Intervenciones nutricionales en edades tempranas para optimizar la función de la microbiota ruminal, la salud digestiva y la productividad animal

Tesis doctoral

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CSIC

UNIVERSITAS GRANATENSIS 1531

Programa de Doctorado Biología Fundamental y de Sistemas

Editor: Universidad de Granada. Tesis Doctorales
Autor: Juan Manuel Palma Hidalgo
ISBN: 978-84-1117-255-4
URI: http://hdl.handle.net/10481/73621

Esta tesis ha sido realizada en el marco de las Ayudas para la formación de profesorado universitario (FPU) 2016, habiendo disfrutado el autor de la tesis, Juan Manuel Palma Hidalgo, de una de dichas ayudas (FPU16/01981).

Los resultados que se presentan en esta Memoria de Tesis Doctoral por Agrupación de Publicaciones se recogen en los siguientes trabajos:

- Belanche A., Palma-Hidalgo J.M., Nejjam I., Serrano R., Jiménez E., Martín-García A.I., Yáñez Ruiz D.R. 2018. In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. Journal of the Science of Food and Agriculture, 15;99(1):163-172. DOI: https://doi.org/10.1002/jsfa.9157

Índice de impacto (Journal Citation Reports): 3.639 (Q1 Agriculture – Multidisciplinary).

- Belanche, A., Palma-Hidalgo, J. M., Nejjam, I., Jiménez, E., Martín-García, A. I., & Yáñez-Ruiz, D. R. (2020). Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. Journal of Dairy Science, 103(6), 5047–5060. DOI: https://doi.org/10.3168/jds.2019-18002

Índice de impacto (Journal Citation Reports): 4.034 (Q1 Agriculture, Dairy & Animal Science).

- Palma-Hidalgo, J.M., Jiménez, E., Popova, M., Morgavi, D.P., Martín-García A.I., Yáñez-Ruiz, D.R., Belanche, A. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. Animal microbiome, 3:11. DOI: https://doi.org/10.1186/s42523-021-00073-9

Índice de impacto (Journal Citation Reports): Sin índice de impacto (revista reciente).

- Palma-Hidalgo, J.M., Belanche, A., Jiménez, E., Martín-García, A.I., Newbold, C.J., Yáñez-Ruiz D.R. 2021. Short Communication: Saliva and salivary components affect goat rumen fermentation in short-term batch incubations. Animal, 15(7): 100267. DOI: https://doi.org/10.1016/j.animal.2021.100267

Índice de impacto (Journal Citation Reports): 3.240 (Q1 Veterinary Sciences).

- Palma-Hidalgo, J.M., Yáñez-Ruiz, D.R., Jiménez, E., Martín-García, A.I., Belanche, A. 2021. Presence of adult companion goats favors the rumen microbial and functional development in artificially reared kids. Frontiers in Veterinary Science, 8:70659. DOI: https://doi.org/10.3389/fvets.2021.706592

Índice de impacto (Journal Citation Reports): 3.412 (Q1 Veterinary Sciences).

- Palma-Hidalgo, J.M., Belanche, A., Jiménez, E., Martín García, A.I., Newbold C.J., Denman, S.E., Yáñez-Ruiz D.R. 2022. Multi-omics study of the salivary modulation of the rumen microbiome. Enviada a la revista Scientific Reports.

Resumen

El rumen es una cámara de fermentación que permite a los rumiantes aprovechar eficientemente alimentos fibrosos. Dicho órgano alberga una compleja y diversa comunidad microbiana, lo que presenta dificultades en cuanto a la modulación de su función. Las primeras semanas de vida del animal en las que el ecosistema microbiano se está formando y tiene una importante plasticidad representa una interesante oportunidad de modulación. Distinto tipo de intervenciones aplicadas durante este período puede permitir la modulación y estimulación de la colonización microbiana del rumen y el desarrollo ruminal, lo cual adquiere gran relevancia especialmente en sistemas de lactancia artificial, donde las crías se separan rápidamente de las madres y por tanto carecen de una fuente natural de colonización. Esta tesis tuvo como objeto evaluar diversas estrategias para acelerar el desarrollo microbiológico ruminal y ahondar en el conocimiento de los factores que modulan dicha microbiota. En un primer experimento se procedió a identificar, en condiciones *in vitro*, la microbiota ruminal madura con un mayor potencial para ser empleada como inóculo en cabritos recién nacidos (**Publicación 1**). El líquido ruminal fresco muestreado en una situación postprandial resultó ser el inóculo que promovió una mayor actividad fermentativa y por tanto fue el elegido para ser utilizado en el siguiente experimento. En el segundo experimento (**Publicaciones 2 y 3**) se procedió a inocular diariamente cabritos recién nacidos hasta el destete en condiciones de lactancia artificial para estudiar los efectos de esta intervención en el desarrollo del rumen. Dicho estudio mostró que la inoculación con líquido ruminal fresco en cabritos mejoró la actividad fermentativa ruminal y aceleró la colonización microbiana del rumen antes del destete, como demuestran la mayor concentración y absorción de productos de fermentación como el butirato (+50 %) y la más abundante y diversa comunidad microbiana ($P < 0.05$). Además, los cabritos inoculados presentaron una mayor ganancia de peso inmediatamente tras el destete, posiblemente debido a la mayor ingesta de forraje (+44 %) y a la más compleja comunidad protozoaria del rumen. Mientras que el crecimiento no se vio afectado por la inoculación, algunos de los efectos positivos de esta persistieron después del destete. En un estudio subsiguiente (**Publicación 4**) se procedió a evaluar los efectos de la transmisión indirecta de microbiota ruminal a cabritos en lactancia artificial mediante la presencia de animales acompañantes adultos no lactantes en el mismo parque. Dicha estrategia de manejo dio lugar a un desarrollo microbiológico del rumen más temprano en los cabritos, representado por la

presencia de unas comunidades de bacterias (+132 filotipos), protozoos y metanógenos más diversas, las cuales se asimilaban más a las presentes en los adultos. Los efectos en la actividad ruminal y el rendimiento animal no fueron tan claros como con la inoculación directa pero las mayores concentraciones de butirato (+45 %) y amonio sugieren una mejora de las actividades fibrolítica y proteolítica. Estos hallazgos sugieren que, en situaciones de lactancia artificial, la inoculación directa de rumiantes jóvenes con líquido ruminal de animales adultos, o indirectamente mediante la mera presencia de acompañantes adultos, permite acelerar el desarrollo microbiológico y funcional del rumen con efectos positivos sobre el periodo de destete.

Paralelamente, en esta tesis se ha estudiado el papel de los componentes bioactivos de la saliva en la regulación de las poblaciones microbianas del rumen y su actividad fermentativa a través de la incubación con distintas fracciones de saliva y de distintos animales donantes. La incubación con una alta proporción de saliva filtrada (sin microorganismos pero con proteínas salivales) aumentó los niveles de actividad fermentativa ruminal (**Publicación 5**). El uso de saliva autoclavada originó una composición de la comunidad microbiana diferente, con una mayor abundancia de *Proteobacteria* y menor de *Prevotellaceae*, en comparación con las comunidades resultantes de la incubación con saliva no autoclavada de cabra u oveja (**Publicación 6**). Estos hallazgos sugieren que la inoculación directa e indirecta (aunque en menor medida) de microbiota ruminal mejoró el desarrollo del rumen de cabritos en el destete, y que los componentes bioactivos de la saliva modulan selectivamente las poblaciones microbianas del rumen y su actividad en pos de una adecuada asociación microbiota-huésped.

Abstract

The rumen is a fermentation chamber that enables ruminants to efficiently digest fibrous diets. This organ harbours a complex and diverse microbial community which makes it difficult to achieve a successful modulation of its activity. The first weeks of life of the animal when the ecosystem is being developed and therefore have high plasticity represent an interesting opportunity of modulation. Different interventions applied during the first weeks of life can allow the modulation and promotion of the rumen microbial colonization and rumen development, which is of great importance specially in artificial milking systems, where kids are quickly separated from their dams and therefore lack a natural source of colonization. This thesis aimed to evaluate several strategies to accelerate the rumen microbial development and to delve into the knowledge of the different factors that modulate the rumen microbiota. The first experiment of the thesis attempted to identify, under *in vitro* conditions, the mature rumen microbiota with a greater potential to be used as inoculum in newborn goats (**Publication 1**). Fresh rumen fluid sampled after feeding was found to be the inoculum that promoted a higher fermentative activity and therefore it was chosen for the following *in vivo* study. In this second experiment (**Publications 2 and 3**) newborn goat kids were daily inoculated until weaning under artificial-rearing conditions to assess the effects of this intervention on rumen development. This study showed that the inoculation with fresh rumen fluid in goat kids improved rumen fermentative activity and accelerated the rumen microbial colonization before weaning, as shown by the greater concentration and absorption of fermentation products such as butyrate (+50 %) and the more abundant and diverse microbial community ($P < 0.05$). Furthermore, Inoculated kids had a greater weight gain immediately after weaning, possibly because of their higher forage intake (+44 %) and complex rumen protozoal community. While animal growth was unaffected by inoculation, some of its positive effects also persisted after weaning. A subsequent study (**Publication 4**) aimed to evaluate the effects of mimicking the former microbial transmission to newborn goat kids until weaning by the presence of non-lactating adult companions in the same pen. This management strategy also resulted in an earlier rumen microbial development in goat kids, as shown by the presence of a diverse bacterial (+132 phylotipes), protozoal and methanogens communities at weaning, which better resembled those of the adults. Effects on rumen activity and animal performance were not as clear as with the direct inoculation but higher butyrate (+ 45 %) and ammonia concentrations

suggest improved fibrolytic and proteolytic activities. These findings suggest that under artificial-rearing conditions, the direct inoculation of young ruminants with rumen fluid from adult animals, or indirectly by the mere presence of adult companions, allows an acceleration of the microbial and functional rumen development with positive effects during the weaning period.

Concurrently, this thesis evaluated the role of the salivary bioactive components in the regulation of the rumen microbial populations and their fermentative activity by incubating with different saliva fractions and from different donors. Incubating with a high proportion of filtrated saliva (without microbiota but with salivary proteins) boosted the levels of rumen fermentative activity (**Publication 5**). The use of autoclaved saliva led to a very divergent microbial community composition, with greater abundance of *Proteobacteria* and lower of *Prevotellaceae*, compared to the communities resulting from incubation with non-autoclaved goat's or sheep's saliva (**Publication 6**). These findings suggest that direct and, to a lesser extent, indirect inoculation of rumen microbiota improved the rumen development in goat kids at weaning and that the bioactive components of saliva selectively modulate the rumen microbial populations and their activity for a suitable host-microbiota association.

Agradecimientos

Es cuanto menos conmovedor cuando uno echa la vista atrás y se da cuenta de todo lo que ha transcurrido durante estos años de tesis y que sin duda me ha hecho crecer personal y profesionalmente. Esta tesis lleva detrás incontables horas de trabajo compartido por muchas personas, las cuales han contribuido de forma decisiva para que este proyecto salga adelante, y a las cuales es preciso agradecer su inestimable apoyo y ayuda.

Y no puedo empezar de otro modo que agradeciendo enormemente a mis dos directores, David Yáñez y Alejandro Belanche por su consejo, apoyo, dedicación y paciencia durante todo este tiempo. Gracias a David por acogerme en el grupo cuando apenas sabía lo que era trabajar en ciencia y por confiar en mi desde entonces, por tu guía y pragmatismo, y por todo el tiempo y recursos invertidos en mí. Gracias a Alejandro por hacerme aprender tantísimas cosas en tantos ámbitos de la ciencia, por fomentar en mí el espíritu crítico y por tus míticos juegos de palabras.

Agradezco a Nuria Ferrol su interés y el ejercer de tutora en el Programa de Doctorado de la Universidad de Granada. Gracias a todo el apoyo que me ha brindado la EEZ y la UGR, en especial el Departamento de Zoología y la Comisión de Doctorado de Biología Fundamental y de Sistemas en mi tarea como docente o supervisor. A las instituciones estatales por concederme un contrato FPU para financiar y llevar a cabo mi proyecto de tesis.

Desde que entré hace años en el ‘grupo de rumiantes’ me he sentido muy a gusto y culpa de ello la tienen las increíbles personas que lo forman. Eli, Isa, sois unas máquinas y es un placer trabajar con vosotras tanto en el laboratorio como en el estabulario; sólo puedo daros las gracias por todo vuestro apoyo desinteresado y esencialmente por estar siempre ahí para ofrecer soluciones. Sin vosotras esta tesis no habría llegado a buen puerto. También tengo mucho que agradecer a Noemí, Rafa y Alfonso por su ayuda y disponibilidad cada vez que necesitaba que me echasen una mano. A Pedro, mi compi de despacho y fatigas predoctorales, por hacer que la última etapa de mi tesis sea más llevadera. A Ignacio Martín por su consejo, por asesorarme en cuestiones de estadística y por su maestría en asuntos logísticos.

Gracias a todos los compañeros, visitantes y estudiantes que han contribuido en mayor o menor medida con su trabajo en la tesis. Gracias Rosa Serrano, Ibtissam, Eugenia, Amira,

Pablo, Alba, Ana, Zulema, Ana Esteban, Eduarda, Juan Vera, Paco, Mª Jesús, Isaac y todo el equipo de técnicos del departamento por vuestra colaboración e interés. Agradezco también el trato que he tenido en las estancias y colaboraciones por parte de los Dr. Leluo Guan y Dr. Eoin O'Hara en Edmonton, Dr. Milka Popova y Dr. Diego Morgavi en Clermont-Ferrand y el personal del López-Neyra.

A mis amigos Salva Torres, Migue, Jorge, Josema, Zúñiga, Lucas y Salva Moreno por estar siempre ahí para lo que sea, especialmente aquellos con los que he compartido aventura predoctoral, y en general por todos los buenos ratos y risas compartidas.

A mi familia: mi hermano, mis tíos, mis suegros y mis cuñadas, os agradezco todo vuestro cariño y apoyo durante estos años. Gracias especialmente a mis padres por marcarme el camino en esta travesía en el mundo científico, por su conocimiento, consejo y apoyo en todo momento.

Por último, a Zaira, mi compañera en todos los sentidos de la palabra. Gracias por estar ahí tanto en los momentos buenos como en los no tan buenos de la tesis. Gracias por tu incondicional ayuda y cariño, y por calmarme en momentos de estrés o poca motivación. Esta tesis ha salido adelante en gran parte gracias a ti.

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ADN: Ácido desoxirribonucleico

AGV: Ácidos grasos volátiles

ARN: Ácido ribonucleico

ARNm: Ácido ribonucleico mensajero

ARNr: Ácido ribonucleico ribosómico

ASV: Variantes de la Secuencia del Amplicón / Amplicon Sequence Variants

BHB: Beta-hidroxibutirato

CH_4: Metano

CO_2: Dióxido de carbono

DMSO: Dimetilsulfóxido

H_2: Hidrógeno molecular

IgA: Inmunoglobulina A

IgG: Inmunoglobulina G

mcrA: Metil co-reductasa A

NGS: Next Generation Sequencing

OTU: Unidades Taxonómicas Operativas / Operative Taxonomic Units

RNA: Ribonucleic acid

TGI: Tracto gastrointestinal

TLR: Receptores tipo Toll / Toll-like receptors

CAPÍTULO I

INTRODUCCIÓN Y OBJETIVOS

1. Tracto digestivo del rumiante.

Los rumiantes, tales como la vaca, cabra y oveja, son animales caracterizados por poseer un tracto digestivo complejo y peculiar que los diferencia respecto a otros animales empleados en ganadería. Lo más significativo del tracto digestivo de los rumiantes es la presencia de un estómago compuesto por 4 cámaras diferenciadas, de las cuales el rumen o panza es la más importante tanto en tamaño como en implicaciones fisiológicas (Figura 1). El retículo, rumen y omaso son considerados pre-estómagos cuyas secreciones a nivel del epitelio son muy limitadas o inexistentes. Por el contrario, la última cámara, el abomaso, es el estómago que más similitudes guarda con el estómago convencional de monogástricos ya que, a diferencia de los tres anteriores, presenta tejido glandular (**Figura 1**).

La cámara del rumen es la más importante funcionalmente para el animal, ya que en ella se lleva a cabo la fermentación microbiana, entre otros, de alimentos fibrosos (que constituyen una parte importante de la dieta de los rumiantes). El rumen consta de un amplio epitelio estratificado cuya capa apical está compuesta por una serie de papilas que aumentan la superficie disponible para la absorción de nutrientes (Steele et al., 2016). Esta disposición del epitelio ruminal permite al rumiante absorber grandes cantidades de ácidos grasos volátiles (AGV) que son generados en el rumen, de forma que apenas un 10% de estos llegan al intestino delgado (Harfoot, 1978). Estos compuestos son en realidad producto de la fermentación anaerobia de la dieta llevada a cabo por la microbiota presente en el rumen, y suponen la principal fuente de energía para el animal. Aparte de la generación de AGV y otros productos de fermentación como el hidrógeno, dióxido de carbono y metano (H_2, CO_2, CH_4; respectivamente), la microbiota del rumen (más concretamente sus proteínas) constituye la principal fuente de proteína metabolizable para satisfacer las necesidades del hospedador. Es por ello que la microbiota ruminal, que vive en simbiosis con el rumiante, es fundamental para su correcta nutrición y bienestar. Por ello, el epitelio ruminal también posee, en coordinación con otros mecanismos, la capacidad de modular a esta microbiota, ofreciendo así protección frente a la entrada de potenciales agentes patógenos (Chen et al., 2012).

Entre los procesos moduladores que ocurren en el rumen, también se encuentran la regulación de la temperatura, presión osmótica y capacidad tamponadora del pH, que conjuntamente contribuyen a mantener la homeostasis (Krause & Oetzel, 2006). Este

último punto resulta esencial en los actuales sistemas de producción intensiva debido a la frecuencia con la que estos animales experimentan acidosis sub-aguda como consecuencia de la fermentación exacerbada de carbohidratos de la dieta (Aschenbach et al., 2011; Apper-Bossard et al., 2010), lo que puede tener repercusiones en el metabolismo, salud y rendimiento productivo del animal (Krause & Oetzel, 2006).

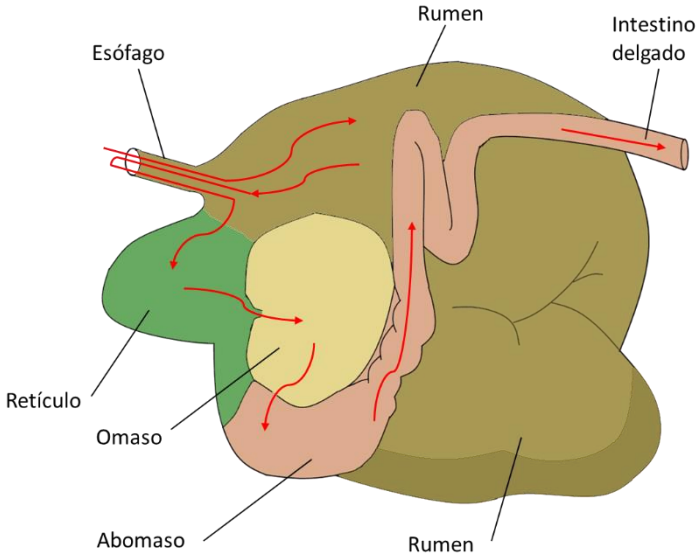

Figura 1. Esquema de las 4 cámaras que componen el estómago de los rumiantes y el recorrido que sigue el alimento.

1.1. Desarrollo anatómico y fisiológico del rumen.

Tras el nacimiento, el rumen es apenas una bolsa afuncional, ya que la mayor parte de la digestión del alimento (leche) durante las primeras semanas de vida se lleva a cabo en el abomaso (Yvon et al., 1985). El desarrollo temporal del rumen es un proceso en el que se produce tanto el crecimiento como la diferenciación de las células, y por tanto la obtención de una función fisiológica por parte de las mismas. El modo en que se produzca este desarrollo determinará, en cierta medida, el patrón de nutrientes que llegará al intestino y, por consiguiente, al resto de tejidos del hospedador (Baldwin et al., 2004). En el desarrollo del rumen se pueden diferenciar en 3 procesos distintos: i) colonización y crecimiento de la microbiota (Fonty et al., 1987, Fouts et al., 2012), ii) desarrollo anatómico (Reynolds et al., 2004) y iii) establecimiento de la función enzimática y actividad microbiana (Rey et al., 2012; Faubladier et al., 2013).

El desarrollo anatómico ocurre a nivel de incremento del tamaño del rumen y el crecimiento de las papilas (Baldwin & Connor, 2017). El rumen del neonato carece de

papilas funcionales y estas no presentan queratina, la cual se va adquiriendo poco a poco conforme el rumen madura y el animal pasa a fase adulta (Gilliland et al., 1962). Sólo cuando el animal deja atrás la fase de pre-rumiante (3-4 semanas de vida) y comienza a ingerir alimento sólido, y la consecuente fermentación ruminal, el rumen comienza a experimentar el desarrollo que permitirá al animal convertirse en rumiante propiamente dicho (Lane et al., 2002, Baldwin et al., 2004). En este sentido, la transición de pre-rumiante a rumiante es un paso crucial ya que el mejor o peor desarrollo del epitelio que ocurre durante la misma va a determinar el grado de absorción de nutrientes y aprovechamiento de los productos de fermentación, especialmente los AGV (Warner et al., 1956; Heinrichs et al., 2005). La presencia de estos AGV, a su vez, estimula el desarrollo y metabolismo epitelial, y por ende el crecimiento del animal (Baldwin & McLeod, 2000), dándose así un proceso de retroalimentación positiva. Precisamente uno de estos AGV, el butirato, provee de energía a las células epiteliales del tracto gastrointestinal (TGI) y está altamente relacionado con su crecimiento (Donohoe et al., 2011), mientras que el acetato y el propionato son fundamentales para la síntesis de grasa y la gluconeogénesis, respectivamente en los tejidos del animal adulto (Harfoot, 1978; Baldwin & Connor, 2017).

En numerosos estudios en los que se emplean rumiantes recién nacidos para comparar su evolución cuando se les ofrece dieta con concentrado y heno o únicamente leche hasta el destete, estos últimos muestran un desarrollo ruminal muy limitado en lo referido a grado de queratinización (Gilliland et al., 1962), pigmentación (Hamada et al. 1976), peso y volumen del rumen, crecimiento papilar, y desarrollo muscular (Tamate et al., 1962; Lane & Jesse, 1997; Connor et al. 2013, Baldwin & Jesse, 1992). Esto se debe principalmente a que la leche o lacto-reemplazante se dirige directamente al abomaso ya que el canal reticulo-esofágico o "gotera esofágica" que da acceso al rumen permanece cerrada (Orskov et al., 1970, Castro et al., 2016). Se ha comprobado que la fermentación de leche en el rumen puede generar una notable producción de AGV en el mismo (Tamate et al., 1962), lo que indica que el hecho de que la leche se derive hacia el abomaso no se debe a un posible bajo rendimiento energético de esta, sino a que no precisa la fermentación anaeróbica propia del rumen para ser digerida y aprovechada más eficientemente. En efecto, pese a que la evidencia científica en ocasiones ha mostrado que la alta ingesta de leche puede reducir el consumo de alimento sólido y por tanto el desarrollo del rumen (Khan et al., 2011), otros autores apuntan a que el consumo elevado de leche es

beneficioso para el crecimiento del animal y que este aun es capaz de digerir dieta sólida a edad temprana (Liang et al. 2016, Schaff et al., 2017).

En cualquier caso, la entrada de alimento en el rumen (principalmente en forma sólida), resulta crucial para que se produzcan AGV y así se promueva el desarrollo del epitelio, así como la adquisición del equipo enzimático necesario para aprovechar cada nutriente (Lane and Jesse 1997, Lane et al., 2002). Es por ello que la ingesta de alimento sólido vaya inherentemente asociada a un mayor nivel de desarrollo del rumen, si bien la naturaleza del mismo puede tener efectos distintos. Así, la introducción de alimento sólido de mala calidad durante el destete no tiene por qué tener un efecto especialmente ventajoso. La administración de pienso de arranque altamente palatable y degradable en edad temprana es positiva para el crecimiento de las papilas y la expresión de genes en el epitelio, a diferencia del reducido efecto del empleo únicamente de forraje de baja calidad (Connor et al., 2013), cuyo uso, por otra parte, suele ser minoritario en la dieta durante esta fase.

1.2. Colonización y desarrollo microbiológico del rumen.

El TGI de cualquier animal en el momento de nacimiento ya presenta cierta microbiota transferida a través del canal del parto de la madre, pero esta es muy limitada en términos de abundancia y muchos de sus constituyentes no forman parte de las comunidades microbianas propias del TGI del rumiante (Alipour et al., 2018). Sin embargo, esta fase es sumamente breve ya que en cuestión de pocas horas, o incluso minutos, comienza la colonización por parte de los microorganismos del ambiente y aquellos provenientes de animales adultos, especialmente de las madres. Las fuentes de microbiota de la madre que potencialmente colonizan el TGI de los recién nacidos incluyen la saliva, el contenido del rumen, las heces, la piel, el calostro y la vagina (Yeoman et al., 2018), albergando esta última el primer ecosistema microbiano con el que los neonatos establecen contacto directo.

En el rumen se produce una secuencia bastante bien definida y progresiva de colonización y establecimiento de la microbiota (Stewart et al., 1988). Los primeros microorganismos que colonizan el rumen son los anaerobios facultativos. Hay evidencia de que apenas 1 o 2 días tras el nacimiento ya existe una importante proporción de estos microorganismos que tendrán cierta relevancia en el rumen del animal adulto (Zhang et al., 2019). En las

primeras 2 semanas de vida, todos los grupos principales de bacterias del rumen ya están establecidos en este órgano aún en desarrollo (Li et al., 2012). Durante estos primeros días, y reafirmando el carácter secuencial de la colonización del rumen, también se produce cierto reemplazo gradual de filos de bacterias: inicialmente hay un predominio del filo *Proteobacteria* (que puede representar hasta el 90% de la microbiota) que son predominantemente anaerobios facultativos, que permite generar un ambiente anaerobio capaz de albergar posteriormente a anaerobios estrictos como *Bacteroidetes* y *Firmicutes*, que irán gradualmente incrementando su abundancia ruminal (Jami et al., 2013; Rey et al., 2014). Este reemplazo es esencial en la maduración del rumen ya que con él se forma una comunidad con capacidad para fermentar anaerobiamente los alimentos sólidos antes incluso de que el rumiante empiece a ingerirlos de forma sustancial. A partir de las 2 semanas y en adelante los cambios en la comunidad bacteriana del rumen a nivel de diversidad de filos se ralentiza, pero sí que se seguirán produciendo variaciones en la abundancia relativa de cada uno de ellos (Yáñez-Ruiz et al., 2015).

