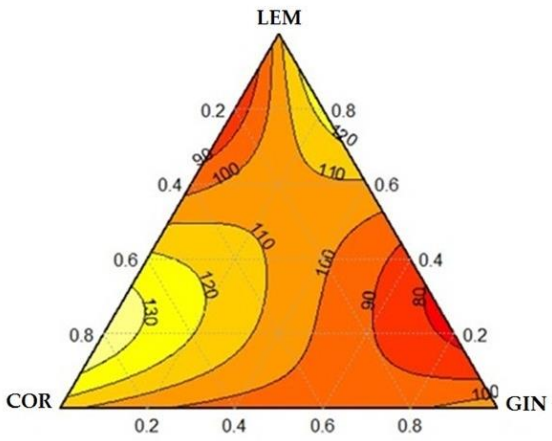

Figure 2 a: Total VFA (mM).

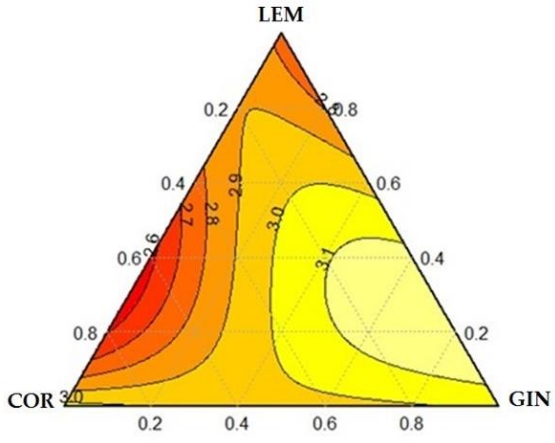

Figure 2 b: A:P ratio.

Figure 3.2. The two-dimensional contour plots for a) total VFA (in m*M*) and b) A:P ratio. The three vertices of the triangle represent individual EO: Lemongrass = (LEM), Coriander = (COR) and Ginger = (GIN). The contour plot provides a 2-dimensional view where all points that have the same response are connected by a line of constant responses.

3.5. Discussion

3.5.1. Effect of phenolic compounds (Experiment 1)

The EO screened in this study can be classified according to their main active compound as phenolic compounds (anis star, black pepper, capsicum, cassia, ginger, thyme, and turmeric oils) and monoterpenes (coriander, geraniol, lemongrass, limonene, and tea tree). Both phenolic and monoterpene compounds have exhibited effective antimicrobial activity in vitro (Calsamiglia et al., 2007; Benchaar et al., 2008). Phenolic compounds have been shown to have antimicrobial activity due to the presence of a hydroxyl group in the phenolic structure (Burt, 2004). These molecules have a wide spectrum of activity against Gram-positive and Gram-negative bacteria (Dorman and Deans, 2000; Lambert et al., 2001). However, in our study, the addition of phenolic compounds at low, medium, or high doses did not improve rumen fermentation profile in the desired direction (increasing propionate and decreasing acetate and the A:P ratio) except for thyme oil which tended to increase total VFA and butyrate proportion, and anise star oil that had a small effect only on butyrate molar proportion. Our results agree with previous studies that used thyme oil at doses ranging between our low and medium doses (at 100 or 200 mg/L) or its main active compound (thymol at 200 mg/L) in in vitro dairy cow rumen microbial fermentation conditions (Benchaar et al., 2008; Hristov et al.,

2008). However, Castillejos et al. (2006) used thymol at higher doses (500 and 5000 mg/L) and observed a decrease in total VFA and an increase in the A:P ratio, but such an effect is not beneficial for improving the energy efficiency. Busquet et al. (2006) used anise oil and its main active compound (anethol) at 300 and 3000 mg/L, and observed a decrease in total VFA, propionate, and acetate molar proportions. However, Chaves et al. (2008) used anethol at low dose (20 mg/L) and did not find effect on rumen microbial profile. The results obtained from capsicum in this study agree with those of Busquet et al. (2006) that used capsicum at different doses (3, 30, 300, and 3000 mg/L) and did not find effect on total VFA and the A:P ratio. Moreover, Busquet et al. (2006) and Nanon et al. (2015) tested ginger oil *in vitro* at similar or higher doses and did not find any effect. In this study, cassia oil did not affect total and individual VFA. Busquet et al. (2006) used cinnamaldehyde, one of the main active compounds of cassia at 3000 mg/L and observed a decrease in total VFA and an increase in propionate molar proportion. The same active compound was used in another in vitro study at 200 mg/L and had no effect on total and individual VFA (Benchaar et al., 2008).

In this study, black pepper and turmeric did not have any effect on total and individual VFA. Moreover, we did not find any study that used black pepper and turmeric oils or their main active compound (piperine and curcumin) in *in vitro* dairy cow rumen microbial fermentation conditions to compare with.

In addition to total and individual VFA, ammonia-N concentration was also measured, and was decreased by capsicum and thyme at low, medium, and high doses, and in cassia oil at high dose. Several studies reported that EO affect deamination and reduce ammonia-N concentration in the rumen (McIntosh et al., 2003; Ferme et al., 2004; Cardozo et al., 2005). The mechanism of action has been attributed to the effect of EO on bacterial groups that intervene in deamination, like hyper-ammoniaproducing bacteria and/or species of the *Prevotella* genus (McIntosh et al., 2003; Ferme et al., 2004). In contrast, cassia oil dosed at low and medium doses increased the concentration of ammonia-N. This effect is not beneficial, because ammonia-N concentration represents a loss of dietary nitrogen and a source of environmental pollution (Kebreab et al., 2002).

3.5.2. Effect of monoterpene compounds (Experiment 1)

A second group of active components used in this study were monoterpenes, which are widespread constituents of essential oils (Bakkali et al., 2008; Cobellis et al., 2016a, b) and also exhibit antimicrobial activity due to their alkyl group that may affect growth and energy metabolism of different microbial populations in the rumen (Benchaar and Greathead, 2011; Herman et al., 2016). Among the monoterpenes screened, only lemongrass oil tended to increase propionate and tended to decrease the A:P ratio. In contrast, Hristov et al. (2008) used lemongrass in vitro at dose slightly higher to our low dose (80 vs. 100 mg/L) and observed a decrease in total VFA and acetate molar proportion. Another study that used lemongrass at doses ranging between 186 and 1491 mg/L *in vitro* but in this case, lemongrass had no effect on the rumen microbial fermentation profile (Nanon et al., 2015). Joch et al. (2016) used citral, the main active compound of lemongrass at high dose (888 mg/L) and observed a decrease in total VFA and propionate proportion and an increase in acetate molar proportion and A:P ratio. The dose of lemongrass (80 mg/L) used in our study seems to be the more adequate to get a positive effect, because higher doses had no effect or negative effects on rumen fermentation profile. Limonene used in this screening did not improve rumen microbial fermentation at low, medium, and high doses. These results agree with those of Joch et al. (2016) that used limonene at higher dose compared with us (844 mg/L) but results were the same, no effects on total and individual VFA. In contrast, Castillejos et al. (2006) used limonene at doses similar or higher than ours (50, 500 and 5000 mg/L) and observed a decrease in total VFA and the A:P ratio. The tea tree oil used in this experiment did not modify the rumen fermentation profile. This result agrees with those of Hristov et al. (2008) that used tea tree oil (100 mg/L) and no effect was observed. Busquet et al. (2006) used tea tree oil at higher dose (3000 mg/L) but total VFA and propionate and acetate molar proportions decreased. We did not find references related to the use of coriander *in vitro* to modify dairy rumen fermentation profile. However, Joch et al. (2016) reported that the use of linalool, one of the main active compounds of coriander oil, in vitro at 870 mg/L decreased total VFA and propionate proportion. Geraniol oil used in this study did not show any effect on total and individual VFA at low, medium, and high doses. However, Joch et al. (2017) used geraniol at 300, 600, and 900 mg/L, and at 300 mg/L observed a decrease in total VFA and acetate proportion, and an increase in butyrate proportion and the A:P ratio.

Ammonia-N concentration was decreased by coriander, and limonene oils at low, medium, and high doses. As discussed previously, these effects have been attributed to the effect of EO on bacterial groups that intervene in deamination, like hyper-ammonia producing bacteria and/or species of the *Prevotella* genus (McIntosh et al., 2003; Ferme et al., 2004). In contrast, geraniol at low and medium doses increased the concentration of ammonia-N, which may reduce the efficiency of dietary nitrogen utilization and increase environmental pollution (Kebreab et al., 2002).

Overall, in the current study, EO supplemented individually did not improve the ruminal fermentation profile by increasing propionate and decreasing acetate and the A:P ratio.

3.5.3. Interaction among different essential oils (Experiment 2)

Our hypothesis was that the combinations of EO at doses that would not have an effect alone would have additive or synergistic effects. For that, in the second experiment, three EO were selected. The selection criteria considered the potential interaction between different types of active compounds (Bassolé et al., 2010). First, LEM containing citral as main active compound, is a monoterpene aldehyde that had positive effect on rumen fermentation in the first experiment. Coriander, containing linalool as main active compound was also selected as a monoterpene for its synergistic effect with phenolic compound (Bassolé et al., 2010). Ginger was selected because its main active compound (gingerols) are phenolics. Although in the first experiment ginger oil did not influence rumen microbial fermentation, we hypothesized that a mix of phenol and monoterpene may result in synergistic effect, and a mix of two monoterpenes, LEM and COR, may also result in additive effect. The determination of interactions between the three EO selected was tested using the Simplex Centroid Design. Our results showed the existence of interactions. Synergy means that the effect of the mixture is higher than the effect of the individual compounds (Burt, 2004). Significant coefficients (Table 3.5) for the two or the three mixed EO indicated that they interacted. Synergies were observed between LEM and COR, where the maximum for total VFA and the minimum for the A:P ratio were obtained by the mix of the two monoterpenes. In contrast, the addition of GIN to the mix decreased the total VFA and increased the A:P ratio suggesting an antagonistic effect of GIN versus LEM and COR on rumen fermentation.

From Figure 3.2a,b, the optimum proportion of each EO in the mix to maximize the surface response for total VFA ranges between 65 and 90% with a majority of COR completed by LEM, and to minimize the surface response of the A:P ratio, the optimum proportion ranges between 55 and 80% with a majority of COR completed by LEM also. The matching zone between these values ranges between 65 and 80% with a maximum of 80% for COR and a minimum of 65% for LEM and must obey other criteria such as price and availability in the market. Results obtained from the analyses of the Simplex Centroid Design, to explore possible additive effect between two monoterpenes and a synergy between a monoterpene and phenol, did not support this hypothesis which was based on the study of Bassolé et al. (2010) that observed an additive effect between monoterpenes and a synergy when using linalool and eugenol. In our case we observed a synergistic effect between two monoterpenes, LEM and COR, and an antagonistic effect when a phenol, GIN, was mixed with monoterpenes. Therefore, not all phenols can interact in the same way, and maybe the synergism found by Bassolé et al. (2010) was specific for eugenol. In another study, Fandiño et al. (2020) also reported antagonistic effect when clove bud oil was mixed with tea-tree, thyme, and (or) oregano oils. It is important to test the potential interactions between EO before mixing them to take advantage of the synergies and avoid the antagonistic effects.

3.6. Conclusions

In the first screening, the use of EO individually had small effects on rumen microbial fermentation. In the second experiment the use of the Simplex Centroid Design demonstrated a synergistic effect between LEM and COR to increase the total VFA concentration and propionate proportion, and to decrease the A:P ratio. However, an antagonistic effect was also observed when GIN oil was added at high doses to the LEM + COR mixture suggesting that only low doses should be used to optimize the ruminal fermentation. This study reveals the importance of taking into consideration the existence of positive and negative interactions among EO.

CHAPTER 4

Essential oils and rumen fermentation

CHAPTER 4

Screening for the effect of some essential oils, with or without carriers, on rumen microbial fermentation and methane production *in vitro*

4.1. Abstract

Two 24-h *in vitro* studies were conducted to evaluate the effect of a blend of anise star and cassia oils (BAC) and the effect of adding lemongrass oil (LEM) with four different carriers: silica-sunflower (SIL), rapeseed oil (RAP), diatomaceous earth (DIAT) and corn cob (CCO), on rumen microbial fermentation. In Exp. 1, treatments were: negative control, monensin (MON) as positive control, BAC, LEM, SIL, RAP, DIAT, CCO, LEM+SIL, LEM+RAP, LEM+DIAT and LEM+CCO. In Exp. 2, BAC and LEM were used to estimate total gas and methane production. All treatments were added to a 1:1 rumen fluid:buffer for Exp.1, and 1:4 rumen fluid:buffer for Exp.2, containing a 50:50 forage: concentrate diet with an initial pH of 6.6. In Exp.1, data were analyzed with the GLM procedures of SAS, and orthogonal contrasts analysis was used for comparison between control, EO and carriers. In Exp. 2, data were analyzed with MIXED procedure of SAS, and differences between means were evaluated using Tukey's multiple comparison test. Significance was declared at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency. In Exp. 1, BAC and LEM increased total volatile fatty acids, and SIL, RAP and CCO tended to increase total volatile fatty acids. Among the combinations of EO+carriers, only the combination LEM-RAP had an effect, decreasing ammonia-N concentration. In Exp. 2, BAC decreased total gas and methane production, and the ratio methane/total gas. In contrast, LEM increased total methane and the ratio methane/total gas.

4.2. Introduction

Volatile fatty acids and methane production are the main end products of rumen microbial fermentation. Methane represents a loss of about 5 to 15% of total metabolizable energy in ruminants (Johnson and Johnson, 1995; Eckard et al., 2010). Methane is also the main greenhouse gas emitted by the livestock sector (Moumen et al., 2016). Ionophors like monensin have been widely used to modulate rumen microbial fermentation and reduce methane production (Appuhamy et al., 2013). However, since the ban on the use of antibiotics as growth promoters in animal feeds in the EU in 2006 (Directive1831/2003/CEE; EC, 2003), the use of natural products has been the focus of research interest (Calsamiglia et al., 2007). Essential oils (EO) are complex mixtures of low molecular weight molecules containing mostly volatile organic compounds synthesized by different parts of plants (Hyldgaard et al., 2012). Several studies reported that EO can modulate rumen microbial fermentation, decreasing acetate, ammonia-N and methane, and increasing propionate and butyrate concentrations (Castillejos et al., 2006; Benchaar et al., 2008; Hristov et al., 2013), because EO are strong antimicrobial against a wide spectrum of Gram-positive and Gram-negative bacteria (Calsamiglia et al., 2007; Newbold et al., 2004). However, the low or insolubility in water, high volatility, and strong odor of EO limit their applications in dairy rations. Hence the need to prepare them, adsorbed or emulsified in a carrier. Several types of carriers (vegetable oils, silica, kaolin, Tween 80…etc; Stevanovic et al., 2020; Simoni et al., 2020) have been used with EO. A good carrier must not interact with or inhibit the EO effect, but on the contrary, it must protect it, by ensuring the oxidative stability, the thermostability, the photostability and the biological activity of the EO. For example, Liang et al. (2012) observed a high antimicrobial activity of peppermint oil emulsified with starch-based substrate against *Listeria monocytogenes* and *Staphylococcus aureus* compared to peppermint oil without carrier. Also, Simoni et al. (2020) observed a high digestibility when different EO were tested with silica and tween 80 carriers compared to EO without carriers. The improvement in the biological activity of EO when it is bound to a carrier could be mainly attributed to the better stability and the less exposure to degradation processes. However, it was hypothesized that in some cases these effects may be due to synergistic interactions of EO with certain carrier such as cashew gum or chitosan (Pandit et al., 2016). In this study, LEM oil, with citral as main active compound, which is a monoterpenes aldehyde, was selected for its positive effect on rumen fermentation (Temmar et al., 2021) and

known for its high antibacterial activity (Bassolé and Juliani, 2012). Lemongrass oil was used with organic (RAP and CCO) and inorganic (SIL and DIAT) carriers *in vitro* to evaluate their effect on rumen microbial fermentation. Others EO were also selected in this study such as anise, with anethol as main active compound, and cassia oils, with cinnamaldehyde as main active compound. Anethol and cinnamaldehyde both belonging to phenolic compounds with aromatic ring and known for their high antibacterial activity (Calsamiglia et al., 2007). It is reported that the combination of two phenolic compounds may resulted in additive and/or synergism effects (Bassolé and Juliani, 2012). In general, compounds with similar structures exhibit additive rather than synergistic effect (Bassolé and Juliani, 2012).

The objective of the present study was to evaluate the effect of BAC without carrier and LEM alone or with carriers on rumen microbial fermentation *in vitro*.

4.3. Materials and Methods

An animal care and use statement was not needed in this study because the rumen fluid was obtained from a slaughterhouse.

4.3.1. Experiment 1: Rumen fermentation

4.3.1.1. Experimental procedures

A blend of anise star and cassia oils (BAC) and lemongrass oil (LEM) with different carriers (silica-sunflower (SIL), rapeseed oil (RAP), diatomaceous earth (DIAT) and corn cob (CCO; Table 4.1) were supplied by TECHNA France Nutrition (Couëron, FR).

The experiment was conducted in *in vitro* batch fermentation conditions (Tilley and Terry, 1963). Rumen fluid was collected from a slaughterhouse from four dairy cows, filtered through four layers of cheesecloth, and mixed and transported to the laboratory in a thermos with no headspace. The rumen fluid was added to a phosphate-bicarbonate buffer (McDougall, 1948) in a 1:1 proportion, purged with N_2 and adjusted to an initial pH of 6.6. The diet used was a 50:50 forage to concentrate dairy cow diet (17.9% CP, 30% NDF, and 21% ADF, DM basis) and consisted (DM basis) of alfalfa hay (50.3%), ground barley grain (19.1%), ground corn grain (19.1%), soybean meal (10.9%) and a vitamin-mineral dairy cow premix (0.6%). The diet was designed to meet or exceed nutrient recommendations for a Holstein cow (650 kg) producing 30 kg of milk (NRC, 2001). Incubations were conducted in 110-ml polypropylene tubes containing 70 ml of

culture fluid with 0.7 g of the diet ground through 1-mm screen (Cyclotec CT 293, Foss, Barcelona, ES). Treatments are presented in Table 4.1. and included a negative control (diet without additive: CTR), a positive control (monensin at 10 mg/L), and a blank (without additive nor diet). The four carriers were also added within each run. The BAC and LEM were dissolved in ethanol in different proportions to reach the appropriate dose, and a total of 0.2 mL were added to the culture fluid in each tube. The equivalent amount of ethanol (0.2 mL) was also added to the CTR, monensin and the blank. Each treatment was used in triplicate, and fermentations were replicated in two independent periods. Anaerobiosis was ensured by the addition of N_2 before sealing tubes with rubber stoppers. Incubations were conducted at 39 ºC in a circulating water bath.

Table 4.1. Identification of EO, carriers and their different doses used in this study.

Composition	Dose in batch (mg/L)	EO (mg/L)	Carrier (mg/L)
Control	-	-	-
Monensin	10	-	-
BAC[1]	80+140	220	-
LEM	140	140	-
LEM+SIL[2]	525	140	383
LEM+RAP	700	140	560
LEM+DIAT	1015	140	873
LEM+CCO	1015	140	873

[1] 36.40% anise + 63.63% cassia; [2] 20% silica + 53% sunflower oil.

4.3.1.2. Measurements, Sampling and Analyses

Samples for volatile fatty acids (VFA) and ammonia-N concentration analyses were collected at 6 and 24h of fermentation. Also, final pH was measured with a pH meter (sensION+, HACH Company, Barcelona, ES). Samples for VFA were analyzed by liquid chromatography. For that, 100 µL of sample were added to 50 µl of 2N HCl and shaken with a multivortex shaker for 5 seconds. Then, 1 mL of chloroform was added and shaken for 1 minute again and centrifuged at 15,000 × g for 5 min at 4 ºC. The organic phase was collected and placed in a 2-mL Eppendorf containing 0.5 mL of 1M NaOH. Samples were shaken again for 3 min and centrifuged at 15,000x g for 5 minutes at 4 ºC. The aqueous phase (0.4 mL) was collected, 50 µL of 2N HCl were added, shaken with a multivortex shaker for 5 minutes and centrifugated at 14,500 × g during 5 minutes at 4 ºC. The

supernatant was placed in chromatographic glass vials before being injected for analysis. The HPLC system (HPLC 1100 series,Walldbroom, Germany) was composed of a quaternary pump, an automated injector, a column (Agilent, Sta. Clara, CA, USA) oven, and a UV detector. Branch-chained VFA were calculated as the sum of isobutyric and isovaleric acids. Ammonia-N concentration was analyzed as described by Chaney and Marbach (1962), where 4 mL sample of fermentation fluid were acidified with 4 mL of 0.2N HCl and frozen. After thawing, samples were centrifuged at 15,000 × g for 15 minutes at 7 ºC, and the supernatant was analyzed by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK).

4.3.1.3. Statistical analyses

The effect of addition of carriers and EO with or without carrier was tested using the general lineal model (GLM) procedure of SAS (v. 9.4 SAS Institute, Inc., Cary, NC). The treatment (12), sampling time (6 vs. 24h) and their interaction were the fixed effects, and the period was the random effect. Orthogonal contrasts analysis was performed to look for differences between control, EO and carriers at different sampling time (6 vs. 24 h). All means are reported as least squares means and were declared significant at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

4.3.2. Experiment 2: Gas and methane production

4.3.2.1. Experimental procedures

A second experiment was conducted to measure total gas and methane production *in vitro*. Treatments were a negative control (diet without additive; CTR), a positive control (monensin at 10 mg/L), LEM (140 mg/L), BAC (220 mg/L) and a blank (without additive nor diet). Incubations were conducted in 60-ml serum bottles in quadruplicate for each treatment (two serum bottles to measure total gas production, and two other serum bottles to measure methane production) in two periods. The *in vitro* incubation media was prepared anaerobically under N_2 atmosphere by mixing the buffered medium and ruminal fluid in 4:1 proportion (Goering and Van Soest, 1970). The rumen fluid was collected from a slaughterhouse from four dairy cows. Bottles were inoculated with 50 mL of buffer-fluid mixture solution, while the bottle headspace was continuously flushed with N_2, and 0.5 g of the same 50:50 forage to concentrate diet used in the first

experiment. Bottles were sealed with rubber stoppers and aluminum caps. Incubations were conducted in a circulating water bath at 39.0 ºC for 24 h.

4.3.2.2. Measurements, Sampling and Analyses

Gas production was measured by recording the pressure (Kpascal) produced in each duplicate bottle for each treatment using a manometer (HD8804, TP804, DELTA OHM, It) coupled to a pressure gauge at 2, 4, 6, 8, 10 and 24 h of incubation. After each sampling time, flasks were vented, mixed and returned to the water bath. Then, this pressure was converted to volume by using a linear equation defined at our laboratory between pressure recorded in the same type of bottles and known inoculated air volume as: $V = 0.067 + 1.172p$. Where V = gas volume (mL); p = measured pressure (Kpascal).

Samples for methane were collected in plain vacuum-sealed tubes (BD Vacutainers®, UK) at 2, 4, 6, 8, 10 and 24 h of incubation time. After each sampling time, flasks were vented, mixed and returned to the water bath. Chromatographic analysis was done by injecting a 100 µL sample (with a special syringe with a 100 µL gas valve) into a split injector at 200 ºC in a 5:1 ratio. Methane was determined using a gas chromatograph (GC6890A, Agilent, Sta. Clara, CA, USA) fitted to a flame ionization detector, using a capillary column (Agilent 19095Z-323 (300ºC Max) HP-1 Methyl Siloxane Capillary 30,0 m × 530 µm × 1,50 µm nominal, Germany) with Helium as carrier gas at a constant flow of 12 mL/min and an oven temperature maintained at 120ºC.

