TESIS DOCTORAL

UNIVERSIDAD D CÓRDOBA

ESTUDIO DE LOS MECANISMOS DE ACCIÓN DE COMPUESTOS QUE MODIFICAN LA FERMENTACIÓN RUMINAL EN PEQUEÑOS RUMIANTES

MECHANISMS OF ACTION OF COMPOUNDS THAT MODIFY RUMINAL FERMENTATION IN SMALL RUMINANTS

CSIC
CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

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Estación Experimental del Zaidín

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TITULO: *Estudio de los mecanismos de acción de compuestos que modifican la fermentación rumial en pequeños rumiantes*

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Los trabajos de investigación que se exponen en la Memoria de Tesis Doctoral, titulada "Estudio de los mecanismos de acción de compuestos que modifican la fermentación ruminal en pequeños rumiantes" han sido realizados bajo nuestra dirección por el Licenciado Gonzalo Martínez Fernández para aspirar al grado de Doctor. Esta memoria refleja fielmente los resultados obtenidos.

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Granada 2013

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Martínez, G.; Abecia, L.; Snelling, T.; Martín-García, A. I.; Ramos-Morales, E.; Molina-Alcaide, E.; Newbold, J.C.; Yañez-Ruiz, D. R. 2012. Pyrosequencing study of the rumen archaeal community reveals an inhbition of the Methanomicrobiales Order associated to decreased methane emissions. En 8th INRA-ROWETT Symposium on Gut Microbiology. Clermont-Ferrand, France.

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Martínez-Fernández, G., Abecia, L., Ramos-Morales, E., Martin-Garcia, I., Molina Alcaide, E. Yáñez-Ruiz, D.R. 2013. Effects of propyl propane thiosulfinate on nutrient utilization, ruminal fermentation, microbial population and methane emissions in goats. Enviada a la revista *Animal Feed Science and Technology*.

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TÍTULO DE LA TESIS: "ESTUDIO DE LOS MECANISMOS DE ACCIÓN DE COMPUESTOS QUE MODIFICAN LA FERMENTACIÓN RUMINAL EN PEQUEÑOS RUMIANTES"

DOCTORANDO/A: GONZALO MARTÍNEZ FERNÁNDEZ

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

Los trabajos de esta tesis, realizados bajo la dirección de David Rafael Yáñez Ruíz y Eduarda Molina Alcaide, se han desarrollado en el plazo de los 4 años de beca-contrato FPI con que el doctorando contaba. EL grado de cumplimiento del plan de trabajo propuesto Gonzalo Martínez Fernández ha sido total. Además, como resultado de sus estancias en el INRA (Francia), la Universidad de Aberystwyth (Reino Unido) y el CSIRO (Australia) se ha completado el nivel de competencia del doctorando en el uso de técnicas moleculares para el estudio de las poblaciones microbianas del rumen. Ha existido una evolución clara del trabajo de Gonzalo Martínez, tanto en la capacidad del doctorando para entender los objetivos y planteamientos de los trabajos como para abordar dichos objetivos con metodologías adecuadas y analizar y discutir los resultados. La evolución en la capacidad científica del doctorando se refleja en las publicaciones enviadas a publicar en revistas SCI (4 de las cuales 1 ya está aceptada y las otras 3 en revisión), en libros así como en sus contribuciones a congresos nacionales e internacionales.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 5 de septiembre de 2013

Firma del/de los director/es

Fdo.: David Rafael Yáñez Ruíz

Fdo.: Eduarda Molina Alcaide

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"Lo que sabemos es una gota de agua; lo que ignoramos es el océano."

Isaac Newton

"*No hay que confundir nunca el conocimiento con la sabiduría. El primero nos sirve para ganarnos la vida; la sabiduría nos ayuda a vivir*."

Sorcha Carey

"Solamente una vida dedicada a los demás merece ser vivida"

Albert Einstein

Y uno aprende...
después de un tiempo,
uno aprende la sutil diferencia
entre sostener una mano
y encadenar un alma.

Y uno aprende
que el amor
no significa recostarse
y una compañía
no significa seguridad.

Y uno empieza a aprender…
que los besos no son contratos
y los regalos no son promesas
y que uno empieza a aceptar sus derrotas
con la cabeza alta y los ojos abiertos.

Y uno aprende a construir
todos sus caminos en el hoy,
porque el terreno del mañana
es demasiado inseguro para planes…
y los futuros tienen una forma
de caerse en la mitad.

Y después de un tiempo uno aprende
que si es demasiado,
hasta el calorcito del sol quema.
Así que uno planta su propio jardín
y decora su propia alma,
en lugar de esperar
que alguien le traiga flores.

Y uno aprende….
que realmente puede aguantar,
que uno realmente es fuerte,
y que con cada adiós uno aprende

Y uno aprende…

(Adaptación atribuida a Jorge Luis Borges)

A mis padres y hermana.

Y a todas las personas que me han acompañado en este camino.

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[1]Listado de abreviaturas

3NP	3-nitrooxypropanol
ADF	Acid detergent fiber
ADL	Acid detergent lignin
ADN	Ácido desoxirribonucleico
AGVs	Ácidos grasos volátiles
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ARN	Ácido ribonucleico
ATP	Adenosin trifosfato
BCM	Bromocloromethane
BW	Body weight
$BW^{0.75}$	Metabolic weight
CAR	Carvacrol
CD-PTS	α-cyclodextrin-propyl propane thiosulfinate complex
CH_4	Metano
CIN	Cinnamaldehyde
CLA	Conjugated linoleic acid
CP	Crude protein
DDS	Diallyl disulfide
DGGE	Denaturant Gradient Gel Electrophoresis
DM	Dry matter
DMI	Dry matter intake

[1]Abreviaturas más destacadas del texto.

DNA	Desoxirribonucleic acid
DNDF	digested neutral detergent fiber
EMNS	Efficiency of microbial nitrogen synthesis
E3NP	Ethyl-3-nitrooxy propionate
EUG	Eugenol
FAD	Fibra ácido detergente
FAO	Food and Agriculture Organization of United Nations
GE	Gross energy
GLM	General linear model
H_2	Hidrógeno
H_2O	Agua
HMG-CoA	3-hydroxy-3-methylglutaryl coenzima A
ME	Metabolizable energy
MNF	Microbial nitrogen flow
N	Nitrógeno
NADH	Nicotinamida adenina dinucleótido reducida
NADPH	Nicotinamida adenina dinucleótido fosfato reducido
NDF	Neutral detergent fiber
$N\text{-}NH_3$	Nitrógeno amoniacal
OM	Organic matter
OTU	Operational Taxonomic Unit
P	Probability
PB	Proteína bruta
pb	Pares de bases
PCR	polymerase chain reaction

PD	Purine derivatives
PTS	Propyl propane thiosulfinate
PTSO	Propyl propane thiosulfonate
qPCR	Real Time PCR
RNA	Ribonucleic acid
SEM	Standard error of the mean
Sed	Standard error desviation
VFA	Volatile fatty acid

Capítulo 1. Introducción y objetivos

Introducción y objetivos

La producción animal en Europa ha de orientarse, como respuesta a la demanda social, hacia sistemas productivos que sean lo más eficientes posibles para, por un lado, optimizar su productividad y competitividad, y por otro, minimizar su impacto ambiental. En el caso de los rumiantes, es la actividad fermentativa que ocurre en el rumen la que determina la eficiencia de utilización de los nutrientes. La fermentación anaerobia ruminal implica que tanto el uso de la energía como el de proteína sean ineficientes para el animal. La fermentación ruminal produce metano, que representa una pérdida (entre el 2 y el 12 %) de la energía ingerida por el animal; también produce un exceso de amonio y una elevada excreción de urea en le orina que representan una perdida de nitrógeno (alrededor del 60 % del ingerido). Esta doble ineficiencia tiene implicaciones medioambientales a escala global y local, respectivamente. La nutrición correcta del rumiante implica, entre otras cosas, minimizar estas ineficicias mediante una formulación adecuada de la dieta. Además, el empleo de sustancias modificadoras de la fermentación microbiana por su capacidad específica de inhibir o potenciar el crecimiento de ciertos grupos microbianos, es una estrategia alimentaria con un gran potencial y que ha experimentado un gran desarrollo. Dada la importancia de la microbiota que existe en el rumen uno de los métodos más efectivos y sencillos para modificar la fermentación ruminal ha sido la utilización de compuestos antimicrobianos, fundamentalmente antibióticos ionóforos, como aditivos considerados como promotores del crecimiento. Sin embargo, desde enero de 2006, se prohibió el

empleo de antibióticos en la alimentación animal en los países de la Unión Europea. El mero anuncio, hace unos años, de esa prohibición impulsó el interés de la investigación pública y privada hacia la búsqueda de alternativas a los mencionados antibioticos. En rumiantes se han estudiado, como alternativas, levaduras, ácidos orgánicos, probióticos, extractos de plantas, aceites esenciales, enzimas, etc. La literatura científica ofrece abundante información sobre los efectos de distintos compuestos sobre la fermentación ruminal. Esta información es difícil de interpretar, y en ocasiones, los resultados obtenidos en los diferentes experimentos son contradictorios. La importancia económica a nivel mundial, del ganado vacuno ha hecho que la mayoría de los estudios llevados a cabo se centren en esa especie ganadera, siendo escasos los estudios relativos al ganado **ovino o caprino.** En nuestro país, sin embargo, los pequeños rumiantes tienen una extraordinaria importancia económica y social.

El desarrollo y empleo práctico de aditivos, con capacidad para modificar la fermentación ruminal y mejorar la productividad del rumiante, se enfrenta en la actualidad a una serie de **limitaciones** científico-técnicas, que conviene considerar en su conjunto para tratar de solventarlas. Entre esas limitaciones, se pueden destacar:

- Variación en el efecto de los aditivos dependiendo del tipo de compuesto activo y su concentración
- Variación del efecto en función de la naturaleza de la dieta que recibe el animal

- Dificultad de utilizar condiciones de experimentación similares en ensayos *in vitro* e *in vivo* y diferencias entre los resultados obtenidos *in vitro* e *in vivo*
- Carencia de estudios que confirmen la persistencia en el tiempo de los efectos de los aditivos

Estas limitaciones, sin duda alguna, están ligadas a la falta de conocimiento que aún se tiene del **ecosistema microbiano del rumen** y de los grupos microbianos que determinan ciertas actividades metabólicas. Ello limita, a su vez, el conocimiento de los mecanismos de acción mediante los que los compuestos estudiados modifican la fermentación ruminal. El objetivo general del presente trabajo es contribuir a superar las limitaciones mencionadas ,en relación a la actividad metabólica que determina la **producción de metano** en el rumen, mediante el estudio de los mecanismos de acción de una gran variedad de compuestos que pueden modificar la fermentación ruminal: i) compuestos organosulfurados derivados del ajo, ii) aceites esenciales y iii) compuestos de síntesis.

Los objetivos especificos que se plantean en este trabajo de Tesis Doctoral son los siguientes:

1. Estudiar, mediante incubaciones de corta duración en cultivos no renovados de microorganismos ruminales, el efecto de una serie de aditivos y dosis de los mismos sobre la fermentación ruminal para establecer su potencial antimetanogénico.

2. Establecer el efecto del tipo de concentrado de la dieta sobre la efectividad de los aditivos estudiados.

3. Estudiar, mediante incubaciones en fermentadores de flujo continuo, el efecto de los aditivos y dosis selccionados anteriormente sobre la fermentación ruminal a tiempos de adiministación más prolongados.

4. Estudiar, en ovino y en caprino, el efecto sobre la fermentación y degradación ruminal, respuesta digestiva y producción de metano de la aplicación de los aditivos y las dosis que mostraron resultados más prometedores en las incubaciones *in vitro* tanto a corto como a más largo plazo.

5. Establecer los mecanismos de acción de aditivios con un mayor potencial antimetanogénico que no comprometan la fermentación ruminal de la dieta, mediante el estudio de los cambios que dichos aditivos promueven, tanto *in vitro* como *in vivo*, en las comunidades microbianas ruminales y, en particular, las arqueas metanogénicas.

Introduction and objectives

Animal production in Europe must focus on most effective production systems in order to improve productivity and competivity of animal husbandry, and in the other hand, minimized its environmental impact. In the case of ruminants, the fermentation activity that occurs in the rumen, determines in a great extent the efficiency of the use of nutrients. The ruminal anaerobic fermentation implies some energy and protein inefficiencies for the animal. The ruminal fermentation produces methane which represents a loss of between 2-12 % of the total gross energy intake for the animal; also in some cases produces an ammonia excess and high urea excretion by the urine that represents a nitrogen loss (up to 60 % intake). This double inefficiency has environmental implications at global and local scale, respectively. A correct nutrition strategy for ruminants implies, among others things, to minimize these inefficiencies by a correct diet formulation. Furthermore, the use of products that modulate the microbial activity due to their specific capacity to inhibit or enhance the growth of different microbial groups, represents a feeding strategy with a high potential and has experimented a strong development in the last decade. Due to the importance of rumen microbiota, one of the most promising methods to modify rumen fermentation has been the use of antimicrobials compounds, principally antibiotic ionophores, as growth promoters. However, since January 2006, the use of antibiotics in animal feeding has been banned in the UE. The announcement, some years ago, of this banning increased the interest of the public and private research to develop alternatives to replace antibiotics. In ruminants different alternatives have been studied: yeast, organic acids,

probiotics, plants extracts, essential oils, enzymes, etc. The research literature shows vast information about the effects of different compounds on rumen fermentation. This information is difficult to understand, and sometimes, the results obtained are contradictory. Most of the nutritional studies in this area of research have focused on cattle production (beef and dairy) due to the global economic importance; however very limited studies about sheep and goats have been performed. Nevertheless, in the Mediterranean area, the small ruminants sector has high economic and social importance.

The development and practical use of additives, with ability to modify rumen fermentation and improve ruminant productivity, is currently facing a number of scientific and technical limitations that should be considered as a whole to try to solve them. Among these limitations, we can highlight:

- Variation in the effect of additives due to the type of active compound and its concentration.
- Variation of the effects depending on the nature of the animal diet.
- Difficulty of using similar experimental conditions *in vitro* and *in vivo* and differences between the results obtained *in vitro* and *in vivo*.
- Lack of studies confirming the persistence over time of the effects of additives.

These limitations are related to the lack of knowledge that still exists about rumen microbial ecosystem and microbial groups that determine certain metabolic activities. At the same time, this limits the knowledge of the mechanisms of action by which the compounds studied modified ruminal fermentation. The aim of this work is to help overcome these limitations, in relation to the metabolic activity that determines the production of methane in

the rumen, through the study of the mechanisms of action of a variety of compounds that can modify rumen fermentation i) garlic organosulphur compounds, ii) essential oils and iii) synthetic compounds.

The specific objectives of this PhD Thesis were, therefore, the following:

1. To study using short-term incubations in batch cultures of mixed rumen microorganisms, the effect of a number of additives and doses on ruminal fermentation to establish their potential antimethanogenic effect.

2. To establish the effect of type of concentrate of the diet in the effectiveness of the additives studied.

3. To study using continuous flow fermenters, the effect of the selected additives in previous stage and doses on ruminal fermentation in longer term incubation times.

4. To study in sheep and goats, the effect on rumen fermentation, feed degradability, digestive response and methane production of the treatment of additives and doses that showed promising results *in vitro*, in short and long administration periods.

5. To establish the mechanisms of action of those additives with high antimethanogenic effect that do not compromise rumen fermentation of the diet, by studying the changes that these additives promote, *in vitro* and in vivo, on rumen microbial communities and, particularly in the methanogenic archaea population.

Capítulo 2. Revisión bibliográfica

1. Fermentación ruminal: ineficiencias y estrategias para optimizarla

El tracto digestivo del rumiante es muy diferente al de los animales monogástricos, con una serie de peculiaridades tanto anatómicas como fisiológicas o metabólicas. Entre las peculiaridades anatómicas del digestivo de rumiantes destaca la presencia de un estómago complejo, formado por 4 cámaras: 3 pre-estómagos (rumen, retículo y omaso) y un estómago glandular (abomaso). Por su volumen y funciones destaca el rumen, que alberga una compleja y diversa microbiota simbiótica, con capacidad para degradar alimentos fibrosos, que permite al rumiante ocupar un nicho ecológico, en relación a su dieta, especial y de gran relevancia (Van Soest, 1982). Como resultado de la fermentación microbiana de los alimentos en el rumen se originan proteína microbiana, ácidos grasos volátiles (AGV) y NH_3, calor y gases, principalmente CO_2, CH_4 e H_2.

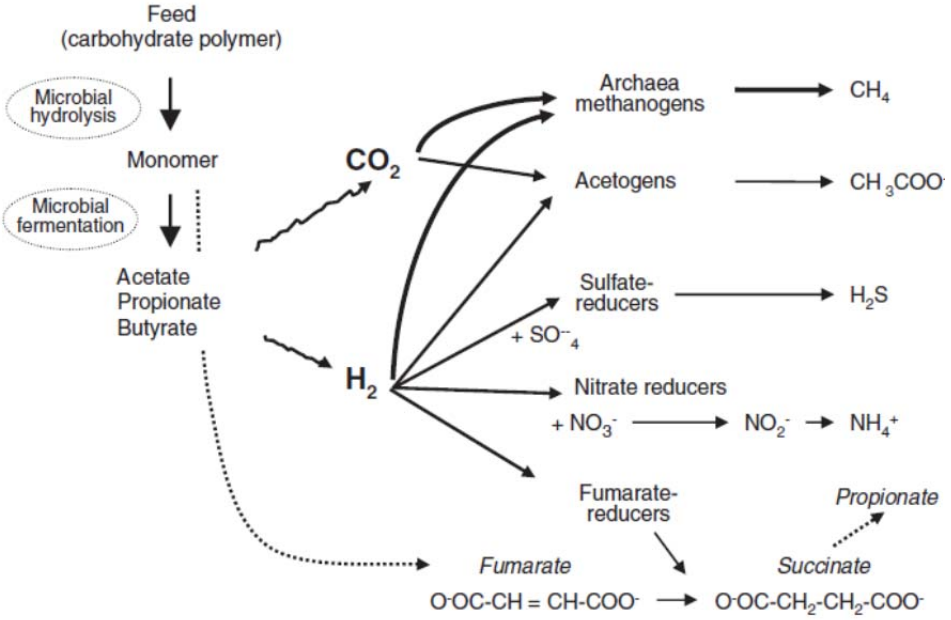

Figura 2.1. Esquema de la fermentación de los polisacáridos y uso del H_2 resultante en el rumen (Morgavi et al., 2010).

La microbiota ruminal está constituida, esencialmente, por microorganismos anaerobios por lo que la oxidación de los substratos en el rumen no es completa y la eficiencia del metabolismo proteico y energético se considera baja (Ørskov y Ryle, 1990). La formación de metano representa una pérdida energética importante (2-12% de energía bruta ingerida; (Johnson y Johnson, 1995)) pero representa la principal vía metabólica para mantener la concentración de H_2 dentro de límites fisiológicos del ecosistema microbiano.

El metano es un gas que contribuye de manera importante al denominado "efecto invernadero", con un potencial radiativo más de 20 veces superior al del CO_2. Los rumiantes domésticos producen más de 80 millones de toneladas de CH_4 anuales, lo que supone alrededor del 33% del total de metano antrópico emitido (Beauchemin et al., 2008) habiéndose estimado que en España ese porcentaje es del 31% (MAGRAMA, 2011).

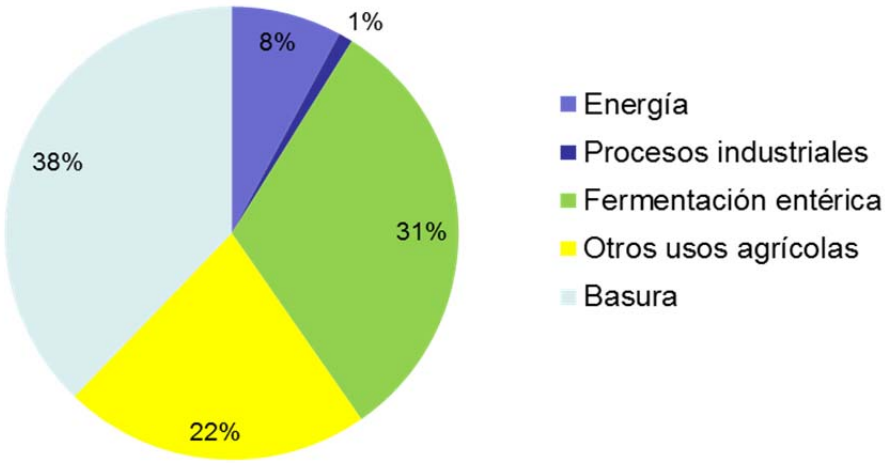

Figura 2.2. Contribución de distintas fuentes a la emisión de metano en España durante el año 2011 (MAGRAMA).

Las emisiones de metano procedentes de ovino y caprino alcanzan 9,5 Tm/año, mientras que 19 Tm/año proceden de vacas de leche y 55,9 de vacuno de carne (McMichael et al., 2007). Una reducción de la producción de metano, derivada de la fermentación entérica (90% del metano total es producido por los rumiantes;(Murray et al., 1976)), permitiría optimizar la eficiencia energética de este grupo animal y reducir el impacto de la ganadería sobre las emisiones de gases de efecto invernadero, lo que constituye un objetivo esencial de la producción animal, como recoge la FAO en su informe ""Livestock's long shadow" (Steinfeld et al., 2006) y la revisión actualizada de la FAO sobre opciones para mitigar el metano en la ganadería (Gerber et al., 2013). La fermentación ruminal es un proceso oxidativo, en el cual cofactores reducidos como NADH, NADPH, FADH son re-oxidados liberándose H_2. Este H_2 es utilizado, fundamentalmente, por las arqueas metanogénicas, grupo microbiano diferenciado de las bacterias, para reducir CO_2 a CH_4 según la ecuación: $CO_2+4H_2=CH_4+2H_2O$, principal ruta metabólica para la formación de metano en el rumen. La metanogénesis en el rumen es de gran importancia ya que evita una acumulación excesiva de H_2, que inhibiría la actividad deshidrogenasa, relacionada con la oxidación de los cofactores reducidos mencionados anteriormente. La fermentación microbiana de la fibra del alimento, que recibe el rumiante, produce acetato y libera H_2. También la formación de butirato, como consecuencia de la fermentación de algunos substratos, produce H_2. En ambos casos, la fermentación ruminal favorecería la formación de metano. Por el contrario, la formación de propionato, como consecuencia de la fermentación ruminal del alimento, compite con la

metanogénesis por el uso de H_2 (Demeyer y Fievez, 2000; McAllister y Newbold, 2008; Martin et al., 2010).

Para el desarrollo de estrategias que permitan reducir las emisiones de metano procedentes de los rumiantes han de considerarse tanto las rutas metabólicas, implicadas en la formación y en la utilización del H_2 en el rumen, como la comunidad de arqueas metanogénicas (Martin et al., 2010). Cualquier estrategia, dirigida a reducir la producción de metano en el rumen, implica (McAllister y Newbold, 2008; Martin et al., 2010) lo siguiente:

- La reducción de la cantidad de H_2 producido en el rumen, sin afectar a la digestión de los alimentos.
- El aumento de la utilización del H_2 en rutas metabólicas alternativas, que permitan obtener productos finales de la fermentación beneficiosos para el animal.
- La reducción de la actividad de las arqueas metanogénicas en el rumen.

Numerosos autores (Beauchemin et al., 2008; McAllister y Newbold, 2008; Eckard et al., 2010; Benchaar y Greathead, 2011) han estudiado diferentes estrategias para disminuir la producción de metano, habiéndose estudiado el efecto que sobre la metanogénesis tienen la relación entre la cantidad de forraje y de concentrado en la dieta, el uso de aditivos (compuestos vegetales, compuestos sintéticos), que modulan la fermentación ruminal o la inmunización de los animales frente a determinados microorganismos metanogénicos. También destacan otras estrategias, tales como, la utilización de lípidos en la dieta, el procesado del alimento para mejorar la digestibilidad, el tipo de forraje o el uso de nitratos (Gerber et al., 2013).

Tabla 2.1. Estrategias para reducir la producción de metano en los rumiantes (Hook et al., 2010).

Methane abatement strategy	Mechanism of abatement activity	Considerations when selecting abatement strategy
Dietary composition Increase hemicellulose/starch Decrease cell wall components Grinding	Increased passage rate; greater proportion propionate versus acetate; reduced ruminal pH	Shift methanogensis to hind gut or manure, risk of subacute ruminal acidosis (SARA)
Lipids Fatty acids Oils Seeds Tallow	Inhibition of methanogens and protozoa; greater proportion propionate versus acetate; biohydrogenation	Effect on palatability, intake, performance, and milk components; varies with diet and ruminant species; long-term studies needed
Defaunation Chemical Feed additives	Removes associated methanogens; less hydrogen for methanogenesis	Adaptation of microbiota may occur; varies with diet; maintenance of defaunated animals
Methanogen Vaccine	Host immune response to methanogens	Vaccine targets; diet and host geographical location differences
Monensin	Inhibits protozoa and gram-positive bacteria; lack of substrate for methanogenesis	Adaptation of microbiota may occur; varies with diet and animal; banned in the EU
Plant Compounds Condensed tannins Saponins Essential oils	Antimicrobial activity; reduced hydrogen availability	Optimum dosage unknown; more *in vivo* research needed; long-term studies needed; may affect digestibility; residues unknown
Organic Acids Fumarate Malate	Hydrogen sink, greater proportion propionate versus acetate	Varies with diet; more *in vivo* research needed; long-term studies needed; may affect digestibility

Como se ha mencionado anteriormente, la fermentación ruminal es también ineficiente en relación a la utilización del nitrógeno dietético. Los rumiantes excretan una gran cantidad de nitrógeno, mayoritariamente a través de la orina (Eckard et al., 2008). Las cantidades que se eliminan al medio dependen de la naturaleza de la dieta y del sistema de explotación del rumiante pero, generalmente, son superiores a las requeridas para un aprovechamiento eficiente del N por el sistema suelo-planta. El aumento de las explotaciones ganaderas extensivas en las últimas décadas ha incrementado las emisiones de compuestos nitrogenados al medio. La necesidad de reducir las emisiones de amonio y óxido nitroso ha determinado el desarrollo de estrategias para este

fin, que también permitan mejorar la eficiencia de utilización digestiva y metabólica del nitrógeno en los rumiantes (Eckard et al., 2010). Estas estrategias se basan, fundamentalmente, en la selección genética de animales especialmente eficientes, el empleo de proteína protegida para evitar su degradación en el rumen, el uso de componentes vegetales como los taninos, la reducción de los niveles de PB en la dieta, o la inclusión de aditivos en el forraje para reducir la degradación de la proteína (Foskolos, 2012).

Las ineficiencias del proceso fermentativo que tiene lugar en el rumen podrían superarse o disminuir mediante estrategias diversas e imaginativas que, además, permitiesen disminuir el impacto ambiental de la producción animal (Gerber et al., 2013).

2. Microbiota y ecología ruminal

La microbiota ruminal constituye una comunidad microbiana diversa, y altamente específica en relación a sus funciones metabólicas, que son esenciales para el desarrollo, salud y nutrición del rumiante (Morgavi et al., 2010). Los principales microorganismos del rumen se clasifican en bacterias, protozoos, arqueas metanogénicas, hongos y virus. Se estima que en el ecosistema ruminal existen más de 1.000 especies distintas (Deng et al., 2008), pertenecientes filogenéticamente a los dominios Bacteria, Archaea y Eucarya. La mayor parte de esos microorganismos no han sido aún cultivados aunque la aplicación de técnicas moleculares ha permitido estimar que, por ejemplo, las bacterias ruminales presentan entre 300 y 400 filotipos (Edwards et al., 2004; Yu et al., 2006). La microbiota ruminal es dinámica y diversos los factores que la pueden afectar, tales como la dieta, la especie o la edad del animal, la presencia de aditivos en la dieta, la zona geográfica en la que se asienta una determinada explotación ganadera o la estación del año (Tajima et al., 2001a; Zhou et al., 2010). Los microorganismos ruminales establecen entre sí relaciones complejas de cooperación, que permiten la degradación del alimento que llega al rumen y, en consecuencia, la utilización de sus nutrientes.. También se establecen relaciones de competencia, intra e inter-específica, y de predación (Ley et al., 2006).

La mayoría de los microorganismos presentes en el rumen son anaerobios estrictos aunque existen anaerobios facultativos, que metabolizan el oxígeno que llega al rumen a través del alimento, del agua de bebida o de las paredes del rumen. La anaerobiosis se mantiene en el rumen gracias a los gases generados durante la fermentación, tales como dióxido de carbono, metano e

hidrógeno. Solo los microorganismos capaces de tolerar un potencial redox bajo (-350 mV) pueden sobrevivir en el rumen (Kamra, 2005), siendo la temperatura óptima de 39ºC.. Además, los microorganismos ruminales disponen de estrategias, tales como: moléculas que permiten la adhesión, colonización y degradación de los sustratos, o que son capaces de inhibir el crecimiento de competidores (bacteriocinas) o de resistir al sistema inmunitario del animal hospedador; plasticidad genética que les permite adaptarse a los cambios en dicho hábitat y; elevadas tasas de multiplicación, que permiten el mantenimiento de densidades estables de microorganismos; (Ley et al., 2006) que favorecen la supervivencia y crecimiento en dicho ecosistema.

2.1. Bacterias

Las bacterias, junto con las arqueas, representan entre un 50 - 60 % de la masa microbiana del rumen (Stewart et al., 1997). En un estudio reciente (Kong et al., 2010) se ha observado que aproximadamente el 65 % de las secuencias de bacterias ruminales estudiadas, se encuentran asociadas a las partículas sólidas del contenido ruminal. El número de bacterias presentes en el rumen asciende a 10^{10} - 10^{11} células/mL de contenido ruminal (Wright y Klieve, 2011) y están implicadas en la degradación de carbohidratos simples y complejos, lípidos, proteínas, etc. Además, la interacción entre ellas y con otros grupos microbianos permite la producción de AGV y proteína microbiana. La mayor parte de las bacterias implicadas en la degradación de dietas con una elevada cantidad de fibra son Gram negativas, mientras que dietas ricas en concentrado determinan el predominio de bacterias Gram positivas en el rumen (Hungate, 1966). El pH óptimo para su crecimiento se encuentra entre 6,0 y

6,9. Además, pueden tolerar niveles elevados de ácidos orgánicos sin que se afecte su metabolismo (Kamra, 2005). Existe poca información acerca de las secuencias de bacterias del rumen aunque, trabajos recientes están haciendo aportaciones relevantes en este aspecto. Kim et al. (2011), mediante meta análisis, han clasificado las bacterias del rumen en 19 *phyla* siendo los más abundantes *Firmicutes*, *Bacteroidetes* y *Proteobacteria* con 57,8; 26,7 y 6,9%, respectivamente, del total de las secuencias analizadas. Aproximadamente el 90% de las secuencias del *phyla Firmicutes* se asignaron a la clase *Clostridia* y, el resto, a las clases *Bacilli* y *Erysipelotrichi* o no se clasificaron. Los géneros predominantes en este *phyla* y pertenecientes a la clase *Clostridia*, son *Butyrivibrio* (4,8%), *Acetivibrio* (4,5%), *Ruminococcus* (4,1%), *Succiniclasticum* (3,7%), *Pseudobutyrivibrio* (2,3%) y *Mogibacterium* (2,3%). El 88,5% de las secuencias del *phyla Bacteroidetes* se asignaron a la clase *Bacteroidia*, y el resto, a la clase *Sphingobacteria* o no se clasificaron. El género mayoritario fue *Prevotella*, representando un 11% del total de secuencias de las bacterias ruminales cuyas secuencias se analizaron. El 7 % de las secuencias bacterianas se asignaron a 16 *phyla* menores, entre los que destacan *Synergistetes*, *Spirochaetes* y *Fibrobacteres*. Sin embargo, las proporciones podrían variar ampliamente en función de factores tales como el manejo de la muestra y su procesado, el número de animales muestreados, el origen geográfico de las muestras analizadas, la especie animal de la que proceden las muestras, la dieta suministrada a los animales donadores etc. (Edwards et al., 2004; Kim et al., 2011).

2.2. Arqueas

Los microorganismos implicados en la metanogenesis ruminal pertenecen al dominio Arquea, un grupo de organismos procariotas filogenéticamente próximo a las bacterias, aunque existen diferencias genotípicas y fenotípicas entre ambos grupos (Lange et al., 2005). Inicialmente, las diferencias entre estos dos dominios procariotas se basaban en su estructura ribosómica pero, posteriormente, se han reconocido otras diferencias (Konings et al., 2002) como la composición de la pared celular (ausencia de mureína en arqueas) y lípidos de la membrana (ésteres de glicerol y isoprenol, en arqueas, y ésteres de glicerol y ácidos grasos, en bacterias). En la actualidad, el dominio Arquea se organiza en dos *phyla*, *Euryarchaeota* y *Crenarchaeota,* en base al análisis de ARN 16S. Los microorganismos responsables de la producción de metano en el rumen pertenecen al grupo de las arqueas y son las únicas arqueas conocidas de este ecosistema aunque es posible que existan otras no cultivadas y con una función diferente a la metanogénesis (Tajima et al., 2001b). La concentración de arqueas metanogénicas varía entre 10^7 y 10^9 células/mL de contenido ruminal, dependiendo del tipo de dieta que recibe el animal y de la proporción de fibra que dicha dieta contiene (Kamra, 2005). Se han observado arqueas asociadas con las fases sólida y líquida del contenido ruminal, con el epitelio ruminal y con los protozoos (Morgavi et al., 2010). Los tres principales substratos, utilizados por las arqueas metanogénicas, para producir metano son: CO_2, compuestos que contienen un grupo metilo y acetato (Liu y Whitman, 2008). En el rumen, la vía predominante para la formación de metano es la hidrogenotrófica en la que se utiliza CO_2 como fuente de carbono e H_2 como principal donador de electrones (Hungate, 1967). El formiato es también un importante donador de electrones, utilizado por parte

de las arqueas metanogénicas ruminales hidrogenotróficas, cuya producción de metano representa hasta el 18% del total producido en el rumen (Hungate et al., 1970). Las arqueas ruminales metilotróficas, del orden *Methanosarcinales* y del *Methanobacteriales* como *Methanosphaera* spp. (Liu y Whitman, 2008) pueden utilizar metilaminas y metanol para formar metano. Por último, el metano se porduce también a partir del acetato mediante la vía acetoclástica, en la que intervienen principalmente arqueas del orden *Methanosarcinales* (Liu y Whitman, 2008).

El metaanálisis de las secuencias de arqueas ruminales de diferentes especies animales, almacenadas en las bases de datos, ha permitido realizar un estudio (Kim et al., 2011) amplio y preciso de la diversidad de estos microorganismos. El 94 % de las secuencias analizadas se han asignado a cuatro clases del *phyla Euryarchaeota*: *Methanobacteria*, *Methanomicrobia*, *Thermoplasmata* y *Methanopyri* (70,3%, 16,4%, 7,4% y 0,04 %, respectivamente, del total de secuencias disponibles). Se han encontrado 12 géneros siendo mayoritarios *Methanobrevibacter*, *Methanosphera* y *Methanomicrobium, que representan* 50%, 13% y 15%, respectivamente, del total de secuencias analizadas. Estudios muy recientes (St-Pierre y Wright, 2013), llevados a cabo en diferentes especies y razas de rumiantes, indican que el género *Methanobrevibacter* es el mayoritario en los rumiantes, aunque dependiendo de factores como la especie o raza u origen geográfico del animal o la dieta que recibe pueden predominar las arqueas de los géneros *Methanosphera* y *Methanomicrobium*. La aplicación de técnicas de secuenciación de nueva generación podría mejorar considerablemente el conocimiento de la estructura de las poblaciones microbianas del rumen, y facilitar el análisis de un mayor

número de muestras o condiciones experimentales (Morgavi et al., 2010; St-Pierre y Wright, 2013).

2.3. Protozoos

Los protozoos son microorganismos eucariotas, unicelulares y heterótrofos, que carecen de pared celular (Lange et al., 2005). La concentración en el rumen de un animal adulto sano es de 10^5-10^6 células/mL de contenido ruminal (Wright y Klieve, 2011). La clasificación de los protozoos se ha realizado en base a su morfología existiendo dos grandes grupos: flagelados y ciliados. Se conoce poco acerca del primero y del segundo se sabe que son los más numerosos e importantes, subdividiéndose en: holotricos y entodiniomorfos (Williams y Coleman, 1997). Diferentes estudios han permitido, mediante técnicas moleculares, profundizar en el conocimiento de las relaciones filogéneticas de los protozoos, su distribución y abundancia en el rumen (Regensbogenova et al., 2004; Shin et al., 2004; Sylvester et al., 2004; Skillman et al., 2006). Los protozoos no son capaces de sintetizar aminoácidos a partir de amonio (Jouany et al., 1990) sino que su crecimiento depende de la proteína disponible en el rumen y de las bacterias a las que predan (Mackie et al., 2002). Aunque su papel en el rumen no está claro debido a las limitaciones existentes hasta ahora para su cultivo *in vitro*, se conoce que hasta un 20% de las arqueas metanogénicas ruminales se asocian a los protozoos ciliados (Mackie et al., 2002). Algunos autores no han encontrado modificaciones importantes del metabolismo ruminal en ausencia de protozoos (Ushida et al., 1991), mientras que otros han observado una disminución significativa de la digestibilidad de la materia orgánica (Eugène et al., 2004), aumento de la

concentración de amonio en rumen y del flujo de proteína bacteriana hacia el duodeno, en animales libres de protozoos (Koenig et al., 2000). También se ha descrito el papel de los protozoos como tamponadores en el rumen ya que algunos fagocitan y metabolizan el almidón, reduciendo la fermentación bacteriana del mismo y, en consecuencia, evitando un descenso brusco del pH (Wakita y Hoshino, 1989). Se ha estimado que los protozoos contribuyen de forma importante al flujo de ácidos grasos insaturados al duodeno (Yanez-Ruiz et al., 2006; Yáñez-Ruiz et al., 2007) y que contienen cantidades elevadas de ácidos grasos insaturados, incluyendo CLA y ácido vacénico, que provienen principalmente de las bacterias que predan (Lourenco et al., 2010).Ciertas especies de protozoos pueden utilizar el oxígeno gracias a la presencia de distintos sistemas enzimáticos (Williams, 1986). Por último, cabe destacar que los protozoos tienden a desaparecer de sistemas *in vitro* como los fermentadores en los primeros estadios de la fermentación (Hannah et al., 1986; Yang et al., 2004; Moumen et al., 2009), aunque algunos autores han conseguido mantener los protozoos durante un periodo más prolongado de incubación mediante modificaciones de dichos sistemas *in vitro* (Muetzel et al., 2009).

2.4. Hongos

En el rumen y en diferentes zonas del tracto gastrointestinal de animales herbívoros, tanto rumiantes como no rumiantes, existen hongos anaerobios estrictos. Estos hongos se diferencian de los hongos aerobios por no tener mitocondrias sino unos orgánulos denominados “hidrogenosomas”, que generan ATP e H_2 (Boxma et al., 2005). Los hongos representan 10^3–10^6

células/mL de contenido ruminal (Wright y Klieve, 2011) aunque su concentración aumenta con el contenido en fibra de la ración, siendo menor cuanto más rica en azúcares solubles y almidón sea la dieta (Son et al., 2006). En el rumen se han identificado hongos pertenecientes a los géneros *Neocallimastix*, *Caecomyces*, *Orpynomices*, *Sphaeromas* y *Piromonas* (Hobson y Stewart, 1997). Los hongos ruminales presentan, en su mayoría, actividad fibrolítica observándose relaciones de sinergismo con las bacterias fibrolíticas (Orpin y Joblin, 1997).

2.5. Virus

La población de virus en el rumen está formada, mayoritariamente, por bacteriófagos, estimándose su concentración en alrededor de 10^9-10^{10} partículas/mL de contenido ruminal (Wright y Klieve, 2011), con variaciones que pueden deberse a la naturaleza de la dieta que recibe el animal o a la abundancia de bacterias en el rumen. El papel que desempeñan los bacteriófagos en el ecosistema ruminal no ha sido estudiado en profundidad, aunque se han descrito diferentes funciones como el reciclado de la proteína bacteriana o el control de la densidad de determinadas especies bacterianas en el rumen. También se han descrito efectos negativos sobre la eficiencia de degradación del alimento y la síntesis de proteína microbiana, derivados de la presencia de virus en el rumen (Swain et al., 1996).

2.6. Empleo de técnicas de secuenciación de última generación para el estudio del ecosistema microbiano del rumen

En los últimos 2-3 años el desarrollo y aplicación de técnicas de secuenciación masiva ha permitido dar un salto cualitativo en la descripción de la comunidad microbiana del rumen ya que ha permitido tanto la descripción de distintos grupos como la identificación de especies minoritarias (Morgavi et al., 2012). Entre las técnicas utilizadas destaca la pirosecuanciación empleando la tecnología desarrollada por Roche 454 ®, que ha permitido el estudio de los cambios que tienen lugar en la comunidad bacteriana del rumen como respuesta a la suplementación con lípidos (Zened et al., 2013), el incremento de cereales en la dieta (Chen et al., 2011) o la caracterización de la población que coloniza el rumen durante el desarrollo de este compartimento del tracto digestivo del rumiante (Li et al., 2012). Estos y otros trabajos han permitido establecer que, además de un grupo mayoritario de *phyla*, común para todas las especies de rumiantes (core population) existen grupos minoritarios (rare population) que en algunos casos son esenciales para entender la adaptación de la funcionalidad del rumen a cambios ambientales (Morgavi et al., 2012). Por otro lado, el avance de las plataformas de secuenciación masiva como Illumina, ha permitido mejorar el conocimiento del ecosistema ruminal mediante el análisis metatranscriptómico de la actividad microbiana. Así, se ha profundizado en el conocimiento del papel de los protozoos en la funcionalidad del rumen (Qi et al., 2011) o de la expresión de ciertos genes esenciales de las arqueas que permite explicar la disminución de la producción de metano cuando se emplea aceite de colza (Poulsen et al., 2012). Sin duda la aplicación de estas tecnologías permitirá entender con mayor profundidad cómo funciona el ecosistema microbiano del rumen y en qué medida ciertos genes pueden ser

esenciales para explicar la respuesta del ecosistema a estrategias alimentarias, cambios ambientales, etc.

3. Moduladores de la fermentación ruminal: potencial y limitaciones de su uso

En los últimos años, y especialmente tras la prohibición en Europa del uso de los antibióticos en la dieta del ganado (Casewell et al., 2003), como promotores del crecimiento, se ha estimulado la búsqueda de aditivos que modulen la actividad ruminal y puedan emplearse como sustitutos de los antibióticos. Asimismo, ha aumentado globalmente la preocupación por las emisiones de gases con efecto invernadero, siendo la actividad ganadera, uno de los actores que contribuye al aumento de este tipo de gases de manera más importante.

En los últimos años se están llevando a cabo numerosos estudios para evaluar los efectos que, sobre la fermentación ruminal y la producción de metano, tienen distintos compuestos, mayoritariamente extractos de plantas (aceites esenciales, compuestos organosulfurados, etc.) así como compuestos diseñados y sintetizados para tal efecto. Estos estudios se han centrado en el ganado vacuno siendo menos numerosos los referidos a ovino y caprino. Los resultados obtenidos son, en ocasiones, contradictorios, difíciles de interpretar y proporcionan poca información sobre los mecanismos de acción de los compuestos estudiados. Ello puede deberse a factores como la gran diversidad y origen de los compuestos empleados, las condiciones de estudio de sus efectos (*in vitro* vs. *in vivo*) y la dieta que el animal recibe (Hart et al., 2008; Patra y Saxena, 2010; Benchaar y Greathead, 2011), entre otros.

Los compuestos utilizados en los estudios realizados pertenecen esencialmente a dos categorías: compuestos derivados de plantas y compuestos sintéticos.

3.1. Compuestos derivados de plantas

Los términos “metabolitos secundarios” o “compuestos derivados de plantas” se usan para describir una amplia gama de compuestos, de origen vegetal, que no están implicados en procesos bioquímicos de crecimiento o reproducción de las plantas (Patra y Saxena, 2010). El uso de plantas, con fines medicinales comenzó hace aproximadamente 5000 años en China, utilizándose también por los egipcios extractos de plantas para preservar alimentos y momias aproximadamente 1500 años A.C. (Davidson y Naidu, 2000). Sin embargo, hasta principios del siglo XX no se obtuvieron evidencias científicas de las propiedades antimicrobianas de estos compuestos (Calsamiglia et al., 2007).

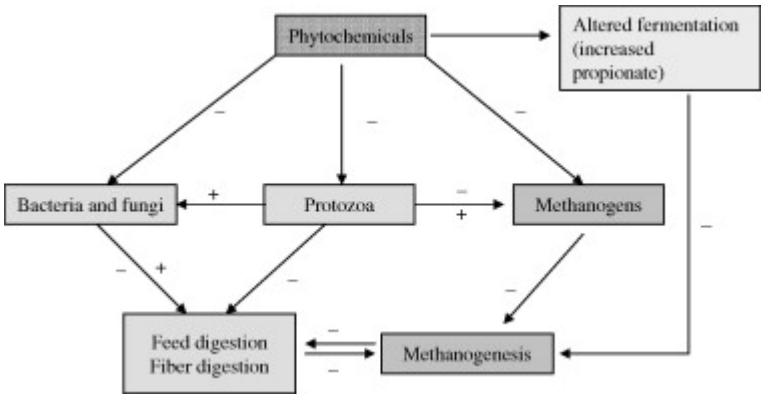

Figura 2.3. Esquema de los posibles mecanismos de acción de compuestos vegetales que inhiben la formación de metano en el rumen (Patra y Saxena, 2010)

La actividad y concentración de los compuestos presentes en las plantas dependen, en gran medida, de su origen geográfico, de la época de año en que se recolecten las plantas, del sistema de recolección o de almacenamiento (Bodas et al., 2008). Sus efectos sobre la fermentación ruminal se encuentran condicionados por el tipo de compuesto, su concentración o del procesado a que se haya sometido la planta que los contiene y de la dieta suministrada al

animal, (Hart et al., 2008). Los "metabolitos secundarios" o "compuestos derivados de plantas" se pueden clasificar en 4 grupos mayoritarios (Hart et al., 2008; Patra y Saxena, 2010; Bodas et al., 2012): saponinas, taninos, aceites esenciales y compuestos organosulfurados.

3.1.1. Saponinas

Las saponinas son compuestos secundarios que se encuentran en raíces, tubérculos, hojas, semillas y frutas (Hart et al., 2008), aunque también pueden encontrarse en algunos animales marinos y bacterias (Riguera, 1997). Son moléculas de glucósido, de alto peso molecular en las que las cadenas de azúcares están unidas a un triterpeno o grupo esteroideo (Patra y Saxena, 2010). Las saponinas presentan amplia variedad de efectos biológicos actuando fundamentalmente, sobre las membranas celulares (Francis et al., 2002).

Los efectos de las saponinas sobre la fermentación ruminal son diversos y, en algunos casos, contradictorios. Así, se ha descrito que la presencia de saponinas en la dieta del rumiante no afecta a la concentración de AGV totales (Hess et al., 2003; Patra et al., 2006; Guo et al., 2008), la disminuye (Pen et al. 2007) o la aumenta (Lila et al., 2003) efectos que pueden ir asociados a la disminución de la producción de metano. Con respecto a las proporciones molares de AGVs individuales la presencia de plantas que contienen saponinas o de extractos de saponinas en la dieta del rumiante provoca, tanto *in vivo* como *in vitro,* un incremento de la proporción de propionato (Lila et al., 2003; Agarwal et al., 2006; Patra et al., 2006; Guo et al., 2008; Holtshausen et al., 2009). Otros estudios no han indicado cambios en la proporción

acético/propiónico, debidos a la presencia de saponinas en la dieta (Hu et al., 2005; Pen et al., 2007; Goel et al., 2008a). Respecto a la producción de metano el efecto de las saponinas tampoco parece claro habiéndose observado la disminución, tanto *in vitro* como *in vivo* (Santoso et al., 2004; Pen et al., 2006; Pen et al., 2007; Holtshausen et al., 2009; Wang et al., 2009) o la ausencia de efecto (Klita et al., 1996; Goel et al., 2008b; Pen et al., 2008). Las contradicciones en los resultados observados pueden deberse al tipo y dosis de saponina, dieta utilizada, así como a la adaptación de los microorganismos del rumen a la presencia de dichos compuestos (Hart et al., 2008; Patra y Saxena, 2010; Benchaar y Greathead, 2011). Por último, cabe destacar, como efectos adversos de algunas saponinas, el desarrollo de fotosensibilización, daños renales y hepáticos, hemolisis y gastroenteritis (Patra y Saxena, 2010).