Estas variaciones de filos y géneros bacterianos durante los primeros días de vida han sido descritas detalladamente en los últimos años (Jami et al., 2013; Rey et al., 2014; Zhang et al., 2019). Así:

- En los dos primeros días de vida, destaca la presencia de bacterias anaerobias facultativas pertenecientes a los géneros *Bacillus* o *Lactococcus* y pronto comienza a haber el ya mencionado reemplazo gradual de *Proteobacteria* (70% de la comunidad inicial) a *Bacteroidetes*.
- Entre los días 2 y 4 los animales cesan su alimentación a base de calostro (en lactancia natural) y ceden paso a dietas a base de leche o reemplazante artificial, con cantidades desdeñables de alimento sólido. En este periodo el género bacteriano más abundante es *Bacteroides*, seguido de *Streptococcus, Prevotella Fusobacterium* y *Actinobacillus*.
- A partir del 7-8 y hasta el día 15 los animales comienzan a consumir cantidades mínimas de pienso, produciéndose cambios reseñables en la microbiota como consecuencia de ello. Concretamente, empieza a haber una disminución en la abundancia de *Bacteroides* y se incrementa la de *Succinivibrio*, que comprende varias especies con un papel importante en la degradación del almidón del pienso (O'Herrin et al., 1993).

- Desde el día 15 hasta el destete (6-8 semanas) apenas se observan cambios notables en la comunidad microbiana, cuya composición se va asimilando cada vez más a la propia de animales adultos al incrementarse la abundancia de *Prevotella* y *Succinivibrio*.
- El destete, sobre todo si este se practica de forma abrupta, es el momento en el que se da el cambio más drástico en la microbiota, ya que esta se desarrolla rápidamente para digerir el alimento sólido. A partir de este punto y en adelante, lo más destacable es el descenso de la abundancia relativa de *Succinivibrio*, lo que contrasta con el continuo aumento de *Prevotella* (que alcanza un ~45% de abundancia relativa), *Fibrobacter* y *Ruminococcus*, entre otros géneros (Jami et al., 2013).

En el caso de los protozoos ciliados del rumen, su colonización sigue un patrón bastante distinto. Estos, a diferencia de las bacterias, no son detectados en el rumen hasta prácticamente las 2 semanas tras el nacimiento en animales con lactancia natural. Debido a la naturaleza anaeróbica estricta de estos microorganismos, la colonización del rumen por parte de los mismos sólo se produce por contacto directo entre distintos animales mediante la saliva (Becker & Hsiung, 1929) o el agua de bebida compartida (Bird et al., 2010), por lo que la falta de contacto con animales adultos evitaría tal transmisión (Bryant & Small, 1960; Eadie, 1962). Al igual que para las bacterias ruminales, se ha descrito la secuencia de colonización de los protozoos ruminales, en la que los primeros colonizadores pertenecen a la familia *Entodiniinae* y seguidamente se establecen especies de *Diplodiniinae* y *Ophryoscolecinae*, siendo los protozoos holotricos los últimos en detectarse en el rumen en condiciones naturales (Williams & Coleman, 1992).

En cuanto a las arqueas metanogénicas del rumen, su colonización ocurre muy tempranamente, a los 2-4 días de vida, antes de que el animal ingiera alimento sólido. Recientemente, incluso se ha detectado la presencia de arqueas en el rumen de neonatos, concretamente de los géneros *Methanobrevibacter* y *Methanomicrobium* (Guzman et al., 2015). A las dos semanas, la cantidad de arqueas alcanza niveles equivalentes a aquellos de los animales adultos en términos de abundancia (Morvan et al., 1994), pero no tanto en diversidad.

El establecimiento de los hongos anaeróbicos también se produce antes de que el animal consuma alimento sólido. Concretamente, a los 8-10 días de vida ya es posible detectar

hongos ruminales en corderos con lactancia natural (Stewart et al., 1988). El hongo más importante en los primeros días de vida en términos de abundancia relativa es todo el género *Neocallimastix* spp. y más concretamente la especie *N. frontalis* (Stewart et al., 1988). En función de la dieta sólida administrada al animal al final del destete, pienso o forraje, estos hongos aumentan o disminuyen en abundancia, respectivamente.

La colonización del rumen ocurre de forma desigual entre sus distintos espacios: epitelio, contenido líquido y macropartículas de alimento. La comunidad epitelial, cuya proliferación es clave para una adecuada interacción con el hospedador, aparece bastante pronto tras el nacimiento y en poco tiempo se alcanza una cantidad de microorganismos equivalente a la de un animal adulto, si bien se producen cambios en su diversidad con el paso del tiempo (Mueller et al., 1984; Rieu et al., 1990). Resulta relevante que de todos los microorganismos identificados inicialmente en terneros de entre 1 y 10 semanas de vida, tan sólo 1/3 de ellos se han detectado más adelante cuando los animales eran ya adultos, siendo por tanto miembros nativos de la comunidad ruminal (Mueller et al., 1984).

Buena parte de los primeros estudios de la comunidad epitelial del rumen determinaron que las diferencias entre esta y la comunidad asociada a la digesta ruminal eran mínimas (Dehority & Grub, 1981, Mead & Jones, 1981). No obstante, en los 15 años últimos años se ha contradicho esa hipótesis y se ha demostrado que la comunidad microbiana asociada al epitelio ruminal difiere considerablemente de aquella presente en el líquido ruminal (Sadet et al., 2007; Malmuthuge et al., 2014). En el desarrollo de la comunidad del contenido ruminal hay una clara influencia de la dieta, pero en la epitelial la dieta apenas afecta al perfil microbiológico, lo que sugiere que hay una mayor influencia del hospedador en la microbiota asociada al epitelio (Sadet et al., 2007). En terneros con 3 semanas de vida *Prevotella* es, con diferencia, el género mayoritario en el contenido del rumen. No obstante, en el entorno de la mucosa epitelial la abundancia de *Prevotella* es más baja y se contrarresta con una mayor preponderancia de otras especies del mismo filo, *Bacteroidetes* (Malmuthuge et al., 2014).

La colonización del rumen no tiene por qué seguir un patrón general sino que hay varios factores, entre los que destaca la dieta y el propio hospedador, que influyen en que se desarrolle de una forma u otra, favoreciendo así el establecimiento de comunidades potencialmente distintas (Jiao et al., 2015). La propia microbiota presente en el animal en

edad temprana también puede tener influencia sobre el establecimiento del ecosistema ruminal maduro en edad adulta. Así, en varios estudios se ha hipotetizado sobre la idoneidad o no de la presencia de protozoos en el rumen en crecimiento, al no considerarse estos esenciales para el correcto funcionamiento del rumen (Santra & Jakhmola, 1998). En efecto, la ausencia o presencia de estos puede afectar sustancialmente a la composición de la comunidad procariota del rumen, lo que a su vez puede dar lugar a distintos patrones de fermentación (Yáñez-Ruiz et al., 2007). Se ha llegado a considerar que, a grandes rasgos, los protozoos ruminales y más concretamente sus especies más dominantes determinan considerablemente el ecosistema ruminal que presentará el animal en edad adulta, y que en gran medida todos los animales pertenecientes al mismo grupo y ubicación geográfica albergarán una comunidad protozoaria similar (Williams & Coleman, 1992). De forma análoga, en estudios con arqueas metanogénicas se ha observado que los corderos recién nacidos guardaban una gran similitud con sus respectivas madres en las poblaciones de estos microorganismos en el rumen (Skillman et al., 2004). De igual modo, el conjunto de los factores antes descritos conduce de una u otra forma al animal en edad temprana a albergar una compleja microbiota ruminal en su madurez, la cual se describe a continuación.

1.2.1. Bacterias del rumen.

Las bacterias ruminales constituyen el grupo microbiano con más abundancia (Choudhury et al., 2015), llegando a concentraciones de hasta 10^{11} células/ml (Wright & Klieve, 2011), y también el más estudiado, dado su papel fundamental en la degradación de la dieta y síntesis de AGV y proteína. La comunidad bacteriana del rumen está dominada por los filos *Bacteroidetes, Firmicutes* y *Proteobacteria*, que conforman más del 90% del total (Kim et al., 2011; Fouts et al., 2012; Henderson et al., 2015). Pueden encontrarse en el entorno del epitelio, en el líquido del rumen o asociadas a las partículas de alimento, donde son el grupo microbiano más importante (McAllister et al., 1994). La mayoría de las bacterias del rumen, sobre todo aquellas presentes en el contenido ruminal, llevan a cabo procesos fermentativos en anaerobiosis, lo que contrasta con el carácter anaerobio facultativo de la comunidad epitelial, que ayuda a mantener las condiciones de anaerobiosis mediante el consumo de oxígeno (Liu et al., 2016a).

Inicialmente, los estudios basados en métodos de cultivo y técnicas de secuenciación de primera generación pudieron describir no más de 400 especies de bacterias ruminales

(Edwards et al., 2004). Más adelante, el estudio de la comunidad bacteriana del rumen mediante secuenciación de alto rendimiento '*Next Generation Sequencing*' (NGS) ha dado lugar a la detección de más de 3000 especies distintas (Fouts et al., 2012; Denman & McSweeney et al., 2015), aunque muchas de ellas aún están por identificar. Se estima que un alto porcentaje de bacterias del rumen, independientemente de la especie, raza o entorno del animal, aún no están clasificadas ni caracterizadas de forma fidedigna a nivel de género o especie, siendo esto especialmente patente en algunas familias bacterianas concretas como *Rikenellaceae*, *Lachnospiraceae* o *Ruminococcaceae* (Creevey et al., 2014; Henderson et al., 2015). No obstante, sí que se ha descrito un conjunto de bacterias común en rumiantes (presencia en ~90% de los animales analizados) conformado por 30 géneros bacterianos dominantes, destacando en términos de abundancia *Prevotella*, *Butyrivibrio* y *Ruminococcus* (Henderson et al., 2015).

Los sustratos empleados por las bacterias ruminales incluyen una amplia variedad de moléculas de origen vegetal con mayor o menor grado de degradación previa. Muchas especies bacterianas actúan, de hecho, como fermentadoras secundarias al utilizar los monómeros u oligómeros producidos fruto de la degradación de la fibra llevada a cabo por otros microorganismos denominados fermentadores primarios (Hobson & Stewart et al., 1997). Aun así, existen bacterias ruminales especializadas en la degradación de cada uno de los principales componentes de la pared celular de las plantas (fibra), que constituye un componente importante del forraje en la dieta del rumiante. Destacan en este aspecto las especies de los géneros *Fibrobacter* y *Ruminococcus* como degradadoras de celulosa (Shi & Weimer, 1997), *Butyrivibrio*, *Prevotella* y *Ruminococcus* como degradadoras de hemicelulosa (Zhou et al., 2015) y *Butyrivibrio* y *Prevotella* como degradadoras de pectina (Marounek & Dusková, 1999). En lo que se refiere a la fermentación del almidón, el cual es un componente habitual de la dieta, son numerosas las bacterias capaces de degradarlo, entre ellas algunas especies de *Prevotella*, *Clostridium*, *Butyrivibrio* y *Ruminobacter* (Zhou et al., 2015). Si existe una bacteria predominante en el rumen, esa es el género *Prevotella*, cuyas especies pueden suponer más del 50% del total de la comunidad bacteriana del rumen (Jami & Mizrahi, 2012) y cuyo metabolismo es tan versátil que les permite la degradación de fibra, azúcares y también péptidos, aunque con grandes diferencias en las diferentes especies del género *Prevotella* (Hobson & Stewart, 1997).

1.2.2. Arqueas del rumen.

Las arqueas presentes en el rumen son en su totalidad metanogénicas y su concentración oscila en torno a 10^7 y 10^8 células/ml (Kamra, 2005), lo que supone no más del 3% del total de la masa microbiana ruminal (Janssen & Kirs, 2008). Los metanógenos del rumen se pueden clasificar en tres grupos en función la ruta metabólica por la que generan metano: 1) hidrogenotrofas, que son las más abundantes y utilizan hidrógeno molecular o ácido fórmico para reducir CO_2 a CH_4, 2) acetoclásticas, las cuales utilizan hidrógeno y acetato, y 3) metilotrofas, que usan metanol o metilaminas (Lessner, 2009).

Los metanógenos del rumen comprenden un número de familias y géneros, todos pertenecientes al filo *Euryarchaeota.* El género *Methanobrevibacter*, que utiliza la ruta hidrogenotrofa, es con diferencia el más abundante, ya que puede suponer hasta el 90% de las secuencias de metanógenos del rumen (Hristov et al., 2012), y en él destacan en abundancia las especies *M. gottshalkii, M. ruminantium* y *M. smithii* (Janssen & Kirs, 2008; Carberry et al., 2014). Otros metanógenos hidrogenotrofos (*Methanosphaera, Methanobacterium, Methanimicrococcus*) y metilotróficos (*Methanosarcinales, Methanomassillicoccaceae*) también están presentes en el rumen pero en menores concentraciones (Janssen & Kirs, 2008; Tapio et al., 2017).

Pese al relevante número de especies de metanógenos presentes en el rumen, todas ellas ocupan un nicho ecológico similar, concretamente aprovechando los productos de fermentación resultantes de la degradación de azúcares, en la cual se genera hidrógeno (Hegarty et al., 2007). Dependiendo de la ruta metabólica concreta llevada a cabo por bacterias, hongos o protozoos se producirán mayores o menores cantidades de este producto de fermentación. En relación a esto, se estima que en torno a un 25% de los metanógenos se suelen encontrar asociados endógena o exógenamente con los protozoos ruminales por su elevada producción de hidrógeno (Newbold et al., 1995; Sharp et al., 1998, Belanche et al., 2014). El consumo de hidrógeno a su vez acarrea la síntesis de metano como producto de fermentación, que es una molécula sin valor nutritivo para el animal y contribuye notablemente a las emisiones de gases de efecto invernadero (IPCC, 2019). En algunos estudios con el interés puesto en investigar si existe ciertamente una relación directa entre la biomasa de metanógenos y la producción de metano, se ha observado que, curiosamente, una mayor abundancia de estos microorganismos no tiene por qué conllevar necesariamente una mayor producción de este gas (Zhou et al., 2011).

Por el contrario, se piensa que es el nivel de actividad de los metanógenos y no su abundancia lo que marca realmente el grado de síntesis de metano (Roehe et al., 2016).

1.2.3. Protozoos del rumen.

Los protozoos ruminales son el principal grupo de eucariotas en el rumen y alcanzan concentraciones de 10^6 células/ml (Wright & Klieve, 2011). En términos de biomasa microbiana, comprenden hasta el 50% del total del rumen y, pese a ello, su papel aún no se conoce en su totalidad (Newbold et al., 2015). Los protozoos ruminales contribuyen a la degradación de carbohidratos de la dieta así como a la predación de bacterias, de las cuales obtienen el nitrógeno necesario para sintetizar sus propias proteínas y, por ende, para su susbsistencia (Mackie et al., 2002). Esto les convierte en uno de los actores principales en la proteólisis que ocurre en el rumen, la cual contribuye al aumento en la concentración ruminal de amonio y a un descenso en el flujo duodenal de proteína microbiana que podría ser utilizada por el animal (Koenig et al., 2000). Como se ha descrito anteriormente, los protozoos son además importantes productores de H_2 durante la fermentación, pudiendo contribuir indirectamente a la producción de CH_4 ya que proporcionan el sustrato necesario a los metanógenos con los que se encuentran asociados (Sharp et al., 1998). Existe un trabajo que apunta que esta relación causa-efecto puede no ser tal, ya que animales defaunados (sin protozoos) producían más metano que los que poseían protozoos (Morgavi et al., 2011). No obstante, la bibliografía en general muestra que, incluso pudiendo haber otros microorganismos distintos de los metanógenos que puedan actuar como sumideros del H_2 producido por los protozoos (Belanche et al., 2015), la asociación entre la abundancia de protozoos ruminales (especialmente los holotricos) y la producción de metano es bastante clara (Morgavi et al., 2008; Belanche et al., 2015; Newbold et al., 2015).

Debido a lo anterior, la importancia de la presencia de estos microorganismos en el rumen para el animal siempre ha estado en entredicho. Muchos estudios se han centrado, por tanto, en evaluar los efectos de la defaunación (eliminación de los protozoos) del rumen en el metabolismo ruminal (Newbold et al., 2015). Se ha observado que la defaunación da lugar a un incremento de un 30% de la proteína bacteriana disponible para el animal y una reducción del 11% en la producción de CH_4 (Newbold et al., 2015). Por otro lado, la eliminación de protozoos ruminales siempre ha sido asociada a un descenso considerable en la producción de butirato en el rumen (Demeyer & Van Nevel, 1979; Eugène et al.,

2004), lo cual puede estar relacionado con una menor degradación de fibra ya que numerosos genes implicados en la actividad fibrolítica han sido identificados en protozoos (Béra-Maillet et al., 2005; Newbold et al., 2005).

Hasta 250 especies han sido identificadas en el rumen en base a su morfología, todas ellas pertenecientes a dos órdenes: *Entodiniomorphida* y *Vestibuliferida* (protozoos holotricos) (Williams & Coleman, 1992). La familia *Entodiniinae* (y más concretamente el género *Entodinium*), son los protozoos más abundantes en el rumen, rondando el 90% de abundancia relativa del total de protozoos (Belanche et al., 2012; Belanche et al., 2019a). Otros géneros destacables en abundancia son *Polyplastron, Diplodinium, Epidinium, Isotricha* y *Dasytricha* (Sylvester et al., 2004; Belanche et al., 2012). De entre ellos, se ha sugerido que *Entodinium* posee una mayor actividad proteolítica sobre bacterias, mientras que los holotricos (*Isotricha, Dasytricha*) tienen poca actividad proteolítica (Belanche et al., 2012) pero una elevada actividad fibrolítica y correlación con la metanogénesis (Newbold et al., 2015).

1.2.4. Hongos del rumen.

Las primeras identificaciones de los hongos anaerobios del rumen han sido relativamente recientes (Orpin, 1974). Son el grupo microbiano menos abundante en el rumen ocupando un 10 % de la biomasa y teniendo una concentración de 10^4-10^5 células/ml (Wright & Klieve, 2011). Puede que por ello sean el grupo microbiano del rumen menos estudiado tras los virus, pese a que juegan un papel importante en la degradación de la dieta, ya que contienen enzimas celulolíticas, hemicelulolíticas, ligninolíticas, proteolíticas y amilolíticas (Choudhury et al., 2015). Esto les convierte en organismos importantes en la hidrólisis de la pared celular vegetal y la degradación de la misma (Yousuf et al., 2013). El metabolismo de los hongos ruminales a partir de los sustratos vegetales resulta en la producción de acetato, formato, lactato, etanol, CO_2 y H_2 (Gordon & Phillips, 1998). Debido a este complejo metabolismo y a su alta capacidad para degradar fibra, el estudio de los hongos anaerobios del rumen se está desarrollando intensamente en los últimos años, no sólo para investigar su papel dentro del microbioma ruminal sino también su potencial uso en fines biotecnológicos (Edwards et al., 2017).

La naturaleza anaerobia de los hongos ruminales, a diferencia de la mayoría de los hongos, hace que todos ellos se encuentren en el orden *Neocallimastigales*, que se

caracteriza por la presencia de hidrogenosomas en lugar de mitocondrias (Marvin-Sikkema et al., 1994). Dentro de este orden se diferenciaban morfológicamente 6 géneros al final del siglo XX, siendo los más abundantes *Neocallimastix, Caecomyces* y *Orpynomices* (Orpin & Joblin, 1997). No obstante, en los últimos años, los avances en biología molecular han permitido describir 3 géneros más en base a sus características genéticas: *Pecoramyces, Buwchfawromyces* y *Oontomyces* (Edwards et al., 2017). Pese a ello, algunos estudios en los que se emplea secuenciación masiva sugieren que una proporción de hongos ruminales aún podrían estar sin identificar o clasificar (Fouts et al. 2012, Kittelmann et al., 2013).

1.2.5. Virus del rumen.

Los virus son el grupo microbiano menos estudiado en el rumen, a pesar de que alcanzan concentraciones de 10^{10} virus/ml (Klieve & Swain, 1993). A pesar de ello, poco se conoce acerca de los fagos del rumen en cuanto a su índice de replicación, su virulencia, su rango de posibles hospedadores, etc., principalmente por el deficiente desarrollo de marcadores genéticos fiables para su cuantificación y caracterización (Gilbert et al., 2020). Estudios recientes donde en los que se utilizan técnicas de secuenciación masiva han demostrado la existencia de una gran cantidad de bacteriófagos y arqueófagos (Gilbert et al., 2017), llegando a identificarse 28000 genotipos distintos (Berg Miller et al., 2012). Un número considerable de esos virus se encontraban asociados a *Firmicutes, Bacteroidetes* y *Proteobacteria*, pero la gran mayoría aún no se han descrito (Berg Miller et al., 2012). El viroma asociado a estos filos bacterianos y a los metanógenos del rumen ha sido analizado para identificar posibles interacciones metabólicas y así explorar el potencial uso de los virus para modular la microbiota ruminal y, por ejemplo, reducir la producción de metano (Islam et al., 2019). La metatranscriptómica también ha permitido identificar virus de ARN específicos de hongos anaerobios (micovirus), pero su funcionalidad aun no ha sido caracterizada (Hitch et al., 2019).

Los fagos del rumen presentan un alto grado de especificidad en cada individuo independientemente de su comunidad bacteriana (Swain et al., 1996), aunque el limitado rango de hospedadores de cada virus hace que la microbiota e indirectamente la dieta tengan una influencia preponderante en el desarrollo de la comunidad vírica del rumen (Gilbert et al., 2020). En general, todos los fagos ruminales poseen una mayor proporción de material genético relacionado con el metabolismo de ADN y proteínas en comparación

a la menor proporción de genes relacionados con el metabolismo de carbohidratos y aminoácidos (Berg Miller et al., 2012). Debido a ello, las funciones principales de los fagos del rumen son: la regulación y mantenimiento de la biodiversidad de la comunidad procariótica, así como el intercambio genético entre las distintas especies de esta (Berg Miller et al., 2012). Dentro de estas funciones, existen diversas funciones específicas como la transferencia de genes como mecanismo de adaptación a la dieta, la infección y lisis de microorganismos, la adquisición de resistencia por parte de estos, la producción de toxinas, la estimulación de la formación de biofilms o incluso la interacción con el sistema inmune del animal (Styriak et al., 1991; Gilbert et al., 2020).

2. Intervenciones en edad temprana.

Existen múltiples estrategias nutricionales y de manejo (dieta, aditivos, probióticos, etc.) que permiten modular la microbiota ruminal del animal en edad adulta de forma limitada en el tiempo. Sin embargo, en las primeras semanas de vida del rumiante la comunidad ruminal aún está en fase de desarrollo y es más variable y por tanto ofrece mayor plasticidad, lo que hace que estas estrategias cobren más sentido (Yáñez-Ruiz et al., 2015). Esto ha dado lugar a plantear intervenciones nutricionales o a nivel de manejo alimentario con el objetivo de programar la microbiota del rumen en edad temprana para mejorar la producción en edad adulta (Yáñez-Ruiz et al., 2015). A raíz de este concepto, varios estudios han sido concebidos en torno a la intervención en edad temprana, sugiriendo que esta puede ejercer efectos positivos sobre el microbioma ruminal más allá del destete, si bien pocos estudios han demostrado la persistencia de los mismos a largo plazo (Yáñez-Ruiz et al., 2010; Abecia et al., 2013; Veneman et al., 2015).

2.1. Tipo de lactancia.

Los sistemas ganaderos de animales rumiantes se pueden clasificar en dos categorías principales: sistemas con animales para producción cárnica o para producción lechera, aunque existen también sistemas mixtos. En los sistemas destinados a la producción de carne las madres suelen alimentarse de pasto con mayor o menor aporte de concentrado y las crías normalmente reciben lactancia materna. Este sistema ofrece un menor grado de control sobre la cantidad o tiempo de ingesta de leche por parte de la cría durante toda la lactancia, si bien esta siempre tiene acceso a la leche materna (Weary et al., 2008) y su consumo no conlleva un coste adicional en forma de lacto-reemplazante. Además, en

estas condiciones, la toma de leche puede incluso prolongarse hasta los 7-8 meses de vida y se caracteriza por un descenso gradual en la frecuencia de mamar y un aumento de la toma de alimento sólido (Weary et al., 2008). En condiciones de lactancia natural, la pronta colonización del rumen favorecida por el contacto con las madres, también permite al rumiante joven adquirir antes la capacidad para digerir alimento sólido y especialmente fibra, gracias a una comunidad bacteriana y protozoaria más diversa (Belanche et al., 2018).