4.3.2.3. Statistical analyses

A nonlinear mathematical model of Gompertz (France et al., 2000) was used to fit the total gas production and methane production curves.

$$Y = a \exp\{-b \exp(-ct)\}$$

Where:

Y: cumulative gas production at time t (mL/ mg DM); *a* is the asymptotic maximal gas production (ml); *b* is a rate constant (h^{-1}); and *c* is a rate constant ($h^{-1/2}$).

The goodness of fit of the model was evaluated by analyzing the observed versus predicted gas and methane production values. The estimated kinetic parameters of methane and total gas productions, and accumulated methane and total gas productions data were analyzed using MIXED procedure of SAS 9.4 (SAS, Inc., Cary, NC). Treatment

was the fixed effects and period was the random effect. Differences between means were evaluated using Tukey's multiple comparison test. All means are reported as least squares means. Significance was declared at $P < 0.05$, and $0.05 < P < 0.10$ was considered a tendency.

4.4. Results

4.4.1. Experiment 1: Rumen fermentation

The results of the different treatments used in experiment 1 are presented in Table 4.2. Monensin, used in this experiment as positive control, did not had effect on total and individual VFA and ammonia-N concentration. The addition of EO, BAC and LEM to the medium resulted in the same response, both increased ($P < 0.05$) total VFA. However, the molar proportions of individual VFA and ammonia-N concentration were not affected by EO supplementation. The addition carriers, SIL, RAP and DIAT to the medium tended to increase total VFA ($P = 0.09$). However, the molar proportions of individual VFA and ammonia-N concentration were not affected by the addition of the carrier. The CCO carrier did not have any effect on rumen microbial fermentation. The addition of EO and carrier did not affect rumen microbial fermentation profile, except for the combination of LEM+RAP which decreased ($P < 0.05$) ammonia-N concentration, and the combination of LEM+SIL which tended to increase ($P = 0.06$) the proportion of BCVFA compared to the CTR.

The results of time of sampling (6 vs. 24 h) are presented in Table 4.3. Time of sampling (6 vs. 24 h) affected ($P < 0.05$) all variables except for butyrate and BCVFA proportions. However, the interaction between sampling time and different treatments was not significant ($P > 0.10$) for all measured variables.

Figure 4.2. Effect of EO with or without carriers on rumen microbial fermentation profile *in vitro*.

Item[1]	CTR	MON	BAC	LEM	SIL	RAP	DIAT	CCO	LEM +SIL	LEM + RAP	LEM +DIAT	LEM +CCO	SEM[2]	Effect (*P*-Value)		
														Trt	Hour	Interaction[3]
Total VFA (m*M*)	50.2	51.7	55.7 [U]	56.2[T]	58.1[V]	56.5[W]	59.5	64.9 [X]	55.4	54.4	57.1	57.8	0.45	0.09	0.01	0.41
Acetate	58.7	57.7	57.4	57	59.1	58.6	58.4	57.7	57.6	58.6	59.8	57.2	0.14	0.46	0.01	0.18
Propionate	20.3	22.2	21.2	19.4	18.7	20.9	19.8	19.4	19.2	20.0	20.0	21.5	0.15	0.28	0.02	0.26
Butyrate	14.6	14.3	14.6	15.1	15	14.6	15.5	15.6	15.4	14.6	14.2	14.8	0.11	0.89	0.66	0.10
BCVFA	4.39	4.05	3.86	6.24	4.49	3.7	4.51	5.03	5.55[Y]	4.72	4.33	3.88	0.118	0.4	0.62	0.44
A: P[4]	2.91	2.61	2.81	2.99	3.17	2.83	2.95	2.99	3.01	2.94	3.00	2.70	0.022	0.31	0.01	0.23
N-NH_3[5]	19.1	16.7	23.3	19.9	18.5	21	21	22.3	18.3	17.6[Z]	20	21.6	0.26	0.16	0.01	0.64
pH	6.25	6.32	6.25	6.26	6.25	6.24	6.24	6.21	6.27	6.24	6.23	6.20	0.025	1.00	-	-

[1] MON: monensin; BAC: Mix of anise star and cassia oils; LEM: lemongrass oil; SIL: silica-sunflower carrier; RAP: rapessed oil carrier; DIAT: diatomaceous earth carrier; CCO: corn cob carrier; LEM+SIL: lemongrass oil + silica-sunflower carrier; LEM+RAP: lemongrass oil + rapessed oil carrier; LEM+DIAT: lemongrass oil + diatomaceous earth carrier; LEM+CCO: lemongrass oil + corn cob carrier; [2] standard error of the mean; [3] interaction between treatment and sampling hour (6 vs. 24 h); [4] acetate to propionate ratio; [5] N-NH_3 (mg/100mL); [T] the contrast of control vs. LEM is different ($P < 0.05$); [U] the contrast of control vs. BAC is different ($P < 0.05$); [V] there is a tendency with the contrast of control vs. SIL ($P = 0.08$); [W] there is a tendency with the contrast of control vs. RAP ($P = 0.07$); [X] there is a tendency with the contrast of control vs. CCO ($P = 0.08$); [Y] there is a tendency with the contrast of control vs. LEM+SIL ($P = 0.06$); [Z] the contrast of control vs. LEM+RAP is different ($P = 0.04$).

Table 4.3. The effect of sampling time (6 vs. 24 h) on rumen microbial fermentation and their interaction with different treatments.

Item	Hour effect		SEM[1]	Effect (*P*-Value)		
	6	24		Treatment	Hour	Interaction[2]
TVFA (m*M*)	48.2	64.7	8.28	0.09	0.01	0.41
Acetate	57.5	58.8	0.65	0.46	0.01	0.18
Propionate	20.9	19.5	0.67	0.28	0.02	0.26
Butyrate	14.8	14.9	0.09	0.89	0.66	0.10
BCVFA	4.46	4.66	0.10	0.40	0.62	0.44
A: P[3]	2.79	3.03	0.12	0.31	0.01	0.23
N-NH_3 [4]	16.4	23.4	3.50	0.16	0.01	0.64
pH	-	6.25	-	1.00	-	-

[1]Standard error of the mean; [2]Interaction between sampling hour (6 vs. 24 h); [3]Acetate to propionate ratio; [4] N-NH_3 (mg/100mL).

4.4.2. Experiment 2: Gas and methane production

The results of the kinetic of gas production model for BAC, LEM monensin and control are presented in Table 4.4. No differences ($P > 0.10$) were observed in the regression coefficients (a, b and c) of the gas production kinetic between different treatments in this study.

Table 4.4. Estimated gas production variables of essential oils *in vitro*.

Gas production characterisrics	CTR	MON[1]	BAC[2]	LEM[3]	SEM[4]	*P*-Value
a, mL	0.004	0.001	0.002	0.006	0.0016	0.21
b, h^{-1}	2.23	2.12	1.51	1.31	0.446	0.42
c, $h^{-1/2}$	0.28	0.29	0.24	0.24	0.022	0.36

[1]Monensin used as positive control; [2] mix of anis star and cassia oils; [3]lemongrass oil; [4]standard error of the mean.

The results of cumulative total gas and methane production are presented in Table 4.5 and Figure 4.1. Monensin used as positive control did not affect the total gas and methane production nor the ratio methane/total gas compared to the CTR. BAC had the lowest cumulative total gas and methane production ($P < 0.05$) and the lowest ratio methane/cumulative gas ($P < 0.05$). LEM had the highest amount of total gas produced but not different from the CTR. However, LEM had the highest cumulative methane production ($P < 0.05$) and the highest ratio methane/total gas ($P < 0.05$). The addition of EO and monensin did not affect the pH compared to the CTR ($P = 0.43$).

Table 4.5. Total gas, methane production, methane to total gas ratio and pH at 24 h *in vitro* microbial fermentation.

Item	CTR	MON[1]	BAC[2]	LEM[3]	SEM[4]	*P*-Value
Cumulative total gas (mL/24h)	765.6 [a]	819.6 [a]	527.4 [b]	906.0 [a]	0.04	0.01
Cumulative CH_4 (mL/24)	55.2 [b]	56.4 [b]	22.7 [c]	81.3 [a]	0.01	0.01
Ratio CH_4/total gas	0.07 [b]	0.07 [b]	0.04 [c]	0.09 [a]	0.01	0.01
pH	6.48	6.45	6.45	6.45	0.01	0.43

[1]Monensin used as positive control; [2]A mix of anis star and cassia oils; [3]lemongrass oil; [4]standard error of the mean; [a,b,c] means in the same row with different superscripts differ significantly ($P < 0.05$).

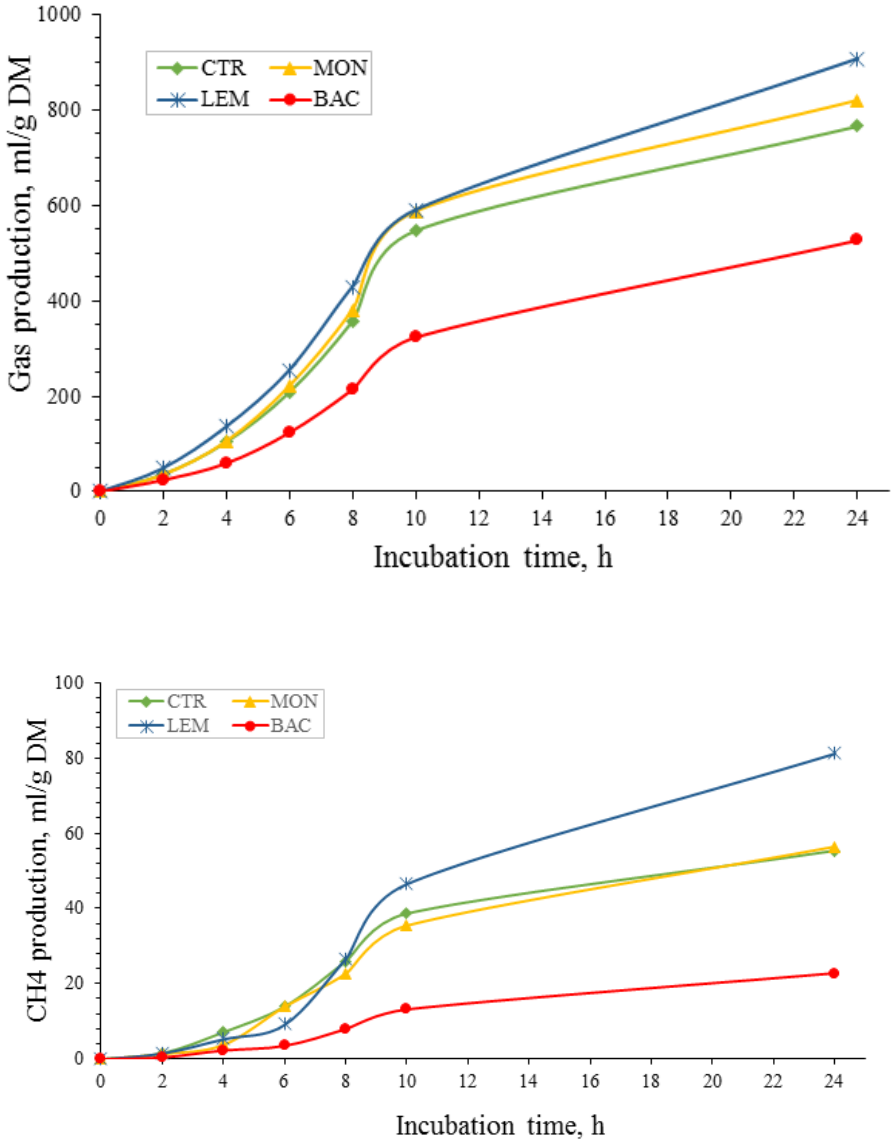

Figure 4.1. Effect of treatments: CTR, monensin (MON), a mix of anise star and cassia oils (BAC) and lemongrass oil (LEM), on cumulative gas and methane production at different incubation times.

4.5. Discussion

The loss of energy ingested by ruminants in the form of methane reduces feed efficiency and pollutes the environment (Eckard et al., 2010; Capper et al., 2009). EO can favorably affect rumen microbial fermentation and gas production *in vitro* (Calsamiglia et al., 2007; Benchaar et al., 2008; Bodas et al., 2012). However, EO could not be incorporated directly into the feed and need to be adsorbed or emulsified to a carrier, almost all *in vitro* studies have been conducted with EO directly without carriers (Joch et al., 2016; Nanon et al., 2015). In the current study we evaluated the effect of a blend of anise and cassia oils without carriers and LEM with four carriers on rumen microbial fermentation *in vitro*.

4.5.1. Rumen microbial fermentation

A mix of anise oil, with anethole as main active compound, and of cassia oil, with cinnamaldehyde as main active compound, was tested in this study. Anethol and cinnamaldehyde both belonging to phenolic compounds with aromatic ring and known for their high antibacterial activity. It is reported that the combination of two phenolic compounds may resulted in additive and/or synergistic effects. In general, compounds with similar structures exhibit additive rather than synergistic effect (Bassolé and Juliani, 2012). In our study, the BAC increased total VFA and decreased gas production. We could not compare our results because we could not find other work that used this mixture *in vitro*. Anise and cassia oils were used individually in Temmar et al. (2021) in which only anise increased butyrate molar proportion. Busquet et al. (2006) used anise oil and its main active compound, anethol, individually and at high dose of 3000 mg/L, observed a decrease in total and individual VFA. Chaves et al. (2008) used anethol at low dose of 20 mg/L and did not observe any effect. Busquet et al. (2006) used cinnamaldehyde, the main active compounds of cassia at high dose of 3000 mg/L and observed a decrease in total VFA and an increase in propionate molar proportion. The same active compound was used in another *in vitro* study at 200 mg/L and no effect on total and individual VFA was observed (Benchaar et al., 2007b). We noted that at high doses (i.e, 3000 mg/L) total VFA decreased, and this result is not in favor to the animal because VFA are their main source of energy and a decrease in VFA means less energy available to the ruminant (Benchaar et al., 2009). However, at lower doses (i.e, 20 mg/L) EO had no effect. Further studies are recommended to find the optimal dose to modulate rumen microbial

fermentation to produce more propionate and less acetate.

LEM, with citral as the main active compound, only increased total VFA. In Temmar et al. (2021), LEM was used individually at 80 mg/L, and tended to increase propionate, and decrease the A:P ratio. However, Hristov et al. (2008) used LEM at a dose slightly higher to the dose used in Temmar et al. (2021; 80 vs. 100 mg/L) and observed a decrease in total VFA and acetate proportion. Another *in vitro* study used LEM at doses ranging from 50 to 1600 mg/kg of DM, reported that 200 mg/ kg of DM of LEM increased total VFA without effect on individual VFA (Nanon et al., 2015). The differences observed between studies may be due to the difference in doses between studies, because EO act in a dose-dependent manner (Benchaar and Greathead, 2011). Furthermore, the dose and the concentration of the main active compound in the EO (Calsamiglia et al., 2007), the fermented substrate (Santos et al., 2010), the type of ruminal liquid incubated, and the pH (Cardozo et al. 2005), also interfere with the effect of the EO.

In addition to total and individual VFA, ammonia-N concentration was also measured in this study, but no effect of BAC and LEM was observed on ammonia-N concentration. In Temmar et al. (2021), anise did not had effect on ammonia-N concentration, but cassia oil tended to decrease ammonia-N concentration at dose of 750 mg/L and tended to increase it at doses of 80 and 300 mg/L. However, the mixture of anise with cassia in this study made all the effects of cassia on ammonia-N concentration disappear, which suggest an antagonist effect between cassia and anise oils.

In our study, the addition of SIL (that contained 20% silica and 53% sunflower oil), RAP and CCO carriers alone tended to increase total VFA. In contrast, the combination EO-carriers had no effect on rumen microbial fermentation, and all effect of EO and carriers alone disappear, suggesting an antagonistic effect between EO and the carriers, except for the combination LEM+RAP which decreased ammonia-N concentration. However, we did not find studies that used the same EO-carriers that we used to compare with.

4.5.2. Total gas and methane production

Accumulation of greenhouse gases (GHG) in the atmosphere which contribute to the increase in average global temperatures is currently one of the major concerns in animal production. Enteric fermentation is the main source of methane emission from dairy cows and represents about 46% of their total emission (Gerber et al., 2013). Several strategies

like the use of propionate enhancers (Karakurt et al., 2012), defaunation, dietary manipulation by the addition of lipids or nitrate (Hristov et al., 2013) and the use of feed additives like monensin (Odongo et al., 2007), have been successful in reducing enteric methane production. Therefore, there is a need for sustainable feed technology able to decrease methane emissions. Several studies have documented a reduction in methane production using EO (Macheboeuf et al., 2008; Castro-Montoya et al., 2015; Hart et al., 2019). In the present study, BAC and LEM were evaluated for their efficacy to mitigate methane production in *in vitro* ruminal microbial fermentation. The BAC was the most effective in reducing total gas, methane, and the ratio methane:total gas. The phenolic nature of BAC active components may explain its high inhibition of methanogen bacteria (Patra and Yu, 2012). In some cases, EO decrease methane production but also decrease total and individual VFA, reducing energy efficiency (Benchaar et al., 2008). For example, 400 mg/L of thymol, the main active compound of thymus and oregano, was reported to be a strong inhibitor of methane *in vitro*, but at the same time decreased acetate and propionate concentrations (Evans and Martin, 2000). Also, Busquet et al. (2005c) found that garlic oil and its component, diallyl disulfide, decreased *in vitro* methane production during 17 hours of incubations at 300 ppm, but also total VFA production was decreased. In our case, BAC increased total VFA, which is an interesting result, because BAC decreased gas and methane production without decreasing total VFA. The effect of BAC in this study allowed as to suggest that BAC reduce methanogen archaea which resulted in less gas and methane production.

The second EO used in this study was LEM, with citral as main active compound. Lemongrass oil increased total VFA in Exp. 1 but also increased methane production and the ratio methane/total gas. This difference between the EO effect of BAC and LEM suggests that BAC and LEM have a different mechanism of action and different target bacteria in the rumen.

4.6. Conclusions

A combination of anise and cassia oils increase total VFA and decrease methane and total gas production. In contrast, LEM increased total volatile fatty acid and methane production. The combination of LEM with different carrier made the whole effect of LEM disappear, which suggested an antagonistic interaction between the LEM, and the carriers used.

CHAPTER 5

Guanidinoacetic acid and rumen fermentation

CHAPTER 5

Evaluation of guanidinoacetic acid in dairy or beef fermentation conditions on rumen microbial fermentation and nutrient flow from a continuous culture system

5.1. Abstract

Eight dual flow continuous culture fermenters were used in 2 periods (7 days adaptation and 3 days sampling) to evaluate the effect of GAA on rumen microbial fermentation and nutrient degradability in dairy and beef cattle. The study was a randomized block design. Treatments (n = 4) were arranged in a 2 × 2 factorial, with factors being the type of fermentation conditions: beef (pH between 5.5 and 6.5; diet 10:90 forage:concentrate, 16.3% CP and 17.6% NDF) or dairy (pH between 5.8 and 6.8; diet 50:50 forage:concentrate, 17.1% CP and 30.0% NDF); and GAA: 0 vs. 2 g/d. Temperature (38.5 ºC), liquid (0.10/h) and solid (0.05/h) dilution rates were kept constant. Diets (90 g/d DM) were fed in 3 portions/d. Effluent samples were collected from a composite of the 3 sampling days, and bacteria were isolated on the last day of each period from fermenters for protein metabolism calculations. Fermenter samples were taken 3 h after the morning feeding of sampling days for microbiome analysis. Fermentation data were analyzed with the PROC MIXED of SAS and the microbiome diversity and composition with R-Studio. Significance was set at $P < 0.05$ and $0.05 < P < 0.10$ was considered a tendency. No differences were observed on true OM degradation. Degradation of NDF, the proportions of acetate, butyrate, the acetate to propionate ratio, NH3-N concentration, the flow of total N and ammonia N, the efficiency of microbial protein synthesis, and alpha and beta diversity of microbial population were higher in dairy than in beef. Total VFA and the propionate proportion were higher in beef than in dairy. The GAA increased NH_3-N concentration, and the flow of total and ammonia N. The microbial degradation of GAA was higher in dairy (69.8%) than in beef (6.30%) fermentation conditions. The GAA supplementation did not affect rumen microbial fermentation. The rate of degradation of GAA should be taken in consideration when GAA is used in dairy cow.

5.2. Introduction

Guanidinoacetic acid is a natural precursor of creatine, which is a compound that allows the storage of high-energy phosphate bonds in muscle (Brosnan and Brosnan, 2010; Murakami et al., 2014). GAA is synthesized from amino acids, mainly from glycine and arginine. The Food and Drug Administarion (FDA, 2016) allowed the use of GAA and declared it as a safe substance that spares arginine and serves as a precursor of creatine in broiler chicken and turkey drinking water and feeds. Also, the European Commission allowed its use as feed additive for growing chickens and pigs (Regulation (EU) 2016/1768; EC, 2016).

Several studies reported that supplementing broiler chicken diets with GAA improved creatine synthesis, feed conversion ratio, growth performance, energy metabolism and gut morphology (Faraji et al., 2018; Amiri et al., 2019; Portocarero and Braun, 2021). He et al. (2019) reported that feeding 600 mg of GAA/kg of feed to broiler chicken increased their feed conversion ratio by 13.3%. Also, feeding GAA to finishing pigs increased their average daily gain by 15.7% (Li et al., 2018).

However, its use in ruminants has not been widely studied. Li et al. (2020) studied the effect of increasing GAA doses (0.3, 0.6 and 0.9 g/kg of DM) on rumen fermentation profile and microbial population in Angus bulls, and observed that increasing GAA supplementation improved microbial growth, especially *R. flavifaciens*, *B. fibrisolvens*, *P. ruminicola* and *Rb. Amylophilus*, and increased the activities of cellobiases, pectinases and proteases. This increase in microbial growth resulted in higher nutrient degradation, higher total VFA and propionate proportion, and lower acetate proportion, acetate to propionate ratio and ammonia-N concentration. Later, Liu et al. (2021) supplemted Angus bull diets with 0.6 g/kg DM of GAA with or without betaine and observed an increase in the population of total bacteria, *R. albus*, *B. fibrisolvens* and *Rb. Amylophilus*, and an increase in the activities of xylanases, cellobiases, proteases and α-amylases which resulted in higher total VFA and ammonia-N concentration without affecting individual VFA or the acetate to propionate ratio.

The rest of the studies dealing with the use of GAA in ruminants are focused more on its post-ruminal effect. Among the positive effects of the post ruminal infusion of GAA are the increase of creatine synthesis in heifers and the spare of arginine utilization (Ardalan et al., 2020; Speer et al., 2020). The structure of the GAA molecule is like an amino acid, suggesting that its ruminal degradation may be high. Speer et al. (2020)

studied the bioavailability of GAA when infused directly into the rumen or abomasum and observed that GAA was 50 % degraded in the rumen of steers.

The aims of this study were to evaluate the effect of adding GAA to beef and dairy rumen fermentation conditions on rumen microbial fermentation, the rumen microbiome, and the microbial degradation of GAA.

5.3. Materials and Methods

An animal care and use statement was not needed in this study because the rumen fluid was obtained from a slaughterhouse.