3.1.2. Taninos

Los taninos son compuestos polifenólicos, solubles en agua y capaces de formar complejos, principalmente con las proteínas. Se encuentran en distintas partes de plantas, frutas y semillas. Los taninos pueden ser hidrolizables o condensados (McMahon et al., 2000). Los taninos hidrolizables se caracterizan por tener un núcleo constituido por un glúcido, cuyos grupos hidroxilos se encuentran esterificados con ácidos fenólicos. Los taninos condensados, también denominados proantocianidinas, son polímeros no ramificados de hidroxiflavonoles como la catequina, unidos mediante enlaces entre carbonos y no contienen el núcleo glucídico, que caracteriza a los taninos hidrolizables.

Los efectos de los taninos sobre la fermentación ruminal en general y sobre la producción de metano, en particular, han sido estudiados en diversos estudios, tanto *in vivo* como *in vitro*. Se ha observado una disminución de 10-30 % de la producción de metano con diferentes tipos de extractos de taninos (Min et al., 2005; Bhatta et al., 2009; Grainger et al., 2009). Sin embargo, al igual que se ha descrito anteriormente para las saponinas, existen otros estudios en los que no se observó efecto de los taninos sobre la producción de metano (Min et al., 2006; Beauchemin et al., 2007). Las diferencias en los efectos descritos pueden deberse a las dosis, concentración o naturaleza de los taninos así como a la dieta utilizada o a la duración del tratamiento (Hart et al., 2008; Patra y Saxena, 2010). Diversos estudios indican que la adición de taninos o extractos de plantas que los contienen reduce la digestibilidad de la dieta tanto *in vitro* (Patra et al., 2006; Bhatta et al., 2009) como *in vivo* (Animut et al., 2008a, b; Grainger et al., 2009). En otros estudios, sin embargo, no se ha observado efecto de los taninos sobre digestibilidad de los nutrientes (Carulla et al., 2005; Patra et al., 2006; Bhatta et al., 2009; Hariadi y Santoso, 2010). Sin embargo, los taninos pueden afectar al crecimiento de determinados microorganismos, tales como las bacterias celulolíticas o los hongos (Patra y Saxena, 2009), pudiendo, en consecuencia, afectar negativamente a la degradación de la fibra. También los efectos de los taninos sobre la concentración de AGV totales en el rumen son diversos y contradictorios, habiéndose observado tanto una disminución (Min et al., 2006; Beauchemin et al., 2007; Grainger et al., 2009) como ausencia de efecto (Carulla et al., 2005; Patra et al., 2006; Animut et al., 2008a, b). La variación en sus efectos sobre la fermentación ruminal depende del tipo de planta del que se extraen (McAllister

et al., 2005), dosis y composición de los taninos (Hervás et al., 2003), así como del tipo de dieta utilizada (Hart et al., 2008; Patra y Saxena, 2010). Por último, cabe destacar que los taninos son también potencialmente tóxicos para los animales cuando estos consumen grandes cantidades y el periodo de adaptación de los animales a la dieta es insuficiente (Patra y Saxena, 2010).

3.1.3. Aceites esenciales

Se denominan aceites esenciales a diferentes mezclas de compuestos secundarios de la fracción volátil de plantas, obtenidos mediante diferentes métodos (Gershenzon y Croteau, 1991). El término "esencial" deriva de "esencia" y, se refiere a la propiedad de estas sustancias de proporcionar olores y sabores a gran cantidad de plantas. Se caracterizan por una gran diversidad en cuanto a su composición y naturaleza así como con respecto a su actividad. Estructuralmente pueden clasificarse como alcoholes, éster o aldehídos derivados de fenilpropanoides o terpenoides (Greathead, 2003). Los compuestos activos más importantes de los aceites esenciales se clasifican en dos grupos: terpenoides y fenilpropanoides. Los terpenoides son los compuestos secundarios derivados de plantas más numerosos y diversos (Gershenzon y Croteau, 1991). Contienen 5 átomos de carbono (C_5H_8) en una estructura denominada isopranoide. Los fenilpropanoides, aunque no son los compuestos más comunes, están presentes, en cantidades significativas, en algunas plantas. Se trata de compuestos con una cadena de 3 carbonos, unida a un anillo aromático de 6 carbonos. La característica más importante de estos compuestos es su poder antiséptico y antimicrobiano ejerciendo su acción sobre la membrana celular bacteriana (Griffin et al., 1999; Davidson y Naidu,

2000; Dorman y Deans, 2000). El carácter hidrofóbico de los hidrocarbonos cíclicos les permite interactuar con la membrana celular y acumularse en la bicapa lipídica, llegando a ocupar el espacio existente entre las cadenas de ácidos grasos (Sikkema et al., 1994; Ultee et al., 1999). Esta interacción promueve cambios estructurales de la membrana, provocando pérdida de iones y, por tanto, del gradiente iónico de la membrana (Griffin et al., 1999). En algunos casos, las bacterias intentan corregir esta disfunción mediante bombas iónicas, que requieren una gran cantidad de energía lo que reduce su crecimiento o incluso provoca la muerte celular (Griffin et al., 1999; Ultee et al., 1999; Cox et al., 2001). El efecto es mayor sobre las bacterias Gram positivas que sobre las Gram negativas, ya que las membranas celulares de las primeras interactúan directamente con los compuestos hidrofóbicos de los aceites esenciales (Smith et al., 1998; Chao y Young, 2000; Cimanga et al., 2002). Por el contrario, la pared celular que existe alrededor de la membrana celular de las bacterias Gram negativas es hidrofílica, impidiendo el paso de las sustancias lipofílicas. Sin embargo, no son completamente impermeables a las sustancias hidrofóbicas, y moléculas de bajo peso molecular que pueden formar puentes de hidrógeno con el agua, atravesar, por difusión, la pared celular e interactuar con la bicapa lipídica de la membrana bacteriana (Griffin et al., 1999; Dorman y Deans, 2000), lo que ocurre, por ejemplo, con compuestos aromáticos como el carvacrol o el timol. Por tanto, el bajo peso molecular de algunos aceites esenciales permite que sean activos frente a bacterias Gram-positivas y Gram-negativas, reduciendo su selectividad frente a poblaciones microbianas específicas (Calsamiglia et al., 2007). Los aceites esenciales

también poseen otros mecanismos de acción como la coagulación citoplasmática y la lisis celular (Burt, 2004).

Tabla 2.2. Aceites esenciales de diferentes plantas, componentes activos y microorganismos sobre los que actuan (Calsamiglia et al., 2007).

Essential oil of	Name	Active components	Susceptible microorganisms	Reference
Allium sativum	Garlic	Allicin, diallyl sulfite	Enteropathogenic bacteria	Ross et al., 2001
Anethum graveolens	Dill	Limonene, carvone	Gram-positive and gram-negative bacteria	Deans and Ritchie, 1987
Capsicum annum	Paprika	Capsaicin	Gram-positive and gram-negative bacteria	Deans and Ritchie, 1987
Cinnamomum cassia	Cassia	Cinnamaldehyde	*Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis*	Ouattara et al., 1997; Mahmoud, 1994; Smith-Palmer et al., 1998
Juniperus oxycedrus	Juniper	Cadinene, pinene	*Aeromonas sobria, Enterococcus fecalis, Staph. aureus*	Hammer et al., 1999
Melaleuca alternifolia	Tea tree	Terpinen-4-ol	*Staph. aureus, E. coli,* gram-positive and gram-negative bacteria	Chao and Young, 2000; Cox et al., 2001
Origanum vulgare	Oregano	Carvacrol, thymol	Gram-positive and gram-negative bacteria	Sivropoulou et al., 1996; Dorman and Deans, 2000
Pimpinella anisum	Anise	Anethol	*Aeromonas hydrophila, Brevibacterium linens, Brochothrics thermosphacta*	Deans and Ritchie, 1987
Rosmarinus officinalis	Rosemary	1,8-Cineole	*Staph. aureus, L. monocytogenes, Campylobacter jejuni*	Ouattara et al., 1997; Smith-Palmer et al., 1998
Syzygium aromaticum	Clove	Eugenol	*E. coli, Staph. aureus, L. monocytogenes, S. enteritidis, C. jejuni*	Ouattara et al., 1997; Smith-Palmer et al., 1998
Thymus vulgaris	Thyme	Thymol, carvacrol	*Salmonella typhimurium, Staph. aureus, Aspergillus flavus*	Juven et al., 1994; Ouattara et al., 1997; Mahmoud, 1994
Zingiber officinale	Ginger	Zingiberene, zingerone	Gram-positive and gram-negative bacteria	Chao and Young, 2000

Existen numerosos trabajos acerca del efecto de los aceites esenciales sobre la fermentación ruminal, siendo los principales compuestos ensayados los siguientes:

3.1.3.1. Anetol

El anetol (1-methoxy-4-propenylbenzene; $C_{10}H_{12}O$) es el principal compuesto activo del aceite de anís (*P. anisum*), responsable de su efecto antimicrobiano. La mayoría de los estudios en los que se ha utilizado se han realizado *in vitro*, con cultivos ruminales no renovados habiéndose observado una disminución de la concentración de AGV totales y de acético (Cardozo et al., 2005; Busquet et al., 2006). En incubaciones más prolongadas, con fermentadores, no se han observado efectos significativos sobre parámetros de la fermentación ruminal (Cardozo et al., 2004; Busquet et al., 2005c). En incubaciones *in vitro* de 6 horas se observó una disminución de la producción de metano (Chaves et al., 2008a). Apenas se han realizado estudios *in vivo* habiéndose observado (Cardozo et al., 2006) un ligero descenso en la ingestión de materia seca así como de la concentración ruminal de acético en terneros de engorde. No se ha estudiado *in vivo* el efecto de este compuesto sobre la producción de metano en el rumen

3.1.3.2. Timol

El timol es un monoterpeno [5-methyl-2-(1-methylethyl)phenol; $C_{10}H_{14}O$], presente en el orégano (*Origanum* spp.) y el tomillo (*Thymes* spp.) con amplio espectro antimicrobiano

(Burt, 2004). Es uno de los aceites esenciales más estudiado en cuanto a su efecto sobre la fermentación ruminal, aunque casi siempre se ha ensayado en condiciones *in vitro*. (Castillejos et al., 2006; Martinez et al., 2006; Benchaar et al., 2007; Castillejos et al., 2008). Algunos autores han estudiado los efectos de este aceite esencial sobre la metanogénesis ruminal (Evans y Martin, 2000; Macheboeuf et al., 2008), observándose una drástica disminución de la producción de metano con dosis elevadas del aceite. Los efectos sobre la fermentación ruminal, descritos en sistemas *in vitro,* son diversos, y el uso de dosis elevadas provoca una disminución de la concentración de AGV y amonio (Evans y Martin, 2000; Castillejos et al., 2006; Martinez et al., 2006; Macheboeuf et al., 2008). Por ello, diversos autores sugieren que la actividad antimicrobiana del timol podría ser demasiado fuerte e inespecífica para modular la fermentación en un ecosistema tan complejo como el rumen (Calsamiglia et al., 2007).

3.1.3.3. Carvacrol

El carvacrol es un compuesto fenólico [2-methyl-5-(1-methylethyl) phenol; $C_6H_3CH_3(OH)(C_3H_7)$], presente en el orégano (*Origanum* spp.) y el tomillo (*Thymes* spp.) al igual que el timol. También al igual que ocurre con el timol, se ha atribuido al carvacrol un importante efecto antimicrobiano debido a la presencia del grupo hidroxilo en su estructura fenólica (Burt, 2004). Los estudios realizados con este compuesto han sido, en su mayoría, realizados en sistemas *in vitro*, observándose con las dosis más altas efectos sobre la fermentación ruminal similares a los descritos para el timol. Busquet et al., (2005c) observaron *in vitro* una disminución de la concentración de los péptidos de cadena larga y un aumento de la concentración de amonio, sugiriéndose una

disminución de la proteólisis. Las dosis más elevadas disminuyen la concentración de AGV totales, efecto que también han observado otros autores usando extracto puro o extractos de orégano y tomillo que contenían carvacrol (Cardozo et al., 2005; Busquet et al., 2006; Castillejos et al., 2006; Macheboeuf et al., 2008). Cabe destacar la ausencia de efectos de este aceite esencial tras incubaciones más prolongadas en fermentadores (Cardozo et al., 2004) o el aumento de la concentración de AGV totales cuando se usaron dosis bajas (Castillejos et al., 2008). El efecto del carvacrol sobre la producción de metano ha sido estudiado en menor medida que sobre otros parámetros ruminales habiéndose observado un descenso linear de la producción de metano con dosis crecientes de carvacrol, en incubaciones *in vitro* de 16 y 24 horas de duración (Macheboeuf et al., 2008; Benchaar y Greathead, 2011). Al igual que ocurre con el timol el efecto antimicrobiano del carvacrol podría ser demasiado fuerte e inespecífico para modular la fermentación en un ecosistema tan complejo como el rumen (Calsamiglia et al., 2007). Uno de los pocos estudios llevados a cabo en corderos (Chaves et al., 2008c) demostró que el uso conjunto de carvacrol y cinamaldehido produjo un leve incremento de la concentración de AGV totales, sin efectos adversos sobre la ganancia de peso diaria y la ingesta de materia seca.

3.1.3.4. Cinamaldehido

El cinamaldehido (3-fenil-2-propenal phenol; C_9H_8O), un fenilpropanoide no fenólico con actividad antimicrobiana, es el principal compuesto activo del aceite de canela (*C. cassia*). Presenta actividad antimicrobiana tanto frente a bacterias Gram-negativas como Gram-positivas (Burt, 2004), siendo su actividad similar a la mostrada tanto por el

timol como por el carvacrol. Sin embargo, al contrario de los dos compuestos anteriores, no presenta grupo hidroxilo o ácido, siendo su actividad microbiana debida a la acción de su grupo carbonilo sobre las enzimas microbianas (Burt, 2004).

Los primeros estudios realizados para establecer el efecto del cinamaldehido y del aceite de canela sobre la fermentación y la microbiota ruminales se han llevado a cabo *in vitro* (Cardozo et al., 2004), con fermentadores de flujo doble continuo, observándose una inhibición de la peptidolisis, con ausencia de efecto sobre los AGV. Ferme et al. (2004) observaron una reducción de bacterias del género *Prevotella* spp. en fermentadores de doble flujo continuo usando cinamaldehido, el principal género de bacterias proteolíticas. Posteriormente, Busquet et al. (2006), en incubaciones más cortas con cultivos no renovados de microorganismos ruminales, observaron que el uso de cinamaldehido y del aceite de canela reduce la concentración de AGV totales un 42,3 % y un 22,8%, respectivamente, afectando a los AGV individuales de forma distinta en función del compuesto considerado. Otros estudios *in vitro*, con incubaciones de 16 y 24 horas han mostrado un descenso de la concentración de AGV totales y una modificación de su perfil con dosis elevadas de cinamaldehido (Macheboeuf et al., 2008; Mateos et al., 2013) , corroborando los resultados de Busquet et al. (2006). Estos resultados sugieren, que aunque el cinamaldehido es el principal componente del aceite de canela, otros compuestos presentes en dicho aceite podrían interactuar con el cinamaldehido o modular su efecto (Calsamiglia et al., 2007). La ausencia de efecto del cinamaldehido o de la canela sobre los AGV, en incubaciones más prolongadas, sugiere una adaptación de los microorganismos ruminales a la presencia de dichos compuestos en la dieta (Busquet et al., 2005c). Sin embargo, otros estudios realizados

en las mismas condiciones que los referidos anteriormente han mostrado su efecto sobre la concentración y el perfil de AGV (Lourenco et al., 2008). Con dosis elevadas de cinamaldehido y en condiciones *in vitro* se ha observado una disminución de la producción de metano (Macheboeuf et al., 2008; Mateos et al., 2013), observándose también un efecto de la dieta incubada. Los escasos estudios llevados a cabo *in vivo*, que abordan el efecto de los aceites esenciales sobre la producción de metano, utilizan mezclas de aceites, no habiéndose estudiado el efecto aislado del cinamaldehido. Ohene-Adjei et al. (2008) estudiaron el efecto de diferentes aceites esenciales, entre ellos el cinamaldehido, sobre las arqueas metanogénicas en el rumen de ovino, observándose un cambio en su diversidad como consecuencia del tratamiento. Como se ha descrito anteriormente para otros compuestos, la variabilidad de los resultados obtenidos con cinamaldehido puede depender, entre otros factores de su pureza y concentración, tipo de extracto y de la dieta utilizada.

3.1.3.5. Eugenol

El eugenol (4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$) es un monoterpeno fenólico con amplio espectro antimicrobiano (Dorman y Deans, 2000), que está presente en grandes cantidades en el clavo (*S. aromaticum*) y la hoja de la canela (*Cinnamomum cassia*).

La mayoría de los estudios *in vitro* que evalúan los efectos del eugenol sobre la fermentación ruminal, muestran una disminución significativa de las concentraciones de AGV totales y amonio así como cambios en el perfil de los AGV individuales, disminuyendo la proporción acético:propiónico y la concentración de AGV ramificados (Busquet et al., 2006; Cardozo et al., 2006; Castillejos et al., 2006; Castillejos et al.,

2008; Chaves et al., 2008a). Por otra parte, en incubaciones prolongadas con fermentadores, el eugenol no afectó a la fermentación ruminal (Busquet et al., 2005c; Lourenco et al., 2008). El eugenol y el aceite de clavo, en ensayos *in vitro,* disminuyen la producción de metano (Chaves et al., 2008a; Benchaar y Greathead, 2011) o no la afectan (Patra et al., 2010). No existen estudios *in vivo* realizados con eugenol como único compuesto.

3.1.3.6. Mezcla de aceites esenciales

El uso de mezclas de aceites esenciales provoca efectos sinérgicos, antagónicos o aditivos de sus componentes (Burt, 2004). Su efecto sobre la fermentación ruminal se ha estudiado, sobre todo *in vitro*, con resultados variables: aumento de la concentración de AGV y de la proporción molar de acético (Castillejos et al., 2005, 2007) y un descenso *marcado de la producción de* metano (Macheboeuf et al., 2008; Agarwal et al., 2009).

Los estudios *in vivo*, especialmente los que inciden en su efecto sobre la producción de metano, son muy escasos. En vacuno, Cardozo et al. (2006) observaron que una mezcla de cinamaldehido y eugenol aumentaba la concentración del ácido propiónico y disminuía la de acético, sin afectar a la concentración total de AGV en el rumen.

Por lo que se refiere a la microbiota ruminal también se han descrito efectos muy diversos. En cultivos puros, se ha observado que algunas bacterias hiperproductoras de amonio y hongos son sensibles a mezclas comerciales de aceites esenciales (Mcintosh et al., 2003). En ovejas lechera,s también se ha observado un efecto de mezclas de

aceites esenciales sobre bacterias hiperproductoras de amonio cuyo número disminuye (Giannenas et al., 2011).

3.1.4. Compuestos organosulfurados

Estos compuestos pertenecen, principalmente, a dos familias vegetales: familia Alliaceae, que incluye diversas especies como el ajo (*Allium sativum*), cebolla (*Allium cepa*) y puerro (*Allium porrum*). Las plantas de esta familia contienen la enzima alliin-alinasa. La otra familia es la Cruciferae a la que pertenecen el wasabi (*Wasabia japonica*), rábano picante (*Armoracia rusticana*) y la coliflor (*Brassica oleracea*), que contienen la enzima glucosinolato-myrosinasa. De esas plantas puede obtenerse una amplia variedad de compuestos organosulfurados, por la acción de las dos enzimas citadas anteriormente (Patra y Saxena, 2010). Los principales compuestos organosulfurados presentes en las especies de la familia Alliaceae son la -glutamyl-S-allyl-l-cysteina y el S-allyl-L-cysteina sulfóxido. Estos compuestos se convierten en tiosulfinatos, como la alicina, por la acción de las correspondientes enzimas. Los tiosulfinatos se degradan dando lugar a un amplio rango de compuestos volátiles, como el dialil disulfuro, dialil trisulfuro, allil metil disulfido, etc. (Lawson, 1996). Respecto a la familia Cruciferae, los principales compuestos sulfurados son los glucosinolatos, existiendo una amplia variedad dependen función del tipo de planta, siendo los principales el allil, 3-butenil glucosinolato y el 2-hidroxi 3-butenil glucosinolato (Patra y Saxena, 2010). El grupo sulfuro de estos compuestos es activo frente a un amplio espectro de microorganismos, como bacterias Gram positivas y Gram negativas (Reuter et al., 1996).

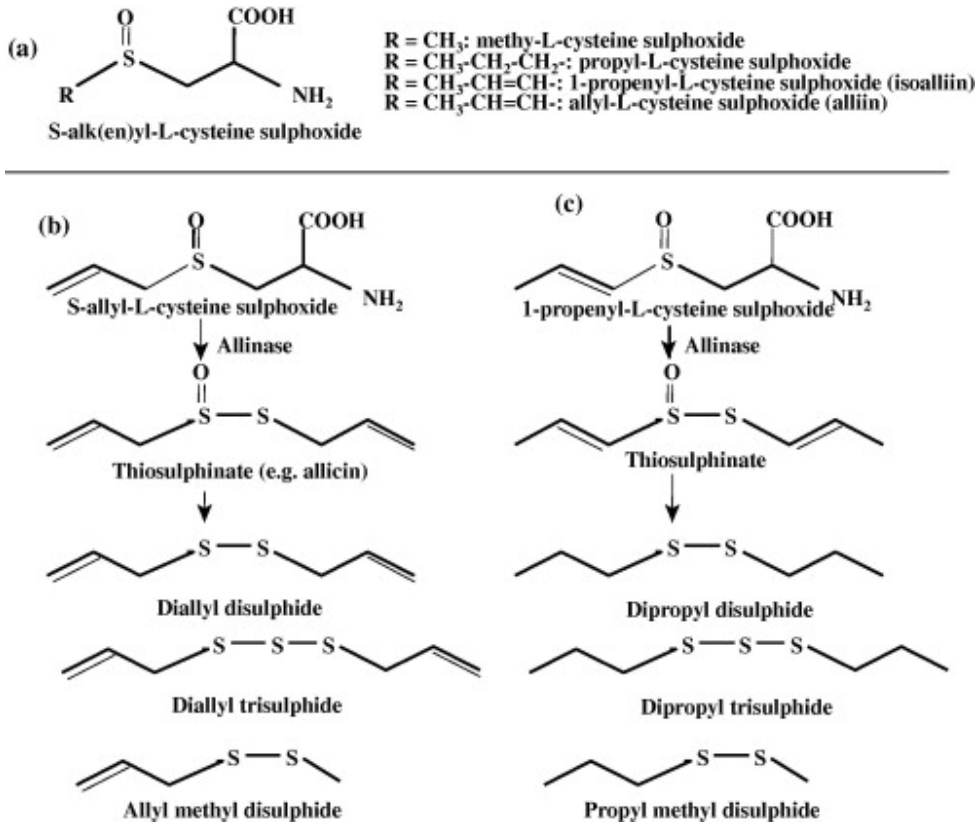

Figura 2.4. Principales compuestos organosulfurados de plantas de la familia *Alliaceae*: a) compuestos organosulfurados, b) compuestos producidos a partir de cystein shulphoxide (en ajo) y, c) 1-propenyl cystein sulphoxide en cebolla (Patra y Saxena, 2010).

Su efecto sobre la fermentación y la microbiota ruminal han comenzado a estudiarse recientemente (Benchaar y Greathead, 2011) aunque ya se dispone de una abundante información, que indica que compuestos organosulfurados de origen muy diverso (aceites del ajo o rábanos, compuestos secundarios como la alicina, dialil disulfuro o tiosulfinatos) inhiben la metanogenesis en el rumen (Hart et al., 2008; Patra y Saxena, 2010; Anassori et al., 2011; Benchaar y Greathead, 2011). Se ha sugerido que los compuestos organosulfurados actúan sobre las arqueas metanogenicas a través de la

inhibición de la actividad del 3-hydroxy-3-methylglutaryl coenzima A reductasa (HMG-CoA reductasa), que tiene un papel importante en la síntesis de éteres isoprenoides, principales componentes de la membrana de dichas arqueas (Busquet et al., 2005b). Mediante estudios i*n vitro* se ha confirmado que la presencia de compuestos organosulfurados, principalmente compuestos del ajo, disminuye la producción de metano (Busquet et al., 2005b; Chaves et al., 2008a; Kamel et al., 2008; Kongmun et al., 2010; Patra y Yu, 2012; Mateos et al., 2013) aunque los efectos de dichos compuestos sobre otros parámetros fermentativos son contradictorios. En algunos casos, se ha observado un descenso drástico de la concentración de AGV totales, o un descenso de la proporción acético:propiónico, mientras que en otros casos no se ha observado ningún efecto (Hart et al., 2006; Chaves et al., 2008a; Mateos et al., 2013). Igual contradicción existe en cuanto al efecto de los compuestos organosulfurados sobre la microbiota ruminal y, específicamente, sobre las arqueas metanogénicas, directamente relacionadas con la producción de metano. En algunos trabajos se ha descrito un descenso de arqueas como consecuencia de la adición de compuestos organosulfurados (Mohammed et al., 2004; Hart et al., 2006; Patra y Yu, 2012), mientras que en otros no se ha observado efecto (Ohene-Adjei et al., 2008; Kongmun et al., 2011). Sin embargo, cabe destacar el trabajo de Ohene-Adjei et al., (2008) quienes describen un cambio en la diversidad de las población de arqueas metanogénicas tras suministrar extracto de ajo. Esta acción de modificación de la estructura de la población se ha observado con otros compuestos y parece explicar de manera más consistente las variaciones en la producción de metano (Abecia et al., 2012; Poulsen et al., 2012). Los estudios *in vivo* con compuestos derivados de plantas y, concretamente, de los

derivados del ajo, son escasos y proporcionan resultados muy contradictorios, con ausencia de efecto del extracto de ajo sobre la producción de metano en ovejas (Klevenhusen et al., 2011b; Patra et al., 2011) o descenso de la misma en búfalos (Kongmun et al., 2011; Verma et al., 2012). También el diallil disulfuro en ovejas (Klevenhusen et al., 2011a) o el aceite de ajo en vacuno de carne (Manasri et al., 2012) han promovido un descenso de la producción de metano.

La contradicción en los resultados encontrados, tanto *in vitro* como *in vivo*, puede deberse a factores como la naturaleza química del compuesto estudiado o su concentración (Hart et al., 2008). Así mismo, la posible adaptación de los microorganismos ruminales a la presencia del compuesto puede contribuir a la contradicción (Benchaar y Greathead, 2011).

3.2. Compuestos sintéticos

Los compuestos sintéticos, que reducen la producción de metano y afectan a la fermentación ruminal, son muy diversos: ionóforos, halogenados y otros.

3.2.1. Ionóforos

Los antibióticos ionóforos, como la monensina y el lasalocid, se usan en producción animal y, concretamente en los rumiantes, para modular la fermentación ruminal y mejorar la eficiencia productiva cárnica y lechera (McGuffey et al., 2001). Son moléculas con átomos de oxígeno en diferentes posiciones, mejorando la captación de iones y la interacción con las membranas. Los compuestos ionóforos se fijan en las membranas celulares de bacterias y protozoos facilitando el movimiento de iones a

través de las mismas. De este modo, se produce una acumulación intracelular de H^+ y Na^+, que aumentaría el uso de los sistemas de transporte intracelular para eliminar el exceso de cationes. El incremento en el consumo de energía, necesaria para equilibrar el nivel intracelular de iones, disminuye la velocidad de división celular. Este mecanismo de acción afecta principalmente a las bacterias Gram positivas, debido a las características de sus membranas. Las bacterias Gram negativas, cuyas membranas son impermeables a moléculas de gran tamaño, son resistentes a los compuestos ionóforos, (McGuffey et al., 2001). Al disminuir el tamaño de la población de bacterias Gram positivas los ionóforos pueden también incrementar la relación acético-propiónico, disminuir la producción de metano mejorando la eficiencia energética del animal y disminuir el número de protozoos en el rumen (Beauchemin et al., 2008). Sin embargo, los ionóforos no tienen un efecto directo sobre las arqueas metanogénicas (Hook et al., 2009) ya que estimulan la propiogénesis en el rumen y, por tanto, la captación de H_2 por el ácido propiónico. Además, disminuyen la producción de H_2 al afectar a las principales productoras de H_2 en el rumen, las bacterias Gram positivas. Diversos estudios indican un descenso en la producción de metano en los rumiantes, tanto *in vitro* como *in vivo* (McGinn et al., 2004; Van Vugt et al., 2005; Benchaar et al., 2006; Odongo et al., 2007), por acción de compuestos ionóforos. Por el contrario, otros indican ausencia de efecto (Waghorn et al., 2007) o la desaparición del mismo tras un tratamiento prolongado con esos compuestos (Guan et al., 2006) lo que podría deberse a la adaptación de los microorganismos ruminales a la presencia de dichos compuestos (Johnson y Johnson, 1995; Guan et al., 2006). No obstante, también existen estudios

que consideran que la adaptación de la microbiota podría no ocurrir (Odongo et al., 2007).

Finalmente, cabe destacar que el uso de antibióticos como los ionóforos, en alimentación animal no está permitido en la Unión Europea (Casewell et al., 2003) y otros países. Ello, junto a la posible falta de efecto tras periodos prolongados de tratamiento con dichos compuestos, hace que no se consideren como una solución práctica para reducir la producción de metano en el rumen (Beauchemin et al., 2008).

3.2.2. Halogenados

Los compuestos halogenados sintéticos, como el bromoclorometano (BCM) o el ácido bromoetanosulfonico (BES), son potentes inhibidores de la metanogénesis en los rumiantes (McAllister y Newbold, 2008). Se ha sugerido que reduce la vitamina B_{12} y, como resultado, inhibe la actividad metil transferasa cobamida-dependiente, implicada en la metanogénesis y responsable de la síntesis de metil coenzima M (Wood et al., 1982). El efecto del BCM se ha estudiado, tanto *in vitro* (Goel et al., 2009) como *in vivo* (Denman et al., 2007; Tomkins et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012), observándose una disminución de la producción de metano y de la proporción acético:propiónico así como un efecto sobre la abundancia de las arqueas metanogénicas. Sin embargo, aunque no se han observado signos de toxicidad o residuos en la carne o vísceras (Tomkins et al., 2009) su uso en producción animal está prohibido debido a su efecto anti-ozono, por lo que no puede considerarse como alternativa práctica a otros aditivos antimetanogénicos.

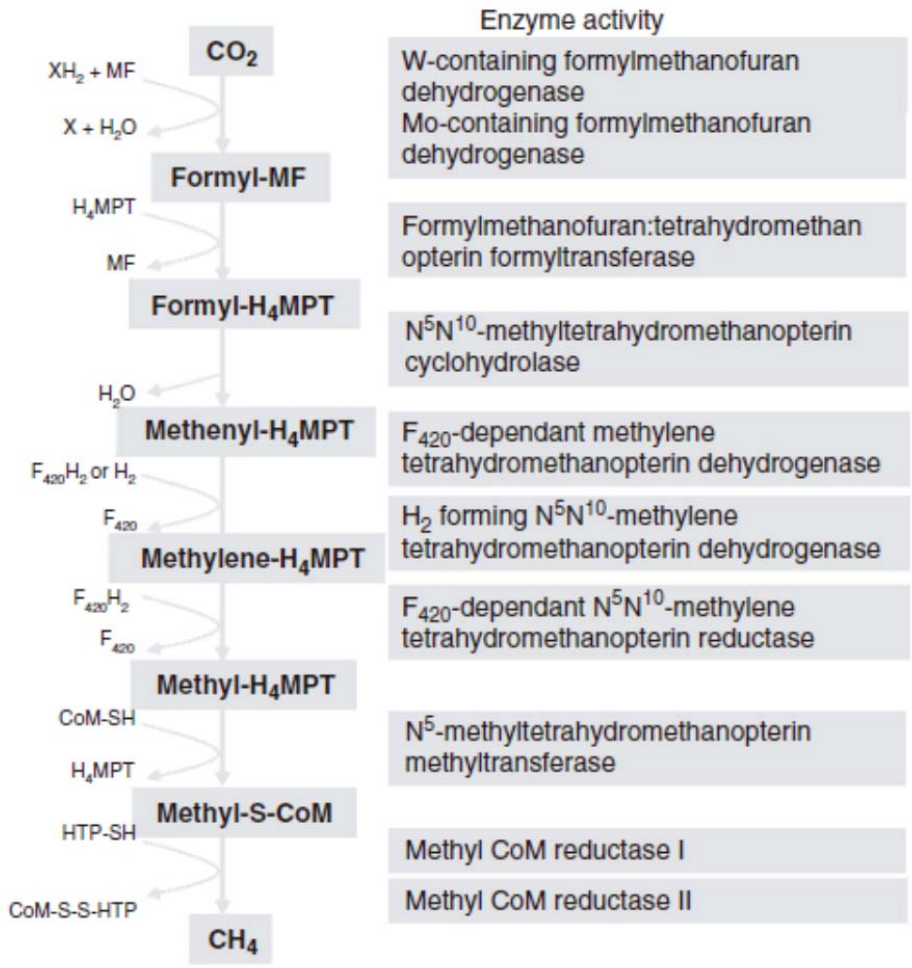

Figura 2.5. Rutas metabólicas y principales enzimas implicadas en la metanogénesis hidrogenotrófica (Attwood y McSweeney, 2008).

El BES es un análogo estructural de la coenzima M (CoM) e inhibe la metilación reductiva de la metil-S-CoM, en el último paso de la metanogénesis (Müller et al., 1993). Se ha estudiado su efecto sobre cultivos puros de arqueas metanogénicas (Ungerfeld et al., 2004) observándose diferencias que dependen de las especies de arqueas metanogénicas consideradas. También se ha observado una disminución de la

producción de metano, tanto *in vitro* (Choi et al., 2004) como *in vivo* (Tomkins y Hunter, 2003), con la adición de BES.

3.2.3. Otros

Los compuestos lovastatin y mevastatin son inhibidores de la enzima hydroxymethylglutaryl-SCoA reductasa, involucrada en la formación del mevolanato, un precursor de la síntesis de isoprenoides, presentes en las membranas de las arqueas metanogénicas. Por ello, estos compuestos afectan al crecimiento de las arqueas metanogénicas, sin afectar a las bacterias ruminales. Otro compuesto, el ácido ethyl ester 3-azido-propiónico es un análogo del bromoethanosulfanato, un potente antimetanogénico. Estos compuestos reducen la producción de metano *in vitro* hasta en un 50% sin afectar a la eficiencia de utilización del alimento (Miller y Wolin, 2001). Por otro lado, Soliva et al. (Soliva et al., 2011) han estudiado, en fermentadores RUSITEC, el efecto del lovastatin y del ácido ethyl ester 3-azido-propiónico, observando también una disminución del metano sin afectar de forma dramática a otros parámetros de la fermentación ruminal. Aunque sus efectos sobre la producción de metano son patentes, para suministrar dichos compuestos sintéticos en condiciones prácticas, han de estudiarse previamente *in vivo* no solo en cuanto a su efecto en el rumen sino también en lo que se refiere a su persistencia en los productos de rumiantes destinados al consumo humano.

3.3. Ácidos orgánicos

Capítulo 2

Existe una gran variedad de ácidos orgánicos, aunque el más estudiado en rumiantes ha sido el fumarato. Los ácidos dicarboxílicos son precursores del propionato en el rumen, actuando como sumideros de hidrógeno, alternativos a la metanogénesis, efecto que se ha observado *in vitro* (Lopez et al., 1999; Newbold et al., 2005; Carro y Ranilla, 2007; García-Martínez et al., 2007). La mayoría de los estudios realizados *in vivo* corroboran la reducción de la producción de metano y su efecto sobre el perfil de AGV encontradas en estudios *in vitro* (Wallace et al., 2006; Wood et al., 2009; Yang et al., 2012) aunque también existen algunos estudios en los que no se observó efecto del fumarato sobre la producción de metano (McGinn et al., 2004; Beauchemin y McGinn, 2006). Aunque la reducción de la producción de metano en los rumiantes por los ácidos orgánicos ha sido ampliamente contrastada, su uso práctico es económicamente inviable (McAllister y Newbold, 2008).

4. Limitaciones actuales al uso de aditivos que modifican la fermentación ruminal

Existen distintas limitaciones al uso de compuestos que modulan la fermentación ruminal como estrategia alimentaria: i) La pureza de los compuestos utilizados, ii) la adaptación de los microorganismos ruminales a su presencia, iii) la naturaleza de la dieta suministrada al rumiante y el pH ruminal, iv) la dificultad para extrapolar las condiciones ensayadas *in vitro* y los resultados obtenidos en este tipo de ensayos a las condiciones *in vivo*, v) la transferencia de posibles residuos de los compuestos a los productos animales, vi) la viabilidad económica, y vii) la estabilidad de los aditivos durante su almacenamiento.

i) Pureza de los compuestos utilizados

Existen evidencias de que dosis similares de los mismos compuestos vegetales, con distinta pureza, provocan efectos diferentes sobre la fermentación ruminal (Hart et al., 2008; Patra y Saxena, 2010; Benchaar y Greathead, 2011; Busquet et al., 2005a; Busquet et al., 2005b; Hart et al., 2006; Chaves et al., 2008b; Soliva et al., 2011). Superar esta limitación requiere estandarizar la concentración y actividad de los compuestos, así como la composición de las mezclas de dichos compuestos, que se utilizan en los diferentes estudios.

ii) Adaptación de los microorganismos ruminales a su presencia

Los microorganismos ruminales poseen la capacidad de adaptarse o degradar un amplio rango de compuestos naturales o sintéticos (Casewell et al., 2003; Benchaar y Greathead, 2011). Algunas especies de bacterias ruminales son capaces

de crecer en presencia de concentraciones elevadas de aceites esenciales y, por tanto, de adaptarse a los mismos (McIntosh et al. 2003). La diversidad de las arqueas metanogénicas del rumen varia cuando los animales se tratan con compuestos derivados de plantas (Ohene-Adjei et al., 2008). También se ha observado que ciertos compuestos no afectan a la fermentación ruminal en ensayos prolongados, tanto *in vitro* como *in vivo* (Cardozo et al., 2004; Molero et al., 2004; Castillejos et al., 2007; Benchaar y Greathead, 2011; Klevenhusen et al., 2011b). Esta variabilidad de resultados se podría explicar por una adaptación de los microorganismos, en función del tiempo de tratamiento. Wang et al. (2000) observaron que, tras un tiempo relativamente corto de tratamiento el efecto de las saponinas sobre los protozoos,en cultivos *in vitro*, desaparecía sugiriéndose una degradación de las mismas por parte de éste grupo microbiano.

iii) Naturaleza de la dieta suministrada al rumiante y pH ruminal.

La naturaleza de la dieta incubada, en los estudios *in vitro,* o suministrada a los animales, en los estudios *in vivo*, así como el pH que promueve la fermentación de las mismas modula el efecto de los aceites esenciales (Molero et al., 2004; Cardozo et al., 2005). Wallace et al. (2002) sugirieron que el efecto de mezclas de aceites esenciales depende de la degradabilidad de la proteína dietética siendo tanto más evidente cuanto mayor es la degradabilidad. El tipo (Duval et al., 2007) y la cantidad (Mateos et al., 2013) de carbohidratos de la dieta también modulan el efecto de aceites esenciales y compuestos organosulfurados.

iv) Dificultad para extrapolar las condiciones ensayadas *in vitro* y los resultados obtenidos en este tipo de ensayos a las condiciones *in vivo*

Los efectos de ciertos compuestos sobre la fermentación ruminal, observados *in vitro,* desaparecen o disminuyen en ensayos *in vivo*. Esta falta de efecto puede deberse a distintos factores; uno de ellos es la dificultad de extrapolar las dosis *in vitro* a condiciones *in vivo* debido, sobre todo, a la complejidad del ecosistema microbiano del rumen. En general, se requieren dosis superiores a las utilizadas *in vitro* para que el efecto de un determinado compuesto se haga patente en el animal. No obstante, ha de tenerse precaución ya que las dosis necesarias para detectar efectos *in vivo* pueden ser demasiado elevadas (Beauchemin et al., 2009). La actividad de los compuestos depende de la probabilidad de que el componente activo interactúe con los microorganismos, lo que es función de la concentración de dicho componente, del tamaño de las poblaciones microbianas y de la complejidad del ecosistema ruminal (Calsamiglia et al., 2007). Otro factor diferencial entre las condiciones *in vitro* e *in vivo* puede ser la homogeneidad con la que el compuesto o compuestos estudiados se distribuyen. Esa homogeneidad es mayor *in vitro* lo que hace que, en esas condiciones, los microorganismos se expongan más rápidamente a la actividad de los compuestos que en el rumen. También ha de considerarse, como se ha señalado anteriormente, la posible adaptación de los microorganismos a algunos compuestos tras su suministro al animal, durante un periodo de tiempo prolongado (Benchaar y Greathead, 2011; Bodas et al., 2012). El tiempo de permanencia de la digesta en el rumen, que depende de un número importante de factores (relación forraje:concentrado, tamaño de partícula, nivel de ingestión, contenido y digestibilidad

de la fibra, etc…), ha de tenerse en cuenta para extrapolar condiciones ensayadas *in vitro* a estudios *in vivo*. Los sistemas cerrados implican, por definición, la no renovación del medio de fermentación, y los fermentadores continuos y semi-continuos pueden no simular totalmente los flujos de salida de las fracciones sólida y líquida de la digesta ruminal. Cuando se trata de aplicar una dosis de un determinado compuesto, previamente estudiada en sistemas de cultivo *in vitro*, a animales alimentados a nivel de mantenimiento (Publicación 1 y 3) debe de tenerse en cuenta el tiempo fraccional de paso de la digesta a través del rumen (3%, Yáñez-Ruiz et al., 2004), siendo posible que el incremento de la dosis haya de ser de hasta el 80% con respecto a la utilizada *in vitro* (Publicación 1 y 3). Ello puede explicar el que una misma dosis de aceite de rábano provoque una disminución de la producción de metano in vitro del 90% y cambios en otros parámetros de la fermentación ruminal mientras que en terneros la disminución sea solo del 19% y no afecte a otros parámetros de la fermentación (Mohammed et al., 2004). Otro aspecto importante es, como señalan Soto et al. (2012), que la microbiota en sistemas *in vitro* es diferente de la que existe en el rumen.

v) Transferencia de posibles residuos de los compuestos a los productos animales

Los estudios sobre la presencia de residuos, en leche o carne, de extractos de plantas son limitados aunque existen indicios de la presencia de terpenos, procedentes de forrajes, en la leche de vaca, que alteran sus propiedades organolépticas (Vialloninsta et al., 2000; Tornambé et al., 2006). Hallier et al. (2013) no han observado residuos, en la leche de vaca, de timol, carvacrol, cinamaldehido o dialil disulfuro, administrados en dosis de 120 mg/día durante 3 semanas. Trabajos recientes, llevados a cabo en vacuno

lechero en el marco del proyecto Europeo FP7-SMEthane (www.smethane.eu), han mostrado mediante cromatografía gases-masas, la ausencia, en la leche, de una mezcla de aceites esenciales, suministrada a razón de 1g/animal/día. Dada la limitada información existente, se requieren estudios más detallados y de larga duración para determinar si existen residuos de aditivos dietéticos en leche o carne.

vi) Viabilidad económica

La viabilidad económica del uso de aditivos en alimentación animal depende, no solo del coste del aditivo sino también de la dosis requerida para que se produzca un efecto cuyo beneficio supere al coste de su uso (Benchaar y Greathead, 2011).

vii) Estabilidad de los aditivos durante su almacenamiento

Un aspecto a considerar y, no menos importante que los señalados anteriormente, es el que se refiere a la estabilidad y conservación de los aditivos a lo largo del tiempo. En el caso de los extractos de plantas, el solvente y su dilución, así como el tiempo, la temperatura, presión, etc., requeridas para la extracción del compuesto objeto de estudio, pueden afectar a la actividad del mismo (Bodas et al., 2012). Así, el cinamaldehido se descompone cuando se calienta a 60ºC mientras que si se mezcla con otros compuestos, como el eugenol o el extracto de canela, se mantiene estable durante 30 minutos, con temperaturas de hasta 200ºC (Friedman et al., 2000).

Capítulo 3. Metodología y resultados

Publication 1

In vitro-in vivo study of the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats

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Abstract

Two *in vitro* and an *in vivo* experiments were conducted to investigate the effects of a selection of plant active compounds on rumen fermentation, microbial concentration and methane emissions in goats. Treatments were: control (no additive), carvacrol (**CAR**), cinnamaldehyde (**CIN**), eugenol (**EUG**), propyl-propane-thiosulfinate (**PTS**), propyl-propane-thiosulfonate (**PTSO**), diallyl disulfide (**DDS**), a mixture (40:60) of PTS and PTSO (**PTS+PTSO**) and bromochloromethane (**BCM**) as positive control with proven antimethanogenic effectiveness. Four doses (40, 80, 160 and 320 µL/L) of the different compounds were incubated *in vitro* for 24 h in diluted rumen fluid from goats using two diets differing in starch and protein source within the concentrate (Experiment 1).The total gas production was linearly decreased ($P < 0.012$) by all compounds, with the exception of EUG and PTS+PTSO ($P \geq 0.366$). Total volatile fatty acids (**VFA**) concentration decreased ($P \leq 0.018$) only with PTS, PTSO and CAR while the acetate:propionate ratio decreased ($P \leq 0.002$) with PTS, PTSO and BCM, and a tendency (P=0.064) was observed for DDS. Based on results from Experiment 1, two doses of PTS, CAR, CIN, and BCM (160 and 320 µL/L), PTSO (40 and 160 µL/L) and DDS (80 and 320 µL/L) were further tested *in vitro* for 72 h (Experiment 2). The gas production kinetics were affected ($P \leq 0.045$) by all compounds, and digested NDF (**DNDF**) after 72 h of incubation was only linearly decreased ($P \leq 0.004$) by CAR and PTS. The addition of all compounds linearly decreased ($P \leq 0.009$) methane production, although the greatest reductions were observed for PTS (up to 96%), DDS (62%) and BCM (95%). No diet x dose interaction was observed. To further test the results obtained *in vitro,* two groups of sixteen adult non-pregnant goats were used to study *in*

vivo the effect of adding PTS (50, 100 and 200 mg/L rumen content per day) and BCM (50, 100 and 160 mg/L rumen content per day) during 9 days on methane emissions (Experiment 3). The addition of PTS and BCM resulted in linear reductions (33% and 64%, respectively, $P \leq 0.002$) of methane production per unit of dry matter intake, which were lower than the maximum inhibition observed *in vitro* (87 and 96 %, respectively). We conclude that applying the same doses *in vivo* as *in vitro* resulted in proportional lower extent of methane decrease and that PTS at 200 mg/L rumen content per day has the potential to reduce methane emissions in goats. Whether the reduction in CH_4 emission observed *in vivo* persists over longer periods of treatments and improves feed conversion efficiency requires further research.

Keywords: additives, goats, methane, plant active compounds, rumen fermentation.

Implications

This study shows that some plant extracts have potential to improve rumen fermentation and hence animal productivity in goats; however, applying *in vivo* the same dosage as used *in vitro* in relation to rumen volume result in a proportional lower extent of improvement. Short term (9 days) *in vivo* trials allowed us to test the potential of different dosages in the diet of ruminants that would need to be further confirmed in longer term trials.

Introduction

Animal production and in particular the ruminant sector carries with it a significant environmental cost as enteric methane from ruminants is responsible for circa 80% of

the methane emissions from the sector (Morgavi *et al*., 2010). In addition, methane production in the rumen may account for as much as 12 % of the gross energy intake in ruminant animals (Johnson and Johnson, 1995), thus representing an energy loss for the animal. If the ruminant livestock sector is to continue to flourish and grow then new technologies to maximize efficiency should be developed. In that context, previous studies showed that plants contain an extensive variety of secondary compounds with antimicrobial activity and potential, in certain amounts, to enhance rumen fermentation (Benchaar and Greathead, 2011). However, effects reported in the literature are variable and contradictory, which may be due to the different concentrations of active ingredients, basal diets used and lack of direct *in vitro* – *in vivo* comparisons (Hart *et al*., 2008). With regards to the diet, Newbold *et al.* (2004) reported that a specific blend of essential oils affected protein degradation to a different extent depending on the protein source used in the diet (rapeseed *vs*. soya bean meal) and Duval *et al.* (2007) suggested that essential oils interfere differently with some key rumen bacteria depending on the starch source (wheat, barley or maize).