En los sistemas intensivos de producción lechera los rumiantes recién nacidos son separados de sus madres poco después de nacer y reciben lactancia artificial a base de lacto-reemplazante (Belanche et al., 2018). La abrupta separación entre madre y cría suele conllevar situaciones de estrés tanto para la madre como para el neonato, que pueden tener consecuencias comportamentales y fisiológicas negativas que afecten a la salud de la cría incluso durante varias semanas después de la separación (Lu & Potchoiba, 1988; Mandel & Nicol, 2017). Esta circunstancia puede verse más exacerbada aún en términos productivos y de desarrollo cuando se produce un aislamiento total de los animales recién nacidos (Belanche et al., 2019a). Las prácticas llevadas a cabo en sistemas intensivos también han sido relacionadas con una colonización microbiana del rumen más tardía, ya que, en comparación con sistemas con lactancia natural, se han observado concentraciones en rumen significativamente más bajas de bacterias y arqueas y una diversidad microbiana hasta tres veces más baja en las 2-4 primeras semanas de vida (Abecia et al., 2014; 2017). Este retraso en la colonización hace que el proceso de destete a nivel de cambios microbiológicos y fisiológicos en el TGI sea más brusco hasta la fase post-destete (Meale et al., 2017). Por ello, las prácticas habituales del sistema lechero suelen originar un desarrollo ruminal sub-óptimo que potencialmente puede acarrear efectos negativos para el animal. El aislamiento de los recién nacidos puede ser aún más crítico cuando se combina con las estrategias de destete precoz u oferta restringida de lacto-reemplazante (típico en terneros mamones). El destete precoz se realiza a las pocas semanas de edad (4-6 semanas en pequeños rumiantes) para reducir costes en lacto-reemplazante y/o facilitar el manejo del ganado (Svensson et al., 2003).

Permitir que los animales lactantes tengan contacto visual con sus congéneres o que directamente co-habiten con ellos puede atenuar los efectos negativos del período de lactancia en sistemas lecheros. La agrupación de animales es la práctica habitual en

pequeños rumiantes y cada vez está más extendida también en el ganado vacuno (Cantor et al., 2019), dado que se facilita notablemente el manejo de los animales (Hötzel et al., 2014). La creación de grupos en vacuno es aplicable en terneros mamones de aptitud cárnica de en torno a 1 mes de edad (Salse-Bernadó & Salse-Bernadó, 2018) pero incluso también (aunque en lotes algo más pequeños) en terneras de recría con no más de tres semanas de vida, que habitualmente se alojan en parques individuales (Fernández, 2011). En estos sistemas, la creación de la dinámica de grupo típica en rumiantes en edad temprana puede reportar diversos beneficios para el animal (Costa et al., 2016) pero, al usarse de forma combinada con sistemas de alimentación automatizados, permite recoger datos de ingesta y facilita la implementación de otras estrategias (Knauer et al., 2017). La organización de una paridera en grupos de 4-8 o hasta 30 animales puede permitir el reparto de animales por grupos de edad (Pedersen et al., 2009), pero esto dificulta que se lleven a cabo estrategias como el destete por grupos en función de la ingesta de cada animal (Benetton et al., 2019). El agrupamiento de animales jóvenes ha demostrado acelerar el desarrollo del comportamiento alimentario y posteriormente la toma de alimento sólido (Costa et al., 2016), además de las interacciones sociales (Abdelfattah et al., 2018). Por contra, el contacto directo entre animales en edad temprana hace que estos compartan microbiota y, por tanto, también posibles patógenos, facilitándose su transmisión (Kung et al., 1997).

2.2. Manejo alimentario.

La nutrición del rumiante en edad temprana afecta al desarrollo y el consiguiente rendimiento productivo del mismo. Como se ha comentado en el apartado 1.1, el distinto tipo de dieta administrada puede promover cambios significativos en la microbiota ruminal, pese a su resiliencia, lo que provoca que esta se adapte a dicha dieta dando lugar a variaciones en el patrón de fermentación (Li et al., 2012; Henderson et al., 2015; Belanche et al., 2019b). Esto hace que, en un mismo animal, la eficiencia digestiva se pueda ver modificada en función de la dieta ofertada (Durunna et al., 2011).

La cantidad de leche o lacto-reemplazante ofertado durante las primeras semanas puede condicionar el desarrollo del animal. Durante la lactancia, los programas con oferta de leche *ad libitum* suelen resultar en una mayor toma de nutrientes que permite a los animales tener una mayor ganancia diaria y peso vivo que las estrategias de alimentación restringida (Maccari et al., 2015; Schäff et al., 2017). El mayor crecimiento en las

primeras semanas de vida tiene efectos a largo plazo sobre el desarrollo y puede promover un buen estado de salud y una mayor producción de leche en edad adulta (Khan et al., 2011). Un consumo elevado de leche artificial ha sido también asociado a un mayor desarrollo de diversos tejidos en edad adulta, especialmente la fracción de células madre del tejido parenquimal de la glándula mamaria (Soberon & Van Amburgh, 2017). Pese a ello, los regímenes de leche o lacto-reemplazante *ad libitum* aun están en entredicho por los posibles efectos negativos sobre la ingesta de alimento sólido, el desarrollo del rumen y la eficiencia digestiva (Khan et al., 2016; Byrne et al., 2017). En este sentido, durante la lactancia es vital combinar la dieta a base de leche (o lacto-reemplazante) con alimento sólido, ya sea concentrado de arranque y/o forraje.

El concentrado de arranque está compuesto principalmente por ingredientes ricos en polisacáridos de reserva como el almidón (enlaces glucosídicos α) y azúcares solubles (así como saborizantes para estimular su pronto consumo), lo que lo convierte en un sustrato más fácilmente degradable en comparación con el forraje, el cual es más rico en fibra (polisacáridos con enlaces glucosídicos β y polímeros más complejos). Por ello, es recomendable ofertar concentrado de arranque a las pocas semanas después del nacimiento, incluso antes de que se introduzca forraje en la dieta, para optimizar así el proceso de destete (Khan et al., 2011). Se ha comprobado que a mayor cantidad de concentrado ofertada durante la lactancia, la ingesta del mismo se ve incrementada, lo que puede permitir adelantar el destete sin consecuencias negativas para el rumiante (Wang et al., 2020). No obstante, es importante limitar o reducir al mismo tiempo y de forma gradual la oferta de leche, ya que si esta se mantiene *ad libitum*, la apetencia por alimento sólido y sus efectos positivos durante el proceso de destete se ven reducidos (Byrne et al., 2017). Asimismo, es fundamental que el rumiante en edad temprana tenga acceso a forraje al menos dos semanas antes del destete. Pese a su más difícil degradación en el rumen aún inmaduro, la suplementación con forraje resulta beneficiosa para el desarrollo anatómico y funcional del rumen y favorece el proceso de destete en comparación a la suplementación únicamente con concentrado de arranque (Khan et al., 2016). La ingesta de forraje o hierba antes del destete en lugar de concentrado puede favorecer especialmente a aquellos animales que en edad productiva estarán sometidos a regímenes de pastoreo (Phillips et al., 2004), al poseer un rumen más desarrollado.

2.3. Tipo de destete.

El destete es la fase más crítica para el desarrollo fisiológico y microbiológico del rumen. El proceso de destete se puede llevar a cabo de forma abrupta o gradual. El destete abrupto suele reducir el gasto en alimentación y facilita el manejo, pero también puede suponer un estrés extra para las crías y una ralentización de su desarrollo (Mikus et al., 2020). Cuando el destete abrupto además se realiza de forma precoz, los efectos negativos se magnifican aún más, lo que puede comprometer incluso la viabilidad de las crías (Belanche et al., 2017; Mikus et al., 2020). Por otro lado, el destete gradual consiste en que las crías reduzcan la ingestión de lacto-reemplazante durante 1 o 2 semanas mediante la reducción de la cantidad de leche ofertada, su concentración o el tiempo en el que está disponible. Esto permite a los animales tener una fase de transición y adaptación a la dieta sólida, que hace que el destete sea menos estresante (Belanche et al., 2017). Dicho esto, recientemente se ha observado que el factor realmente determinante para un destete gradual óptimo es que haya una ingesta alta de concentrado, y que cuando no se da esta circunstancia el destete puede ser similar al destete abrupto en términos de estrés (Bittar et al., 2020).

El destete también puede llevarse a cabo en función de la edad o el peso. El destete por edad permite organizar a los animales en grupos, con las consecuentes ventajas mencionadas en el apartado 2.1, entre las que se incluyen la reducción del estrés al no mezclar individuos entre grupos y la facilidad para implementar el ya mencionado destete gradual y otras estrategias nutricionales (Mikus et al., 2020). El gran inconveniente de esta práctica es que, en función del tipo de lactancia y alimentación, resulta complicado determinar a qué edad es preciso realizar el destete debido a la heterogeneidad de pesos entre animales congéneres. Lo ideal es que el animal alcance 2,5 veces el peso de nacimiento y que ya ingiera una cantidad considerable de alimento sólido antes de ser destetado (Benetton et al., 2019). El destete por edad llevado a cabo de forma estricta puede por tanto someter a ciertos animales a un destete extremadamente temprano para su condición y desarrollo. Por ello, existe la estrategia alternativa del destete por peso, basado en seleccionar animales con un peso determinado (p.e. 2,5 veces el peso inicial) de forma individual, lo que evita lo antes descrito y asegura que la cría esté mejor preparada para la digestión de alimento sólido. Uno de los inconvenientes de esta práctica es que puede conllevar la reubicación de la cría en un grupo distinto al de la lactancia, por lo que es recomendable que al menos se mantenga el contacto visual y auditivo con

sus congéneres, a fin de reducir el estrés resultante de la suma del proceso de destete y la reubicación (Belanche et al., 2017).

2.4. Uso de probióticos y microbiota ruminal.

El empleo de antibióticos como promotores de crecimiento en los animales de granja en la UE se prohibió hace ya 15 años debido a los posibles perjuicios que estos podrían acarrear en la salud y bienestar de los animales y en última instancia también de los humanos por la posible aparición de resistencias microbianas (FAO, 2016). Como sustitutos de estas sustancias, han surgido diversas estrategias. Una de ellas se basa en el uso de probióticos o de sus metabolitos (prebióticos), algunos de los cuales, como las bacteriocinas, ejercen la función de antimicrobianos naturales (Hernández-González et al., 2021). Los probióticos o '*Direct-Fed Microbials*' son microorganismos inoculados en el TGI del animal que están cobrando cada vez más importancia en el sector ganadero como alternativa al uso de antibióticos debido a que, además de ejercer un control sobre el crecimiento de patógenos, incluso se mejora el rendimiento productivo del animal (McAllister et al., 2011; Buntyn et al., 2016). El principal modo de acción de los probióticos o prebióticos es la modificación de la dinámica poblacional del TGI, modulando el equilibrio entre los microorganismos beneficiosos y los potencialmente dañinos (An et al., 2008; Mountzouris et al., 2009). Dependiendo del probiótico en sí, el mecanismo por el que pueden llevar a cabo dicha modificación puede basarse en la exclusión competitiva, la ocupación previa de un nicho ecológico-digestivo, la producción de compuestos antimicrobianos (eg. bacteriocinas) o la estimulación del sistema inmune del hospedador (Buntyn et al., 2016). Según la sección del tracto digestivo sobre el que actúan (diana) a los que van destinados los microorganismos probióticos, se pueden diferenciar dos grandes grupos: los que actúan a nivel de rumen y los que lo hacen en el intestino (FAO, 2016). En rumiantes, es común el uso de probióticos de origen fúngico, destacando entre todos ellos las levaduras (*Saccharomyces cerevisiae*) (Chaucheyras-Durand et al., 2008). Las levaduras suscitan gran interés ya que posibilitan la modulación de la microbiota ruminal para mejorar considerablemente la eficiencia. Las levaduras actúan principalmente a nivel ruminal, consumiendo oxígeno y creando un ambiente ruminal más anaerobio que favorece el crecimiento de microorganismos anaerobios extrictos como las bacterias fibrolíticas (Chaucheyras-Durand et al., 2008). La mayoría de los estudios en los que se han empleado estos microorganismos, o una

mezcla de ellos y las bacterias mencionadas en el párrafo siguiente, están centrados en evaluar los efectos en edad adulta especialmente sobre la mitigación de la acidosis ruminal y la mejora de la producción lechera, donde aunque se han observado resultados variables dependiendo del tipo de dieta, en general existe una mejora de la producción (Stella et al., 2007; Desnoyers et al., 2009).

Los probióticos bacterianos más empleados para modular la microbiota son las bacterias del ácido láctico y las bifidobacterias, particularmente los géneros *Lactobacillus* y *Bifidobacterium*, que actúan principalmente en el intestino y cuya presencia está asociada a la disminución de las poblaciones de *E. coli* y *Clostridium* spp. (Yang et al 2012; Cao et al., 2013). Estos microorganismos compiten con los potenciales patógenos mediante la producción de ácido láctico y bacteriocinas y la adhesión al epitelio intestinal para inducir así la respuesta inmune del hospedador (Shim et al., 2012; Hung et al., 2012). En relación a esto, también hay abundante investigación centrada en el efecto probiótico de las bacterias presentes en los ensilados (forraje conservado en humedad donde se produce fermentación láctica) suplementados en ocasiones a los animales (Weinberg et al., 2004).

En referencia al estudio del uso de probióticos en edad temprana, este se centra en evaluar los efectos sobre el crecimiento del animal, el desarrollo microbiológico del rumen y el intestino, la eficiencia digestiva y la limitación en la colonización de patógenos (Frizzo et al., 2011). La administración de mezclas constituidas por bacterias del ácido láctico, bifidobacterias y/o levaduras durante las primeras semanas de vida del animal se ha relacionado con una mayor ingesta de alimento sólido, una mayor ganancia de peso diaria y un mayor peso vivo hasta el momento de destete (Lesmeister et al., 2004; Frizzo et al., 2011; Cantor et al., 2019). Estos efectos beneficiosos también se producen con la inoculación de cepas únicas de especies autóctonas del rumen menos utilizadas como *Propionibacterium jensenii* o *Megasphaera elsdenni*, que contribuyen al desarrollo ruminal mientras que aumentan la producción de propionato y butirato, respectivamente (Adams et al., 2008; Yohe et al., 2018). Un poco más tarde, justo después del destete, el empleo de bacterias productoras de ácido láctico presentes en el rumen no sólo se ha relacionado con una mayor ganancia de peso (+19%) en corderos durante esta fase (Devyatkin et al., 2021), sino que ha dado lugar a una mejora del desarrollo epitelial del rumen (Izuddin et al., 2019) y del estado inmune del animal (Apás et al., 2010).

En los últimos años se ha ido un paso más allá al plantearse la utilización del conjunto de la microbiota del rumen adulto como probiótico. El trasplante de contenido ruminal se ha empleado históricamente como una alternativa eficaz para el tratamiento puntual de animales con disbiosis ruminal o indigestiones por ingesta de compuestos tóxicos (DePeters & George, 2014). La puesta en marcha de una estrategia de este tipo a gran escala plantea ciertas dudas debido a la complicación que supone la obtención y conservación del inóculo de microbiota ruminal manteniendo sus características y posibles efectos beneficiosos. Por ello, uno de los retos en esta materia ha sido buscar alternativas o métodos de conservación para preservar el inóculo en condiciones óptimas. En ensayos *in vitro*, se ha comprobado que la congelación durante 24h del inóculo a -18 o -20 °C afectaba moderadamente al patrón de fermentación microbiana (Hervás et al., 2005), aunque la congelación en nitrógeno líquido resultaba ser más efectiva (Prates et al., 2010). La refrigeración a 0-4 °C durante unas horas también se ha postulado como alternativa, pero los resultados no han sido lo suficientemente satisfactorios si además se tiene en cuenta el poco tiempo durante el que el inóculo se mantendría viable (Prates et al., 2010). Paralelamente, algunos autores han sugerido el empleo de crio-conservadores del inóculo congelado como el glicerol o el etilenglicol, obteniéndose niveles de fermentación ruminal in vitro por encima de la actividad fermentativa generada sin crio-conservador, si bien los mejores resultados al respecto se obtuvieron con el uso de dimetilsulfóxido (DMSO) (El-Raof et al., 2007; Denek et al., 2010). La liofilización de la microbiota también ha resultado ser un interesante método de conservación al no observarse diferencias notables en el pH ni en las concentraciones de amonio y AGV en el contenido ruminal con respecto al inóculo fresco (Zhong et al., 2014). Estudios como el de Abo-Donia et al. (2011) incluso sugieren que la liofilización apenas afecta a la abundancia relativa de bacterias y protozoos en el contenido ruminal, si bien se antoja necesario seguir estudiando esta estrategia para obtener resultados más consistentes.

Recientemente, distintos estudios han investigado el potencial de la inoculación de contenido ruminal de animales adultos en rumiantes jóvenes para contrarrestar el deficiente proceso de colonización que ocurre en condiciones de lactancia artificial (Zhong et al., 2014; De Barbieri et al., 2015a,b; Li et al., 2019; Yu et al., 2020). Los resultados obtenidos al respecto han sido variados, en parte por los distintos modos de inoculación empleados, que en general han sido intermitentes, pero también por la falta de un análisis completo de los efectos en todos los grandes grupos microbianos del rumen,

la fermentación ruminal y el rendimiento del animal. Pese a ello, en general, esta estrategia ha mostrado ser efectiva en la mejora de la biodiversidad y actividad bacteriana en animales jóvenes inoculados (De Barbieri et al., 2015a; Yu et al., 2020), lo cual sugiere que en esta ventana de tiempo se puede acelerar la colonización microbiana del rumen y en principio poder "programar" el microbioma ruminal del animal adulto (Yáñez-Ruiz et al., 2015).

2.5. Otras intervenciones.

Además de las prácticas señaladas en apartados anteriores, también existen las intervenciones en edad temprana centradas en el uso de aditivos alimenticios y en las mejoras en el manejo/comportamiento.

Al igual que en el caso de los probióticos, los aditivos alimenticios han cobrado gran importancia como potenciales agentes moduladores de la microbiota y metabolismo del rumen especialmente a partir de la prohibición de los antibióticos como promotores de crecimiento. Entre los aditivos empleados, destacan los extractos de plantas, especialmente los aceites esenciales (Hart et al., 2008). Los aceites esenciales actúan selectivamente sobre microorganismos concretos y de manera muy general sus principales funciones son las de modular el patrón de colonización bacteriana en las partículas de digesta en el rumen e inhibir el crecimiento de bacterias productoras de amonio para reducir así la degradación de almidón y aminoácidos, respectivamente (Hart et al., 2008). Sus efectos son muy variables dependiendo de los compuestos incluidos, la dosificación y la dieta que recibe el animal (Yáñez-Ruiz & Belanche, 2020). Debido al efecto positivo que supone la presencia en el rumen de productos de la fermentación ruminal como los AGV, los derivados algunos de ellos también se han empleado como aditivos para mejorar el desarrollo anatómico y funcional del rumen. Además de los AGV más abundantes (acetato y propionato), destaca el uso de isobutirato y butirato sódico como promotores del crecimiento de las papilas en el rumen y estimuladores de la producción de AGV del desarrollo de la microbiota en el intestino (Górka et al., 2018; O'Hara et al., 2018).

En relación a las intervenciones a nivel de manejo, algunas están destinadas a la mejora del enriquecimiento ambiental para los animales, con efectos poco concluyentes sobre el desarrollo y parámetros productivos. En relación a ello, recientemente se ha investigado

acerca del efecto que puede tener la colocación del comedero de pienso de arranque en distintas localizaciones del parque sobre la ingesta y crecimiento de las crías durante el destete. En terneros alojados en parque individuales, la colocación del comedero justo en el lado opuesto a la toma de leche fomenta un mayor movimiento del animal y mayor ingesta de leche, pero por el contrario esto no supone mejoras en indicadores de estrés y además conlleva una menor y más lenta ingesta de pienso, lo que puede comprometer su desarrollo (Parsons et al., 2020). En el caso de animales jóvenes alojados en grupos durante la lactancia, es posible implementar una práctica basada en la introducción de acompañantes adultos en el mismo parque, que fomente el aprendizaje de alimentación de sustrato sólido en las crías. En efecto, se ha visto que esta estrategia supone un aumento de las interacciones sociales entre los animales y, sobre todo, una mayor ingesta de concentrado y un mayor crecimiento antes del destete (De Paula Vieira et al., 2012). La presencia de animales adultos podría a su vez actuar como fuente de inóculación indirecta de microbiota ruminal, salival, de la piel, etc. que favorezca la colonización del TGI en las crías (Yeoman et al., 2018). Sin embargo, los efectos de esta estrategia todavía no están suficientemente descritos.

2.6. Efectos de la microbiota sobre los parámetros productivos y su persistencia en el tiempo.

La eficiencia digestiva y el rendimiento del animal vienen determinados en gran medida por un complejo conjunto de factores genéticos y fisiológicos del propio rumiante (Cantalapiedra-Híjar et al., 2018; Lima et al., 2019). Como parte de estos procesos, la microbiota también interviene de forma trascendental en la eficiencia digestiva, ya que es la principal responsable de la transformación del alimento ingerido a sustrato energético y proteico (AGV y proteína microbiana) para el hospedador. Diversos estudios han mostrado una relación directa entre distintos patrones en la composición y actividad de la microbiota ruminal y la eficiencia digestiva del animal (Guan et al., 2008; Carberry et al., 2012; Shabat et al., 2016), indicando que los animales más eficientes poseían una microbiota ruminal similar entre ellos y distinta a los animales menos eficientes. Estos animales con microbioma ruminal y eficiencia digestiva diferenciadas también han presentado un patrón de fermentación variable en lo que se refiere a concentraciones de AGV y otros metabolitos ruminales, aunque en la producción de estos también hay una importante influencia de la dieta administrada (Guan et al., 2008; Shabat et al., 2016). No obstante, también se han observado correlaciones claras entre la microbiota ruminal y el

rendimiento digestivo y productivo del animal independientemente de la dieta (Hernández-Sanabria et al., 2012; Carberry et al., 2012), y que hasta cierto punto existe un núcleo de microorganismos compartido entre la mayoría de los animales ('core community') responsables de la mejor o peor utilización del alimento (Li & Guan, 2017). Se ha sugerido que entre estos microorganismos puede haber miembros de las familias *Veillonellaceae, Lachnospiraceae y Prevotellaceae* (Myer et al., 2015; Li & Guan, 2017) y que taxones de metanógenos pertenecientes a *Methanomassiliicoccales* o *Methanobrevibacter* pueden estar relacionados con una mayor eficiencia digestiva (Carberry et al., 2014; Li & Guan, 2017).

En el animal adulto, las alteraciones en el microbioma ruminal que conllevan cambios en el rendimiento del animal son posibles, pero debido al elevado grado de especificidad del individuo y la resiliencia de la microbiota en esta fase, son a veces difíciles de alcanzar y se mantienen siempre que el tratamiento se siga aplicando (Weimer et al., 2010; Weimer, 2015). Como se ha comentado en el apartado anterior, la fase de neonato o juvenil ofrece una oportunidad para modular esta microbiota en el rumen aun en desarrollo debido a su cambio constante y por tanto plasticidad (Yáñez-Ruiz et al., 2015). Se ha demostrado que llevar a cabo ciertas estrategias de manejo (p.e. el tipo de lactancia) o intervenciones nutricionales en edad temprana (p.e. aditivos o dieta) tienen impacto directo sobre la microbiota del rumen y su actividad fermenativa (Yáñez et al., 2010; Abecia et al., 2013), así como efectos a nivel fisiológico sobre el animal (Abecia et al., 2017; Belanche et al., 2019a). Partiendo de esta premisa, uno de los grandes retos en este campo ha sido concebir posibles intervenciones que tengan efectos sobre el rendimiento del animal no sólo durante la intervención temprana, sino a medio-largo plazo. El uso de distintas dietas (concentrado vs. forraje) durante el destete ha demostrado dar lugar a comunidades procarióticas distintas pero con similares patrones de fermentación a los 4 meses (Yáñez-Ruiz et al., 2010). Intervenciones destinadas a la reducción de las poblaciones de arqueas en edad temprana han tenido el efecto esperado sobre la producción de metano a medio (3 meses post-destete; Abecia et al., 2013; 2014) y largo plazo (12 meses post-destete; Fonty et al., 2007), si bien los ligeros efectos sobre el patrón de fermentación y el peso de los animales apenas persistieron tras el destete. Por otro lado, las diferencias sustanciales reportadas durante el destete en corderos con distintos tipos de lactancia en términos de eficiencia, microbiota y fermentación ruminal apenas tuvieron persistencia pasados 4 meses, si bien los animales con lactancia natural presentaron un mayor

crecimiento a esa edad cuando se alimentaban a base de pasto (Belanche et al., 2019a). La variabilidad de los resultados obtenidos en estos estudios y el potencial que este tipo de intervenciones pueden tener a largo plazo, invitan a seguir indagando sobre los factores que determinan la posible persistencia de los efectos a nivel del ecosistema ruminal y el consecuente rendimiento del animal.

3. Interacción microbiota-hospedador.

El epitelio de todo el TGI y especialmente del rumen juega un papel fundamental en la absorción de nutrientes (principalmente AGV), a la vez que realiza el proceso de filtrado de moléculas tóxicas para que estas no lleguen al sistema circulatorio (Plaizier et al., 2018). Además, este epitelio posee una serie de mecanismos por los que se promueve la coexistencia con la microbiota autóctona del TGI en una relación simbiótica donde existe un equilibrio entre las poblaciones mutualistas y comensalistas con el hospedador, relegando a los microorganismos patógenos a apariciones esporádicas (Kuhn & Stappenbeck, 2013).

3.1. Regulación de la microbiota por el sistema inmune.

El sistema inmune juega un papel esencial para que haya una interacción óptima entre la microbiota del TGI y el hospedador. Para ello, ha de darse un proceso previo de 'aprendizaje' o 'maduración' del sistema inmune que consiste en establecer contacto con cada uno de los microorganismos que colonizan al hospedador, lo cual cobra más importancia en edad temprana (Collado et al., 2012). La mucosa del digestivo suele estar muy involucrada en esta tarea de reconocimiento de microorganismos, en parte gracias a la presencia de prolongaciones de tejido linfoide asociadas a las capas más apicales del epitelio. Sin embargo, el rumen carece de tejido linfoide organizado como tal en el epitelio (Sharpe et al., 1977), y además el elevado grosor y baja permeabilidad del mismo impide un diálogo fluido entre las macromoléculas o microorganismos del rumen y los líquidos circundantes (Trevisi et al., 2014). Quizá por esta razón, al menos parcialmente, los estudios e información referentes a los mecanismos de defensa del epitelio ruminal son muy escasos en comparación a la información disponible acerca del epitelio del intestino, especialmente en monogástricos (Dommett et al., 2005). En efecto, se ha observado que existe cierto transporte selectivo de microorganismos entre el intestino y el torrente sanguíneo a través de la barrera intestinal y que estos microorganismos circulan

en la sangre o incluso dentro de células sanguíneas conformando un patrón microbiano común único distinto al del intestino (Peña-Cearra et al., 2021). En cuanto al rumen, la evidencia científica ha demostrado que existen varios mecanismos implicados que actúan conjuntamente en la respuesta del hospedador frente a la presencia de microorganismos en el rumen, entre los cuales los anticuerpos y los receptores de tipo Toll (TLR) son los más estudiados (Malmuthuge et al., 2012).