Eight 1340 mL dual flow continuous culture fermenters (Hoover et al., 1976) were used in two replicated periods. Each experimental period consisted of 7 days for adaptation of the ruminal fluid to the continuous culture system and 3 days for sampling. Treatments (n = 4) were arranged in a 2 × 2 factorial design, the main factors being the fermentation conditions: beef (pH between 5.5 and 6.5; diet 10:90 forage:concentrate, 16.3% of crud protein (CP) and 17.6% of neutral detergent fiber (NDF)) or dairy (pH between 5.8 and 6.8; diet 50:50 forage:concentrate, 17.1% CP and 30.0% NDF), and the addition of 0 vs. 2 g/d of GAA (Creamino®, Alzchem SA®, Trostberg, Germany).

5.3.1. Diet

A total of two diets, one for beef (10:90 forage:concentrate; NRC, 2016) and another for dairy (60:40 forage:concentrate; NRC, 2001) cattle, were formulated to meet or exceed their nutrient recommendations. The ingredients were ground through a 1-mm mesh screen to obtain a homogeneous mixture. Ingredients and chemical composition of diets are presented in Table 5.1. Diets were fed at 90 g/d of DM in three equal portions at 06:00, 14:00 and 22:00 h to the fermenters.

Table 5.1. Ingredient and chemical composition of dietary treatments.

	Diet type	
Item	Beef	Dairy
Ingredient composition (% DM)		
Corn silage	-	28.3
Grass silage	-	16.5
Barely grain	-	8.27
Corn Grain Ground Fine	69.1	12.6
Soybean Meal 44 Solvent	15.2	23.9
Fat Soybean Oil	2.21	-
Wheat Straw	9.67	1.97
Urea 281 CP	0.83	-
Limestone Ground	0.69	-
Sodium Bicarbonate	0.69	-
Calcium Carbonate	-	0.81
MinVit[1]	-	7.71
Salt Trace Min	1.10	-
Salt White	0.55	-
Chemical composition (% DM)		
DM	89.9	92.5
OM	93.6	92.3
CP	16.3	17.1
NDF	17.6	30.0
ADF	9.0	17.3

[1]Mineral and Vitamin mix contained per kg DM: Ca 0.61%, P 0.38%, Mg 0.21%, K 1.45%, S 0.21%, Na 0.02%, Cl 0.27%, Fe 154.97 ppm, Zn 32.65 ppm, Cu 9.98 ppm, Mn 38.04 ppm, Se 0.07 ppm, Co 0.07 ppm, I 0.01 ppm, 1000 KIU of Vit A, 200 KIU of Vit D_3, 1330 mg of Vit E.

5.3.2. Continuous culture conditions

On the first day of each period, four fermenters were inoculated with undiluted ruminal fluid taken from a slaughterhouse from four beef cattle and another four fermenters were inoculated with undiluted ruminal fluid taken from a slaughterhouse from four dairy cattle. Fermentation conditions were maintained constant with a temperature of 38.5 ºC. The pH was left to fluctuate between 5.5 and 6.5 for beef, and between 5.8 and 6.8 for dairy cow. The pH limits were controlled by the infusion of 3N HCl or 5N NaOH. Fermenter pH was continuously recorded using a 1/3 Sony CCD IR camera. Anaerobic conditions were maintained by the infusion of N_2 gas at a rate of 40 mL/min. Artificial saliva (Weller and Pilgrim, 1974) containing 0.4 g/L of urea was continuously infused to simulate recycled N. Infusion of saliva and flows of filtered liquid were set to maintain liquid and solid dilution rates at 0.10 and 0.05/h respectively.

5.3.3. Sample collection

During sampling days, collection vessels were maintained at 4 ºC to prevent microbial activity. Every 24-h, solid and liquid effluents were mixed and homogenized for 1 min at 1700 rpm (RZR 2052 control, Heidolph, Germany) and a 500 mL sample was removed by aspiration and frozen at −20 ºC. Upon completion of each period, effluents from the three sampling days were composited and mixed within fermenter and homogenized for 1 min. Subsamples were taken for total N, ammonia-N, VFA analyses and the degree of rumen GAA degradation. The remainder of the sample was lyophilized. Dry samples were analyzed for DM, ash, NDF, ADF, and purine contents. In addition, another sample of approximately 10 mL was collected directly from the fermenter 3 h after the morning feeding of sampling days and frozen at − 80 ºC for subsequent microbial population analyses. Bacterial cells were obtained from fermenter flasks on the last day of each experimental period. Solid and liquid associated bacteria were isolated using a combination of several detachment procedures, selected to obtain the maximum detachment without affecting cell integrity (Whitehouse et al., 1994). One hundred milliliters of a 2 g/L methylcellulose solution and small marbles (30 of 2 mm and 15 of 4 mm of diameter) were added to each fermenter and incubated in the same fermenter flasks at 38.5 ºC and mixed for 1 h to remove attached bacteria. After incubation, fermenter flasks were refrigerated for 24 h at 4 ºC. Then, fermenter contents were agitated for 1 h to dislodge loosely attached bacteria. Finally, the fermenter contents were filtered through cheesecloth and washed with saline solution (8.5 g/L NaCl). Bacterial cells were isolated from fermenter contents within 4 h by differential centrifugation at 1000 × g for 10 min at 10 ºC to separate feed particles, and the supernatant was centrifuged at 20,000 × g for 20 min at 10 ºC to isolate bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 × g for 20 min at 10 ºC. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N and purine contents. Degradation of OM, NDF, ADF, CP, and flows of total, non-ammonia, microbial and dietary N were calculated as described by Stern and Hoover (1990).

5.3.4. Chemical analyses

Effluent DM was determined by lyophilizing 200 mL aliquots in triplicate with subsequent drying at 103 ºC in a forced air oven for 24 h. The DM content of diets and bacterial samples was determined by drying samples for 24 h in a 103 ºC forced air oven (950.01; AOAC, 1990). Dry samples of diets, effluents and bacteria were ashed at 550 ºC in a muffle furnace (942.05; AOAC, 1990), and OM was determined by difference. Fibre components of diets and effluents were analyzed sequentially (Van Soest et al., 1991) using a thermostable alpha-amylase and sodium sulfite and expressed without residual ash. Total N of diets, effluents and bacterial samples was determined by the Kjeldhal method (976.05; AOAC, 1990) Ammonia-N was analyzed by colorimetry as described by Chaney and Marbach (1962), where 4 mL of a 0.2 N HCl solution were added to 4 mL of filtered rumen fluid and frozen. After thawing, samples were centrifuged at 15,000 g for 15 min at 7 ºC, and the supernatant was used to determine ammonia-N by spectrophotometry (Libra S21, Biochrom Technology, Cambridge, UK). Samples for VFA analysis were analyzed by gas chromatography (model HP 6890 Series II GC System, with flame ionization detection (FID), Agilent Technologies, USA, equiped with a Column BP21 30 m × 0.25 mm x 250 µm, SGE, pre-column apolar 5 m × 0.32 mm Technochrome). Samples of lyophilized effluent and bacterial cells were analyzed for purine content (adenine and guanine) by HPLC as described by Balcells et al. (1992), using allopurinol as internal standard. The GAA content in fermenter fluid samples were analyzed, using an ion chromatography system (Column: Hypersil Hypercarb 4.6 × 100 mm, pre-column Aminopac PA1 and Aminopac PA1, 4 × 250 mm (in stated order). Eluent: Deionised and vacuum degassed water. Injection volume: 50 µL. Flow: 1.0 mL/min. Column temperature: 30 ºC. Detection: UV, 200 nm).

5.3.5. Microbial population analyses

Results of total a marker-based approach using the 16S ribosomal RNA subunit gene (rRNA16S) was used to study bacterial diversity of fermenter samples. The composition and structure of the sampled microbial communities was assessed through the amplification and sequencing the V3-V4 variable regions of the 16S rRNA gene. The Illumina Miseq sequencing 300 × 2 approach was used. Amplification was performed after 25 PCR cycles. A negative control of the DNA extraction was included as well as a positive Mock Community control to ensure quality control. Taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva

database version 138 (99% OTUs full-length sequences). All steps of the microbiota analysis, from DNA extraction, amplification and sequencing, description and quantification, diversity, and taxonomic study profile bioinformatics processing and analysis, were carried out in Microomics Systems S.L. (Barcelona, Spain).

5.3.6. Statistical Analyses

Results of total concentration and individual proportions of VFA, N fractions, nutrient degradation and flows were analyzed as a completely randomized block design using the PROC MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). The model accounted for the effects of fermentation conditions (beef vs. dairy) and GAA addition, and the interaction of both as fixed effects. Period was considered as a random effect. Treatment means (n = 4 for each treatment) are reported as least squares means and differences were tested using the Tukey's multiple comparison test and declared significant at $P < 0.05$, and a tendency at $0.05 < P < 0.10$.

Biostatistical analysis for the microbiota was performed using the open-source software RStudio (V3.6.0). Alpha diversity comparisons were performed using the Kruskal-Wallis non-parametric test. Beta diversity distance matrices were used to calculate principal coordinates analysis (PCoA) and to make ordination plots. The significance of groups present in the community structure was tested using the Permanova and ANOSIM tests. The Permdisp test was used to identify location vs. dispersion effects. The significant threshold was set at $P < 0.05$. Differential relative abundance of taxa was tested using two methods: ANCOM and Kruskal Wallis non-parametric test. After the Kruskal Wallis, Conover's test with FDR Benjamini-Hochberg correction was added for pairwise comparisons. The significant threshold was set at $P < 0.05$. BiodiversityR version 2.11-1, PMCMR version 4.3, RVAideMemoire version 0.9-7 and vegan version 2.5-5 packages were used for the different statistical analysis of the microbiome.

5.4. Results

The interaction between the fermentation conditions and the addition of GAA was not significant except few exceptions and, therefore, results of nutrient degradation, total and individual VFA, and nutrient flows are presented as main factors.

5.4.1. Effects of fermentation conditions

Fermentation conditions had no effect on organic matter truly degraded. In contrast, NDF and ADF degradation was higher ($P < 0.05$; Table 5.2) in dairy than beef fermentation conditions.

Table 5.2. Effect of the fermentation conditions and the addition of a guanidineaceteic acid (GAA) on organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradation in continuous culture.

Item	Treatments				SEM[1]	*P*-Value	
	Fermentation conditions		GAA (g)				
	Beef	Dairy	0	2		Fermentation conditions	GAA
True degradation, %							
OM	61.6	57.1	62.1	56.7	7.94	0.15	0.10
Degradation, %							
NDF	31.2	49.9	40.6	39.6	3.75	0.04	1.00
ADF	28.2	50.6	39.8	39.1	5.19	0.04	0.96

[1] SEM standard error of the mean.

The beef fermentation conditions resulted in higher total VFA and propionate proportion than dairy fermentation conditions. In contrast, dairy fermentation conditions resulted in higher acetate and butyrate proportions, and acetate to propionate ratio (Table 5.3).

The effects of beef or dairy fermentation conditions on N metabolism of rumen microbial fermentation are presented in Table 5.4. Ammonia-N concentration, flow of total and ammonia N, and efficiency of microbial protein synthesis (EMPS) were higher ($P < 0.05$) in dairy than in beef. Also, CP degradation tended to be higher ($P < 0.10$) in dairy than in beef. In contrast, no effect ($P > 0.05$) between beef and dairy fermentation conditions was observed on non-ammonia, bacterial and dietary N flow.

Table 5.3. Effect of the fermentation conditions and the addition of guanidineaceteic acid (GAA) on total and individual volatile fatty acid (VFA) concentration in continuous culture.

Item	Treatments				SEM[1]	*P*-Value	
	Fermentation conditions		GAA (g)				
	Beef	Dairy	0	2		Fermentation conditions	GAA
Total VFA (m*M*)	112.6	100.1	107.2	105.5	1.85	0.01	0.48
VFA (mol/100 mol)							
Acetate	44.1	51.2	47.3	48.0	1.65	0.02	0.72
Propionate	40.9	25.6	33.2	33.2	1.76	0.01	1.00
Butyrate	10.6	19.3	14.8	15.1	1.48	0.01	0.81
BCVFA[2] (mM)	0.58	1.36	0.92	1.02	0.138	0.02	0.62
Acetate:propionate	1.12	2.02	1.51	1.63	0.091	0.01	0.39

[1] SEM standard error of the mean; [2] Branched chain VFA includes isobutyrate and isovalerate.

Table 5.4. Effect the fermentation conditions and the addition of guanidineaceteic acid (GAA) on N metabolism of ruminal microorganisms in continuous culture.

Item	Treatments				SEM[1]	*P*-Value	
	Fermentation conditions		GAA (g)				
	Beef	Dairy	0	2		Fermentation conditions	GAA
N-NH_3 (mg/100mL)	2.92	8.18	3.78	7.31	0.662	0.01	0.02
N flow (g/d)							
Total	2.67	2.83	2.71	2.79	0.023	0.01	0.02
Ammonia	0.09	0.27	0.12	0.24	0.021	0.01	0.02
Non-ammonia	2.58	2.56	2.58	2.56	0.033	0.71	0.58
Bacterial	0.87	1.00	1.00	0.87	0.239	0.43	0.43
Dietary	1.71	1.56	1.58	1.68	0.249	0.31	0.48
CP degradation, %	33.1	43.7	32.5	44.3	9.95	0.10	0.08
EMPS[2]	22.1	30.6	25.7	27.0	2.20	0.02	0.67
GAA degradation (%)	6.3	69.8	0	76.0	0.04	0.01	0.01

[1] SEM standard error of the mean; [2] EMPS efficiency of microbial protein synthesis (g bacterial N/kg organic matter truly digested).

5.4.2. Effects of GAA supplementation

Neutral detergent fiber and ADF degradation were not affected by the addition of GAA. However, organic matter tended ($P < 0.10$) to decrease in fermenters with GAA compared with control (Table 5.2). No effect of GAA was observed on total and individual VFA (Table 5.3). However, the addition of GAA to fermenters resulted in higher ($P < 0.05$) ammonia-N concentration, and total and ammonia-N flow (Table 5.4).

The interaction between rumen fermentation conditions and GAA supplementation was significant ($P = 0.03$) by increasing non-ammonia N flow for beef and decreasing it for dairy fermentation conditions (2.62 vs. 2.49 ± 0.047 g/d; respectively). The same interaction tended ($P > 0.10$) to increase ammonia-N concentration (3.89 vs. 10.7 ± 0.905 mg/100 mL), total N (2.75 vs. 2.84 ± 0.032 g/d), and ammonia-N flow (0.12 vs. 0.35 ± 0.029 g/d) for beef and dairy fermentation conditions respectively. In contrast, the interaction between fermentation conditions and GAA supplementation tended ($P = 0.06$) to increase dietary N for beef and decrease it for dairy fermentation conditions (1.90 vs. 1.46 ± 0.264 g/d; respectively).

The average GAA degradation was higher (69.8%) in fermenters inoculated with dairy conditions than in beef conditions (6.3%).

5.4.3. Rumen microbial population

Alpha and beta diversity

Bacterial population diversity was measured by alpha diversity indices that explain variability within samples and are presented in Figure 5.1. The number of observed operational taxonomic units (OTUs), which indicate the microbial community richness, was higher ($P < 0.05$) for dairy compared with beef conditions. The addition of GAA and their interaction with fermentation conditions did not affect the alpha diversity indices. The Evenness and the Shannon indices were not different among dairy or beef rumen fermentation conditions nor for the addition of GAA.

The beta diversity, that measures the change in the diversity of species between samples, is visualized by the dispersion of principal coordinate analysis (PcoA; Figure 5.2) that allows to explore similarities or dissimilarities of microbial diversity among treatments.

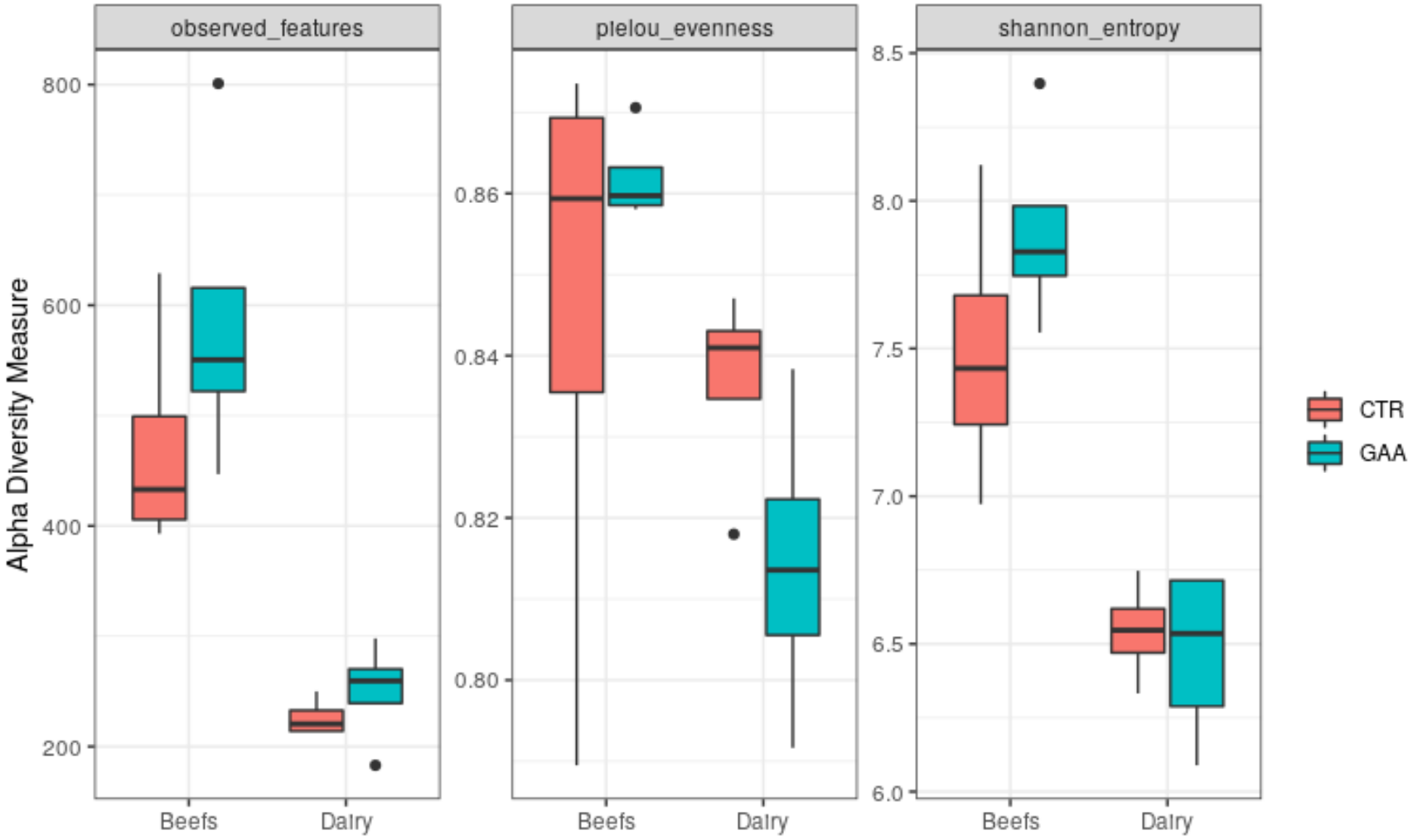

Figure 5.1. Alpha diversity indices of beef and dairy rumen microbiota (Red color, CTR; Bleu color, GAA).

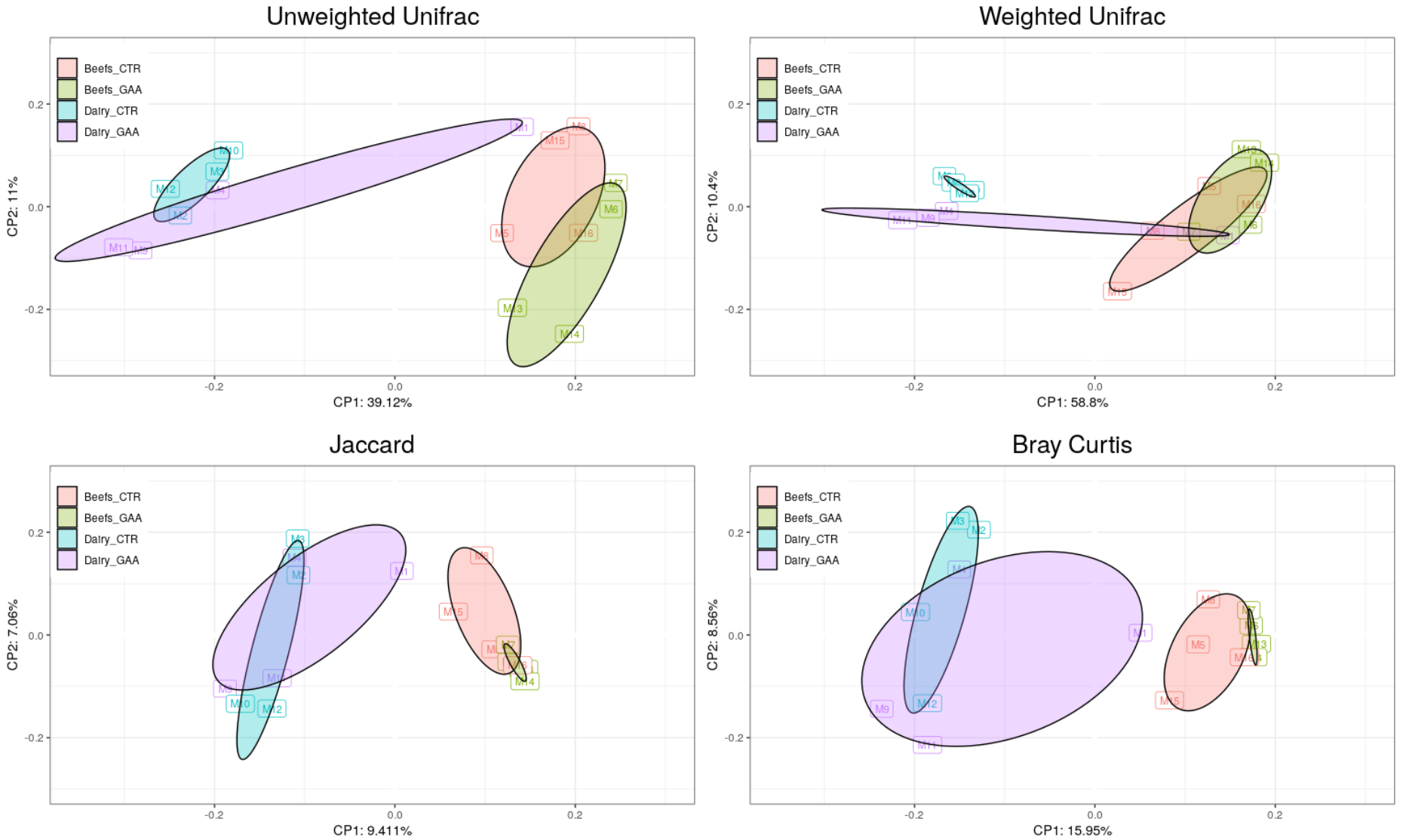

Figure 5.2. Principal Coordinate Analysis to visualize similarities or dissimilarities of microbial Beta diversity of different treatments.

Symbols (from M1 to M 16) represent fermenter samples, colors peach and pistachio represent dairy conditions, and green and purple represent beef conditions. The PcoA dispersion using a Curtis distance showed that samples with or without GAA were similar ($P > 0.10$). However, differences were observed between beef and dairy rumen fluid samples ($P < 0.05$). Beta diversity indices (Figure 5.2) demonstrated that dairy fermentation conditions had higher diversity compared with beef conditions.