Although *in vitro* methodologies are useful to assess the effects of a wide variety of plant extracts and their bioactive compounds on rumen fermentation, there are limitations related to the extrapolation of compound doses tested *in vitro* to *in vivo* conditions (i.e. heavily buffered rumen fluid *in vitro*, different solid and liquid turnover rates, changes induced in the microbial ecosystem when incubated *in vitro* such as decrease in total biomass and community structure as well as limited presence of fungi and protozoa; Soto *et al*., 2012). Also, *in vitro* and *in vivo* studies are normally performed separately and often no reference compound with a known, consistent effect is included. The

majority of *in vivo* research has been conducted with cattle or sheep, with limited information concerning goats, which sometimes respond to antimicrobial compounds differently from other ruminants (i.e. presence of condensed tannins; Yáñez-Ruiz *et al*., 2004). Based on recent literature (Benchaar and Greathead, 2011; Bodas *et al*., 2012) and results in our group, eight plant active compounds that have shown promise in decreasing methane production in the rumen were selected to evaluate *in vitro* (24 and 72 h) the effects of different doses on rumen fermentation and microbiota when two substrates differing in starch and protein source are fermented. The objective was to identify the compounds with more antimethanogenic potential and to validate to what extent the activity is confirmed *in vivo* in goats using the same range of dosage as *in vitro*.

Material and methods

Two *in vitro* experiments were conducted in batch cultures to assess the effects of different concentrations of a range of plant active compounds on rumen fermentation by the incubation of two experimental diets over 24 h (**Exp. 1**) or 72 h (**Exp. 2**). Based on results obtained in Experiments 1 and 2, an *in vivo* experiment (**Exp. 3**) was conducted with goats to further test *in vitro* results.

Diets, additives and animals

The experimental diets (Table 3.1.1) used in Exp. 1 and Exp. 2 consisted of a 50:50 forage (alfalfa hay): concentrate, in which the concentrate included maize gluten meal (116 g/kg) and rumen-inert fat (70 g/kg) plus different protein and starch sources with

high rumen degradability: barley (349 g/kg) and faba beans (465 g/kg) in concentrate (diet barley-beans); and medium degradability: maize (349 g/kg) and sunflower meal (465 g/kg) in concentrate (diet maize-sunflower). The diet used in Exp. 3 was alfalfa hay:concentrate (55:45), in which the concentrate was a mix (non-pelleted) of all the ingredients used in Experiments 1 and 2 (gluten meal 116 g/kg, rumen-inert fat 70 g/kg, maize 174 g/kg, barley 174 g/kg, faba beans 233 g/kg and sunflower meal 233 g/kg). Animals had access to mineral-vitamin blocks and clean drinking water.

The active compounds tested were carvacrol (**CAR**, 5-isopropyl-2-methylphenol, 97 % purity), cinnamaldehyde (**CIN**, (2E)-3-phenylprop-2-enal, 93% purity), eugenol (**EUG**, 4-Allyl-2-methoxyphenol, 98% purity) and four garlic compounds: diallyl disulfide (**DDS**, 3-prop-2-enyldisulfanyl prop-1-ene, 80% purity), propyl propane thiosulfinate 75% purity (**PTS**), propyl propane thiosulfonate 85% purity (**PTSO**) and a commercial mixture (40:60) of PTS and PTSO (**PTS+PTSO)**. Additionally bromochloromethane (**BCM**, halogenated aliphatic hydrocarbon) was included as a positive control (Goel *et al*., 2009; Abecia *et al*., 2012). The CAR, CIN, EUG and DDS were obtained from Sigma-Aldrich Chemical; PTS and PTSO were provided by DMC Research Center SL (Granada, Spain); the commercial mixture of PTS and PTSO (Garlicon) was obtained from Prebia Feed Extracts S.L. (Toledo, Spain); BCM (Sigma-Aldrich Chemical) was entrapped in an α-cyclodextrin matrix (May *et al*., 1995). The formulation was prepared in our laboratory as dry white powder in 1-2 kg batches and contained 10–12% (wt/wt) of BCM.

Four female Murciano-granadina goats fitted with permanent rumen cannula were used as donors of rumen content for the *in vitro* experiments. Goats were fed *ad libitum* once a day (0900 h) with equal amounts of alfalfa hay and concentrate (same as in Exp.

3) and had free access to water and mineral salt blocks (Pacsa Sanders, Sevilla, Spain). In Exp. 3, thirty two adult, female, non-pregnant Murciano-granadina goats (33.2 ± 5.2 kg), fed at maintenance level with alfalfa hay and concentrate (55:45), were used. Animals were cared for by trained personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree No. 1201/2005) and the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Directive 86/609). All the experimental procedures involved in this study were approved by the Animal Welfare Committee at the Institute of Animal Nutrition (CSIC, Spain).

In vitro experiments

Rumen contents were collected and pooled from the four goats before the morning feeding, and immediately taken in thermal flasks to the laboratory where they were filtered through two layers of cheesecloth whilst bubbled with CO_2. The buffered mineral solution (Menke and Steingass, 1988) was heated in a water bath at 39ºC and bubbled continuously with CO_2, 2 h before rumen contents collection. The filtered rumen fluid was mixed with the buffer mineral solution in a 1:3 ratio (Menke and Steingass, 1988). Time required from rumen content collection to inoculation of bottles was less than 30 minutes.

Experiment 1

Three 24 h incubation runs were carried out with two bottles (per diet, treatment and dose) and two blanks in each run. Average values from two bottles in each run were

used as experimental replicate. Treatments were: control (dose 0), CAR, CIN, EUG, DDS, PTS, PTSO, PTS+PTSO and BCM. Doses were 0, 40, 80, 160 and 320 µL/L with the exception of BCM that was added at 160 and 320 µL/L doses. A commercial wireless system (Ankom^{RF} Gas Production, Ankom Technology, NY, USA) consisting of bottles equipped with pressure sensors modules and a reception base station connected to a computer was used to measure pressure as described by Cornou *et al*. (2013). After 24 h of incubation the fermentation was stopped by placing the bottles in ice and the content filtered to collect a sub-sample: 0.8 ml for VFA analysis was collected and was kept at -20ºC.

Experiment 2

Three 72 h incubation runs were carried out with three bottles (per diet, treatment and dose) and three blanks in each run. Average values from two bottles in each run were used as experimental replicate. Based on Exp. 1 results, a selection of compounds and doses was made: 160 and 320 µL/L for CA, CIN and PTS; 40 and 160 µL/L for PTSO; 80 and 320 µL/L for DDS; 160 and 320 µL/L for BCM and 0 µL/L as control. Experimental diets incubated were those used in Experiment 1. The experimental procedure was based on Theodorou *et al.* (1994). Volume of gas and headspace pressure were measured with a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ) and a glass calibrated syringe (Ruthe®, Normax, Marinha Grande, Portugal) respectively, at 2, 4, 6, 8, 12, 24, 36, 48 and 72 h after inoculation. At 2, 4, 6, 8, 12 and 24 h a sample of the gas in each bottle was collected in a graduated syringe and transferred to a 5 ml vacuum tube and then kept at room temperature before

methane content was measured by GC. One of the replicate bottles per treatment was opened at 24 hours to collect 1 ml from the content for DNA extraction. After 72 h, fermentation was stopped in the two remaining bottles and the bottle content was lyophilized for DM and NDF determination.

Experiment 3

Two groups of 16 adult goats were used to test effects of PTS and BCM, respectively, on methane emissions. Within each group of 16 goats, four experimental blocks of four animals were made according to body weights and within each block, one goat was randomly assigned one of the four experimental treatments (control plus three doses) for nine consecutive days. Therefore, four goats per treatment were used. Goats were held in individual pens of 2 x 2 m. Doses of PTS and BCM were selected based on results from Experiments 1 and 2, assuming a similar rumen content volume in both experimental groups of 11% of body weight (Abecia *et al*., 2012). Doses were equivalent to: 0, 50, 100 and 200 mg/L rumen content per day of PTS and 0, 50, 10 and 160 mg/L rumen content per day of BCM. The experimental diet consisted of alfalfa hay and concentrate provided at a ratio of 55:45 to cover the maintenance energy requirements (Prieto *et al*., 1990). The experiment included 7 days for adaptation of animals to the additives and 2 days for feed intake and methane emissions measurements. Diet and additives were provided to animals in two equal meals at 0900 h and 1400 h. The corresponding dose of PTS and BCM was pipetted and weighed, respectively, into 10 g of ground oats and wrapped in cellulose paper coated with molasses immediately before oral administration. On day 8, each animal was transferred into a cage within a

respiration chamber for methane measurements for 2 consecutive days. A set of four identical chambers (1.8 m wide × 1.8 m deep × 1.5 m tall) were used as described by Abecia *et al*. (2012).

Chemical analyses

Dry matter (method ID 934.01), ash (method ID 942.05), ether extract (method ID 7.045) and crude protein by Kjeldhal (method ID 984.13) in samples were determined by the procedures of the Association of Official Analytical Chemists (AOAC, 2005). Gross energy was measured with an adiabatic calorimeter (Model 1356, Parr Instrument Co., Moline, IL). Neutral detergent fibre with heat-stable amylase and expressed inclusive of residual ash (NDF), acid detergent fibre expressed inclusive of residual ash (ADF) and ADL contents were analysed following the methodology described by Van Soest *et al*. (1991) using an ANKOM Model 220 Fibre Analyser (Macedon, NY, USA). The individual VFA concentrations were analysed using the gas chromatography technique described by Isac *et al*. (1994). The CH_4 concentration was determined by gas chromatography (GC) using a HP Hewlett 5890, Packard Series II gas chromatograph (Waldbronn, Germany). A sample of 0.5 ml of gas was injected using a 1 ml Sample-Lock® syringe (Hamilton, Nevada, USA).

Real-Time PCR Analysis

Samples collected in Experiment 2 after 24 h of incubation were freeze –dried and used to isolate DNA using QIAamp DNA Stool Mini Kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions but with higher temperature (95ºC) for lysis

incubation. The DNA samples were used as templates for quantitative real-time PCR (qPCR) amplification. The corresponding gene copies of total bacteria, protozoa and methanogenic archaea were quantified by qPCR as described by Abecia *et al.* (2012).

Calculations

The volume of gas produced in Exp. 1 was calculated from the readings of gas pressure in the 24 h of fermentation as described by Cornou *et al*. (2013). The gas produced in batch cultures (Exp. 2) was adjusted to the model: $y=A[1-e^{-c*t}]$ (France *et al*., 2000) where *y* represents the cumulative gas production (ml); *t*, the incubation time (h); *A*, the asymptote (potential gas volume at steady state; ml) and *c*, the gas production rate (h^{-1}). Digested NDF after 72 h of incubation was calculated as described by Van Soest *et al*. (1966) as (NDF input – NDF output)/NDF input, NDF output being NDF content in the residue after 72 h incubation. The volume of gas produced (ml) was corrected for standard conditions (10^5 Pa, 298 K), and the amount (µmol) of methane produced was calculated by multiplying the gas produced (µmol) by the concentration of methane in the analysed sample. The flux of methane (Exp. 3) for each chamber was calculated for the two day periods of measurement from the difference of fresh-air intake and chamber exhaust methane concentrations and mean air flux. The air stream in each of the five ducts (chamber one to chamber four and background) was sub-sampled, and methane concentration was measured continuously using a gas analyser ADM MGA3000 (Spurling works, Herts, UK). It took 14 min to sequentially sample the airflow in each intake and exhaust ducts in the four chambers (3 min in chambers, 2 min for background).

Statistical analysis

Data from Exp. 1 and Exp. 2 were analysed as univariate model using the MIXED procedure of SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). The statistical model included the fixed effects of diet, dose and their interaction, with the period as a random effect. Data from Exp. 3 were analysed using the MIXED procedure of SAS, with the animal as the experimental unit. Linear (L), quadratic (Q) and cubic (C) components of the response to incremental amounts of each compound were evaluated using orthogonal polynomial contrasts. The CONTRAST option of the MIXED procedure used the coefficient matrix generated in PROC IML for the unequally spaced treatments. In addition, the flux of methane emissions measured in Exp. 3 was subjected to ANOVA for repeated measures using the MIXED procedure of SAS and assuming a covariance structure fitted on the basis of Schwarz's Bayesian information model fit criterion. The statistical model included the fixed effects of dose, hour, and their interaction, and the initial record measured at 0h (covariate). Differences were declared significant at $P < 0.05$ and considered as tendencies towards significance at $P < 0.10$.

Results

Experiments 1 and 2 (in vitro)

In Exp. 1, total gas production was linearly decreased ($P \leq 0.012$) by all compounds, with the exception of EUG and PTS+PTSO (Table 3.1.2). Significant ($P \leq 0.014$) Diet x Dose interaction was detected for CAR, PTS, PTSO and DDS, which consisted of a stronger decrease in gas production mainly for PTS and DDS at doses 40 and 160,

respectively, when diet Maize-Sunflower was used. Total VFA concentration was linearly reduced ($P \leq 0.002$) by the increasing addition of CAR, PTS and PTSO while no effect ($P \geq 0.05$) was observed for the other compounds. Diet x Dose interaction was only detected ($P = 0.0138$) for PTS (28 % decrease in bottles with Maize-Sunflower substrate, while no effect on Barley-Beans diet). The acetate:propionate ratio was modified by all compounds with the exception of PTS+PTSO; however, the response differed among molecules: increasing levels of CAR, CIN and EUG linearly increased ($P \leq 0.002$) the ratio, while the opposite pattern ($P \leq 0.003$) was observed for PTS, PTSO and BCM, and a tendency was observed for DDS ($P = 0.064$).

In Exp. 2, no Diet x Dose interaction ($P \geq 0.05$) was observed for any of the studied parameters (Table 3.1.3). The potential gas volume (A) was significantly lowered ($P \leq 0.001$) by all treatments, and as a tendency ($P = 0.095$) was observed with PTS. The gas production rate (c) was linearly increased ($P \leq 0.008$) by CAR, DDS and BCM and reduced ($P \leq 0.004$) by CIN, PTS and PTSO. In spite of the effects on (A) and (c), the DNDF after 72 h of incubation was only linearly decreased ($P \leq 0.004$) by CAR and PTS and as a dose tendency ($P \leq 0.089$) by DDS and BCM. Methane production measured after 24 h of incubation was linearly decreased by all compounds ($P \leq 0.009$), although the greatest reductions were observed for PTS (up to 96%), DDS (62%) and BCM (95%).

In experiment 2, no effect of treatment on population size of bacteria, protozoa and archaea was observed (Table 3.1.4), with the exception ($P \leq 0.003$) of DDS and PTS that caused, respectively, a reduction of concentration of archaea (10.5 vs. 10.1) and protozoa (9.1 vs. 7.4).

Experiment 3 (in vivo)

The addition of PTS and BCM linearly decreased ($P \leq 0.002$) methane produced per kg of DMI, respectively up to 33% and 64%, compared with control (Table 3.1.5). The same effect was observed for the proportion of gross energy lost as methane. On the other hand, for both compounds, the postprandial pattern of methane emissions through the day (Figures 3.1.1 and 3.1.2) consisted of larger differences among treatments over the first five hours after the morning feeding, and then they gradually came closer towards the end of the day. When animals were treated with BCM, the hourly emissions showed differences among the three levels ($P < 0.001$) right from the first measurements and there was no dose x time interaction ($P = 0.683$). As for PTS, the same pattern ($P = 0.011$) was only observed for the highest dose (200 mg/L rumen content) compared with the other two doses (50 and 100 mg/L), which resulted in a tendency to significant ($P = 0.099$) dose x time interaction.

Discussion

The literature on the use of plant extracts to manipulate rumen fermentation is large and mostly involves *in vitro* assays (Benchaar and Greathead, 2011). The reported effects are variable and often contradictory, which is most likely due to differences in the plant extracts used, dose and possibly the basal diet (Hart *et al*., 2008). The variability in concentration of active compounds in plant extracts generates confusion because the effects can be contradictory, according to the content of the active component in the extract and the dose used. Therefore, it is necessary either to report the concentrations

of these active compounds in the plant extracts used in research, or to use pure products to define activities, doses, and mechanisms of action in an unequivocal form. The latest is the option chosen for this work. On the other hand, the effect of additives on rumen fermentation, and in particular on methane emissions, has been shown to depend to a certain extent on the substrate fermented (e.g. forage:concentrate ratio (Mateos *et al*., 2013) and type of forage (Castro-Montoya *et al*., 2012)). The *in vitro* experiments in this work included two diets formulated for dairy goats and based on previous observations made when testing different types of essential oils (Newbold *et al*., 2004; Duval *et al*., 2007). The ultimate goal was to test whether there was an interaction of additive x diet to ensure that the *in vivo* experiment was conducted using a diet that maximizes the effectiveness of the compounds and contribute to fill the gap of the lack of *in vitro* and *in vivo* experiments designed and carried out together for robust comparisons.

Experiments 1 and 2 (in vitro)

Exp. 1 was designed to screen four doses of eight compounds over 24 h incubations using two different substrates with the aim of selecting narrower dosage levels to be further tested over longer incubation periods (72 h). Although the reduction of gas production *in vitro* may indicate that the rumen fermentation could be compromised, most of the antimicrobial compounds tested in the literature have exhibited a depression of fermentation at a certain level of dosage (Benchaar and Greathead, 2011; Bodas *et al*., 2012). The challenge is to identify the dosage range to maximize the beneficial effect without compromising the overall fermentation. Only three compounds (PTS, CAR and

PTSO) showed a negative effect on VFA concentration. Volatile fatty acids represent the main supply of metabolisable energy for ruminants, and therefore a reduction in their production would be nutritionally unfavourable for the host animal. On the other hand, for most of the compounds (except DDS and PTS+PTSO), the acetate to propionate ratio was linearly modified as the doses increased. The increase in this ratio has been reported previously for different essential oils such as eugenol, cinnamaldehyde and carvacrol (Macheboeuf *et al*., 2008; Mateos *et al*., 2013), while the lowered acetate to propionate ratio observed here for PTS and PTSO has been reported for different organosulphour compounds such as allicin, diallyl disulphide and allyl mercaptan (Hart *et al*., 2008). This different effect on the fatty acid profile might be a consequence of the distinct modes of action exhibited by each compound: essential oils seem to have a broader antimicrobial activity by affecting the membrane integrity and disturbing energy metabolism in the cell, while organosulphour compounds such as thiosulphinates specifically inhibit the growth of archaea by affecting the synthesis of their glycerol containing lipids in membranes (Busquet *et al.*, 2005).

In Exp. 2, as discussed above, the selection of doses was made trying to cover the dosage window to maximize the effects without compromising overall fermentation. Contrary to what was observed in Exp 1. none of the studied parameters exhibited significant Diet x Dose interaction. This might indicate that over short-term incubations (24 h), the effect of some compounds may be influenced by the rumen degradability of starch and protein sources; however, when the incubation lasts for 72 h this diet-dependent effect (including the gas production rate) is no longer apparent. The selection of the substrates in this work was made so different starch (barley *vs*. maize) and

protein (fava beans *vs*. sunflower meal) sources differing in their degradability patterns in the rumen to test this hypothesis as described previously (Newbold *et al*., 2004; Duval *et al*., 2007). The lack of significant interaction might be due to the fact that these ingredients are not the sole substrates but were used to formulate the concentrate of the diet that had the same forage (alfalfa hay). In that sense, it has been shown that it is the type of forage and the forage:concentrate ratio that are the main diet-related factors driving the effectiveness of antimethanogenic compounds (Castro Montoya *et al*., 2012). In the light of these results, a sole diet was used in the subsequent *in vivo* experiment, which included a concentrate that contained all starch and protein sources used in the *in vitro* experiment.

In Exp. 2 gas production was affected by all studied compounds (PTS, P = 0.095); however, the values obtained for dose I were very close to control, or even numerically higher. This was also observed for the gas production rate and might suggest that at that level of dosage the fermentation was not compromised. Indeed, the DNDF was only significantly affected by CAR and PTS with a reduction of 28 % in both cases, which is consistent with values reported using other garlic compounds and essential oils *in vitro* (Busquet *et al.*, 2005). Although a possible overestimation of the *in vitro* method to estimate digestibility based on NDF residue has been reported (Getachew *et al*., 2004), this method is accepted for comparative purposes; nevertheless, these results need to be confirmed *in vivo* in producing animals with faster passing digesta rates than the 72 h used in this *in vitro* assay.

The addition of CAR and CIN affected methane concentration, which is in agreement with Macheboeuf *et al*. (2008) and Mateos *et al*., (2013), who reported a linear decrease

in CH_4 production using similar compounds and doses. The addition of PTS and DDS inhibited methane emission up to 90% and 60%, respectively, which are comparable to values reported in other *in vitro* studies (Busquet *et al.*, 2005, Soliva *et al.*, 2011). In despite of the methane reduction observed with the addition of PTS, DDS and BCM, only protozoal and archaeal abundances were affected by the highest dose of PTS and DDS, respectively, while the concentration of total bacteria remained unchanged for all treatments. A decrease in protozoa abundances in batch cultures was also observed by Kongmun *et al*. (2010) when garlic powder was added. Similarly, *in vivo* results reported by Ohene-Adjei *et al*. (2008) showed that garlic oil did not affect total number of methanogenic archaea in sheep as quantified by archaeal 16S *rRNA* gene copies. However, these authors showed an increased phylogenetic diversity of methanogenic archaea, which may have resulted from changes in associated protozoal species. The overall lack of effect on archaea concentration supports recent observations which suggest that methanogenesis in the rumen depends to a large extent, on the distribution of different archaea species rather than their absolute numbers (Zhou *et al*., 2010).

The results observed for PTS and DDS on rumen fermentation and methane production were similar to those observed when adding BCM, although the highest dose (320 µL/L) of PTS tended to negatively affect rumen fermentation.

Experiment 3 (in vivo)

Based on results obtained in Exp. 1 and 2, PTS was selected as the compound to be tested with BCM as the positive control. The doses used were 50, 100 and 200 mg/L rumen content for PTS, which were within the dosage range needed to decrease

methane production but below the high dose tested *in vitro* (320 µL/L) that potentially may compromise rumen fermentation. Although *in vitro* and *in vivo* experiments were not run in parallel with the same rumen samples and animals in this study, they were sequentially designed using the same diets, type of animals, active compounds and same positive control (BCM). This may allow *in vitro* versus *in vivo* comparison of effectiveness in methane inhibition. Methane emissions measured *in vivo* (L/ kg DMI) decreased over 33% with PTS at the highest dose (200 mg/L). This is equivalent to the reduction (27%) observed *in vitro* with the dose of 160 µL/L and far less than the reduction (87%) achieved with a dose of 320 µL/L *in vitro*. On the other hand, the reduction observed with BCM *in vivo* (34 to 64%) was not as high as that obtained *in vitro* (96%); however, it was similar to the decrease (30%) achieved in our group with dairy goats treated over 2 months using the same compound and similar dosage (Abecia *et al*., 2012). Similar differences between *in vitro* and *in vivo* studies have been observed by Mohammed *et al.* (2004) using Japanese horseradish oil, who reported substantially greater inhibitions of methane production *in vitro* (89%) than *in vivo* (18.7%). The disagreement in the effectiveness observed between results obtained *in vitro* and *in vivo* with the same doses strongly supports the need for testing *in vivo* what it is previously observed *in vitro* and may be explained by a number of factors: i) the compounds used in this study had very low solubility in water and therefore the homogenous distribution across the rumen compartments might have not been fully achieved; ii) the degradation rate of the active compounds may differ *in vitro* and i*n vivo*; iii) there is a reported decrease in microbial densities and changes in bacterial community structure when rumen content is processed prior to inoculation *in vitro*, which

could be attributed to the exposure of microorganisms to oxygen and the discard of the main part of solids during the filtration process (Soto *et al*., 2012). In addition, the direct extrapolation of concentrations from *in vitro* to *in vivo* did not take into account the rumen outflow, which in our conditions, with animals fed restricted intake, was estimated to be around 3%/h (Yáñez-Ruiz *et al*., 2004). This would require an increase of the daily dosage of about 80% *in vivo* in comparison with the dose used in *in vitro* conditions and would explain the proportionally lower reduction achieved *in vivo* as compared with *in vitro*.

The pattern of methane emissions throughout the day reveals a distinctive pattern that consists of larger differences between treatments over the first 5 hours after the morning feeding, and then they gradually came closer towards the end of the day. This distinct pattern between control and effective treatments agrees with Thornton and Owens (1981), who using monensin in steers observed that inhibition of methane production declined with time postprandially. However, in our case the effective treatment of BCM (dose 100 and 160 mg/L rumen content) showed a slight recovery towards the end of the day. This raises the question of the difficulty of achieving a sustained anti-methanogenic activity in the rumen throughout the day. In the case of BCM, cyclodextrin was used as an encapsulating material aiming at reducing the volatile nature of the ingredient; however, it is likely that the use of two ‘shots’ in this study may have diminished the potential methane reduction effect as compared with a more homogeneous application if the additive was completely mixed with the diet.

With regards to intakes, the highest dose of PTS (200 mg/L) showed a numerical reduction in DMI (478 vs. 601 g/day) that was not observed with BCM. These data

needs to be taken with caution given that the animals had been adapting to the treatment for only 7 days and therefore it cannot be considered as a conventional intake experiment. The literature shows that the effect of garlic derived compounds on feed intake is rather variable. Yang *et al.* (2007) did not observe detrimental effects of garlic oil on daily intakes by growing lambs and dairy cows, while Patra and Saxena (2010) reported that the addition of garlic bulb (10 g/kg DM intake) to a concentrate mixture reduced its intake for initial 10–15 days in buffaloes and sheep probably due to pungent smell of garlic oil. Once animals were adapted to garlic, feed intake was not affected. In this study we attempted to confirm *in vivo* the results obtained *in vitro* using short term (9 days) treatments. This has the advantage of allowing the study of different levels of inclusion and once an optimum dosage is identified, longer treatment periods should be used.

In conclusion, the results obtained in this work suggest that applying *in vivo* the same dosage as used *in vitro* in relation to rumen volume results in a proportional lower extent of methane inhibition. This may be related to the lack of homogenous distribution of compounds within the rumen, lower microbial concentration *in vitro* than *in vivo* and to the higher dilution rate *in vivo compared* to *in vitro* conditions. Among the compounds tested here, propyl propane thiosulfinate (PTS) at doses between 50 and 200 mg/L of rumen contents has the potential to reduce methane emissions in goats. Whether the reduction in CH_4 observed *in vivo* persists over longer periods of treatments and improves feed conversion efficiency requires further research.

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References

Abecia L, Toral P, Martín-García A, Martínez G, Tomkins N, Molina-Alcaide E, Newbold C and Yáñez-Ruiz D 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. Journal of Dairy Science 95, 2027-2036.

Association of Official Analytical Chemists (AOAC) 2005. Official Methods of Analysis 18th ed. AOAC, Gaithersburg, MD.

Benchaar C and Greathead H 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology 166-167, 338-355.

Bodas R, Prieto N, García-González R, Andrés S, Giráldez F and López S 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animal Feed Science and Technology 176, 78-93.

Busquet M, Calsamiglia S, Ferret A, Carro M and Kamel C 2005. Effect of garlic oil and four of its compounds on rumen microbial fermentation. Journal of Dairy Science 88, 4393-4404.

Capítulo 3

Castro-Montoya J, De Campeneere S, Van Ranst G, and Fievez V 2012. Interactions between methane mitigation additives and basal substrates on in vitro methane and VFA production. Animal Feed Science and Technology 176, 47-60.

Cornou C, Storm ID, Hindrichsen I, Worgan H, Bakewell E, Yáñez-Ruiz D and Abecia L 2013. A ring test of a wireless in vitro gas production system. Animal Production Science (In press).

Duval SM, McEwan NR, Graham RC, Wallace RJ and Newbold CJ 2007. Effect of a blend of essential oil compounds on the colonization of starch-rich substrates by bacteria in the rumen. Journal of Applied Microbiology 103, 2132-2141.

European Directive 86/609 2007. Commission recommendations of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Annex II to European Council Directive 86/609. The Commission of the European Communittes Publishing, Brussels, Belgium.

France J, Dijkstra J, Dhanoa M, Lopez S and Bannink A 2000. 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. British Journal of Nutrition 83, 143-150.

Getachew G, Robinson P, DePeters E and Taylor S 2004. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. Animal Feed Science and Technology 111, 57-71.

Goel G, Makkar HPS and Becker K 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation

using batch and continuous fermentations. British Journal of Nutrition 101, 1484-1492.

Hart KJ, Yáñez-Ruiz DR, Duval SM, McEwan NR and Newbold CJ 2008. Plant extracts to manipulate rumen fermentation. Animal Feed Science and Technology 147, 8-35.

Isac M, García M, Aguilera J and Molina AE 1994. A comparative study of nutrient digestibility, kinetics of digestion and passage and rumen fermentation pattern in goats and sheep offered medium quality forages at the maintenance level of feeding. Archiv für Tierernährung 46, 37-50.

Johnson KA and Johnson DE 1995. Methane emissions from cattle. Journal of Animal Science 73,2483–2492.

Kongmun P, Wanapat M, Pakdee P and Navanukraw C 2010. Effect of coconut oil and garlic powder on in vitro fermentation using gas production technique. Livestock Science 127, 38-44.

Macheboeuf D, Morgavi D, Papon Y, Mousset J and Arturo-Schaan M 2008. Dose-response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Animal Feed Science and Technology 145, 335-350.

Mateos I, Ranilla M, Tejido M, Saro C, Kamel C and Carro MD 2013. The influence of diet type (dairy versus intensive fattening) on the effectiveness of garlic oil and cinnamaldehyde to manipulate in vitro ruminal fermentation and methane production. Animal Production Science 53, 299-307.

Mohammed, N, N. Ajisaka, Z. A. Lila, Koji Hara, K. Mikuni, K. Hara, S. Kanda and H. Itabashi. 2004. Effect of Japanese horseradish oil on methane production and

ruminal fermentation in vitro and in steers. Journal of Animal Science 82, 1839-1846.

May C, Payne AL, Stewart PL and A. EJ 1995. A delivery system for agents. AU International Patent PCT/AU95/00733. Australian Industrial Property Organisation,Canberra, ACT, Australia.

Menke KH and Steingass H 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development 28, 7- 55.

Morgavi DP, Forano E, Martin C and Newbold CJ 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4,1024–1036.

Newbold C, McIntosh F, Williams P, Losa R and Wallace R 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 114, 105-112.

Ohene-Adjei S, Chaves A, McAllister T, Benchaar C, Teather R and Forster R 2008. Evidence of increased diversity of methanogenic archaea with plant extract supplementation. Microbial Ecology 56, 234-242.

Patra AK and Saxena J 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198-1222.

Prieto C, Aguilera J, Lara L and Fonollá J 1990. Protein and energy requirements for maintenance of indigenous Granadina goats. British Journal of Nutrition 63, 155-163.

Royal Decree No. 1201/2005 of 10 October on the protection of animals used for experimentation and other scientific purposes. Boletin del Estado, pp. 34367-34391. Spanish Government Publishing, Madrid, Spain.

Soliva CR, Amelchanka SL, Duval SM and Kreuzer M 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). British Journal of Nutrition 106, 114-122.

Soto E, Yáñez-Ruiz D, Cantalapiedra-Hijar G, Vivas A and Molina-Alcaide E 2012. Changes in ruminal microbiota due to rumen content processing and incubation in single-flow continuous-culture fermenters. Animal Production Science 52, 813-822.

Theodorou MK, Williams BA, Dhanoa MS, McAllan AB and France J 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48, 185-197.

Thornton, J. H., and F. N. Owens. 1981. Monensin supplementation and in vivo methane production by steers. Journal of Animal Science 52, 628-634.

Van Soest P, Wine R and Moore L 1966. Estimation of the true digestibility of forages by the in vitro digestion of cell walls. In International Grassland Congress 10, 438-441.

Van Soest PJ, Robertson J and Lewis B 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583-3597.

Capítulo 3

Yang W, Benchaar C, Ametaj B, Chaves A, He M and McAllister T 2007. Effects of garlic and juniper berry essential oils on ruminal fermentation and on the site and extent of digestion in lactating cows. Journal of Dairy Science 90, 5671-5681.

Yáñez-Ruiz DR, Moumen A, Martin Garcia IA and Molina Alcaide E 2004. Ruminal fermentation and degradation patterns, protozoa population and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: Effect of PEG supply. Journal of Animal Science 82, 2023-2032.

Zhou M, Hernandez-Sanabria E and Guan LL 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Applied and Environmental Microbiology 76, 3776-3786.

Tables and figures

Table 3.1.1. *Chemical composition of alfalfa hay and concentrates (g/kg DM) and ingredients (g/kg) of concentrates.*

Item	Alfalfa hay	Concentrate Barley-Beans	Concentrate Maize-Sunflower	Concentrate *In vivo*
DM (g/kg fresh matter)	905	917	919	915
OM	893	942	940	884
NDF	504	293	329	244
ADF	315	102	155	117
ADL	89	19	54	36
CP	206	219	213	197
Ether Extract	9.5	17.2	19.0	18.1
GE (MJ/Kg DM)	18.4	20.6	20.1	19.5
Ingredients				
Barley		349		174
Faba beans		465		233
Maize			349	174
Sunflower meal			465	233
Maize gluten meal		116	116	116
Rumen-inert fat		70	70	70

Table 3.1.2. *Effects of diet and additive doses on gas production (GP, ml gas/g incubated DM), volatile fatty acids (VFA) concentration (mM) and acetic:propionic ratio (A/P) after 24 h of incubation in batch cultures (Experiment 1).*

		Diet		Dose[1]						*P-value*[2]			
Item	Compound[3]	Barley Beans	Maize Sunflower	0	40	80	160	320	s.e.d.	Diet	Dose	DxDo	*Contrast*[4]
GP	CAR	167	151	169	170	170	153	134	5.9	***	***	*	LC
	CIN	173	155	169	170	166	165	151	8.7	***	*	t	L
	EUG	172	156	169	163	167	163	157	9.0	***	ns	ns	
	PTS	161	141	169	163	157	145	120	9.3	***	***	*	L
	PTSO	172	152	169	160	165	160	157	5.4	***	*	***	L
	DDS	163	146	169	167	154	143	140	5.9	***	***	***	LQ
	PTS+PTSO	169	161	169	160	167	165	164	9.5	t	ns	ns	
	BCM	166	157	169	n.d.[5]	n.d.[5]	159	158	4.9	*	*	t	L
VFA	CAR	61.1	59.0	63.4	63.1	62.7	59.2	51.8	3.13	ns	***	ns	L
	CIN	63.2	60.7	63.4	63.6	62.8	60.0	60.1	5.20	ns	ns	ns	
	EUG	61.7	64.0	63.4	67.7	64.2	58.2	60.7	4.80	ns	ns	ns	
	PTS	58.5	55.3	59.9	60.8	58.1	56.4	49.3	2.98	*	***	*	L
	PTSO	59.2	55.6	59.9	57.8	57.1	56.6	55.4	1.64	***	*	ns	L
	DDS	59.1	57.8	59.9	59.4	57.2	57.6	57.4	2.25	ns	ns	ns	
	PTS+PTSO	59.8	61.2	59.9	59.9	60.8	60.2	61.8	2.57	ns	ns	ns	
	BCM	60.6	59.8	59.9	n.d.[5]	n.d.[5]	60.5	60.1	2.30	ns	ns	ns	
A/P	CAR	3.36	3.32	3.07	3.15	3.30	3.51	3.69	0.152	ns	***	ns	L
	CIN	3.21	3.13	3.07	3.18	3.02	3.21	3.37	0.122	ns	**	ns	L
	EUG	3.29	3.27	3.07	3.19	3.25	3.26	3.63	0.135	ns	***	ns	L
	PTS	3.25	3.23	3.36	3.37	3.18	3.29	3.02	0.146	ns	*	t	L
	PTSO	3.28	3.19	3.36	3.36	3.24	3.20	3.01	0.125	ns	**	ns	L
	DDS	3.13	3.23	3.36	3.30	3.21	3.04	3.01	0.188	ns	t	ns	
	PTS+PTSO	3.36	3.57	3.36	3.37	3.52	3.52	3.55	0.212	*	ns	ns	
	BCM	2.82	2.76	3.36	n.d.[5]	n.d.[5]	2.60	2.41	0.237	ns	***	ns	L

[1] Doses are expressed in µL/L of buffered inoculum.

[2] Probability of significance effects due to diet, dose and their interaction (DxDo). ns = $P > 0.10$; t = $P < 0.10$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[3] Compounds: CAR (carvacrol), CIN (cinnamaldehyde), EUG (eugenol), PTS (propyl propane thiosulfinate), PTSO (propyl propane thiosulfonate), DDS (diallyl disulfide) and BCM (Bromochloromethane).

[4] Indicates significant ($P < 0.05$) linear (L), quadratic (*Q)* or cubic (*C)* effects of the response to incremental dose of each compound estimated by orthogonal polynomial contrast.

[5] n.d.: not determined.

Table 3.1.3. *Effects of diet and additive doses on kinetics gas parameters (A: potential gas volume at steady state, ml; c: gas production rate, h^{-1}), digested NDF (DNDF, g/g) after 72 h incubation and on methane production (µmol) after 24 h of incubation in batch cultures (Experiment 2).*

		Diet		Dose[1]				*P-value*[2]			
Item	Compound[3]	Barley Beans	Maize Sunflower	0	I	II	s.e.d.	Diet	Dose	DxDo	*Contrast*[4]
A	CAR	103	87	111	100	73	3.4	***	***	ns	LQ
	CIN	118	103	111	113	108	2.7	***	*	ns	Q
	PTS	102	95	111	98	87	13.9	ns	t	ns	
	PTSO	116	102	111	112	105	1.8	***	**	ns	L
	DDS	100	90	111	94	76	5.5	ns	***	ns	LQ
	BCM	117	97	111	103	107	2.4	***	**	ns	LQ
c	CAR	0.108	0.109	0.089	0.100	0.137	0.0068	ns	***	ns	LQ
	CIN	0.084	0.083	0.089	0.085	0.077	0.0043	ns	*	ns	L
	PTS	0.063	0.060	0.089	0.073	0.024	0.0081	ns	***	ns	LQ
	PTSO	0.083	0.083	0.089	0.090	0.070	0.0042	ns	***	ns	L
	DDS	0.109	0.110	0.089	0.103	0.127	0.0075	ns	***	ns	LQ
	BCM	0.091	0.098	0.089	0.097	0.098	0.0041	**	*	ns	L
DNDF	CAR	0.51	0.52	0.64	0.50	0.42	0.082	ns	*	ns	L
	CIN	0.59	0.64	0.64	0.59	0.61	0.040	t	ns	ns	
	PTS	0.49	0.54	0.64	0.48	0.43	0.067	ns	**	ns	L
	PTSO	0.56	0.62	0.64	0.56	0.57	0.055	ns	ns	ns	
	DDS	0.57	0.58	0.64	0.58	0.51	0.065	ns	t	ns	
	BCM	0.56	0.63	0.64	0.57	0.59	0.042	*	t	ns	
CH_4	CAR	332	258	353	329	203	18.4	***	***	ns	LQ
	CIN	363	305	353	351	298	24.3	**	*	ns	L
	PTS	227	185	353	251	14	23.5	*	***	ns	LQ
	PTSO	352	291	353	333	279	20.4	***	***	ns	L
	DDS	239	202	353	175	133	26.0	*	***	ns	LQ
	BCM	141	118	353	20	15	28.6	ns	***	ns	LQ

[1] Dose I for CAR, CIN, PTS and BCM was 160 µL/L, for PTSO was 40 µL/L and for DDS was 80 µL/L; Dose II for CAR, CIN, PTS, DDS and BCM was 320 µL/L and for PTSO was 160 µL/L.

[2] Probability of significance effects due to diet, dose and their interaction (DxDo). ns = $P > 0.10$; t = $P < 0.10$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[3] Compounds: CAR (carvacrol), CIN (cinnamaldehyde), PTS (propyl propane thiosulfinate), PTSO (propyl propane thiosulfonate), DDS (diallyl disulfide) and BCM (Bromochloromethane).

[4] Indicates significant ($P < 0.05$) linear (L) or quadratic (Q) effects of the response to incremental dose of each compound estimated by orthogonal polynomial contrast.

Table 3.1.4. *Effects of diet and additive dose on the concentration (log gene copies/ml fresh matter) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea (mcrA gene) in batch cultures after 24 h incubation (Experiment 2).*

		Diet		Dose[1]			*P-value*[2]		
Item	Compound[3]	Barley Beans	Maize Sunflower	Control	Active	s.e.d.	Diet	Dose	DxDo
Bacteria	CAR	12.6	12.7	12.7	12.6	0.22	ns	ns	ns
	CIN	12.7	12.7	12.7	12.7	0.14	ns	ns	ns
	PTS	12.4	12.4	12.4	12.4	0.19	ns	ns	ns
	PTSO	12.3	12.5	12.4	12.5	0.13	t	ns	ns
	DDS	12.3	12.5	12.4	12.4	0.08	**	ns	ns
	BCM	12.4	12.5	12.4	12.5	0.09	ns	ns	*
Archaea	CAR	11.5	11.7	11.8	11.4	0.26	ns	t	ns
	CIN	11.8	11.9	11.8	11.9	0.11	ns	ns	ns
	PTS	10.1	10.2	10.5	9.9	0.39	ns	ns	ns
	PTSO	10.4	10.5	10.5	10.4	0.14	ns	ns	ns
	DDS	10.2	10.3	10.5	10.1	0.12	ns	**	ns
	BCM	10.0	9.2	10.5	8.8	1.12	ns	t	ns
Protozoa	CAR	9.0	8.8	9.0	8.8	0.43	ns	ns	ns
	CIN	9.0	9.1	9.0	9.1	0.13	ns	ns	ns
	PTS	8.5	7.9	9.1	7.4	0.50	ns	**	t
	PTSO	9.1	9.2	9.1	9.2	0.15	ns	t	ns
	DDS	9.2	9.1	9.1	9.2	0.08	ns	ns	ns
	BCM	9.2	9.1	9.1	9.2	0.08	ns	ns	ns

[1] Control correspond to dose 0; Active correspond to dose 320 µL/L in CAR, CIN, PTS and DDS and 160 µL/L in BCM and PTSO.

[2] Probability of significance effects due to diet, dose and their interaction (DxDo). ns = $P>0.10$; t = $P<0.10$; * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$.

[3] Compound: CAR (carvacrol), CIN (cinnamaldehyde), PTS (propyl propane thiosulfinate), PTSO (propyl propane thiosulfonate), DDS (diallyl disulfide) and BCM (Bromochloromethane)

Table 3.1.5. *Effects of different doses of PTS and BCM on daily methane (CH_4) emissions as litres/kg DMI and percentage of gross energy intake (GE intake) by goats. (Experiment 3)*

Item	Compound[2]	Dose[1]				s.e.d.	*P-value*[3]	Contrast[4]
		0	I	II	III			
CH_4, l/day	PTS	21.1	17.9	19.0	10.9	2.56	*	L
	BCM	17.3	12.1	13.4	6.4	2.10	**	L
CH_4, l/kg DMI	PTS	34.5	29.8	30.4	23.1	1.81	***	L
	BCM	43.9	28.6	24.1	15.7	4.97	**	L
CH_4, % of GE intake	PTS	5.0	4.3	4.4	3.3	0.26	***	L
	BCM	6.3	4.1	3.5	2.2	0.71	**	L

[1] Doses I, II and III in PTS were 50, 100 and 200 mg/L rumen content respectively. Doses I, II and III in BCM were 50, 100 and 160 mg/L rumen content respectively.

[2] PTS: propyl propane thiosulfinate, BCM: Bromochloromethane.

[3] Probability of significance effects due to dose. ns = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

[4] Indicates significant ($P < 0.05$) linear (L), quadratic (Q) or cubic (C) effects of the response to incremental dose of each compound estimated by orthogonal polynomial contrast.

Figure captions

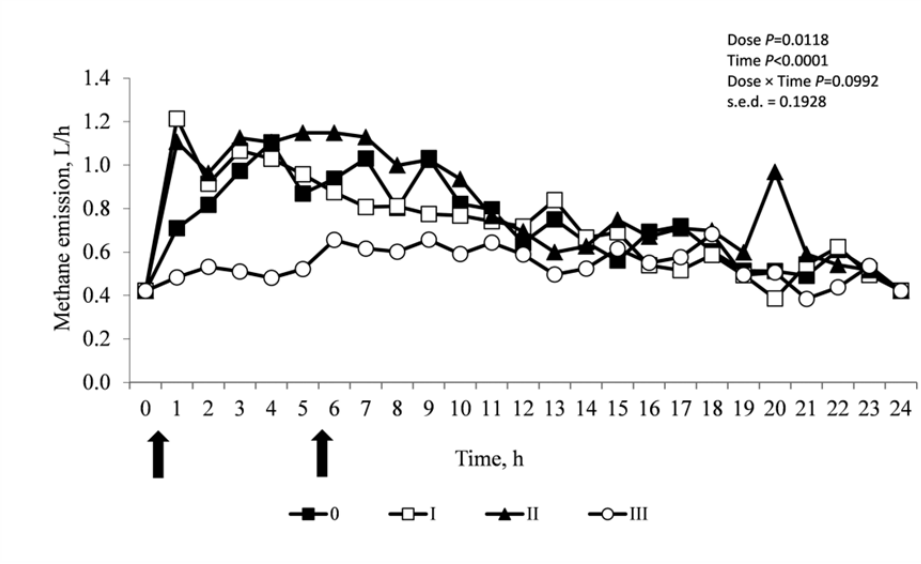

Figure 3.1.1. Effect of propyl propane thiosulfinate (PTS) addition on methane emissions by goats over 24 h. Doses I, II and III in PTS were 50, 100 and 200 mg/L rumen content respectively. The arrows show feeding and treatment addition times.

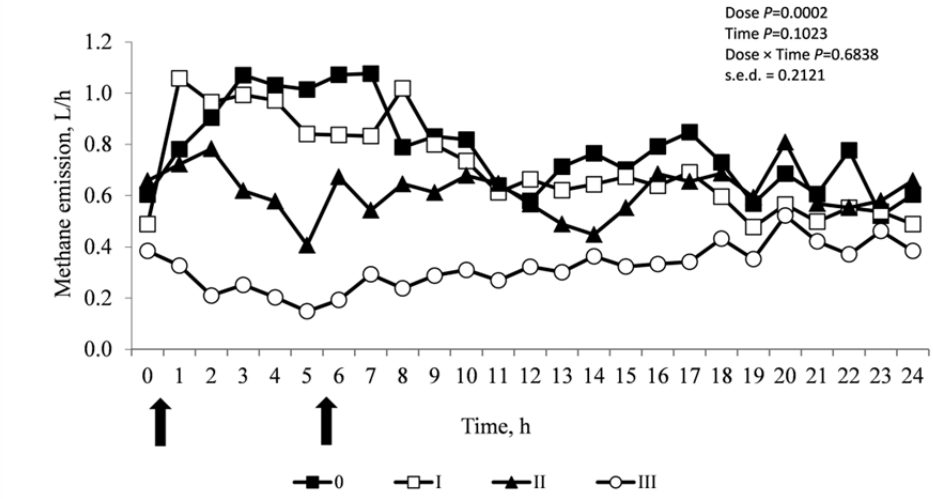

Figure 3.1.2. Effect of bromochloromethane (BCM) addition on methane emissions by goats over 24 h. Doses I, II and III in BCM were 50, 100 and 160 mg/L rumen content respectively. The arrows show feeding and treatment addition times.

Publication 2

Response of the rumen microbial ecosystem to anti-methanogenic organosulphur compounds in continuous-culture fermenters

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Abstract

Eight continuous flow fermenters were used to study the effect of propyl propane thiosulfinate (PTS) and dialyl disulfide (DDS) on rumen fermentation, methane production and microbial population. Fermenters were inoculated with ruminal fluid from goats and treated with DDS (80 µL/L), PTS (200 µL/L) and BCM (160 mg/L), as positive control, in two runs of 12 incubation days. The additives and diet (alfalfa hay:concentrate 1:1) used, were selected based on previous in vitro results. In each incubation run fermenters contents were taken on day 12 to inoculate 24 hours batch cultures using the same diet and treatments to measure methane production. No effects on rumen fermentation were observed with PTS and DDS, while total VFA concentration decreased with BCM. Only amylase activity increased on day 4 and 10 with BCM compared to the control and the other treatments. A decrease on methane production was observed with PTS (48%) and BCM (94%), compared to control and no effect was observed with DDS, The concentration of methanogenic archaea decreased with BCM from day 4 onward, while with PTS the effect disappeared after 12 days of incubation although a methane reduction was observed. The 454 pyrosequencing analysis, from fermenters content samples obtained after 12 days of incubation, revealed that PTS and BCM decreased the relative abundance of archaea belonging to *Methanomicrobiales* order and *Methanomicrobium* genus and increased the relative abundance of *Methanobrevibacter* and *Methanosphera* genus. The change in the community composition was more pronounced for BCM than for PTS. The concentration of total bacteria was not modified by any treatment, although pyrosequencing analysis revealed, with BCM treatment, a change in the structure of the bacteria population, increasing the relative abundance of

Prevotella and decreasing that of *Ruminococcus*. These results suggest that the inhibition of methane production in the rumen exhibited by BCM and PTS is associated with a shift in the archaeal biodiversity that involves an increase in *Methanosphaera* and a decrease in *Methanomicrobium* and changes in the structure of the bacterial community when treating with BCM.