Los TLR son proteínas presentes en una amplia variedad de células a lo largo de todo el TGI, tanto en la superficie celular como en compartimentos intracelulares (Chang, 2010). Se han identificado un total de 13 TLR, cuya función es reconocer moléculas concretas de la cápsula o pared celular de las bacterias o incluso el material genético de las mismas (Guan et al., 2010). Se ha demostrado que estos receptores son capaces de diferenciar entre microorganismos comensales y los potencialmente patógenos, manteniendo la homeostasis y el epitelio en estado óptimo (Rakoff-Nahoum et al., 2004). Los niveles de expresión de los TLR a lo largo del TGI, así como de otras moléculas con función inmune como los receptores de reconocimiento de patrones, las proteínas de reconocimiento de peptidoglicanos y las péptidos antimicrobianos tipo defensinas (Meade et al., 2014), resultan ser, en general, más elevados durante los primeros meses de vida (Malmuthuge et al., 2012). Esta información sugiere que las interacciones entre la microbiota comensal del TGI y el sistema inmune a través de estas moléculas es necesaria para mantener al animal en estado sano mientras que se fomentan las respuestas inmunes innata y adaptativa (Guan et al., 2010).

El sistema inmune innato del rumiante se empieza a adquirir a través de la madre y alcanza un mayor grado de desarrollo cuando el recién nacido entra en contacto con el exterior y empieza a tomar calostro y leche maternos (Wheeler et al., 2007). Desde hace más de un siglo se sabe que parte de la inmunidad de la madre se transfiere al suero sanguíneo de su descendencia gracias a la toma de calostro o directamente de suero materno (transferencia de inmunidad materna) (Famulener, 1912). Lo que al principio era ‘algo’ desconocido que se transfería a la descendencia, luego se comprobó que eran en realidad anticuerpos (Orcutt & Howe et al., 1922). Los anticuerpos presentes en el calostro y la leche proceden del suero y se secretan a través de la glándula mamaria, donde además pueden cobrar importancia en caso de mastitis (Lascelles, 1979). Se ha demostrado que el anticuerpo mayoritario en el calostro es la inmunoglobulina G (IgG), pero también se han detectado

cantidades variables de inmunoglobulina M (IgM) e inmunoglobulina A (IgA), estando el origen de esta última próximo al TGI, donde hay un mayor contacto con antígenos (Lascelles, 1979).

Los anticuerpos e incluso los linfocitos de origen materno pasan de esta forma al neonato, el cual los adquiere al menos parcialmente y los incorpora a su propio suero para así dar pie a la respuesta inmune frente a los microorganismos del TGI y de otros sistemas (Sheldrake & Husband, 1985). Se han detectado anticuerpos en suero específicos para cepas concretas de bacterias ruminales con tal grado de especificidad que no forman complejos anticuerpo-antígeno cuando se enfrentan a cepas similares presentes en monogástricos (Sharpe et al., 1969). Pese a que se ha comprobado que en el calostro ya hay anticuerpos frente a microorganismos del rumen concretos como *Butyrivibrio* y bacterias del ácido láctico (Sharpe et al., 1977), la gran especificidad de los mismos indica que el sistema inmune se adapta más allá de aquellos componentes conferidos directamente por la madre. En efecto, en animales gnotobióticos (microbiológicamente estériles o con microbioma reducido) sin toma de calostro e inoculados con especies bacterianas concretas, se produjo un desarrollo de anticuerpos frente a estas en cuestión de pocos meses (Sharpe et al., 1977). Más allá del calostro y la leche, existe por tanto otro mecanismo o vehículo por el cual el rumiante en edad temprana consigue inmunidad adquirida frente a estos microorganismos, favoreciendo o inhibiendo su crecimiento (Fouhse et al., 2017).

Dada la baja presencia de tejido linfático en la pared ruminal, el fluido con potencial función inmune que entra en el rumen en mayor cantidad es la saliva (Duric et al., 1994). La secreción de moléculas salivales con función inmune permite, en cierto modo, difundir la respuesta inmune adquirida en base al reconocimiento de microorganismos específicos en los distintos epitelios del TGI. Muestra de ello es que el anticuerpo mayoritario en el rumen es IgA, el cual es también el más abundante en la saliva a diferencia del calostro y el suero, que presentan una mayor concentración de IgG (Lascelles, 1979; Subharat et al., 2015). La razón principal por la que se da esta circunstancia puede deberse a la presencia de un componente secretor en la IgA que le confiera una mayor resistencia a la actividad proteolítica y la acidez del rumen (Snoeck et al., 2006).

En el proceso de modulación del sistema inmune del neonato en base a la microbiota presente en el mismo también intervienen una serie de mediadores que están en continuo

contacto con las células con función inmune. Estos mediadores no son otros que las citoquinas e interleucinas, polipéptidos de bajo peso molecular también presentes en la leche (Keller et al., 1981) especialmente cuando hay infección (Sordillo et al., 1997). Entre otras funciones específicas, se ha evidenciado que las citoquinas están implicadas en la reducción de las reacciones inflamatorias o alérgicas en el TGI (Donnet-Hughes et al., 2000), así como en promover la producción de anticuerpos, especialmente IgA (Eckmann et al., 1992; Hansen et al., 2019). Recientemente se han identificado otros mediadores no peptídicos del sistema inmune, los micro RNAs, con un efecto regulador especialmente en edad temprana. Algunos de estos fragmentos de ARN (miR15/16, miR-29) intervienen en el desarrollo de la mucosa y de células con función inmune y también se les ha relacionado directamente con el crecimiento de algunas bacterias del TGI, o la inhibición del mismo (Liang et al., 2014). Así, varios de estos micro RNAs se han correlacionado positivamente con bacterias, a priori, beneficiosas como *Lactobacillus* y *Bifidobacterium* (Liang et al., 2014). Por el contrario, se ha demostrado que otros micro RNAs inhiben el crecimiento de *Fusobacterium nucleatum* y *Escherichia coli* mediante la regulación directa sobre sus transcritos bacterianos (Liu et al., 2016b).

La exposición inmediata y sostenida en el tiempo del rumiante joven a la comunidad microbiana del TGI es por tanto de suma importancia para establecer un correcto patrón de comunicación entre el microbioma, las citoquinas y demás mediadores y el resto de componentes del sistema inmune (Taschuk & Griebel, 2012). De hecho, en modelos de animales gnotobióticos, se ha visto que estos sufren severas consecuencias en el desarrollo inmunológico a nivel de alteraciones en el epitelio, la circulación de citoquinas y el reconocimiento de antígenos (Falk et al., 1998). Por esta razón, el correcto desarrollo de la función de barrera frente a patógenos de la mucosa del TGI a través de la acción conjunta de anticuerpos y el resto de componentes del sistema inmune, depende en gran medida del contacto con microorganismos en las primeras horas o días de vida (Malmuthuge et al., 2011; Taschuk & Griebel, 2012). Pese a ello, se desconoce el efecto concreto de la saliva y sus componentes como elementos moduladores de la microbiota ruminal y su actividad.

3.2. Composición de la saliva.

La saliva del rumiante es secretada en las glándulas submaxilares, sublinguales y parótidas, siendo estas últimas las que más saliva producen y de forma más continua

(Bailey, 1961). La producción de saliva en todas las glándulas se ve incrementada notablemente cuando el animal ingiere alimento sólido ya que esta es esencial para garantizar la correcta masticación, deglución, regurgitación y rumia del alimento. Por ello, los rumiantes producen grandes cantidades de saliva llegando a volúmenes de hasta 9 litros diarios en pequeños rumiantes como las ovejas (Duric et al., 1994). La composición iónica de la saliva del rumiante, ya sea de la saliva total de la boca como la propia de cada glándula, ha sido estudiada ampliamente desde hace décadas (Mcdougall, 1948; Somers, 1957) por su importancia en el mantenimiento de la presión osmótica y el pH del rumen (Warner & Stacy, 1977). En cambio, el estudio de los componentes bioactivos de la saliva, donde se incluyen los péptidos y proteínas, ha sido mucho más escaso de modo que hasta la fecha no existe un conocimiento completo del conjunto del proteoma de la saliva del rumiante. El estudio del proteoma de la saliva con distintos métodos y enfoques ha demostrado que la albúmina es, como ocurre en el suero, la proteína mayoritaria llegando a suponer el 50% de la proteína total (Lamy et al., 2009; Haigh et al., 2010). Además de la albúmina, se han detectado un número considerable de proteínas implicadas en el transporte de sustancias extra e intracelularmente, destacando en abundancia las distintas subunidades de la hemoglobina (Lamy et al., 2009). El proteoma salival del rumiante incluye, además, un gran número de proteínas y polipéptidos de menor peso molecular, siendo en estos últimos donde radica el mayor grado de variabilidad y especificidad del proteoma entre individuos (Lamy et al., 2009). A parte del transporte, todas estas moléculas están implicadas en otros muchos procesos y funciones, entre ellos: función estructural del citoesqueleto, desarrollo celular, función enzimática, regulación del metabolismo de nutrientes y función inmunológica (Ang et al., 2011; Escribano et al., 2019).

3.2.1. La saliva como moduladora de la microbiota en rumiantes.

Como se ha mencionado en apartados anteriores, la saliva juega un papel fundamental en la regulación de la microbiota del TGI gracias a que contiene moléculas con función inmune entre las que destacan los anticuerpos. La IgA secretora es el anticuerpo mayoritario en la saliva, con una concentración variable dependiendo de la glándula salival muestreada, pero que en el conjunto de la saliva bucal puede alcanzar los 5,95 mg/ml (Fouhse et al., 2017). La IgG también está presente en la saliva pero en concentraciones en torno a 4 veces menor que la IgA (Lascelles & McDowell, 1974).

Ambas, pero especialmente la IgA secretora al ser más abundante en el rumen, tienen la capacidad de modular el crecimiento de las bacterias ruminales, ya que se ha comprobado que la comunidad bacteriana que forma agregados con IgA es más similar al conjunto de la microbiota del rumen que al de la microbiota total de la saliva (Fouhse et al., 2017). A este respecto, y pese a lo que históricamente se podría pensar acerca del papel de las inmunoglobulinas, la IgA no sólo tiene la capacidad de dificultar el desarrollo y anclaje de ciertas bacterias o parásitos al epitelio del TGI sino que además puede marcar a otras comensales o simbióticas para estimular su proliferación y su contacto estrecho con el hospedador (Donaldson et al., 2018). En cualquiera de los casos, las inmunoglobulinas se unen al agente en cuestión mediante aglutinación y, si efectivamente es patógeno, este quedará inmovilizado hasta su eliminación por medio de la digestión o la fagocitosis (Brandtzaeg et al., 1968; Carrero et al., 2007). Tras esta última, y previa a la lisis, se producirá la presentación de antígenos a las células inmuno-competentes (células dendríticas y neutrófilos) presentes en la mucosa con las citoquinas como mediadoras en el proceso (Wines & Hogarth, 2006), estableciéndose así la inmunidad adquirida. Ejemplo de este proceso de inmunización son los mayores niveles de IgG y IgA específicos para *Methanobrevibacter ruminantium* detectados en la saliva y en el rumen de vacas en respuesta a la previa vacunación con antígenos de este metanógeno (Subharat et al., 2015).

La saliva también posee proteínas de menor peso molecular como las pertenecientes a la familia de proteínas de choque térmico HSP70/HSPA (Asea, 2005). Las HSP70/HSPA son proteínas de defensa secretadas en las glándulas salivales (Fábián et al., 2003) que, al igual que las inmunoglobulinas, son capaces de anclarse a la superficie de bacterias concretas dando lugar a complejos de aglutinación mediante la formación de oligómeros (Soares et al., 2004). Las bacterias quedan así inmovilizadas y las HSP70/HSPA evitan así su propagación a la vez que inician la señalización para la liberación de citoquinas proinflamatorias y la activación del sistema del complemento (Campisi et al., 2002; Prohászka et al., 2002).

Entre los polipéptidos catiónicos salivales con función inmune encontramos las defensinas, histatinas, catelicidinas, adrenomedulina y la lactoferrina. Tanto las α- como las β-defensinas presentan una actividad antibacteriana de amplio espectro que se basa en su carácter catiónico (Gorr, 2009; Wiesner & Vilcinskas, 2010). La carga positiva de estos

péptidos les sirve para entrar en contacto con las cargas negativas típicas de las membranas bacterianas en un proceso orquestado por otras proteínas como la lisozima que asiste a las defensinas mediante la lisis de las capas más externas de la bacteria (Fabian et al., 2012). Las defensinas se integran así en la membrana bacteriana, abren canales dentro de ella y finalmente provocan la ruptura de la misma (Brogden, 2005). Al igual que las defensinas, las histatinas y la lactoferrina son polipéptidos catiónicos de alto espectro con propiedades antibacterianas, antifúngicas e incluso antivíricas (Wiesner & Vilcinskas, 2010; White et al., 2010). Tanto histatinas como lactoferrina actúan de forma similar a las defensinas (Brogden, 2005), pero además pueden unirse a iones de cobre y níquel o hierro, respectivamente, de forma que pueden inhibir la actividad de enzimas o cofactores importantes en el crecimiento bacteriano y fúngico o en la metanogénesis de arqueas por medio de la Coenzima M (Grogan et al., 2001, Wiesner & Vilcinskas, 2010) o potencialmente la metanogénesis de arqueas por medio de la metil-coenzima M reductasa (Wongnate & Ragsdale, 2015). Además de esto, la lactoferrina salival ha sido recientemente propuesta como un biomarcador en situaciones de estrés agudo (p.e. en el proceso de esquilado) al disminuir su concentración significativamente tras dicho episodio de estrés (Escribano et al., 2019). Las catelicidinas comprenden una gran variedad de oligopéptidos con cierta prevalencia en la saliva de rumiantes (Lamy et al., 2009). Estas tienen su origen en los neutrófilos y tienen propiedades inmuno-moduladoras al unirse al lipopolisacárido de las bacterias Gram- y a partir de ahí promover la escisión de la membrana celular (Gorr, 2009).

También existen otra serie de proteínas salivales que, a diferencia de las anteriores, no son de naturaleza catiónica. En este grupo se incluye la lisozima, la proteína por excelencia del sistema inmunitario innato en la saliva (Veerman et al., 1996). El principal modo de acción de esta, como se ha comentado anteriormente, es la hidrólisis de los enlaces β-glucosídicos entre los monosacáridos que conforman el peptidoglucano de la pared bacteriana, teniendo una mayor efectividad frente a bacterias Gram+ por su estructura (Wiesner & Vilcinskas, 2010). La lisozima además tiene la propiedad de aumentar la permeabilidad de las membranas y, al actuar conjuntamente con los péptidos catiónicos, se produce la ruptura de estas membranas también en bacterias Gram- y hongos (Ibrahim et al., 2001). La α-amilasa salival, además de su función enzimática, tiene la capacidad de unirse a bacterias a través del pili o el lipopolisacárido para promover la adhesión de estas a la superficie de los dientes (Scannapieco et al., 1995;

Rogers et al., 2001), lo que puede llevar a su inmovilización para que no entren en el digestivo, pero también puede tener un efecto negativo al formar agregaciones bacterianas en la superficie dental. Funciones similares a las descritas son llevadas a cabo por las cistatinas y las 'proteínas ricas en prolina', ya que ambos grupos de proteínas se unen a las bacterias y hongos a través del lipopolisacárido principalmente, lo que conlleva su exclusión en superficie y por tanto la no entrada de estos hacia el estómago (Tenovuo, 2002; Choi et al., 2011). Las cistatinas además participan como inhibidores de las proteasas bacterianas y de protozoos parásitos, impidiendo así su crecimiento (Dickinson, 2002). Por último, se encuentran las mucinas, que no poseen propiedades antimicrobianas en sí mismas pero son las principales responsables de la formación de la película salival alrededor de los dientes ya que promueven la adhesión de más proteínas a las superficies bucales y aumentan la viscosidad de la saliva (Pramanik et al., 2010). Esto, y la cierta afinidad de algunas mucinas (MUC7) para unirse a los microorganismos, hacen que estos queden atrapados y aglutinados dentro de partículas donde pueden actuar otras proteínas (Fábián et al., 2008; Gorr, 2009).

Sin embargo, la identidad, abundancia y funcionalidad de muchos de estos componentes de la saliva en rumiantes aún se desconoce. Los efectos directos de estos componentes sobre la proliferación y el metabolismo de la microbiota propia de la saliva y sobre todo del rumen no se han estudiado suficientemente aún, pese al papel trascendental que podrían jugar en la dinámica poblacional del microbioma ruminal. La investigación acerca de cómo la saliva y sus componentes intervienen en la relación hospedador-microbiota se plantea, por tanto, fundamental para comprender como el sistema inmune modula a esta última.

4. Avances en el estudio de la microbiota ruminal.

Durante prácticamente todo el siglo XX el estudio de la microbiota del rumen se ha basado en observaciones microscópicas y el cultivo selectivo de poblaciones microbianas (Hungate, 1969). Todo esto se consiguió gracias a las investigaciones destinadas al desarrollo de cultivos *in vitro* en condiciones de anaerobiosis y con sustratos específicos del rumen. Aún se siguen empleando este tipo de técnicas de cultivo hoy en día, sobre todo para caracterizar mejor a los microorganismos ruminales parcialmente desconocidos, haciendo especial hincapié en su metabolismo (Creevey et al., 2014; Miltko et al., 2015).

En décadas posteriores, a partir de los años 80 y hasta la actualidad, empezaron a desarrollarse técnicas moleculares que permitían la detección y caracterización de comunidades microbianas a gran escala. Estas técnicas se basaron en la utilización de los genes de ARNr 16S, ARNr 18S o genes del espaciador transcrito interno del ARNr para medir la composición de procariotas, protozoos y hongos del rumen, respectivamente (Woese et al., 1983; Kittelmann et al., 2013). El gen del ARNr 16S se considera hoy en día el marcador genético estándar para los estudios de ecosistemas procarióticos, debido a que contiene regiones altamente conservadas y, entre ellas, 9 regiones hipervariables que permiten diferenciar entre las distintas especies bacterianas y de metanógenos al usar cebadores específicos para estas regiones (Chakravorty et al., 2007). En estudios centrados específicamente en la comunidad metanógena, otros fragmentos genéticos como el gen de la metil co-reductasa A (mcrA) también se han utilizado para la amplificación y secuenciación (Denman et al., 2007).

Basándose en los amplicones antes mencionados, surgieron las primeras técnicas de secuenciación basadas en la clonación de los fragmentos de ADN de interés y en el uso de didesoxinucleótidos (Sanger et al., 1977), con las que se llevaron a cabo los primeros estudios de la composición del microbioma ruminal. Paralelamente, se desarrollaron otra serie de técnicas como la PCR cuantitativa para medir la concentración de las distintas comunidades al utilizar cebadores universales de las regiones antes mencionadas, o de cada una de las poblaciones al utilizar cebadores específicos para ellas (Maeda et al., 2003). Otros métodos encaminados a mejorar el poder de discriminación entre especies como la electroforesis en gel con gradiente de desnaturalización (DGGE) (Sadet et al., 2007), polimorfismo de longitud de fragmentos de restricción-terminación (T-RFLP) (Yáñez-Ruiz et al., 2010) o la hibridación fluorescente *in situ* (FISH) (Newbold et al., 2005) han sido utilizados para el estudio de la microbiota ruminal. No obstante, todos los anteriores fueron pronto sustituidos, debido a su bajo rendimiento, por los métodos más eficientes de secuenciación masiva (NGS), que permitían la generación de muchas más secuencias o lecturas de ADN más cortas (Liu et al., 2007).

La pirosecuenciación 454 ha sido el primer gran sistema de NGS basado en los amplicones (‘*Amplicon Sequencing’*) de los genes antes mencionados empleado para la caracterización de la composición del microbioma del TGI en mamíferos (Arrieta et al., 2014). Sin embargo, el gran coste y el relativamente alto índice de error de la

secuenciación 454 ha desencadenado su desuso en los últimos años en favor de la secuenciación por Illumina (Luo et al., 2012). Con el método Illumina se obtienen secuencias resultantes de la unión de lecturas correspondientes a los dos extremos finales del fragmento del amplicón (p.e. región del gen del ARNr 16S) con menores tasas de error. Las secuencias resultantes pueden agruparse en base a distintos umbrales de similaridad dando lugar a Unidades Taxonómicas Operativas (OTU) o Variantes de la Secuencia del Amplicón (ASV), en función del método empleado, lo que equivale aproximadamente a las distintas especies (>97% similaridad) o filotipos microbianos (100% similaridad), respectivamente. Estos OTU o ASV se identifican con la utilización de bases de datos y a partir de ello se puede incluso inferir el perfil funcional de una comunidad microbiana con herramientas bioinformáticas como PICRUSt (Langille et al., 2013) o la reciente CowPi, que es epecífica para el microbioma del rumen (Wilkinson et al., 2018).

El método descrito en el párrafo anterior (‘*Amplicon Sequencing’*) ha sido ampliamente utilizado para describir la composición microbiana del rumen en una gran diversidad de estudios. Recientemente, también ha cobrado importancia la secuencación no dirigida del metagenoma (‘*Metagenomics or shotgun sequencing’*) para describir de forma directa la funcionalidad de la microbiota del rumen en base a todos los genes pertenecientes a esta (Sunagawa et al., 2013). Este enfoque, a diferencia del anterior, permite saber exactamente los genes (y por extrapolación las funciones) que poseen las distintas poblaciones del rumen en base a la secuenciación profunda de fragmentos aleatorios del metagenoma microbiano. Eso sí, además de su elevado coste, el hecho de ser una técnica basada en fragmentos aleatorios de ADN, es más posible que haya contaminación del hospedador y, a diferencia de las técnicas basadas en el ARN donde queda patente la actividad real de la comunidad, esta evalúa solo la posible funcionalidad de la misma (Li et al., 2018).

Por ello, últimamente también han cobrado especial trascendencia otros métodos como la secuenciación del metatranscriptoma (‘*RNA seq’*), que técnicamente es similar a la secuenciación del metagenoma pero en este caso se realiza en ADN complementario procedente de la retrotranscripción de ARNm microbiano. Esta tecnología permite conocer la parte activa y el tipo de actividad que ejercen las poblaciones microbianas del rumen (Kim et al., 2017) en cada momento. Cabe mencionar, no obstante, que los niveles

de expresión del ARNm no siempre están relacionados con la presencia y actividad de las proteínas, debido a la regulación postranscripcional y a las modificaciones postraduccionales que puedan ocurrir (Maier et al., 2009). Así, el estudio del proteoma o el metaboloma para describir el contenido total de proteínas o metabolitos del ecosistema ruminal (Deusch et al., 2015), respectivamente, podría complementar la información obtenida con los métodos anteriores. No obstante, estos dos últimos enfoques han sido, de momento, poco empleados para el estudio del rumen.

5. Justificación y objetivos.

Trabajos previos muestran que el rumen comienza a ser colonizado en las primeras horas de vida, mucho antes de que comience la ingesta de alimento sólido y que se alcanzan concentraciones microbianas similares a las del rumen adulto tras el primer mes de vida. Estos procesos de colonización se ven alterados de manera notable en sistemas de producción lechera, en los que las crías se separan de las madres tras nacer y carecen de contacto con otros animales adultos que les puedan servir de fuentes de colonización. En contraposición a la estabilidad y resistencia del ecosistema microbiano existente en el animal adulto, el animal pre-rumiante ofrece una ventana de tiempo con alta plasticidad durante el proceso primero de colonización tras el nacimiento y hasta que se instaura de manera definitiva el proceso de rumia y función ruminal (a los 2 meses de edad aproximadamente). Se ha comprobado que la manipulación de la población microbiana que coloniza el rumen desde el nacimiento puede jugar un papel clave en el desarrollo metabólico del animal y que potencialmente puede ejercer efectos que perduren en el animal adulto. Una de estas manipulaciones implica la inoculación de microbiota ruminal proveniente de animales adultos a rumiantes jóvenes. Sin embargo, aún se desconocen diversos aspectos como:

a) La naturaleza de la microbiota ruminal y las condiciones de conservación en las que esta puede tener mayor potencial para su utilización como inóculo en rumiantes jóvenes.

b) Los efectos de intervenciones microbiológicas (inoculación de microbiota ruminal) o a nivel de manejo (presencia de adultos) sobre el desarrollo del rumen y su persistencia en el tiempo.

c) Los mecanismos por los que el sistema inmunitario del animal hospedador, a través de vehículos como la saliva, permite el establecimiento de la microbiota ruminal comensal y su modulación.

En base a esto, los objetivos de este proyecto de tesis fueron los siguientes:

1) Evaluar la importancia de distintos factores (dieta del donante, el tiempo de muestreo, la fracción empleada, el sustrato de incubación y la conservación) para maximizar la actividad de la microbiota ruminal para aplicarla como inóculo en otros ensayos *in vitro* e *in vivo*.
2) Analizar si la inoculación directa de microbiota ruminal en cabritos en edad temprana tiene efectos sobre el desarrollo microbiológico y fisiológico del rumen.
3) Analizar si la presencia de animales adultos y la posible transmisión de microbiota tiene efectos sobre el desarrollo microbiológico y fisiológico del rumen de cabritos en edad temprana.
4) Evaluar el papel de la saliva del rumiante y sus componentes bioactivos como regulador del establecimiento de las distintas poblaciones microbianas del rumen y su actividad en función de la fracción empleada o el animal donante.