The relative abundance of the main genera in cattle rumen fluid is shown in Figure 5.3. No significant ($P > 0.10$) differences due to the fermentation conditions nor to GAA supplementation or their interaction were observed. In beef conditions with or whithout GAA, the most abundant genera were *Prevotella*, *Disulfovibrio* and *Acidaminococcus*. In dairy conditions with or without GAA, in addition to *Prevotella* and *Disulfovibrio*, genera, *Enterococcus* and *Lactobacillus* were also relatively abundant.

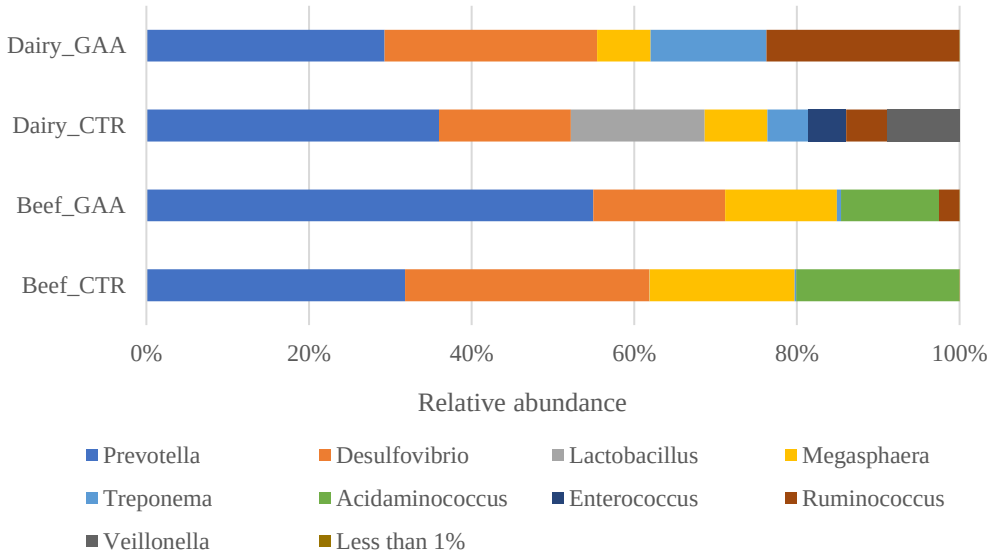

Figure 5.3. Relative abundance (%) of the main genera found in different treatments.

5.5. Discussion

The use of GAA in ruminants is still in the experimental phase, unlike its use in monogastric, which has been authorized as a feed additive since 2016 in the EU (Regulation (EU) 2016/1768, 2016) and the USA (FDA, 2016). In this study, two different fermentation media, namely beef and dairy rumen conditions, were used to determine the effect of adding GAA on rumen microbial fermentation.

5.5.1. Effect of fermentation conditions

The degradation of OM was not different among diets. This result agrees with those of Castillejos et al. (2005) who found no difference in OM degradation (60.2% and 58.1%) between a 10:90 vs 60:40 forage to concentrate diets, respectively. In contrast, degradation of NDF and ADF was higher in dairy than in beef fermentation conditions, as expected. The beef diet rich in concentrate decreased ruminal pH and, consequently, fiber degradation also decreased. The low degradation of NDF in the case of beef may be explained by the type of substate fermented and the low pH. A study conducted by Calsamiglia et al. (2008) to assess the effect of diet type and rumen pH on microbial fermentation and nutrient flow, reported that fiber degradation decreased below pH 6. At low pH, fibrolytic bacteria find it difficult to attach to feed particles and their growth slows down (Russell and Dombrovski, 1980; Cheng et al., 1984). In our case, the average pH was 5.80 for beef and 6.26 for dairy, and this difference in pH may explain the difference in fiber degradation.

Total VFA was higher with beef compared with dairy fermentation conditions. Calsamiglia et al. (2008) reported that variation in total VFA can be explained by diet composition, pH, and their interaction. Also, Brandao and Faciola (2019) observed that the concentration of total VFA increased as the concentration of rapidly fermentable carbohydrates increased. However, in our case, the OM degradation was the same for beef and dairy fermentation conditions. Thus, the higher total VFA observed in beef fermentation conditions may be explained by the low utilization of carbon skeletons by microbes for their growth, since the microbial N flow was lower in beef than in dairy fermentation conditions, which led to the accumulation of VFA.

Molar proportion of acetate was lower with beef compared with dairy fermentation conditions. This result agrees with the lower content and degradation of NDF in beef diet. Typically, acetate molar proportion increases with high forage diet, which is characterized by a high content of NDF and associated with a higher pH (Brandao and Faciola, 2019). In contrast, propionate molar proportion was higher in beef compared with dairy fermentation conditions. Diets rich in starch and grain rapidly fermented lower the pH and favor the development of amylolytic bacteria at the expense of cellulolytic bacteria, which results in an increase in propionate at the expense of acetate. These effects are pH and diet type dependent. Calsamiglia et al. (2008) studied the effect of pH (range of pH from 4.9 to 7) and diet type (forage: concentrate 60:40 vs. 10: 90) and their

interaction using a dual flow continuous culture system, observed that the proportion of acetate was mainly affected by pH variation. However, they observed that propionate concentration was affected by both, diet type and pH variation. In our study, the proportion of butyrate was lower in beef compared with dairy fermentation conditions, and this is due to the low pH observed in beef fermentation conditions compared with dairy fermentation conditions (Calsamiglia et al., 2008).

Branched chain VFA proportion was lower in beef compared with dairy fermentation conditions. The BCVFA are derived from the deamination of branched chain amino acids, and the low pH in the case of beef fermentation conditions decreases the deamination of amino acids resulting in a lower concentration of ammonia, which may explain the low proportion of BCVFA in beef. (Oltjen et al., 1971).

The acetate to propionate ratio was lower with beef than with dairy fermentation conditions, as expected, and results from the increase in propionate and the decrease in acetate (Calsamiglia et al., 2008). This variation in microbial fermentation is largely explained by the type of fermented substrate, which is responsible for about 75% of this change, and pH is only responsible for the remaining 25% (Calsamiglia et al., 2008; Russell, 1998).

Ammonia-N concentration decreased in beef fermentation conditions resulting in lower total and ammonia-N flow compared with dairy fermentation conditions, which was an expected result, because beef diet contain less CP compared with dairy diet and the amount of CP in the diet is mainly responsible of ammonia-N concentration. Also, pH may affect ammonia-N production, and at low pH ammonia-N production is decreased (Calsamiglia et al., 2008). Non-ammonia, dietary and bacterial N flows degradation were the same in beef and dairy fermentation conditions. However, CP degradation tended to decrease in beef compared to dairy fermentation conditions. This effect may be due to the lower degradability of NDF and lower pH in the case of beef fermentation conditions that reduced the access of microbes and enzymes to protein, which reduced protein degradability (Devant et al., 2000).

Also, the efficiency of microbial protein synthesis (EMPS) was lower in beef compared with dairy fermentation conditions. Calsamiglia et al. (2008) reported that EMPS is mainly affected by diet composition and pH had only small effect. Russell et al. (2009) suggested that low pH in the case of beef fermentation conditions may increase

bacterial maintenance energy requirements at the expense of bacterial growth energy requirements.

The degradability of GAA was lower in beef fermentation conditions (6.3%) compared with dairy fermentation conditions (69.8 %). Speer et al. (2020) reported that GAA was about 50% degraded in the rumen of steers. It is generally accepted that beef rumen microbial fermentation is less efficient than dairy because of its low pH and result in less deamination and less protein degradation. Also, at low pH, the proteolytic enzymes produced by bacteria are inhibited (Wallace and Cotta, 1989). The GAA molecule has a structure similar to AA and we suggested that GAA in the rumen could be metabolized like an AA, which explains its lower degradability in the case of beef fermentation conditions. In the case of dairy fermentation conditions, we suggested that the higher degradation of GAA may be due to the presence of hyper-ammonia producing bacteria (HAP) that metabolize GAA as an AA, which resulted in higher ammonia-N concentration, because the high rates of ammonia production have been cited as evidence for the presence and the significant role of HAP bacteria in ruminal nitrogen metabolism (Russell and Strobel, 1988). Further studies are needed to understand the metabolism of GAA in the rumen.

5.5.2. Effect of GAA supplementation

Few studies have been done on the effect of GAA on dairy or beef fermentation, and the majority focused on post ruminal effect (Ardalan et al., 2020; Speer et al., 2020; Li et al., 2021). Therefore, it is difficult to compare our results with others. In general, the supplementation of 2 g/fermenter/d of GAA to both dairy and beef rumen fermentation conditions did not affect nutrient degradability nor total and individual VFA. However, ammonia-N concentration increased with GAA addition and resulted in an increase in total N flow, ammonia N flow and CP degradation. The GAA was added on top of iso-N diets, and this addition of GAA (which contains 3 atoms of N, equivalent to 35.9 % of N) to the fermenter was responsible for this increase in N flows.

In contrast, Li et al. (2020) reported that increasing GAA levels (0.3, 0.6 and 0.9 g/kg of DM) in Angus bulls resulted in a higher nutrient degradation, higher total VFA and propionate proportion, and lower acetate proportion, acetate to propionate ratio and ammonia-N concentration. Liu et al. (2021) supplemented Angus bull diets with 0.6 g/kg DM of GAA with or without betaine (betaine is used to provide the methyl group needed

for the reaction to convert GAA to creatine) reported a higher total VFA and ammonia-N concentration without affecting individual VFA or the acetate to propionate ratio.

5.5.3. Rumen microbial population

Rumen microbial population is mainly affected by diet and pH. However, diet is the most important factor of variation in ruminal microbiota (Malmuthuge and Guan, 2017). The purpose of the microbial population analysis was to study the composition and the abundance of bacteria in both dairy and beef rumen fermentation conditions, and to determine if the addition of GAA affected microbial population. The number of observed OTUs and beta diversity indices were higher in dairy compared with beef fermentation conditions. This was an expected result, because beef diet type is rich in cereals which leads to a decrease in the diversity of microbial bacteria (Ishaq et al., 2017). When comparing the relative abundance, no difference was detected between treatments and *Prevotella, Disulfovibrio* and *Acidaminococcus* numerically were identified as the dominant genera across beef rumen fermentation conditions and *Prevotella, Disulfovibrio, Enterococcus* and *Lactobacillus* across dairy rumen fermentation conditions. Though, in the literature, Zened et al. (2020), reported that forage to concentrate ratio can determine the dominant group of bacteria in the rumen. For example, in the case of beef fermentation conditions, the amylolytic bacteria (e.g., *Streptococcus bovis, Ruminobacter amylophilus*) are the most abundant. In contrast, in the case of dairy fermentation conditions the fibrolytics bacteria are the more abundant (e.g., *Fibrobacter succinogenes, Butyrivibrio fibrisolvens;* Zened et al., 2020), which was not our case in this study.

In this study, the addition of GAA to beef or dairy fermentation conditions did not modify the diversity and the relative abundance of microbial population. However, Li et al. (2020) reported that supplementing Angus bull diets with increasing levels of GAA (0.3, 0.6 and 0.9 g/kg of DM) improved microbial growth, especially *R. flavifaciens, B. fibrisolvens, P. ruminicola* and *Rb. amylophilus*, and enzyme activities of cellobiases, pectinases and proteases. Also, Liu et al. (2021) supplemented Angus bull diets with 0.6 g/kg DM of GAA with or without betaine and observed an increase in the population of total bacteria, *R. albus, B. fibrisolvens* and *Rb. amylophilus* and an increase in the activities of xylanases, cellobiases proteases and α-amylases. The lack of response in our case, maybe due to the difference in experimental conditions starting with the method used. For example, Li et al. (2020) tested GAA on animals (*in vivo*) during long period

(104 days:14 days of adaptation and 90 days for data collection), and with doses higher compared to the dose used in our study. Further studies on animals are needed to better understand the role of GAA on the rumen microbial population.

5.6. Conclusion

Rumen fermentation conditions of beef and dairy affected nutrient digestion, fermentation end- products, N metabolism and microbial diversity. However, the addition of GAA did not affect rumen microbial fermentation. The degradation of GAA was higher in dairy than in beef conditions. The use of GAA in dairy cow, but not in beef, may require protection from ruminal degradation.

CHAPTER 6

General discussion

CHAPTER 6

General discussion and implications

The results of each experiment are discussed separately in different sections of this work. Therefore, here we discuss together the main results obtained to highlight the most interesting findings and implications for generating new knowledge to improve rumen fermentation efficiency.

In this thesis, we used different *in vitro* techniques to evaluate the effect of two different types of feed additives with the common objective of improving the efficiency of rumen microbial fermentation in cattle, because rumen microbial fermentation is a biological process that is not 100% effective. The main by-product resulting from the inefficiencies of the rumen are methane, which is considered a greenhouse gas, and nitrogen, which is a pollutant compound. These two by-products are an economic loss for the producer and are products that threaten the environment (air, water and soil pollution).

Improving rumen microbial fermentation means improving the synthesis of volatile fatty acids and ruminal microbial proteins because they represent, respectively, the main energy and protein supply for ruminants. However, the great challenge today is improving rumen microbial fermentation, to enhance animal growth and productivity, without increasing enteric CH_4 and N emissions. The addition of feed additives to diets improves dietary nutrient utilization (Adesogan, 2009). Ideal feed additives should have one or more of the following qualities, among others: 1) modulate ruminal pH and reduce lactate accumulation, 2) reduce the risk of developing metabolic and digestive diseases, 3) reduce ruminal methanogenesis and decrease the acetate to propionate ratio without affecting milk fat synthesis, 4) improve the efficiency of ruminal nitrogen utilization by (i) reducing proteolysis, peptidolysis and amino acid deamination, thus minimizing production and losses of NH_3 to the environment; (ii) enhancing the synthesis of microbial protein; and 5) increase ruminal organic matter and fiber degradability. Finally, feed additives should be cost effective and approved by legal authorities (Adesogan, 2009).

Several feed additives have been used to modulate rumen microbial fermentation towards the production of more propionate and less acetate, to reduce the acetate to propionate ratio and to reduce the ammonia-N concentration. Among these additives, ionophores like monensin, which is an organic compound obtained mainly from

Streptomyces species, cause selective transportation of ions across the outer cell membrane of Gram-positive bacteria which result in slower growth of Gram-positive bacteria compared to Gram-negative bacteria (McGuffey et al., 2001; Duffield et al., 2008). The mode of action of monensin gives variable results in *in vitro* and *in vivo* studies, but in general results with positive effect on rumen microbial fermentation which make monensin the reference against which other feed additives are evaluated (DiLorenzo, 2011). However, the prohibition of the use of antibiotics as growth promotor in animal feeds in the European Union since January 2006 led to increased interest in finding alternatives to modulate rumen microbial fermentation. In this thesis (in Chapter 3 and 4), monensin was used as positive control for their expected results, at 10 mg/L in *in vitro* batch fermentation culture. However, in our case, monensin had no effect on rumen microbial fermentation profile, or total gas and methane production. In contrast, Chaves et al. (2008) used monensin at 5 mg/L in short-term fermentation system and observed a decrease in total VFA, ammonia-N concentration and methane production. Also, Hristov et al. (2008) used monensin at 5 mg/L in short-term fermentation system and observed that monensin reduced ammonia-N concentrations. The dose of 10 mg/L was used in our study because according to the FDA (2005) the effective dose for dairy cattle (185 to 660 mg/d) corresponded to a dose of 1.8 to 6.6 mg /L in a rumen of 100 L, which is a dose lower than 10 mg/L. Therefore, our hypothesis was that with 10 mg/L we should see an effect although it was not observed in our case.

Essential oils

Over the past two-decades, various studies have been done on the of use EO as a feed additive to improve the efficiency of ruminants and to avoid the use of antibiotics. In this thesis, the evaluation of the efficacy of EO on rumen microbial fermentation was tested *in vitro* through the quantification of some indicators of rumen fermentation such as total and individual VFA, and ammonia production. Results showed that some EO have small effects on rumen microbial fermentation such as lemongrass (LEM) that tended to increase the propionate proportion and tended to decrease the acetate to propionate ratio. Anise star, coriander seed oil (COR), and thyme tended to increase butyrate proportion, but this is not a desired result. Capsicum, COR and thyme also decreased ammonia-N concentration. Other EO did not have any effect on rumen microbial fermentation such as ginger, geraniol, limonene oils …etc. However, results between studies in this thesis were different. For example, LEM used in Chapter 3 at 80 mg/L tended to increase the

propionate proportion and tended to decrease the acetate to propionate ratio, and in Chapter 4 the use of LEM at 140 mg/L only increased total VFA. This difference may be due to the rumen fluid used in each study because rumen fluid was the only different variable between studies. The concentration of the main active compound of LEM used in each experiment and experimental conditions (diet, pH…etc.) were the same.

Another important approach studied in this thesis is the combination of EO. Bassolé and Juliani (2012) reported that some EO oils and their active compounds can have additive, synergistic or antagonistic effects when are in combination. These interactions between EO components can produce positive changes in rumen fermentation, but the combination that successfully produces such changes remains unknown (Burt, 2004; Benchaar et al., 2009). Many products based on the combination of EO are marketed (CRINA-Ruminants®, DSM, Switzerland; AGOLIN®, Agolin Sa, Switzerland, XTRACT®; see Chapter 1). However, evidence of their additivity or synergy is lacking. Recently, Fandiño et al. (2020) addressed this issue in high concentrate feedlot-type fermentation conditions and found an antagonistic effect when clove bud oil was mixed with tea-tree, thyme and (or) oregano oils. And to our knowledge, no study was specifically designed to study the relationship between EO when are in combination in dairy fermentation conditions. In Chapter 3 of this thesis, the evaluation of EO individually and in combination to modify rumen microbial fermentation profile was carried out, and a synergy was observed when lemongrass and coriander oils were mixed. However, the addition of a high dose of ginger oil to the mix resulted in an antagonistic effect. These experiments (Fandiño et al., 2020; Temmar et al. 2021) highlighted the importance of testing combinations of EO.

Essential oils can specifically inhibit methanogens, hyper-ammonia producing bacteria and other bacteria, thereby positively affecting rumen fermentation by reducing methane production and ammonia concentrations (McIntosh et al., 2003). In Exp. 2 in Chapter 4, LEM and a blend of anise and cassia oils (BAC) were evaluated for their effect on gas and methane production. The BAC was more effective in reducing total gas and methane production, which confirm that EO can inhibit methanogens. In contrast, LEM increased total VFA but also increased methane production, which suggests that different EO had different mechanisms of action.

Essential oils are characterized by their low or insolubility in water, high volatility, and strong odor, which limit their applications in dairy rations. Therefore, the need for

prior dilution, and they need to be adsorbed or emulsified in a carrier. For these reasons, in Exp. 1 of Chapter 4, LEM was tested with four different carries: rapeseed oil (RAP), corn cob (CCO), diatomaceous earth (DIAT) and a mixed carrier that contained 20% silica and 53% sunflower oil (SIL). The carrier and the EO were added separately to the incubated medium to evaluate their effect on rumen microbial fermentation *in vitro.* LEM increased total VFA, and SIL, RAP and CCO tended to increase total volatile fatty acids. Normally, the addition of carriers should not interfere with the effect of EO. However, in our study, when SIL, RAP and CCO were used alone, they increased total VFA. The nature of these carriers, which are all vegetable oils, may be the cause of the observed effect, because the addition of fat to the ration can cause changes in the rumen microbiota. Ikwuegbu and Sutton (1982) and Bauchart et al (1985) reported the elimination of protozoa and an increase in the total number of bacteria when vegetable oils were added to dairy cow diet. This increase in bacterial mass increased ruminal fermentation which resulted in our case in a high production of total VFA. Also, when LEM was added with the carriers, all effect disappeared, which suggested an antagonistic effect between LEM and CCO, SIL and DIAT. Only the LEM-RAP combination had an effect resulting in a decrease in ammonia-N concentration. Also, Careful selection of adequate carriers is essential to ensure the antimicrobial activity of EO.

In some cases, results obtained in these studies showed that careful selection and mixture of EO and EOAC can promote positive results. However, these results should be taken with precaution because we did not study the adaptation of microorganisms to EO. There is evidence from results of continuous culture system (Busquet et al., 2005a, b; Cardozo et al., 2004) and long-term *in* vivo (Benchaar et al., 2008) studies that the positive effects associated with the use of EO as feed additive may decrease due to microbial adaptation to EO or changes in microbial populations. Also, doses used *in vivo* cannot be higher as *in vitro* or experimental doses because of potentially deleterious effects on the efficiency of rumen microbial fermentation, palatability, possible toxicity, and high cost (Benchaar et al., 2009).

In vitro system to screen EO have been used as the main tool for evaluating their effects on rumen fermentation characteristics, due to the high number of EO and their active compounds (Benchaar et al., 2009). This method was used to simulate and predict *in vivo* results, which is not easy, because in *in vitro* studies doses used are higher compared to *in vivo* studies. Also, in batch culture system, it is difficult to remove

fermentation end-products which led to its subsequent accumulation (Benchaar et al., 2009). Therefore, it is necessary to determine the optimal doses of EO and their active compounds in ruminant animal models (Calsamiglia et al., 2007; Chaves et al., 2008).

Several *in vivo* studies have been done to evaluate the effect of EO on dairy performance, production and methanogenesis. In a meta-analysis conducted to evaluate the effect of feeding 1g/cow/d for more than 28 days of Agolin® Ruminant, that contains coriander seed oil, eugenol, geranyl acetate and geraniol as the main active compounds, on milk yield, rumen fermentation, methane emissions and health. Agolin® Ruminant showed inconsistent results among studies, but in general feeding Agolin® Ruminant increased milk yield (+3.6%, equivalent to 1.12 kg/d), fat and protein corrected milk (+4.1%) and feed efficiency (+4.4%) without any effect on rumen microbial fermentation (Belanche et al., 2020). Also, Calsamiglia et al. (2023) evaluated the effect of EO supplementation on dairy cow performance based on sixteen peer review papers that used 28 different treatments (Table 6.1). Results showed an increase in milk production (about +0.98 kg/d), dry matter intake (+0.36 kg/d) and small effects in fat and protein content. Another *in vivo* study conducted by Yang et al. (2007) where he tested garlic oil (5 g/cow/d), and juniper oil (2 g/cow/d) on dairy cows and observed that ruminal digestibility of dry matter and organic matter were higher for garlic and juniper oils compared to the control. However, no effects of garlic and juniper were observed on milk production, ruminal microbial protein synthesis, ruminal pH and ruminal concentrations of volatile fatty acids and ammonia-N.

Table 6.1. Summary of the effects of supplementing on dairy cattle performance (Calsamiglia et al., 2023).

Item	Average	Difference	SE
DMI, kg/d	22.0	+0.36	1.17
Milk, kg/d	32.6	+0.98	2.01
FCM, kg/d	34.9	+0.22	1.04
FE, kg milk/kg DMI	1.56	+0.03	0.07
Fat, %	3.70	+0.05	0.33
Protein, %	3.09	+0.03	0.07
MUN, mg/dL	14.3	-0.47	1.16

The discrepancy in results of *in vitro* and *in vivo* studies may be due to several factors, among others: 1) the adaptation of rumen microbes to EO which represents a major challenge for the use of these compounds to improve rumen microbial efficiency; 2) some EO like terpenes can disappear from the digestive tract due to their bioconversion by the rumen microflora, transferred to the gas phase of the rumen due to their volatility and loss during eructation or absorbed across the ruminal wall into the blood system and excretion in the urine (Benchaar and Greathed, 2011), 3) the low doses used in the case of *in vivo* studies.

These results lead us to ask the question on whether the use of EO is profitable in dairy farms.