Introduction

Enteric microbial fermentation in ruminants is an important source of anthropogenic methane (CH_4), a potent greenhouse gas (Steinfeld et al., 2006). Its production represents a loss of around 2-12% energy for the animal, and consequently, a decrease of productivity (Morgavi et al., 2010). The microbial populations responsible of fermentation in the rumen are comprised of an extremely diverse and complex mix of bacteria, protozoa, fungi and archaea. This enables the animal to digest and metabolize plant structural carbohydrates that otherwise could not do with only its digestive enzymes. Methanogenic archaea fill the role of terminal reducers of carbon, producing CH_4 from H_2 and CO_2.

In the last decade, a wide range of compounds have been tested for their ability to reduce methane emissions (Benchaar and Greathead, 2011). Some plant secondary metabolites have shown promise due to their antimicrobial activity, including garlic derived compounds. (Hart et al., 2008). However, inconsistent results together with adverse effects on fiber digestion and fermentation have also been reported, with the magnitude of these adverse effects varying depending upon the types and doses and diet composition. Part of the inconsistency in the effects has been associated to the variety of compounds included in the plant extracts, which highlights the importance of using pure active compounds. In some cases, the effect is reversed after a few days of treatment (Soliva et al., 2011). Thus, the research aiming to decrease CH_4 emissions from ruminants has to be built upon a correct understanding on the mechanisms of action involved in relation to the microbial groups targeted (Bacteria and Archaea).

We have recently observed that two Organosulphur compounds (propyl propane tiosulfonate PTS and dialyl disulphide DDS) strongly inhibit methane production (%, respectively) in batch culture after 24 hours incubation of goats' rumen fluid.

However, the mechanisms behind such effect and the persistency in time are unknown. Therefore, the aim of this work was to evaluate the effects of DDS and PTS on rumen fermentation, microbial abundances and community structure and on methane production using longer incubation times in continuous-culture fermenters (CCF) inoculated with goats' rumen fluid.

Materials and methods

Fermenters, treatments, diet and animals

Eight continuous-culture fermenters (CCF) following the model of Muetzel et al. (2009) with an effective volume of 1000 mL were used. The treatments were control (without additive), diallyl disulfide (DDS, 3-prop-2-enyldisulfanyl prop-1-ene purity of 80%), propyl propane thiosulfinate (PTS, purity of 75%) and bromochloromethane (BCM, halogenated aliphatic hydrocarbon, purity of 12%) that was included as antimethanogenic reference compound (positive control). The additives and doses were selected from previous results obtained in batch cultures (Martinez et al. 2011ab): 80 µl/L/day for DDS, 200 µl/L/day for PTS and 160 mg/L/day for BCM. The DDS and BCM were provided by Sigma-Aldrich Chemical; PTS was provided by DMC Research Center SL (Granada, Spain). The BCM was entrapped in α-cyclodextrin matrix (May et al., 1995) before included in the diet to ensure its stability. The experimental diet (Table 3.2.1), was composed of alfalfa hay and concentrate in a 50:50 ratio.

Eight Murciano-granadina goats fitted with permanent rumen cannula were used as donors of rumen content for the experiment. Goats were adapted for 21 days to the experimental diet and were fed once a day (9:00 h) with free access to water and mineral salt block (Pacsa Sanders, Sevilla, Spain). Animals were

cared by trained personnel in accordance with the Spanish guidelines for animal protection (Royal decree, 2005) and the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Directive, 2007). All the experimental procedures involved in this study were approved by the Animal Welfare Committee at the Institute of Animal Nutrition (CSIC, Spain).

Experimental procedure and sampling

Eight continuous-culture fermenters (1L), as described by Muetzel et al. (2009), were used in two replicated incubation runs of 12 days each. Eight adult Murciano-Granadina goats fitted with ruminal canula were used in total as donors of ruminal contents. For each incubation run, two groups of three fermenters were inoculated with a different pool (700 mL) each one obtained from three different animals selected randomly. This resulted in four different pools as follows: pool 1 (goats 1, 2and 3), pool 2 (goats 4, 5 and 6), pool 3 (goats 2, 5 and 7) and pool 4 (goats 3, 6 and 8) dietary treatments (control, DDS, PTS and BCM) were randomly supplied to one of the 4 fermenters with each of the rumen content pool. Every fermenter was fed 16 g of fresh matter per day of the basal diet ground at 1 mm, in two equal portions at 09:00 and 14:00 h. Flow through fermenters was maintained by continuous infusion of artificial saliva (Muetzel et al., 2009) at a rate of 40 mL/h and CO_2 was continuously infused to keep anaerobic conditions. Fermenters were maintained in a water bath at 39ºC. On days 0, 4, 8 and 12 of incubation 10 mL of the fermenters content were collected before the morning feeding for VFA analysis and DNA extraction and kept at -20ºC and -80 ºC, respectively. On days 0, 4 and 8 of incubation 1 mL of the

fermenters content was collected before feeding and frozen at -80ºC for determination of amylase, xylanase and endoglucanase activities. On day 12 10 mL of fermenter contents were collected at 0, 2 and 4 h after the morning feeding and kept at -80ºC for DNA extraction.

On day 12 of each incubation run a 24 hours batch culture trial, based on Theodorou et al (1994) protocol, was used by incubating fermenters content to measure CH4 production. The content of each fermenter was filtered through two layers of cheesecloth while bubbling with CO2. The substrate incubated consisted in 500 mg of the diet fed to fermenters in 120 ml serum bottles with 60 ml of the fermenter content. Three replicates and a blank of each fermenter and treatment were used. Bottles were sealed with rubber stoppers and aluminium caps and incubated at 39ºC in a water bath. At 24 h after inoculation, the gas volume was measured in each bottle and a sample of the gas was collected in a graduated syringe and transferred to a 5 mL vacuum tube (Venoject, Terumo Europe N.V., Leuven, Belgium) and then kept at room temperature before methane concentration was measured by gas chromatography (GC).

Chemical analysis and calculations

Dry matter (method ID 934.01), ash (method ID 942.05), ether extract (method ID 7.045) and crude protein (method ID 984.13) in samples were determined by the procedures of the Association of Official Analytical Chemists (AOAC, 2005). Gross Energy was measured with an adiabatic calorimeter (Model 1356, Parr Instrument Co., Moline, IL). The NDF, ADF and lignin (sa) contents were analysed following the methodology described by Van Soest et al. (1991) using an ANKOM Model 220 Fiber Analyzer (Macedon, NY). The α-amylase enzyme

was used for NDF analysis in the concentrate, and both NDF and ADF were expressed exclusive of residual ash. The ADL content was determined by solubilization of cellulose in the ADF residue with 72% sulphuric acid.

To measure the enzymatic activities in fermenters content, cells were lysed using a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) to release intracellular enzymes. Cell material was removed by centrifugation (10,000×g, 10 min, 4 ºC) and the supernatant was used for analyses. Carboxymethylcellulase (EC 3.2.1.4.), xylanase (EC 3.2.1.8.) and amylase (EC 3.2.1.1.) activities were determined (Giraldo et al., 2008) using carboxymethylcellulose, oat beachwood xylan and soluble starch, respectively, as substrates. Enzymatic activities were expressed as mol of glucose or xylose released in 1 min from the corresponding substrates per mL of sample at 39 ºC and pH 6.5.

The individual VFA concentrations were analysed using the gas chromatography technique described by Isac et al. (1994).

The CH_4 concentration was determined by GC using a HP Hewlett 5890, Packard Series II gas chromatograph (Waldbronn, Germany). A sample of 0.5 mL of gas was injected using a 1 mL Sample-Lock® syringe (Hamilton, Nevada, USA).

The volume of gas produced (mL) was corrected for standard conditions (105 Pa, 298 K), and the amount of methane produced (micromoles) was calculated by multiplying the gas produced (micromoles) with the concentration of methane in the analysed sample.

Real-Time PCR Analysis

Samples from the content of fermenters collected on days 0, 4, 8 and 12 (0, 2 and 4 hours after feeding) of the trials were freeze-dryed and used to isolate DNA

using QIAGEN QIAamp® DNA stool mini kit (Qiagen Ltd., UK) following the manufacturer's instructions but with higher temperature (95ºC) for lysis incubation. The DNA samples were used as templates for quantitative real-time PCR (qPCR) amplification. The numbers of total *Bacteria*, protozoa and methanogenic *Archaea* were quantified by qPCR. Primer sets used and the amplification program were based on Abecia et al (2012).

Pyrosequencing and sequence analysis

otal DNA was extracted from 0.5 g of each sample on day 12 at hour 0, using QIAGEN QIAamp® DNA stool mini kits (Qiagen Ltd, West Sussex, UK). The yield and purity of the extracted DNA were assessed using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Amplification of the bacterial V1-V2 regions of 16SrRNA was performed using the primer pair 27F and 357R (Liu et al., 2007) a 330 bp amplicon . The archaeal hypervariable V6 region of the 16S rRNA gene was amplified using the primer pair 958F and 1048 Rmajor (Galand et al., 2009). Primers incorporated 10 nt barcode tags allowing samples to be multiplexed and Roche/454 adaptors. The PCR was performed in triplicate, in a total volume each one of 25 µL containing 10x PCR buffer, 10 mM dNTP mix, 10 pmol/µL of forward and reverse primers, 1U FastStart Polymerase, and 1 µL of DNA template. The amplification conditions were: an initial denaturation step at 95ºC for 2 min; 30 cycles of denaturation at 95ºC for 30s, annealing at 55ºC for 30s, and elongation at 72ºC for 2 min; and a final extension step at 72ºC for 7 min. The size of the PCR products was then checked on a 1% agarose gel electrophoresis. Then triplicates were pooled together and products were then purified using the short fragment removal method described by Roche

using their GS FLX amplicon DNA preparation guide and AMPure beads. The purified PCR products were quantified using Quant-iT PicoGreen dsDNA quantification kit (Invitrogen) and mixed in equimolar amounts to 10^7 molecules/ µL sample. The amplicon pooled libraries were pyrosequenced on a Roche 454 FLX Titanium. The flowgram (sff) files were converted to fasta DNA (fna) and quality score (qual) file on the 454 cluster and transferred onto a Linux based workstation running the Quantitative Insights Into Microbial Ecology (QIIME) per scripted modules and workflow scripts (Caporaso et al., 2010b). Sequences were filtered to exclude those with mismatches to the primer sequence exceed 6 homopolymer runs or ambiguities and include minimum sequence length down to 80nt for *Archaea* amplicons and 150 nt for *Bacteria* amplicons. The libraries were split according to the 10nt barcode incorporated into the forward primer. The error-corrections of amplicon pyrosequences were made using Acacia (Bragg et al., 2012). Libraries sequenced at different times or different lanes of the 454 were pooled. The OTUs were generated by aligning the reads to the GreenGenes database (DeSantis Jr et al., 2006) using PyNAST(Caporaso et al., 2010a) and clustered at 97% sequence identity using UCLUST (Edgar, 2010) for *Bacteria* and CD-hit for *Archaea* (Li and Godzik, 2006). Taxonomic classification was assigned using the Basic Local Alignment Search Tool (BLAST). Unifrac (Lozupone and Knight, 2005), a phylogenetic method, was used for comparing populations by using the trees constructed during the OTU picking script.

Statistical analysis

Fermentation parameters and microbial population were analysed as a repeated measures univariate analysis using GLM procedure of SPSS (IBM SPSS

Statistics v.19, IBM Corp., Somers, NY). The linear model used for each dependent variable accounted for the effects of treatment (T), time (t) and Txt interaction. Effects were considered significant at $P \leq 0.05$. When significant differences were detected, differences among means were studied using the LSD comparison test. An ANOVA analysis was used to establish differences in OTUS due to the treatments, using the QIIME software.

Results

Ruminal fermentation

Methane produced in batch cultures after 24 hours of incubation (Table 3.2.2) was decreased ($P \leq 0.005$) by PTS (48 %) and BCM (94 %) compared with control. The pH values in fermenters were not affected ($P = 0.308$) by the treatment or time after inoculation ($P = 0.072$). Within treatment total VFA and individual VFA molar proportions differed ($P \leq 0.044$) for days 0 and 4 and then remained unchanged from the day 4 onward. Total VFA concentration decreased ($P < 0.001$) only with BCM addition from day 4 onward compared with control. Acetate molar proportion decreased ($P \leq 0.038$) from day 4 with all the treatments compared with control. Propionate molar proportion increased ($P \leq 0.044$) with all the treatments on day 4, although from day 4 remained higher ($P < 0.001$) only for BCM treatment. *Iso*-butyrate molar proportion was no affected ($P = 0.222$) by any of the treatments. Molar proportion of butyrate was higher ($P \leq 0.034$) with BCM compared with control and the other two treatments from day 4 onward,. Valerate and *Iso*-valerate molar proportions were higher ($P < 0.001$) from day 4 and day 8 onward, respectively, with BCM compared with control and all the

treatments. Acetate topropionate ratio decreased ($P < 0.001$) from day 4 onward with BCM compared with other treatments and control. Only on day 4 the ratio was higher ($P = 0.036$) for PTS and on days 4 and 12 it was lower ($P \leq 0.040$) for DDS compared to the control.

Enzymatic activities

The carboxymethylcellulase, xylanase and amylase activities (Table 3.2.3) decreased ($P \leq 0.036$) in day 4 compared with day 0 for all the treatments. Only amylase activity increased ($P \leq 0.031$) on day 4 and 10 with BCM compared to the control and the other treatments.

Microbial community

The effects of the studied additives during 12 days of treatment on the numbers of bacteria, archaea and protozoa are shown in table 3.2.4. The size of bacterial population increased ($P \leq 0.040$) yon days 4 and 12, increased by BCM treatment as compared with control. The number of archaeal mcrA gene copies was lower ($P \leq 0.035$) with BCM and PTS from day 4 and from day 4 to 8, respectively, compared with control. No effect ($P = 0.547$) on the numbers of protozoa was observed with any treatment. However, a reduction ($P < 0.001$) of protozoa numbers was observed for all the treatments at day 4 compared to 0. The treatment x time interaction was only significant ($P < 0.001$) for the archaeal gene copy numbers.

The Roche/454 pyrosequencing analysis exhibited 21,530 and 36,183 input sequence reads, of *Bacteria* and *Archaea*, respectively. After the removal the low-quality reads, 18,182 bacterial and 29,404 archaeal reads were used for the

analysis.

Bacterial Taxonomic analysis (Figure 3.2.1) showed a substantial shift in the relative abundance of some families in fermenters treated with BCM: lower*Anaeroplasmataceae* (2.6%) and *Ruminococcaceae* (6.1%) than in comparison to the other treatments 5.4% and 10.2%, respectively. The BCM treatment induced greater abundances of *Prevotellaceaea* (15.1%) and *Streptococcaceae* (3.83%) families and of an unclassified belonging to the order *Bacteriodales* (17.1%) in comparison to the other treatments (9.1%, 1.3% and 12.3%, respectively). Treatment with PTS resulted in higher relative abundance of the *Spirochaetaceae* (5.9%) family compared to the other treatments (2.63%).

Taxonomic analysis of the archaeal community (Figure 3.2.2) using the BLAST classifier revealed higher relative abundance of *Methanobrevibacter* genus in samples from fermenters treated with PTS (79.2%) and BCM (73.7%) than for control (60.6%) and DDS (48.7%) treatments. The relative abundance of Archaea belonging to the *Methanosphaera* genus was higher for BCM (25.3%) than for the other three treatments (6.5%). However, the relative abundance of *Methanomicrobium* genus, from the *Methanomicrobiaceae* family was higher for control (32.9%) and DDS (41.4%) than for PTS (11.7%) and BCM (0.3%).

The main OTUs increasing with BCM were classified as *Butyrivibrio* and *Prevotella* genus, while those decreasing with BCM treatment were classified as *Ruminococcus* genus. The PTS treatment increased ($P \leq 0.040$) the abundance of some OTUs classified as *Ruminococcus* and *Prevotella* genus. The DDS only affected ($P < 0.001$) one OTU classified as *Clostridium* genus.

In relation to the archaeal community, BCM and PTS affected ($P \leq 0.023$) the

relative abundance of different OTUs compared with the control. The relative abundance of OTUs classified as *Methanosphaera* and *Methanobrevibacter* genus were increased, while decreased the abundance of *Methanomicrobium* genus.

Unifrac was used to state phylogenetic-based beta diversity based on weighted Unifrac of rarified samples a PCoA plot of archaeal and bacterial microbial communities are shown in Figure 3.2.3 (A and B), respectively. The composition of weighted *Archaea* population showed a different spacial distribution of samples treated with PTS and BCM compared with control and those treated with DDS. Weighted *Bacteria* PCoA plot shows that samples treated with BCM and PTS grouped separately from the other treatments.

Discussion

The interest on the study of potential antimethanogenic effect plant active compounds that modify the microbial ecosystem has increased in last years. A wealth of information on the subject has been generated but knowledge on the mechanisms of action and effects of plant derived compounds on rumen microbial populations is still very limited.

Ruminal fermentation

Halogenated compounds (McAllister and Newbold, 2008), such as BCM and organosulphur compounds (Patra and Yu, 2012; Mateos et al., 2013), such as PTS, have shown antimethanogenic effect in ruminants. In the present work the

addition of PTS and BCM reduced methane production 48% and 98%, respectively, values comparable to those reported *in vitro* (Goel et al., 2009; Patra and Yu, 2012). The lack of effect of DDS on methane production using fermenter content taken on day 12 of treatment may suggest an adaptation of the microorganisms to the presence of this compound, which agrees with the results obtained in sheep by Klevenhusen et al (2011). Those authors suggested that some antimicrobial additives may be degraded by rumen microorganisms. The antimethanogenic effect of PTS found in the present work agrees with previous results obtained *in vitro* and *in vivo* in our group (Martinez et al., 2011a). The treatment x time interaction for some individual VFA molar proportions suggests that some microbial populations could adapt to the presence of the studied compounds. Total VFA concentration and profile was only affected by BCM, in contrast to other observations (Goel et al., 2009; Mitsumori et al., 2011; Abecia et al., 2012)., which could be due to the dose used and suggests that microbial activity could be slightly affected. Busquet et al. (2005) reported no effect of DDS and garlic oil after 9 days of incubation in continuous-culture fermenters on total VFA and modifications of VFA profile with decreased acetate and increased butyrate molar proportions. In the present work acetate:propionate ratio decreased with BCM from day 4 to 12, in agreement with results obtained in previous experiments (Mitsumori et al., 2011; Abecia et al., 2012). Treatment with DDS decreased acetate:propionate ratio only after 12 days of incubation. This reduction has been considered (McAllister et al., 2008) a common feature for antimethanogenic compounds as a result of a redirection of hydrogen from methane to propionic metabolic pathways.

Only BCM treatment increased amylolitic activity on day 4 and 10. Other authors (Hristov et al., 2003) reported decreased amylolytic activity by several bioactive agents using bovine rumen fluid, in contrast to our results. These differences could be due to the different activity, chemical structure or mechanism of action of the compounds used in both studies. Janssen (2010) suggested that cellulolytic microbes produce more acetate and H_2, while amylolytic microbes produce less H_2 and more propionate, so more CH_4 is formed from forage based diets. That could explain the different mechanisms of action of PTS and BCM, so BCM could affect competitors of amylolytic microorganisms, thus increases amylolytic activity and produces less H_2, while PTS could affect other microorganisms. In relation to the time effect, our results were in agreement with results obtained by Martínez et al., (2010) who observed decreased enzymatic activities in Rusitec fermenters after 10 days of incubation.

Microbial community

In despite of the methane reduction observed with the addition of PTS and BCM the abundance of methanogenic *Archaea* was reduced on days 4 and 8 (and on day 12 only with BCM). Goel et al. (2009) reported a sharp decrease in biomass of methanogenic *Archaea* as a result of adding BCM in batch and continuous-culture fermenters, in agreement with our results. On the contraryAbecia et al. (2012) reported no changes in the concentration of methanogenic Archaea in goats treated with BCM for 60 days. This disagreement could be due to the different duration of the treatments (Williams et al., 2009) and the inherent differences between *in vivo*- and *in vitro* conditions. We have recently reported

that the microbial ecosystem that develops in contrinuous culture fermenters differ to that in the original rumen inoculum, which could influence the sensitivity of some species depending on their ability grow *in vitro* (Soto et al., 2012; 2013). some authors reported that adding garlic compounds does not induce changes in the abundance of methanogenic *Archaea* (Ohene-Adjei et al., 2008; Kongmun et al., 2011) , while othersreported decreased archaea population size (Patra and Yu, 2012). The disagreement between our results and other studies using organosulphur compounds could be due again to the duration of the trials, the type and dose of compounds (Patra and Saxena, 2009) or primers set used to quantify the archaeal numbers. In this sense, the variability in concentration of active compounds in plant extracts generates confusion because the effects can be contradictory, according to the content of the active component in the extract and the dose used (Patra and Saxena, 2009). Therefore,It seems necessary either to report concentrations and active compounds in plant extracts or to use pure products to unequivocally define activities, doses, and mechanisms of action.

The pyrosequencing study of *Bacteria* revealed that the most abundant families were *Lachnospiraceae, Ruminococcaceae*, *Prevotellaceae* and unclassified *Bacteroidales* in accordance with other studies (Kong et al., 2010; Zened et al., 2012). The treatment with BCM increased the relative abundance of *Prevotella* and decreased those belonging to *Ruminococcus,* which is in the line of what Mitsumory et al. (2011) observed. Increased abundance of *Prevotella* promoted by treatment with BCM is likely associated to the increasein branched chain fatty acids, propionate and amylolitic activity observed in this study (Mitsumori et al., 2011). The abundance in *Prevotella* could be associated to hydrogen accumulation due to decreased methane production (Goel et al., 2009;Likewise,

the effect of BCM on *Ruminococcus* is in agreement with Mitsumori et al. (2011) that reported a decrease in *R. albus* as a result of treating goats with BCM, due to the high sensitivity to high partial pressure of hydrogen. This decrease might be compensated by greater abundances of other fibrolytic bacteria such as *F. succinogenes* that does not produce H_2 and is not susceptible to H_2 accumulation. With regards to PTS, although a decrease in methane production was observed, it did not induce detectable changes in the bacterial taxonomic distribution, which could be explained by the fact that the reduction in methane was not as dramatic as for BCM (48 and 94 %, respectively for PTS and BCM) and therefore no major shift in metabolic H_2 transfer. Indeed, this is confirmed by the PCA plot in which a distinct group including BCM samples was recognized and separated from the rest.

The dominant archaea belonged to the orders *Methanobacteriales* and *Methanomicrobiales,* in accordance with previous reports (Janssen and Kirs, 2008; Zhou et al., 2009; GU et al., 2011). Both BCM and PTSincreased the relative abundance of *Methanobrevibacter* and decreased that of *Mathenomicorbium* compared with control and DDS. The BCM treatment also increased archaea from *Methanosphaera*. Our results are the first to confirm that BCM reduces methane production in the rumen via a major shift in the distribution of archaeal groups. They also support previous observations by Ohene-Adjei et al. (2008) who reported changes in the archaeal banidng profile by DGGE as a result of treating sheep with garlic oil. Based on the taxonomy derived from pyrosequencing we hypothesize that the extent in which groups are shift is directly associatedto the extent of methane inhibition. However, the different mechanisms of action of BCM and PTS could also explain the different impact

observed. Bromochloromethane directly reacts with reduced vitamin B12 and results in the inhibition of cobamide-dependent methyl group transfer in methanogenesis (Wood et al., 1968). On the other hand the antimicrobial effect of thiosulfinates (Focke et al., 1990; Ruiz et al., 2010) is associated with chemical reaction with thiol groups of various enzymes such as the acetyl-CoA-forming system. Some authors (Busquet et al., 2005; Benchaar and Greathead, 2011) reported the relationship of antimethanogenic effect of organosulphur and the inhibition of HMG-CoA reductase, which play an important role in the synthesis of isoprenoid ethers, the main component of archaeal cell membranes. Therefore, the sensitivity of key archaeal groups may be explained by the different mechanisms of action exhibited, which would need to be further tested using deep-sequencing combined with pure culture in vitro incubations.

In conclusion, the inhibition of methane production in the rumen by BCM (94%) and PTS (48%) is associated with a shift in the archaeal biodiversity that involves an increase in *Methanosphaera* and a decrease in *Methanomicrobium*. In the case of BCM the effect causes changes in the bacterial population that are clearly reflected in the fermentation pattern. The microbial ecosystem adapted to the presence of DDS after 12 days of treatment.

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References

Abecia L., Toral P., Martín-García A., Martínez G., Tomkins N., Molina-Alcaide E., Newbold C., Yáñez-Ruiz D. (2012) Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. Journal of dairy science 95:2027-2036.

Association of Official Analytical Chemists (AOAC) 2005. Official Methods of Analysis 18th ed. AOAC, Gaithersburg, MD.

Benchaar C., Greathead H. (2011) Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology. 166-167: 338-335.

Bragg, L., Stone, G., Imelfort, M., Hugenholtz, P., and Tyson, G. W. (2012). Fast, accurate error-correction of amplicon pyrosequences using Acacia. Nature Methods 9, 425-426.

Busquet M., Calsamiglia S., Ferret A., Carro M., Kamel C. (2005) Effect of garlic oil and four of its compounds on rumen microbial fermentation. Journal of Dairy Science 88:4393-4404.

Caporaso J.G., Bittinger K., Bushman F.D., DeSantis T.Z., Andersen G.L., Knight R. (2010a) PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics 26:266-267.

Caporaso J.G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F.D., Costello E.K., Fierer N., Pena A.G., Goodrich J.K., Gordon J.I. (2010b) QIIME

allows analysis of high-throughput community sequencing data. Nature methods 7:335-336.

DeSantis Jr T., Hugenholtz P., Keller K., Brodie E., Larsen N., Piceno Y., Phan R., Andersen G. (2006) NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. Nucleic acids research 34:W394-W399.

Edgar R.C. (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461.

European Directive 86/609. Commission recommendations of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Annex II to European Council Directive 86/609. The Commission of the European Communittes Publishing, Brussels, Belgium.

Focke M., Feld A., Lichtenthaler H.K. (1990) Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. FEBS letters 261:106-108.

Galand P.E., Casamayor E.O., Kirchman D.L., Potvin M., Lovejoy C. (2009) Unique archaeal assemblages in the Arctic Ocean unveiled by massively parallel tag sequencing. The ISME journal 3:860-869.

Giraldo L., Tejido M., Ranilla M., Carro M. (2008) Effects of exogenous fibrolytic enzymes on in vitro ruminal fermentation of substrates with different forage: concentrate ratios. Animal Feed Science and Technology 141:306-325.

Goel G., Makkar H.P.S., Becker K. (2009) Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen

fermentation using batch and continuous fermentations. British Journal of Nutrition 101:1484-1492.

Gu M.J., Alam M., Kim S.H., Jeon C.O., Chang M.B., Oh Y.K., Lee S.C., Lee S.S. (2011) Analysis of methanogenic archaeal communities of rumen fluid and rumen particles from Korean black goats. Animal Science Journal 82:663-672.

Hart K.J., Yáñez-Ruiz D.R., Duval S.M., McEwan N.R., Newbold C.J. (2008) Plant extracts to manipulate rumen fermentation. Animal Feed Science and Technology 147:8-35.

Isac M., García M., Aguilera J., Molina A.E. (1994) A comparative study of nutrient digestibility, kinetics of digestion and passage and rumen fermentation pattern in goats and sheep offered medium quality forages at the maintenance level of feeding. Archiv für Tierernährung 46:37.

Janssen P.H., Kirs M. (2008) Structure of the archaeal community of the rumen. Applied and environmental microbiology 74:3619-3625.

Klevenhusen F., Zeitz J.O., Duval S., Kreuzer M., Soliva C.R. (2011) Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Animal Feed Science and Technology 166–167:356-363.

Kong Y., Teather R., Forster R. (2010) Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. FEMS microbiology ecology 74:612-622.

Kongmun P., Wanapat M., Pakdee P., Navanukraw C., Yu Z. (2011) Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livestock Science 135:84-92.

Li W., Godzik A. (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22:1658-1659.

Liu Z., Lozupone C., Hamady M., Bushman F.D., Knight R. (2007) Short pyrosequencing reads suffice for accurate microbial community analysis. Nucleic acids research 35:e120.

Lourenco M., Cardozo P., Calsamiglia S., Fievez V. (2008) Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. Journal of animal science.

Lozupone C., Knight R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. Applied and environmental microbiology 71:8228-8235.

Martinez M.E., Ranilla M.J., Tejido M.L., Ramos S., Carro M.D. (2010) Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. Journal of dairy science 93:3684-3698.

Martinez G., Abecia L., Martin-Garcia A.I., Ramos E., Molina-Alcaide E., Ranilla M., Yañez-Ruiz D.R. (2011a) In vitro evaluation of some plant extracts as a methane-inhibiting agents with different degradability diets and rumen inoculum from goats. In: Poster presentations. Advances in Animal Biosciences. 2, pp. 488.

Martínez, G.;Abecia, L.;Martín García, A.I.;Soto, E.C.;Molina Alcaide, E.;García Pareja, M.P.;Ranilla, M.J.;Yáñez Ruiz, D.R. (2011b). Estudio de la adición de aditivos organosulfurados sobre la fermentación ruminal *in vitro* de

dietas de distinta degradabilidad empleando líquido ruminal de caprino. In: XIV Jornadas sobre Producción Animal. Edited by Asociación Interprofesional para el Desarrollo Agrario (AIDA). ISBN: 978-84-615-0062-8. Zaragoza, Spain.

Mateos I., Ranilla M., Tejido M., Saro C., Kamel C. and Carro M.D. (2013). The influence of diet type (dairy versus intensive fattening) on the effectiveness of garlic oil and cinnamaldehyde to manipulate in vitro ruminal fermentation and methane production. Animal Production Science 53, 299-307.

May C., Payne A.L., Stewart P.L., A. E.J. (1995) A delivery system for agents, International Patent Application.

McAllister T., Newbold C.J. (2008) Redirecting rumen fermentation to reduce methanogenesis. Australian Journal of Experimental Agriculture 48:7-13.

Mitsumori M., Shinkai T., Takenaka A., Enishi O., Higuchi K., Kobayashi Y., Nonaka I., Asanuma N., Denman S.E., McSweeney C.S. (2011) Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. British journal of nutrition 108:482-491.

Morgavi D., Forano E., Martin C., Newbold C.J. (2010) Microbial ecosystem and methanogenesis in ruminants. animal 4:1024-1036.

Muetzel S., Lawrence P., Hoffmann E.M., Becker K. (2009) Evaluation of a stratified continuous rumen incubation system. Animal Feed Science and Technology 151:32-43.

Ohene-Adjei S., Chaves A., McAllister T., Benchaar C., Teather R., Forster R. (2008) Evidence of increased diversity of methanogenic archaea with plant extract supplementation. Microbial ecology 56:234-242.

Patra, A. K. and Saxena, J. (2009). Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie van Leeuwenhoek 96, 363-375.

Patra A.K., Yu Z. (2012) Effects of Essential Oils on Methane Production and Fermentation by, and Abundance and Diversity of, Rumen Microbial Populations. Applied and environmental microbiology 78:4271-4280.

Royal Decree No. 1201/2005 of 10 October on the protection of animals used for experimentation and other scientific purposes. Boletin del Estado, pp. 34367-34391. Spanish Government Publishing, Madrid, Spain.

Ruiz R., García M., Lara A., Rubio L. (2010) Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. Veterinary microbiology 144:110-117.

Soliva C.R., Amelchanka S.L., Duval S.M., Kreuzer M. (2011) Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). British Journal of Nutrition 106:114-122.

Soto E., Yáñez-Ruiz D., Cantalapiedra-Hijar G., Vivas A. and Molina-Alcaide E. (2012). Changes in ruminal microbiota due to rumen content processing and incubation in single-flow continuous-culture fermenters. Animal Production Science 52, 813-822.

Soto, E., E. Molina-Alcaide, H. Khelil, and D. Yáñez-Ruiz (2013). Ruminal microbiota developing in different in vitro simulation systems inoculated with goats' rumen liquor. Animal Feed Science and Technology http://dx.doi.org/10.1016/j.anifeedsci.2013.06.003

Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M and de Haan C (2006) Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations, Rome, Italy.

Theodorou M.K., Williams B.A., Dhanoa M.S., McAllan A.B., France J. (1994) A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Animal Feed Science and Technology 48:185-197.

Van Soest P.J., Robertson J., Lewis B. (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of dairy science 74:3583-3597.

Zened A., Combes S., Cauquil L., Mariette J., Klopp C., Bouchez O., Troegeler, Meynadier A., Enjalbert F. (2012) Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. R2. FEMS microbiology ecology.

Zhou M., Hernandez-Sanabria E., Guan L.L. (2009) Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Applied and environmental microbiology 75:6524-6533.

Tables and figures

Table 3.2.1. Chemical composition of alfalfa hay and concentrate (g/kg dry matter) and ingredients (g/kg) of concentrate.

Item	Alfalfa hay	Concentrate
DM (g/kg fresh matter)	907	915
OM	875	884
NDF	513	245
ADF	330	118
ADL	99.2	36.3
CP	203	168
Ether Extract	8.1	15.3
GE (MJ/Kg DM)	18.4	19.5
Ingredients		
Barley		174
Faba beans		233
Maize		174
Sunflower meal		233
Maize gluten meal		116
Rumen-inert fat		70

Table 3.2.2. Effects of additives on CH_4 production (µmol) after 24 hours of incubation in batch culture inoculated with fermenters content after 12 days of incubation and on VFA concentration and profiles and pH on days 0, 4, 8 and 12 of incubation in continuous-culture fermenters.

		Treatment[1]				SEM	*P*-value[2]		
Item	Day	Control	BCM	DDS	PTS		T	t	Txt
Total VFA (mM)	**0**	30.6^{B}	31.6^{B}	28.8^{B}	28.7^{B}	11.4	0.015	<0.001	0.013
	4	76.2^{aA}	62.9^{bA}	74.9^{aA}	73.1^{aA}				
	8	73.9^{aA}	60.2^{bA}	74.82^{aA}	72.4^{aA}				
	12	77.4^{aA}	65.7^{bA}	78.1^{aA}	78.9^{aA}				
Acetate mol/100 mol	**0**	69.0^{A}	68.4^{A}	68.2^{A}	68.4^{A}	1.8	<0.001	<0.001	<0.001
	4	63.7^{aB}	50.0^{cB}	61.4^{bB}	62.8^{abB}				
	8	64.9^{aB}	49.4^{cB}	62.6^{bB}	62.6^{bB}				
	12	64.6^{aB}	49.6^{bB}	61.9^{aB}	62.1^{aB}				
Propionate mol/100 mol	**0**	13.8^{B}	14.1^{C}	14.1^{B}	13.9^{B}	1.5	<0.001	<0.001	<0.001
	4	16.0^{bA}	25.8^{aA}	17.6^{bA}	17.1^{bA}				
	8	17.0^{cA}	24.1^{aAB}	17.5^{bA}	17.8^{bA}				
	12	17.8^{bA}	23.4^{aB}	18.6^{bA}	18.0^{bA}				
Isobutyrate	**0**	1.88^{A}	1.85^{A}	1.80^{A}	1.83^{A}	0.16	0.163	0.013	0.118
	4	1.48^{B}	1.23^{B}	1.38^{B}	1.43^{B}				
	8	1.33^{B}	1.25^{B}	1.35^{B}	1.23^{B}				
	12	1.30^{B}	1.22^{B}	1.35^{B}	1.35^{B}				
butyrate	**0**	11.2	11.4^{B}	12.1^{B}	11.9	0.7	0.002	<0.001	<0.001
	4	13.8^{b}	16.3^{aA}	14.5^{bA}	13.9^{b}				
	8	12.2^{b}	15.4^{aA}	13.3^{bAB}	13.8^{ab}				
	12	11.7^{c}	15.6^{aA}	12.9^{bcAB}	13.7^{b}				
Isovalerate	**0**	2.93	3.03	2.70	2.83	0.39	<0.001	0.405	<0.001
	4	2.78	2.73	2.75	2.50				
	8	2.38^{b}	5.38^{a}	2.68^{b}	2.20^{b}				
	12	2.38^{b}	5.90^{a}	2.58^{b}	2.33^{b}				
Valerate	**0**	1.28^{B}	1.28^{B}	1.25^{C}	1.20^{B}	0.32	<0.001	<0.001	<0.001
	4	2.20^{bA}	4.00^{aA}	2.38^{bB}	2.35^{bA}				
	8	2.20^{cA}	4.43^{aA}	2.50^{bAB}	2.38^{bcA}				
	12	2.33^{bA}	4.38^{aA}	2.68^{bA}	2.55^{bA}				
Acetate:Propionate ratio	**0**	5.02^{A}	4.88^{A}	4.90^{A}	4.97^{A}	0.46	<0.001	<0.001	<0.001
	4	3.98^{aB}	1.94^{dB}	3.48^{cB}	3.67^{bB}				
	8	3.85^{aB}	2.06^{bB}	3.57^{aB}	3.52^{aB}				
	12	3.67^{aB}	2.13^{cB}	3.34^{bB}	3.46^{abB}				
pH	**0**	6.55^{A}	6.58	6.55^{A}	6.58	0.22	0.138	0.033	0.668
	4	6.39^{AB}	6.45	6.43^{AB}	6.39				

	8	6.31^{B}	6.44	6.41^{AB}	6.40				
	12	6.36^{AB}	6.44	6.38^{B}	6.40				
Methane production	**13**	249^{a}	14^{c}	248^{a}	129^{b}	1	<0.001	n.d.	n.d.

[1] Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane thiosulfinate) and BCM (Bromochloromethane).

[2] T: treatment effect; t: time effect; Txt: Treatment x time interaction.

[a-c] within a row treatment means without a common superscript differ, $P < 0.05$.

[A-B] within a column treatment without a common superscript differ, $P < 0.05$.

Table 3.2.3. Effect of the additives on xylanase, amylase and endogluconase activities in single-flow continuous-culture fermenters content sampled on day 0, 4 and 10 days after inoculation.

		Treatment[1]				SEM	*P*-value[2]		
Item	Day	Control	BCM	DDS	PTS		T	t	Txt
Xylanase	**0**	19.4^{A}	18.9^{A}	18.0^{A}	17.7^{A}	3.58	0.410	0.029	0.996
	4	5.72^{B}	6.52^{B}	5.65^{B}	5.14^{B}				
	10	6.91^{B}	7.10^{B}	6.08^{B}	5.22^{B}				
Amylase	**0**	5.04^{A}	5.72^{A}	4.68^{A}	4.66^{A}	0.75	0.010	0.010	0.051
	4	1.03^{bB}	1.33^{aB}	1.02^{bB}	1.04^{abB}				
	10	1.10^{bB}	1.23^{aB}	1.06^{bB}	1.08^{bB}				
Endoglucanase	**0**	2.54^{A}	2.51^{A}	2.08^{A}	2.37^{A}	0.38	0.169	<0.001	0.086
	4	0.19^{B}	0.14^{B}	0.19^{B}	0.14^{B}				
	10	-	-	-	-				

[1] Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane thiosulfinate) and BCM (Bromochloromethane).

[2] T: treatment effect; t: time effect; Txt: Treatment x time interaction. $^{a-c}$ within a row treatment means without a common superscript differ, $P < 0.05$. $^{A-B}$ within a colum treatment without a common superscript differ, $P < 0.05$.

Amylase activity is expressed as nanomoles of glucose released from soluble starch by 1 mL of ruminal fluid in 1 min at 39ºC and pH=6.5. Xylanase activity is expressed as nanomoles of xylose liberated from oat beachwood xylan by 1 mL of ruminal fluid in 1 min at 39ºC and pH=6.5.

Table 3.2.4. Effects of the additives on the concentration (log copy gene numbers/g fresh matter) of total Bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic Archaea (mcrA gene) in fermenters after 0, 4, 8 and 12 days of incubation.

Item	Day	Treatment[1]				SEM	*P*-value[2]		
		Control	BCM	DDS	PTS		T	t	Txt
Archaea	**0**	8.72	8.63^{A}	8.69^{AB}	8.68^{A}	0.10	<0.001	0.027	<0.001
	4	8.62^{a}	7.91^{bB}	8.17^{abB}	7.96^{abAB}				
	8	8.69^{a}	7.19^{cC}	8.37^{bAB}	8.26^{bB}				
	12	8.81^{a}	6.90^{bC}	8.71^{aA}	8.26^{aAB}				
Protozoa	**0**	8.11^{A}	7.96^{A}	8.14^{A}	8.05^{A}	0.51	0.551	<0.001	0.033
	4	7.23^{B}	7.13^{B}	7.28^{B}	6.96^{B}				
	8	6.93^{B}	7.02^{B}	6.48^{C}	6.74^{B}				
	12	6.79^{B}	6.99^{B}	6.63^{BC}	6.92^{B}				
Bacteria	**0**	9.86^{A}	9.82^{A}	9.88^{A}	9.79^{A}	0.09	0.010	0.002	0.099
	4	9.49^{bB}	9.76^{aA}	9.58^{abB}	9.54^{bABC}				
	8	9.50^{B}	9.56^{B}	9.45^{B}	9.44^{C}				
	12	9.51^{B}	9.74^{A}	9.53^{B}	9.68^{AB}				

[1] Treatment: control (without additive), DDS (diallyl disulfide), PTS (propyl propane thiosulfinate) and BCM (Bromochloromethane).

[2] T: treatment effect; t: time effect; Txt: Treatment x time interaction.

[a-c] within a row treatment means without a common superscript differ, $P < 0.05$.

[A-B] within a colum treatment without a common superscript differ, $P < 0.05$.

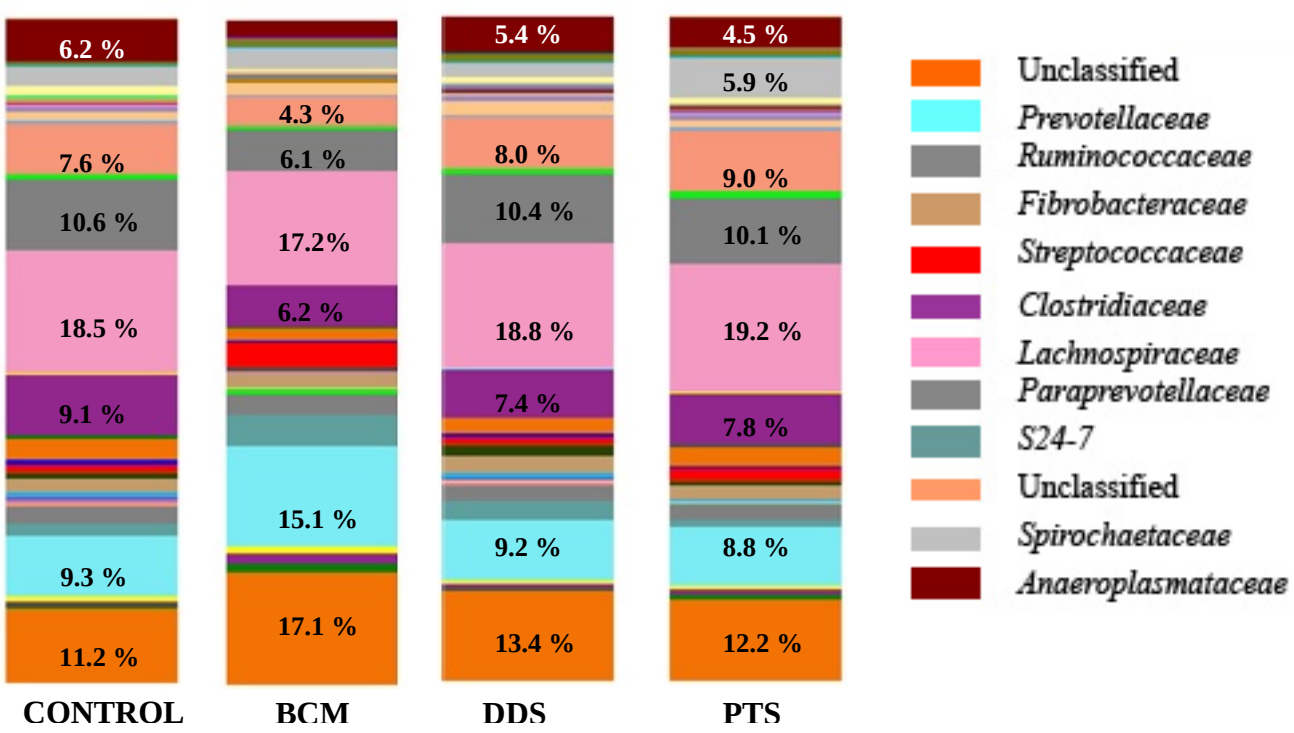

Figure 3.2.1. Bacterial taxonomic compositions of the fermenters' content without treatment (CONTROL), or treated with BCM, DDS and PTS after 12 days of incubation.at family level. Sequences were classified using BLAST with a 97% similarity level.

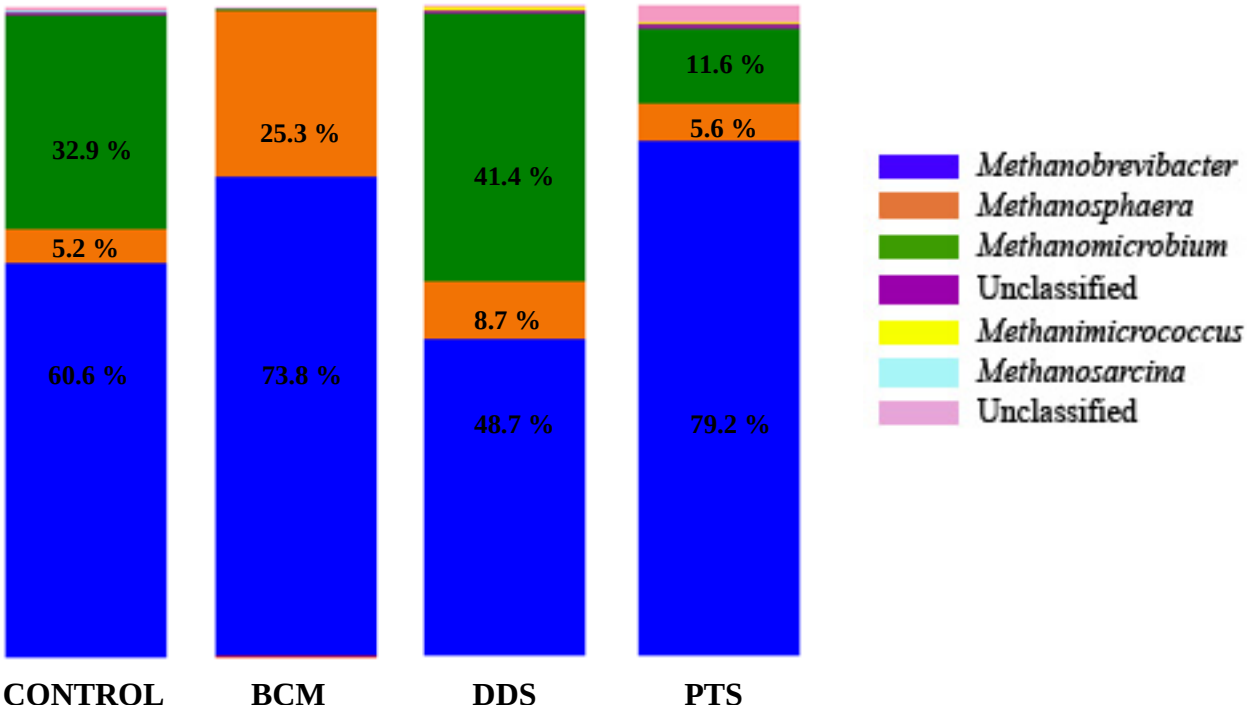

Figure 3.2.2. Archaeal taxonomic composition of the fermenters' content without treatment (CONTROL), or treated with BCM, DDS and PTS after 12 days of incubation. Sequences were classified using BLAST with a 97% similarity level.