6. Referencias.

Abdelfattah, E. M., Karousa, M. M., Lay, D. C., Marchant-Forde, J. N., & Eicher, S. D. (2018). Short communication: Effect of age at group housing on behavior, cortisol, health, and leukocyte differential counts of neonatal bull dairy calves. Journal of Dairy Science, 101(1), 596–602. https://doi.org/10.3168/jds.2017-12632

Abecia, L., Martín-García, A. I., Martínez, G., Newbold, C. J., & Yáñez-Ruiz, D. R. (2013). Nutritional intervention in early life to manipulate rumen microbial colonization and methane output by kid goats postweaning. Journal of Animal Science, 91(10), 4832–4840. https://doi.org/10.2527/jas2012-6142

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., & Yáñez-Ruiz, D. R. (2014). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Animal Production Science, 54(9), 1449–1454. https://doi.org/10.1071/AN14337

Abecia, L., Jiménez, E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., Denman, S. E., Newbold, C. J., & Yáñez-Ruiz, D. R. (2017). Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. PLoS ONE, 12(8), 1–19. https://doi.org/10.1371/journal.pone.0182235

Abo-Donia, F. M., Ghada, S. I., Nadi, S., & Sayah, M. S. (2011). Effect of Inoculating New Born Lambs with Fresh or Lyophilized Rumen Fluid on Rumen Activity and Lamb Performance. Journal of American Science, 7(9), 409–422. http://www.americanscience.orghttp//www.americanscience.org.

Adams, M. C., Luo, J., Rayward, D., King, S., Gibson, R., & Moghaddam, G. H. (2008). Selection of a novel direct-fed microbial to enhance weight gain in intensively reared calves. Animal Feed Science and Technology, 145(1–4), 41–52. https://doi.org/10.1016/j.anifeedsci.2007.05.035

Alipour, M. J., Jalanka, J., Pessa-Morikawa, T., Kokkonen, T., Satokari, R., Hynönen, U., Iivanainen, A., & Niku, M. (2018). The composition of the perinatal intestinal microbiota in cattle. Scientific Reports, 8(1), 1–14. https://doi.org/10.1038/s41598-018-28733-y

An, B. K., Cho, B. L., You, S. J., Paik, H. D., Chang, H. I., Kim, S. W., Yun, C. W., & Kang, C. W. (2008). Growth performance and antibody response of broiler chicks fed yeast derived β-glucan and single-strain probiotics. Asian-Australasian Journal of Animal Sciences, 21(7), 1027–1032. https://doi.org/10.5713/ajas.2008.70571

Ang, C. S., Binos, S., Knight, M. I., Moate, P. J., Cocks, B. G., & McDonagh, M. B. (2011). Global survey of the bovine salivary proteome: Integrating multidimensional prefractionation, targeted, and glycocapture strategies. Journal of Proteome Research, 10(11), 5059–5069. https://doi.org/10.1021/pr200516d

Apás, A. L., Dupraz, J., Ross, R., González, S. N., & Arena, M. E. (2010). Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. Journal of Bioscience and Bioengineering, 110(5), 537–540. https://doi.org/10.1016/j.jbiosc.2010.06.005

Apper-Bossard, E., Faverdin, P., Meschy, F., & Peyraud, J. L. (2010). Effects of dietary cation-anion difference on ruminal metabolism and blood acid-base regulation in dairy cows receiving 2 contrasting levels of concentrate in diets. Journal of Dairy Science, 93(9), 4196–4210. https://doi.org/10.3168/jds.2009-2975

Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E., & Finlay, B. (2014). The intestinal microbiome in early life: Health and disease. Frontiers in Immunology, 5(AUG), 1–18. https://doi.org/10.3389/fimmu.2014.00427

Aschenbach, J. R., Penner, G. B., Stumpff, F., & Gäbel, G. (2011). Ruminant nutrition symposium: Role of fermentation acid absorption in the regulation of ruminal pH. Journal of Animal Science, 89(4), 1092–1107. https://doi.org/10.2527/jas.2010-3301

Asea, A. (2005). Stress proteins and initiation of immune response: Chaperokine activity of Hsp72. Exercise Immunology Review, 11(254), 34–45.

Bailey, C. B. (1961). Saliva secretion and its relation to feeding in cattle. British Journal of Nutrition, 15(4), 489–498. https://doi.org/10.1079/bjn19610062

Baldwin VI, R. L., McLeod, K. R., Klotz, J. L., & Heitmann, R. N. (2004). Rumen Development, Intestinal Growth and Hepatic Metabolism In The Pre- and Postweaning Ruminant. J. Dairy Sci., 87(13_suppl), E55-65. https://doi.org/10.3168/jds.S0022-0302(04)70061-2

Baldwin VI, R. L., & Jesse, B. W. (1992). Developmental changes in glucose and butyrate metabolism by isolated sheep ruminal cells. Journal of Nutrition, 122(5), 1149–1153. https://doi.org/10.1093/jn/122.5.1149

Baldwin VI, R. L., & McLeod, K. R. (2000). Effects of diet forage:Concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. Journal of Animal Science, 78(3), 771–783.

Baldwin, R. L., & Connor, E. E. (2017). Rumen Function and Development. Veterinary Clinics of North America - Food Animal Practice, 33(3), 427–439. https://doi.org/10.1016/j.cvfa.2017.06.001

Becker, E. R., and Hsiung, T. S. (1929). The method by which ruminants acquire their fauna of infusoria, and remarks concerning experiments on the host-specificity of these protozoa. Proceedings of the National Academy of Sciences of the U.S.A., 15(8), 684–690. https://doi.org/10.1073/pnas.15.8.684

Belanche, A., Cooke, J., Jones, E., Worgan, H. J., & Newbold, C. J. (2018). Short- and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. Animal, 13(4), 740–749. https://doi.org/10.1017/S1751731118002100

Belanche, A., De la Fuente, G., Moorby, J. M., & Newbold, C. J. (2012). Bacterial protein degradation by different rumen protozoal groups. Journal of Animal Science, 90C(12), 4495–4504. https://doi.org/10.2527/jas.2012-5118

Belanche, A., Martín-García, A. I., & Yáñez-Ruiz, D. R. (2017). La importancia del desarrollo del rumen. Ganadería, 112, 68–73.

Belanche, A., de la Fuente, G., & Newbold, C. J. (2014). Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiology Ecology, 90(3), 663–677. https://doi.org/10.1111/1574-6941.12423

Belanche, A., De La Fuente, G., & Newbold, C. J. (2015). Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. FEMS Microbiology Ecology, 91(3). https://doi.org/10.1093/femsec/fiu026

Belanche, A., Kingston-Smith, A. H., Griffith, G. W., & Newbold, C. J. (2019). A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. Frontiers in Microbiology, 10(FEB). https://doi.org/10.3389/fmicb.2019.00122

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., & Newbold, C. J. (2019). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environmental Microbiology, 21(11), 4360–4377. https://doi.org/10.1111/1462-2920.14801

Benetton, J. B., Neave, H. W., Costa, J. H. C., von Keyserlingk, M. A. G., & Weary, D. M. (2019). Automatic weaning based on individual solid feed intake: Effects on behavior and performance of dairy calves. Journal of Dairy Science, 102(6), 5475–5491. https://doi.org/10.3168/jds.2018-15830

Béra-Maillet, C., Devillard, E., Cezette, M., Jouany, J. P., & Forano, E. (2005). Xylanases and carboxymethylcellulases of the rumen protozoa Polyplastron multivesiculatum,

Eudiplodinium maggii and Entodinium sp. FEMS Microbiology Letters, 244(1), 149–156. https://doi.org/10.1016/j.femsle.2005.01.035

Berg Miller, M. E., Yeoman, C. J., Chia, N., Tringe, S. G., Angly, F. E., Edwards, R. A., Flint, H. J., Lamed, R., Bayer, E. A., & White, B. A. (2012). Phage-bacteria relationships and CRISPR elements revealed by a metagenomic survey of the rumen microbiome. Environmental Microbiology, 14(1), 207–227. https://doi.org/10.1111/j.1462-2920.2011.02593.x

Bird, S. H., Hegarty, R. S., & Woodgate, R. (2010). Modes of transmission of rumen protozoa between mature sheep. Animal Production Science, 50(6), 414–417. https://doi.org/10.1071/AN09216

Brandtzaeg, P., Fjellanger, I., & Gjeruldsen, S. T. (1968). Adsorption of immunolgobulin A onto oral bacteria in vivo. Journal of Bacteriology, 96(1), 242–249. https://doi.org/10.1128/jb.96.1.242-249.1968

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nature Reviews Microbiology, 3(3), 238–250. https://doi.org/10.1038/nrmicro1098

Bryant, M. P., & Small, N. (1960). Observations on the Ruminal Microorganisms of Isolated and Inoculated Calves. Journal of Dairy Science, 43(5), 654–667. https://doi.org/10.3168/jds.S0022-0302(60)90216-2

Buntyn, J. O., Schmidt, T. B., Nisbet, D. J., & Callaway, T. R. (2016). The role of direct-fed microbials in conventional livestock production. Annual Review of Animal Biosciences, 4, 335–355. https://doi.org/10.1146/annurev-animal-022114-111123

Byrne, C. J., Fair, S., English, A. M., Johnston, D., Lonergan, P., & Kenny, D. A. (2017). Effect of milk replacer and concentrate intake on growth rate, feeding behaviour and systemic metabolite concentrations of pre-weaned bull calves of two dairy breeds. Animal, 11(9), 1531–1538. https://doi.org/10.1017/S1751731117000350

Camacho, A., Guitian, J., Pallas, E., Gestal, J., Olmeda, S., Goethert, H., Telford, S., & Spielman, A. (2005). Serum protein response and renal failure in canine Babesia annae infection. Veterinary Research, 36(5–6), 713–722. https://doi.org/10.1051/vetres

Cantalapiedra-Hijar, G., Abo-Ismail, M., Carstens, G. E., Guan, L. L., Hegarty, R., Kenny, D. A., Mcgee, M., Plastow, G., Relling, A., & Ortigues-Marty, I. (2018). Review: Biological determinants of between-animal variation in feed efficiency of growing beef cattle. Animal, 12(s2), S321–S335. https://doi.org/10.1017/S1751731118001489

Cantor, M. C., Stanton, A. L., Combs, D. K., & Costa, J. H. C. (2019). Effect of milk feeding strategy and lactic acid probiotics on growth and behavior of dairy calves fed using an automated feeding system. Journal of Animal Science, 97(3), 1052–1065. https://doi.org/10.1093/jas/skz034

Cao, G. T., Zeng, X. F., Chen, A. G., Zhou, L., Zhang, L., Xiao, Y. P., & Yang, C. M. (2013). Effects of a probiotic, Enterococcus faecium, on growth performance, intestinal morphology, immune response, and cecal microflora in broiler chickens

challenged with Escherichia coli K88. Poultry Science, 92, 2949–2955. https://doi.org/http://dx.doi.org/ 10.3382/ps.2013-03366

Carberry, C. A., Kenny, D. A., Han, S., McCabe, M. S., & Waters, S. M. (2012). Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Applied and Environmental Microbiology, 78(14), 4949–4958. https://doi.org/10.1128/AEM.07759-11

Carberry, C. A., Waters, S. M., Kenny, D. A., & Creevey, C. J. (2014). Rumen methanogenic genotypes differ in abundance according to host residual feed intake phenotype and diet type. Applied and Environmental Microbiology, 80(2), 586–594. https://doi.org/10.1128/AEM.03131-13

Carrero, J. C., Cervantes-Rebolledo, C., Aguilar-Díaz, H., Díaz-Gallardo, M. Y., Laclette, J. P., & Morales-Montor, J. (2007). The role of the secretory immune response in the infection by Entamoeba histolytica. Parasite Immunology, 29(7), 331–338. https://doi.org/10.1111/j.1365-3024.2007.00955.x

Castro, J. J., Gomez, A., White, B. A., Mangian, H. J., Loften, J. R., & Drackley, J. K. (2016). Changes in the intestinal bacterial community, short-chain fatty acid profile, and intestinal development of preweaned Holstein calves. 1. Effects of prebiotic supplementation depend on site and age. Journal of Dairy Science, 99(12), 9682–9702. https://doi.org/10.3168/jds.2016-11006

Chakravorty, S., Helb, D., Burday, M., Connell, N., & Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. Journal of Microbiological Methods, 69(2), 330–339. https://doi.org/10.1016/j.mimet.2007.02.005

Chang, Z. L. (2010). Important aspects of Toll-like receptors, ligands and their signaling pathways. Inflammation Research, 59(10), 791–808. https://doi.org/10.1007/s00011-010-0208-2

Chaucheyras-Durand, F., Walker, N. D., & Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. Animal Feed Science and Technology, 145(1–4), 5–26. https://doi.org/10.1016/j.anifeedsci.2007.04.019

Chen, Y., Oba, M., & Guan, L. L. (2012). Variation of bacterial communities and expression of Toll-like receptor genes in the rumen of steers differing in susceptibility to subacute ruminal acidosis. Veterinary Microbiology, 159(3–4), 451–459. https://doi.org/10.1016/j.vetmic.2012.04.032

Choi, S., Baik, J. E., Jeon, J. H., Cho, K., Seo, D. G., Kum, K. Y., Yun, C. H., & Han, S. H. (2011). Identification of Porphyromonas gingivalis lipopolysaccharide-binding proteins in human saliva. Molecular Immunology, 48(15–16), 2207–2213. https://doi.org/10.1016/j.molimm.2011.06.434

Choudhury, P. K., Salem, A. Z. M., Jena, R., Kumar, S., Singh, R., & Puniya, A. K. (2015). Rumen Microbiology: An Overview. In A. K. Puniya, R. Singh, & D. N. Kamra (Eds.), Rumen Microbiology: From Evolution to Revolution (pp. 1–379). Springer India. https://doi.org/10.1007/978-81-322-2401-3

Collado, M. C., Cernada, M., Bäuerl, C., Vento, M., & Pérez-martínez, G. (2012). Microbial ecology and host-microbiota interactions during early life stages © 2012 Landes Bioscience . Do not distribute © 2012 Landes Bioscience . Do not distribute. Gut Microbes, 3(4), 352–365.

Collins, B. (2003). Ground layer competition and herbivory effects on cherrybark oak (Quercus pagoda Raf.) regeneration in experimental canopy gaps. Journal of the Torrey Botanical Society, 130(3), 147–157. https://doi.org/10.2307/3557550

Connor, E. E., Baldwin, R. L., Li, C. J., Li, R. W., & Chung, H. (2013). Gene expression in bovine rumen epithelium during weaning identifies molecular regulators of rumen development and growth. Functional and Integrative Genomics, 13(1), 133–142. https://doi.org/10.1007/s10142-012-0308-x

Costa, J. H. C., von Keyserlingk, M. A. G., & Weary, D. M. (2016). Invited review: Effects of group housing of dairy calves on behavior, cognition, performance, and health. Journal of Dairy Science, 99(4), 2453–2467. https://doi.org/10.3168/jds.2015-10144

Creevey, C. J., Kelly, W. J., Henderson, G., & Leahy, S. C. (2014). Determining the culturability of the rumen bacterial microbiome. Microbial Biotechnology, 7(5), 467–479. https://doi.org/10.1111/1751-7915.12141

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., Klieve, A. V., & Ouwerkerk, D. (2015). Programming rumen bacterial communities in newborn Merino lambs. Small Ruminant Research, 129, 48–59. https://doi.org/10.1016/j.smallrumres.2015.05.015

De Barbieri, I., Hegarty, R. S., Silveira, C., & Oddy, V. H. (2015). Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. Small Ruminant Research, 129, 37–47. https://doi.org/10.1016/j.smallrumres.2015.05.017

De Paula Vieira, a, von Keyserlingk, M. a G., & Weary, D. M. (2012). Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before and after weaning from milk. Journal of Dairy Science, 95(6), 3218–3224. https://doi.org/10.3168/jds.2011-4821

Dehority, B. A., & Grubb, J. A. (1981). Bacterial Population Adherent to the Epithelium on the Roo of the Dorsal Rumen of Sheep. Applied and Environmental Microbiology, 41(6), 1424–1427. https://doi.org/10.1128/aem.41.6.1424-1427.1981

Demeyer, D. I., & Van Nevel, C. J. (1979). Effect of defaunation on the metabolism of rumen micro-organisms. British Journal of Nutrition, 42(3), 515–524. https://doi.org/10.1079/bjn19790143

Denek, N., Can, A., & Avci, M. (2010). Frozen rumen fluid as microbial inoculum in the two-stage in vitro digestibility assay of ruminant feeds. South African Journal of Animal Sciences, 40(3), 251–256. https://doi.org/10.4314/sajas.v40i3.9

Denman, S. E., & McSweeney, C. S. (2015). The early impact of genomics and metagenomics on ruminal microbiology. Annual Review of Animal Biosciences,

3(September 2014), 447–465. https://doi.org/10.1146/annurev-animal-022114-110705

Denman, S. E., Tomkins, N. W., & McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiology Ecology, 62(3), 313–322. https://doi.org/10.1111/j.1574-6941.2007.00394.x

DePeters, E. J., & George, L. W. (2014). Rumen transfaunation. Immunology Letters, 162(2), 69–76. https://doi.org/10.1016/j.imlet.2014.05.009

Desnoyers, M., Giger-Reverdin, S., Bertin, G., Duvaux-Ponter, C., & Sauvant, D. (2009). Meta-analysis of the influence of Saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants. Journal of Dairy Science, 92(4), 1620–1632. https://doi.org/10.3168/jds.2008-1414

Deusch, S., Tilocca, B., Camarinha-Silva, A., & Seifert, J. (2015). News in livestock research - Use of Omics-technologies to study the microbiota in the gastrointestinal tract of farm animals. Computational and Structural Biotechnology Journal, 13, 55–63. https://doi.org/10.1016/j.csbj.2014.12.005

Devyatkin, V., Mishurov, A., & Kolodina, E. (2021). Probiotic effect of Bacillus subtilis B-2998D, B-3057D, and Bacillus licheniformis B-2999D complex on sheep and lambs. Journal of Advanced Veterinary and Animal Research, 8(1), 146–157. https://doi.org/10.5455/javar.2021.h497

Dommett, R., Zilbauer, M., George, J. T., & Bajaj-Elliott, M. (2005). Innate immune defence in the human gastrointestinal tract. Molecular Immunology, 42(8), 903–912. https://doi.org/10.1016/j.molimm.2004.12.004

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W. C., Conner, M. E., Earl, A. M., Knight, R., Bjorkman, P. J., & Mazmanian, S. K. (2018). Gut microbiota utilize immunoglobulin a for mucosal colonization. Science, 360(6390), 795–800. https://doi.org/10.1126/science.aaq0926

Donnet-Hughes, A., Duc, N., Serrant, P., Vidal, K., & Schiffrin, E. J. (2000). Bioactive molecules in milk and their role in health and disease: The role of transforming growth factor-β. Immunology and Cell Biology, 78(1), 74–79. https://doi.org/10.1046/j.1440-1711.2000.00882.x

Donohoe, D. R., Garge, N., Zhang, X., Sun, W., O'Connell, T. M., Bunger, M. K., & Bultman, S. J. (2011). Microbiome, The Regulate, Butyrate Metabolism, Energy. Cell Metabolism, 13(5), 517–526. https://doi.org/10.1016/j.cmet.2011.02.018.The

Duric, M., Zhao, G., Orskov, E., & Chen, X. (1994). Indirect measurement of saliva secretion in sheep fed diets of different structures and the effect of such diets on ruminal fluid kinetics and fermentation pattern. Experimental Physiology, 79(5), 823–830. https://doi.org/10.1113/expphysiol.1994.sp003810

Durunna, O. N., Wang, Z., Basarab, J. A., Okine, E. K., & Moore, S. S. (2011). Phenotypic and genetic relationships among feeding behavior traits, feed intake, and

residual feed intake in steers fed grower and finisher diets. Journal of Animal Science, 89(11), 3401–3409. https://doi.org/10.2527/jas.2011-3867

Eadie, J. M. (1962). The Development of Rumen Microbial Populations in Lambs and Calves Under Various Conditions of Management. Journal of General Microbiology, 29(4), 563–578. https://doi.org/10.1099/00221287-29-4-563

Eckmann, L., Morzycka-Wroblewska, E., Smith, J. R., & Kagnoff, M. F. (1992). Cytokine-induced differentiation of IgA B cells: studies using an IgA expressing B-cell lymphoma. Immunology, 76(2), 235–241. http://www.ncbi.nlm.nih.gov/pubmed/1634247%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1421533

Edwards, J. E., McEwan, N. R., Travis, A. J., & Wallace, R. J. (2004). 16S rDNA library-based analysis of ruminal bacterial diversity. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology, 86(3), 263–281. https://doi.org/10.1023/B:ANTO.0000047942.69033.24

El-Raof, A. Y. M., Ghanem, M. M., & Galbat, S. (2007). Cryopreservation of rumen protozoa using three different cryoprotectants with successful refaunation of defaunated sheep. Proceeding of the Second Scientific Onference, January, 314–332.

Escribano, D., Horvatić, A., Contreras-Aguilar, M. D., Guillemin, N., Cerón, J. J., Lopez-Arjona, M., Hevia, M. L., Eckersall, P. D., Manteca, X., & Mrljak, V. (2019). Identification of possible new salivary biomarkers of stress in sheep using a high-resolution quantitative proteomic technique. Research in Veterinary Science, 124(March), 338–345. https://doi.org/10.1016/j.rvsc.2019.04.012

Eugène, M., Archimède, H., & Sauvant, D. (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. https://doi.org/10.1016/S0301-6226(03)00117-9

Fábián, T. K., & Fejérdy, P. (2008). Saliva in Health and Disease, Chemical Biology of. Wiley Encyclopedia of Chemical Biology, 1. https://doi.org/10.1002/9780470048672.wecb643

Fábián, T. K., Gáspár, J., Fejérdy, L., Kaán, B., Bálint, M., Csermely, P., & Fejérdy, P. (2003). Hsp70 is present in human saliva. Medical Science Monitor, 9(1), 62–66.

Fábián, T. K., Hermann, P., Beck, A., Fejérdy, P., & Fábián, G. (2012). Salivary defense proteins: Their network and role in innate and acquired oral immunity. International Journal of Molecular Sciences, 13(4), 4295–4320. https://doi.org/10.3390/ijms13044295

Falk, P. G., Hooper, L. V., Midtvedt, T., & Gordon, J. I. (1998). Creating and Maintaining the Gastrointestinal Ecosystem: What We Know and Need To Know from Gnotobiology. Microbiology and Molecular Biology Reviews, 62(4), 1157–1170. https://doi.org/10.1128/mmbr.62.4.1157-1170.1998

Famulener, L. W. (1912). On the transmission of immunity from mother to offspring. a study upon serum hemolysins in goats. Journal of Infectious Diseases, 10(3), 332–368. https://doi.org/10.1093/infdis/10.3.332

Faubladier, C., Julliand, V., Danel, J., & Philippeau, C. (2013). Bacterial carbohydrate-degrading capacity in foal faeces: Changes from birth to pre-weaning and the impact of maternal supplementation with fermented feed products. British Journal of Nutrition, 110(6), 1040–1052. https://doi.org/10.1017/S0007114512006162

Fernández, M. (2012). Cría y recría de terneras (y II). Mundo Ganadero, 244(y II), 42–45.

Fleshner, M., & Campisi, J. (2003). Stress-Induced Extracellular Hsp72 Is a Functionally Significant Danger Signal To the Immune System. Medicine & Science in Sports & Exercise, 35(Supplement 1), S9. https://doi.org/10.1097/00005768-200305001-00040

Fonty, G., Gouet, P., Jouany, J.-P., & Senaud, J. (1987). Establishment of the Microflora and Anaerobic Fungi in the Rumen of Lambs. Microbiology, 133(7), 1835–1843. https://doi.org/10.1099/00221287-133-7-1835

Fonty, G., Joblin, K., Chavarot, M., Roux, R., Naylor, G., & Michallon, F. (2007). Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. Applied and Environmental Microbiology, 73(20), 6391–6403. https://doi.org/10.1128/AEM.00181-07

Fouhse, J. M., Smiegielski, L., Tuplin, M., Guan, L. L., & Willing, B. P. (2017). Host immune selection of rumen bacteria through salivary secretory IgA. Frontiers in Microbiology, 8(MAY), 1–9. https://doi.org/10.3389/fmicb.2017.00848

Fouts, D. E., Szpakowski, S., Purushe, J., Torralba, M., Waterman, R. C., MacNeil, M. D., Alexander, L. J., & Nelson, K. E. (2012). Next Generation Sequencing to Define Prokaryotic and Fungal Diversity in the Bovine Rumen. PLoS ONE, 7(11). https://doi.org/10.1371/journal.pone.0048289

Frizzo, L. S., Zbrun, M. V., Soto, L. P., & Signorini, M. L. (2011). Effects of probiotics on growth performance in young calves: A meta-analysis of randomized controlled trials. Animal Feed Science and Technology, 169(3–4), 147–156. https://doi.org/10.1016/j.anifeedsci.2011.06.009

Gilbert, R. A., Kelly, W. J., Altermann, E., Leahy, S. C., Minchin, C., Ouwerkerk, D., & Klieve, A. V. (2017). Toward understanding phage: Host interactions in the rumen; complete genome sequences of lytic phages infecting rumen bacteria. Frontiers in Microbiology, 8(DEC), 1–17. https://doi.org/10.3389/fmicb.2017.02340

Gilbert, R. A., Townsend, E. M., Crew, K. S., Hitch, T. C. A., Friedersdorff, J. C. A., Creevey, C. J., Pope, P. B., Ouwerkerk, D., & Jameson, E. (2020). Rumen Virus Populations: Technological Advances Enhancing Current Understanding. Frontiers in Microbiology, 11(March), 1–26. https://doi.org/10.3389/fmicb.2020.00450

Gilliland, R. L., Bush, L. J., & Friend, J. D. (1962). Relation of Ration Composition to Rumen Development in Early-Weaned Dairy Calves with Observations on Ruminal

Parakeratosis. Journal of Dairy Science, 45(10), 1211–1217. https://doi.org/10.3168/jds.S0022-0302(62)89598-8

Gorr, S. U. (2009). Antimicrobial peptides of the oral cavity. Periodontology 2000, 51(1), 152–180. https://doi.org/10.1111/j.1600-0757.2009.00310.x

Grasty, S., & FAO. (1999). Reducing enteric methane and livelihoods Win - Win opportunities for farmers. In Most (Vol. 14, Issue 2).