Based on the results reported by Calsamiglia et al. (2023; Table 6.1.) and assuming that: the price of a liter of milk under normal conditions in Spain is 0.36 euro, and for each tenth more of fat (+ 0.1%) the price of milk increases by 0.002 euros, and for each tenth more in protein (+ 0.1%), the price of milk increases by 0.006 euros (Calsamiglia et al., 2018). The calculation of the economic profit of using EO to improve rumen microbial fermentation efficiency is performed in steps: 1) we estimated the additional cost that the farmer spends in the increase in diet intake and treatment (EO) 2) we estimated the new income resulting from feeding EO which contain the increase in milk production and the proportion of fat and protein, 3) by subtracting what the farmer earns from what has invested we will have the gain, and by dividing the gain by the cost we will have the return of using this feed additive.

Therefore, the calculations result in:

The cost of feeding 1g of EO/cow/d assuming that the price of 1kg of EO is 60 euro:

EO cost = (60 euro/1000 g) = 0.06 euro/cow/d

The cost of the increase in DMI

DMI (kg/d) cost = (the increase in DMI kg/d * price of the diet euro/kg)

DMI (kg/d) cost = (0.36 kg/cow/d * 0.25 euro/kg) = 0.09 euro/cow/d

Total cost of investment = (EO cost + DMI cost)

Total cost of investment = (0.06 + 0.09) = 0.15 euro/cow/d

The price of milk with bonification after feeding EO to dairy cow

Total income = (the increase in milk production kg/d * price of liter of milk) + (the increase in fat % * price of fat) + (the increase in protein % * price of protein)

Total income = (0.95 l/d * 0.342) + (0.05 * 0.002/0.1) + (0.03 * 0.006/0.1)

Total income = 0.328 euro/cow/d

Profit = total income - total cost of investment

Profit = 0.328 euro/cow/d - 0.15 euro/cow/d = 0.178 euro/cow/d

Return = profit /investment

Return = 0.178/0.15 = 1.185

In summary, the supplementation of 1 g of EO to dairy cow diet, resulted in gain of 0.178 euro/cow/d to the farmer.

- ***Future perspectives***

The use of EO is widespread in animal feed but with different purpose. As shown in Figure 6.1. monogastric occupy the higher percentage with the main objective to improve gut health, host immunity, antioxidant stress…etc. that improve functionality and ensure sustainable livestock production. In ruminants, unlike monogastric and despite the increased interest in using EO as feed additive, most of studies were focused only on modulating rumen microbial fermentation.

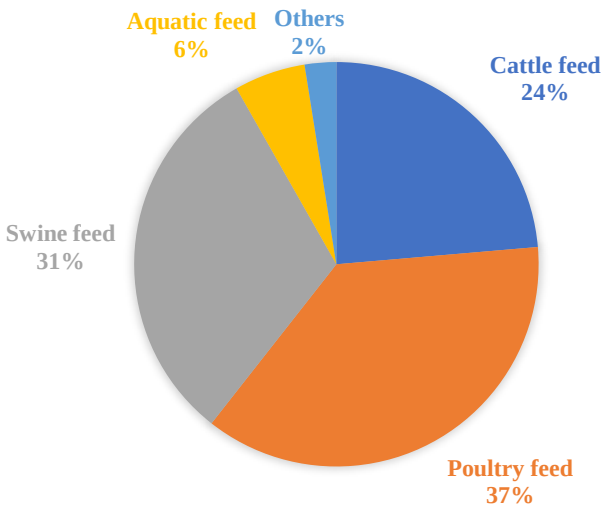

Figure 6.1. The percentage of utilization of essential oils in livestock feed (IFIF, 2021).

Recently, researchers started to investigate the effect of EO on immune system, oxidative stress and hormonal status such as insulin regulation in ruminants based on their effect in monogastric (Oh et al., 2017). Also, Oh et al. (2015) used EO in *in vivo* studies in ruminants and observed changes in performance at doses that did not affect ruminal fermentation, suggesting that the changes could be attributed to a post-ruminal effect. For example, garlic, curcumin and capsicum were infused postruminally at 2 g/cow per day, had an effect on immune-stimulatory system by activating and inducing the expansion of CD4 cells (Oh et al., 2013). Also, Ahmed et al. (2015) tested different doses of green tea by-products (0, 0.5, 1.0, and 2.0% of DM) in goats and observed an increase in the proliferation of T and B cells in spleen cells and concluded that green tea by-products exhibited proinflammatory effects in the spleen of goats. This approach of using EO beyond the rumen, opens new opportunities for research on the post-rumen effect of EO which also requires appropriate technologies to protect EO from rumen microbes.

- ***Guanidinoacetic acid***

Another feed additive studied in Chapter 5 of this thesis is the guanidinoacetic acid (GAA) to improve dairy and beef cattle feed efficiency. The GAA is the natural precursor of creatine, which serve as an energy-storing molecule in muscle (Murakami et al., 2014). Creatine and GAA are synthesized by dairy and beef cattle but not in enough quantities to maximize performance. Several studies report that the addition of GAA to broiler and pig diets improved creatine synthesis, feed conversion ratio, growth performance, energy metabolism and gut morphology (Faraji et al., 2018; Amiri et al., 2019; Portocarero and Braun, 2021). In contrast in cattle, the addition of GAA to dairy and beef diet is still in the experimental phase. Ardalan et al. (2020) studied the effect of supplementing GAA on post-ruminal effects by using increased doses of GAA with or without methionine and observed that GAA supplementation increased creatine supply to cattle and spared arginine, but without any effect on methionine.

Recently, Liu et al. (2021) and Li et al. (2020) were interested in evaluating the effect of adding GAA on rumen microbial fermentation. For that, in our study we evaluated the addition of GAA to dairy and beef fermentation conditions in a dual flow continuous culture system to compare the different fermentation conditions. Our results indicated that GAA in general did not affect rumen microbial fermentation. However, the degradation of GAA was higher in dairy than in beef fermentation conditions. Speer et al. (2020) also

studied the bioavailability of GAA when infused directly into the rumen or abomasum and observed that GAA was 50% degraded in the rumen of steers. These results can be explained by the fact that GAA has the same structure as an amino acid and in the rumen the GAA is deaminated as AA. Another hypothesis to explain the difference in degradation between dairy and beef is the difference in rumen efficiency, where beef rumen is less efficient than dairy rumen (Ishaq et al., 2017). But further in-depth studies are needed to confirm this hypothesis. To escape rumen degradation, two options can be considered, protects the GAA but this technology is very expensive, at about 5.9 euro/kg. This price was obtained by the difference between the price of a protected lysine (ranging between 6.8 and 8 euro/kg, with an average of 7.4 euro/kg) and the price of lysine without protection (ranging between 1.7 and 2.3 euro/kg, with an average of 1.5 euro/kg), which is an estimation since GAA is similar to an AA. The second option is to supply larger quantities of GAA to dairy cattle considering its rate of degradation in the rumen as the GAA is not expensive (6 euro/kg). In our study (Chapter 5) we found that about 70% of GAA was degraded, and based on the dose of 40 g/d of GAA fed to Holstein heifers in a study of Ardalan et al. (2020), we get:

- *First option:* the use of protected GAA

Total cost of using protected GAA= (dose of GAA g/cow/d * price of protection euro/kg)

Total cost of using protected GAA= (40 g/cow/d * 5.9)/1000 g

Total cost of using protected GAA= 0.236 euro/cow/d

- *Second option:* supplementation of more quantity of GAA

Total dose needed to replace the amount of GAA degraded in the rumen, when assuming that GAA is 70% degraded in the rumen:

GAA g = (dose of GAA g/cow/d * rate of degradation %)/ 100%

GAA g = (40 g/cow/d * 70 %)/100% = 28 g

Therefore, we should give 28 g/cow/d more of GAA, which cost:

The cost of GAA = (28 g/cow/d * 6 euro/1000 g) = 0.168 euro/cow/d

Comparing the two option, 0.236 euro/cow/d vs. 0.168 euro/cow/d, the best one is to give more amount of GAA/cow/d.

As for EO, we evaluated the profitability of the incorporation of GAA as feed additive in ruminant diets. For dairy cows we found no studies on the effect of GAA on milk production and its components. In the case of beef cattle, few studies are available on the effect of supplementing GAA in beef diet. For example, Li et al. (2020) reported that feeding 0.6 g of GAA/kg DM increased DMI (+ 1.4 kg/d), BW (+ 31 kg/90d), and ADG (+ 0.39 kg/d). Based on these values, and knowing that the price of 1kg of meat in Spain is about 4 euro, and the price of beef diet cost 0.40 euro/kg, we have:

Therefore, results were:

The cost of feeding 0.6g of GAA/beef/d assuming that the price of 1kg of GAA is 6 euro:

GAA cost = (0.6 * 6/1000 g) = 0.0036 euro/beef/d

The cost of the increase in DMI

DMI (kg/d) cost = (the increase in DMI kg/d * price of the diet euro/kg)

DMI (kg/d) cost = (1.4 kg/beef/d * 0.40 euro/kg) = 0.56 euro/beef/d

Total cost of investment = (GAA cost + DMI cost)

Total cost of investment = (0.0036 + 0.56) = 0.564 euro/beef/d

The supplementation of 0.6g of GAA resulted in an increase in ADG (+ 0.39 kg/d) and assuming that the price of kg of meat is about 4 euro:

Total income = (the increase in ADG kg/d * price of kg of meat)

Total income = (0.39 kg/d * 4 euro) = 1.56 euro/beef/d

Profit = total income - total cost of investment

Profit = 1.56 euro/beef/d - 0.564 euro/beef/d = 0.996 euro/beef/d

Return = profit /investment

Return = 0.996 /0.564 = 1.766

In summary, the supplementation of 0.6 g of GAA to beef diet, resulted in gain of 0.996 euro/beef/d to the farmer.

CHAPTER 7

Conclusions

CHAPTER 7

Conclusions

The conclusions obtained in the different experiments carried out in this thesis are the following:

7.1. Specific Conclusions

7.1.1. Interaction effects among EO

- Lemongrass oil tended to increase propionate proportion and tended to decrease the acetate to propionate ratio.
- Anise star, coriander and thyme oils tended to increase butyrate proportion.
- Capsicum, coriander and thyme oils decreased ammonia-N concentration.
- A synergy was observed between lemongrass and coriander oils that resulted in an increase in total VFA and propionate proportion, and a decrease in the acetate to propionate ratio.
- The addition of high doses of GIN to the mix of lemongrass and coriander oils had an antagonistic effect on the rumen microbial fermentation profile.

7.1.2. EO carriers' effects on microbial fermentation and methane production

- A blend of anise star and cassia oils, and lemongrass oil increased total volatile fatty acids.
- Carriers (silica-sunflower, rapeseed oil and corn cob) tended to increase total volatile fatty acids.
- Among the combinations of EO-carriers, only the combination lemongrass oil-rapeseed oil had an effect, decreasing ammonia-N concentration.
- A blend of anise star and cassia oils decreased total gas and methane production, and the ratio methane/total gas.
- Lemongrass oil increased total methane and the ratio methane/total gas.

7.1.3. GAA effects on dairy-beef rumen microbial fermentation and nutrient flow from a continuous culture system

Dairy and beef fermentation conditions

- No differences were observed on true OM degradability.
- Degradation of NDF, the proportions of acetate and butyrate, the acetate to propionate ratio, NH_3-N concentration, the flow of total N and ammonia N, the efficiency of microbial protein synthesis, and alpha and beta diversity of microbial population were higher in dairy than in beef.
- Total VFA and the propionate proportion were higher in beef than in dairy.

The GAA supplementation

- The addition of GAA to dairy and beef diets did not affect nutrient digestion, total and individual VFA, and rumen microbial population and diversity.
- The GAA only increased NH_3-N concentration and the flow of total N and ammonia N.
- The microbial degradation of GAA was higher in dairy (69.8%) than in beef (6.30%).

7.2. General Conclusions

Some of the EO used in the previous studies show a small result as feed additives to modulate rumen microbial fermentation profile. However, the use of GAA did not had effect on modulating rumen microbial fermentation. In this thesis we highlight:

- The importance of taking into consideration the existence of positive and negative interactions among EO.
- The use of carriers also may result in antagonistic effect with the EO, and a careful selection is needed.
- Some EO can decreased methane production, while others increased it.
- Rumen fermentation conditions of beef and dairy affect differently microbial fermentation profile, nitrogen metabolism and microbial diversity.
- GAA did not affect rumen microbial fermentation nor microbial population diversity.

- The degradation of GAA was higher (69.8 %) in dairy than in beef conditions (6.30 %), which it must be taken into consideration in the case where the GAA is added to dairy cow diet and if the post-ruminal effect is sought.

CHAPTER 8

References

CHAPTER 8

References

Adesogan, A.T. Using dietary additives to manipulate rumen fermentation and improve nutrient utilization and animal performance. In: Proc. of the 20th Florida Ruminant Nutrition Symposium, Gainesville, Florida, USA, 10–11 Feb. **2009**; pp. 13–38. https://animal.ifas.ufl.edu/dairy/conferences--meetings/florida-ruminant-nutrition-symposium/florida-ruminant-nutrition-symposium-archive/.

Akin, D.E.; Benner, R. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. *Appl. Environ. Microbiol.* **1988**, 54, 1117–1125. https://doi.org/10.1128/aem.54.5.1117-1125.1988.

Akin, D.E.; Borneman, W.S.; Lyon, C.E. Degradation of leaf blades and stems by monocentric and polycentric isolates of ruminal fungi. *Anim. Feed Sci. Technol.* **1990**, 31, 205–221. https://doi.org/10.1016/0377-8401(90)90125-R.

Alexander, G., Singh, B.; Sahoo, A.; Bhat, T.K. *In vitro* screening of plant extracts to enhance the efficiency of utilization of energy and nitrogen in ruminant diets. *Anim. Feed Sci. Technol.* **2008**, 145, 229–244. https://doi.org/10.1016/j.anifeedsci.2007.05.036.

Amiri, M.; Ghasemi, H.A.; Hajkhodadadi, I.; Farahani, A.H.K. Efficacy of guanidinoacetic acid at different dietary crude protein levels on growth performance, stress indicators, antioxidant status, and intestinal morphology in broiler chickens subjected to cyclic heat stress. *Anim. Feed Sci. Technol.* **2019**, 254, 114208. https://doi.org/10.1016/j.anifeedsci.2019.114208.

AOAC. Official Methods of Analysis. 15th ed.; *Association of Official Analytical Chemists*, Washington DC, USA, **1990.**

Appuhamy, J.A.D.; Strathe, A.B.; Jayasundara, S.; Wagner-Riddle, C.; Dijkstra, J.; France, J.; Kebreab, E. Anti-methanogenic effects of monensin in dairy and beef cattle: A meta-analysis. *J. Dairy Sci.* **2013**, 96, 5161–5173. https://doi.org/10.3168/jds.2012-5923.

Ardalan, M.; Batista, E.D.; Titgemeyer, E.C. Effect of post-ruminal guanidinoacetic acid supplementation on creatine synthesis and plasma homocysteine concentrations in cattle. *J. Anim. Sci.* **2020**, 98, 1–9. https://doi.org/10.1093/jas/skaa072.

Aruoma, O.I. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, 75, 199–212. https://doi.org/10.1007/s11746-998-0032-9.

Bach, S.J.; McAllister, T.A.; Veira, D.M.; Gannon, V.P.J.; Holley, R.A. Effect of bacteriophage DC22 on Escherichia coli O157:H7 in an artificial rumen system

(Rusitec) and inoculated sheep. *Anim. Res.* **2003**, 52, 89–101. https://doi.org/10.1051/animres:2003009.

Bajpai, V.K.; Baek, K.H.; Baek, S.C. Control of *Salmonella* in foods by using essential oils: A review. *Food Res. Int.* **2012**, 45, 722–734. https://doi.org/10.1016/j.foodres.2011.04.052.

Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological effects of essential oils – A review. *Food Chem. Toxicol.* **2008**, 46, 446–475. https://doi.org/10.1016/j.fct.2007.09.106.

Balcells, J.; Guada, J.A.; Peiro, J.M.; Parker, D.S. Simultaneous determination of allantoin and oxypurines in biological fluids by high performance liquid cromatography. *J. Chromatogr. B.* **1992**, 575, 153–157. https://doi.org/10.1016/0378-4347(92)80517-T.

Baracho, N.C.; Castro, L.P.; Borges, N.D.C.; Laira, P.B. Study of renal and hepatic toxicity in rats supplemented with creatine. *Acta Cir Bras.* **2015**, 30, 313–318. https://doi.org/10.1590/S0102-865020150050000002.

Bassolé, I.H.N.; Juliani, H.R. Essential oils in combination and their antimicrobial properties. *Molecules* **2012**, 17, 3989–4006. https://doi.org/10.3390/molecules17043989.

Bassolé, I.H.N.; Lamien-Meda, A.; Bayala, B.; Tirogo, S.; Franz, C.; Novak, J.; Nebié, R.C.; Dicko, M.H. Composition and antimicrobial activities of Lippia Multiflora Moldenke, Mentha x piperita L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. *Molecules* **2010**, 15, 7825–7839. https://doi.org/10.3390/molecules15117825.

Bauchart, D.; Aurousseau, B. ; Auclair, E. Addition of sorbitol to a milk substitute for veal calves. — I. Effects on health, growth and feed conversion. *Reprod. Nutr. Develop.* **1985**, 25, 399–409. https://doi.org/10.1051/rnd:19850306.

Béard, E.; Braissant, O. Synthesis and transport of creatine in the CNS: Importance for cerebral functions. *J. Neurochem.* **2010**, 115, 297–313. https://doi.org/10.1111/j.1471-4159.2010.06935.x.

Beauchemin, K.A.; McGinn, S.M. Methane emissions from beef cattle: effects of fumaric acid, essential oil and canola oil. *J. Anim. Sci.* **2006**, 84, 1489–1496. https://doi.org/10.2527/2006.8461489x.

Belanche, A.; Newbold, C.J.; Morgavi, D.P.; Bach, A.; Zweifel, B.; Yáñez-Ruiz, D.R. A meta-analysis describing the effects of the essential oils blend agolin ruminant on performance, rumen fermentation and methane emissions in dairy cows. *Animals* **2020**, 10, 620. https://doi.org/10.3390/ani10040620.

Bemben, M.G.; Lamont, H.S. Creatine supplementation and exercise performance: Recent findings. *Sports Med.* **2005**, 35, 107–125. https://doi.org/10.2165/00007256-200535020-00002.

Benchaar, C.; Petit, H.V.; Berthiaume, R.; Ouellet, D.R.; Chiquette, J.; Chouinard, P.Y. Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *J. Dairy Sci.* **2007a**, 90, 886–897. https://doi.org/10.3168/jds.S0022-0302(07)71572-2.

Benchaar, C.; Chaves, A.V.; Fraser, G.R.; Wang, Y.; Beauchemin, K.A.; McAllister, T.A. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Can. J. Anim. Sci.* **2007b**, 87, 413–419. https://doi.org/10.4141/CJAS07012.

Benchaar, C.; Calsamiglia, S.; Chaves, A.V.; Fraser, G.R.; Colombatto, D.; Mcallister, T.A.; Beauche-min, K.A. A review of plant-derived essential oils in ruminant nutrition and production. *Anim. Feed Sci. Technol.* **2008**, 145, 209–228. https://doi.org/10.1016/j.anifeedsci.2007.04.014.

Benchaar, C.; Hristov, A.N.; Greathead, H. Essential oils as feed additives in ruminant nutrition. In: Steiner, T. ed. Phytogenics in Animal Nutrition. *Nottingham University Press*, Nottingham, UK, **2009**; pp. 111–146.

Benchaar, C.; Greathead, H. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Anim. Feed Sci. Technol.* **2011**, 166/167, 338–355. https://doi.org/10.1016/j.anifeedsci.2011.04.024.

Bergman, E.N. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* **1990**, 70, 567–590. https://doi.org/10.1152/physrev.1990.70.2.567.

Bladen, H.A.; Bryant, M.P.; Doetsch, R.N. A study of bacterial species from the rumen which produce ammonia from protein hydrolyzate. *Appl. Microbiol.* **1961**, 9, 175–80. https://doi.org/10.1128/am.9.2.175-180.1961.

Blanch, M.; Carro, M.; Ranilla, M.J.; Viso, A.; Vazquez-Anon, M.; Bach, A. Influence of a mixture of cinnamaldehyde and garlic oil on rumen fermentation, feeding behavior and performance of lactating dairy cows. *Anim. Feed Sci. Technol.* **2016**, 219, 313–323. https://doi.org/10.1016/j.anifeedsci.2016.07.002.

Boadi, D.A.; Wittenberg, K.M. Methane production from dairy and beef heifers fed forages differing in nutrient density using the sulphur hexafl uoride (SF_6) tracer gas technique. *Can. J. Anim. Sci.* **2002**, 82, 201–206. https://doi.org/10.4141/A01-017.

Bodas, R.; Prieto, N.; Garcia-Gonzalez, R.; Andres, S.; Giraldez, F.J.; Lopez, S. Manipulation of rumen fermentation and methane production with plant secondary

metabolites. *Anim. Feed Sci. Technol.* **2012**, 176: 78–93. https://doi.org/10.1016/j.anifeedsci.2012.07.010.

Bolla, J.M.; Alibert-Franco, S.; Handzlik, J.; Chevalier, J.; Mahamoud, A.; Boyer, G.; Kieć-Kononowicz, K.; Pagès, J.M. Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria. *FEBS Lett.* 2011, 585, 1682–1690. https://doi.org/10.1016/j.febslet.2011.04.054.

Brandao, V.L.N.; Faciola, A.P. Unveiling the relationships between diet composition and fermentation parameters response in dual-flow continuous culture system: a meta-analytical approach. *J. Anim. Sci.* **2019**, 3, 1064–1075. https://doi.org/10.1093/tas/txz019.

Brenner, D.M. Perilla: botany, uses and genetic resources. In: Janick, J., Simon, J.E. ed. New Crops. *Wiley*, New York, USA, **1993**; pp. 322–328.

Brosnan, J.T.; Brosnan, M.E. Creatine metabolism and the urea cycle. *Mol. Gene. Metab.* **2010**, 100, S49–S52. https://doi.org/10.1016/j.ymgme.2010.02.020.

Brosnan, M.E; Brosnan, J.T. The role of dietary creatine. *Amino Acids* **2016**, 48, 1785–1791. https://doi.org/10.1007/s00726-016-2188-1.

Bruni, R.; Medici, A.; Andreotti, E.; Fantin, C.; Muzzoli, M.; Dehesa, M.; Romagnoli, C.; Sacchetti, G. Chemical composition and biological activities of Ishpingo essential oil, a traditional Ecuadorian spice from *Ocotea quixos* (Lam.) Kosterm. (Lauraceae) flower calices. *Food Chem.* **2004**, 85, 415–421. https://doi.org/10.1016/j.foodchem.2003.07.019.

Bryant, M.P. Nutritional requirements of the predominant rumen cellulolytic bacteria. *Fed. Proc.* **1973,** 32, 1809–1813. https://pubmed.ncbi.nlm.nih.gov/4718898/.

Buddle, B.M.; Denis, M.; Attwood, G.T.; Altermann, E.; Janssen, P.H.; Ronimus, R.S.; Pinares-Patiño, C.S.; Muetzel, S.; Wedlock, D.N. Strategies to reduce methane emissions from farmed ruminants grazing on pasture. *Vet. J.* **2011**, 188, 11–17. https://doi.org/10.1016/j.tvjl.2010.02.019.

Burt, S. Essential oils: Their antibacterial properties and potential applications in foods: A review. *Int. J. Food Microbiol.* **2004**, 94, 223–253. https://doi.org/10.1016/j.ijfoodmicro.2004.03.022.