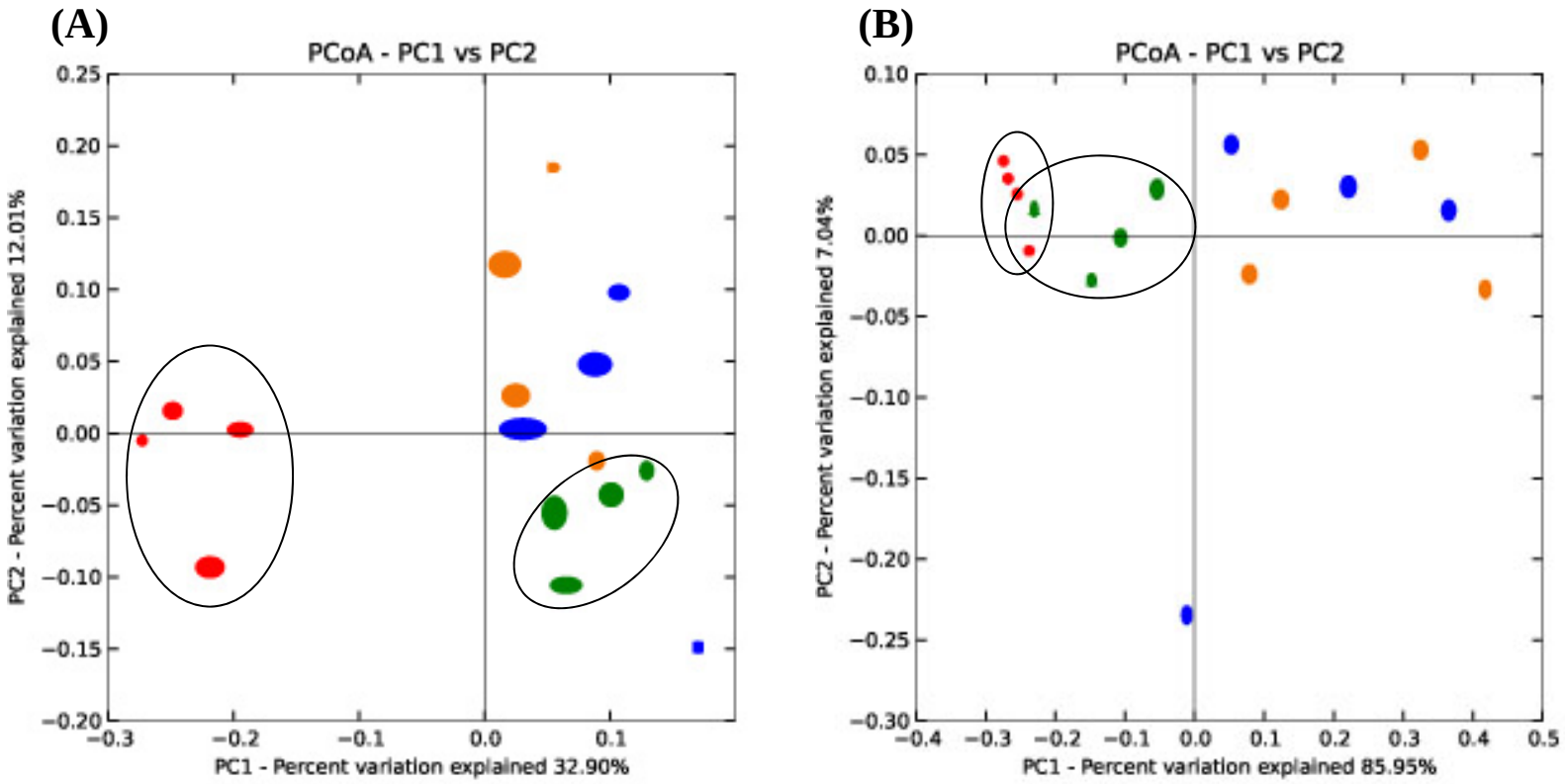

Figure 3.2.3. PCoA plot results showing the relationships of bacterial (A) and archaeal (B) communities of the fermenters treated with BCM (Red), PTS (Green), DDS (Orange) and without treatment (Blue) after 12 days of incubation. The PCoA plots were constructed using the weighted UniFrac method.

Publication 3

Effects of propyl propane thiosulfinate on nutrient utilization, ruminal fermentation, microbial population and methane emissions in goats

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Abstract

The aim of this work was to investigate the effect of α-cyclodextrin-propyl propane thiosulfinate complex (CD-PTS), an organosulphur compound with potential antimethanogenic activity, in goats on ruminal methane production, fermentation pattern, the major microbial groups and nutrient utilization. Additionally, a methanogen pure culture trial was conducted to test propyl propane thiosulfinate (PTS) at a dose of 200 ppm and bromoclorомethane (BCM) at a concentration of 10 against three different archaeal strains: *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii* and *Methanobrevibacter millerae*, showing a substantial reduction with both compounds of methane production in all the strains. The *in vivo* trial was conducted over 36 days, to study the effect of CD-PTS in goat. Twelve adult goats and fed with alfalfa hay and concentrate (50:50), were grouped in two group and treated with α-cyclodextrin-PTS complex at one dose (200 mg/L rumen content per day) or without any treatment (control) over 36 d. On d 7, 14, 21 and 28 methane emissions were recorded in chambers and rumen fluid samples were collected for pH, VFA and NH_3 analysis. On day 14 and 28 rumen samples were collected for quantification of bacterial, protozoal and archaeal numbers by real-time PCR and study the archaeal structure population by denaturing gradient gel electrophoresis. In addition, on days 17 and 18 samples of alfalfa hay were placed into nylon bags and incubated in the rumen of each goat for 24 and 48 h, respectively, to determine the ruminal degradation of DM and aNDFom. Finally, on day 30 animals were moved to individual metabolism crates to determine nutrient digestibility, N and energy use and urinary purine derivative (PD) excretion. No significant ($P \leq 0.17$) reduction on

methane production was observed although a numerical reduction on day 28 was observed with CD-PTS. The DMI, apparent digestibility of nutrients, N and energy use, purine derivatives, creatinine and N microbial flow were no affected by the treatment (P≤0.18). Likewise, total concentration of the analyzed microbial groups in the rumen, showed no difference (P≤0.33) between treated and non-treated goats. However, on day 28 a change in archaeal structure population was observed in goats treated with CD-PTS compared with control goats. The effect observed *in vivo* does not confirm what previously observed *in vitro*, which could be related to the means of administration of the active conpound to the animals. Further research is needed to evaluate the longer-term efficacy of α-cyclodextrin-PTS complex for inhibiting methanogenesis and to examine animal performance trials.

Keywords: propyl propane thiosulfinate, rumen, methane, archaea, goat

Abbreviations: ADFom, acid detergent fiber expressed exclusive of residual ash; aNDFom, neutral detergent fiber assayed with heat stable amylase and expressed exclusive of residual ash; BCM, bromocloromethane; BW, body weight; $BW^{0.75}$, metabolic weight; CD-PTS, α-cyclodextrin-propyl propane thiosulfinate complex; CP, crude protein, DGGE, denaturing gradient gel electrophoresis; DM, dry matter; DMI, dry matter intake; Lignin(sa), lignin measured by solubilization of cellulose with sulphuric acid; OM, organic matter; PD, purine derivative; PTS, propyl propane thiosulfinate; qPCR, quantitative polymerase-chain reaction; VFA, volatile fatty acid.

Introduction

The enteric fermentation in ruminants contributes to around 30% of global methane emissions (Steinfeld et al., 2006). In addition methane represents a loss of around 2-12 % of energy ingested by the animal (Johnson and Johnson, 1995). Since reduction in methane emissions could improve productivity in ruminants and decrease the environmental impact of animal production (McAllister and Newbold, 2008), significant efforts are being made to develop nutritional strategies, based on the use of active compounds with antimicrobial activity, to reduce methane production in ruminants (Benchaar and Greathead, 2011). The majority of the studies focus on identifying additives that specifically affect the activity of methanogenic archaea, responsible for the synthesis of methane in the rumen. The results obtained are mainly based on *in vitro* studies and have been shown to be contradictory (Hart et al., 2008; Patra and Saxena, 2010; Benchaar and Greathead, 2011). The few results obtained *in vivo* are variable and in most cases do not confirm *in vitro* observations. This could be explained by different factors: the effective concentration of active compound in the rumen, the effect of the type of diet fed to animals and the time of treatment to allow adaptation of microbiota to the compound. The lack of direct comparative *in vitro* – *in vivo* studies is definitely limiting the understanding of the factors involved (Benchaar and Greathead, 2011).

On the other hand, the antimethanogenic effect of some garlic-derived compounds *in vitro* has been widely reported (Busquet et al., 2005b; Kamel et al., 2008; Mateos et al., 2013), but the few studies conducted *in vivo* not always confirm such potential (Patra and Saxena, 2010; Klevenhusen et al., 2011). Williams et al. (2009) suggested that ruminal methanogens take longer than 4

weeks to adapt to dietary changes, compared with only 10 to 15 days needed for bacterial community adaptation. Therefore, medium-term *in vivo* experiments would be necessary to state the effects of different compounds on methanogens and on methane emissions. The propyl propane thiosulfinate (PTS), a novel organosulphur garlic-derived compound, has been tested *in vitro* and short-term (9 days) *in vivo* trials in our group (Martinez-Fernandez, under review), exhibiting a substantial decrease (up to 33 %) of methane production. However, the persistency of such an effect in a longer-term study needs to be confirmed.

Therefore, the present study was designed to investigate the effects of treating nonproductive goats over a month with α-cyclodextrin-PTS complex (CD-PTS) on nutrient utilization, ruminal fermentation, methane emissions and rumen archaeal population.

Material and methods

Animals, diet and organosulphur compound

Twelve adult dry Murciano-granadina goats (37.8 ± 5.73) fitted with permanent rumen cannula were used. Animals had free access to water and were fed twice a day (0900h and 1600h) at approximately 1.1 times the energy requirements for maintenance level (Prieto et al., 1990) a diet that consisted of alfalfa hay chopped at 15-20 cm and concentrate in a 50:50 ratio and a mineral-vitamin supplement. The concentrate ingredients composition (g/kg) were wheat meal (350), barley (210), sunflower meal (150), corn meal (90), sorghum (80), soybean meal (50), soybean peel (40), NaCl (20.5) and a vitamin–mineral mixture (10.5) (table 3.3.1). Animals were cared by trained personnel and

managed in accordance with the Spanish guidelines for experimental animal protection (Royal Decree No. 1201/2005) and the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Directive 86/609). All the experimental procedures involved in this study were approved by the Animal Welfare Committee at the Institute of Animal Nutrition (CSIC, Spain). In chambers used for methane emissions measurement temperature, humidity and air turn out were carefully monitored according to the animal welfare conditions. The CO_2 concentration was also continuously monitored to ensure a good air quality and renovation rate in the chambers. Animals did not show any stress-related behavior while they were allocated in chambers.

The PTS is an organosulphur compound derived from garlic that was provided by DMC Research Center SL (Granada, Spain). In order to avoid volatilization the PTS, was entrapped in an α-cyclodextrin matrix, the mixture containing 9 % (wt/wt) of PTS.

Experimental design and sampling

A 36 days trial was carried out. During the whole trial individual daily intake of dry matter (DMI) was registered. Animals were randomly distributed into 2 experimental groups: control (without additive) and treated (0.208 g/kg of BW of CD-PTS mixture per day to achieve a concentration of 200 mg/kg rumen content). Twice a day before feeding (9:00 and 15:00 h), the corresponding amounts of CD-PTS 9% mixture plus 1 g of ground oats placed in a cellulose package were placed directly into the rumen via cannula Animals in the control group received oats as well. Treated animals were progressively adapted to the

CD-PTS dose by administering 25, 50, 75 and 100 % of the total dose, respectively over the first 4 days of the trial.

On days 0, 7, 14, 21 and 28 of the trial, around 50 mL of rumen content were collected from each goat 4 h after feeding. On day 14 and 28 fifty 10 mL were immediately kept at −80°C before DNA extraction. The remaining sample was strained through 2 layers of cheesecloth for pH measurements and stored at -20ºC for VFA and NH_3-N analyses. On days 17 and 18 samples of alfalfa hay were placed into nylon bags and incubated in the rumen of each goat for 24 and 48 h, respectively, to determine the ruminal degradation of DM and aNDFom. On days 7, 14, 21 and 28 animals body weight was recorded and then individually placed in the respiration chambers for two days and methane measured over 24 h as described by Abecia et al. (2012).

On day 30 animals were moved to individual metabolism crates as described by Romero-Huelva et al. (2013). After 2 days of adaptation to the crates, total feces and urine produced were individually collected for 5 days to determine nutrient digestibility, N and energy balances and urinary purine derivative (PD) excretion.

Ruminal degradability of alfalfa hay

Ruminal degradability was measured on 3-g of 2 mm ground alfalfa hay. Samples were placed in 5 cm x 10 cm nylon bags with a pore size of 50 µm (#R510 Ankom *in situ* bags, Macedon NY). Four bags were incubated in the rumen of each goat immediately before the morning feeding two of them were withdrawn after 24 h and the other two after 48 h of incubation. The incubations times were chosen based on average rumen passage rate as stated for

different feedstuffs in goats (Yáñez-Ruiz et al., 2004). At the end of corresponding incubation time bags were washed with cold water and maintained at -20 ºC before to be washed in a washing machine using a short cold water program including two bags per feed that had not been incubated in the rumen to account for solubility. After washing, the bags were placed in the oven at 60 ºC for 48 h. Ruminal degradability (%) was calculated as the loss of dry matter and aNDFom over the corresponding incubation time.

Real-time PCR analysis

Samples of rumen contents were freeze-dried and thoroughly mixed by physical disruption using a bead beater (Mini-bead Beater; BioSpec Products, Bartlesville, OK, USA). The DNA extraction was performed using the QIAamp® DNA Stool Mini Kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions with a higher (95ºC) than the recommended temperature for lysis incubation. The DNA samples were used as templates for quantitative real-time PCR (qPCR) amplification. The numbers of total bacteria, protozoa and methanogenic archaea were quantified as described by Abecia et al*.,* (2012) in a previous work.

PCR-DGGE analysis of the methanogenic archaeal population

For denaturing gradient gel electrophoresis (DGGE) analysis of archaeal community, the mcrA gene was amplified and biodiversity indices were calculated as described by Abecia et al. (2013).

Chemical Analyses and calculations

The VFA were analyzed by gas chromatography and ammonia N concentration by using a colorimetric methodology following the protocols described by (Cantalapiedra-Hijar et al., 2009).

Feed samples were ground through a 1-mm sieve before analysis, and DM, ash, ether extract and N contents were determined following the AOAC official methods 934.01, 942.05, 920.39 and 984.13 respectively (AOAC, 2005). Gross energy was determined in an adiabatic bomb calorimeter (Gallenkamp & Co. Ltd., London, UK) according to the methodology described by Prieto et al. (1990). The aNDFom, ADFom and lignin (sa) contents analyses were performed by the sequential procedure of Van Soest et al. (1991) using the Ankom 2000 Fiber Analyzer (Ankom Technology Corp., Macedon, NY). The α-amylase enzyme was used for aNDFom analysis in concentrate, and both aNDFom and ADFom were expressed exclusive of residual ash. The lignin (sa) content was determined by solubilization of cellulose in the ADFom residue with 72% sulphuric acid. The N values were determined by the Kjeldahl procedure were converted to crude protein (CP) by multiplying by 6.25. Urinary PD (allantoin, hypoxanthine, uric acid, and xanthine) and creatinine were determined following the procedures described by Balcells et al. (1992).

The apparent nutrient digestibility was calculated from nutrient intakes and losses in feces. Microbial N flow was calculated as described by Belenguer et al. (2002) for goats: MNF (g/d) = (PD excretion/0.76)/ (0.92 × PB:N), where 0.76 is the incremental recovery of PD (Belenguer et al., 2002), 0.92 is the true digestibility of duodenal PB (Chen et al., 1990), and PB:N (0.83) is the ratio between PB and N content recorded in bacterial pellets isolated from the rumen

of goats in different previous works using similar diets (Cantalapiedra-Hijar et al., 2009; Romero-Huelva and Molina-Alcaide, 2013).

Methanogens Pure Cultures

In addition to the study of the archaeal community in the rumen of goats, a pure culture in vitro experiment was carried to investigate the effect of including CD-PTS on the growth of three methanogenic strains: *Methanobrevibacter ruminantium* DSM1093, ATCC 35063 and JCM13430; *Methanobrevibacter smithii* DSM 861 and ATCC 35061 and *Methanobrevibacter millerae,* DSM 16643 and OCM 820. The pure cultures were acquired from DSMZ - German Collection of microorganisms and cell culture.

Archaea culture was carried out in Hungate tubes with medium and growing conditions as specified by DSMZ for anaerobes. Media (119, 120 and 161), described in detail at the web site (i.e.: www.dsmz.de/media/med119.htm) were prepared anaerobically, aseptically and under an atmosphere consisting of 80 % H_2 and 20 % CO_2.

For the inoculation, ampoules with the different pure cultures were handled within an anaerobic chamber and under an atmosphere consisting of 80 % H_2 and 20 % CO_2 as specified by DSMZ (www.dsmz.de).

Four Hungate tubes containing 5 mL of the corresponding specific medium of every strain were used for the following treatments: control, PTS treatment (75% of purity), tween20 and bromoclоromethane (BCM). Tween20 was used as carrier to dilute PTS before application in the culture in a ratio 1:2 and BCM as positive control with proved antimethanogenic effect (Goel et al., 2009; Abecia et al., 2012). The doses used were 200 ppm for PTS, a concentration of

10 µM for BCM and 100 ppm for tween20 an equivalent concentration that was used to dilute PTS treatment. Cultures were incubated for 12 d and the corresponding treatment was applied on d 2.

Pressurization with H_2/CO_2 gas in anaerobic chamber was applied to achieve 1 bar in the tubes headspace. Tubes were horizontally placed in a shaking incubator at 37ºC at 120 rev min-1 in the dark.

Methanogen growth was followed by CH_4 production (Balch & Wolfe, 1976; Balch et al., 1979). On days 2, 4, 6, 8, 10 and 12 of incubation 0.5 mL-samples of gas produced were taken, H_2 was added to maintain 1 bar pressure in the tubes and then CH_4 concentration was measured (Chong-Song et al., 2002) by manually injecting the gas in a flame ionization-detection GC (HP Hewlett 5890, Packard Series II, Waldbronn, Germany) using a 1 mL Sample-Lock® syringe (Hamilton, Nevada, USA). The concentration of CH_4 was determined using a standard curve generated by injecting different volumes of 99.9 % pure CH_4 pre and post the injection of samples.

Statistical Analyses

Data were analyzed as a mixed model using the PROC MIXED (version 9.3, SAS Institute Inc., Cary, NC, USA). Data for BW, DMI, methane emissions, pH, VFA, and NH_3-N were analyzed as repeated measures. The effects of the treatment (treat), time and treat × time interaction were considered fixed, and animal effect was considered random. Other parameters were analyzed as univariate model using the MIXED procedure of SAS. The statistical model included treatment as fixed effect and the animal as a random effect. Effects

were declared significant at P < 0.05 and P-values between 0.05 and 0.10 were considered as a trend.

Results

Neither BW (P=0.41) or DMI (P=0.83) were affected by CD-PTS treatment. Methane emissions were no modified by neither the treatment (P≤0.17) nor day of administration (P≤0.76), although a numerical reduction of 10% compared with the control, was observed, on days 14, 21 and 28 (Table 3.3.2). The average values of pH, ammonia, total VFA and VFA profile were similar (P≤0.27) for goats treated with CD-PTS and control over the trial. As pointed for methane, a numerical reduction of the acetate to propionate ratio was observed in animals treated with CD-PTS from day 14 onwards, compared with goats fed the control diet.

The nutrients intake and apparent digestibility of DM, OM, fat, CP, aNDFom and ADFom was no modified (P≤0.49) by CD-PTS treatment (Table 3.3.3). No effect of CD-PTS (P≤0.46) was observed on DM and NDF degradability of alfalfa hay at 24 and 48 h.

The metabolic BW, N and energy use were similar (P≤0.33) for treated and non-treated animals. The average values of purine derivatives and creatinine urinary excretion were not affected (P≤0.18) by CD-PTS (Table 3.3.4). As a result, the estimated microbial N flow to the duodenum and efficiency of microbial protein synthesis were similar (P≥0.33) between treatments.

The abundance of total bacteria, protozoa and methanogens on days 14 and 28 (Table 3.3.5) were not affected by the treatment (P≤0.33) and the time (P≤0.10) although a time x effect (P=0.029) interaction was observed for methanogens.

On the other hand, the richness and Shannon index obtained from the DGGE analysis of methanogens banding profile (Figure 3.3.2) were not (P≤0.44) affected by PTS treatment. The dendrogram obtained from the study of the similarity in the banding profile is shown in Figure 3.3.1. The dendrogram showed that on day 14 samples were separated by the animal and not by the treatment and grouped in two different clusters. However, on day 28 four of the samples belonging to the animals treated with CD-PTS were grouped in a sole cluster with 60% of similarity, while non-treated animals were grouped in different clusters showing lower similarity.

The *in vitro* growth of different Archaea strains over 12 days is shown in Figure 3.3.3. A steady state growth was achieved around 7 days post inoculation of culture, probably due to the limitation of some nutrients or the saturation with metabolic end-products. Both BCM and PTS had potent inhibition effect on all strains with no sign of adaptation or recovery. The addition of Tween 80 to the PTS did not affect the growth of any strain.

Discussion

Garlic contains a variety of organosulphur compounds, that have shown *in vitro* inhibitory effect on rumen methanogenesis (Busquet et al., 2005b; Patra et al., 2010; Soliva et al., 2011; Mateos et al., 2013). However *in vivo* studies and the effects on ruminal fermentation patterns, methane production and microbial population are not yet fully understood. This study was designed to examine a novel organosulphur compound, (PTS) that had been previously evaluated *in vitro* and in a short-term *in vivo* trial in our group (Martínez-Fernández et al. 2013) with promising results: decreased methane production (up to 96 %) at an

effective dose that ranges from 160 to 320 µL/L of rumen contents in batch culture. A significant linear decrease in methane production was also observed *in vivo* over 9 d treatment of three increasing doses: 50, 100 and 200 µL of PTS/L of rumen content in adult goats (Martínez-Fernández et al., 2013). In our study, PTS was entrapped in a CD matrix to avoid losses of active compound over the course of the trial and to increase the distribution across the rumen compartments. Although the CD-PTS dosage in relation to rumen contents was 200 mg of PTS/L, the effects on methane emissions on days 7, 14, 21 and 28 were not significant. Similar differences between *in vitro* and *in vivo* results have been reported with another garlic compound, diallyl disulfide, a compound with antimethanogenic effect *in vitro* (Busquet et al., 2005b; Kamel et al., 2008) but with no effect on methane emissions when similar doses were tested in sheep (Klevenhusen et al., 2011) or dairy cows (van Zijderveld et al., 2011). The same has been observed with Japanese horseradish oil, which exhibited greater inhibitions of methane production *in vitro* (89%) than *in vivo* (18.7%) using similar doses (Mohammed et al., 2004). Although in the present work a non-significant effect on methane production and acetate/propionate ratio was found for CD-PTS, a reduction of 10% in methane production and acetate/propionate ratio existeed on day 28 of treatment (10%), which is in agreement with the reported relation between methane production and propionate concentration in the rumen. In this sense, Mitsumori et al (2012) observed a significant linear relationship between methane production and the (C2+C4)/C3 ratio (y = 9.65x – 25.7 (r^2 0.698)) and Moss et al. (2000) also observed a negative correlation between methane and C2/C3 ratio (r^2 = 0.772). The lack of significant effect of CD-PTS in this trial as compared to previous short-term *in vitro* and *in vivo*

studies could be explained by some or a combination of different factors: *i*) the compound administration was gradual during the first days of the trial, so the effects could be less marked than in the short *in vivo* study in which the dosage was the same from the beginning, *ii*) a possible adaptation of the rumen microorganisms to the compound as a result of a prolonged administration; *iii*) the different distribution in the rumen of CD-PTS across the rumen compartments or a potential wash out effect when it is included in cyclodextrine compared with an application of purified compound (Martínez-Fernández et al., 2013). Passage of CD-PTS out of the rumen *in vivo* therefore seems likely to exert some influence. In addition, the direct extrapolation of the compound dose from *in vitro* to *in vivo* did not take into account the complexity of the microbial community in the animals compared with the *in vitro* systems (Soto et al., 2012) and the rumen outflow, which would require an increase of the daily dosage to be tested in *in vivo* conditions.

A decrease of methane production is associated with greater rumen H_2 concentrations, and an increased of propionate concentrations as a fermentation end-product. In this sense, some authors (McAllister and Newbold, 2008; Janssen, 2010) reported that partial inhibition of methanogens results in higher H_2 concentrations, less CH_4, and more propionate formation. Other authors (Van Nevel et al., 1969; Russell, 1998; Moss et al., 2000) also reported a negative correlation between the propionate formation in the rumen and the CH_4 produced productions in the rumen on it. In relation to this, organosulphur compounds have shown an increased the acetate/propionate ratio and a decreased in methane production when were tested in *in vitro* and *in vivo*

conditions (Busquet et al., 2005b; Kongmun et al., 2010; Kongmun et al., 2011; Patra and Yu, 2012).

The fermentation parameters, nutrient apparent digestibility, N and energy use and alfalfa hay degradation were not affected by CD-PTS. However, different studies support improved nutrient digestibility with garlic oils or garlic derived compounds in sheep (Klevenhunsen et al.(2011), and lactating cows (Yang et al., 2007). Other studies (Manasri et al., 2012) reported a decrease in fiber digestibility in cattle when were supplemented with mangosteen peel and garlic pellet, although these results could be due to the interaction of different compounds and not only to the supplemented garlic compounds. In our study, the lack of effect could be due, in addition to the factors previously described, to a different chemical structure and activity of CD-PTS and other studied garlic-derived compounds.

The urinary excretion of creatine and PD was within the range of values reported for goats fed at maintenance level with similar diets (Yáñez-Ruiz et al., 2004; Cantalapiedra-Hijar et al., 2009). The lack of effect of CD-PTS on microbial N flow and efficiencies of microbial N synthesis is in agreement with results obtained by Busquet et al. (2005a; 2005b) in dual flow fermenters using garlic oils and diallyl disulfide. However our results may be in contrast with previous studies (McSweeney and Denman, 2007; McSweeney et al., 2009) which proposed that adding organic sulfur compounds to diets of ruminants might improve ruminal fermentation by selectively increasing microbial protein synthesis.

Although the antimicrobial activity of garlic and organosulphur compounds is known (Reuter et al., 1996), no differences in total bacteria, protozoa and

methanogen were observed in our study on days 14 and 28. This lack of effect could indicate an adaptation of the microbiota to the compound. A dosage of the compound lower than the expected and the complexity of the rumen ecosystem compared with the *in vitro* system may also contribute to the lack of CD-PTS effect on different microbial groups. On the other hand, methanogens abundance was not affected by the treatment but the structure of the archaeal population at 28 days of CD-PTS treatment changed in 4 treated animals as compared with the control animals. In addition, on day 28 a numerical reduction of 10% of methane production was observed in those treated animals. This is in accordance with the hypothesis that rather than the total archaeal biomass it is the species distribution that correlates to changes in methane production in the rumen (Zhou et al., 2010; Abecia et al., 2012). Mitsumori et al. (2012) reported a half-log reduction in the normal methanogens population correlated with > 50% reduction in methane, indicating that the relative methanogenic activity of different archaeal species in the rumen plays a greater role in determining methane output than absolute number of methanogens. Therefore, any study of the methanogenesis inhibition should account for the effect on a wide range of ruminal methanogenic Archaea species and not only on those that are believed to be numerically dominant. Ohene-Adjei et al. (2008) also observed a shift in the structure and diversity of archaeal population in sheep treated with antimethanogenic plant extracts. Our results on archaeal population structure on day 28, are in accordance with Williams et al., (2009) study who suggested that methanogens take longer than 4 week to adapt to rumen changes, compared with the eubacterial community which need only 10 to 15 day.

In order to state the effect of PTS on some methanogens as compared to other well-known antimethanogenic compound (BCM, Abecia et al., 2012), pure cultures of different strains of 3 species were used. A strong inhibitory effect of PTS and BCM on all methanogen strains was observed. The first five steps of the methanogenic pathway result in the sequential reduction of CO_2 by electrons from H_2 to form N5-methyl-tetrahydromethanopterin methyl transferase (Thauer et al., 1993). The methyl group is then transferred to coenzyme M via the action of methyl-H4MPT:CoM-methyltransferase which is encoded by the mtr gene cluster (Attwood and McSweeney, 2008). The BCM interferes with the cobamide-dependent methyl transferase step which explains the high sensitivity of methanogens to BCM. However, to our knowledge, there is no data on the specific mechanism of action of PTS in relation to the biochemical pathways involved in the reduction of CO_2 to CH_4. Antimicrobial activity of PTS against pathogenic bacteria, mainly coliforms and enterobacteria, in pigs has been observed (Ruiz et al., 2010) and associated to the chemical reaction of thiosulfinates with thiol groups of different enzymes (Focke et al., 1990; Ruiz et al., 2010; Benchaar and Greathead, 2011). Ramos-Morales et, al. (2013) reported changes in the structure and diversity of Butyrivibrio species in continuous-culture fermenters treated with PTS. Thiosulfinates like PTS, as other authors suggested (Busquet et al., 2005b) for other organosulphur compounds, could be involved in the inhibition of enzymes of rumen methanogenic archaea, such as 3-hydroxy-3-methyl-glutaryl coenzyme A (HMGCoA) reductase that acts in the synthesis of isoprenoid ethers, the main components of archaeal cell membranes or another enzymes

implicated in the methanogenic pathway as coenzyme M reductase (Liu and Whitman, 2008). This need to be confirmed in future studies.

Conclusion

In conclusion, the present study has shown that an α-cyclodextrin-PTS complex offers the potential to inhibit ruminal methane production in goats by causing a shift in the distribution of archaeal groups in the rumen. However the effect observed *in vivo* does not quantitatively confirm what previously observed *in vitro*, which could be related to the means of administration of the active compound to the animals. Further research is needed to evaluate the longer-term efficacy of α-cyclodextrin-PTS complex for inhibiting methanogenesis and to increase animal performance.

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References

Abecia, L., Fondevila, M., Rodríguez-Romero, N., Martínez, G., Yáñez-Ruiz, D., 2013. Comparative study of fermentation and methanogen community structure in the digestive tract of goats and rabbits. J. Anim. Physiol. Anim. Nutr. 97, 80-88.

Abecia, L., Toral, P., Martín-García, A., Martínez, G., Tomkins, N., Molina-Alcaide, E., Newbold, C., Yáñez-Ruiz, D., 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95, 2027-2036.

AOAC, 2005. Official Methods of Analysis 18th ed. Assoc. Off. Anal. Chem., Gaithersburg, MD.

Attwood, G., McSweeney, C., 2008. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H2 utilisation in the rumen. Anim. Prod. Sci. 48, 28-37.

Balcells, J., Guada, J., Peiró, J., Parker, D., 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. J. Chromatogr. B. Biomed. Sci. Appl. 575, 153-157.

Belenguer, A., Yanez, D., Balcells, J., Ozdemir Baber, N., Gonzalez Ronquillo, M., 2002. Urinary excretion of purine derivatives and prediction of rumen microbial outflow in goats. Livest. Prod. Sci. 77, 127-135.

Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol. 166-167, 338-335.

Busquet, M., Calsamiglia, S., Ferret, A., Cardozo, P., Kamel, C., 2005a. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J. Dairy Sci. 88, 2508-2516.

Busquet, M., Calsamiglia, S., Ferret, A., Carro, M., Kamel, C., 2005b. Effect of garlic oil and four of its compounds on rumen microbial fermentation. J. Dairy Sci. 88, 4393-4404.

Cantalapiedra-Hijar, G., Yáñez-Ruiz, D., Martín-García, A., Molina-Alcaide, E., 2009. Effects of forage: concentrate ratio and forage type on apparent digestibility, ruminal fermentation, and microbial growth in goats. J. Anim. Sci. 87, 622-631.

European Directive 86/609. Commission recommendations of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Annex II to European Council Directive 86/609. The Commission of the European Communittes Publishing, Brussels, Belgium.

Focke, M., Feld, A., Lichtenthaler, H.K., 1990. Allicin, a naturally occurring antibiotic from garlic, specifically inhibits acetyl-CoA synthetase. FEBS letters 261, 106-108.

Goel, G., Makkar, H.P.S., Becker, K., 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. of Nutr. 101, 1484-1492.

Hart, K.J., Yáñez-Ruiz, D.R., Duval, S.M., McEwan, N.R., Newbold, C.J., 2008. Plant extracts to manipulate rumen fermentation. Anim. Feed Sci. Technol. 147, 8-35.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Technol. 160, 1-22.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483-2492.

Kamel, C., Greathead, H., Tejido, M., Ranilla, M., Carro, M., 2008. Effects of allicin and diallyl disulfide on in vitro rumen fermentation of a mixed diet. Anim. Feed Sci. Technol. 145, 351-363.

Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M., Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Anim. Feed Sci. Technol. 166–167, 356-363.

Kongmun, P., Wanapat, M., Pakdee, P., Navanukraw, C., 2010. Effect of coconut oil and garlic powder on in vitro fermentation using gas production technique. Livest. Sci. 127, 38-44.

Kongmun, P., Wanapat, M., Pakdee, P., Navanukraw, C., Yu, Z., 2011. Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livest. Sci. 135, 84-92.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171-189.

Manasri, N., Wanapat, M., Navanukraw, C., 2012. Improving rumen fermentation and feed digestibility in cattle by mangosteen peel and garlic pellet supplementation. Livest. Sci. 148, 291-295.

Mateos, I., Ranilla, M., Tejido, M., Saro, C., Kamel, C., Carro, M.D., 2013. The influence of diet type (dairy versus intensive fattening) on the effectiveness of garlic oil and cinnamaldehyde to manipulate in vitro ruminal fermentation and methane production. Anim. Prod. Sci. 53, 299-307.

McAllister, T., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7-13.

McSweeney, C., Denman, S., Conlan, L., Prasad, C., Anandan, S., Chandrasekharaiah, M., Sampath, K., 2009. The stimulatory effect of the organic sulfur supplement, mercaptopropane sulfonic acid on cellulolytic rumen microorganisms and microbial protein synthesis in cattle fed low sulfur roughages. Animal 3, 802-809.

McSweeney, C.S., Denman, S.E., 2007. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. J. Appl. Microbiol. 103, 1757-1765.

Mitsumori, M., Shinkai, T., Takenaka, A., Enishi, O., Higuchi, K., Kobayashi, Y., Nonaka, I., Asanuma, N., Denman, S.E., McSweeney, C.S., 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 108, 482-491.

Moss, A.R., Jouany, J.-P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootec. 49, 231-254.

Ohene-Adjei, S., Chaves, A., McAllister, T., Benchaar, C., Teather, R., Forster, R., 2008. Evidence of increased diversity of methanogenic archaea with plant extract supplementation. Microb. Ecol. 56, 234-242.

Patra, A.K., Kamra, D.N., Agarwal, N., 2010. Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds in vitro. J. Sci. Food Agri. 90, 511-520.

Patra, A.K., Saxena, J., 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198-1222.

Patra, A.K., Yu, Z., 2012. Effects of Essential Oils on Methane Production and Fermentation by, and Abundance and Diversity of, Rumen Microbial Populations. Appl. Environm. Microbiol. 78, 4271-4280.

Prieto, C., Aguilera, J., Lara, L., Fonollá, J., 1990. Protein and energy requirements for maintenance of indigenous Granadina goats. Br. J. Nutr. 63, 155-163.

Ramos-Morales, E., Martínez-Fernández, G., Abecia, L., Martin-García, A.I., Molina-Alcaide, E., Yáñez-Ruiz, D.R., 2013. Garlic derived compounds modify ruminal fatty acid biohydrogenation and induce shifts in the Butyrivibrio community in continuous-culture fermenters. Anim. Feed Sci. Technol. http://dx.doi.org/10.1016/j.anifeedsci.2013.05.017

Reuter, H.D., Koch, H., Lawson, L., 1996. Therapeutic effects and applications of garlic and its preparations, In: Koch, H., Lawson, L. (Eds.), Garlic. The Science and Therapeutic Application of Allium sativum L. and Related Species, Williams and Wilkins. Baltimore, MD, USA, pp. 135-212.

Romero-Huelva, M., Molina-Alcaide, E., 2013. Nutrient utilization, ruminal fermentation, microbial nitrogen flow, microbial abundances, and methane emissions in goats fed diets including tomato and cucumber waste fruits. J. Anim. Sci. 91, 914-923.

Royal Decree No. 1201/2005 of 10 October on the protection of animals used for experimentation and other scientific purposes. Boletín del Estado, pp. 34367-34391. Spanish Government Publishing, Madrid, Spain.

Ruiz, R., García, M., Lara, A., Rubio, L., 2010. Garlic derivatives (PTS and PTS-O) differently affect the ecology of swine faecal microbiota in vitro. Vet. microbiol. 144, 110-117.

Russell, J., 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. J. Dairy Sci. 81, 3222-3230.

Soliva, C.R., Amelchanka, S.L., Duval, S.M., Kreuzer, M., 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106, 114-122.

Soto, E., Yáñez-Ruiz, D., Cantalapiedra-Hijar, G., Vivas, A., Molina-Alcaide, E., 2012. Changes in ruminal microbiota due to rumen content processing and incubation in single-flow continuous-culture fermenters. Anim. Prod. Sci. 52, 813-822.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., De Haan, C., 2006. Livestock's long shadow: environmental issues and options. Food and Agriculture Organization of the United Nations, Rome, Italy.

Thauer, R.K., Hedderich, R., Fischer, R., 1993. Reactions and enzymes involved in methanogenesis from CO2 and H2, In: Ferry, J.G. (Eds.) Methanogenesis. Springer, New York, pp. 209-252.

Van Nevel, C., Henderickx, H., Demeyer, D., Martin, J., 1969. Effect of chloral hydrate on methane and propionic acid in the rumen. Appl. Microbiol. 17, 695-700.

Van Zijderveld, S.M., Dijkstra, J., Perdok, H.B., Newbold, J.R., Gerrits, W.J.J., 2011. Dietary inclusion of diallyl disulfide, yucca powder, calcium

fumarate, an extruded linseed product, or medium-chain fatty acids does not affect methane production in lactating dairy cows. J.Dairy Sci. 94, 3094-3104.

Williams, Y.J., Popovski, S., Rea, S.M., Skillman, L.C., Toovey, A.F., Northwood, K.S., Wright, A.D.G., 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. Appl. Environm. Microbiol. 75, 1860-1866.

Yang, W., Benchaar, C., Ametaj, B., Chaves, A., He, M., McAllister, T., 2007. 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. 90, 5671.

Yáñez-Ruiz, D., Moumen, A., Garcia, A.M., Alcaide, E.M., 2004. Ruminal fermentation and degradation patterns, protozoa population, and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: Effect of PEG supply. J. Anim. Sci. 82, 2023-2032.

Zhou, M., Hernandez-Sanabria, E., Guan, L.L., 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Environm. Microbiol. 76, 3776-3786.

Tables and figures

Table 3.3.1. Chemical composition of alfalfa hay and concentrate (g/kg dry matter) and ingredients (g/kg fresh matter) composition of the concentrate.

Item	Alfalfa hay	Concentrate
DM (g/kg fresh matter)	923	914
OM	891	904
aNDFom	475	338
ADFom	349	129
Lignin(sa)	71.6	27.4
CP	217	185
Ether Extract	14.1	28.9
Gross energy (MJ/Kg DM)[1]	18.9	19.1

Table 3.3.2. Effect of CD-PTS on body weight, dry matter intake, methane emissions, pH, concentrations of NH_3-N and total and individual VFA in the rumen of goats at different sampling days.

	Time											
	7		14		21		28			*P*-value[1]		
Item	Control	CD-PTS	Control	CD-PTS	Control	CD-PTS	Control	CD-PTS	SED	Treat	time	Treatxtime
BW kg	36.2	39.1	36.6	39.4	36.1	39.0	37.1	39.5	3.3	0.41	0.12	0.82
DMI g/d	664	694	688	714	695	651	720	761	92	0.83	0.31	0.75
CH_4 L/d	19.8	21.5	21.3	20.0	21.1	19.6	22.3	21.5	1.9	0.78	0.76	0.72
CH_4 L/kg DMI	30.4	30.9	31.4	27.9	30.6	28.3	31.4	27.6	3.1	0.17	0.79	0.41
pH	6.0	6.3	6.3	6.4	6.3	6.4	6.3	6.3	0.2	0.40	0.36	0.83
NH_3-N mg/100mL	25.6	24.4	21.6	21.4	21.1	23.5	24.6	25.1	4.3	0.88	0.52	0.93
Total VFA, mM	76.9	74.2	71.0	70.7	75.5	73.1	75.7	71.5	10.1	0.36	0.92	0.95
Individual, mol/100mL												
Acetate	66.5	64.5	67.9	67.1	68.7	67.9	65.8	65.4	1.4	0.27	0.010	0.87
Propionate	18.1	16.9	16.8	17.3	16.1	17.3	17.0	18.1	0.9	0.49	0.43	0.16
Isobutyrate	2.1	2.3	2.1	1.9	1.9	1.9	2.0	1.8	0.3	0.87	0.29	0.58
Butyrate	10.5	12.5	10.5	10.9	10.5	9.8	10.6	10.9	1.2	0.46	0.45	0.40
Isovalerate	1.2	1.5	1.4	1.5	1.1	1.1	1.3	1.2	0.23	0.52	0.28	0.65
Valerate	1.8	1.9	1.5	1.5	2.0	1.6	2.2	2.2	0.3	0.59	<0.001	0.41
Acetate:Propionate	3.69	3.83	4.07	3.88	4.30	3.93	3.9	3.64	0.25	0.32	0.081	0.43

[1] Treat: treatment effect; Treatxtime: Interaction between treatment and time.

Table 3.3.3. Effect of CD-PTS on nutrient apparent digestibility and degradation of dry matter (DMD) and aNDFom (NDFD) of alfalfa hay at 24 and 48 hours in goats.

	Treatment			
Item	Control	CD-PTS	SED	*P*-value
Intake g/d				
Total DM	743	775	44	0.49
OM	678	704	41	0.54
Fat	16.2	16.9	1.0	0.51
CP	151	157	9	0.52
aNDFom	319	331	19	0.51
ADFom	179	187	11	0.49
Apparent digestibility coefficent				
DM	0.66	0.67	0.02	0.91
OM	0.69	0.69	0.02	0.96
Fat	0.76	0.75	0.02	0.67
CP	0.79	0.80	0.01	0.57
aNDFom	0.50	0.51	0.03	0.65
ADFom	0.42	0.44	0.03	0.66
Alfalfa hay 24 h DMD	0.53	0.50	0.04	0.46
Alfalfa hay 48 h DMD	0.59	0.60	0.03	0.97
Alfalfa hay 24 h NDFD	0.31	0.29	0.05	0.71
Alfalfa hay 48 h NDFD	0.40	0.40	0.08	0.99

Table 3.3.4. Effect of CD-PTS on metabolic weight, N and energy use, urinary excretion of creatinine and purine derivatives, microbial N flow and efficiency in goats.

Item	Treatment		SED	*P*-value
	Control	CD-PTS		
BW $^{0.75}$	15	16	1	0.33
N, g/kg BW$^{0.75}$	1.58	1.53	0.07	0.55
Fecal N, g/kg BW$^{0.75}$	0.33	0.30	0.03	0.31
Urine N, g/kg BW$^{0.75}$	0.79	0.79	0.07	0.92
Digestible N[1], g/kg BW$^{0.75}$	1.24	1.23	0.06	0.84
N retention[2], g/kg BW$^{0.75}$	0.45	0.43	0.05	0.72
N use,				
Digestible N/intake N	0.79	0.80	0.02	0.43
N retention/digestible N	0.37	0.35	0.04	0.79
N retention/ N intake	0.29	0.28	0.03	0.89
Energy intake, MJ/kg of BW$^{0.75}$	0.92	0.90	0.05	0.75
Fecal energy, MJ/kg of BW$^{0.75}$	0.286	0.283	0.023	0.87
Digestible Energy[3], MJ/kg of BW$^{0.75}$	0.636	0.625	0.033	0.73
Energy use,				
DE/energy intake	0.69	0.69	0.02	0.90
Creatinine, µmol/kg$^{0.75}$	326	330	33	0.91
Allantoin, µmol/kg$^{0.75}$	403	394	43	0.84
Xanthine, µmol/kg$^{0.75}$	11.2	10.9	4.9	0.95
Hypoxanthine, µmol/kg$^{0.75}$	29.2	31.1	9.9	0.85
Uric acid, µmol/kg$^{0.75}$	26.8	31.9	3.6	0.18
Total purine derivatives, µmol/kg$^{0.75}$	464	453	47	0.82
Microbial N flow, g/d	12.0	13.2	1.5	0.42
EMNS, g/kg OM[4]	20.3	18.2	2.1	0.33
EMNS, g/kg DOM[5]	29.2	26.4	2.9	0.36

[1] Digestible N = intake N – fecal N

[2] N retention = digestible N – urine N

[3] Digestible Energy = energy intake – fecal energy

[4]EMNS= efficiency of microbial N synthesis; OM= organic matter.

[5]EMNS= efficiency of microbial N synthesis; DOM= digested organic matter.

Table 3.3.5. Effect of CD-PTS on the concentration (log copy gene numbers/g fresh matter) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea (mcrΔ gene) and Richness and Shannon indexes in the rumen of goats on days 14 and 28.

	Day 14		Day 28			*P*-value[1]		
Item	Control	CD-PTS	Control	CD-PTS	SED	treat	time	treatxtime
Bacteria	9.85	9.85	9.91	9.93	0.12	0.89	0.38	0.93
Protozoa	8.51	8.58	8.84	8.65	0.12	0.59	0.10	0.27
Methanogenic archaea	8.16[b]	8.26[b]	8.35[a]	8.45[a]	0.09	0.33	0.029	1.00
Richness	23.0	22.0	19.2	21.6	1.4	0.49	0.054	0.11
Shannon index	3.13	3.09	2.95	3.07	0.07	0.44	0.049	0.097

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

[1] Treat: treatment effect; Treatxtime: Interaction between treatment and day.

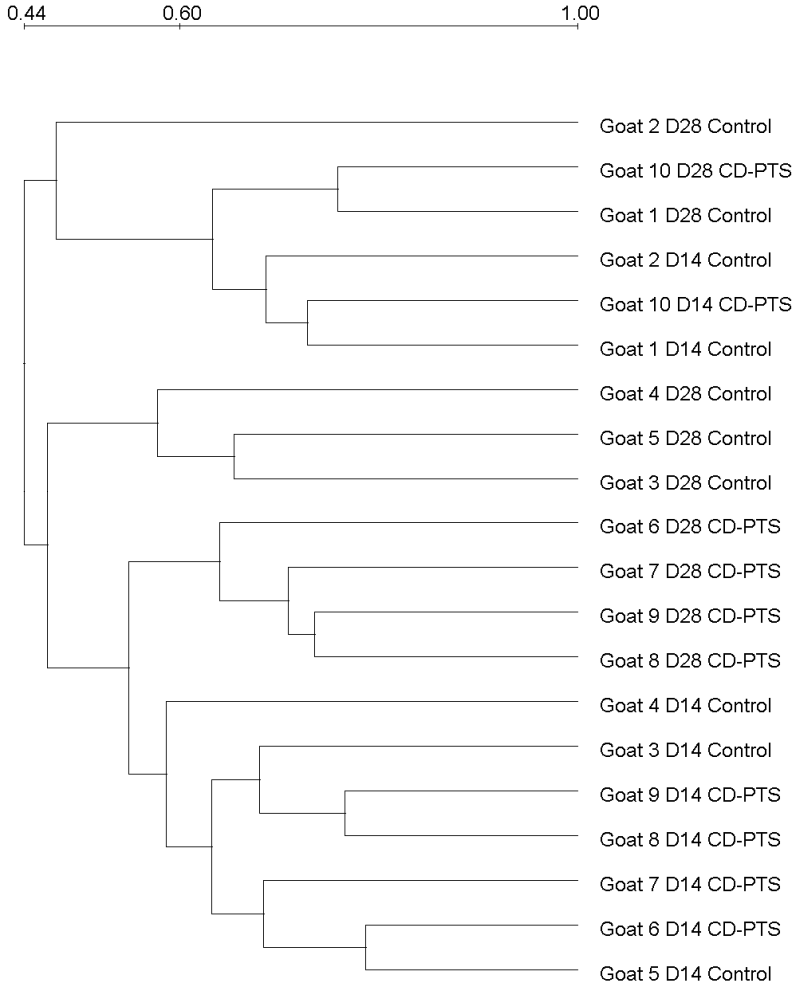

Figure 3.3.1. Dendogram obtained from the DGGE banding profile analysis of rumen samples from goats (n = 10) treated with CD-PTS and non-treated on days 14 and 28.