Grogan, J., McKnight, C. J., Troxler, R. F., & Oppenheim, F. G. (2001). Zinc and copper bind to unique sites of histatin 5. FEBS Letters, 491(1–2), 76–80. https://doi.org/10.1016/S0014-5793(01)02157-3

Guan, L. L., Nkrumah, J. D., Basarab, J. A., & Moore, S. S. (2008). Linkage of microbial ecology to phenotype: Correlation of rumen microbial ecology to cattle's feed efficiency. FEMS Microbiology Letters, 288(1), 85–91. https://doi.org/10.1111/j.1574-6968.2008.01343.x

Guan, Y., Ranoa, D. R. E., Jiang, S., Mutha, S. K., Li, X., Baudry, J., & Tapping, R. I. (2010). Human TLRs 10 and 1 Share Common Mechanisms of Innate Immune Sensing but Not Signaling. The Journal of Immunology, 184(9), 5094–5103. https://doi.org/10.4049/jimmunol.0901888

Guzman, C. E., Bereza-Malcolm, L. T., De Groef, B., & Franks, A. E. (2015). Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. PLoS ONE, 10(7), 1–11. https://doi.org/10.1371/journal.pone.0133048

Haigh, B. J., Stewart, K. W., Whelan, J. R. K., Barnett, M. P. G., Smolenski, G. A., & Wheeler, T. T. (2010). Alterations in the salivary proteome associated with periodontitis. Journal of Clinical Periodontology, 37(3), 241–247. https://doi.org/10.1111/j.1600-051X.2009.01525.x

Hamada, T., Maeda, S., & Kameoka, K. (1976). Factors Influencing Growth of Rumen, Liver, and Other Organs in Kids Weaned from Milk Replacers to Solid Foods. Journal of Dairy Science, 59(6), 1110–1118. https://doi.org/10.3168/jds.S0022-0302(76)84330-5

Hansen, I. S., Baeten, D. L. P., & den Dunnen, J. (2019). The inflammatory function of human IgA. Cellular and Molecular Life Sciences, 76(6), 1041–1055. https://doi.org/10.1007/s00018-018-2976-8

Harfoot, C. G. (1978). Anatomy, physiology and microbiology of the ruminant digestive tract. Progress in Lipid Research, 17(1), 1–19. https://doi.org/10.1016/0079-6832(78)90003-4

Harrer, H., Graves, R., Street, H., York, N., & Putnam, J. P. T. (2002). S Even Y Ears in T Ibet. 485–508.

Hegarty, R. S., Goopy, J. P., Herd, R. M., & McCorkell, B. (2007). Cattle selected for lower residual feed intake have reduced daily methane production. Journal of Animal Science, 85(6), 1479–1486. https://doi.org/10.2527/jas.2006-236

Heinrichs, A. J., Heinrichs, B. S., Harel, O., Rogers, G. W., & Place, N. T. (2005). A prospective study of calf factors affecting age, body size, and body condition score at first calving of holstein dairy heifers. Journal of Dairy Science, 88(8), 2828–2835. https://doi.org/10.3168/jds.S0022-0302(05)72963-5

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P. H., Abecia, L., Angarita, E., Aravena, P., Arenas, G. N., Ariza, C., Attwood, G. T., Avila, J. M., Avila-Stagno, J., Bannink, A., Barahona, R., Batistotti, M., Bertelsen, M. F., Brown-Kav, A., … Zunino, P. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Scientific Reports, 5(September). https://doi.org/10.1038/srep14567

Hernández-González, J. C., Martínez-Tapia, A., Lazcano-Hernández, G., García-Pérez, B. E., & Castrejón-Jiménez, N. S. (2021). Bacteriocins from lactic acid bacteria. A powerful alternative as antimicrobials, probiotics, and immunomodulators in veterinary medicine. Animals, 11(4). https://doi.org/10.3390/ani11040979

Hernandez-Sanabria, E., Goonewardene, L. A., Wang, Z., Durunna, O. N., Moore, S. S., & Guan, L. L. (2012). Impact of feed efficiency and diet on adaptive variations in the bacterial community in the rumen fluid of cattle. Applied and Environmental Microbiology, 78(4), 1203–1214. https://doi.org/10.1128/AEM.05114-11

Hervás, G., Frutos, P., Javier Giráldez, F., Mora, M. J., Fernández, B., & Mantecón, Á. R. (2005). Effect of preservation on fermentative activity of rumen fluid inoculum for in vitro gas production techniques. Animal Feed Science and Technology, 123-124 Pa, 107–118. https://doi.org/10.1016/j.anifeedsci.2005.05.004

Hitch, T. C. A., Edwards, J. E., & Gilbert, R. A. (2019). Metatranscriptomics reveals mycoviral populations in the ovine rumen. FEMS Microbiology Letters, 366(13), 1–8. https://doi.org/10.1093/femsle/fnz161

Hobson, P. N., & Stewart, C. S. (1997). The Rumen Microbial Ecosystem. In The Rumen Microbial Ecosystem. https://doi.org/10.1007/978-94-009-1453-7

Hötzel, M. J., Longo, C., Balcão, L. F., Cardoso, C. S., & Costa, J. H. C. (2014). A survey of management practices that influence performance and welfare of dairy calves reared in southern Brazil. PLoS ONE, 9(12), 1–17. https://doi.org/10.1371/journal.pone.0114995

Hristov, A. N., Callaway, T. R., Lee, C., & Dowd, S. E. (2012). Rumen bacterial, archaeal, and fungal diversity of dairy cows in response to ingestion of lauric or myristic acid. Journal of Animal Science, 90(12), 4449–4457. https://doi.org/10.2527/jas.2011-4624

Hung, A. T., Lin, S. Y., Yang, T. Y., Chou, C. K., Liu, H. C., Lu, J. J., Wang, B., Chen, S. Y., & Lien, T. F. (2012). Effects of Bacillus coagulans ATCC 7050 on growth performance, intestinal morphology, and microflora composition in broiler chickens. Animal Production Science, 52(9), 874–879. https://doi.org/10.1071/AN11332

Hungate, R. E. (1969). Chapter IV A Roll Tube Method for Cultivation of Strict Anaerobes. Methods in Microbiology, 3(PART B), 117–132. https://doi.org/10.1016/S0580-9517(08)70503-8

Ibrahim, H. R., Thomas, U., & Pellegrini, A. (2001). A Helix-Loop-Helix Peptide at the Upper Lip of the Active Site Cleft of Lysozyme Confers Potent Antimicrobial Activity with Membrane Permeabilization Action. Journal of Biological Chemistry, 276(47), 43767–43774. https://doi.org/10.1074/jbc.M106317200

IPCC. (2019). 2019 Refinement to the 2006 IPCC guidelines for national greenhouse gas inventories. In Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Islam, M. M., Fernando, S. C., & Saha, R. (2019). Metabolic modeling elucidates the transactions in the rumen microbiome and the shifts upon virome interactions. Frontiers in Microbiology, 10(OCT), 1–17. https://doi.org/10.3389/fmicb.2019.02412

Izuddin, W. I., Loh, T. C., Foo, H. L., Samsudin, A. A., & Humam, A. M. (2019). Postbiotic L. plantarum RG14 improves ruminal epithelium growth, immune status and upregulates the intestinal barrier function in post-weaning lambs. Scientific Reports, 9(1), 1–10. https://doi.org/10.1038/s41598-019-46076-0

Jami, E., Israel, A., Kotser, A., & Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME Journal, 7(6), 1069–1079. https://doi.org/10.1038/ismej.2013.2

Jami, E., & Mizrahi, I. (2012). Composition and similarity of bovine rumen microbiota across individual animals. PLoS ONE, 7(3), 1–8. https://doi.org/10.1371/journal.pone.0033306

Janssen, P. H., & Kirs, M. (2008). Structure of the archaeal community of the rumen. Applied and Environmental Microbiology, 74(12), 3619–3625. https://doi.org/10.1128/AEM.02812-07

Jensen, M. B., Duve, L. R., & Weary, D. M. (2015). Pair housing and enhanced milk allowance increase play behavior and improve performance in dairy calves. Journal of Dairy Science, 98(4), 2568–2575. https://doi.org/10.3168/jds.2014-8272

Jiao, J., Huang, J., Zhou, C., & Tan, Z. (2015). Taxonomic identification of ruminal epithelial bacterial diversity during rumen development in goats. Applied and Environmental Microbiology, 81(10), 3502–3509. https://doi.org/10.1128/AEM.00203-15

Kamra, D. N. (2005). Rumen microbial ecosystem. Current Science, 89(1), 123–135.

Keller, M. A., Kidd, R. M., Bryson, Y. J., Turner, J. L., & Carter, J. (1981). Lymphokine production by human milk lymphocytes. Infection and Immunity, 32(2), 632–636. https://doi.org/10.1111/j.1600-0765.1986.tb01468.x

Khan, M. A., Bach, A., Weary, D. M., & von Keyserlingk, M. A. G. (2016). Invited review: Transitioning from milk to solid feed in dairy heifers. Journal of Dairy Science, 99(2), 885–902. https://doi.org/10.3168/jds.2015-9975

Khan, M. A., Weary, D. M., & Von Keyserlingk, M. A. G. (2011). Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. Journal of Dairy Science, 94(3), 1071–1081. https://doi.org/10.3168/jds.2010-3733

Kim, M., Morrison, M., & Yu, Z. (2011). Status of the phylogenetic diversity census of ruminal microbiomes. FEMS Microbiology Ecology, 76(1), 49–63. https://doi.org/10.1111/j.1574-6941.2010.01029.x

Kim, M., Park, T., & Yu, Z. (2017). Metagenomic investigation of gastrointestinal microbiome in cattle. Asian-Australasian Journal of Animal Sciences, 30(11), 1515–1528. https://doi.org/10.5713/ajas.17.0544

Kittelmann, S., Seedorf, H., Walters, W. A., Clemente, J. C., Knight, R., Gordon, J. I., & Janssen, P. H. (2013). Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities. PLoS ONE, 8(2). https://doi.org/10.1371/journal.pone.0047879

Klieve, A. V., & Swain, R. A. (1993). Estimation of ruminal bacteriophage numbers by pulsed-field gel electrophoresis and laser densitometry. Applied and Environmental Microbiology, 59(7), 2299–2303. https://doi.org/10.1128/aem.59.7.2299-2303.1993

Knauer, W. A., Godden, S. M., Dietrich, A., & James, R. E. (2017). The association between daily average feeding behaviors and morbidity in automatically fed group-housed preweaned dairy calves. Journal of Dairy Science, 100(7), 5642–5652. https://doi.org/10.3168/jds.2016-12372

Koenig, K. M., Newbold, C. J., McIntosh, F. M., & Rode, L. M. (2000). Effects of protozoa on bacterial nitrogen recycling in the rumen. Journal of Animal Science, 78(9), 2431–2445. https://doi.org/10.2527/2000.7892431x

Krause, K. M., & Oetzel, G. R. (2006). Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Animal Feed Science and Technology, 126(3–4), 215–236. https://doi.org/10.1016/j.anifeedsci.2005.08.004

Kruger Ben Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., & Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME Journal, 10(12), 2958–2972. https://doi.org/10.1038/ismej.2016.62

Kuhn, K. A., & Stappenbeck, T. S. (2013). Peripheral education of th eimmune system by the colonic microbiota. Seminars in Immunology, 25(5), 364–369. https://doi.org/10.1016/j.smim.2013.10.002.Peripheral

Kung, L., Demarco, S., Siebenson, L. N., Joyner, E., Haenlein, G. F. W., & Morris, R. M. (1997). An Evaluation of Two Management Systems for Rearing Calves Fed Milk Replacer. Journal of Dairy Science, 80(10), 2529–2533. https://doi.org/10.3168/jds.S0022-0302(97)76206-4

Lamy, E., da Costa, G., Santos, R., Capela e Silva, F., Potes, J., Pereira, A., Coelho, A. V., & Sales Baptista, E. (2009). Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research? Physiology and Behavior, 98(4), 393–401. https://doi.org/10.1016/j.physbeh.2009.07.002

Lane, M. A., Baldwin VI, R. L., & Jesse, B. W. (2002). Developmental changes in ketogenic enzyme gene expression during sheep rumen development. Journal of Animal Science, 80(6), 1538–1544. https://doi.org/10.2527/2002.8061538x

Lane, M. A., & Jesse, B. W. (1997). Effect of Volatile Fatty Acid Infusion on Development of the Rumen Epithelium in Neonatal Sheep. Journal of Dairy Science, 80(4), 740–746. https://doi.org/10.3168/jds.S0022-0302(97)75993-9

Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Vega Thurber, R. L., Knight, R., Beiko, R. G., & Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nature Biotechnology, 31(9), 814–821. https://doi.org/10.1038/nbt.2676

Lascelles, A. K. (1979). The Immune System of the Ruminant Mammary Gland And Its Role in the Control of Mastitis. Journal of Dairy Science, 62(1), 154–160. https://doi.org/10.3168/jds.S0022-0302(79)83216-6

Lascelles, A. K., & McDowell, G. H. (1974). Localized Humoral Immunity with Particular Reference to Ruminants. Immunological Reviews, 19(1), 170–208. https://doi.org/10.1111/j.1600-065X.1974.tb00132.x

Lesmeister, K. E., Heinrichs, A. J., & Gabler, M. T. (2004). Effects of supplemental yeast (Saccharomyces cerevisiae) culture on rumen development, growth characteristics, and blood parameters in neonatal dairy calves. Journal of Dairy Science, 87(6), 1832–1839. https://doi.org/10.3168/jds.S0022-0302(04)73340-8

Lessner, D. J. (2009). Methanogenesis Biochemistry. Encyclopedia of Life Sciences, 1–11. https://doi.org/10.1002/9780470015902.a0000573.pub2

Li, F., & Guan, L. L. (2017). Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. Applied and Environmental Microbiology, 83(9), 1–16. https://doi.org/10.1128/AEM.00061-17

Li, F., Neves, A. L. A., Ghoshal, B., & Guan, L. L. (2018). Symposium review: Mining metagenomic and metatranscriptomic data for clues about microbial metabolic functions in ruminants1. Journal of Dairy Science, 101(6), 5605–5618. https://doi.org/10.3168/jds.2017-13356

Li, R. W., Connor, E. E., Li, C., Baldwin Vi, R. L., & Sparks, M. E. (2012). Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environmental Microbiology, 14(1), 129–139. https://doi.org/10.1111/j.1462-2920.2011.02543.x

Li, W., Edwards, A., Riehle, C., Cox, M., Raabis, S., Skarlupka, J., Steinberger, A., Walling, J., Bickhart, D., & Suen, G. (2019). Transcriptomics analysis of host liver and meta-transcriptome analysis of rumen epimural microbial community in young calves treated with artificial dosing of rumen content from adult donor cow. Scientific Reports, 9(1), 1–11. https://doi.org/10.1038/s41598-018-37033-4

Liang, G., Malmuthuge, N., McFadden, T. B., Bao, H., Griebel, P. J., Stothard, P., & Guan, L. L. (2014). Potential regulatory role of microRNAs in the development of

bovine gastrointestinal tract during early life. PLoS ONE, 9(3), 1–13. https://doi.org/10.1371/journal.pone.0092592

Liang, Y., Carroll, J. A., & Ballou, M. A. (2016). The digestive system of 1-week-old Jersey calves is well suited to digest, absorb, and incorporate protein and energy into tissue growth even when calves are fed a high plane of milk replacer. Journal of Dairy Science, 99(3), 1929–1937. https://doi.org/10.3168/jds.2015-9895

Lima, J., Auffret, M. D., Stewart, R. D., Dewhurst, R. J., Duthie, C. A., Snelling, T. J., Walker, A. W., Freeman, T. C., Watson, M., & Roehe, R. (2019). Identification of rumen microbial genes involved in pathways linked to appetite, growth, and feed conversion efficiency in cattle. Frontiers in Genetics, 10(JUL), 1–18. https://doi.org/10.3389/fgene.2019.00701

Liu, J. hua, Zhang, M. ling, Zhang, R. yang, Zhu, W. yun, & Mao, S. yong. (2016). Comparative studies of the composition of bacterial microbiota associated with the ruminal content, ruminal epithelium and in the faeces of lactating dairy cows. Microbial Biotechnology, 9(2), 257–268. https://doi.org/10.1111/1751-7915.12345

Liu, S., Da Cunha, A. P., Rezende, R. M., Cialic, R., Wei, Z., Bry, L., Comstock, L. E., Gandhi, R., & Weiner, H. L. (2016). The Host Shapes the Gut Microbiota via Fecal MicroRNA. Cell Host and Microbe, 19(1), 32–43. https://doi.org/10.1016/j.chom.2015.12.005

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(18). https://doi.org/10.1093/nar/gkm541

Lu, C. D., & Potchoiba, M. J. (1988). Milk feeding and weaning of goat kids - A review. Small Ruminant Research, 1(2), 105–112. https://doi.org/10.1016/0921-4488(88)90025-9

Luo, C., Tsementzi, D., Kyrpides, N., Read, T., & Konstantinidis, K. T. (2012). Direct comparisons of Illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. PLoS ONE, 7(2). https://doi.org/10.1371/journal.pone.0030087

Maccari, P., Wiedemann, S., Kunz, H. J., Piechotta, M., Sanftleben, P., & Kaske, M. (2015). Effects of two different rearing protocols for Holstein bull calves in the first 3 weeks of life on health status, metabolism and subsequent performance. Journal of Animal Physiology and Animal Nutrition, 99(4), 737–746. https://doi.org/10.1111/jpn.12241

Mackie, R. I. (2002). Mutualistic fermentative digestion in the gastrointestinal tract: Diversity and evolution. Integrative and Comparative Biology, 42(2), 319–326. https://doi.org/10.1093/icb/42.2.319

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., & Takashiba, S. (2003). Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. FEMS

Immunology and Medical Microbiology, 39(1), 81–86. https://doi.org/10.1016/S0928-8244(03)00224-4

Maier, T., Güell, M., & Serrano, L. (2009). Correlation of mRNA and protein in complex biological samples. FEBS Letters, 583(24), 3966–3973. https://doi.org/10.1016/j.febslet.2009.10.036

Malmuthuge, N., Griebel, P. J., & Guan, L. L. (2014). Taxonomic identification of commensal bacteria associated with the mucosa and digesta throughout the gastrointestinal tracts of preweaned calves. Applied and Environmental Microbiology, 80(6), 2021–2028. https://doi.org/10.1128/AEM.03864-13

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P. J., Baurhoo, B., Zhao, X., & Guan, L. L. (2012). Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiology Ecology, 79(2), 337–347. https://doi.org/10.1111/j.1574-6941.2011.01220.x

Malmuthuge, N., Li, M., Fries, P., Griebel, P. J., & Guan, L. L. (2012). Regional and age dependent changes in gene expression of Toll-like receptors and key antimicrobial defence molecules throughout the gastrointestinal tract of dairy calves. Veterinary Immunology and Immunopathology, 146(1), 18–26. https://doi.org/10.1016/j.vetimm.2012.01.010

Mandel, R., & Nicol, C. J. (2017). Re-direction of maternal behaviour in dairy cows. Applied Animal Behaviour Science, 195(June 2018), 24–31. https://doi.org/10.1016/j.applanim.2017.06.001

Marounek, M., & Dušková, D. (1999). Metabolism of pectin in rumen bacteria Butyrivibrio fibrisolvens and Prevotella ruminicola. Letters in Applied Microbiology, 29(6), 429–433. https://doi.org/10.1046/j.1472-765X.1999.00671.x

McAllister, T. A., Bae, H. D., Jones, G. A., & Cheng, K. J. (1994). Microbial attachment and feed digestion in the rumen. Journal of Animal Science, 72(11), 3004–3018. https://doi.org/10.2527/1994.72113004x

McAllister, T. A., Beauchemin, K. A., Alazzeh, A. Y., Baah, J., Teather, R. M., & Stanford, K. (2011). Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Canadian Journal of Animal Science, 91(2), 193–211. https://doi.org/10.4141/cjas10047

Mcdougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal, 43, 99–109.

Mead, L. J., & Jones, G. A. (1981). Isolation and presumptive identification of adherent epithelial bacteria ("epimural" bacteria) from the ovine rumen wall. Applied and Environmental Microbiology, 41(4), 1020–1028. https://doi.org/10.1128/aem.41.4.1020-1028.1981

Meade, K. G., Cormican, P., Narciandi, F., Lloyd, A., & O'Farrelly, C. (2014). Bovine β-defensin gene family: Opportunities to improve animal health? Physiological Genomics, 46(1), 17–28. https://doi.org/10.1152/physiolgenomics.00085.2013

Miltko, R., Pietrzak, M., Bełz˙ecki, G., Wereszka, K., Michałowski, T., & Hackstein, J. H. P. (2015). Isolation and in vitro cultivation of the fibrolytic rumen ciliate Eremoplastron (Eudiplodinium) dilobum. European Journal of Protistology, 51(1), 109–117. https://doi.org/10.1016/j.ejop.2014.11.002

Morgavi, D. P., Jouany, J. P., & Martin, C. (2008). Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. Australian Journal of Experimental Agriculture, 48(1–2), 69–72. https://doi.org/10.1071/EA07236

Morgavi, D. P., Martin, C., Jouany, J. P., & Ranilla, M. J. (2012). Rumen protozoa and methanogenesis: Not a simple cause-effect relationship. British Journal of Nutrition, 107(3), 388–397. https://doi.org/10.1017/S0007114511002935

Morvan, B., Dore, J., Rieu-Lesme, F., Foucat, L., Fonty, G., & Gouet, P. (1994). Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. FEMS Microbiology Letters, 117(3), 249–256. https://doi.org/10.1111/j.1574-6968.1994.tb06775.x

Mountzouris, K. C., Balaskas, C., Xanthakos, I., Tzivinikou, A., & Fegeros, K. (2009). Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with Salmonella enteritidis. British Poultry Science, 50(4), 467–478. https://doi.org/10.1080/00071660903110935

Mueller, R. E., Asplund, J. M., & Iannotti, E. L. (1984). Successive changes in the epimural bacterial community of young lambs as revealed by scanning electron microscopy. Applied and Environmental Microbiology, 47(4), 715–723.

Nations, F. and A. O. of the U. (2005). Probiotics in animal nutrition. In FAO Animal Production and Health (Vol. 179). http://archive.nbuv.gov.ua/portal/Chem_Biol/TvUkr/2012_5/12oirpif.pdf

Newbold, C. J., Lassalas, B., & Jouany, J. P. (1995). The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. Letters in Applied Microbiology, 21(4), 230–234. https://doi.org/10.1111/j.1472-765X.1995.tb01048.x

Newbold, C. J., McEwan, N. R., Calza, R. E., Chareyron, E. N., Duval, S. M., Eschenlauer, S. C. P., McIntosh, F. M., Nelson, N., Travis, A. J., & Wallace, R. J. (2005). An NAD+-dependent glutamate dehydrogenase cloned from the ruminal ciliate protozoan, Entodinium caudatum. FEMS Microbiology Letters, 247(2), 113–121. https://doi.org/10.1016/j.femsle.2005.04.034

Newbold, C. J., De la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. Frontiers in Microbiology, 6(NOV), 1–14. https://doi.org/10.3389/fmicb.2015.01313

Ninomiya, S., & Sato, S. (2009). Effects of “five freedoms” environmental enrichment on the welfare of calves reared indoors. Animal Science Journal, 80(3), 347–351. https://doi.org/10.1111/j.1740-0929.2009.00627.x

O'Herrin, S. M., & Kenealy, W. R. (1993). Glucose and carbon dioxide metabolism by Succinivibrio dextrinosolvens. Applied and Environmental Microbiology, 59(3), 748–755. https://doi.org/10.1128/aem.59.3.748-755.1993

Orcutt, B. Y. M. L., & Howe, P. E. (1922). The relation between the accumulation of globulins and the appearance of agglutinins in the blood of new-born calves. The Journal of Experimental Medicine, 36(3), 291–308.

Orpin, C. G. (1974). The rumen flagellate Callimastix frontalis: does sequestration occur? Journal of General Microbiology, 84(2), 395–398. https://doi.org/10.1099/00221287-84-2-395

Orpin, C. K., & Joblin, K. N. (1997). The rumen anaerobic fungi. In P. N. Hobson & C. Stewart (Eds.), The rumen microbial ecosystem (Springer, pp. 140–195).