Busquet, M.; Calsamiglia, S; Ferret, A.; Cardozo, P.W.; Kamel, C. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. *J. Dairy Sci.* **2005a**, 88, 2508–2516. https://doi.org/10.3168/jds.S0022-0302(05)72928-3.

Busquet, M.; Calsamiglia, S; Ferret, A.; Kamel, C. Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous culture. *Anim. Feed Sci. Technol.* **2005b**, 123/124, 597–613. https://doi.org/10.1016/j.anifeedsci.2005.03.008.

Busquet, M.; Calsamiglia, S.; Ferret, A.; Carro, M.D.; Kamel, C. Effect of garlic oil and four of its compounds on rumen microbial fermentation. *J. Dairy Sci.* **2005c**, 88, 4393–4404. https://doi.org/10.3168/jds.S0022-0302(05)73126-X.

Busquet, M.; Calsamiglia, S.; Ferret, A.; Kamel, C. Plant extracts affect *in vitro* rumen microbial fermentation. *J. Dairy Sci.* **2006**, 89, 761–771. https://doi.org/10.3168/jds.S0022-0302(06)72137-3.

Calsamiglia, S.; Busquet, M.; Cardozo, P.W.; Castillejos, L.; Ferret, A. Invited review: Essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* **2007**, 90, 2580–2595. https://doi.org/10.3168/jds.2006-644.

Calsamiglia, S.; Cardozo, P.W.; Ferret, A.; Bach, A. Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. *J. Anim. Sci.* **2008**, 86, 702–711 https://doi.org/10.2527/jas.2007-0146.

Calsamiglia, S.; Astiz, S.; Baucells, J.; Castillejos, L. A stochastic dynamic model of a dairy farm to evaluate the technical and economic performance under different scenarios. *J. Dairy Sci.* **2018**, 101, 7517–7530. https://doi.org/10.3168/jds.2017-12980.

Calsamiglia, S.; Rodríguez-Prado, M.; Fernández-Turren, G.; Castillejos, L. The use of plant extracts as dietary supplements in dairy cow nutrition: Plant essential oils. **2023**. Book chapter (In press).

Capper, J.L.; Cady, R.A.; Bauman, D.E. The environmental impact of dairy production: 1944 compared with 2007. *J. Anim. Sci.* **2009**, 87, 2160–2167. https://doi.org/10.2527/jas.2009-1781.

Cardozo, P.W.; Calsamiglia, S.; Ferret, A.; Kamel, C. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. *J. Anim. Sci.* **2004**, 82, 3230–3236. https://doi.org/10.2527/2004.82113230x.

Cardozo, P.W.; Calsamiglia, S.; Ferret, A.; Kamel, C. Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. *J. Anim. Sci.* **2005**, 83, 2572–2579. https://doi.org/10.2527/2005.83112572x.

Carneiro de Barros, J.; Lúcia da Conceição, M.; Gomes Neto, N.J.; Vieira da Costa, A.C.; Siqueira, J.P.; Basílio, I.D.; Leite de Souza, E. Interference of Origanum vulgare L. essential oil on the growth and some physiological characteristics of *Staphylococcus aureus* strains isolated from foods. *LWT - Food Sci. Technol.* **2009**, 42, 1139–1143. https://doi.org/10.1016/j.lwt.2009.01.010.

Carson, C.F.; Mee, B.J.; Riley, T.V. Mechanism of action of Melaleuca alternifolia (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. *Antimicrob. Agents Chemother.* **2002**, 46, 1914–1920. https://doi.org/10.1128/AAC.46.6.1914-1920.2002.

Castillejos, L.; Calsamiglia, S.; Ferret, A.; Losa, R. Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. *J. Anim. Feed Sci. Technol.* **2005**, 119, 29–41. https://doi.org/10.1016/j.anifeedsci.2004.12.008.

Castillejos, L.; Calsamiglia, S.; Ferret, A. Effect of essential oils active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. *J. Dairy Sci*. **2006**, 89, 2649–2658. https://doi.org/10.3168/jds.S0022-0302(06)72341-4.

Castillejos, L.; Calsamiglia, S.; Ferret, A.; Losa, R. Effects of dose and adaptation time of a specific blend of essential oils compounds on rumen fermentation. *Anim. Feed Sci. Technol.* **2007**, 132, 186–201. https://doi.org/10.1016/j.anifeedsci.2006.03.023.

Castro-Montoya, J.; Peiren, N.; Cone, J.W.; Zweifel, B.; Fievez, V.; De Campeneere, S. *In vivo* and *in vitro* effects of a blend of essential oils on rumen methane mitigation. *Livestock Sci.* **2015**, 180, 134–142. https://doi.org/10.1016/j.livsci.2015.08.010.

Chaney, A.L.; Marbach, E.P. Modified reagents for determination of urea and ammonia. *Clin. Chem.* **1962**, 8, 130–132. https://doi.org/10.1093/clinchem/8.2.130.

Chao, S.C.; Young, D.G.; Oberg, C.J. Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *J. Essent. Oil Res.* **2000**, 12, 639–649. https://doi.org/10.1080/10412905.2000.9712177.

Chaves, A.V.; He, M.L.; Yang, W.Z.; Hristov, A.N.; McAllister, T.A.; Benchaar, C. Effects of essential oils on proteolytic, deaminate and methanogenic activities of mixed ruminal bacteria. *Can. J. Anim. Sci.* **2008**, 88, 117–122. https://doi.org/10.4141/CJAS07061.

Chen, G.; Russell, J.B. More monensin-sensitive, ammonia-producing bacteria from the rumen. *Appl. Environ. Microbiol*. **1989**, 55, 1052–1057. https://doi.org/10.1128/aem.55.5.1052-1057.1989.

Chen, Y.L.; Huang, S.T.; Sun, F.M.; Chiang, Y.L.; Chiang, C.J.; Tsai, C.M.; Weng, C.J. Transformation of cinnamic acid from trans- to cis-form raises a notable bactericidal and synergistic activity against multiple-drug resistant Mycobacterium tuberculosis. *Eur. J. Pharm. Sci.* **2011**, 43, 188–194. https://doi.org/10.1016/j.ejps.2011.04.012.

Cheng, K.J.; Costerton, J.W. Ultrastructure of Butyrivibrio fibrisolvens: a Gram-positive bacterium. *J. Bacteriol.* **1977**, 129, 1506–1512. https://doi.org/10.1128/jb.129.3.1506-1512.1977.

Cheng, K.J.; Stewart, C.S.; Dinsdale, D.; Costerton, J.W. Electron microscopy of bacteria involved in the digestion of plant all walls. *Anim. Feed Sci. Technol.* **1984**, 10, 93–120. https://doi.org/10.1016/0377-8401(84)90002-6.

Cobellis, G.; Trabalza-Marinucci, M.; Yu, Z. Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: A review. *Sci. Total Environ.* **2016a**, 545/546, 556–568. https://doi.org/10.1016/j.scitotenv.2015.12.103.

Cobellis, G.; Trabalza-Marinucci, M.; Marcotullio, M.C.; Yu, Z. Evaluation of different essential oils in modulating methane and ammonia production, rumen fermentation, and rumen bacteria *in vitro*. *Anim. Feed Sci. Technol.* **2016b**, 215, 25–36. https://doi.org/10.1016/j.anifeedsci.2016.02.008.

Cornell, J.A. Experiments with Mixtures: Designs, Models, and the Analysis of Mixture Data. 2nd ed. *Wiley*, New York, USA, **1990**.

Cox, S.D.; Mann, C.M.; Markham, J.L.; Bell, H.C.; Gustafson, J.E.; warmington, J.R.; Wyllie, S.G. The mode of antimicrobial action of essential oil of Melaleuca alternifolia (tea tree oil). *J. Appl. Microbiol.* **2000**, 88, 170–175. https://doi.org/10.1046/j.1365-2672.2000.00943.x.

Cox, S.D.; Mann, C.M.; Markham, J.L. Interaction between components of the essential oil of Melaleuca alternifolia. *J. Appl. Microbiol.* **2001a,** 91, 492–497. https://doi.org/10.1046/j.1365-2672.2001.01406.x.

Cox, S.D.; Mann, C.M.; Markham, J.L.; Gustafson, J.E.; warmington, J.R.; Wyllie, S.G. Determining the antimicrobial actions of tea tree oil. *Molecules* **2001b**, 6, 87–91. https://doi.org/10.3390/60100087.

Czerkawski, J.W.; Cheng, K.J. Compartmentation in the rumen. In: Hobson, P.N. ed. The Rumen Microbial Ecosystem, *Elsevier Appl. Sci.*, New York, USA, **1988**; pp. 361–386.

da Silva, R.P.; Nissim, I.; Brosnan, M.E.; Brosnan, J.T. Creatine synthesis: hepatic metabolism of guanidinoacetate and creatine in the rat *in vitro* and *in vivo*. *Am. J. Physiol. Endocrinol. Metab.* **2009**, 296, E256–261. https://doi.org/10.1152/ajpendo.90547.2008.

Dai, X.; Tian, Y.; Li, J.; Luo, Y.; Liu, D.; Zheng, H.; Wang, J.; Dong, Z.; Hu, S.; Huang, L. Metatranscriptomic analyses of plant cell wall polysaccharide degradation by microorganisms in the cow rumen. *Appl. Environ. Microbiol.* **2015**, 81, 1375–1386. https://doi.org/10.1128/AEM.03682-14.

Davidson, P.M.; Parish, M.E. Methods for testing the efficacy of food antimicrobials. *Food Technol.* **1989**, 43, 148–155.

de Azeredo, G.A.; Stamford, T.L.M.; Nunes, P.C.; Neto, N.J.G.; de Oliveira, M.E.G.; de Souza, E.L. Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. *Food Res. Int.* **2011**, 44, 1541–1548. https://doi.org/10.1016/j.foodres.2011.04.012.

Delaquis, P.J.; Stanich, K.; Girard, B.; Mazza, G. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int. J. Food Microbiol.* **2002**, 74, 101–109. https://doi.org/10.1016/s0168-1605(01)00734-6.

Devant, M., Ferret, A.; Calsamiglia, S.; Casals, R.; Gasa, J. Effects of protein concentration and degradability on performance, ruminal fermentation, and nitrogen metabolism in rapidly growing heifers fed high-concentrate diets from 100 to 230 kg body weight. *J. Anim. Sci.* **2000**, 78, 1667–1676. https://doi.org/10.2527/2000.7861667x.

Di Pasqua, R.; Betts, G.; Hoskins, N.; Edwards, M.; Ercolini, D.; Mauriello, G. Membrane toxicity of antimicrobial compounds from essential oils. *J. Agric. Food Chem.* **2007**, 55, 4863–4870. https://doi.org/10.1021/jf0636465.

Di Pasqua, R.; Mamone, G.; Ferranti, P.; Ercolini, D.; Mauriello, G. Changes in the proteome of Salmonella enterica serovar Thompson as stress adaptation to sublethal concentrations of thymol. *Proteomics* **2010**, 10, 1040-1049. https://doi.org/10.1002/pmic.200900568.

Dilger, R.N.; Bryant-Angeloni, K.; Payne, R.L.; Lemme, A.; Parsons, C.M. Dietary guanidino acetic acid is an efficacious replacement for arginine for young chicks. *Poult. Sci.* **2013**, 92, 171–177. https://doi.org/10.3382/ps.2012-02425.

DiLorenzo, N. Manipulation of the rumen microbial environment to improve performance of beef cattle. In: Proc. of the 22nd Florida Ruminant Nutrition Symposium, Gainesville, Florida, USA, 1–2 Feb. **2011**; pp. 118–133. https://animal.ifas.ufl.edu/apps/dairymedia/rns/2011/11diLorenzo.pdf.

Dorman, H.J.D.; Deans, S.G. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **2000**, 88, 308–316. https://doi.org/10.1046/j.1365-2672.2000.00969.x.

Dryden, G.McL. Animal Nutrition Sciences. *CABI*, Oxford, UK. **2008**.

Duffield, T.F.; Rabiee, A.R.; Lean, I.J. A meta-analysis of the impact of monensin in lactating dairy cattle. Part 2. Production effects. *J. Dairy Sci.* **2008**, 91, 1347–1360. https://doi.org/10.3168/jds.2007-0608.

EC. European Commission. Regulation Nº 1831/2003 of the European parliament and of the council of 22 September 2003 on additives for use in animal nutrition. *Off. J. Eur. Union* **2003**, L268/229. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003R1831&rid=10.

EC. European Commission. Regulation Nº 904/2009 of the of the Commission Implementing Regulation 2016/1768 of 4 October 2016 concerning the authorisation of guanidinoacetic acid as a feed additive for chickens for fattening,

weaned piglets, and pigs for fattening. *Off. J. Eur. Union* **2016**, L268/4. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R1768&from=EN.

Eckard, R.J.; Grainger, C.; de Klein, C.A.M. Options for the abatement of methane and nitrous oxide from ruminant production. *Livestock Sci.* **2010**, 130, 47–56. https://doi.org/10.1016/j.livsci.2010.02.010.

Edris, A.E. Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents. *Phytother. Res.* **2007**, 21, 308–323. https://doi.org/10.1002/ptr.2072.

Edwards, J.E.; McEwan, N.R.; Travis, A.J.; Wallace, R.J. 16S rDNA library-based analysis of ruminal bacterial diversity. *Antonie van Leeuwenhoek* **2004**, 86, 263–281. https://doi.org/10.1023/B:ANTO.0000047942.69033.24.

European pharmacopoeia. European Directorate for the Quality of Medicines. 6th ed.; *Council of Europe*, London, UK, **2008**.

Evans, J.D.; Martin, S.A. Effects of thymol on ruminal microorganisms. *Curr. Microbiol.* **2000**, 41, 336–340. https://doi.org/10.1007/s002840010145.

Fallis, B.D.; Lam, R.L. Betaine and glycocyamine therapy for the chronic residuals of poliomyelitis. *J. Am. Med. Assoc.* **1952**, 150, 851–853. http://doi.org/10.1001/jama.1952.03680090015007.

Fandiño, I.; Fernandez-Turren, G.; Ferret, A.; Moya, D.; Castillejos, L.; Calsamiglia, S. Exploring additive, synergistic or antagonistic effects of natural plant extracts on in vitro beef feedlot-type rumen microbial fermentation conditions. *Anim.* **2020**, 10, 173. http://dx.doi.org/10.3390/ani10010173.

Faraji, M.; Dehkordi, S.K.; Moghadam, A.K.Z.; Ahmadipour, B.; Khajali, F. Combined effects of guanidinoacetic acid, coenzyme Q_{10} and taurine on growth performance, gene expression and ascites mortality in broiler chickens. *J. Anim. Physiol. Anim. Nut.* **2018**, 103, 162–169. https://doi.org/10.1111/jpn.13020.

FDA. Food and Drug Administration. Direct food substances affirmed as generally recognized as safe (GRAS). *U.S. Food and Drug Administration*, Maryland, USA, **2004**. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=184.

FDA. Food and Drug Administration. Freedom of information summary. Supplemental new animal drug application. NADA 095-735. Monensin Sodium (Rumensin 80): Type A Medicated Article for Dairy Cattle. *U.S. Food and Drug Administration*, Maryland, USA, **2005**. https://animaldrugsatfda.fda.gov/adafda/app/search/public/document/downloadFoi/334.

FDA. Food and Drug Administration. Food additives permitted in feed and drinking water of animals: Guanidinoacetic acid. *U.S. Food and Drug Administration*, Maryland, USA, **2016**. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=573.496.

Fei, L.; Hao, L.; Qipeng, Y.; Chunfang, L. *In vitro* antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Res. Int.* **2011**, 44, 3057–3064. https://doi.org/10.1016/j.foodres.2011.07.030.

Ferme, D.; Banjac, M.; Calsamiglia, S.; Busquet, M.; Kamel, C.; Avgustin, G. The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. *Folia Microbiol.* **2004**, 49, 151–155. https://doi.org/10.1007/BF02931391.

Firkins, J.; Yu, Z. Characterisation and quantification of the microbial populations in the rumen. In: Sejrsen, K., Hvelplund, T., Nielsen, M.O. ed. Ruminant Physiology, Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress, *Wageningen Academic Publishers*, Wageningen, Netherlands, **2006**; pp. 19–54. https://doi.org/10.3920/978-90-8686-566-6.

Fisher, K.; Phillips, C. The mechanism of action of a citrus oil blend against Enterococcus faecium and Enterococcus faecalis. *J. Appl. Microbiol.* **2009**, 106, 1343–1349. https://doi.org/10.1111/j.1365-2672.2008.04102.x.

Foskolos, A.; Ferret, A.; Siurana, A.; Castillejos, L.; Calsamiglia, S. Effects of capsicum and propyl-propane thiosulfonate on rumen fermentation, digestion, and milk production and composition in dairy cows. *Anim.* **2020**, 10, 859. http://dx.doi.org/10.3390/ani10050859.

France, J.; Dijkstra, J.; Dhanoa, M.S.; Lopez, S.; Bannink, A. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed *in vitro*: derivation of models and other mathematical considerations. *Br. J. Nutr.* **2000**, 83, 143–150. https://doi.org/10.1017/S0007114500000180.

Frey, J.C.; Pell, A.N.; Berthiaume, R.; Lapierre, H.; Lee, S.; Ha, J.K.; Mendell, J.E.; Angert, E.R. Comparative studies of microbial populations in the rumen, duodenum, ileum and faeces of lactating dairy cows. *J. Appl. Microbiol.* **2010**, 108, 1982–1993. https://doi.org/10.1111/j.1365-2672.2009.04602.x.

Fu, Y.J.; Zu, Y.G.; Chen, L.Y.; Shi, X.G.; Wang, Z.; Sun, S.; Efferth, T. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother. Res.* **2007**, 21, 989–994. https://doi.org/10.1002/ptr.2179.

Gallucci, M.N.; Oliva, M.; Casero, C.; Dambolena, J.; Luna, A.; Zygadlo, J.; Demo, M. Antimicrobial combined action of terpenes against the food-borne

microorganisms *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. *Flavour Fragr. J.* **2009**, 24, 348–354. https://doi.org/10.1002/ffj.1948.

Garrity, G.M.; Lilburn, T.G.; Cole, J.R.; Harrison, S.H.; Euzéby, J.; Tindall, B.J. Taxonomic Outline of Bacteria and Archaea. Part 1 - The Archaea: Phyla Crenarchaeota and Euryarchaeota. *Michigan State University*, Lansing, USA, **2007**, 7, 6–31. http://www.taxonomicoutline.org/content/7/7/6/pdf.

Gerber, P.J.; Steinfeld, H.; Henderson, B.; Mottet, A.; Opio, C.; Dijkman, J.; Falcucci, A.; Tempio, G. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. *Food and Agriculture Organization FAO*, Rome, Italy, **2013**. https://www.fao.org/3/i3437e/i3437e.pdf.

Gershenzon, J.; Croteau, R. Terpenoids. In: Rosenthal, G.A., Berenbaum, M.R. ed. Herbivores: Their Interactions with Secondary Plant Metabolites, *Academic Press*, California, USA, **1991**; pp. 165–219.

Giannenas, I.; Christaki-Sarikaki, E.; Bonos, E.; Florou-Paneri, P. Essential oils and their applications in animal nutrition. *Med. Arom. Plan.* **2013**, 2, 6. https://doi.org/10.4172/2167-0412.1000140.

Gill, A.O.; Delaquis, P.; Russo, P.; Holley, R.A. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *Int. J. Food Microbiol.* **2002**, 3, 83–92. https://doi.org/10.1016/s0168-1605(01)00712-7.

Gill, A.O.; Holley, R.A. Mechanisms of bactericidal action of Cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. *Appl. Environ. Microbiol.* **2004**, 70, 5750–5755. https://doi.org/10.1128/AEM.70.10.5750-5755.2004.

Gill, A.O.; Holley, R.A. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant essential oils aromatics. *Int. J. Food Microbiol.* **2006a**, 108, 1–9. https://doi.org/10.1016/j.ijfoodmicro.2005.10.009.

Gill, A.O.; Holley, R.A. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. *Int. J. Food Microbiol.* **2006b**, 3, 170–174. https://doi.org/10.1016/j.ijfoodmicro.2006.04.046.

Goering, H.K.; Van Soest, P.J. Forage Fiber Analyses (apparatus, Reagents, Procedures, and Some Applications). *U.S. Agricultural Research Service*, Washington, USA, **1970**. https://naldc.nal.usda.gov/download/CAT87209099/PDF.

Gold, J.J.; Heath, L.B.; Bauchop, T. Ultrastructural description of a new chytrid genus of caecum anaerobes, Caecomyces equi gen. nov., sp. nov., assigned to the Neocallimasticaceae. *Biosystems* **1988**, 21, 403–415. https://doi.org/10.1016/0303-2647(88)90039-1.

Goñi, P.; López, P.; Sánchez, C.; Gómez-Lus, R.; Becerril, R.; Nerín, C. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. *Food Chem.* **2009**, 116, 982–989. https://doi.org/10.1016/j.foodchem.2009.03.058.

Greathead, H. Plants and plant extracts for improving animal productivity. *Proc. Nutr. Soc.* **2003**, 62, 279–290. https://doi.org/10.1079/pns2002197.

Griffin, S.G.; Wyllie, S.G.; Markham, J.L.; Leach, D.N. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour Fragr. J.* **1999**, 14, 322–332. https://doi.org/10.1002/(SICI)1099-1026(199909/10).

Guenther, E. The Essential Oils. *D. van Nostrand Company*, New York, USA, **1948**.

Guimaraes-Ferreira, L. Role of the phosphocreatine system on energetic homeostasis in skeletal and cardiac muscles. *Einstein* **2014**, 12, 126–131. https://doi.org/10.1590/s1679-45082014rb2741.

Gutierrez, J.; Barry-Ryan, C.; Bourke, P. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int. J. Food Microbiol.* **2008**, 124, 91–97. https://doi.org/10.1016/j.ijfoodmicro.2008.02.028.

Hammer, K.A.; Carson, C.F.; Riley, T.V. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **1999**, 86, 985–990. https://doi.org/10.1046/j.1365-2672.1999.00780.x.

Haney, M.E.; Hoehn, M.M. Monensin, a new biologically active compound. I. Discovery and isolation. *Antimicrob. Agents Chemother.* **1967**, 7, 349–352. http://doi.org/10.1128/AAC.7.3.349.

Hart, K.J.; Jones, H.G.; Waddams, K.E.; Worgan, H.J.; Zweifel, B.; Newbold, C.J. An essential oil blend decreases methane emission and increases milk yield in dairy cows. *Open J. Anim. Sci.* **2019**, 9, 259–267. https://doi.org/10.4236/ojas.2019.93022.

Hart, K.J.; Yanez-Ruiz, D.R.; Duval, S.M.; McEwan, N.R.; Newbold, C.J. Plant extract to manipulate rumen fermentation. *Anim. Feed Sci. Technol.* **2008**, 147, 8–35. https://doi.org/10.1016/j.anifeedsci.2007.09.007.

He, D.; Yang, L.; Li, J.; Dong, B.; Lai, W.; Zhang, L. Effects of guanidinoacetic acid on growth performance, creatine metabolism and plasma amino acid profile in broilers. *J. Anim. Physiol. and Anim. Nutr.* **2019**, 103, 766–773. https://doi.org/10.1111/jpn.13081.

He, D.T.; Gai, X.R.; Yang, L.B.; Li, J.T.; Lai, W.Q.; Sun, X.L.; Zhang, L.Y. Effects of guanidinoacetic acid on growth performance, creatine and energy metabolism, and carcass characteristics in growing-finishing pigs. *J. Anim. Sci.* **2018**, 96, 3264–3273. https://doi.org/10.1093/jas/sky186.