Scale bar shows percentage of similarity.

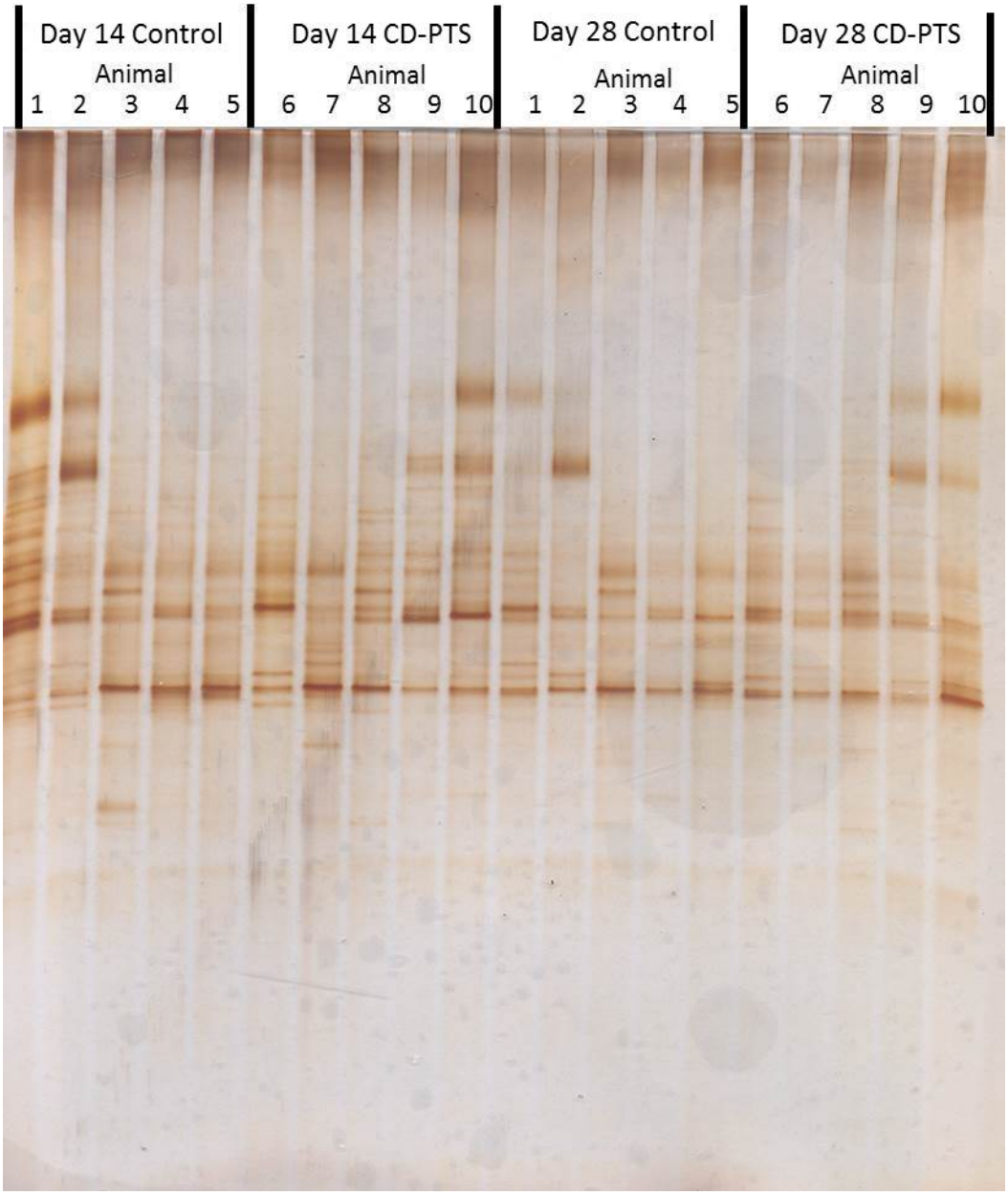

Figure 3.3.2. Comparison of archaeal DGEE profiles of the amplicons of rumen samples from goats (n = 10) treated with CD-PTS and non-treated on days 14 and 28.

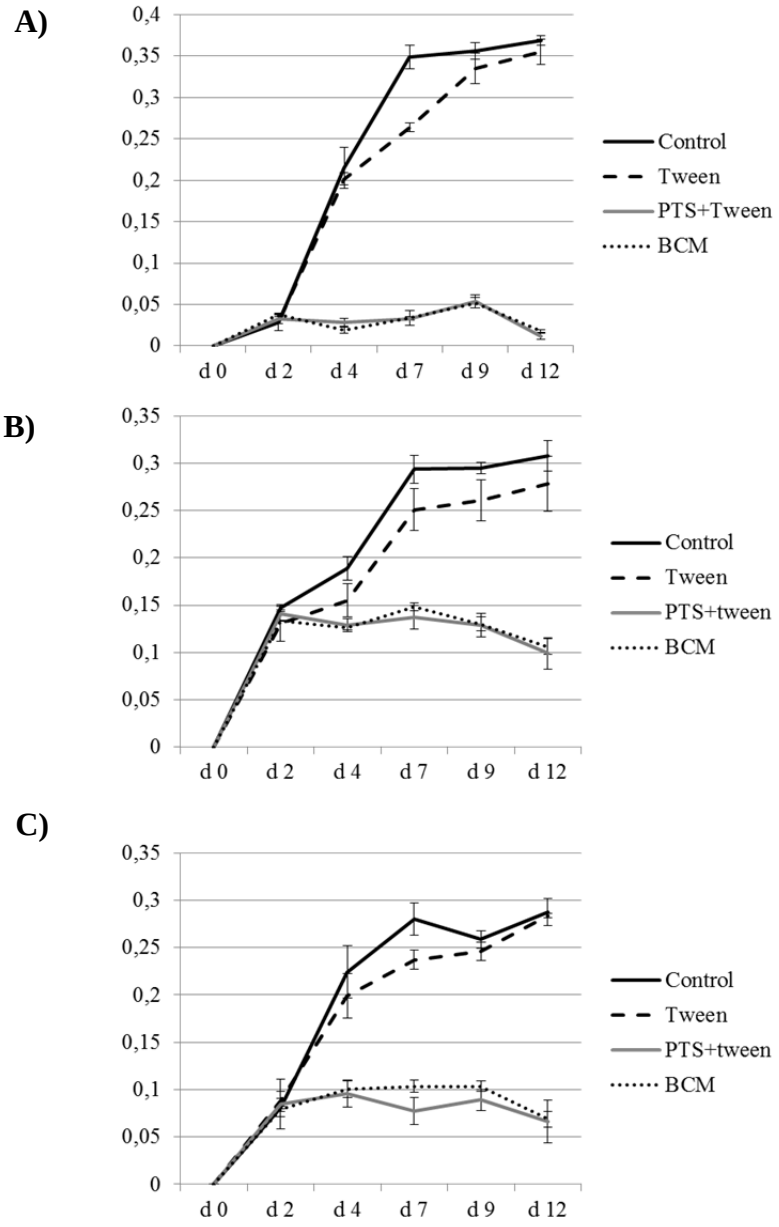

Figure 3.3.3. Effect of PTS+tween and BCM on the growth (mL CH_4/mL gas) of *Methanobrevibacter ruminantium* (A), *Methanobrevibacter smithii* (B) and *Methanobrevibacter millerae* (C).

Publication 4

Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundances and methane emissions in sheep

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INTERPRETIVE SUMMARY: New Methane Inhibitor in sheep. By Martínez-Fernández et al. In the last decade an intensive research effort has being made to develop nutritional strategies to reduce methane emissions from ruminants resulting in contradictory and highly variable results. This work used nitrooxy alkanoic acids as antimethanogenic compounds and observed a 29% and 21% reduction in methane yield after 15 days 30 days of treatment, respectively. Both effects were accompanied by a decrease in acetate:propionate ratio and no detrimental effect on dry matter degradability in the rumen.

NEW METHANE INHIBITOR IN SHEEP

Abstract

The aim of this work was to investigate the effect of feeding ethyl-3-nitrooxy propionate (**E3NP**) and 3-nitrooxypropanol (**3NP**), two recently developed compounds with potential antimethanogenic activity, *in vitro* and *in vivo* in non-lactating sheep on ruminal methane production, fermentation pattern, the abundance of major microbial groups, and feed degradability. Three experiments were conducted, one *in vitro* and two *in vivo*. The *in vitro* batch culture trial (experiment 1) tested two doses of E3NP and ENP (40 and 80 µL/L) which showed a substantial reduction of methane production (up to 95 %) without affecting VFA concentration. The two *in vivo* trials were conducted over 16 (experiment 2) and 30 days (experiment 3) respectively, to study their effects in sheep. In experiment 2, six adult non-pregnant sheep with permanent rumen cannula and fed with alfalfa hay and oats (60:40), were treated with ethyl-3-nitrooxy propionate at two doses (50 and 500 mg/animal per day). After 7 and 14-15 d of treatment methane emissions were recorded in chambers and individual rumen fluid samples were collected for VFA analysis and quantification of bacterial, protozoal and archaeal numbers by real-time PCR. Methane production decreased by 29% compared with the control with the highest dose on d 14-15. A decrease in the acetate to propionate ratio was reflected without detrimental effects in DMI. In experiment 3, nine adult non-pregnant sheep with permanent rumen cannula and fed with alfalfa hay and oats (60:40), were treated with ethyl-3-nitrooxy propionate and 3-nitrooxypropanol at one dose (100 mg/animal per day) over 30 d. On d 14 and

29-30 methane emissions were recorded in chambers. Rumen fluid samples were collected on d 29 and 30 for VFA analysis and quantification of bacterial, protozoal and archaeal numbers by real-time PCR. In addition, on days 22 and 23 samples of oats and alfalfa hay were incubated in the rumen of sheep to determine the dry matter ruminal degradation over 24 and 48 hours, respectively. A reduction on methane production was observed with both additives at d 14 and 29-30. In both treatments the acetate to propionate ratio was significantly reduced. No significant effect on the alfalfa hay and oats degradability was observed. Likewise, total concentration of the analyzed microbial groups in the rumen, showed no difference among treatments and doses for both experiments. Both tested compounds showed promising potential as methane inhibitors in the rumen with no detrimental effect on fermentation and intake, which would need to be confirmed in producing animals.

Key words: ethyl-3-nitrooxy propionate, 3-nitrooxypropanol, rumen, methane, sheep

Introduction

The inhibition of methane (CH_4) production by ruminants has long been an objective of ruminant nutritionists. Methane is produced within the rumen by methanogenic archaea. It is a byproduct of ruminal fermentation and constitutes a pathway for the disposal of metabolic hydrogen produced by microbial metabolism. The production of CH_4 represents an energy loss comprised between 2 and 12 % of the dietary gross energy (Johnson and Johnson, 1995) and contributes significantly to around 30 % of anthropogenic greenhouse gases emissions (Steinfeld et al., 2006). It is obvious that, if the energy lost as methane was to be conserved as fermentation products, improved energy retention would lead to increased productivity in addition to decreasing the production of this important greenhouse gas (Moss, 1993). Various approaches aiming at reducing methane emissions from enteric fermentation have been studied in many countries (McAllister and Newbold, 2008). The development of new feed additives (mainly based on plant extracts) to decrease methane production within the rumen has attracted research efforts over the last 20 years. The results remain variable and contradictory (Benchaar and Greathead, 2011), hereby restricting the uptake and use of these new compounds in the animal feeding market. The reasons behind such restriction may be related to a number of factors amongst which the variability in concentration of active compounds in plant extracts, the stability of the active compound within the rumen, the side effects compromising overall ruminal fermentation and the lack of persistency of the effects when they are tested *in vivo* due to the adaptation of the microbial ecosystem (Hart et al., 2008).

The development of synthetic compounds with activity specific to metabolic pathways essential to ruminal archaea has recently shown promising results. This may overcome the restrictions associated to the use plant derived compounds (Liu et al., 2011; Soliva et al., 2011). The methyl-CoM reductase catalyzes the last step of reduction of CO_2 to CH_4 by hydrogenotrophic methanogenic archaea (Attwood and McSweeney, 2008). Preliminary observations, using an *in silico* screening approach (Halgren et al., 2004) identified some nitrooxy carboxilic acids with a potential to dock into the active site of methyl-CoM reductase.

Therefore, the present study was designed to investigate the effects of two compounds (ethyl-3-nitrooxy propionate and 3-nitrooxypropanol), on antimethanogenic activity *in vitro* and *in vivo*, on ruminal fermentation and on microbial abundances.

Material and methods

One *in vitro* and two *in vivo* experiments were conducted. In experiment 1, the antimethanogenic activity of ethyl-3-nitrooxy propionate (**E3NP**) and 3-nitrooxypropanol (**3NP**) was assessed in batch cultures over 24 h. In experiment 2, the effects of two doses of E3NP on ruminal fermentation, methane production and microbial abundances were studied in sheep over 16 days, while in experiment 3 single doses of E3NP and 3NP were tested in sheep over 30 days.

Animals, diet and compound

Fifteen adult and dry Segureña sheep (44.3±4.7) fitted with permanent rumen cannula were used for experiments 1, 2 and 3. Over the duration of the trials they had free access to water and were fed twice a day (0900h and 1600h) a diet that consisted of alfalfa hay chopped at 15-20 cm and grain oats in a proportion 60:40 at approximately 1.1 times the energy requirements for maintenance level (Aguilera et al., 1986) and a mineral-vitamin supplement (Table 3.4.1). The same diet was used as substrate for the *in vitro* experiment. Animals were cared for by trained personnel in accordance with the Spanish guidelines for experimental animal protection (Royal Decree No. 1201/2005) and the European Convention for the Protection of Vertebrates used for Experimental and other Scientific Purposes (European Commission, 2007). All the experimental procedures involved in this study were approved by the Animal Welfare Committee at the Institute of Animal Nutrition (CSIC, Spain). The temperature, humidity and air turn out in chambers were carefully monitored according to the animal welfare conditions. The CO_2 concentration was also continuously monitored to ensure a good air quality and renovation rate in the chambers. Animals did not show any stress-related behavior while they were allocated in chambers.

The compounds to be tested were ethyl-3-nitrooxy propionate (**E3NP**) with 99.7% of purity and 3-nitrooxypropanol (**3NP**) with 99.5% of purity, both classified as nitrooxy alkanoic derivates (Figure 3.4.1). Both compounds were provided by DSM (Saint-Louis Cedex, France), (Duval and Kindermann, 2012). In experiment 1, the compounds were directly pipetted into the bottles before inoculation. In experiments 2 and 3 the compounds were provided twice a day through the ruminal cannula at the time the animals were fed. The

corresponding amount to each additive was pipetted onto 10 grams of grounded oats and wrapped in cellulose paper immediately before it was placed in the rumen.

Experimental designed and sampling

Experiment 1. Three 24 h incubation runs were carried out with two bottles per treatment (including blanks). Treatments were: control (CO, no additive treatment), E3NP at 40 and 80 µL/L, 3NP at 40 and 80 µL/L and bromochloromethane (**BCM**) at 160 and 320 µL/L as positive antimethanogenic control (Goel et al., 2009). The substrate incubated was the same diet provided to animals and was ground to 1-mm before being weighed in the bottles. The experimental procedure was based on Theodorou et al. (1994). The substrate (500 mg DM) was weighed into 120-mL serum bottles. Ruminal contents were obtained immediately before the morning feeding from two different sheep in each run (six in total), pooled and strained through four layers of cheesecloth into an Erlenmeyer flask with an O_2-free headspace. Particle-free fluid was mixed with the buffer solution of Menke and Steingass (1988) in a proportion 1 : 3 (vol:vol) at 39 °C under continuous flushing with CO_2 and 60 mL inoculated in each bottle. Time required from rumen content collection to the inoculation of bottles was less than 30 minutes. Additives were directly pipetted into the bottles prior to inoculation with buffered rumen fluid. Bottles were sealed with rubber stoppers and aluminum caps, incubated at 39 °C, and withdrawn 24 h after inoculation. At that point a gas sample (10 mL) was removed from each bottle and stored in a Haemoguard Vacutainer (Terumo Europe N.V., Leuven,

Belgium) before analysis for methane. Bottles were then uncapped, the pH was measured immediately with a pH meter, and the fermentation was stopped by swirling the bottles in ice. One mL of content was added to 1 mL of deproteinising solution [i.e. metaphosphoric acid (20 g/L) and crotonic acid (4 g/L)] for volatile fatty acid (VFA) determination.

***Experiment 2*.** Six sheep were used. The design consisted of three experimental periods in which pairs of animals were assigned to each of the three treatments: control, E3NP 50 mg/d and 500 mg/d. The trial consisted in 14 days of adaptation to the dose, followed by two consecutive days of methane measurements in chambers (d 14 and 15). A period of two weeks was then allowed as washout in between experimental periods. Over the course of the adaptation period, methane was also measured at d 7. On d 14 and 15 samples of ruminal contents were collected two hours after the morning feeding, sub-sampled and immediately frozen prior to DNA extraction and determination of VFA and N-NH_3 concentration. The BW was measured on d 7 and 16 and the DMI was registered daily while in the chambers (d 7, 14, 15 and 16).

***Experiment 3*.** Nine sheep were used. The experimental design consisted of a 3 x 3 Latin square with 3 sheep per treatment (control, E3NP 100 mg/d and 3NP 100 g/d) in each period**.** Experimental animals were allocated to three sub-groups of 3 animals each and within sub-groups were randomly assigned to one of the three treatments. Each period included 28 days of adaptation to the treatment followed by two consecutive days of methane measurements in chambers and collection of rumen samples (d 29 and 30). Over the course of

the adaptation phase, methane was measured on d 14. In addition, at d 22 and 23, samples of alfalfa hay and oats placed in nylon bags were incubated in the rumen of sheep to determine the dry matter ruminal degradation over 48 and 24 h, respectively. During the two days of methane measurements in chambers (d 29 and 30) rumen contents samples were collected two hours after the morning feeding, sub-sampled and immediately frozen prior to DNA extraction and determination of volatile fatty acids and $N\text{-}NH_3$ concentration. The BW was measured on d 14 and 30 and the DMI was registered daily while in the chambers (d 14, 29 and 30).

Methane Measurement

Three chambers (1.8 m wide × 1.8 m deep × 1.5 m tall) were used in experiments 2 and 3. Chamber air temperature was maintained between 15 and 20 °C. Within each chamber, the animals were individually restrained in the same cages as during adaptation. Interruptions occurred daily at 0900h and 1400h, when the chamber floor was cleaned, and the animals were fed. These interruptions had little impact on the daily methane emissions because fluxes were calculated three times per day and then averaged to derive the 23-h emission value. Airflow and concentration of methane was measured for the inflow and outflow ducts of each chamber. Air velocity was continuously monitored over the day in the exhaust duct for each chamber. The air stream in each of the 4 ducts (chambers 1, 2 and 3 and background) was sub-sampled, and methane concentration was measured continuously using a gas analyzer ADM MGA3000 (Spurling works, Herts, UK). It took 11 min to sequentially

sample the airflow in all inflow and exhausts ducts in the chambers (3 min in chambers 1, 2 and 3, and 2 min for background). In summary, the flux of methane for each chamber was calculated for each measuring day from the difference of fresh-air inflow and chamber exhaust methane concentrations and mean air velocities.

Chemical Analyses

Volatile fatty acids were analyzed by gas chromatography and ammonia N concentration by colorimetry following the protocol described by established in our laboratory (Weatherburn, 1967).

Volatile fatty acids were analyzed by gas chromatography and ammonia N concentration by colorimetry following the protocols established in our laboratory (Isac et al. 1994).

Feed samples were ground through a 1-mm sieve before analysis, and DM, ash, ether extract and N contents were determined following AOAC official methods 934.01, 942.05, 920.39 and 984.13 respectively (AOAC, 2005). Gross energy was determined in an adiabatic bomb calorimeter (Gallenkamp & Co. Ltd., London, UK) according to the methodology described by Prieto et al. (1990). The NDF, ADF, and ADL analyses were performed by the sequential procedure of Van Soest et al. (1991) using the Ankom 2000 Fiber Analyzer (Ankom Technology Corp., Macedon, NY). The NDF was assayed with sodium sulfite and without α-amylase. Both NDF and ADF were expressed without residual ash. The N values were determined by the Kjeldahl procedure, were converted to CP by multiplying by 6.25.

After 24 h of incubation an aliquot of the gas produced was taken in a 10 mL vacuum tube (Venoject, Terumo Europe N.V., Leuven, Belgium) for CH4 concentration analysis. The CH4 concentration was determined by gas chromatography (GC) using a HP Hewlett 5890, Packard Series II gas chromatograph (Waldbronn, Germany). A sample of 0.5 mL of gas was injected using a 1 mL Sample-Lock syringe (Hamilton, Nevada, USA).

The volume of gas produced (mL) was corrected for standard conditions (105 Pa, 298 K), and the amount of methane produced (micromol) was calculated by multiplying the gas produced (micromol) by the concentration of methane in the analyzed sample.

Ruminal degradability was measured on three grams of 2 mm ground feed placed in 5 cm x 10 cm nylon bags with a pore size of 50 µm (#R510 Ankom *in situ* bags, Macedon NY). The two ingredients used in the animals´ diets were tested: oats and alfalfa hay. Bags with oats were incubated in the rumen for 24 hours, while those with alfalfa hay for 48 hours. The incubations times were chosen based on average residing times in the rumen of different feedstuffs. Bags (two bags per feed and period) were placed in the rumen immediately before the morning feeding. At 24 or 48 hours they were taken out of the rumen, washed with cold water and frozen at -20 ºC. At the end of every period the frozen bags were washed in a washing machine using a short cold water program including two bags per feed that had not been incubated in the rumen to account for solubility. After washing, the bags were placed in the oven at 60 ºC for 48 hours. Ruminal degradability (%) was calculated as the loss of dry matter over the incubation time.

Real-time PCR analysis

Samples of rumen contents were freeze-dried and thoroughly mixed by physical disruption using a bead beater (Mini-bead Beater; BioSpec Products, Bartlesville, OK, USA). DNA extraction was performed using the QIAamp® DNA Stool Mini Kit (Qiagen Ltd, West Sussex, UK) following the manufacturer's instructions with the modification that a higher temperature (95ºC) was used for lysis incubation. DNA samples were used as templates for quantitative real-time PCR (qPCR) amplification. The numbers of total bacteria, protozoa and methanogenic archaea were quantified by qPCR as described by Abecia et al. (2012).

Statistical Analyses

Three incubation runs with two replication batch (per treatment and dose) in each run were carried out in experiment 1. The statistical model included the fixed effects of dose, with the period as a random effect. Data from Exp. 1 were analyzed as an univariate model using the GLM procedure of SPSS version 19.0 (2010; SPSS Inc., Chicago, IL). Data in experiment 2 and 3 were analyzed as a repeated measures analysis using GLM procedure of SPSS version 19.0 with the animal as the experimental unit. In experiment 2 dose, time (as days of treatment) and dose x time interaction were considered as fixed effects for the analysis of CH_4 production and DMI. The effect of dose was analyzed for VFA, ammonia, total bacteria, total protozoa and methanogenic archaea. In experiment 3 CH_4 measurements and DMI were analyzed considering the the effects of additive, time (as days of treatment) and additive x time interaction.

The effect of additive was accounted for VFA, ammonia, dry matter degradation, total bacteria, total protozoa and methanogenic archaea. Effects were considered significant at $P \leq 0.05$. When significant differences were detected, differences among means were tested using a least significant difference (LSD).

Results

Experiment 1

The effects of supplementing batch cultures with E3NP, 3NP and BCM are shown in Table 3.4.2. None of the treatments applied affected the concentration of total VFA; however, all treatment and doses significantly decreased the acetate:propionate ($P = 0.002$) ratio and CH_4 production ($P = 0.001$).

Experiment 2

Overall, DMI (table 3.4.3) was modified by the addition of E3NP ($P = 0.025$) compared to control along the trial, although E3NP-50 produced higher DMI ($P = 0.001$) than E3NP-500 at d 14. Methane emissions per unit of DMI, was significantly reduced (up to 29 %) compared to control with E3NP ($P = 0.033$).

The study of the rumen fermentation parameters showed a shift in the fermentation pathways (table 3.4.4) towards a more propionic type profile in the rumen of animals receiving E3NP, especially those treated with the highest dose. This was reflected by a decrease in the acetate to propionate ratio ($P = 0.026$) with the treatment. An increase ($P = 0.013$) in the butyric and iso-valeric acids was observed with the highest dose. However, total VFA concentration

decreased (P = 0.025) in animals treated with E3NP-500 as compared to E3NP-50, but no significant effect was observed compared with control. Concentration of ammonia was similar among treatments (P = 0.904).

The study of the concentration of the main microbial groups in the rumen showed no difference ($P \geq 0.409$) among treatments (table 3.4.4).

Experiment 3

Dry matter intake (table 3.4.5) was not affected ($P \geq 0.172$) by the treatment and time. Methane emissions per kg of DMI were significantly ($P \leq 0.015$) reduced on day 14 when both compounds were incorporated in the diet. The reduction observed against the control was 14 % and 25 % for E3NP and 3NP respectively. When methane emissions per kg of DMI were recorded two weeks later, on d 29 and 30, the decrease in methane emissions persisted ($P \leq 0.040$) and was around 21 % with both compounds.

The study of the rumen fermentation parameters (table 3.4.6) collected on d 29 and 30 showed no effect on total VFA concentration, decreased the molar proportion of acetate and increasedpropionate. As a consequence, the acetate to propionate ratio was significantly (P = 0.001) reduced in both treatments. The concentration of ammonia was similar among treatments (P = 0.972). The ruminal degradation of DM of alfalfa hay and oats assessed *in sacco* showed no effect of the additive treatment on the rumen degradability. The biomass of the three microbial groups quantified in rumen samples was not ($P \geq 0.298$) affected by any of the compound used.

Discussion

Several studies have documented substantial reduction in methane production by chemical agents such as ionophores, sulphate, nitrate, fumarate and halogenated methane analogues (Soliva et al., 2011). However, the practical use of such compounds is limited by either the costs of production or legal restrictions. We report here on the potential of two new molecules to modify rumen fermentation across *in vitro* and *in vivo* assessment.

Methane Production and Ruminal Fermentation

The results obtained in **experiment 1** confirmed the antimethanogenic activity of E3NP and 3NP. The lack of effect on total VFA concentration at both tested doses suggests no alteration of overall fermentation.

In **experiment 2**, E3NP was provided to sheep at 50 and 500 mg/animal/d. These doses correspond to around 10 and 100 mg/L rumen content, assuming 11 % of rumen content in relation to body weight (Abecia et al., 2012). Based on the results obtained *in vitro*, in which a substantial decrease in CH_4 was observed, we decided to test *in vivo* two doses that were, respectively, slightly lower and higher than the doses tested in batch cultures.

E3NP-500 resulted in a significant ($P < 0.05$) reduction of methane production at d 14 (29 %), although not as high as that obtained *in vitro* (95%). It was however similar to the decrease (30 %) achieved in our group with dairy goats treated over 2 months using BCM (Abecia et al., 2012). Similar differences between *in vitro* and *in vivo* studies have been observed by Mohammed et al. (2004) using Japanese horseradish oil, who reported substantially greater inhibitions of methane production *in vitro* (89%) than *in vivo* (18.7%). The

discrepancy in the extent of effectiveness with similar doses may be explained by a number of factors: i) the compounds used in this study had moderate solubility in water and therefore the homogenous distribution across the rumen compartments might have not been fully achieved, especially when provided in two pulses through ruminal cannula; ii) the degradation rate of the active compounds, if a degradation takes place, may differ *in vitro* and *in vivo*; iii) there is a decrease in microbial densities and changes in bacterial community structure when rumen content is processed prior inoculation *in vitro*, which could be attributed to the exposure of microorganisms to oxygen and the removal of solids during the filtration process (Soto et al., 2012). In addition, the direct extrapolation of concentrations from *in vitro* to *in vivo* did not take into account the rumen outflow, which in our conditions, with animals fed restricted intake, was estimated to be around 3%/h (Yáñez-Ruiz et al., 2004). This would require an increase of the daily dosage of about 80% *in vivo* in comparison with the dose used in *in vitro* conditions and would in part explain the proportionally lower reduction achieved *in vivo* as compared with *in vitro*. However, such increase in the dosage might not be feasible.

On the other hand a shift in the VFA profile was observed. The monomers resulting from the fermentation of different nutrient in the rumen are further converted into VFAs, CO_2 and H_2 by both the primary fermenters and other microbes that do not have the capacity to hydrolyze complex polymers by themselves (secondary fermenters). Methanogens are at the bottom of this trophic chain and use the end products of fermentation as substrates. Methanogenesis in the rumen contributes to the efficiency of the system as it avoids the accumulation of H_2 to levels that might inhibit the microbial enzymes

involved in electron transfer reactions (Morgavi et al., 2010). Inhibition of methanogenesis is usually associated with an increase in propionate due to the competition for hydrogen (Hungate, 1967). With more hydrogen available, reductive acetogenesis become thermodynamically favorable. Acetate concentrations were unchanged by E3NP-50 but decreased by E3NP-500. In this trial, the proportional contribution of reductive acetogenesis and the production of acetate by oxidative pathways could not be assessed, which makes difficult to conclude whether reductive acetogenesis contributed in a greater or lesser extent. In any case the decrease in acetate and total VFA concentrations by E3NP-500 could indicate a decrease in fiber degradation as some fibroltytic microorganisms are more sensitive to high hydrogen partial pressure (Morgavi et al., 2010). Wolin et al. (1997) demonstrated that a high concentration of hydrogen was able to influence the metabolism of *Ruminococcus. flavefaciens* and *R. albus*, two important fiber-degrading species and was responsible for inhibiting the re-oxidation of NADH in these two species. Consequently, hydrogen was redirected towards other products such as succinate and ethanol (Wolin et al., 1997). The adaptive changes in rumen microbial ecology due to substantial inhibition of methanogenesis are still unknown, although the increase in propionate production has been observed in most studies. Also, we observed an increased isovalerate production with E3NP-500 and not with the lower dose (E3NP-50), probably as a consequence of disruption of interspecies hydrogen transfer in concordance with what Goel et al. (2009) and Mitsumori et al. (2011) observed using BCM.

In **experiment 3**, the same compound used in experiment 2 (E3NP) and a related molecule (3NP) were tested over a longer period (30 d) at a single dose (100 mg/d). This dose was chosen to avoid the potential negative effect of 500 mg/d used in experiment 2 on fermentation but doubling the 50 mg/d dose to ensure effectiveness. Both compounds decreased methane production per kg of DMI, with similar VFA profile to that obtained in experiment 2 at dose 50. The reduction on methane production observed at d 14 persisted on d 29-30, which suggests no rapid adaptation of the microbial ecosystem to revert the antimethanogenic activity. A standard 14 d period is used in most of *in vivo* studies to allow the rumen ecosystem to adapt to the tested treatment. However, recent observations suggest that methanogenic archaea community in the rumen needs a period of around one month to fully adapt to changes in the rumen environment (Williams et al., 2009). In this study both compounds showed effect over a month treatment in methane emissions that was further confirmed by a shift in the fermentation pattern. Indeed the effectiveness in the persistency of synthetic compounds to decrease methane emissions vary in the literature when *in vitro* results are further tested *in vivo*. Abecia et al. (2012) reported persistency over 2 months in decreased methane emissions in dairy goats treated with BCM, while Van Nevel and Demeyer (1996) observed reversed effects in sheep treated with a similar halogenated compound, 2-bromoethanesulphonate (BES). The reversion of the effects could be based on at least two mechanisms: selection of species of methanogens with greater resistance to the inhibitor or development of microbial species capable of degrading the molecule (Ungerfield et al., 2004). None of the above mechanisms seemed to happen in the case of E3NP and 3NP supplementation.

On the other hand, as outlined above, the lowered acetate concentration observed in experiment 2 might be due to compromised fiber degradation (Newbold et al., 2005). The inhibition of methanogenesis is expected to increase the partial pressure of H_2 and potentially leading to an inhibition of H_2 producing microorganisms such as *Ruminococci*, protozoa and fungi. The data in the literature suggests that in the complex rumen microbiota the mechanisms involved in H_2 balance are not straightforward (Morgavi et al., 2010). In order to rule out this occurring, in experiment 3, ruminal degradability of the two ingredients of the diet (alfalfa hay and oats) was assessed by incubating nylon bags in the rumen of the animals. The results showed no such effect on dry matter degradation. These results could be explained by a mechanism in which *Fibrobacter* increases in biomass to compensate the decrease in fibrolytic rumnococci and rumen fungi as shown by Mitsumori et al., (2012) in goats treated with BCM. Mitsumori et al. (2012) also revealed that the rumen ecosystem appeared to adapt to the high H_2 levels by shifting fermentation to propionate, and increasing the population of hydrogen-consuming *Prevotella* spp, which could be in accordance with our results on fermentation profiles.

Rumen Microbial population

The quantification of the three main microbial groups in the rumen showed that the reduction of methane emissions was not accompanied by lowered concentration of any group. With regards to the archaeal numbers, no effect was observed for neither of the treatments. The research available on the effect of methane inhibitors on methanogenic archaea in the rumen is variable and

sometimes contradictory (Morgavi et al., 2010). Nevertheless, there is a general consensus nowadays that rather than the total archaeal biomass it is the species distribution that correlates to changes in methane emissions (Zhou et al., 2010). Kongmun et al. (2011) reported no changes on the archaeal abundance on buffalo treated for 21 days with coconut oil although a reduction of methane production was observed. Recent studies have observed that a reduction of 30 % in methane production in dairy goats treated during two months with bromochloromethane (Abecia et al., 2012) was not accompanied by an effect on the concentration of the methanogenic archaea, but the distribution of archaeal species assessed by DGGE was affected (Abecia et al., 2011). Both compounds tested here are thought to target the final step of CO_2 reduction to CH_4, and specifically the final reduction of methyl-Coenzyme M (CoM) to methane by methyl CoM reductase. In that sense, Ungerfield et al. (2004) reported that molecules with similar activity in the rumen such as 2-bromoethanesulphonate (BES), 3-bromopropanesulphonate (BPS), lumazine, propynoic acid and ethyl 2-butynoate inhibited differently some of the known key methanogens in the rumen, namely *Methanobrevibacter ruminantium*, *Methanosarcina mazei* and *Methanomicrobium mobile*). If this was the case for E3NPand 3NP, this could suggests that a substantial shift in the species distribution may have occurred. Thus the decrease of the more sensitive groups would be compensated by an increase in relative abundance of the more resistant archaeal populations. There is also the possibility that other hydrogenotrophic microorganisms such as the acetogens would make reductive acetogenesis an alternative sink in the rumen (Attwood and McSweeny, 2008).

Conclusions

Both molecules tested in this study, E3NP and 3NP, showed potential to be used as antimethanogeic additives in the diet of ruminants. The decrease in methane emissions observed *in vivo* was lower than that *in vitro*, but persisted over the entire experimental period (one month) and was not associated to a reduction of archaeal biomass. It also did not appear to compromise rumen fermentation and intake. This would need to be confirmed in producing animals. A more sustained supply of the compound to the rumen (i.e. mixed with diet) deserves investigation in order to achieve greater reduction in methane production.

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References

Abecia, L., A. Martín-García, G. Martínez, N. Tomkins, C. Newbold, and D. Yáñez-Ruiz. 2011. Manipulation of the rumen microbial ecosystem to reduce methane emissions in ruminants through the intervention at early life stage of pre-ruminants and their mothers. Adv Anim. Biosci. 2:271.

Abecia, L., P. Toral, A. Martín-García, G. Martínez, N. Tomkins, E. Molina-Alcaide, C. Newbold, and D. Yáñez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. J. Dairy Sci. 95:2027-2036.

AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical Chemists International, Gaithersburg, MD.

Attwood, G. and C. McSweeney. 2008. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H_2 utilisation in the rumen. Anim. Prod. Sci. 48:28-37.

Benchaar, C. and H. Greathead. 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol. 166-167:338-355.

Duval, S. and M. Kindermann. 2012. Nitrooxy alkanoic acids and derivatives thereof in feed for reducing methane emission in ruminants, and/or to improve ruminant performance. US Pat. No. 20,120,315,339.

European Commission 2007. Commission recommendations of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes. Annex II to European Council Directive 86/609. The Commission of the European Communittes Publishing, Brussels, Belgium.

Goel, G., H. P. S. Makkar, and K. Becker. 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. Br. J. Nutr. 101:1484-1492.

Halgren, T. A., R. B. Murphy, R. A. Friesner, H. S. Beard, L. L. Frye, W. T. Pollard, and J. L. Banks. 2004. Glide: A New Approach for Rapid, Accurate Docking and Scoring. 2. Enrichment Factors in Database Screening. J. Medic. Chem. 47:1750-1759.

Hart, K. J., D. R. Yáñez-Ruiz, S. M. Duval, N. R. McEwan, and C. J. Newbold. 2008. Plant extracts to manipulate rumen fermentation. Anim. Feed Sci. Technol. 147:8-35.

Hungate, R. 1967. Hydrogen as an intermediate in the rumen fermentation. Archi. Mikrobiol. 59:158-164.

Isac, M., M. García, J. Aguilera, and A. E. Molina. 1994. A comparative study of nutrient digestibility, kinetics of digestion and passage and rumen fermentation pattern in goats and sheep offered medium quality forages at the maintenance level of feeding. Archiv für Tierernährung 46:37-50.

Johnson, K. A. and D. E. Johnson. 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.

Kongmun, P., M. Wanapat, P. Pakdee, C. Navanukraw, and Z. Yu. 2011. Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livest. Sci. 135:84-92.

Liu, H., J. Wang, A. Wang, and J. Chen. 2011. Chemical inhibitors of methanogenesis and putative applications. Appl. Microbiol. biotechnol. 89:1333-1340.

McAllister, T. and C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. AgriC. 48:7-13.

Mitsumori, M., T. Shinkai, A. Takenaka, O. Enishi, K. Higuchi, Y. Kobayashi, I. Nonaka, N. Asanuma, S. E. Denman, and C. S. McSweeney. 2012. Responses in digestion, rumen fermentation and microbial populations to inhibition of methane formation by a halogenated methane analogue. Br. J. Nutr. 1:1-10.

Mohammed, N., N. Ajisaka, Z. Lila, K. Hara, K. Mikuni, K. Hara, S. Kanda, and H. Itabashi. 2004. Effect of Japanese horseradish oil on methane production and ruminal fermentation in vitro and in steers. J. Animal Sci. 82:1839-1846.

Morgavi, D., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal. 4:1024-1036.

Moss A.R. 1993. Methane-Global Warming and Production by Animals. Page 105. Chalcombe Publications, Canterbury, UK.

Newbold, C., S. López, N. Nelson, J. Ouda, R. Wallace, and A. Moss. 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. Br. J. Nutr. 94:27-35.

Prieto, C., J. Aguilera, L. Lara, and J. Fonollá. 1990. Protein and energy requirements for maintenance of indigenous Granadina goats. Br. J. Nutr. 63:155-163.

Royal Decree No. 1201/2005 of 10 October on the protection of animals used for experimentation and other scientific purposes. Boletin del Estado. Pages 34367-34391. Spanish Government Publishing, Madrid, Spain.

Soliva, C. R., S. L. Amelchanka, S. M. Duval, and M. Kreuzer. 2011. Ruminal methane inhibition potential of various pure compounds in comparison

with garlic oil as determined with a rumen simulation technique (Rusitec). Br. J. Nutr. 106:114-122.

Soto, E., D. Yáñez-Ruiz, G. Cantalapiedra-Hijar, A. Vivas, and E. Molina-Alcaide. 2012. Changes in ruminal microbiota due to rumen content processing and incubation in single-flow continuous-culture fermenters. Anim. Prod. Sci. 52:813-822.

Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, and C. de Haan. 2006. Livestock's long shadow: environmental issues and options. Food and Agriculture Organization of the United Nations, Rome, Italy.

Theodorou, M. K., B. A. Williams, M. S. Dhanoa, A. B. McAllan, and J. France. 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48:185-197.

Ungerfeld, E., S. Rust, D. Boone, and Y. Liu. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. J. Appl. Microbiol. 97:520-526.

Van Nevel, C., and Demeyer, D. 1996. Control of rumen methanogenesis. Environ. Monit. Assessm. 42:73-97.

Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597.

Wolin, M., T. Miller, and C. Stewart. 1997. Microbe-microbe interactions. Pages 467-491 in The rumen microbial ecosystem. P.N. Hobson and C. Stewart, ed. Springer, London, UK.

Yáñez-Ruiz, D., A. Moumen, A. M. Garcia, and E. M. Alcaide. 2004. Ruminal fermentation and degradation patterns, protozoa population, and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: Effect of PEG supply. J. Anim. Sci. 82:2023-2032.

Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. 39:971–974.

Williams, Y. J., S. Popovski, S. M. Rea, L. C. Skillman, A. F. Toovey, K. S. Northwood, and A. D. G. Wright. 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. Appl. Environm. Microbiol. 75:1860-1866.

Zhou, M., E. Hernandez-Sanabria, and L. L. Guan. 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Appl. Environm. Microbiol. 76:3776-3786.

Tables and figures

Table 3.4.1. Chemical composition of alfalfa hay and oats (g/kg DM)

	Alfalfa hay	Oats
DM (g/kg fresh matter)	907	912
Organic matter	875	975
CP	193	103
NDF	517	263
ADF	334	67.4
ADL	103	13.2
EE[1]	9.4	19.9
GE[1] (MJ/kg DM)	18.5	21.1

[1]EE = ether extract; GE = gross energy

Table 3.4.2. Effect of the inclusion of two doses of BCM, E3NP and 3NP on total volatile fatty acids, acetate:propionate and methane production after 24 hour *in vitro* fermentation in batch culture (experiment 1).

		BCM[1]				E3NP[1]				3NP[1]			
	Control	160	320	SEM	*P*-value	40	80	SEM	*P*-value	40	80	SEM	*P*-value
Total VFA, mM	30.7	30.2	29.5	1.6	0.952	29.0	*27.5*	0.7	0.210	28.6	29.6	0.7	0.479
Acetate/Propionate	3.47[a]	2.55[b]	2.46[b]	0.07	0.002	2.34[b]	2.28[b]	0.06	0.001	2.32[b]	2.40[b]	0.08	0.002
Methane, µmol	389[a]	16[b]	16[b]	13	0.001	36[b]	19[b]	14	0.001	54[b]	18[b]	15	0.001
Methane/VFA, µmol/µmol	0.213[a]	0.009[b]	0.010[b]	0.009	0.001	0.021[b]	0.011[b]	0.006	0.001	0.032[b]	0.010[b]	0.008	0.001

[a-b] Means within a row with different superscripts differ ($P < 0.05$).

[1]Compounds were: bromochloromethane (BCM), ethyl-3-nitrooxy propionate (E3NP) and 3-nitrooxypropanol (3NP). Doses were expressed in µL/L.

Table 3.4.3. Effect of the addition of two doses of E3NP on body weight, DMI and methane emissions by sheep on d 7 and 14-15 (experiment 2).

	d 7			d 14-15				*P*-value		
Item	Control	E3NP50[1]	E3NP500[1]	Control	E3NP50[1]	E3NP500[1]	SEM	Dose	Time	DxT[2]
BW, kg	44.2	44.0	43.1	43.3	43.5	43.3	2.5	0.221	0.147	0.432
DMI, kg	0.857	0.904	0.802	0.810	0.859	0.809	0.054	0.025	0.111	0.283
CH_4 L/d	24.2	20.7	17.8	25.5	22.3	18.0	1.8	0.063	0.495	0.870
CH_4 L/kg DMI	29.1	23.4	23.3	31.8	26.3	22.7	3.2	0.033	0.401	0.542

[1] Doses for ethyl-3-nitrooxy propionate (E3NP) were 50 mg/d/animal (E3NP50) and 500 mg/d/animal (E3NP500)

[2] Interaction between dose and time.

Table 3.4.4. Effect of adding two doses of E3NP on total volatile fatty acid concentration (mmol/L), VFA profile (mol/100 mol), ammonia concentration (mg/100 mL), the concentration (copy gene numbers/g fresh matter) of total bacteria (16SrRNA), protozoa (18S rRNA) and methanogenic archaea (mcrΔ gene) in the rumen of sheep after 15 d of treatment (experiment 2).

	Control	E3NP50[1]	E3NP500[1]	SEM	*P*-value
Total VFA, *mM*	111.2^{ab}	123.3^{a}	104.1^{b}	5.5	0.025
Individual, mol/100 mol					
Acetate	70.6^{a}	71.0^{a}	65.5^{b}	0.9	0.011
Propionate	15.7	16.8	18.8	0.8	0.056
Butyrate	6.7^{ab}	5.6^{a}	8.1^{b}	0.6	0.013
iso-butyrate	3.7	3.5	3.4	0.2	0.696
Valerate	1.6	1.5	1.8	0.1	0.247
iso-valerate	1.7^{a}	1.6^{a}	2.4^{b}	0.2	0.012
C_2:C_3	4.59^{a}	4.26^{ab}	3.57^{b}	0.23	0.026
N-NH_3, mg/100 mL	42.5	40.5	40.1	5.5	0.904
Total bacteria, log_{10}	9.03	9.08	9.18	0.10	0.491
Total protozoa, log_{10}	6.35	6.28	6.43	0.06	0.409
Methanogenic archaea, log_{10}	8.49	8.57	8.45	0.22	0.951

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1] Doses for ethyl-3-nitrooxy propionate (E3NP) were 50 mg/d/animal (E3NP50) and 500 mg/d/animal (E3NP500)

Table 3.4.5. Effect of the addition of E3NP and 3NP (100 mg/animal/d) on body weight, DMI and methane emissions by sheep measured after 14 and 29-30 d of treatment (experiment 3).

	d 14			d 29-30				*P*-value		
Item	Control	E3NP	3NP	Control	E3NP	3NP	SEM	Additive	Time	AxT[2]
BW, kg	44.6	44.1	43.9	44.3	44.0	43.6	1.7	0.280	0.723	0.700
DMI, kg	0.819	0.848	0.870	0.840	0.938	0.899	0.041	0.172	0.120	0.375
CH_4 L/d	24.3	21.8	19.7	22.3	20.6	18.8	1.7	0.137	0.323	0.876
CH_4 L/kg DMI	30.0	25.6	22.3	27.4	21.5	20.9	1.8	0.003	0.043	0.618

[1] Dose for ethyl-3-nitrooxy propionate (E3NP) was 100 mg/d/animal and for 3-nitrooxypropanol (3NP) was 100 mg/d/animal

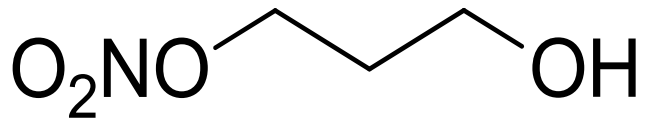
[2] Interaction between additive and time.

Table 3.4.6. Effect of the addition E3NP and 3NP (100 mg/animal/d)on volatile fatty acid profile (mol/100 mol), total VFA concentration (mmol/L), ammonia concentration (mg/100 mL), concentration (log copy gene numbers/g fresh matter) of total bacteria (16S rRNA), protozoa (18S rRNA) and methanogenic archaea (mcrΔ gene) in the rumen of sheep on days 29 and 30; and dry matter degradation (DMD, %) of oats (24 hours) and alfalfa hay (48 hours) in the rumen of sheep on d 22 and 23 (experiment 3).

	Control	E3NP[1]	3NP[1]	SEM	*P value*
Total VFA, *mM*	114.4	115.1	114.2	9.2	0.996
Individual, mol/100 mol					
Acetate	69.2[a]	67.5[b]	64.5[c]	0.6	0.000
Propionate	14.3[a]	16.6[b]	17.5[b]	0.7	0.027
Butyrate	11.0	10.1	12.3	0.8	0.069
iso-butyrate	2.1	2.0	2.1	0.2	0.834
Valerate	1.5	1.8	1.8	0.2	0.317
iso-valerate	2.0	1.9	1.8	0.2	0.790
C_2:C_3	4.9[a]	4.1[b]	3.9[b]	0.2	0.000
N-NH_3, mg/100 mL	49.3	49.8	51.0	4.6	0.972
alfalfa hay DMD, %	78.6	78.3	78.8	1.3	0.963
oats DMD, %	74.2	74.0	70.6	1.9	0.405
Total bacteria, $\log_{10}$	10.74	10.91	10.96	0.09	0.346
Total protozoa, $\log_{10}$	5.41	5.24	5.18	0.13	0.484
Methanogenic archaea, $\log_{10}$	8.54	8.45	8.34	0.07	0.298

[a-c]Means within a row with different superscripts differ ($P < 0.05$).