Ørskov, E. R., Benzie, D., & Kay, R. N. B. (1970). The effects of feeding procedure on closure of the oesophageal groove in young sheep. British Journal of Nutrition, 24(3), 785–795. https://doi.org/10.1079/bjn19700080

Parsons, S. D., Steele, M. A., Leslie, K. E., Renaud, D. L., & DeVries, T. J. (2020). Investigation of weaning strategy and solid feed location for dairy calves individually fed with an automated milk feeding system. Journal of Dairy Science, 103(7), 6533–6556. https://doi.org/10.3168/jds.2019-18023

Pedersen, R. E., Sørensen, J. T., Skjøth, F., Hindhede, J., & Nielsen, T. R. (2009). How milk-fed dairy calves perform in stable versus dynamic groups. Livestock Science, 121(2–3), 215–218. https://doi.org/10.1016/j.livsci.2008.06.007

Peña-Cearra, A., Belanche, A., Gonzalez-Lopez, M., Lavín, J. L., Pascual-Itoiz, M. Á., Jiménez, E., Rodríguez, H., Aransay, A. M., Anguita, J., Yáñez-Ruiz, D. R., & Abecia, L. (2021). Peripheral blood mononuclear cells (PBMC) microbiome is not affected by colon microbiota in healthy goats. Animal Microbiome, 3(1). https://doi.org/10.1186/s42523-021-00091-7

Phillips, C. J. C. (2004). The effects of forage provision and group size on the behavior of calves. Journal of Dairy Science, 87(5), 1380–1388. https://doi.org/10.3168/jds.S0022-0302(04)73287-7

Plaizier, J. C., Mesgaran, M. D., Derakhshani, H., Golder, H., Khafipour, E., Kleen, J. L., Lean, I., Loor, J., Penner, G., & Zebeli, Q. (2018). Review: Enhancing gastrointestinal health in dairy cows. Animal, 12(s2), S399–S418. https://doi.org/10.1017/S1751731118001921

Pramanik, R., Osailan, S. M., Challacombe, S. J., Urquhart, D., & Proctor, G. B. (2010). Protein and mucin retention on oral mucosal surfaces in dry mouth patients. European Journal of Oral Sciences, 118(3), 245–253. https://doi.org/10.1111/j.1600-0722.2010.00728.x

Prates, A., de Oliveira, J. A., Abecia, L., & Fondevila, M. (2010). Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation. Animal Feed Science and Technology, 155(2–4), 186–193. https://doi.org/10.1016/j.anifeedsci.2009.12.005

Prohászka, Z., Singh, M., Nagy, K., Kiss, E., Lakos, G., Duba, J., & Füst, G. (2002). Heat shock protein 70 is a potent activator of the human complement system. Cell Stress and Chaperones, 7(1), 17–22. https://doi.org/10.1379/1466-1268(2002)007<0017:HSPIAP>2.0.CO;2

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., & Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. Journal of Applied Microbiology, 116(2), 245–257. https://doi.org/10.1111/jam.12405

Rey, M., Enjalbert, F., & Monteils, V. (2012). Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. Journal of Dairy Science, 95(3), 1500–1512. https://doi.org/10.3168/jds.2011-4902

Reynolds, C. K., Dürst, B., Lupoli, B., Humphries, D. J., & Beever, D. E. (2004). Visceral Tissue Mass and Rumen Volume in Dairy Cows During the Transition from Late Gestation to Early Lactation. Journal of Dairy Science, 87(4), 961–971. https://doi.org/10.3168/jds.S0022-0302(04)73240-3

Rieu, F., Fonty, G., Gaillard, B., & GouetP. (1990). Electron Microscopy Study of the Bacteria Adherent to the Rumen Wall in Young Conventional Lambs. Canadian Journal of Microbiology, 36(2), 140–144.

Roehe, R., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., McKain, N., Ross, D. W., Hyslop, J. J., Waterhouse, A., Freeman, T. C., Watson, M., & Wallace, R. J. (2016). Bovine Host Genetic Variation Influences Rumen Microbial Methane Production with Best Selection Criterion for Low Methane Emitting and Efficiently Feed Converting Hosts Based on Metagenomic Gene Abundance. PLoS Genetics, 12(2), 1–20. https://doi.org/10.1371/journal.pgen.1005846

Rogers, J. D., Palmer, J., Kolenbrander, P. E., & Scannapieco, F. A. (2001). Role of Streptococcus gordonii amylase-binding protein A in adhesion to hydroxyapatite, starch metabolism, and biofilm formation. Infection and Immunity, 69(11), 7046–7056. https://doi.org/10.1128/IAI.69.11.7046-7056.2001

Sadet, S., Martin, C., Meunier, B., & Morgavi, D. P. (2007). PCR-DGGE analysis reveals a distinct diversity in the bacterial population attached to the rumen epithelium. Animal, 1(7), 939–944. https://doi.org/10.1017/S1751731107000304

Salse-Bernadó, P., & Salse-Bernadó, X. (2018). Manejo en una granja de producción de terneros mamones & cebo de terneros. Ruminews, 26–35.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the U.S.A., 74(12), 5463–5467. https://doi.org/10.1097/00006250-199004001-00013

Santra, A., & Jakhmola, R. C. (1998). Effect of defaunation on animal productivity. Journal of Applied Animal Research, 14(2), 103–116. https://doi.org/10.1080/09712119.1998.9706689

Scannapieco, F. A., Levine, M. J., & Torres, G. I. (1995). Salivary Amylase Promotes Adhesion of Oral Streptococci to Hydroxyapatite. Journal of Dental Research, 74(7), 1360–1366. https://doi.org/10.1177/00220345950740070701

Schäff, C. T., Gruse, J., Maciej, J., Pfuhl, R., Zitnan, R., Rajsky, M., & Hammon, H. M. (2018). Effects of feeding unlimited amounts of milk replacer for the first 5 weeks of age on rumen and small intestinal growth and development in dairy calves. Journal of Dairy Science, 101(1), 783–793. https://doi.org/10.3168/jds.2017-13247

Sharp, R., Ziemer, C. J., Stern, M. D., & Stahl, D. A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiology Ecology, 26(1), 71–78. https://doi.org/10.1016/S0168-6496(98)00024-5

Sharpe, M. E., Latham, M. J., & Reiter, B. (1969). The occurrence of natural antibodies to rumen bacteria. Journal of General Microbiology, 56(3), 353–364. https://doi.org/10.1099/00221287-56-3-353

Sharpe, M. E., Latham, M. J., & Reiter, B. (1977). The immune response of the hosts animal to bacteria in the rumen and caecum. In A. C. I. I. W. McDonald (Ed.), Proceedings of the IV International Symposium on Ruminant Physiology: Digestion and Metabolism in the Ruminant.

Sheldrake, R. F., & Husband, A. J. (1985). Intestinal uptake of intact maternal lymphocytes by neonatal rats and lambs. Research in Veterinary Science, 39(1), 10–15. https://doi.org/10.1016/s0034-5288(18)31762-4

Shi, Y., Odt, C. L., & Weimer, P. J. (1997). Competition for cellulose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited conditions. Applied and Environmental Microbiology, 63(2), 734–742. https://doi.org/10.1128/aem.63.2.734-742.1997

Shim, Y. H., Ingale, S. L., Kim, J. S., Kim, K. H., Seo, D. K., Lee, S. C., Chae, B. J., & Kwon, I. K. (2012). A multi-microbe probiotic formulation processed at low and high drying temperatures: effects on growth performance, nutrient retention and caecal microbiology of broilers. British Poultry Science, 53(4), 482–490. https://doi.org/10.1080/00071668.2012.690508

Skillman, L. C., Evans, P. N., Naylor, G. E., Morvan, B., Jarvis, G. N., & Joblin, K. N. (2004). 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe, 10(5), 277–285. https://doi.org/10.1016/j.anaerobe.2004.05.003

Soares, R. V., Lin, T., Siqueira, C. C., Bruno, L. S., Li, X., Oppenheim, F. G., Offner, G., & Troxler, R. F. (2004). Salivary micelles: Identification of complexes containing MG2, sIgA, lactoferrin, amylase, glycosylated proline-rich protein and lysozyme. Archives of Oral Biology, 49(5), 337–343. https://doi.org/10.1016/j.archoralbio.2003.11.007

Somers, B. M. (1957). Salivary secretion and its functions in ruminants. The Australian Veterinary Journal, 33(11), 297–301.

Sordillo, L. M., Shafer-Weaver, K., & DeRosa, D. (1997). Immunobiology of the Mammary Gland. Journal of Dairy Science, 80(8), 1851–1865. https://doi.org/10.3168/jds.S0022-0302(97)76121-6

Steele, M. A., Penner, G. B., Chaucheyras-Durand, F., & Guan, L. L. (2016). Development and physiology of the rumen and the lower gut: Targets for improving gut health. Journal of Dairy Science, 99(6), 4955–4966. https://doi.org/10.3168/jds.2015-10351

Stella, A. V., Paratte, R., Valnegri, L., Cigalino, G., Soncini, G., Chevaux, E., Dell'Orto, V., & Savoini, G. (2007). Effect of administration of live Saccharomyces cerevisiae on milk production, milk composition, blood metabolites, and faecal flora in early lactating dairy goats. Small Ruminant Research, 67(1), 7–13. https://doi.org/10.1016/j.smallrumres.2005.08.024

Stewart, C. S., Fonty, G., & Gouet, P. (1988). The establishment of rumen microbial communities. Animal Feed Science and Technology, 21(2–4), 69–97. https://doi.org/10.1016/0377-8401(88)90093-4

Ŝtyriak, I., Gálfi, P., & Kmeł, V. (1991). Preliminary observations of interaction between bacteriophages and Streptococcus bovis bacteria on ruminal epithelium primoculture. Veterinary Microbiology, 29(3–4), 281–287. https://doi.org/10.1016/0378-1135(91)90135-3

Subharat, S., Shu, D., Zheng, T., Buddle, B. M., Janssen, P. H., Luo, D., & Wedlock, D. N. (2015). Vaccination of cattle with a methanogen protein produces specific antibodies in the saliva which are stable in the rumen. Veterinary Immunology and Immunopathology, 164(3–4), 201–207. https://doi.org/10.1016/j.vetimm.2015.02.008

Subharat, S., Shu, D., Zheng, T., Buddle, B. M., Kaneko, K., Hook, S., Janssen, P. H., & Wedlock, D. N. (2016). Vaccination of sheep with a methanogen protein provides insight into levels of antibody in saliva needed to target ruminal methanogens. PLoS ONE, 11(7), 1–14. https://doi.org/10.1371/journal.pone.0159861

Sunagawa, S., Mende, D. R., Zeller, G., Izquierdo-Carrasco, F., Berger, S. A., Kultima, J. R., Coelho, L. P., Arumugam, M., Tap, J., Nielsen, H. B., Rasmussen, S., Brunak, S., Pedersen, O., Guarner, F., De Vos, W. M., Wang, J., Li, J., Doré, J., Dusko Ehrlich, S., … Bork, P. (2013). Metagenomic species profiling using universal phylogenetic marker genes. Nature Methods, 10(12), 1196–1199. https://doi.org/10.1038/nmeth.2693

Svensson, C., Lundborg, K., Emanuelson, U., & Olsson, S. O. (2003). Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. Preventive Veterinary Medicine, 58(3–4), 179–197. https://doi.org/10.1016/S0167-5877(03)00046-1

Swain, R. A., Nolan, J. V., & Klieve, A. V. (1996). Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. Applied and Environmental Microbiology, 62(3), 994–997.

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., & Firkins, J. L. (2004). Development of an Assay to Quantify Rumen Ciliate Protozoal Biomass in Cows Using Real-Time PCR. The Journal of Nutrition, 134(12), 3378–3384. https://doi.org/10.1093/jn/134.12.3378

Tamate, H., McGilliard, A. D., Jacobson, N. L., & Getty, R. (1962). Effect of Various Dietaries on the Anatomical Development of the Stomach in the Calf. Journal of Dairy Science, 45(3), 408–420. https://doi.org/10.3168/jds.S0022-0302(62)89406-5

Tapio, I., Snelling, T. J., Strozzi, F., & Wallace, R. J. (2017). The ruminal microbiome associated with methane emissions from ruminant livestock. Journal of Animal Science and Biotechnology, 8(1), 1–11. https://doi.org/10.1186/s40104-017-0141-0

Taschuk, R., & Griebel, P. J. (2012). Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. Animal Health Research Reviews / Conference of Research Workers in Animal Diseases, 13(1), 129–141. https://doi.org/10.1017/S1466252312000096

Tenovuo, J. (2002). Antimicrobial agents in saliva - Protection for the whole body. Journal of Dental Research, 81(12), 807–809. https://doi.org/10.1177/154405910208101202

Trevisi, E., Amadori, M., Riva, F., Bertoni, G., & Bani, P. (2014). Evaluation of innate immune responses in bovine forestomachs. Research in Veterinary Science, 96(1), 69–78. https://doi.org/10.1016/j.rvsc.2013.11.011

Veerman, E. C. I., Van Den Keybus, P. A. M., Vissink, A., & Nieuw Amerongen, A. V. (1996). Human glandular salivas: Their separate collection and analysis. European Journal of Oral Sciences, 104(4 PART 1), 346–352. https://doi.org/10.1111/j.1600-0722.1996.tb00090.x

Veneman, J. B., Muetzel, S., Hart, K. J., Faulkner, C. L., Moorby, J. M., Perdok, H. B., & Newbold, C. J. (2015). Does dietary mitigation of enteric methane production affect rumen function and animal productivity in dairy cows? PLoS ONE, 10(10), 1–18. https://doi.org/10.1371/journal.pone.0140282

Wang, C., Li, D., Yang, J., Xia, Y., Tu, Y., White, R., Gao, H., Diao, Q., & Mao, H. (2020). Weaning performance of beef cattle calves based on concentrate intake. Animals, 10(1), 1–12. https://doi.org/10.3390/ani10010018

Warner, A. C. I., & Stacy, B. D. (1977). Influence of ruminal and plasma osmotic pressure on salivary secretion in sheep. Quarterly Journal of Experimental Physiology, 62, 133–142.

Warner, R. G., Flatt, W. P., & Loosli, J. K. (1956). Dietary factors influencing the development of the ruminant stomach. Journal of Agricultural and Food Chemistry, 4(9), 788–792. https://doi.org/10.1021/jf60067a003

Weary, D. M., Jasper, J., & Hötzel, M. J. (2008). Understanding weaning distress. Applied Animal Behaviour Science, 110(1–2), 24–41. https://doi.org/10.1016/j.applanim.2007.03.025

Weimer, P. J., Stevenson, D. M., Mantovani, H. C., & Man, S. L. C. (2010). Host specificity of the ruminal bacterial community in the dairy cow following near-total exchange of ruminal contents1. Journal of Dairy Science, 93(12), 5902–5912. https://doi.org/10.3168/jds.2010-3500

Weinberg, Z. G., Muck, R. E., Weimer, P. J., Chen, Y., & Gamburg, M. (2004). Lactic acid bacteria used in inoculants for silage as probiotics for ruminants. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology, 118(1–3), 1–9. https://doi.org/10.1385/abab:118:1-3:001

Wheeler, T. T., Hodgkinson, A. J., Prosser, C. G., & Davis, S. R. (2007). Immune components of colostrum and milk - A historical perspective. Journal of Mammary Gland Biology and Neoplasia, 12(4), 237–247. https://doi.org/10.1007/s10911-007-9051-7

White, M. R., Helmerhorst, E. J., Ligtenberg, A., Karpel, M., Tecle, T., Siqueira, W. L., Oppenheim, F. G., & Hartshorn, K. L. (2009). Multiple components contribute to ability of saliva to inhibit influenza viruses. Oral Microbiology and Immunology, 24(1), 18–24. https://doi.org/10.1111/j.1399-302X.2008.00468.x

Wiesner, J., & Vilcinskas, A. (2010). Antimicrobial peptides: The ancient arm of the human immune system. Virulence, 1(5), 440–464. https://doi.org/10.4161/viru.1.5.12983

Wilkinson, T. J., Huws, S. A., Edwards, J. E., Kingston-Smith, A. H., Siu-Ting, K., Hughes, M., Rubino, F., Friedersdorff, M., & Creevey, C. J. (2018). CowPI: A rumen microbiome focussed version of the PICRUSt functional inference software. Frontiers in Microbiology, 9(MAY), 1–10. https://doi.org/10.3389/fmicb.2018.01095

Williams, A.G., and Coleman, G. S. (1992). The Rumen Protozoa (Springer-V).

Wines, B. D., & Hogarth, P. M. (2006). IgA receptors in health and disease. Tissue Antigens, 68(2), 103–114. https://doi.org/10.1111/j.1399-0039.2006.00613.x

Woese, C. R., Gutell, R., Gupta, R., & Noller, H. F. (1983). Detailed analysis of the higher-order structure of 16S-like ribosomal ribonucleic acids. Microbiological Reviews, 47(4), 621–669. https://doi.org/10.1128/mmbr.47.4.621-669.1983

Wongnate, T., & Ragsdale, S. W. (2015). The reaction mechanism of methyl-coenzyme M reductase. Journal of Biological Chemistry, 290(15), 9322–9334. https://doi.org/10.1074/jbc.M115.636761

Wright, A. D. G., & Klieve, A. V. (2011). Does the complexity of the rumen microbial ecology preclude methane mitigation? Animal Feed Science and Technology, 166–167, 248–253. https://doi.org/10.1016/j.anifeedsci.2011.04.015

Yang, C. M., Cao, G. T., Ferket, P. R., Liu, T. T., Zhou, L., Zhang, L., Xiao, Y. P., & Chen, A. G. (2012). Effects of probiotic, Clostridium butyricum, on growth performance, immune function, and cecal microflora in broiler chickens. Poultry Science, 91(9), 2121–2129. https://doi.org/10.3382/ps.2011-02131

Yáñez-Ruiz, D. R., Abecia, L., & Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: A review. Frontiers in Microbiology, 6(OCT), 1–12. https://doi.org/10.3389/fmicb.2015.01133

Yáñez-Ruiz, D. R., & Belanche, A. (2020). Plant secondary compounds: beneficial roles in sustainable ruminant nutrition and productivity. June, 727–774. https://doi.org/10.19103/as.2020.0067.25

Yáñez-Ruiz, D. R., MacÍas, B., Pinloche, E., & Newbold, C. J. (2010). The persistence of bacterial and methanogenic archaeal communities residing in the rumen of young lambs. FEMS Microbiology Ecology, 72(2), 272–278. https://doi.org/10.1111/j.1574-6941.2010.00852.x

Yáñez-Ruiz, D. R., Williams, S., & Newbold, C. J. (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. https://doi.org/10.1017/S0007114507675187

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., & Aldridge, B. M. (2018). Biogeographical Differences in the Influence of Maternal Microbial Sources on the Early Successional Development of the Bovine Neonatal Gastrointestinal tract. Scientific Reports, 8(1), 1–14. https://doi.org/10.1038/s41598-018-21440-8

Yohe, T. T., Enger, B. D., Wang, L., Tucker, H. L. M., Ceh, C. A., Parsons, C. L. M., Yu, Z., & Daniels, K. M. (2018). Short communication: Does early-life administration of a Megasphaera elsdenii probiotic affect long-term establishment of the organism in the rumen and alter rumen metabolism in the dairy calf? Journal of Dairy Science, 101(2), 1747–1751. https://doi.org/10.3168/jds.2017-12551

Yousuf, S., Sudha, N., Murugesan, G., & Enoch, I. V. M. V. (2013). Isolation of Prunin from the fruit shell of Bixa orellana and the effect of β-cyclodextrin on its binding with calf thymus DNA. Carbohydrate Research, 365, 46–51. https://doi.org/10.1016/j.carres.2012.10.003

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., & Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. BMC Microbiology, 20(1), 1–15. https://doi.org/10.1186/s12866-020-1716-z

Yvon, M., Pélissier, J., Guilloteau, P., & Toullec, R. (n.d.). III . Amino acid compositions of the digesta leaving the abomasum. 1.

Zhang, K., Li, B., Guo, M., Liu, G., Yang, Y., Wang, X., Chen, Y., & Zhang, E. (2019). Maturation of the goat rumen microbiota involves three stages of microbial colonization. Animals, 9(12), 1–17. https://doi.org/10.3390/ani9121028

Zhong, R. Z., Sun, H. X., Li, G. D., Liu, H. W., & Zhou, D. W. (2014). Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. Livestock Science, 162(1), 154–158. https://doi.org/10.1016/j.livsci.2013.12.021

Zhou, M., Chen, Y., & Guan, L. L. (2015). Rumen Bacteria. In A. K. Puniya, R. Singh, & D. N. Kamra (Eds.), Rumen Microbiology: From Evolution to Revolution (pp. 79–95). Springer India.

Zhou, M., Chung, Y. H., Beauchemin, K. A., Holtshausen, L., Oba, M., McAllister, T. A., & Guan, L. L. (2011). Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. Journal of Applied Microbiology, 111(5), 1148–1158. https://doi.org/10.1111/j.1365-2672.2011.05126.x

CAPÍTULO II

MATERIAL Y MÉTODOS

El material y métodos empleados en cada uno de los ensayos que conforman la tesis se describen con detalle en las publicaciones presentes en el Capítulo III. Por ello, en esta sección se muestran únicamente los esquemas de los diseños experimentales correspondientes a los distintos ensayos de la tesis. En primer lugar, se presentan los esquemas de los 3 experimentos *in vitro* llevados a cabo en la **Publicación 1**. En el Experimento 1 de esta publicación se evaluó la actividad fermentativa de la microbiota ruminal utilizando distintos tampones de incubación y dietas como sustrato (**Figura 2**). En esta misma publicación se incluyó el Experimento 2 para evaluar el efecto del tiempo de muestreo y la fracción de contenido ruminal empleada (**Figura 3**) y el Experimento 3 para determinar el método de conservación de la microbiota ruminal más adecuado en función de la dieta a la que está adaptada esta microbiota (**Figura 4**). La **Figura 5** representa el diseño experimental del subsiguiente ensayo *in vivo* (**Publicaciones 2 y 3**) en el que se procedió a inocular a cabritos en edad temprana con líquido ruminal autoclavado o fresco adaptado a dieta concentrada o forrajera. A continuación, y siguiendo la línea de intervenciones en edad temprana, se acometió un ensayo (**Publicación 4)** para promover una inoculación indirecta de microbiota ruminal en cabritos durante la lactancia por medio de la presencia de animales adultos (**Figura 6**).

Paralelamente, se realizaron ensayos *in vitro* para estudiar el papel de la saliva del rumiante en la modulación de la microbiota ruminal y su actividad. La **Publicación 5** comprendió dos incubaciones no renovadas destinadas a analizar los efectos sobre la fermentación ruminal del empleo de distintas proporciones de saliva (Experimento 1, **Figura 7**) y de las distintas fracciones de esta (Experimento 2, **Figura 8**) en base a distintos procesos de filtrado o autoclavado. Por último, se llevó a cabo un ensayo (**Publicación 6**) basado en una incubación semi-continua de mayor duración para ver el efecto sobre la microbiota ruminal de los componentes bioactivos de la saliva en función del animal donante (**Figura 9**).

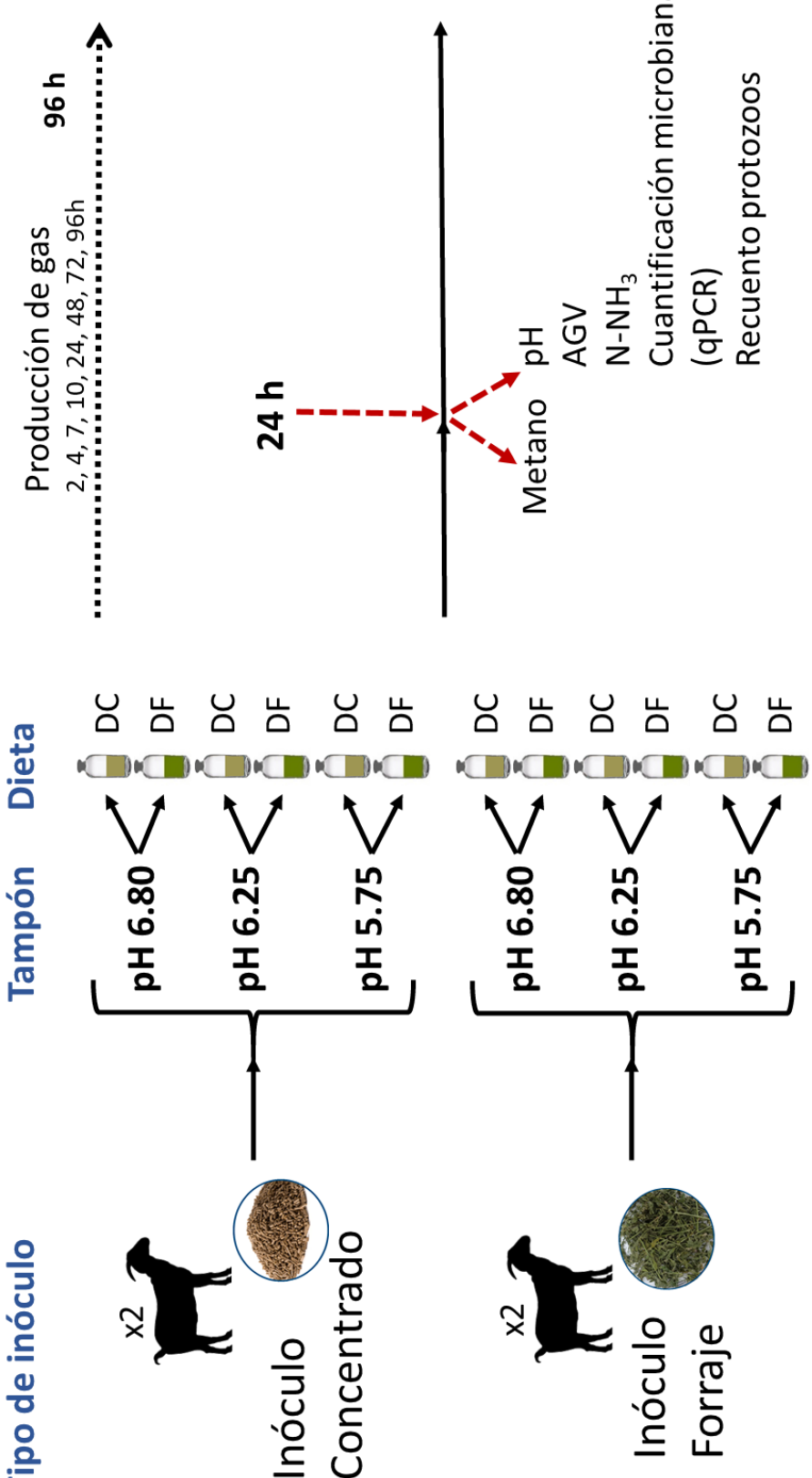

Figura 2. Diseño experimental correspondiente a la Publicación 1 (Experimento 1). DC: Sustrato de incubación a base de concentrado. DF: Sustrato de incubación a base de forraje. AGV: Ácidos Grasos Volátiles.