Helander, I.M.; Alakomi, H.; Latva-Kala, K.; Mattila-Sandholm, T.; Pol, I., Smid, E.J.; Gorris, L.G.M.; Wright, A. Characteritzation of the action of selected essential oil components on gram-negative bacteria. *J. Agric. Food Chem.* **1998**, 46, 3590–3595. https://doi.org/10.1021/jf980154m.

Hemaiswarya, S.; Doble, M. Synergistic interaction of eugenol with antibiotics against gram negative bacteria. *Phytomed.* **2009**, 16, 997–1005. https://doi.org/10.1016/j.phymed.2009.04.006.

Hemaiswarya, S.; Doble, M. Synergistic interaction of phenylpropanoids with antibiotics against bacteria. *J. Med. Microbiol.* **2010**, 59, 1469–1476. https://doi.org/10.1099/jmm.0.022426-0.

Henderson, G.; Cox, F.; Ganesh, S.; Jonker, A.; Young, W. Global Rumen Census Collaborators; Janssen, P.H. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* **2015**, 5, 14567. https://doi.org/10.1038/srep14567.

Herman, A.; Tambor, K.; Herman, A. Linalool affects the antimicrobial efficacy of essential oils. *Curr. Microbiol.* **2016**, 72,165–172. http://dx.doi.org/10.1007/s00284-015-0933-4.

Hili, P.; Evans, C.S.; Veness, R.G. Antimicrobial action of essential oils: the effect of dimethylsulphoxide on the activity of cinnamon oil. *Lett. Appl. Microbiol.* **1997**, 24, 269–275. https://doi.org/10.1046/j.1472-765x.1997.00073.x.

Hook, S.E.; Wright, A.D.G.; McBride, B.W. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* **2010**, 945785. https://doi.org/10.1155/2010/945785.

Hoover, W.H.; Crooker, B.A.; Sniffen, C.J. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. *J. Anim. Sci.* **1976**, 43, 528–534. https://doi.org/10.2527/jas1976.432528x.

Hristov, A.N.; Ropp, J.K.; Zaman, S.; Melgar, A. Effects of essential oils on in vitro ruminal fermentation and ammonia release. *Anim. Feed Sci. Technol.* **2008**, 144, 55–64. https://doi.org/10.1016/j.anifeedsci.2007.09.034.

Hristov, A.N.; Oh, J.; Firkins, J.L.; Dijkstra, J.; Kebreab, E.; Waghorn, G.; Makkar, H.P.; Adesogan, A.T.; Yang, W.; Lee, C.; Gerber, P.J.; Henderson, B.; Tricarico, J.M. Special topics – Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *J. Anim. Sci.* **2013**, 91, 5045–5069. https://doi.org/10.2527/jas2013-6583.

Hungate, R.E. The Rumen and its Microbes. *Academic Press*, New York, USA, **1966**. https://doi.org/10.1016/C2013-0-12555-X.

Hungate, R.E. Hydrogen as an intermediate in the rumen fermentation. *Arch. Mikrobiol.* **1967**, 59, 158–164. https://doi.org/10.1007/BF00406327.

Hungate, R.E. The ruminant and the rumen. In: Hobson, P.N. ed. The Rumen Microbial Ecosystem. *Elsevier Appl. Sci.*, New York, USA,**1988**; pp. 1–19.

Hyldgaard, M.; Mygind, T.; Meyer, R.L. Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. *Front. Microbiol.* **2012**, 3, 12. https://doi.org/10.3389/fmicb.2012.00012.

IFIF. International Feed Industry Federation, Global feed statistics. **2021**. https://ifif.org/global-feed/statistics/.

Ikwuegbu, O.A.; Sutton, J. D. The effect of varying the amount of linseed oil supplementation on rumen metabolism in sheep. *Br. J. Nutr.* **1982**, 48, 365–375. https://doi.org/10.1079/bjn19820120.

Ishaq, S.L.; Alzahal, O.; Walker, N.; McBride, B. An investigation into rumen fungal and protozoal diversity in three rumen fractions, during high-fiber or grain-induced sub-acute ruminal acidosis conditions, with or without active dry yeast supplementation. *Front. Microbiol.* **2017**, 8, 1943. https://doi.org/10.3389/fmicb.2017.01943.

ISO. The International Organization for Standardization. Aromatic natural raw materials, ISO 9235, **2013**. https://www.iso.org/obp/ui/#iso:std:iso:9235:ed-2:v1:en.

Janssen, P.H.; Kirs, M. Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* **2008**, 74, 3619–3625. https://doi.org/10.1128/AEM.02812-07.

Jin, W.; Cheng, Y.F.; Mao, S.Y.; Zhu, W.Y. Discovery of a novel rumen methanogen in the anaerobic fungal culture and its distribution in the rumen as revealed by real-time PCR. *BMC Microbiol*. **2014**, 14, 104. http://www.biomedcentral.com/1471-2180/14/104.

Joch, M.; Cermak, L.; Hakl, J.; Hucko, B.; Duskova, D.; Marounek, M. *In vitro* screening of essential oil active compounds for manipulation of rumen fermentation and methane mitigation. *Asian Aust. J. Anim. Sci.* **2016**, 29, 952–959. https://doi.org/10.5713/ajas.15.0474.

Joch, M.; Kudrna, V.; Hucko, B.; Marounek, M. Effects of geraniol and camphene on *in vitro* rumen fermentation and methane production. *Anim. Sci.* **2017**, 48, 63–69. https://doi.org/10.1515/sab-2017-0012.

Johnson, K.A.; Johnson, D.E. Methane emissions from cattle. *J. Anim. Sci.* **1995**, 73, 2483–2492. https://doi.org/10.2527/1995.7382483x.

Jones, A.; Porter, M. Vegetable oils in fermentation: beneficial effects of low-level supplementation. *J. Ind. Microbiol. Biotech.* **1998**, 21, 203–207. https://doi.org/10.1038/sj.jim.2900571.

Jouany, J.P. Les fermentations dans le rumen et leur optimisation. *INRA Prod. Anim.* **1994**, 7, 207–225. https://hal.archives-ouvertes.fr/hal-00896087.

Jouany, J.P. Effect of rumen protozoa on nitrogen utilization by ruminants: altering ruminal nitrogen metabolism to improve protein utilization. *J. Nutr.* **1996**, 126, 1335S–1346S. https://doi.org/10.1093/jn/126.suppl_4.1335S.

Kamra, D.N. Rumen microbial ecosystem. *Curr. Sci.* **2005**, 89, 124–135. https://www.jstor.org/stable/24110438.

Kamra, D.N.; Pawar, M.; Singh, B. Manipulation of ruminal biohydrogenation by the use of plants bioactive compounds. In: Patra, A.K. ed. Dietary Phytochemicals and Microbes, *Springer*, New York, USA, **2012**; 351–370. https://doi.org/10.1007/978-94-007-3926-0.

Karakurt, I.; Aydin, G.; Aydiner, K. Sources and mitigation of methane emissions by sectors: A critical review. *Renewable Energy* **2012**, 39, 40–48. http://dx.doi.org/10.1016/j.renene.2011.09.006.

Kebreab, E.; France, J.; Mills, J.A.N.; Allison, R.; Dijkstra, J. A dynamic model of N metabolism in the lactating dairy cow and an assessment of impact of N excretion on the environment. *J. Anim. Sci.* **2002**, 80, 248–259. https://doi.org/10.2527/2002.801248x.

Khajali, F.; Lemme, A.; Rademacher-Heilshorn, M. Guanidinoacetic acid as a feed supplement for poultry, *World's Poult. Sci. J.* **2020**, 76, 270–291. https://doi.org/10.1080/00439339.2020.1716651.

Kley, R.A.; Tarnopolsky, M.A.; Vorgerd, M. Creatine for treating muscle disorders. *Cochrane Database Syst. Rev.* **2013**, 6, CD004760. https://doi.org/10.1002/14651858.CD004760.pub4.

Klieve, A.V.; Swain, R.A. Estimation of ruminal bacteriophage numbers by pulsed-field gel electrophoresis and laser densitometry. *Appl. Environ. Microbiol.* **1993**, 59, 2299–2303. https://doi.org/10.1128/aem.59.7.2299-2303.1993.

Klieve, A.V.; Heck, G.L.; Prance, M.A.; Shu, Q. Genetic homogeneity and phage susceptibility of ruminal strains of *Streptococcus bovis* isolated in Australia. *Lett. Appl. Microbiol.* **1999**, 29, 108–112. https://doi.org/10.1046/j.1365-2672.1999.00596.x.

Krause, D.O.; Russell, J.B. An rRNA approach for assessing the role of obligate amino acid-fermenting bacteria in ruminal amino acid deamination. *Appl. Environ. Microbiol.* **1996**, 62, 815–821. https://doi.org/10.1128/aem.62.3.815-821.1996.

Krause, D.O.; Nagaraja, T.G.; Wright, A.D.G. Callaway, T.R. Board-invited review: Rumen microbiology: Leading the way in microbial ecology. *J. Anim. Sci.* **2013**, 91, 331–341. https://doi.org/10.2527/jas.2012-5567.

Lambert, R.J.W.; Skandamis, P.N.; Coote, P.J.; Nychas, G.J.E. A study of the minimum inhibitory concentration and mode of action of oregano essential oil,

thymol and carvacrol. *J. Appl. Microbiol.* **2001**, 91, 453–462. https://doi.org/10.1046/j.1365-2672.2001.01428.x.

Lan, W.; Yang, C. Ruminal methane production: Associated microorganisms and the potential of applying hydrogen-utilizing bacteria for mitigation, *Sci. Total Environ.* **2019**, 654, 1270–1283. https://doi.org/10.1016/j.scitotenv.2018.11.180.

Langeveld, W.T.; Veldhuizen, E.J.A.; Burt, S.A. Synergy between essential oil components and antibiotics: a review. *Crit. Rev. Microbiol.* **2013**, 40, 76–94. https://doi.org/10.3109/1040841X.2013.763219.

Li, J.L.; Zhang, L.; Fu, Y.A.; Li, Y.J.; Jiang, Y.; Zhou, G.H.; Gao, F. Creatine monohydrate and guanidinoacetic acid supplementation affect the growth performance, meat quality and creatine metabolism of finishing pigs. *J. Agri. Food Chem.* **2018**, 86, 9952–9959. https://doi.org/10.1021/acs.jafc.8b02534.

Li, S.Y.; Wang, C.; Wu, Z.Z.; Liu, Q.; Guo, G.; Huo, W.J.; Zhang, J.; Chen, L.; Zhang, Y.L.; Pei, C.X.; Zhang, S.L. Effects of guanidinoacetic acid supplementation on growth performance, nutrient digestion, rumen fermentation and blood metabolites in Angus bulls. *Animal* **2020**, 14, 2535–2542. https://doi.org/10.1017/S1751731120001603.

Li, Z.; Liang, H.; Xin, J.; Xu, L.; Li, M.; Yu, H.; Zhang, W.; Ge, Y.; Li, Y.; Qu, M. Effects of dietary guanidinoacetic acid on the feed efficiency, blood measures, and meat quality of Jinjiang bulls. *Front. Vet. Sci.* **2021**, 9, 684295. https://doi.org/10.3389/fvets.2021.684295.

Liang, R.; Xu, S.; Shoemaker, C.F.; Li, Y.; Zhong, F.; Huang, Q. Physical and antimicrobial properties of peppermint oil nano emulsions. *J. Agric. Food Chem.* **2012**, 60, 7548–7555. https://doi.org/10.1021/jf301129k.

Liu, Y.; Li, J.L.; Li, Y.J.; Gao, T.; Zhang, L.; Gao, F.; Zhou, G.H. Effects of dietary supplementation of guanidinoacetic acid and combination of guanidinoacetic acid and betaine on postmortem glycolysis and meat quality of finishing pigs. *Anim. Feed Sci. Technol.* **2015**, 205, 82–89. https://doi.org/10.1016/j.anifeedsci.2015.03.010.

Liu, C.; Wang, C.; Zhang, V.; Liu, Q.; Guo, G.; Huo, W.J.; Pei, C.X.; Chen, L.; Zhang, Y.L. Guanidinoacetic acid and betaine supplementation have positive effects on growth performance, nutrient digestion and rumen fermentation in Angus bulls. *J. Anim. Feed Sci. Technol.* **2021**, 276, 114923. https://doi.org/10.1016/j.anifeedsci.2021.114923.

Lobo, R.R.; Faciola, A.P. Ruminal phages - A review. *Front. Microbiol.* **2021**, 12, 763416. https://doi.org/10.3389/fmicb.2021.763416.

Macheboeuf, D.; Morgavi, D.P.; Papon, Y.; Mousset, J.L.; Arturo-Schaan, M. Dose-response effects of essential oils on *in vitro* fermentation activity of the rumen

microbial population. *Anim. Feed Sci. Technol.* **2008**, 145, 335–350. https://doi.org/10.1016/j.anifeedsci.2007.05.044.

Mackie, R.I.; McSweeney, C.S.; Aminov, R.I. Rumen. *Encyclo. Life Sci.* **2001**, 1–11. https://doi.org/10.1038/npg.els.0000404.

Mackie, R.I.; McSweeney, C.S.; Aminov, R.I. Rumen. In: Battista, J. ed. eLS. *John Wiley & Sons Ltd*, Chichester, UK, **2013**. https://doi.org/10.1002/9780470015902.a0000404.pub2.

Malmuthuge, N.; Guan L. Understanding host-microbial interactions in rumen: searching the best opportunity for microbiota manipulation. *J. Anim. Sci. Biotechnol.* **2017**, 8, https://doi.org/10.1186/s40104-016-0135-3.

Marino, M.; Bersani, C.; Comi, G. Impedance measurements to study the antimicrobial activity of essential oils from Lamiacea and Compositae. *Int. J. Food Microbiol.* **2001**, 67, 187–195. https://doi.org/10.1016/s0168-1605(01)00447-0.

Maruyama, N.; Sekimoto, Y.; Ishibashi, H.; Inouye, S.; Oshima, H.; Yamaguchi, H.; Abe, S. Suppression of neutrophil accumulation in mice by cutaneous application of geranium essential oil. *J. Inflamm.* **2005**, 2, 1. https://doi.org/10.1186/1476-9255-2-1.

McAllister, T.A.; Newbold, C.J. Redirecting rumen fermentation to reduce methanogenesis. *Aust. J. Exp. Agri.* **2008**, 48, 7–13. https://doi.org/10.1071/EA07218.

McDougall, E.I. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochem. J.* **1948**, 43, 99–109. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1274641/pdf/biochemj00946-0114.pdf.

McGuffey, R.K.; Richardson, L.F.; Wilkinson, J.I.D. Ionophores for dairy cattle: Current status and future outlook. *J. Dairy Sci.* **2001**, 84, E194–E203. https://doi.org/10.3168/jds.S0022-0302(01)70218-4.

McIntosh, F.M.; Williams, P.; Losa, R.; Wallace, R.J.; Beever, D.A.; Newbold, C.J. Effects of essential oils on ruminal microorganisms and their protein metabolism. *Applied Environ. Microbiol.* **2003**. 69, 5011–5014. https://doi.org/10.1128/AEM.69.8.5011-5014.2003.

Michiels, J.; Maertens, L.; Buyse, J.; Lemme, A.; Rademacher, M.; Dierick, N.A.; De Smet, S. Supplementation of guanidinoacetic acid to broiler diets: effects on performance, carcass characteristics, meat quality, and energy metabolism. *Poult. Sci.* **2012**, 91, 402–412. https://doi.org/10.3382/ps.2011-01585.

Miller, R.G. Simultaneous Statistical Inference. 2nd ed. *Springer*, New York, USA, **1981**. https://doi.org/10.1007/978-1-4613-8122-8.

Mohammed, N.; Ajisaka, N.; Lila, Z.A.; Mikuni, K.; Hara, K.; Kanda, S.; Itabashi, H. Effect of Japanese horseradish oil on methane production and ruminal fermentation *in vitro* and in steers. *J. Anim. Sci.* **2004**, 82, 1839–1846. https://doi.org/10.2527/2004.8261839x.

Moumen, A.; Azizi, G.; Ben Chekroun, K.; Baghour, M. The effects of livestock methane emission on the global warming: A review. *Int. J. Global Warming* **2016**, 9, 229–253. https://doi.org/10.1504/IJGW.2016.074956.

Mourey, A.; Canillac, N. Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Cont.* **2002**, 13, 289–292. https://doi.org/10.1016/S0956-7135(02)00026-9.

Murakami, A.E.; Rodrigueiro, R.J.B.; Santos, T.C.; Ospina-Rojas, I.C.; Rademacher, M. Effects of dietary supplementation of meat-type quail breeders with guanidinoacetic acid on their reproductive parameters and progeny performance. *Poult. Sci*. **2014**, 93, 2237–2244 https://doi.org/10.3382/ps.2014-03894.

Nanon, A.; Suksombat, W.; Yang, W. Use of essential oils for manipulation of rumen microbial fermentation using batch culture. *Thai. J. Vet. Med.* **2015**, 45, 167–180. https://www.thaiscience.info/journals/Article/TJVM/10961970.pdf.

Neu, A.; Neuhoff, H.; Trube, G.; Fehr, S.; Ullrich, K.; Roeper, J.; Isbrandt, D. Activation of GABA(A) receptors by guanidinoacetate: a novel pathophysiological mechanism. *Neurobiol. Dis.* **2002**, 11, 298–307. https://doi.org/10.1006/nbdi.2002.0547.

Newbold, C.J. Proposed mechanisms for enzymes as modifiers of ruminal fermentation. In: Proc. of the 8th Florida Ruminant Nutrition Symposium, Gainesville, Florida, 16–17 Jan. **1997**; pp. 146–159. https://animal.ifas.ufl.edu/dairy/conferences--meetings/florida-ruminant-nutrition-symposium/florida-ruminant-nutrition-symposium-archive/.

Newbold, C.J.; McIntosh, F.M.; Williams, P.; Losa, R.; Wallace, R.J. Effects of a specific blend of essential oil compounds on rumen fermentation. *Anim. Feed Sci. Technol.* **2004**, 114, 105–112. https://doi.org/10.1016/j.anifeedsci.2003.12.006.

Newbold, C.J.; Rode, L.M. Dietary additives to control methanogenesis in the rumen. *Int. Cong. Ser.* **2006**, 1293, 138–147. https://doi.org/10.1016/j.ics.2006.03.047.

Nolan, J.V. Quantitative models of nitrogen metabolism in sheep. In: MacDonald, I.W., Warner, A.C.I. ed. Digestion and Metabolism in the Ruminant, *University of New England Publishing*, Arimdale, Australia, **l975**. pp. 416–431.

NRC. National Research Council. Nutrient Requirements of Beef Cattle, 8th ed. *National Academic Press*, Washington DC, USA, **2016**. https://doi.org/10.17226/19014.

NRC. National Research Council. Nutrient Requirements of Dairy Cattle. 7th ed. *National Academic Press*, Washington DC, USA, **2001**. https://doi.org/10.17226/9825.

Odongo, N.E.; Bagg, R.; Vessie, G.; Dick, P.; Or-Rashid, M.M.; Hook, S.E.; Gray, J.T.; Kebreab, E.; France, J.; McBride, B.W. Long-term effects of feeding monensin on methane production in lactating dairy cows. *J. Dairy Sci.* **2007**, 90, 1781–1788. https://doi.org/10.3168/jds.2006-708.

Oltjen, R.R.; Slyter, L.L.; Williams, E.E.; Kern, J.R. Influence of the branched-chain volatile fatty acids and phenylacetate on ruminal microorganisms and nitrogen utilization by steers fed urea or isolated soy protein. *J. Nut.* **1971**, 101, 101-112. https://doi.org/10.1093/jn/101.1.101.

Orpin, C.G. Studies on the rumen flagellate *Neocallimastix frontalis*. *J. Gen. Microbiol.* **1975**, 91, 249–262. https://doi.org/10.1099/00221287-91-2-249.

Orpin, C.G. The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. *J. Gen. Microbiol.* **1977**, 99, 215–218. https://doi.org/10.1099/00221287-99-1-215.

Ørskov, E.R. Recent advances in understanding of microbial transformation in ruminants. *Livest. Prod. Sci.* **1994**, 39: 53–60. https://doi.org/10.1016/0301-6226(94)90153-8.

Ostojic, S.M.; Niess, B.; Stojanovic, M.; Obrenovic, M. Creatine metabolism and safety profiles after 6-week oral guanidinoacetic acid administration in healthy humans. *Int. J. Med. Sci.* **2013**, 10, 141–147. https://doi.org/10.7150/ijms.5125.

Ostojic, S.M. An alternative mechanism for guanidinoacetic acid to affect methylation cycle. *Med. Hypotheses* **2014**, 83, 847–848. https://doi.org/10.1016/j.mehy.2014.11.001.

Ostojic, S.M. Advanced physiological roles of guanidinoacetic acid. *Eur. J. Nutr.* **2015**, 54, 1211–1215. https://doi.org/10.1007/s00394-015-1050-7.

Ostojic, S.M. Tackling guanidinoacetic acid for advanced cellular bioenergetics. *Nutr.* **2017**, 34, 55–57. https://doi.org/10.1016/j.nut.2016.09.010.

Oussalah, M.; Caillet, S.; Saucier, L.; Lacroix, M. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Staphylococcus aureus* and *Listeria monocytogenes*. *Food Cont.* **2007**, 18, 414–420. https://doi.org/10.1016/j.foodcont.2005.11.009.

Owens, F.N.; Goetsch, A.L. Ruminal fermentation. In; Church, D.C. ed. The Ruminant Animal: Digestive Physiology and Nutrition. *Prentice-Hall*, Englewood Cliffs, Nueva Jersey, USA, **1988**.

Oyedemi, S.O.; Okoh, A.I.; Mabinya, L.V.; Pirochenva, G.; Afolayan, A.J. The proposed mechanism of bactericidal action of eugenol, alpha-terpineol and gamma-

terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*. *African J. Biotechnol.* **2009**, 8, 1280–1286. https://www.ajol.info/index.php/ajb/article/view/60106.

Pandit, J.; Aqil, M.; Sultana, Y. Nanoencapsulation technology to control release and enhance bioactivity of essential oils. *Encapsulations* **2016**, 597–640. https://doi.org/10.1016/B978-0-12-804307-3.00014-4.

Paster, B.; Russell, J.B.; Yang, C.M.J.; Chow, J.M.; Woese, C.R.; Tanner, R. Phylogeny of ammonia-producing ruminal bacteria, *Peptostreptococcus anaerobius*, *Clostridium sticklandii* and *Clostridium aminophilum* sp. nov. *Int. J. Syst. Bacteriol.* **1993**, 43, 107–110. https://doi.org/10.1099/00207713-43-1-107.

Patra, A.K.; Saxena, J. A new perspective on the use of plant secondary mebolites to inhibit methanogenesis. *Phytochem.* **2010**, 71, 1198–1222. https://doi.org/10.1016/j.phytochem.2010.05.010.

Patra, A.K.; Yu, Z. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. *Appl. Environ. Microbiol.* **2012**, 78, 4271–4280. https://doi.org/10.1128/AEM.00309-12.

Patra, A.M. Effects of essential oils on rumen fermentation, microbial ecology and ruminant production. *Asian J. Anim. Vet. Adv.* **2011**, 10, 1683–9919. https://doi.org/10.3923/ajava.2011.416.428.