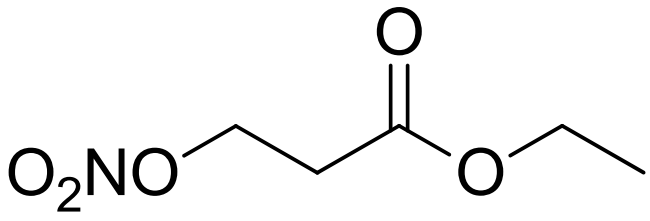
[1] Dose for ethyl-3-nitrooxy propionate (E3NP) was 100 mg/d/animal and for 3-nitrooxypropanol (3NP) was 100 mg/d/animal



$C_3H_7NO_4$

MW: 121.1

3NP



$C_5H_9NO_5$

MW: 163.1

E3NP

Figure 3.4.1. Chemical structure of 3-nitrooxypropanol (3NP) and ethyl-3-nitrooxy propionate (E3NP).

Capítulo 4. Discusión general

Durante la última década ha aumentado el interés por desarrollar aditivos que modifiquen la fermentación ruminal como alternativa a los antibióticos ionóforos, promotores del crecimiento, cuyo uso en alimentación animal está prohibido en la UE desde Enero de 2006; (Casewell et al., 2003) y como respuesta al creciente interés social por minimizar el impacto ambiental de la ganadería.

En los rumiantes, la actividad fermentativa que ocurre en el rumen determina, en gran medida, la eficiencia de utilización de los nutrientes y genera una serie de productos finales, entre ellos diversos gases como el metano (21 % del total de gas en el rumen) que contribuye significativamente a las emisiones de gases con efecto invernadero y representa una ineficiencia energética para el metabolismo del animal. Para tratar de reducir la producción de metano en rumiantes se están investigando diversos tipos de estrategias, entre ellas las basadas en el uso de aditivos, compuestos derivados de plantas o sintéticos, cuyo efecto sobre la fermentación ruminal, en general, y sobre la producción de metano, en particular, es objeto de numerosos estudios en la actualidad. Los resultados obtenidos hasta el momento indican que el uso de compuestos que modulan la fermentación ruminal, como estrategia alimentaria, tiene una serie de limitaciones: la distinta pureza de los compuestos utilizados y la estabilidad de los aditivos durante su almacenamiento; la adaptación de los microorganismos ruminales a la presencia del aditivo; la naturaleza de la dieta suministrada al rumiante; el pH ruminal; la dificultad para extrapolar las condiciones y resultados *in vitro* a las propias de experimentos *in vivo*; la posible presencia de residuos de los aditivos en los productos animales (carne o leche) y la viabilidad económica de su implantación en el sector. Además, los

mecanismos de acción de esos compuestos y sus efectos a largo plazo en el animal, no se conocen bien. Por ello, en el presente trabajo de Tesis doctoral se aborda el estudio del efecto de distintos aditivos, tanto naturales como sintéticos, sobre la fermentación, la producción de metano y las poblaciones microbianas en el rumen así como la relación entre los efectos de los aditivos y el tiempo de tratamiento con los mismos. Se han realizado ensayos *in vitro,* utilizando distintos sistemas de incubación, e *in vivo* utilizando pequeños rumiantes como animales experimentales.

El objetivo de la presente Tesis doctoral es contribuir a entender y, en consecuencia, a superar las limitaciones que afectan al uso de aditivos que modulan la fermentación ruminal y reducen la producción de metano. Para ello se ha abordado el estudio de los mecanismos de acción de compuestos organosulfurados derivados del ajo, aceites esenciales y compuestos sintéticos.

Se ha estudiado el efecto antimetanogénico de una amplia gama de compuestos derivados de plantas, en cultivos no renovados de microorganismos ruminales y se ha tratado de confirmar el efecto de aquellos que mostraron un mayor potencial mediante ensayos de corta duración en caprino (Publicación 1). También se ha estudiado el efecto de una serie de aditivos sobre la fermentación ruminal, producción de metano y la abundancia y estructura de los principales grupos microbianos del rumen en ensayos *in vitro* de media duración utilizando fermentadores de flujo continuo (Publicación 2). En caprino se ha estudiado, a medio-largo plazo, el efecto de la dosis del compuesto que resultó más prometedora en los ensayos mencionados anteriormente (Publicación 3). Por último, se ha estudiado el efecto de dos

compuestos sintéticos, mediante ensayos en cultivos no renovados de microorganismos ruminales y posteriormente en ovino a medio-largo plazo, sobre la producción de metano, parámetros fermentativos y poblaciones microbianas. Los estudios se han centrado en la acción de los compuestos sobre las poblaciones microbianas y en las diferencias en los efectos observados *in vitro* e *in vivo*.

1) Influencia de la dieta y de la dosis de un compuesto sobre sus efectos *in vitro*

La información acerca el uso de compuestos derivados de plantas y sintéticos como moduladores de la fermentación ruminal es extensa y ha sido obtenida fundamentalmente utilizando sistemas *in vitro* (Calsamiglia et al., 2007; Hart et al., 2008; Benchaar y Greathead, 2011). Los efectos observados son variables en función de la naturaleza y la dosis del compuesto empleado y del tipo de sustrato incubado, (Hart et al., 2008). En los ensayos *in vitro* realizados durante 24 y 72 h se incubaron dos dietas diferentes, constituidas por heno de alfalfa y concentrado a partes iguales siguiendo el tipo de alimentación más extendido en caprino lechero en España. Los componentes del concentrado diferían en cuanto a la degradabilidad ruminal del almidón (cebada vs maíz) y proteína (habas vs harina de girasol). Con ambas dietas se estudió el efecto de la dosis de una serie de aceites esenciales y derivados del ajo sobre la fermentación ruminal y la producción de metano para tratar de contrastar la hipótesis, propuesta en trabajos anteriores, acerca de la influencia del patrón de fermentación del substrato incubado sobre el efecto de los aditivos (Newbold et al., 2004; Duval et al., 2007). Cuando la incubación se realizaba durante 24 h

había una interacción entre el tipo de substrato y la dosis de aditivo, que afectaba a la producción de gas; si la incubación se prolonga durante 72 h, no ocurría tal interacción. Es probable que estas diferencias en la respuesta a aditivos, dependiente del tiempo de incubación, esté relacionada con la velocidad y extensión de la degradación ruminal del substrato y, por tanto, del tiempo de permanencia del mismo (Van Soest, 1982) en el que en el caso de los concentrados es, generalmente, rápida e inferior a 24 horas. Es probable que el efecto de los aditivos estudiados hubiese variado aún más si, además de distintos concentrados, la dieta hubiese incluido distintos tipos y proporción de forraje, como se ha indicado recientemente (Castro-Montoya et al., 2012). Sin embargo, se decidió emplear una mezcla estándar que representase el tipo de dieta práctica más utilizada en los sistemas de producción caprino del sur peninsular (Mena Guerrero et al., 2005; Mena et al., 2005). En incubaciones de 24 h de duración se ha observado una disminución lineal de la concentración de AGV y de la producción de gas con dosis crecientes de compuestos como PTS, PTSO y CAR. También la producción de metano disminuía linealmente al incrementarse la dosis de compuestos derivados de plantas (CAR, CIN, PTS, PTSO y DDS) y de compuestos sintéticos (E3NP y 3NP). La relación dosis-efecto se ha observado en otros trabajos con compuestos organosulfurados (Busquet et al., 2005b; Kamel et al., 2008; Mateos et al., 2013) y sintéticos (Soliva et al., 2011). El mecanismo de acción de los compuestos organosulfurados (Busquet et al., 2005b) se ha relacionado con la inhibición del crecimiento de las arqueas metanogénicas, debida a la acción del aditivo en el metabolismo de lípidos específicos de la membrana de estos microorganismos. No obstante, en el presente trabajo no se han observado variaciones en la

abundancia de arqueas metanogénicas como consecuencia de la presencia de la mayoría de los compuestos organosulfurados estudiados, con la excepción del DDS, lo que concuerda con observaciones recientes que sugieren que la metanogénesis en el rumen depende de la abundancia relativa de las especies de arqueas metanogénicas y no de su biomasa total (Zhou et al., 2010; Mitsumori et al., 2012). Los compuestos sintéticos estudiados, fueron seleccionados mediante un screening *in silico* para inhibir la enzima methyl-CoM reductasa, presente en arqueas metanogénicas e implicada en el último paso de la ruta metabólica de la reducción de CO_2 a CH_4. Tampoco estos compuestos afectaron a la abundancia de arqueas metanogénicas aunque sí promovían un descenso en la producción de metano, lo que apoyaría la teoría anteriormente expuesta acerca del efecto de los aditivos sobre la distribución de especies en la población de arqueas metanogénicas.

2) Persistencia del efecto de los aditivos

Los resultados *in vitro* obtenidos en cultivos no renovados de microorganismos ruminales, permitieron seleccionar la dosis y los compuestos más interesantes en cuanto a su efecto sobre la fermentación ruminal y la producción de metano, para estudiar si tales efectos persistían o no en incubaciones más prolongadas, utilizando fermentadores de flujo continuo simple. La dieta consistía en una mezcla de heno de alfalfa y concentrado en proporción 1:1, siendo el concentrado utilizado una mezcla de los dos empleados en las incubaciones de corta duración. Las dosis y compuestos estudiados fueron los siguientes: 80, 200 y 160 µL/L de contenido del fermentador/día de DDS, PTS y BCM, respectivamente. Este último, un compuesto halogenado sintético, se utilizó

como control positivo por su demostrado efecto antimetanogénico tanto en condiciones *in vitro* como *in vivo*. El tratamiento con PTS y BCM durante 12 días redujo la producción de metano 48% y 98%, respectivamente, lo que concuerda con los resultados observados en incubaciones *in vitro* de corta duración (24-72 h). Sin embargo, el tratamiento con DDS no afectó a la producción de metano en contraposición a lo observado en las incubaciones de corta duración. Tampoco se observó un efecto de los compuestos PTS y DDS sobre la concentración de AGVs, lo que contrasta con los resultados obtenidos en las incubaciones cortas. La ausencia de efecto del DDS ha sido también observada *in vivo* con ovejas (Klevenhusen et al., 2011) y puede indicar una adaptación a la presencia del compuesto en el rumen de los microorganismos, bien de las arqueas metanogénicas bien de otros grupos como bacterias o protozoos, que puedan degradar o modificar el compuesto activo de tal manera que pierda su actividad como ocurre con las saponinas (Hart et al., 2008). Sin embargo, el tratamiento con BCM provocó un descenso en la concentración de AGV totales, en contradicción con otros trabajos llevados a cabo por diversos autores (Goel et al., 2009; Abecia et al., 2012; Mitsumori et al., 2012), lo que puede deberse al uso de una dosis más elevada del compuesto en el presente trabajo en comparación con los llevados a cabo por esos autores. Los tratamientos con PTS y BCM promovían un descenso de la abundancia de las arqueas metanogénicas, que no se observó en las incubaciones de corta duración, aunque es de destacar que en el caso del PTS ese efecto solo se observó los días 4 y 8 de tratamiento desapareciendo a los 12 días.

La controversia en cuanto a los resultados obtenidos con distintos sistemas *in vitro* puede deberse al sistema per se (Soto et al., 2012), siendo los cultivos de

microorganismos ruminales no renovados sistemas más simples que los fermentadores de flujo continuo, y con capacidad diferente para simular la fermentación ruminal. Recientemente (Soto et al., 2013, en prensa) hemos descrito en nuestro grupo una disminución de la abundancia de bacterias totales en sistemas no renovados de microorganismos ruminales tras 48 y 72 horas de incubación, en comparación con el inóculo inicial; por el contrario, en fermentadores de flujo continuo no se observó ningún cambio en la abundancia de bacterias tras 4 días de incubación, en comparación con el inóculo inicial. Sin embargo, también la estructura de la población de bacterias cambió en los sistemas no renovados tras 24 y 48 horas de incubación, con respecto a la del inóculo de partida, mientras que en los fermentadores a los 4 días de incubación era similar a la observada en el inóculo de partida. Estos resultados indican una mayor capacidad de los fermentadores de mantener un ecosistema microbiano más cercano en composición y biomasa al del rumen.

En la Publicación 2, la abundancia de bacterias y protozoos no fue afectada por ninguno de los tratamientos a los 12 días de incubación en fermentadores, si bien el estudio, mediante el empleo de pirosecuenciación del gen 16sRNA de bacterias, reveló un cambio en la estructura de la población de dicho grupo en los fermentadores tratados con BCM. Se observó un aumento de la abundancia relativa de bacterias del género *Prevotella* y una disminución de las del género *Ruminococcus* lo que concuerda con los resultados observados *in vivo* por otros autores (Mitsumori et al., 2012), los cuales trataron con tres dosis de BCM durante 22 días a cabras alimentadas a nivel de mantenimiento, observando una disminución lineal de la producción de metano con dosis crecientes del compuesto y un aumento, también lineal, de la producción de H_2. Estos

cambios podrían explicar los resultados observados, como por ejemplo el aumento de los ácidos grasos de cadena ramificada, del propionato y de la actividad amilolítica; también el aumento de bacterias del género *Prevotella* (algunas de cuyas especies se asocian con la producción de propionato) y una disminución de las del género *Ruminococcus* (sensibles a un aumento de la presión parcial de H_2) dirigiendo la fermentación ruminal hacia la formación de propionato como sumidero de H_2, alternativo a la formación de CH_4 (Goel et al., 2009; Mitsumori et al., 2012).

3) Relación entre los resultados obtenidos *in vitro* e *in vivo*

Las dosis y compuestos con efectos más interesantes (mayor depresión de la producción de metano y ausencia de efecto o mejora de la fermentación ruminal), se seleccionaron de entre todos los estudiados *in vitro* para llevar a cabo estudios *in vivo*. Se realizaron ensayos de corta duración del tratamiento con PTS, E3NP y 3NP, tanto en caprino como en ovino (9 y 15 días, respectivamente). Los resultados fueron variables y diferentes de los obtenidos *in vitro*. En caprino se observó una reducción del 33% de la producción de metano por kg de materia seca ingerida, tras 9 días de tratamiento con 200 µL/L de contenido del rumen/día de PTS. La reducción era menor que la observada (48 %) tras 12 días de tratamiento en fermentadores de flujo continuo y también inferior a la observada (33-87% de reducción) con dosis similares (160-320 µL/L), en incubaciones a 24 horas en cultivos no renovados de microorganismos ruminales. Sin embargo, tras 28 días de tratamiento de cabras en mantenimiento con la misma dosis de PTS no se modificó ni la producción de metano, ni la fermentación ruminal ni la digestibilidad de los

nutrientes. El tratamiento de ovejas durante un periodo más prolongado (30 días), con 100 mg/L de contenido del rumen y día de los compuestos E3NP y 3NP redujo, menos de lo esperado, la producción de metano (24 % y 23%, respectivamente, a los 15 y 30 días de tratamiento), siendo estos valores inferiores al descenso observado *in vitro* (96 %) con una dosis inferior (80 µL/L). Las discrepancias entre los resultados obtenidos en ensayos *in vitro* e *in vivo* se han descrito también para el aceite de rábano, que disminuía un 90% la producción de metano *in vitro* (Mohammed et al., 2004) mientras que, en terneros, la disminución fue sólo del 19%; compuestos organosulfurados sin efecto sobre la producción de metano en ensayos *in vivo* (Klevenhusen et al., 2011b) aunque con un descenso de ésta *in vitro* (Busquet et al., 2005b; Kamel et al., 2008) y compuestos sintéticos halogenados como el BCM con un marcado descenso de la producción de metano in vitro (96%) y mucho menor (30%) en cabras en lactación tratadas durante 60 días (Abecia et al., 2012). Las discrepancias *in vitro–in vivo* pueden deberse a un comportamiento diferente, en cuanto a degradación y actividad de los compuestos, como consecuencia de factores como el pH, relativamente constante en sistemas *in vitro* y variable en el rumen. En este sentido, se ha observado que los tiosulfinatos son más estables a pH entre 4,5 y 5,5 y menos en un rango de pH de 6,5-7,5 (Shen y Parkin Kirk, 2002). Otra causa de las discrepancias entre los resultados obtenidos *in vitro* e *in vivo* puede radicar en la diferente forma de administración del compuesto en cuestión. Los cambios en la composición de la microbiota ruminal, como consecuencia del procesado del contenido del rumen durante la preparación del inoculo para los ensayos *in vitro*, que implica la eliminación de la mayor parte de los microorganismos asociados a las

partículas sólidas del contenido ruminal y de los anaerobios estrictos (Soto et al., 2012) podrían explicar también parte de las discrepancias observadas entre los resultados obtenidos *in vitro* e *in vivo*. La homogeneidad con la que los compuestos se distribuyen en los distintos compartimentos del ecosistema ruminal, mayor en los sistemas *in vitro* que en el rumen, puede determinar una exposición más rápida de los microorganismos a la acción del aditivo en los sistemas *in vitro*, en comparación con el rumen. La posible adaptación temporal de los microorganismos a algunos compuestos (Benchaar y Greathead, 2011) puede ser causa de discrepancias, pero es un factor que requiere mayor estudio para discernir qué grupos y en qué medida son los determinantes en cada caso (Hart et al., 2008). El tiempo de paso de la digesta a través del rumen, que depende de diversos factores (relación forraje:concentrado, tamaño de partícula, nivel de ingestión, contenido y digestibilidad de la fibra, etc.) también puede causar discrepancia entre los resultados de ensayos *in vitro* e *in vivo.* Además, factores como la concentración del principio activo de un compuesto, el tamaño de las poblaciones microbianas y la complejidad del ecosistema ruminal pueden determinar el efecto de dicho principio activo (Calsamiglia et al., 2007).

4) Papel de las arqueas en la producción de metano

Los microorganismos responsables de la producción de metano en el rumen pertenecen al grupo de las arqueas, principalmente las del *phyla* Euryarchaeota y la clase *Methanobacteria*. En cuanto a géneros, son pocos los que se han identificado, siendo los mayoritarios *Methanobrevibacter*, *Methanosphera* y *Methanomicrobium* (Kim et al., 2011).

La mayoría de los resultados obtenidos por diversos autores (Kongmun et al., 2011; Abecia et al., 2012; Romero-Huelva et al., 2012) indican que la producción de metano en los rumiantes no está directamente relacionada con la biomasa total de arqueas metanogénicas presentes, aunque algunos trabajos han observado modificaciones de la biomasa de arqueas asociadas a una disminución en la producción de metano en cabras (Mitsumori et al., 2012). Sí que parece existir un acuerdo general acerca de la relación directa entre la producción de metano en rumen y cambios en la diversidad de las especies de arqueas (Ohene-Adjei et al., 2008; Zhou et al., 2010). Los resultados observados, en cuanto a la concentración de arqueas, concuerdan con lo apuntado por Abecia et al. (2012), que no observaron efecto del BCM sobre la biomasa de arqueas del rumen de cabras adultas en lactación tratadas durante 2 meses, pero sí un descenso de la producción de metano de un 33 %. Otros autores (Kongmun et al., 2011) tampoco han observado en búfalos efectos significativos de compuestos derivados del ajo sobre la biomasa de arqueas en el rumen aunque la producción de metano se redujo. Los resultados de los trabajos que constituyen esta tesis doctoral, corroboran la hipótesis de que la producción de metano está más estrechamente relacionada con la estructura de la población de arqueas en el rumen que con la abundancia total de las mismas. No se ha observado efecto de los aditivos ensayados, que reducen la producción de metano, sobre la abundancia de arqueas metanogénicas ni en sistemas *in vitro* ni en ensayos *in vivo*, realizados con cabras tratadas con compuestos organosulfurados (PTS) o con ovejas tratadas con compuestos sintéticos (E3NP y 3NP). Sin embargo, la estructura de la población de arqueas en cabras tratadas durante 27 días con PTS, en los que se observó un

descenso numérico de la producción de metano de alrededor de un 10 %, se diferenció de la de los animales no tratados (Publicación 3) en concordancia con las observaciones realizadas por otros autores (Ohene-Adjei et al., 2008; Zhou et al., 2010). Por último, cabe destacar que el análisis, mediante pirosecuenciación (Publicación 2), del contenido de fermentadores de flujo continuo reveló, que el tratamiento con PTS durante 12 días que promovía una reducción de la metanogenesis, un descenso de la abundancia relativa de las arqueas del orden Methanomicrobiales y del género *Methanomicrobium y* un aumento de la abundancia relativa de arqueas de los géneros *Methanobrevibacter* y *Methanosphera*. El control positivo utilizado en dicho experimento, el BCM, también produjo un efecto similar al observado con el PTS sobre las arqueas metanogénicas, aunque más marcado que el del PTS, lo que podría estar ligado a un efecto más pronunciado del BCM sobre la producción de metano. Aun cuando el fragmento de ADN amplificado y secuenciado en ele studio de pirosecuenciación es limitado en su extensión (100 pares de bases), es posible hipotetizar que los cambios en la estructura de la población de arqueas reflejen un descenso en los grupos de arqueas más sensibles a un determinado aditivo, que sería compensado por un aumento en la abundancia relativa de aquellas arqueas más resistentes a dicho compuesto. Mitsumori et al. (2012) observaron una reducción de solo media unidad logarítmica de la biomasa de arqueas metanógenicas, asociada a una reducción de la producción de metano de más del 50%, en cabras tratadas con BCM, lo que apoya la hipótesis de que la actividad metanogénica relativa de distintas especies de arqueas en el rumen juega un papel más importante en la producción de metano que el número absoluto de metanogénicas. En este

sentido, Poulsen et al. (2012) han observado mediante meta-transcriptómica que sólo las arqueas metanogénicas pertenecentes al grupo RCC (Rumen Cluster C), del Orden Thermoplasmatales, cambian en actividad metanogénica en el rumen de vacas tratadas con aceite de colza, mientras que los grupos hasta ahora considerados mayoritarios no se vieron afectados. Por tanto los estudios de diversidad microbiana o expresión génica deben considerar la detección de grupos minoritarios para poder explicar cambios en la producción de metano. Entre las especies, conocidas y cultivadas de arqueas metanogénicas ruminales, se han observado diferencias en cuanto a su sensibilidad al aumento de la presión parcial de H_2 en el rumen y en cuanto a la codificación de distintas enzimas relacionadas con la metanogénesis. Como ejemplos más destacados de estas diferencias destaca: *Mb. ruminantium* que no codifica la coenzima methyl reductasa II (*mcrII* o *mtr*),implicada en la metanogénesis y si codifica la methil CoM reductasa I, lo que la capacita para crecer en presencia de niveles bajos de H_2 en el rumen (Attwood y McSweeney, 2008). Las especies *Mb. smithii* y *Ms.stadtmanae*, pertenecientes al orden Metanobacteriales comparten regiones del gen *mcr* II semi-conservadas con la especie *Mb. ruminantium* (Attwood y McSweeney, 2008) perteneciente al mismo orden, aunque las dos primeras especies poseen genes que codifican proteínas de superficie en sus membranas celulares, diferenciándolas de *Mb. ruminantium,* y permitiéndoles interactuar con el medio y con otros posibles microorganimos y haciéndolas más resistentes a posibles cambios en el ecosistema. Otra especie, *Ms. Barkeri*, perteneciente al orden Methanosarcinales posee más de una methilcobamide:CoM methiltranferasa (Ferguson et al., 1996; Harms y Thauer,

1996; Tallant y Krzycki, 1996), permitiéndole adaptarse mejor a cambios en el medio que otras especies. Existe también la posibilidad de que otros microorganismos hidrogenotróficos, como los acetogénicos, puedan utilizar la acetogénesis reductiva como un sumidero de electrones, alternativo a la formación de metano (Attwood y McSweeney, 2008).

Por último, podría considerarse que el efecto antimetanogénico de los compuestos organosulfurados se deba bien a la inhibición de la actividad de la enzima HMG-CoA reductasa, con un importante papel en la síntesis de éteres isoprenoides, uno de los principales componentes de las membranas celulares de las arqueas (Busquet et al., 2005b; Benchaar y Greathead, 2011) o bien a la inhibición, mediante reacción del grupo tiol, de enzimas relacionadas con la metanogénesis en las arqueas, como la coenzima M reductasa (Liu y Whitman, 2008).

5) Uso de sustancias modificadoras de la fermentación ruminal en el futuro

En los últimos años han aumentado los estudios sobre compuestos antimetanogénicos, tanto derivados de plantas como sintéticos, y sus efectos sobre la fermentación ruminal (Benchaar y Greathead, 2011; Soliva et al., 2011; Abecia et al., 2012; Bodas et al., 2012; Mateos et al., 2013). La mayoría de estos estudios se han realizado en sistemas *in vitro,* con periodos de incubación cortos, aunque están aumentando los estudios *in vivo* que tratan de establecer el efecto de los aditivos, a largo plazo en los rumiantes, y su posible uso en condiciones prácticas. Así, está aumentando el conocimiento de los efectos de dichos compuestos sobre la fermentación ruminal, y sus posibles

mecanismos de acción y las causas de las diferencias entre los resultados obtenidos *in vitro* e *in vivo*. Además, los avances tecnológicos a nivel molecular (ej. secuenciación de última generación), microbiológico, genético y bioinformático están permitiendo analizar y conocer en mayor profundidad el ecosistema ruminal, los efectos de distintos aditivos sobre éste, e identificar microorganismos implicados y sus posibles mecanismos de acción.

Se ha observado que intentar extrapolar las condiciones ensayadas *in vitro* de la mayoría de los aditivos a situaciones *in vivo* implicaría el uso de dosis muy elevadas en animales, lo que limita su utilización práctica y su viabilidad económica (Proyecto FP7-SMEthane). También existen indicios acerca de la posible adaptación de los microorganismos a los compuestos antimetanogénicos y de la transferencia de residuos de los compuestos a los productos animales, destinados al consumo humano así como de la toxicidad que ciertos aditivos pudieran ocasionar en los animales a largo plazo (Benchaar y Greathead, 2011). Por todo ello, sería necesario realizar estudios *in vivo* con tratamientos de larga duración para estudiar no solo los efectos de los aditivos sino también para obtener información precisa sobre su viabilidad práctica y la transferencia de residuos a los productos animales destinados al consumo humano.

Capítulo 5 .Conclusiones

Conclusiones

Teniendo en cuenta los objetivos abordados y los resultados obtenidos se pueden establecer las siguientes conclusiones:

1. Las incubaciones de corta duración realizadas *in vitro* mostraron que entre los aditivos estudiados los de mayor potencial para reducir la producción de metano, fueron los compuestos organosulfurados propil propano tiosulfinato y diallyl disulfuro, y los compuestos sintéticos ethyl-3-nitrooxy propionato y 3-nitrooxypropanol. El tipo de concentrado moduló el efecto del aditivo a las 24 horas y no a las 72, lo que parece relacionarse con su degradación en el rumen, generalmente rápida e inferior a 24 horas.

2. El efecto antimetanogénico del propil propano tiosulfinato se mantuvo tras 12 días de incubación en fermentadores de flujo continuo. Sin embargo, el del diallyl disulfuro desapareció, posiblemente debido a una adaptación de la microbiota ruminal a la presencia de éste compuesto.

3. El suministro de propil propano tiosulfinato a caprino, durante 9 días, y de ethyl-3-nitrooxy propionato y 3-nitrooxypropanol a ovino durante 15 días disminuyó la producción de metano un 33% y 27%, respectivamente. Tras un tiempo más prolongado de tratamiento (28 y 30 días, respectivamente), el efecto antimetanogénico desapareció en el caprino tratado con propil propano tiosulfinato y se mantuvo (21%) en ovino tratado con ethyl-3-nitrooxy propionato y 3-nitrooxypropanol. La naturaleza

del compuesto estudiado y su dosis, la forma de administración al animal, el tiempo de tratamiento y la especie animal pueden condicionar el efecto antimetanogénico de los aditivos.

4. En fermentadores de flujo continuo la reducción de la producción de metano producida por el propil propano tiosulfinato está asociado a un cambio en la estructura de la población de arqueas aumentando la abundancia relativa de especies de los géneros *Methanobrevibacter* y *Methanosphaera* y disminuyendo las del género *Methanomicrobium*, sin afectar a la biomasa total de la población de arqueas. En incubaciones cortas *in vitro* y en el rumen de caprino y ovino tampoco se observaron cambios de la biomasa total de arqueas con aditivos con efecto antimetanogénicos. Los cambios en la estructura de las arqueas en el rumen de caprino se ponen de manifiesto tras la administración del propil propano tiosulfinato durante al menos 4 semanas.

5. La efectividad de la reducción en la metanogénesis de una determinada dosis de un aditivo es 35-80% menor *in vivo* que *in vitro*, lo que podría deberse a factores tales como la homogeneidad de la distribución del aditivo en el rumen, la adaptación temporal de los microorganismos al mismo, la forma en que se administra al animal, su degradación diferencial en el rumen y en condiciones *in vitro* o el procesado del contenido ruminal, previo a su inoculación en los sistemas *in vitro*.

Conclusions

Based on the objectives and results obtained in this work we can conclude:

1. Short term in vitro incubations runs showed that among the additives studied, those with most potential for reducing methane production were organosulphur compounds (propyl propane thiosulfinate and diallyl disulfide), and synthetic compounds ethyl-3-propionate and 3-nitrooxy nitrooxypropanol. The type of concentrate modulated the effect of additives at 24 hours but not at 72 hours, which seems to be related to the degradation time in the rumen, usually rapid and lower than 24 hours.

2. The antimethanogenic effect of propyl propane thiosulfinate persisted after 12 days of incubation in continuous flow fermenters. However, the effect of diallyl disulfide disappeared, possibly due to an adaptation of the ruminal microbiota to the presence of this compound.

3. The treatment with Propyl propane thiosulfinate to goats, for 9 days, and ethyl-3-propionate and 3-nitrooxypropanol to sheep for 15 days, reduced methane production by 33% and 27%, respectively. After a longer period of treatment (28 and 30 days, respectively), the antimethanogenic effect disappeared in goats treated with propyl propane thiosulfinate and persistes (21%) in sheep treated with ethyl-3-propionate and 3-nitrooxypropanol. The nature of the compound and dosage, the form to

administrate it to the animal, the treatment time and the animal species may influence the effect of the antimethanogenic additives.

4. In singe flow Continuous culture fermentors the reduction of methane yield, produced by propane thiosulfinate propyl, was associated to changes in the archaeal population structure, increasing the relative abundance of *Methanobrevibacter* and *Methanosphaera* and decreasing Methanomicrobium without affecting the total biomass of the archaeal population. In short in vitro incubations and in the rumen of goats and sheep, the decrease in methane production did not involve reduction in the total archaea biomass. Changes in the archaeal population structure in the rumen of goats treated with propyl propane thiosulfinate were first evident after 4 weeks.

5. The effectiveness of the reduction of the methanogenesis by a given dose of an additive is 35-80% lower in vivo than in vitro, which may be due to a number of factors such as the homogeneity of the distribution of the additive in the rumen, the temporal adaptation of some microorganisms, the means of administration to the animal, the differential degradation in the rumen and in vitro or the processing of rumen contents, prior to inoculation in in vitro systems.

Capítulo 6. Resumen

Resumen

Se han llevado a cabo cuatro experimentos *in vitro* y cuatro *in vivo* para estudiar el efecto de aditivos, derivados de plantas o sintéticos, sobre la fermentación ruminal, la producción de metano y distintos grupos microbianos del rumen. El estudio de aditivos *in vivo,* tras conocer sus efectos *in vitro,* está sujeto a una serie de limitaciones. Igualmente el uso de aditivos en condiciones prácticas tiene una serie de limitaciones. Entre esas limitaciones destacan la variación en el efecto de los aditivos dependiendo del tipo de compuesto activo y su concentración así como de la dieta que recibe el animal; las diferencias entre los resultados obtenidos *in vitro* e *in vivo*; la falta de estudios acerca de la persistencia temporal de los efectos de los aditivos y; el desconocimiento de los mecanismos de acción de la mayoría de los compuestos antimetanogénicos estudiados recientemente. En la presente tesis se pretende responder a los interrogantes que existen acerca del uso de aditivos en alimentación de rumiantes, tratando de conocer los posibles mecanismos de acción de los compuestos que presentan un efecto antimetanogénico más claro sin comprometer la fermentación ruminal. La Publicación 1 recoge los resultados obtenidos en dos experimentos *in vitro* en los que se estudiaron los efectos de una serie de aceites esenciales y compuestos organosulfurados sobre la fermentación ruminal, la producción de metano y las poblaciones microbianas. Esta publicación también recoge los resultados de un experimento *in vivo,* de corta duración (9 días) realizado en caprino, en el que se estudió el efecto del compuesto que se mostró más prometedor en los experimentos *in vitro*, en cuanto a su potencial para reducir la producción de metano. El primer

experimento *in vitro* consistió en incubaciones de 24 horas, en cultivos no renovados de microorganismos ruminales con líquido ruminal de caprino como inóculo, de carvacrol (CAR), cinamaldehido (CIN), eugenol (EUG), propyl propano tiosulfinato (PTS), propyl propano tiosulfonato (PTSO), diallyl disulfuro (DDS), una mezcla (40:60) de PTS y PTSO (PTS+PTSO) y bromoclorometano (BCM), un compuesto sintético antimetanogénico, que se utilizó como control positivo. Se ensayaron 4 dosis (40, 80, 160 y 320 µL/L) de los diferentes compuestos, todas ellas con 2 tipos de dieta (50:50, heno de alfalfa:concentrado), que diferían en cuanto a la fuente de almidón y proteína del concentrado en cada una de ellas. La producción de gas a las 24 horas descendió linealmente con dosis crecientes de todos los compuestos, excepto del EUG y de la mezcla PTS+PTSO, observándose una interacción de la dosis del compuesto con la dieta con CAR, PTS, PTSO y DDS. La concentración de AGV totales descendió también linealmente con dosis crecientes de PTS, PTSO y CAR, mientras que la relación acético:propiónico descendió al aumentar la dosis de PTS, PTSO y BCM. Estos resultados, obtenidos *in* vitro con incubaciones de 24 h, permitieron seleccionar dos dosis de los siguientes compuestos: PTS, CAR, CIN y BCM (160 y 320 µL/L), PTSO (40 y 160 µL/L) y DDS (80 y 320 µL/L) para su estudio en incubaciones de 72 horas (experimento 2) utilizando, también, cultivos no renovados de microorganismos ruminales. La cinética de producción de gas se vio afectada por todos los compuestos, al igual que la producción de metano a las 24 horas, aunque es cierto que el mayor descenso se observó con el PTS (96%), DDS (62%) y BCM (95%). La digestibilidad de la fibra neutro detergente tras 72 horas de incubación solo descendió con el CAR y PTS. La concentración a las 24 horas de arqueas y

protozoos, descendieron únicamente con las dosis de DDS y PTS respectivamente.

Por último cabe destacar, la ausencia de interacción entre las dosis y las dietas utilizadas. A partir de los resultados derivados de las incubaciones durante 72 h se seleccionó el PTS para estudiar su efecto sobre la producción de metano en caprino durante 9 días de tratamiento. En este experimento también se utilizó el BCM como control positivo. Se utilizaron 32 cabras de raza murciano-granadina alimentadas, a nivel de mantenimiento energético, con una única dieta (55:45 heno de alfalfa:concentrado) en la que el concentrado era una mezcla de los dos utilizados en los experimentos *in vitro* previamente descritos. Las dosis utilizadas fueron 50, 100 y 200 µL/L de contenido ruminal y día para el PTS y 50, 100 y 160 mg/L de contenido ruminal y día para el BCM. Se observó un descenso de la producción de metano, expresada en L/kg de MS ingerida, de alrededor del 33% con el PTS y del 64% con el BCM, siendo en ambos casos inferior a la reducción observada *in vitro* con dosis similares (87 y 96%, respectivamente). Estos resultados sugieren que los efectos observados con dosis similares son inferiores en el rumen que en los sistemas *in vitro*, lo que ha de tenerse en cuenta.

La publicación 2 recoge los resultados obtenidos en un experimento *in vitro* realizado con 8 fermentadores de flujo continuo, inoculados con liquído ruminal de caprino, a los que se adicionó DDS (80 µL/L), PTS (200 µL/L) y BCM (160 mg/L), este último como control positivo, durante dos periodos de 12 días de incubación. Los compuestos y la dieta (heno de alfalfa y concentrado en proporción 1:1) utilizada se seleccionaron a partir de los resultados obtenidos en los ensayos *in vitro*, incluidos en la publicación 1. No se observaron efectos

ni del DDS ni del PTS sobre los parámetros fermentativos, mientras que con el BCM se observó un descenso de la concentración de AGV totales. En cada periodo el último día de incubación se obtuvo contenido de cada fermentador, que se utilizó como inóculo de cultivos de microorganismos ruminales en los que se incubó durante 24 h la misma dieta que recibía el fermentador del que procedía el inóculo, para estudiar el efecto de los distintos tratamientos sobre la producción de metano. Se observó un descenso de la producción de metano con el PTS (48%) y BCM (94%), comparados con el control y una ausencia de efecto con el DDS, con las mismas dosis utilizadas en los fermentadores. Respecto a las poblaciones microbianas se observó una disminución de la concentración de arqueas metanogénicas a partir del dia 4 de tratamientiento con el BCM mientras que con el PTS el efecto sobre este grupo microbiano desapareció a los 12 días de incubación aunque se observase una disminución de metano. El análisis mediante pirosecuenciación de las muestras de los fermentadores recogidas a los 12 días de incubación reveló que el tratamiento con PTS promovía una reducción de la abundancia relativa de las arqueas del orden Methanomicrobiales y del género *Methanomicrobium y* un aumento de la abundancia relativa de arqueas de los géneros *Methanobrevibacter* y *Methanosphera*. El BCM también produjo un efecto similar al observado con el PTS sobre las arqueas metanogénicas, aunque más marcado que el del PTS, lo que podría estar ligado a un efecto más pronunciado del BCM sobre la producción de metano. Respecto a las bacterias totales tampoco se vio modificada su concentración aunque el análisis mediantes pirosecuenciación, reveló únicamente en el caso del BCM un cambio en la estructura de la población de bacterias, aumentando la abundancia relativa de las bacterias del

género *Prevotella* y una disminución de las del género *Ruminococcus.* Dichos resultados sugieren una adaptación o degradación de los microorganismos al compuesto DDS, al desaparecer el efecto sobre la producción de metano tras 12 de incubación, así como cambios en la estructura de la población de arqueas metanogénicas en los fermentadores a los 12 días de incubación ligados a un descenso de la producción de metano, sin afectar en el caso del PTS a la concentración de arqueas metanogénicas.

La Publicación 3 recoge los resultados obtenidos en un experimento *in vivo,* de larga duración (36 días) realizado para estudiar el efecto del PTS fijado en α-cyclodextrina (CD-PTS) (200 mg/L de volumen ruminal). Se utilizaron 12 cabras adultas, de raza murciano-granadina, canuladas en rumen, alimentadas a nivel mantenimiento energético con una dieta constituida por heno de alfalfa y concentrado en relación 1:1. Seis cabras se trataron con CD-PTS y las otras 6 no recibían aditivo. No se observó efecto del CD-PTS sobre la producción de metano, AGV, abundancia de distintos grupos microbianos y la digestibilidad de los nutrientes, si bien la estructura de la población de arqueas metanogénicas de los animales tratados con CD-PTS durante 28 días, se agrupó en un clado diferenciado, lo que podría asociarse con un descenso numérico, no significativo, de la producción de metano en los animales tratados de alrededor de un 10% considerando el mismo tiempo de tratamiento. Además, se estudió el efecto del PTS (200 ppm) sobre las siguientes especies de arqueas metanogénicas: *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanobrevibacter millerae*, realizándose incubaciones de cultivos puros durante 12 días. El PTS redujo la cantidad de metano producido por todas las especies estudiadas. Este resultado apoya la hipótesis de que dicho

compuesto afecta a las arqueas metanogénicas a corto plazo, observándose efecto antimetanogénico tanto en fermentadores (12 días) como en el experimento *in vivo* de corta duración (9 días), y por tanto la falta de efecto sobre la producción de metano en el *in vivo* de larga duración pudiera deberse a una adaptación de las arqueas a la acción del compuesto o a una degradación del compuesto por parte de otros microorganismos. Al igual que ocurre con otros compuestos derivados del ajo el PTS afecta a la distribución de las especies de arqueas en el ecosistema ruminal, sin afectar a la biomasa total de arqueas en el rumen, manifestándose dichos cambios tras un tiempo relativamente prolongado de tratamiento (28 días). Por tanto, se requieren nuevos estudios para corroborar si dosis superiores a las utilizadas en este trabajo o vías más eficientes de administración del compuesto, tendrían un efecto diferente sobre las emisiones de metano y la fermentación ruminal *in vivo*.

La publicación 4 recoge los resultados de un experimento *in vitro* y dos *in vivo*, realizados para estudiar el efecto de dos compuestos sintéticos, ethyl-3-nitrooxy propionate (E3NP) y 3-nitrooxypropanol (3NP), con potencial antimetanogénico, sobre la producción de metano, parámetros fermentativos y los principales grupos microbianos del rumen. Mediante incubaciones de 24 h en cultivos no renovados de microorganismos ruminales se ensayaron dos dosis (40 and 80 µL/L) de E3NP y ENP observándose un reducción considerable (hasta un 95%) de la producción de metano con ambos compuestos sin afectar a la concentración de AGV totales. Los experimentos *in vivo* se llevaron a cabo durante 15 (experimento 2) y 30 días (experimento 3). En el experimento 2, con un diseño de intercambio, se utilizaron 6 ovejas

canuladas en rumen alimentadas, a nivel de mantenimiento energético, con una dieta de heno de alfalfa y cebada (60:40) y dos dosis de E3NP (50 and 500 mg/animal y día) . A los 15 días de tratamiento con la dosis más alta se redujo (29 %) la producción de metano observándose también un descenso en la relación acético:propiónico. En el experimento 3, también con un diseño de intercambio, se utilizaron 9 ovejas canuladas en rumen alimentadas de la manera ya descrita para el experimento 2 y una sola dosis (100 mg/animal día) de los compuestos E3NP y ENP durante 30 días. Se observó una reducción de la producción de metano a los 14 (14 y 25 % para E3NP y 3NP respectivamente) y 30 días (21 % para ambos compuestos) y una reducción de la relación acético:propionico en los dos días. Por último, la concentración de bacterias totales, arqueas y protozoos en el rumen de ovejas no se vio afectada por ninguna de las dosis ni compuestos ensayados. Los resultados obtenidos muestran que ambos compuestos tienen un potencial antimentanogénico *in vivo,* tanto a corto como a largo plazo, sin afectar a la fermentación ruminal negativamente, si bien es cierto que la reducción de metano observada *in vivo* es menor que la observada *in vitro,* con dosis similares de los mismos compuestos.

Debido a las discrepancias observadas entre los resultados *in vitro* e *in vivo*, con compuestos y dosis similares, cobra especial importancia ratificar *in vivo* lo observado *in vitro* en cuanto al efecto de aditivos de carácter antimetanogenico. Diversos factores pueden explicar las discrepancias *in vitro* vs *in vivo*: la baja solubilidad en agua de algunos compuestos y, por tanto, la homogeneidad de su distribución en todos los compartimentos del rumen podría no lograrse plenamente; una posible adaptación temporal de los microorganismos a los

aditivos; la vía de administración de los compuestos podría limitar su permanencia en el rumen; la degradación de los compuestos podría ser distinta *in vitro* que *in vivo*; el procesado del contenido ruminal, previo a su inoculación en los sistemas *in vitro,* también podría influir en los efectos observados puesto que dicho procesado modifica la microbiota ruminal.

Por último, es importante señalar que los resultados obtenidos refuerzan la hipótesis de que la abundancia relativa de distintas especies de arqueas en el rumen juega un papel más importante en la producción de metano que el número absoluto de microorganismos metanogénicos. Serían necesarios nuevos experimentos para confirmar dichos efectos en animales en producción.

Summary

Four in vitro and four in vivo experiments have been conducted to study the effect of additives, synthetic or plants extract compounds, on rumen fermentation, methane production and microbial ecosystem. The development of an additive to its application in practical conditions normally is supported by in vitro and in vivo studies. Such studies face challenges that constraint the interpretation of results: the variation in the effects due to the type of active compound and its concentration; the diet fed to animals; the differences between the results obtained in vitro and in vivo; the lack of studies about the temporal persistence of the effects and; the lack of knowledge of the mechanisms of action of most of the compounds recently investigated. In the present thesis we aim to provide more insight into these issues by trying to understand the possible mechanisms of action of the antimethanogenic compounds without compromising rumen fermentation.

Publication 1 shows the results obtained from two in vitro experiments conducted to investigate the effects of different essential oils and organosulphur compounds on rumen fermentation, methane production and microbial concentration. This publication also shows the results of a short-term in vivo trial (9 days) conducted with goats, which studied the effect of the most promising antimethanogenic compound selected from in vitro experiments. The first experiment consisted of 24 hours in vitro batch cultures incubations using rumen fluid from goats as inoculum. The compounds tested were carvacrol (CAR), cinnamaldehyde (CIN), eugenol (EUG), propyl propane thiosulfinate (PTS), propyl propane thiosulfonate (PTSO), diallyl disulfide (DDS), a mixture

(40:60) of PTS and PTSO (PTS + PTSO) and bromochloromethane (BCM), a synthetic antimethanogenic compound, was used as positive control. Four doses (40, 80, 160 and 320 µl/l) of the different compounds were tested, using two diets (50:50 alfalfa hay:concentrate) differing in starch and protein source within the concentrate. The total gas production was linearly decreased by all compounds, with the exception of EUG and PTS+PTSO, a diet x dose interaction was observed with CAR, PTS, PTSO and DDS. Total volatile fatty acids (VFA) concentration decreased linearly only with PTS, PTSO and CAR while the acetate:propionate ratio decreased with PTS, PTSO and BCM. Based on results from Experiment 1, two doses of PTS, CAR, CIN, and BCM (160 and 320 µl/l), PTSO (40 and 160 µl/l) and DDS (80 and 320 µl/l) were further tested in vitro for 72 hours (Experiment 2), using also batch cultures with rumen fluid from goat as inoculum. The gas production kinetics were affected by all compounds, and digested NDF (DNDF) after 72 h of incubation was only linearly decreased by CAR and PTS. The addition of all compounds linearly decreased methane production, although the greatest reductions were observed for PTS (up to 96%), DDS (62%) and BCM (95%). The archaea and protozoa concentration only decreased at 24 hours with DDS and PTS, respectively. No diet x dose interaction was observed. To further test the results obtained in vitro, PTS was selected to study its effect on methane production in goats treated over 9 days. In this experiment BCM was also used as positive control. Thirty-two Murciano-granadina goats were used, fed at maintenance level, with a diet of alfalfa hay and concentrate (55:45), where the concentrate was a mixture of both concentrates previously described in vitro. The doses used were 0, 50, 100 and 200 µL/L rumen content per day of PTS and 0, 50, 10

and 160 mg/L rumen content per day of BCM. The addition of PTS and BCM resulted in a linear reduction (33% and 64%, respectively) of methane production per unit of dry matter intake, which were lower than the maximum inhibition observed in vitro (87 and 96 %, respectively). These results suggest that the effectiveness of a compound in vivo is lower than in vitro (at the same initial concentration). This needs to be considered when designing in vivo trial based on in vivo data and it is further discussed in this thesis.

Publication 2 shows the results obtained from an in vitro experiment carried out using eight single flow continuous flow fermenters, inoculated with ruminal fluid from goat and treated with DDS (80 µL/L), PTS (200 µL/L) and BCM (160 mg/L), as positive control, in two runs of 12 days. The additives and diet (alfalfa hay:concentrate 1:1) used, were selected based on previous in vitro results, described in publication 1. No effects on rumen fermentation were observed with PTS and DDS, while total VFA concentration decreased with BCM. In each incubation run fermenters contents were taken on day 12 to inoculate 24 hours batch cultures using the same diet an treatments to measure methane production.. No effects on rumen fermentation were observed with PTS and DDS, while total VFA concentration decreased with BCM. Only amylase activity increased on day 4 and 10 with BCM compared to the control and the other treatments. A decrease on methane production was observed with PTS (48%) and BCM (94%), compared to control and no effect was observed with DDS, . The concentration of methanogenic archaea decreased with BCM from day 4 onward, while with PTS the effect disappeared after 12 days of incubation although a methane reduction was observed. The 454 pyrosequencing analysis, from fermenters content samples obtained after 12 days of incubation, revealed

that PTS and BCM decreased the relative abundance of archaea belonging to Methanomicrobiales order and *Methanomicrobium* genus and increased the relative abundance of *Methanobrevibacter* and *Methanosphera* genus. The change in the community composition was more pronounced for BCM than for PTS. The concentration of total bacteria was not modified by any treatment, although pyrosequencing analysis revealed, with BCM treatment, a change in the structure of the bacteria population, increasing the relative abundance of *Prevotella* and decreasing that of *Ruminococcus*. These results suggest that the inhibition of methane production in the rumen exhibited by BCM and PTS is associated with a shift in the archaeal biodiversity that involves an increase in *Methanosphaera* and a decrease in *Methanomicrobium* and change sin the structure of the bacterial community when treating with BCM.