Patra, A.M. An overview of antimicrobial properties of different classes of phytochemicals. In: Patra, A.M. ed. Dietary Phytochemicals and Microbes, *Springer*, Dordrecht, Netherlands. **2012**; pp. 1–32. https://doi.org/10.1007/978-94-007-3926-0.

Pei, R.S.; Zhou, F.; Ji, B.P.; Xu, J. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved Method. *J. Food Sci.* **2009**, 74, 379–383. https://doi.org/10.1111/j.1750-3841.2009.01287.x.

Portocarero, N.; Braun, U. The physiological role of guanidinoacetic acid and its relationship with arginine in broiler chickens. *Poult. Sci.* **2021**, 100, 101203. https://doi.org/10.1016/j.psj.2021.101203.

Qiu, J.; Feng, H.; Lu, J.; Xiang, H.; Wang, D.; Dong, J.; Wang, J.; Wang, X.; Liu, J.; Deng, X. Eugenol reduces the expression of virulence-related exoproteins in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **2010**, 76, 5846–5851. https://doi.org/10.1128/AEM.00704-10.

Richardson, L.F.; Raun, A.P.; Potter, E.L.; Cooley, C.O.; Rathmacher, R.P. Effect of monensin on rumen fermentation in vivo and in vitro. *J. Anim. Sci.* **1976**, 43, 657–664. https://doi.org/10.2527/jas1976.433657x.

Riesberg, L.A.; Weed, S.A.; McDonald, T.L.; Eckerson, J.M.; Drescher, K.M. Beyond muscles: The untapped potential of creatine. *Int. Immunopharmacol*. **2016**, 37, 31–42. https://doi.org/10.1016/j.intimp.2015.12.034.

Rodríguez-Prado, M.; Ferret, A.; Zwieten, J.; Gonzalez, L.; Bravo, D.; Calsamiglia, S. Effects of dietary addition of capsicum extract on intake, water consumption, and rumen fermentation of fattening heifers fed a high-concentrate diet. *J. Anim. Sci*. **2012**, 90, 1879–1884 https://doi.org/10.2527/jas.2010-3191.

Russell, J.B.; Dombrowski, D.B. Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. *Appl. Environ. Microbiol.* **1980**, 39, 604–610. https://doi.org/10.1128/aem.39.3.604-610.1980.

Russell, J.B.; Strobel, H.J. Effects of additives on *in vitro* ruminal fermentation: a comparison of monensin and bacitracin, another Gram-positive antibiotic. *J. Anim. Sci*. **1988**, 66, 552–558. https://doi.org/10.2527/jas1988.662552x.

Russell, J.B.; Strobel, H.J. Effect of ionophores on ruminal fermentation. *Appl. Environ. Microbiol.* **1989**, 55, 1-6. https://doi.org/10.3390/ani11102871.

Russell, J.B.; Onodera, R.; Hino, T. Ruminal protein fermentation: new perspectives on previous contradictions. IN: Proc. of the 7th International Symposium on Ruminant Physiology, *Academic Press*, Washington DC, USA, **1991**; pp. 681–697. https://doi.org/10.1016/B978-0-12-702290-1.50034-5.

Russell, J.B. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production *in vitro*. *J. Dairy Sci.* **1998**, 81, 3222–3230. https://doi.org/10.3168/jds.S0022-0302(98)75886-2.

Russell, J.B.; Muck, R.E.; Weimer, P.J. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. *FEMS. Microbiol. Ecol.* **2009**, 67, 183–197. https://doi.org/10.1111/j.1574-6941.2008.00633.x.

Rychlik, J.L.; Russell, J.B. Mathematical estimations of hyper-ammonia producing ruminal bacteria and evidence for bacterial antagonism that decreases ruminal ammonia production. *FEMS Microbiol. Ecol.* **2000**, 32, 121–128. https://doi.org/10.1111/j.1574-6941.2000.tb00706.x.

Saad, N.Y.; Muller, C.D.; Lobstein, A. Major bioactivities and mechanism of action of essential oils and their components. *Flavour Fragr. J.* **2013**, 28, 269–279. https://doi.org/10.1002/ffj.3165.

Sacchetti, G.; Maietti, S.; Muzzoli, M.; Scaglianti, M.; Manfredini, S.; Radice, M.; Bruni, R. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods. *Food Chem.* **2005**, 91, 621–632. https://doi.org/10.1016/j.foodchem.2004.06.031.

Sangwan, N.S.; Farooqi, A.H.A.; Shabih, F.; Sangwan, R.S. Regulation of essential oil production in plants. *Plant Growth Reg.* **2001**, 34, 3–21. https://doi.org/10.1023/A:1013386921596.

Santos, M.B.; Robinson, P.H.; Williams, P.; Losa, R. Effects of addition of an essential oil complex to the diet of lactating dairy cows on whole tract digestion of nutrients and productive performance. *Anim. Feed Sci. Technol.* **2010**, 157, 64–71. https://doi.org/10.1016/j.anifeedsci.2010.02.001.

Schären, M.; Frahm, J.; Kersten, S.; Meyer, U.; Hummel, J.; Breves, G.; Dänicke, S. Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic, and immunological attributes of dairy cows. *J. Dairy Sci.* **2018**, 101, 4615–4637. https://doi.org/10.3168/jds.2017-13736.

Sikkema, J.; Bont, J.A.M.; Poolman, B. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **1994**, 269, 8022–8028. https://www.jbc.org/article/S0021-9258(17)37154-5/pdf.

Simoni, M.; Temmar, R.; Bignamini, D.A.; Foskolos, A.; Sabbioni, A.; Ablondi, M.; Quarantelli, A.; Righi, F. Effects of the combination between selected phytochemicals and the carriers silica and Tween 80 on dry matter and neutral detergent fiber digestibility of common feeds. *Ital. J. of Anim. Sci.* **2020**, 19, 723–738. https://doi.org/10.22358/jafs/76754/2017.

Skandamis, P.N.; Nychas, G.J.E. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *J. Appl. Microbiol.* **2001**, 91, 1011–1022. https://doi.org/10.1046/j.1365-2672.2001.01467.x.

Skillman, L.C.; Evans, P.N.; Naylor, G.E.; Morvan, B.; Jarvis, G.N.; Joblin, K.N. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe* **2004**, 10, 277–285. https://doi.org/10.1016/j.anaerobe.2004.05.003.

Speer, H.F.; Pearl, K.A.; Titgemeyer, E.C. Relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally to cattle. *J. Anim. Sci.* **2020**, 98, 1–6. https://doi.org/10.1093/jas/skaa282.

Stern, M.D.; Hoover, W.H. The dual flow continuous culture system. In: Proc. of the Continuous Culture Fermenters: Frustation or Fermentation, *ADSA-ASAS meeting*, Chazy, New York, USA, **1990**; pp. 17–32.

Stevanovic, Z.D.; Sieniawska, E.; Glowniak, K.; Obradovic, N.; Pajic-Lijakovic, I. Natural macromolecules as carriers for essential oils: from extraction to biomedical application. *Front. Bioeng. Biotechnol.* **2020**, 8, 563. http://dx.doi.org/10.3389/fbioe.2020.00563.

Szumacher-Strabel, M.; Cieślak, A. Potential of phytofactors to mitigate rumen ammonia and methane production. *J. Anim. Feed Sci.* **2010**, 19, 319–337. https://doi.org/10.22358/jafs/66296/2010.

Szumacher-Strabel, M.; Cieslak, A. Essential oils and rumen microbial populations. In: Patra, A.M. ed. Dietary Phytochemicals and Microbes, *Springer*, Dordrecht, Netherlands. **2012**; pp. 157–183. https://doi.org/10.1007/978-94-007-3926-0.

Tamminga, S. Nutrition management of dairy cows as a contribution to pollution control. *J. Dairy Sci.* **1992**, 75, 345–357. https://doi.org/10.3168/jds.S0022-0302(92)77770-4.

Tedeschi, L.O.; Fox, D.G.; Tylutki, T.P. Potential environmental benefits of ionophores in ruminant diets. *J. Environ. Qual.* **2003**, 32, 1591–1602. https://doi.org/10.2134/jeq2003.1591.

Teixeira, K.A.; Mascarenhas, A.G.; de Carvalho Mello, H.H.; Arnhold, E.; da Silva Assunção, P.; Carvalho, D.P.; Lopes, S.G. Effect of diets with different levels of guanidinoacetic acid on newly weaned piglets. *Semina Ciências Agrárias* **2017**, 38, 3887–3896. https://doi.org/10.5433/1679-0359.2017v38n6p3887.

Temmar, R.; Rodríguez-Prado, M.; Forgeard, G.; Rougier, C.; Calsamiglia, S. Interactions among natural active ingredients to improve the efficiency of rumen fermentation *in vitro*. *Anim.* **2021**, 11, 1205. http://dx.doi.org/10.3390/ani11051205.

Tilley, J.M.A.; Terry, R.A. A two-stage technique for the in vitro digestion of forage crops. *J. Brit. Grassland Soc.* **1963**, 18, 104–111. https://doi.org/10.1111/j.1365-2494.1963.tb00335.x.

Tossenberger, J.; Rademacher, M.; Nemeth, K.; Halas, V.; Lemme, A. Digestibility and metabolism of dietary guanidino acetic acid fed to broilers. *Poult. Sci.* **2016**, 95, 2058–2067. https://doi.org/10.3382/ps/pew083.

Trombetta, D.; Castelli, F.; Sarpietro, M.G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano, G. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* **2005**, 49, 2474–2478. https://doi.org/10.1128/AAC.49.6.2474-2478.2005.

Tserennadmid, R.; Takó, M.; Galgóczy, L.; Papp, T., Pesti, M.; Vágvölgyi, C.; Almássy, K.; Krisch, J. Anti-yeast activities of some essential oils in growth medium, fruit juices and milk. *Int. J. Food Microbiol.* **2011**, 144, 480–486 https://doi.org/10.1016/j.ijfoodmicro.2010.11.004.

Ultee, A.; Kets, E.P.; Smid, E.J. Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus. *Appl. Environ. Microbiol.* **1999**, 65, 4606–4610. https://doi.org/10.1128/aem.65.10.4606-4610.1999.

Ultee, A.; Bennik, M.H.J.; Moezelaar, R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol.* **2002**, 68, 1561–1568. https://doi.org/10.1128/AEM.68.4.1561-1568.2002.

Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fibre, neutral detergent fibre and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Van Soest, P.J. Digestive flow. In: Nutritional Ecology of The Ruminant. 2nd ed. *Cornell University Press*, Ithaca, New York, USA, **1994**.

Vander Heiden, M.G.; Cantley, L.C.; Thompson, C.B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Sci.* **2009**, 324, 1029–1033. http://doi.org/10.1126/science.1160809.

Voon, H.C.; Bhat, R.; Rusul, G. Flower extracts and their essential oils as potential antimicrobial agents for food uses and pharmaceutical applications. *Comprehensive Rev. Food Sci. Food Saf.* **2012**, 11, 34–56. https://doi.org/10.1111/j.1541-4337.2011.00169.x.

Wallace, R.J.; Cotta, M.A. Metabolism of nitrogen-containing compounds. In: Hobson, P.N. ed. The Rumen Microbial Ecosystem. *Elsevier Appl. Sci.*, New York, USA, **1989**; pp. 217–250.

Wallace, R.J.; McEwan, N.R.; McIntosh, F.M.; Teferedegne, B.; Newbold, C.J. Natural products as manipulators of rumen fermentation. *Asian Aust. J. Anim. Sci.* **2002**, 15, 1458–1468. https://doi.org/10.5713/ajas.2002.1458.

Wallace, R.J. Antimicrobial properties of plant secondary metabolites. *Proc. Nutr. Soc.* **2004**, 63, 621–629. https://doi.org/10.1079/pns2004393.

Wang, C.J.; Wang, S.P.; Zhou, H. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Anim. Feed Sci. Technol. **2009**, 148, 157–166. https://doi.org/10.1016/j.anifeedsci.2008.03.008.

Weber, C.J. Isolation of glycocyamine from urine. *Exp. Biol. Med.* **1934**, 32, 172–174. https://doi.org/10.3181/00379727-32-7602.

Weller, R.A.; Pilgrim, A.F. Passage of protozoa and volatile fatty acids from the rumen of sheep and from a continuous *in vitro* fermentation system. *Br. J. Nutr.* **1974**, 32, 341–351. https://doi.org/10.1079/bjn19740087.

Whitehouse, N.L.; Olson, V.M.; Schwab, C.G.; Chesbro, W.R.; Cunninghan, K.D.; Lycos, T. Improved techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. *J. Anim. Sci.* **1994**, 72, 1335–1343. https://doi.org/10.2527/1994.7251335x.

Williams, C.L.; Thomas, B.J.; McEwan, N.R.; Rees Stevens, P.; Creevey, C.J.; Huws, S.A. Rumen protozoa play a significant role in fungal predation and plant carbohydrate breakdown. *Front. Microbiol.* **2020**, 11, 720. https://doi.org/10.3389/fmicb.2020.00720.

Wright, A.D.G.; Klieve, A.V. Does the complexity of the rumen microbial ecology preclude methane mitigation. *Anim. Feed Sci. Technol.* **2011**, 166/167, 248–253. https://doi.org/10.1016/j.anifeedsci.2011.04.015.

Wyss, M.; Kaddurah-Daouk, R. Creatine and creatinine metabolism. *Physiol. Rev.* **2000**, 80, 1107–1213. https://doi.org/10.1152/physrev.2000.80.3.1107.

Yang, W.Z.; Benchaar, C.; Ametaj, B.N.; Chaves, A.V.; He, M.L.; McAllister, T.A. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. *J. Dairy Sci.* **2007**, 90, 5671–5681. https://doi.org/10.3168/jds.2007-0369.

Zened, A.; Forano, E.; Delbes, C.; Verdier-metz, I.; Morgavi, D.; Popova, M.; Ramayo-Caldas, Y.; Bergonier, D.; Meynadier, A.; Marie-Etancelin, C. Les microbiotes des ruminants : état des lieux de la recherche et impacts des microbiotes sur les performances et la santé des animaux. *INRAE Prod. Anim.* **2020**, 33, 249–260. https://doi.org/10.20870/productions-animales.2020.33.4.4597.

Zhang, S.; Zang, C.; Pan, J.; Ma, C.; Wang, C.; Li, X.; Cai, W.; Yang, K. Effects of dietary guanidinoacetic acid on growth performance, guanidinoacetic acid absorption and creatine metabolism of lambs. *PLOS ONE* **2022**, 17, e0264864. https://doi.org/10.1371/journal.pone.0264864.

Zhou, F.; Ji, B.; Zhang, H.; Jiang, H.; Yang, Z.; Li, J.; Li, J.; Ren, Y.; Yan, W. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella Typhimurium*. *J. Food Prot.* **2007**, 70, 1704–1709. https://doi.org/10.4315/0362-028x-70.7.1704.

CHAPTER 9

Annex

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Annex

In this section are presented all supplementary Tables (9.1–9.7) of Exp. 1 of Chapter 3, in which are screened twelve EO (anis star, black pepper, capsicum, cassia, ginger, thyme, turmeric, coriander, geraniol, lemongrass, limonene, and tea tree) at three different doses each one (low, medium and high doses, calculated according to their main active compounds) to evaluate their effect on rumen microbial fermentation in *in vitro* batch culture system, but since the majority of EO did not have effect on rumen microbial fermentation, we did not presented in the corresponded Chapter but as annex to this thesis.

Table 9.1. Effect of essential oils on total VFA concentration (m*M*) compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	76.3	77.5	77.5	69.9	8.98	0.72
Capsicum	76.3	74.4	74.6	76.8	8.19	0.91
Cassia oil	76.3	79.7	66.8	54.9	11.9	0.25
Lemongrass oil	76.3	73.1	66.6	65.2	10.8	0.44
Garlic oil	76.3	71.9	71.1	71.1	5.04	0.49
Geraniol oil	76.3	79.2	70.2	77.9	11.3	0.68
Ginger oil	76.3	76.3	78.0	77.6	6.56	0.89
Limonene oil	76.3	73.7	72.4	74.6	10.3	0.93
Coriander seed oil	76.3	76.2	76.3	72.9	7.90	0.85
Monensin	76.3	78.6	78.4	-	6.00	0.82
Black pepper	76.3	78.1	78.4	80.1	7.12	0.85
Tea tree oil	76.3	72.0	78.3	75.5	8.47	0.66
Thyme oil	76.3	81.3	72.4	43.9	9.13	0.08
Turmeric oil	76.3	76.4	77.9	73.9	6.69	0.82

[1]SEM = Standard error of the means.

*Means within a row differ significantly from control ($P < 0.05$).

Table 9.2. Effect of essential oils on acetate concentration (m*M*) compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	54.7	52.9	51.0	52.6	2.29	0.52
Capsicum	54.7	50.9	51.5	51.3	1.41	0.33
Cassia oil	54.7	51.6	50.7	57.4	3.87	0.64
Lemongrass oil	54.7	51.9	54.1	56.0	2.29	0.67
Garlic oil	54.7	44.2	42.5	42.8	1.49	0.02
Geraniol oil	54.7	53.6	51.5	49.3	1.70	0.21
Ginger oil	54.7	49.7	50.9	50.0	2.19	0.44
Limonene oil	54.7	51.6	50.5	47.4	2.43	0.33
Coriander seed oil	54.7	49.9	49.9	49.2	2.05	0.34
Monensin	54.7	51.7	51.4	-	2.80	0.68
Black pepper	54.7	50.4	51.4	52.1	1.96	0.52
Tea tree oil	54.7	48.8	51.9	50.6	2.31	0.43
Thyme oil	54.7	52.4	49.6	54.9	1.75	0.25
Turmeric oil	54.7	52.2	51.4	51.9	2.33	0.76

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).

Table 9.3. Effect of essential oils on propionate concentration (m*M*) compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	17.2	18	18.2	14	1.85	0.31
Capsicum	17.2	18.2	18	18.3	1.77	0.72
Cassia oil	17.2	17.8	18	17.4	2.61	0.97
Lemongrass oil	17.2	18.8	14.3	12.4	1.65	0.07
Garlic oil	17.2	19.4	19.6	19	1.3	0.09
Geraniol oil	17.2	18.2	17.7	14.5	2.3	0.23
Ginger oil	17.2	17.6	18.3	18.5	1.83	0.65
Limonene oil	17.2	18.9	17.9	15.1	2.13	0.26
Coriander seed oil	17.2	18.1	18.6	17.2	2.08	0.71
Monensin	17.2	23.3	24.5		3.03	0.17
Black pepper	17.2	18.1	18.2	17.5	2	0.83
Tea tree oil	17.2	16.3	18	18.4	1.62	0.57
Thyme oil	17.2	16.7	14.3	16.3	1.72	0.45
Turmeric oil	17.2	18.2	17.9	18.6	1.41	0.47

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).

Table 9.4. Effect of essential oils on butyrate concentration (m*M*) compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	19.1	18.5	20.0	24.7	1.30	0.09
Capsicum	19.1	19.4	19.2	19.1	0.66	0.98
Cassia oil	19.1	19.6	20.3	17.3	3.67	0.94
Lemongrass oil	19.1	18.8	24.4	24.7	2.04	0.20
Garlic oil	19.1	24.4	25.9	26.5	1.53	0.08
Geraniol oil	19.1	19.3	20.2	24.7	1.40	0.13
Ginger oil	19.1	21.3	19.1	19.7	1.06	0.49
Limonene oil	19.1	18.3	20.3	26.1	1.75	0.11
Coriander seed oil	19.1	20	19.3	21.3	0.65	0.07
Monensin	19.1	14.8	13.9	-	0.83	0.04
Black pepper	19.1	19.8	19.0	19.1	0.44	0.63
Tea tree oil	19.1	23.5	18.8	19.6	1.83	0.36
Thyme oil	19.1	19.2	24.5	19.2	0.92	0.03
Turmeric oil	19.1	19.8	18.9	18.4	0.89	0.77

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).

Table 9.5. Effect of essential oils on BCVFA concentration (m*M*) compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	6.27	8.03	7.88	5.42	3.18	0.48
Capsicum	6.27	9.03	8.93	8.95	1.97	0.56
Cassia oil	6.27	7.75	6.88	5.2	2.7	0.59
Lemongrass oil	6.27	7.66	3.9	3.56	2.47	0.63
Garlic oil	6.27	9.02	8.93	8.75	2.05	0.52
Geraniol oil	6.27	5.95	6.79	7.03	3.66	0.86
Ginger oil	6.27	8.51	9.32	9.25	1.99	0.56
Limonene oil	6.27	8.39	7.88	7.71	2.21	0.67
Coriander seed oil	6.27	9.69	9.91	9.64	2.11	0.36
Monensin	6.27	7.12	7.11	-	2.29	0.92
Black pepper	6.27	9.29	9.1	9.04	1.91	0.56
Tea tree oil	6.27	7.85	8.98	9.07	2.08	0.64
Thyme oil	6.27	9.37	8.67	6.51	2.02	0.49
Turmeric oil	6.27	7.36	9.39	8.49	2.29	0.79

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).

Table 9.6. Effect of essential oils on C2:C3 ratio compared with control in *in vitro* rumen microbial fermentation of 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	3.18	2.97	2.87	3.77	0.31	0.30
Capsicum	3.18	2.84	2.89	2.84	0.31	0.62
Cassia oil	3.18	2.96	2.88	3.39	0.39	0.54
Lemongrass oil	3.18	2.83	3.84	4.51	0.39	0.09
Garlic oil	3.18	2.29	2.18	2.26	0.14	0.02
Geraniol oil	3.18	2.99	2.98	3.49	0.41	0.57
Ginger oil	3.18	2.87	2.84	2.75	0.38	0.64
Limonene oil	3.18	2.80	2.90	3.20	0.45	0.70
Coriander seed oil	3.18	2.81	2.76	2.95	0.42	0.71
Monensin	3.18	2.28	2.17	-	0.37	0.21
Black pepper	3.18	2.83	2.90	3.02	0.41	0.79
Tea tree oil	3.18	3.00	2.93	2.81	0.33	0.71
Thyme oil	3.18	3.23	3.59	3.37	0.44	0.85
Turmeric oil	3.18	2.90	2.92	2.83	0.32	0.67

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).

Table 9.7. Effect of plant extracts on pH compared with control in *in vitro* rumen microbial fermentation of a 50:50 forage: concentrate diet.

Treatment	CTR	Dose (mg/L)			SEM[1]	*P*-Value (Dose Effect)
		Low (0.4-80)	Medium (3-300)	High (7.5-750)		
Anise star oil	6.69	5.98*	5.98*	6.06	0.132	0.002
Capsicum	6.69	6.02	6.01	6.02	0.146	0.05
Cassia oil	6.69	5.99*	6.03	6.05	0.145	0.05
Lemongrass oil	6.69	5.98*	6.07	6.11	0.113	0.01
Garlic oil	6.69	6.14	6.18	6.19	0.1243	0.01
Geraniol oil	6.69	5.97*	6.08	6.08	0.137	0.005
Ginger oil	6.69	6.08	6.007	5.99*	0.138	0.003
Limonene oil	6.69	6.01	6.04	6.11	0.131	0.004
Coriander seed oil	6.69	6.25	6.01	6.02	0.145	0.004
Monensin	6.69	5.99	6.09	-	0.164	0.02
Black pepper	6.69	6.06	6.03	6.03	0.144	0.005
Tea tree oil	6.69	6.10	6.03	5.99	0.133	0.003
Thyme oil	6.69	6.03	6.06	6.24	0.166	0.022
Turmeric oil	6.69	5.98*	5.95*	5.98*	0.164	0.005

[1]SEM = Standard error of the means.
*Means within a row differ significantly from control ($P < 0.05$).