Publication 3 shows the results obtained from a long term (36 days) in vivo trial to study the effect of α-cyclodextrin-PTS complex (CD-PTS) given to goats at 200 mg/L rumen content. Twelve adult goats and fed with alfalfa hay and concentrate (50:50) at maintenance level, were grouped in two group and treated with CD-PTS or without treatment (control). No significant effect on methane production, VFA, microbial abundances and apparent digestibility of nutrients were observed, although archaeal structure population was modified on day 28 in animals treated with CD-PTS, grouped them in a sole cluster, which could be associated with a non-significant methane production decreased (10%) on treated animals at 28 days. Additionally, a methanogen pure culture trial for 12 days was conducted to test PTS (200 ppm) effect against three different archaeal strains: *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii* and *Methanobrevibacter millerae*, showing a

substantial reduction of methane production in all the strains. This result supports the hypothesis that this compound affects the methanogenic archaea in short term, showing this antimethanogenic effect in fermenters (12 days) and in vivo short-term trial (9 days), therefore the lack of effect on methane production in the in vivo long-term trial could be due to an adaptation of the archaeal population to the compound or a compound degradation by other microorganisms. As well as other garlic-derived compounds, PTS affected the distribution of the species of archaea in the rumen ecosystem, without affecting the total archaea biomass in the rumen, showing these changes after a relatively long time of treatment (28 days). Therefore, further studies are required to confirm whether higher doses than those used in this work or most efficient way of administering the compound to the animal could have a different effect on methane emissions and rumen fermentation.

Publication 4 shows the results of three experiments, one in vitro and two in vivo, to investigate the effect of two synthetic compounds, ethyl-3-nitrooxy propionate (E3NP) and 3-nitrooxypropanol (3NP), with potential antimethanogenic activity, on ruminal methane production, fermentation pattern and the abundance of major microbial groups. The in vitro batch culture trial of 24 hours of incubation was carried out to test two doses of E3NP and ENP (40 and 80 µL/L), which showed a substantial reduction of methane production (up to 95 %) without affecting VFA concentration. The two in vivo trials were conducted over 15 (experiment 2) and 30 days (experiment 3), respectively, to study their effects in sheep. In experiment 2, six adult non-pregnant sheep with permanent rumen cannula and fed with alfalfa hay and oats (60:40), were treated with ethyl-3-nitrooxy propionate at two doses (50 and 500 mg/animal per

day). Methane production decreased by 29% compared with the control with the highest dose on day 15 and a decrease in the acetate to propionate ratio was also reflected. In experiment 3, nine adult non-pregnant sheep with permanent rumen cannula and fed with alfalfa hay and oats (60:40), were treated with ethyl-3-nitrooxy propionate and 3-nitrooxypropanol at one dose (100 mg/animal per day) over 30 days. A reduction on methane production was observed with both additives at day 14 (14 and 25 % for E3NP and 3NP, respectively) and 30 days (21% with both compounds) and a reduction of acetate to propionate ratio in both treatments was observed. Likewise, total concentration of the analysed microbial groups in the rumen, showed no difference among treatments and doses for both experiments. Both tested compounds showed promising potential as methane inhibitors in the rumen with no detrimental effect on fermentation and intake, although the methane reduction observed in vivo is lower than in vitro, with similar compounds and doses.

Due to the discrepancies observed between in vitro and in vivo results, with similar compounds and doses, is important to confirm in vivo the results observed in vitro in relation to the antimethanogenic effect. Several factors could explain the discrepancies observed in vivo vs. in vitro: the low water solubility of some compounds and, therefore, its homogeneous distribution in all rumen compartments could not be fully realized; a possible adaptation of the microorganisms to the additives; the way of administration of the compounds could limit their presence on the rumen, the degradation of the compounds could be different in vitro than in vivo; processing of the rumen contents, prior to in vitro systems inoculation could also influence in the observed effects because this processing modifies ruminal microbiota.

Finally, it is important to note that these results support the hypothesis that the relative abundance of different species of archaea in the rumen plays a more determinant role in the production of methane than their absolute numbers.

[1]Capítulo 7. Bibliografía

[1]Referencias de todo el texto, exceptuando las que aparecen exclusivamente en el capítulo de metodología y resultados.

Abecia, L., P. Toral, A. Martín-García, G. Martínez, N. Tomkins, E. Molina-Alcaide, C. Newbold, y D. Yáñez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. Journal of Dairy Science 95(4):2027-2036.

Agarwal, N., D. Kamra, L. Chaudhary, y A. Patra. 2006. Effect of Sapindus mukorossi extracts on in vitro methanogenesis and fermentation characteristics in buffalo rumen liquor. Journal of Applied Animal Research 30(1):1-4.

Agarwal, N., C. Shekhar, R. Kumar, L. Chaudhary, y D. Kamra. 2009. Effect of peppermint (*Mentha piperita*) oil on in vitro methanogenesis and fermentation of feed with buffalo rumen liquor. Animal Feed Science and Technology 148(2):321-327.

Anassori, E., B. Dalir-Naghadeh, R. Pirmohammadi, A. Taghizadeh, S. Asri-Rezaei, M. Maham, S. Farahmand-Azar, y P. Farhoomand. 2011. Garlic: A potential alternative for monensin as a rumen modifier. Livestock Science 142(1–3):276-287.

Animut, G., R. Puchala, A. Goetsch, A. Patra, T. Sahlu, V. Varel, y J. Wells. 2008a. Methane emission by goats consuming diets with different levels of condensed tannins from lespedeza. Animal Feed Science and Technology 144(3):212-227.

Animut, G., R. Puchala, A. Goetsch, A. Patra, T. Sahlu, V. Varel, y J. Wells. 2008b. Methane emission by goats consuming different sources of condensed tannins. Animal Feed Science and Technology 144(3):228-241.

Attwood, G. y C. McSweeney. 2008. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H_2 utilisation in the rumen. Animal Production Science 48(2):28-37.

Beauchemin, K. y S. McGinn. 2006. Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. Journal of Animal Science 84(6):1489.

Beauchemin, K., S. McGinn, T. Martinez, y T. McAllister. 2007. Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. Journal of Animal Science 85(8):1990-1996.

Beauchemin, K. A., M. Kreuzer, F. O'Mara, y T. A. McAllister. 2008. Nutritional management for enteric methane abatement: a review. Australian Journal of Experimental Agriculture 48(2):21-27.

Beauchemin, K., T. McAllister, y S. McGinn. 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 4(035):1-18.

Benchaar, C., H. V. Petit, R. Berthiaume, T. D. Whyte, y P. Y. Chouinard. 2006. Effects of Addition of Essential Oils and Monensin Premix on Digestion, Ruminal Fermentation, Milk Production, and Milk Composition in Dairy Cows. Journal of Dairy Science 89(11):4352-4364.

Benchaar, C., A. Chaves, G. Fraser, K. Beauchemin, y T. McAllister. 2007. Effects of essential oils and their components on *in vitro* rumen microbial fermentation. Canadian Journal of Animal Science 87(3):413-419.

Benchaar, C. y H. Greathead. 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology 166-167:338-355.

Bhatta, R., Y. Uyeno, K. Tajima, A. Takenaka, Y. Yabumoto, I. Nonaka, O. Enishi, y M. Kurihara. 2009. Difference in the nature of tannins on in vitro ruminal methane and volatile fatty acid production and on methanogenic archaea and protozoal populations. Journal of Dairy Science 92(11):5512-5522.

Bodas, R., S. Lopez, M. Fernandez, R. García-González, A. Rodríguez, R. Wallace, y J. González. 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Animal Feed Science and Technology 145(1-4):245-258.

Bodas, R., N. Prieto, R. García-González, S. Andrés, F. Giráldez, y S. López. 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Animal Feed Science and Technology 176(1-4):78-93.

Boxma, B., R. M. de Graaf, G. W. van der Staay, T. A. van Alen, G. Ricard, T. Gabaldón, A. H. van Hoek, S. Y. Moon-van der Staay, W. J. Koopman, y J. J. van Hellemond. 2005. An anaerobic mitochondrion that produces hydrogen. Nature 434(7029):74-79.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods: a review. International Journal of Food Microbiology 94(3):223-253.

Busquet, M., S. Calsamiglia, A. Ferret, P. Cardozo, y C. Kamel. 2005a. Effects of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. Journal of Dairy Science 88(7):2508-2516.

Busquet, M., S. Calsamiglia, A. Ferret, M. Carro, y C. Kamel. 2005b. Effect of garlic oil and four of its compounds on rumen microbial fermentation. Journal of Dairy Science 88(12):4393-4404.

Busquet, M., S. Calsamiglia, A. Ferret, y C. Kamel. 2005c. Screening for effects of plant extracts and active compounds of plants on dairy cattle rumen microbial fermentation in a continuous culture system. Animal Feed Science and Technology 123:597-613.

Busquet, M., S. Calsamiglia, A. Ferret, y C. Kamel. 2006. Plant extracts affect in vitro rumen microbial fermentation. Journal of Dairy Science 89(2):761-771.

Calsamiglia, S., M. Busquet, P. W. Cardozo, L. Castillejos, y A. Ferret. 2007. Invited Review: Essential Oils as Modifiers of Rumen Microbial Fermentation. Journal of Dairy Science 90(6):2580-2595.

Cardozo, P., S. Calsamiglia, A. Ferret, y C. Kamel. 2004. Effects of natural plant extracts on ruminal protein degradation and fermentation profiles in continuous culture. Journal of Animal Science 82(11):3230.

Cardozo, P., S. Calsamiglia, A. Ferret, y C. Kamel. 2005. 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. Journal of Animal Science 83(11):2572.

Cardozo, P. W., S. Calsamiglia, A. Ferret, y C. Kamel. 2006. Effects of alfalfa extract, anise, capsicum, and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high-concentrate diet. Journal of Animal Science 84(10):2801-2808.

Carro, M. y M. Ranilla. 2007. Influence of different concentrations of disodium fumarate on methane production and fermentation of concentrate feeds by rumen micro-organisms in vitro. British Journal of Nutrition 90(03):617-623.

Carulla, J., M. Kreuzer, A. Machmüller, y H. Hess. 2005. Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Crop and Pasture Science 56(9):961-970.

Casewell, M., C. Friis, E. Marco, P. McMullin, y I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. Journal of Antimicrobial Chemotherapy 52(2):159-161.

Castillejos, L., S. Calsamiglia, A. Ferret, y R. Losa. 2005. 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. Animal Feed Science and Technology 119(1-2):29-41.

Castillejos, L., S. Calsamiglia, y A. Ferret. 2006. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. Journal of Dairy Science 89(7):2649-2658.

Castillejos, L., S. Calsamiglia, A. Ferret, y R. Losa. 2007. Effects of dose and adaptation time of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 132(3-4):186-201.

Castillejos, L., S. Calsamiglia, J. Martin-Tereso, y H. Ter Wijlen. 2008. *In vitro* evaluation of effects of ten essential oils at three doses on ruminal fermentation of high concentrate feedlot-type diets. Animal Feed Science and Technology 145(1-4):259-270.

Castro-Montoya, J., S. De Campeneere, G. Van Ranst, y V. Fievez. 2012. Interactions between methane mitigation additives and basal substrates on *in vitro* methane and VFA production. Animal Feed Science and Technology 176:47-60.

Cimanga, K., K. Kambu, L. Tona, S. Apers, T. De Bruyne, N. Hermans, J. Totté, L. Pieters, y A. Vlietinck. 2002. Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. Journal of Ethnopharmacology 79(2):213-220.

Cox, S. D., C. M. Mann, y J. L. Markham. 2001. Interactions between components of the essential oil of *Melaleuca alternifolia.* Journal of Applied Microbiology 91(3):492-497.

Chao, S., y D. G. Young. 2000. Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. Journal of Essential Oil Research 12(5):639-649.

Chaves, A., M. He, W. Yang, A. Hristov, T. McAllister, y C. Benchaar. 2008a. Effects of essential oils on proteolytic, deaminative and methanogenic activities of mixed ruminal bacteria. Canadian Journal of Animal Science 88(1):117-122.

Chaves, A., K. Stanford, M. Dugan, L. Gibson, T. McAllister, F. Van Herk, y C. Benchaar. 2008b. Effects of cinnamaldehyde, garlic and juniper berry essential oils on rumen fermentation, blood metabolites, growth performance, and carcass characteristics of growing lambs. Livestock Science 117(2-3):215-224.

Chaves, A. V., K. Stanford, L. L. Gibson, T. A. McAllister, y C. Benchaar. 2008c. Effects of carvacrol and cinnamaldehyde on intake, rumen fermentation, growth performance, and carcass characteristics of growing lambs. Animal Feed Science and Technology 145(1-4):396-408.

Chen, Y., G. B. Penner, M. Li, y M. Oba. 2011. Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet. Applied and environmental microbiology 77(16):5770-5781.

Choi, N., S. Lee, H. SUNG, S. Lee, y J. Ha. 2004. Effects of halogenated compounds, organic acids and unsaturated fatty acids on in vitro methane production and fermentation characteristics. Asian-australasian journal of animal sciences 17(9):1255-1259.

Davidson, P. y A. Naidu. 2000. Phyto-phenols. Pages 265–293 in Natural Food Antimicrobial Systems. AS Naidu, ed. CRC Press, Boca Raton, FL.

Deans, S. G., and G. Ritchie. 1987. Antibacterial properties of plant essential oils. International Journal of Food Microbiology 5:165–180.

Demeyer, D. y V. Fievez. 2000. Ruminants et environnement: la méthanogenèse. Annales de Zootechnie 49:95-112.

Deng, W., D. Xi, H. Mao, y M. Wanapat. 2008. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: a review. Molecular Biology Reports 35(2):265-274.

Denman, S. E., N. W. Tomkins, y C. S. McSweeney. 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiology Ecology 62(3):313-322.

Dorman, H. J. D. y S. G. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology 88(2):308-316.

Duval, S. M., N. R. McEwan, R. C. Graham, R. J. Wallace, y C. J. Newbold. 2007. Effect of a blend of essential oil compounds on the colonization of starch-rich substrates by bacteria in the rumen. Journal of applied microbiology 103(6):2132-2141.

Eckard, R., D. Chapman, y R. White. 2008. Nitrogen balances in temperate perennial grass and clover dairy pastures in south-eastern Australia. Crop and Pasture Science 58(12):1167-1173.

Eckard, R., C. Grainger, y C. De Klein. 2010. Options for the abatement of methane and nitrous oxide from ruminant production: A review. Livestock Science 130(1):47-56.

Edwards, J. E., N. R. McEwan, A. J. Travis, y R. J. Wallace. 2004. 16S rDNA library-based analysis of ruminal bacterial diversity. Antonie Van Leeuwenhoek 86(3):263-281.

Eugène, M., H. Archimède, y D. Sauvant. 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livestock Production Science 85(1):81-97.

Evans, J. y S. Martin. 2000. Effects of thymol on ruminal microorganisms. Current microbiology 41(5):336-340.

Ferguson, D. J., J. A. Krzycki, y D. A. Grahame. 1996. Specific roles of methylcobamide: coenzyme M methyltransferase isozymes in metabolism of methanol and methylamines in Methanosarcina barkeri. Journal of Biological Chemistry 271(9):5189-5194.

Ferme, D., M. Banjac, S. Calsamiglia, M. Busquet, C. Kamel, y G. Avguštin. 2004. The effects of plant extracts on microbial community structure in a rumen-simulating continuous-culture system as revealed by molecular profiling. Folia Microbiologica 49(2):151-155.

Foskolos, A. 2012. Strategies to Reduce Nitrogen Excretion from Ruminants: Targeting the Rumen.Tesis Docotoral. Universidad Autónoma de Barcelona.

Francis, G., Z. Kerem, H. P. Makkar, y K. Becker. 2002. The biological action of saponins in animal systems: a review. British Journal of Nutrition 88(6):587-605.

Friedman, M., N. Kozukue, y L. A. Harden. 2000. Cinnamaldehyde content in foods determined by gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 48(11):5702-5709.

García-Martínez, R., M. Ranilla, M. Tejido, y M. Carro. 2007. Effects of disodium fumarate on in vitro rumen microbial growth, methane production and fermentation of diets differing in their forage: concentrate ratio. British Journal of Nutrition 94(01):71-77.

Gerber, P., A. Hristov, B. Henderson, H. Makkar, J. Oh, C. Lee, R. Meinen, F. Montes, T. Ott, y J. Firkins. 2013. Technical options for the mitigation of direct methane and nitrous oxide emissions from livestock: a review. Animal 7(s2):220-234.

Gershenzon, J. y R. Croteau. 1991. Terpenoids. Herbivores: their interactions with secondary plant metabolites 1:165–219.

Giannenas, I., J. Skoufos, C. Giannakopoulos, M. Wiemann, O. Gortzi, S. Lalas, y I. Kyriazakis. 2011. Effects of essential oils on milk production, milk

composition, and rumen microbiota in Chios dairy ewes. Journal of Dairy Science 94(11):5569-5577.

Goel, G., H. Makkar, y K. Becker. 2008a. Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin□rich fractions from different plant materials. Journal of applied microbiology 105(3):770-777.

Goel, G., H. P. S. Makkar, y K. Becker. 2008b. Effects of Sesbania sesban and Carduus pycnocephalus leaves and Fenugreek (*Trigonella foenum-graecum L.*) seeds and their extracts on partitioning of nutrients from roughage- and concentrate-based feeds to methane. Animal Feed Science and Technology 147(1–3):72-89.

Goel, G., H. P. S. Makkar, y K. Becker. 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. British Journal of Nutrition 101(10):1484-1492.

Grainger, C., T. Clarke, M. Auldist, K. Beauchemin, S. McGinn, G. Waghorn, y R. Eckard. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Canadian Journal of Animal Science 89(2):241-251.

Greathead, H. 2003. Plants and plant extracts for improving animal productivity. Proceedings of the Nutrition Society 62(02):279-290.

Griffin, S. G., S. G. Wyllie, J. L. Markham, y D. N. Leach. 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour and Fragrance Journal 14(5):322-332.

Guan, H., K. Wittenberg, K. Ominski, y D. Krause. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. Journal of Animal Science 84(7):1896-1906.

Guo, Y., J. X. Liu, Y. Lu, W. Zhu, S. Denman, y C. McSweeney. 2008. Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen microorganisms. Letters in Applied Microbiology 47(5):421-426.

Hallier, A., V. Noirot, B. Medina, L. Leboeuf, y S. Cavret. 2013. Development of a method to determine essential oil residues in cow milk. Journal of Dairy Science 96(3):1447-1454.

Hammer, K. A., C. F. Carson, y T. V. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology 86:985–990.

Hannah, S., M. Stern, y F. Ehle. 1986. Evaluation of a dual flow continuous culture system for estimating bacterial fermentation *in vivo* of mixed diets containing various soya bean products. Animal Feed Science and Technology 16(1):51-62.

Hariadi, B. T. y B. Santoso. 2010. Evaluation of tropical plants containing tannin on in vitro methanogenesis and fermentation parameters using rumen fluid. Journal of the Science of Food and Agriculture 90(3):456-461.

Harms, U. y R. K. Thauer. 1996. Methylcobalamin: coenzyme M methyltransferase isoenzymes MtaA and MtbA from *Methanosarcina barkeri.* European Journal of Biochemistry 235(3):653-659.

Hart, K., S. Girdwood, S. Taylor, D. Yanez-Ruiz, y C. Newbold. 2006. Effect of allicin on fermentation and microbial populations in the rumen simulating fermentor RUSITEC. Reproduction Nutrition Development 46(1):S97.

Hart, K. J., D. R. Yáñez-Ruiz, S. M. Duval, N. R. McEwan, y C. J. Newbold. 2008. Plant extracts to manipulate rumen fermentation. Animal Feed Science and Technology 147(1-3):8-35.

Hervás, G., P. Frutos, F. Javier Giráldez, Á. R. Mantecón, y M. A. C. Álvarez Del Pino. 2003. Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Animal Feed Science and Technology 109(1):65-78.

Hess, H., L. Monsalve, C. Lascano, J. Carulla, T. Díaz, y M. Kreuzer. 2003. Supplementation of a tropical grass diet with forage legumes and *Sapindus saponaria* fruits: effects on in vitro ruminal nitrogen turnover and methanogenesis. Crop and Pasture Science 54(7):703-713.

Hobson, P. N. y C. S. Stewart. 1997. Rumen Microbial Ecosystem. Ed. Springer, London, UK..

Holtshausen, L., A. V. Chaves, K. A. Beauchemin, S. M. McGinn, T. A. McAllister, N. E. Odongo, P. R. Cheeke, y C. Benchaar. 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. Journal of Dairy Science 92(6):2809-2821.

Hook, S. E., K. S. Northwood, A. D. G. Wright, y B. W. McBride. 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. Applied and Environmental Microbiology 75(2):374-380.

Hook, S. E., A.-D. G. Wright, y B. W. McBride. 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010. 1-10.

Hu, W.-L., J.-X. Liu, J.-A. Ye, Y.-M. Wu, y Y.-Q. Guo. 2005. Effect of tea saponin on rumen fermentation *in vitro*. Animal Feed Science and Technology 120(3):333-339.

Hungate, R. E. 1966. Rumen and its microbes. Academic Press, Inc., New York.

Hungate, R. 1967. Hydrogen as an intermediate in the rumen fermentation. Archiv für Mikrobiologie 59(1-3):158-164.

Hungate, R., W. Smith, T. Bauchop, I. Yu, y J. Rabinowitz. 1970. Formate as an intermediate in the bovine rumen fermentation. Journal of bacteriology 102(2):389-397.

Johnson, K. A. y D. E. Johnson. 1995. Methane emissions from cattle. Journal of Animal Science 73(8):2483-2492.

Jouany, J., K. Ushida, S. Hoshino, R. Onodera, H. Minato, y H. Itabashi. 1990. Protozoa and fibre digestion in the rumen. The rumen ecosystem: the microbial metabolism and its regulation 139-150.

Kamel, C., H. Greathead, M. Tejido, M. Ranilla, y M. Carro. 2008. Effects of allicin and diallyl disulfide on in vitro rumen fermentation of a mixed diet. Animal Feed Science and Technology 145(1-4):351-363.

Kamra, D. 2005. Rumen microbial ecosystem. Current Science 89(1):124-135.

Kim, M., M. Morrison, y Z. Yu. 2011. Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiology Ecology 76(1):49-63.

Klevenhusen, F., S. Duval, J. O. Zeitz, M. Kreuzer, y C. R. Soliva. 2011a. Diallyl disulphide and lovastatin: effects on energy and protein utilisation in, as

well as methane emission from, sheep. Archives of Animal Nutrition 65(4):255-266.

Klevenhusen, F., J. O. Zeitz, S. Duval, M. Kreuzer, y C. R. Soliva. 2011b. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Animal Feed Science and Technology 166–167(0):356-363.

Klita, P., G. Mathison, T. Fenton, y R. Hardin. 1996. Effects of alfalfa root saponins on digestive function in sheep. Journal of Animal Science 74(5):1144-1156.

Koenig, K., C. Newbold, F. McIntosh, y L. Rode. 2000. Effects of protozoa on bacterial nitrogen recycling in the rumen. Journal of Animal Science 78(9):2431-2445.

Kong, Y., R. Teather, y R. Forster. 2010. Composition, spatial distribution, and diversity of the bacterial communities in the rumen of cows fed different forages. FEMS Microbiology Ecology 74(3):612-622.

Kongmun, P., M. Wanapat, P. Pakdee, y C. Navanukraw. 2010. Effect of coconut oil and garlic powder on *in vitro* fermentation using gas production technique. Livestock Science 127(1):38-44.

Kongmun, P., M. Wanapat, P. Pakdee, C. Navanukraw, y Z. Yu. 2011. Manipulation of rumen fermentation and ecology of swamp buffalo by coconut oil and garlic powder supplementation. Livestock Science 135(1):84-92.

Konings, W. N., S.-V. Albers, S. Koning, y A. J. Driessen. 2002. The cell membrane plays a crucial role in survival of bacteria and archaea in extreme environments. Antonie Van Leeuwenhoek 81(1-4):61-72.

Lange, M., P. Westermann, y B. K. Ahring. 2005. Archaea in protozoa and metazoa. Applied Microbiology and Biotechnology 66(5):465-474.

Lawson, L. 1996. The composition and chemistry of garlic cloves and processed garlic. Pages 37–107 in Garlic: The science and therapeutic application of *Allium sativum* L and related species. H. P. Koch, and L. D. Lawson, ed. Williams & Wilkins, Baltimore, MD.

Ley, R. E., D. A. Peterson, y J. I. Gordon. 2006. Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124(4):837-848.

Li, R. W., E. E. Connor, C. Li, V. Baldwin, L. Ransom, y M. E. Sparks. 2012. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environmental Microbiology 14(1):129-139.

Lila, Z., N. Mohammed, S. Kanda, T. Kamada, y H. Itabashi. 2003. Effect of sarsaponin on ruminal fermentation with particular reference to methane production in vitro. Journal of Dairy Science 86(10):3330-3336.

Liu, Y. y W. B. Whitman. 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Annals of the New York Academy of Sciences 1125(1):171-189.

Lopez, S., C. Valdés, C. Newbold, y R. Wallace. 1999. Influence of sodium fumarate addition on rumen fermentation in vitro. British Journal of Nutrition 81(01):59-64.

Lourenco, M., P. Cardozo, S. Calsamiglia, y V. Fievez. 2008. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow

continuous culture fermenters. Journal of Animal Science. 86(11): 3045-3053.

Lourenco, M., E. Ramos-Morales, y R. Wallace. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. Animal 4(07):1008-1023.

Mackie, R., C. McSweeney, y A. Klieve. 2002. 4 Microbial Ecology of the Ovine Rumen. Pages 71–94 in Sheep Nutrition ed. Freer, M. and Dove, H.. Oxon New York: CAB International.

Macheboeuf, D., D. Morgavi, Y. Papon, J. Mousset, y M. Arturo-Schaan. 2008. Dose-response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Animal Feed Science and Technology 145(1-4):335-350.

MAGRAMA, Ministerio de Agricultura, Alimentación y Medioambiente. 2011

Mahmoud, A. L. E. 1994. Antifungal action and antiaflatoxigenic properties of some essential oil constituents. Letter Applied Microbiology19:110–113.

Manasri, N., M. Wanapat, y C. Navanukraw. 2012. Improving rumen fermentation and feed digestibility in cattle by mangosteen peel and garlic pellet supplementation. Livestock Science 148(3):291-295.

Martin, C., D. Morgavi, y M. Doreau. 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4(3):351-365.

Martinez, S., J. Madrid, F. Hernandez, M. Megias, J. Sotomayor, y M. Jordans. 2006. Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on in vitro ruminal degradation and volatile fatty acid production. Journal of Agricultal and Food Chemistry 54 (18):6598–6602

Mateos, I., M. Ranilla, M. Tejido, C. Saro, C. Kamel, y M. D. Carro. 2013. The influence of diet type (dairy versus intensive fattening) on the effectiveness of garlic oil and cinnamaldehyde to manipulate in vitro ruminal fermentation and methane production. Animal Production Science 53:299-307.

McAllister, T. y C. J. Newbold. 2008. Redirecting rumen fermentation to reduce methanogenesis. Australian Journal of Experimental Agriculture 48(2):7-13.

McAllister, T. A., T. Martinez, H. D. Bae, A. D. Muir, L. J. Yanke, y G. A. Jones. 2005. Characterization of condensed tannins purified from legume forages: chromophore production, protein precipitation, and inhibitory effects on cellulose digestion. Journal of Chemical Ecology 31(9):2049-2068.

McGinn, S., K. Beauchemin, T. Coates, y D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. Journal of Animal Science 82(11):3346.

McGuffey, R., L. Richardson, y J. Wilkinson. 2001. Ionophores for dairy cattle: current status and future outlook. Journal of Dairy Science 84:194-203.

McIntosh, F., P. Williams, R. Losa, R. Wallace, D. Beever, y C. Newbold. 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Applied and Environmental Microbiology 69(8):5011.

McMahon, L., T. McAllister, B. Berg, W. Majak, S. Acharya, J. Popp, B. Coulman, Y. Wang, y K.-J. Cheng. 2000. A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Canadian Journal of Plant Science 80(3):469-485.

McMichael, A. J., J. W. Powles, C. D. Butler, y R. Uauy. 2007. Food, livestock production, energy, climate change, and health. The Lancet 370(9594):1253-1263.

Mena Guerrero, Y., J. Castel Genís, F. Caravaca Rodríguez, J. Guzmán Guerrero, y P. González Redondo. 2005. Current situation, evolution and diagnosis of goat semiextensive production systems in Middle-Western Andalusia [Spain]. Junta de Andalucía, Sevilla, España.

Mena, Y., J. Castel, F. Romero, M. García, y J. Micheo. 2005. Caracterización técnico-económica de los sistemas caprinos lecheros de raza Malagueña. XXX Jornadas científicas y IX Internacionales de la Sociedad Española de Ovinotecnia y Caprinotecnica:175-177.

Miller, T. L. y M. J. Wolin. 2001. Inhibition of Growth of Methane-Producing Bacteria of the Ruminant Forestomach by Hydroxymethylglutaryl SCoA Reductase Inhibitors. Journal of Dairy Science 84(6):1445-1448.

Min, B., W. Pinchak, R. Anderson, J. Fulford, y R. Puchala. 2006. Effects of condensed tannins supplementation level on weight gain and *in vitro* and *in vivo* bloat precursors in steers grazing winter wheat. Journal of animal science 84(9):2546-2554.

Min, B., W. Pinchak, J. Fulford, y R. Puchala. 2005. Wheat pasture bloat dynamics, in vitro ruminal gas production, and potential bloat mitigation with condensed tannins. Journal of Animal Science 83(6):1322-1331.

Mitsumori, M., T. Shinkai, A. Takenaka, O. Enishi, K. Higuchi, Y. Kobayashi, I. Nonaka, N. Asanuma, S. E. Denman, y C. S. McSweeney. 2012. Responses in digestion, rumen fermentation and microbial populations to

inhibition of methane formation by a halogenated methane analogue. British Journal of Nutrition 108(3):482-491.

Mohammed, N., N. Ajisaka, Z. Lila, K. Hara, K. Mikuni, K. Hara, S. Kanda, y H. Itabashi. 2004. Effect of Japanese horseradish oil on methane production and ruminal fermentation in vitro and in steers. Journal of Animal Science 82(6):1839-1846.

Molero, R., M. Ibars, S. Calsamiglia, A. Ferret, y R. Losa. 2004. Effects of a specific blend of essential oil compounds on dry matter and crude protein degradability in heifers fed diets with different forage to concentrate ratios. Animal Feed Science and Technology 114(1):91-104.

Morgavi, D., E. Forano, C. Martin, y C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. Animal 4(07):1024-1036.

Morgavi, D. P., C. Martin, J.P. Jouany, y M. J. Ranilla. 2012. Rumen protozoa and methanogenesis: not a simple cause–effect relationship. British Journal of Nutrition 107(3):388.

Moumen, A., D. Yáñez-Ruiz, M. Carro y E. Molina-Alcaide. 2009. Protozoa evolution in single-flow continuous culture and Rusitec fermenters fed high-forage diets. Pages 303-308 in Proc. Nutritional and foraging ecology of sheep and goats. Proceedings of the 12th Seminar of the Sub-Network on Nutrition of the FAO-CIHEAM Cooperative Research and Development Network on Sheep and Goats, Thessaloniki, Greece, 11-13 October 2007. Centre International de Hautes Etudes Agronomiques Méditerranéennes.

Muetzel, S., P. Lawrence, E. M. Hoffmann, y K. Becker. 2009. Evaluation of a stratified continuous rumen incubation system. Animal Feed Science and Technology 151(1-2):32-43.

Müller, V., M. Blaut, y G. Gottschalk. 1993. Bioenergetics of methanogenesis. Pages 360-406 in Methanogenesis. Ed. J. G. Ferry. Springer.

Murray, R., A. Bryant, y R. Leng. 1976. Rates of production of methane in the rumen and large intestine of sheep. British Journal of Nutrition 36(01):1-14.

Newbold, C., S. López, N. Nelson, J. Ouda, R. Wallace, y A. Moss. 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. British Journal of Nutrition 94(01):27-35.

Newbold, C. J., F. M. McIntosh, P. Williams, R. Losa, y R. J. Wallace. 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Animal Feed Science and Technology 114(1-4):105-112.

Odongo, N. E., R. Bagg, G. Vessie, P. Dick, M. M. Or-Rashid, S. E. Hook, J. T. Gray, E. Kebreab, J. France, y B. W. McBride. 2007. Long-Term effects of feeding monensin on methane production in lactating dairy cows. Journal of Dairy Science 90(4):1781-1788.

Ohene-Adjei, S., A. Chaves, T. McAllister, C. Benchaar, R. Teather, y R. Forster. 2008. Evidence of increased diversity of methanogenic archaea with plant extract supplementation. Microbial Ecology 56(2):234-242.

Orpin, C. y K. Joblin. 1997. The rumen anaerobic fungi. Pages 140-195 in The rumen microbial ecosystem. P.N. Hobson and C. Stewart, ed. Springer, London, UK.

Ørskov, E. R. y M. Ryle. 1990. Energy nutrition in ruminants. Elsevier Science Publishers Ltd. London and New York.

Ouattara, B., R. E. Simard, R. A. Holley, G. J.-P. Piette, y A. Begin. 1997. Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. International Journal of Food Microbiology 37:155–162.

Patra, A., D. Kamra, y N. Agarwal. 2006. Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Animal Feed Science and Technology 128(3-4):276-291.

Patra, A. K. y J. Saxena. 2009. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie van Leeuwenhoek 96(4):363-375.

Patra, A. K., D. N. Kamra, y N. Agarwal. 2010. Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds in vitro. Journal of the Science of Food and Agriculture 90(3):511-520.

Patra, A. K. y J. Saxena. 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71(11–12):1198-1222.

Patra, A., D. Kamra, R. Bhar, R. Kumar, y N. Agarwal. 2011. Effect of *Terminalia chebula* and *Allium sativum* on in vivo methane emission by sheep. Journal of Animal Physiology and Animal Nutrition 95(2):187-191.

Patra, A. K. y Z. Yu. 2012. Effects of essential oils on methane production and fermentation by, and abundance and diversity of, rumen microbial populations. Applied and Environmental Microbiology 78(12):4271-4280.

Pen, B., C. Sar, B. Mwenya, K. Kuwaki, R. Morikawa, y J. Takahashi. 2006. Effects of Yucca schidigera and Quillaja saponaria extracts on in vitro ruminal fermentation and methane emission. Animal Feed Science and Technology 129(3–4):175-186.

Pen, B., K. Takaura, S. Yamaguchi, R. Asa, y J. Takahashi. 2007. Effects of Yucca schidigera and Quillaja saponaria with or without β 1–4 galacto-oligosaccharides on ruminal fermentation, methane production and nitrogen utilization in sheep. Animal Feed Science and Technology 138(1):75-88.

Pen, B., C. Sar, B. Mwenya, y J. Takahashi. 2008. Effects of Quillaja saponaria extract alone or in combination with Yucca schidigera extract on ruminal fermentation and methanogenesis in vitro. Animal Science Journal 79(2):193-199.

Poulsen, M., C. Schwab, B.B. Jensen, R.M. Engberg, A. Spang, N. Canibe, O. Højberg, G. Milinovich, L. Fragner, C. Schleper, W. Weckwerth, P. Lund, A. Schramm, y Urich T. 2012. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nature Communications. 4:1428.

Qi, M., P. Wang, N. O'Toole, P. S. Barboza, E. Ungerfeld, M. B. Leigh, L. B. Selinger, G. Butler, A. Tsang, y T. A. McAllister. 2011. Snapshot of the eukaryotic gene expression in muskoxen rumen a metatranscriptomic approach. PLoS One 6(5):e20521.

Regensbogenova, M., S. Kisidayova, T. Michalowski, P. Javorsky, S.-v. Staay, G.-v. Staay, J. H. Hackstein, N. R. McEwan, J.-P. Jouany, y J. C. Newbold. 2004. Rapid identification of rumen protozoa by restriction

analysis of amplified 18S rRNA gene. Acta Protozoologica 43(3):219-224.

Reuter, H. D., H. Koch, y L. Lawson. 1996. Therapeutic effects and applications of garlic and its preparations. Pages 135-212 in Garlic. The Science and Therapeutic Application of *Allium sativum* L. and Related Species. H. Koch y L. Lawson, ed. Williams and Wilkins. Baltimore, MD, USA.

Riguera, R. 1997. Isolating bioactive compounds from marine organisms. Journal of Marine Biotechnology 5:187-193.

Romero-Huelva, M., E. Ramos-Morales, y E. Molina-Alcaide. 2012. Nutrient utilization, ruminal fermentation, microbial abundances, and milk yield and composition in dairy goats fed diets including tomato and cucumber waste fruits. Journal of Dairy Science. 95(10):6015-6026.

Ross, Z. M., E. A. O'Gara, D. J. Hill, H. V. Sleightholme, y D. J. Maslin. 2001. Antimicrobial properties of garlic oil against human enteric bacteria: Evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. Applied of Environmental Microbiology 67:475–480.

Santoso, B., B. Mwenya, C. Sar, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, y J. Takahashi. 2004. Effects of supplementing galacto-oligosaccharides, Yucca schidigera or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livestock Production Science 91(3):209-217.

Shen, C. y L. Parkin Kirk. 2002. In Vitro Biogeneration and Stability of Pure Thiosulfinates from Alliums: Stability and Reactivity of Thiosulfinates. Pages 44-57 in Bioactive Compounds in Foods. Vol. 816. American Chemical Society.

Shin, E., K. Cho, W. Lim, S. Hong, C. An, E. Kim, Y. Kim, B. Choi, J. An, y J. Kang. 2004. Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences. Journal of Applied Microbiology 97(2):378-383.

Sikkema, J., J. A. de Bont, y B. Poolman. 1994. Interactions of cyclic hydrocarbons with biological membranes. Journal of Biological Chemistry 269(11):8022-8028.

Sivropoulou, A., E. Papanikolaou, C. Nikolaou, S. Kokkini, T. Lanaras, y M. Arsenakis. 1996. Antimicrobial and cytotoxic activities of *Origanum* essential oils. Journal of Agriculture Food and Chemistry 44:1202–1205.

Skillman, L. C., A. F. Toovey, A. J. Williams, y A.-D. G. Wright. 2006. Development and validation of a real-time PCR method to quantify rumen protozoa and examination of variability between Entodinium populations in sheep offered a hay-based diet. Applied and Environmental Microbiology 72(1):200-206.

SMEthane. Technological platform to develop nutritional additives to reduce methane emissions from ruminants. FP7-SME- 262270. www.smethane.eu

Smith, P., J. Stewart, y L. Fyfe. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Letters in Applied. Microbiology 26(2):118-122.

Soliva, C. R., S. L. Amelchanka, S. M. Duval, y M. Kreuzer. 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as determined with a rumen simulation technique (Rusitec). British Journal of Nutrition 106(01):114-122.

Son, H., J. Song, N. Choi, J. Ha, y J. Chang. 2006. Comparison of the Influence of Carbon Substrates on the Fibrolytic Activities of *Neocallimastix* sp. NLPI-3. Journal of Animal Science and Technology 48(3):415-424.

Soto, E., D. Yáñez-Ruiz, G. Cantalapiedra-Hijar, A. Vivas, y E. Molina-Alcaide. 2012. Changes in ruminal microbiota due to rumen content processing and incubation in single-flow continuous-culture fermenters. Animal Production Science 52(9):813-822.

Soto, E., E. Molina-Alcaide, H. Khelil, y D. Yáñez-Ruiz. 2013. Ruminal microbiota developing in different in vitro simulation systems inoculated with goats' rumen liquor. Animal Feed Science and Technology http://dx.doi.org/10.1016/j.anifeedsci.2013.06.003

St-Pierre, B. y A.-D. G. Wright. 2013. Diversity of gut methanogens in herbivorous animals. Animal 7(1):49-56.

Steinfeld H, Gerber P, Wassenaar T, Castel V, Rosales M and de Haan C 2006. Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations, Rome, Italy.

Stewart, C., H. Flint, y M. Bryant. 1997. The rumen bacteria. Pages 10-72 in The rumen microbial ecosystem. P.N. Hobson and C. Stewart, ed. Springer, London, UK.

Swain, R. A., J. V. Nolan, y A. V. Klieve. 1996. Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. Applied and Environmental Microbiology 62(3):994-997.

Sylvester, J. T., S. K. R. Karnati, Z. Yu, M. Morrison, y J. L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. The Journal of Nutrition 134(12):3378.

Tajima, K., R. Aminov, T. Nagamine, H. Matsui, M. Nakamura, y Y. Benno. 2001a. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Applied and Environmental Microbiology 67(6):2766-2774.

Tajima, K., T. Nagamine, H. Matsui, M. Nakamura, y R. I. Aminov. 2001b. Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. FEMS Microbiology Letters 200(1):67-72.

Tallant, T. C. y J. A. Krzycki. 1996. Coenzyme M methylase activity of the 480-kilodalton corrinoid protein from *Methanosarcina barkeri*. Journal of Bacteriology 178(5):1295-1301.

Tomkins, N. y R. Hunter. 2003. Methane mitigation in beef cattle using a patented anti-methanogen. in Proc. Proceedings: 2nd joint Australia and New Zealand forum on Non-CO2 greenhouse gas emissions from agriculture. CRC for Greenhouse Accounting, Canberra.

Tomkins, N., S. Colegate, y R. Hunter. 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Animal Production Science 49(12):1053-1058.

Tornambé, G., A. Cornu, P. Pradel, N. Kondjoyan, A. Carnat, M. Petit, y B. Martin. 2006. Changes in terpene content in milk from pasture-fed cows. Journal of Dairy Science 89(6):2309-2319.

Ultee, A., E. P. W. Kets, y E. J. Smid. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. Applied Environmental Microbiology 65(10):4606-4610.

Ungerfeld, E., S. Rust, D. Boone, y Y. Liu. 2004. Effects of several inhibitors on pure cultures of ruminal methanogens. Journal of Applied Microbiology 97(3):520-526.

Ushida, K., J. Jouany, y D. Demeyer. 1991. Effects of the presence or absence of rumen protozoa on the efficiency of utilisation of concentrate and fibrous feeds. Pages 625-654 in Proc. Physiological Aspects of Digestion and Metabolism in Ruminants', Proc. 7th Intern. Symp. on Ruminant Physiol., eds. T. Tsuda', Y. Sasaki & R. Kawashima, Acad. Press, New York.

Van Soest, P. J. 1982. Nutritional ecology of the ruminant: Ruminant metabolism, nutritional strategies, the cellulolytic fermentation and the chemistry of forages and plant fibres. O and B Books, Corvallis, Oregon.

Van Vugt S.J., G.C. Waghorn, D.A. Clark, y S.L. Woodward. 2005. Impact of monensin on methane production and performance of cows fed forage diets. Proc. N.Z. Soc. Anim. Prod., 65: 362–366

Verma, V., L. C. Chaudhary, N. Agarwal, R. Bhar, y D. N. Kamra. 2012. Effect of feeding mixture of garlic bulb and peppermint oil on methane emission, rumen fermentation and microbial profile in buffaloes. Animal Nutrition and Feed Technology 12(2):157-164.

Vialloninsta, C., B. Martin, I. Verdier-Metz, P. Pradel, J.-P. Garel, J.-B. Coulon, y J.-L. Berdagué. 2000. Transfer of monoterpenes and sesquiterpenes from forages into milk fat. Le lait 80(6):635-641.

Waghorn, G. C., H. Clark, V. Taufa, y A. Cavannagh. 2007. Monensin controlled release capsules for improved production and mitigating methane in

dairy cows fed pasture. Proceedings of the New Zealand Society of Animal Production, 67, 266–271.

Wakita, M. y S. Hoshino. 1989. Quantitative observation of starch accumulation and degradation by rumen ciliates genus Entodinium *in vitro* and *in vivo*. Japanese Journal of Zootechnical Science 60.

Wallace, R. J., N. R. McEwan, F. M. McIntosh, B. Teferedegne, y C. J. Newbold. 2002. Natural products as manipulators of rumen fermentation. Asian Australasian Journal of Animal Sciences 15(10):1458-1468.

Wallace, R., T. Wood, A. Rowe, J. Price, D. Yanez, S. Williams, y C. Newbold. 2006. Encapsulated fumaric acid as a means of decreasing ruminal methane emissions. Pages 148-151 in Proc. International Congress Series. Elsevier.

Wang, C., S. Wang, y H. Zhou. 2009. Influences of flavomycin, ropadiar, and saponin on nutrient digestibility, rumen fermentation, and methane emission from sheep. Animal Feed Science and Technology 148(2):157-166.

Wang, Y., T. A. McAllister, L. J. Yanke, Z. J. Xu, P. R. Cheeke, y K. J. Cheng. 2000. In vitro effects of steroidal saponins from Yucca schidigera extract on rumen microbial protein synthesis and ruminal fermentation. Journal of the Science of Food and Agriculture 80(14):2114-2122.

Williams, A. G. 1986. Rumen holotrich ciliate protozoa. Microbiological Reviews 50(1):25.

Williams, A. y G. Coleman. 1997. The rumen protozoa. Pages 73-139 in The rumen microbial ecosystem. P.N. Hobson and C. Stewart, ed. Springer, London, UK.

Wood, J., I. Moura, J. Moura, M. Santos, A. Xavier, J. LeGall, y M. Scandellari. 1982. Role of vitamin B12 in methyl transfer for methane biosynthesis by *Methanosarcina barkeri*. Science 216(4543):303-305.

Wood, T., R. Wallace, A. Rowe, J. Price, D. Yáñez-Ruiz, P. Murray, y C. Newbold. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Animal Feed Science and Technology 152(1-2):62-71.

Wright, A.-D. G. y A. V. Klieve. 2011. Does the complexity of the rumen microbial ecology preclude methane mitigation? Animal Feed Science and Technology 166:248-253.

Yang, W., K. Beauchemin, D. Vedres, G. Ghorbani, D. Colombatto, y D. Morgavi. 2004. Effects of direct-fed microbial supplementation on ruminal acidosis, digestibility, and bacterial protein synthesis in continuous culture. Animal Feed Science and Technology 114(1):179-193.

Yang, C., S. Mao, L. Long, y W. Zhu. 2012. Effect of disodium fumarate on microbial abundance, ruminal fermentation and methane emission in goats under different forage: concentrate ratios. Animal 1(1):1-7.

Yáñez-Ruiz, D.R., A. Moumen, I.A. Martin Garcia y E. Molina Alcaide. 2004. Ruminal fermentation and degradation patterns, protozoa population and urinary purine derivatives excretion in goats and wethers fed diets based on two-stage olive cake: Effect of PEG supply. Journal of Animal Science 82:2023-2032.

Yañez-Ruiz, D. R., N. D. Scollan, R. J. Merry, y C. J. Newbold. 2006. Contribution of rumen protozoa to duodenal flow of nitrogen, conjugated linoleic acid and vaccenic acid in steers fed silages differing in their

water-soluble carbohydrate content. British Journal of Nutrition 96(05):861-869.

Yáñez-Ruiz, D. R., S. Williams, y C. J. Newbold. 2007. The effect of absence of protozoa on rumen biohydrogenation and the fatty acid composition of lamb muscle. British Journal of Nutrition 97(5):938-948.

Yu, Z., M. Yu, y M. Morrison. 2006. Improved serial analysis of V1 ribosomal sequence tags (SARSTV1) provides a rapid, comprehensive, sequence based characterization of bacterial diversity and community composition. Environmental Microbiology 8(4):603-611.

Zened, A., S. Combes, L. Cauquil, J. Mariette, C. Klopp, O. Bouchez, A. Troegeler-Meynadier, y F. Enjalbert. 2013. Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. FEMS Microbiology Ecology 83(2):504-514.

Zhou, M., E. Hernandez-Sanabria, y L. L. Guan. 2010. Characterization of variation in rumen methanogenic communities under different dietary and host feed efficiency conditions, as determined by PCR-denaturing gradient gel electrophoresis analysis. Applied and Environmental Microbiology 76(12):3776-3786.