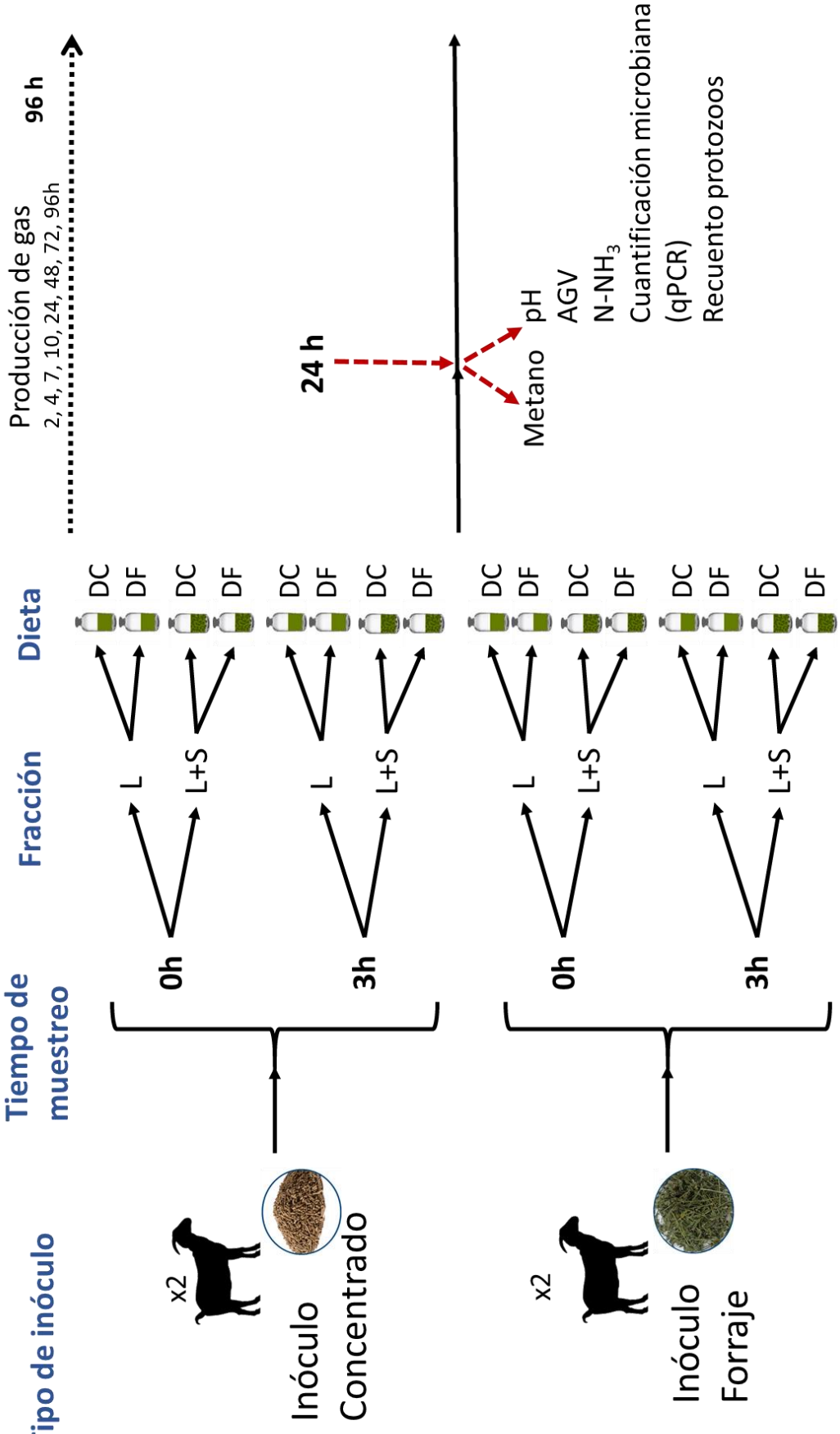

Figura 3. Diseño experimental correspondiente a la Publicación 1 (Experimento 2). L: Fracción líquida. L+S: Fracción líquida + sólida. DC: Sustrato de incubación a base de concentrado. DF: Sustrato de incubación a base de forraje. AGV: Ácidos Grasos Volátiles.

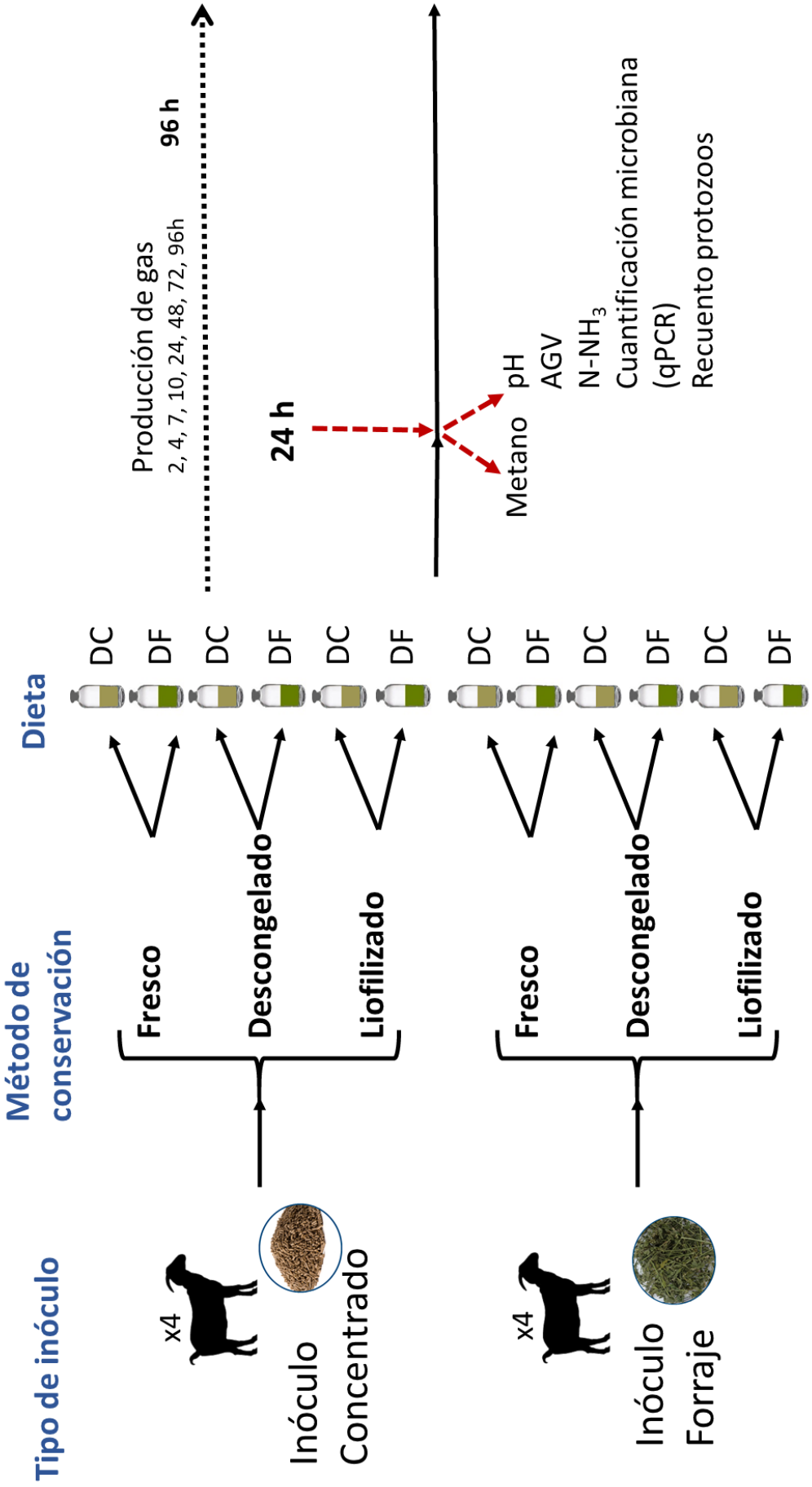

Figura 4. Diseño experimental correspondiente a la Publicación 1 (Experimento 3). DC: Sustrato de incubación a base de concentrado. DF: Sustrato de incubación a base de forraje. AGV: Ácidos Grasos Volátiles.

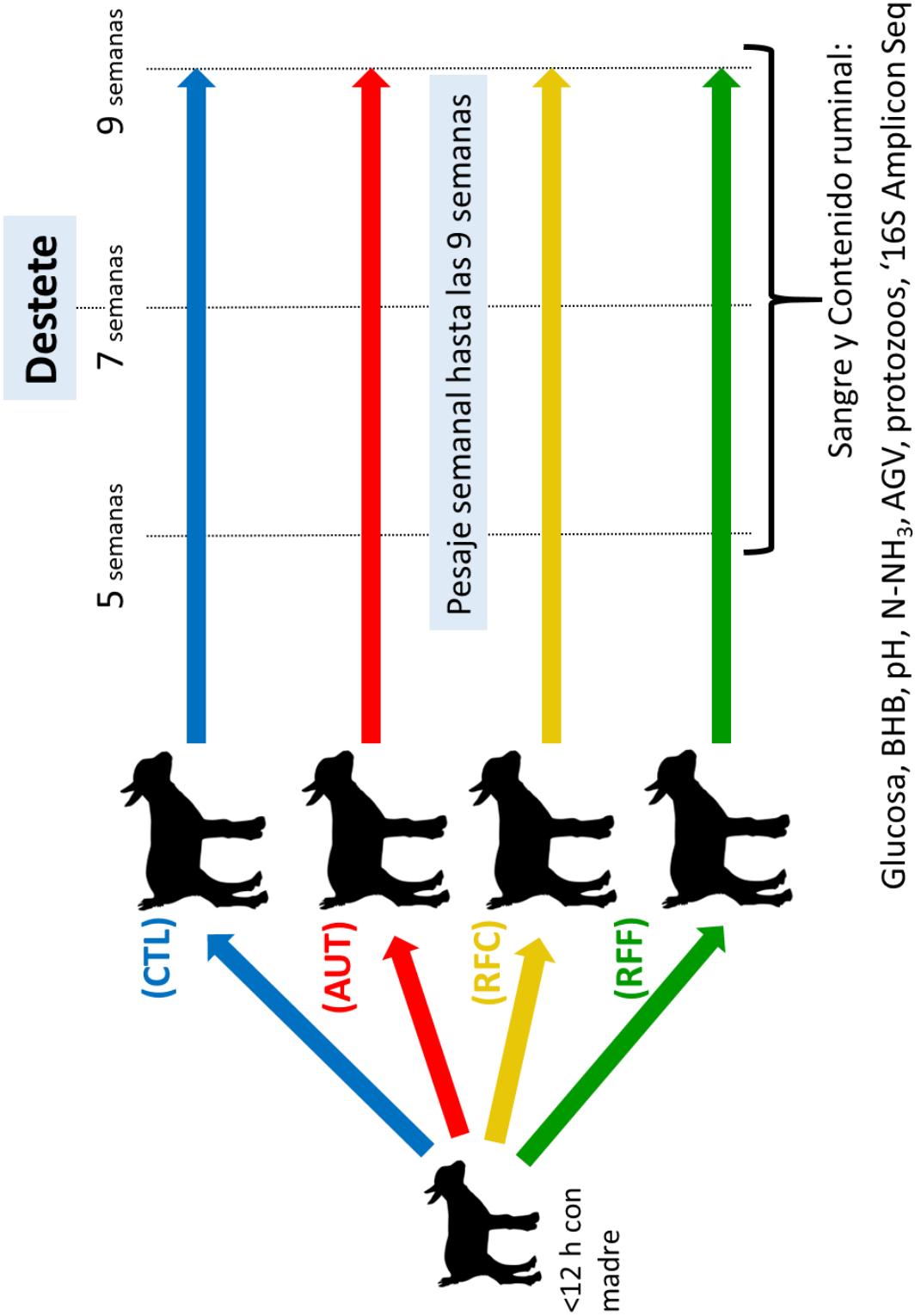

Figura 5. Diseño experimental correspondiente a las Publicaciones 2 y 3. CTL: Grupo control. AUT: Grupo inoculado con líquido ruminal autoclavado. RFC: grupo inoculado con líquido ruminal fresco adaptado a dieta concentrada. RFF: Grupo inoculado con líquido ruminal fresco adaptado a dieta forrajera. BHB: Beta-hidroxi-butirato. AGV: Ácidos Grasos Volátiles.

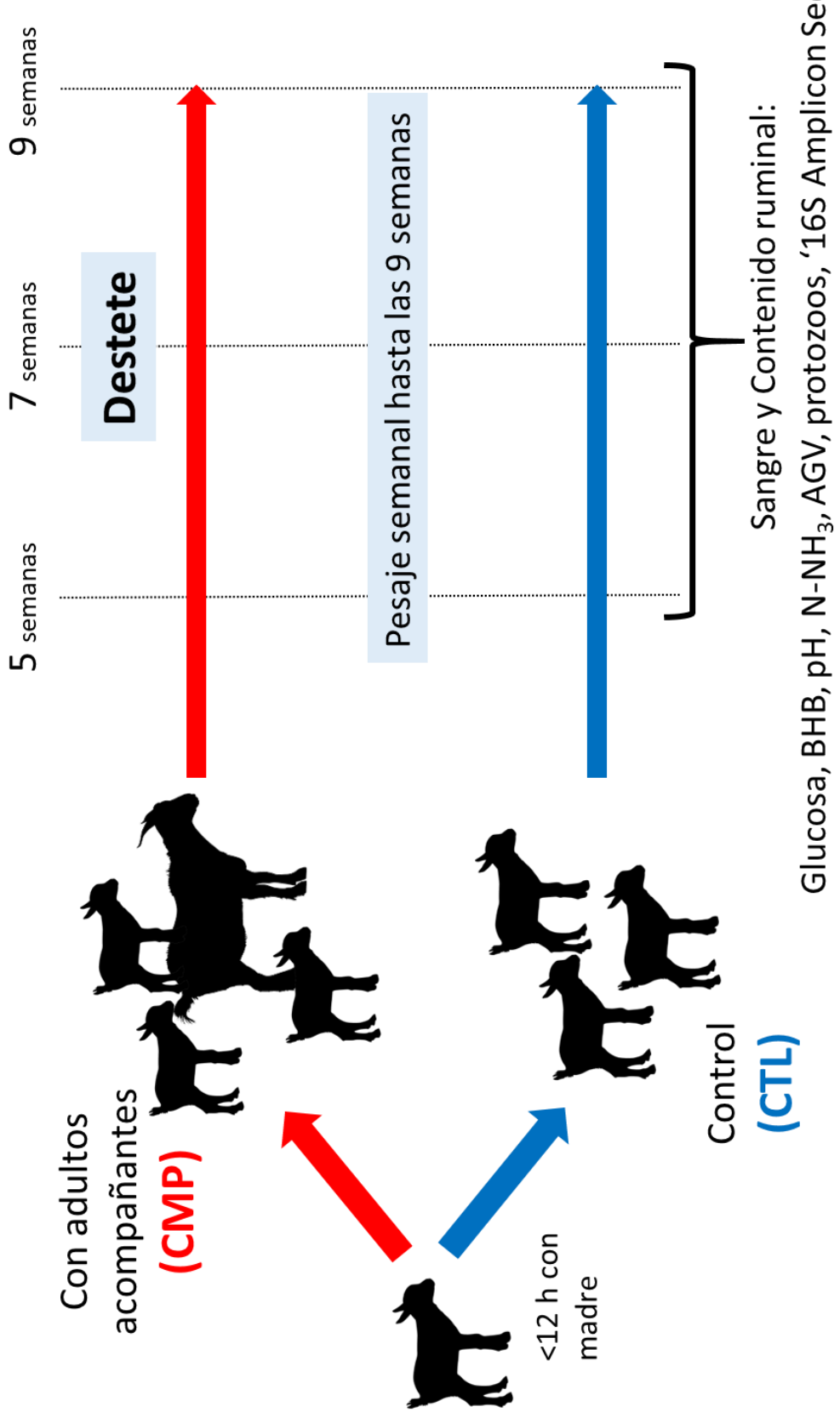

Figura 6. Diseño experimental correspondiente a la Publicación 4. BHB: Beta-hidroxi-butirato. AGV: Ácidos Grasos Volátiles.

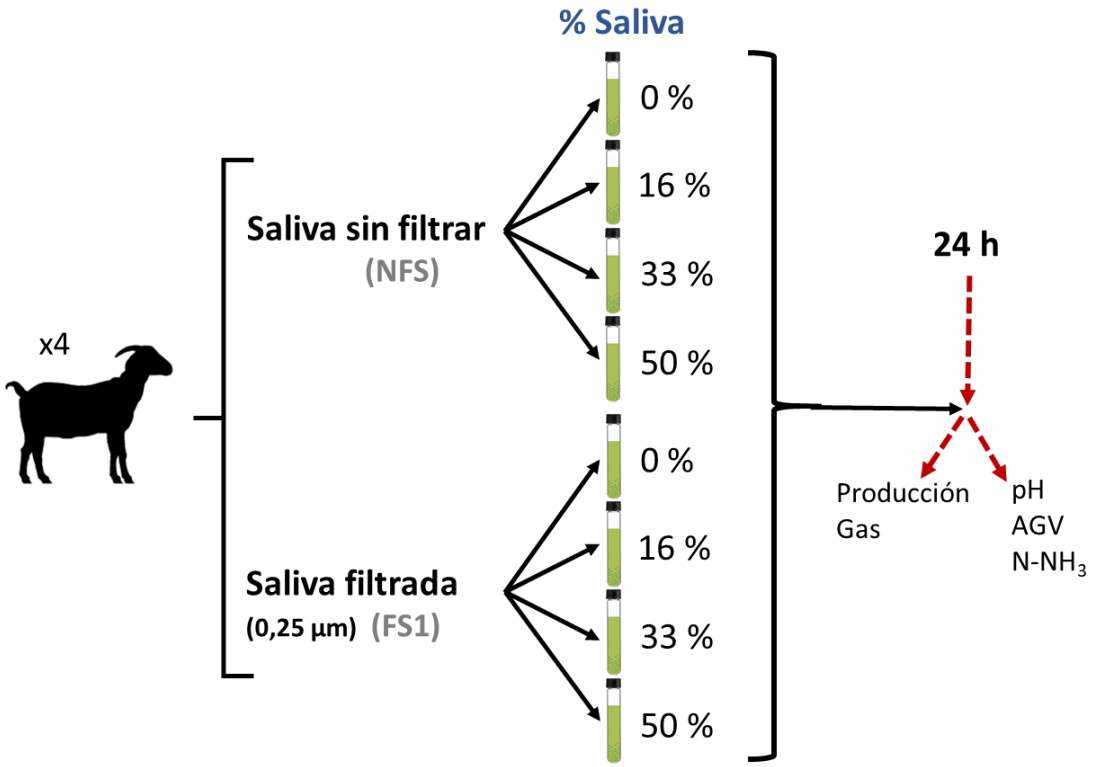

Figura 7. Diseño experimental correspondiente a la Publicación 5 (Experimento 1). AGV: Ácidos Grasos Volátiles.

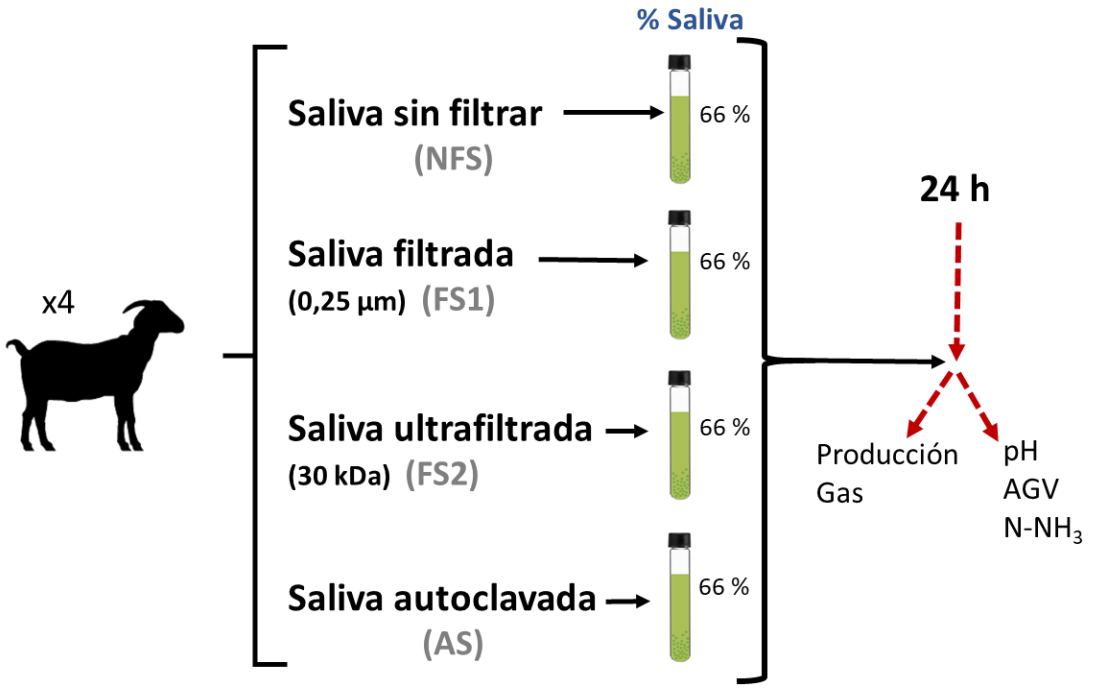

Figura 8. Diseño experimental correspondiente a la Publicación 5 (Experimento 2). AGV: Ácidos Grasos Volátiles.

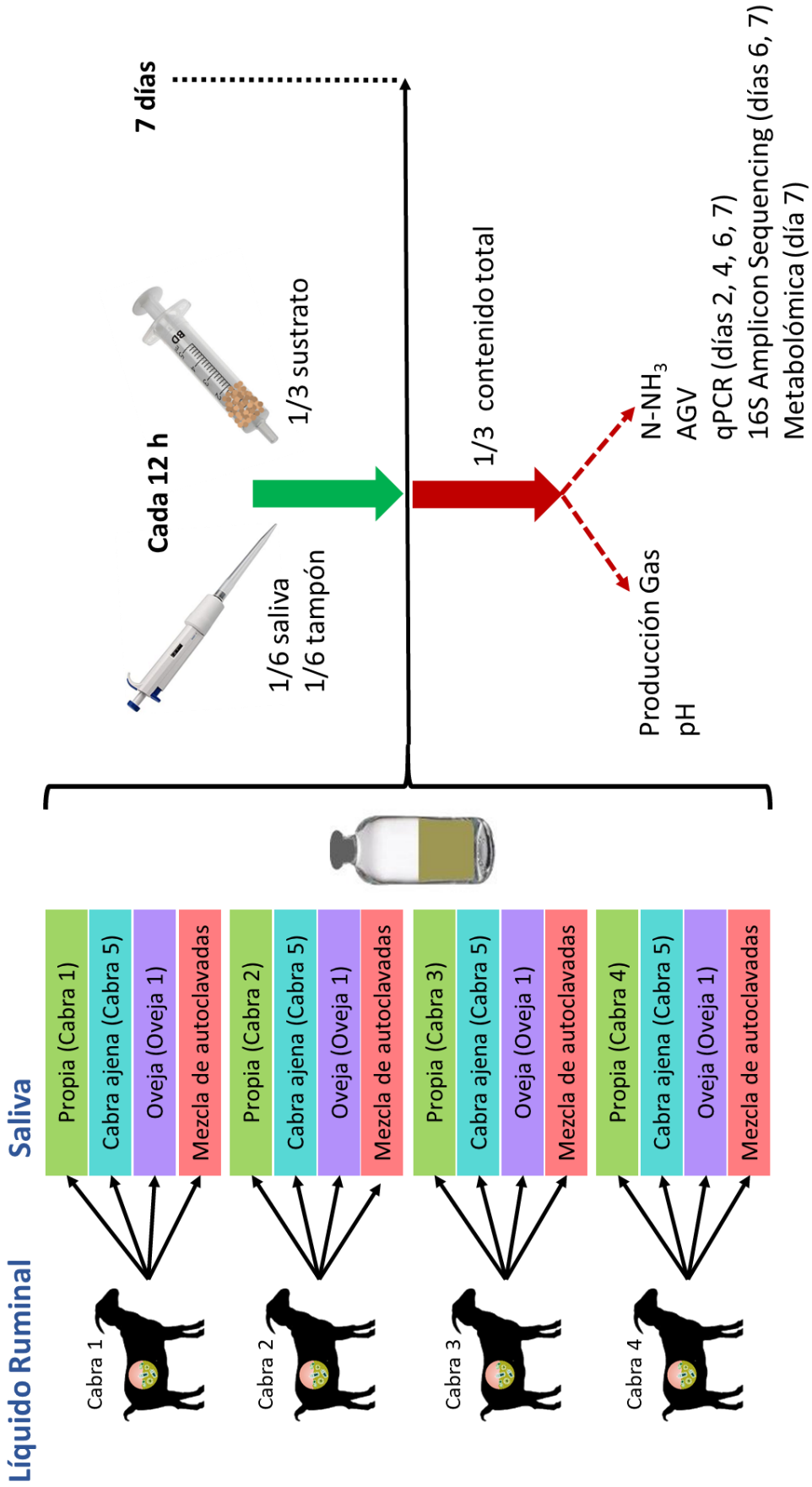

Figura 9. Diseño experimental correspondiente a la Publicación 6. AGV: Ácidos Grasos Volátiles.

CAPÍTULO III

RESULTADOS

Publication 1

In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum

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Journal of the Science of Food and Agriculture, 99(1): 163-172

DOI: https://doi.org/10.1002/jsfa.9157

ABSTRACT

BACKGROUND: The rumen microbiota has been used as inoculum for in vitro studies and as a probiotic to improve productivity in young animals. However, great variability across studies has been noted depending on the inoculum considered. The present study aims to assess the relevance of different factors (microbial fraction, collection time, donor animal diet, fermentation substrate and inoculum preservation method) to maximize the rumen inoculum activity and set the standards for further in vitro and in vivo applications.

RESULTS: Rumen inoculum sampled at 3 h after feeding led to greater microbial growth and activity [+12% volatile fatty acid (VFA), +17% ammonia] compared to before feeding. Similar results were noted when rumen liquid or rumen content were used as inocula. Rumen inoculum adapted to concentrate diets increased microbial activity (+19% VFA) independently of the substrate used in vitro. Freezing-thawing the inoculum, in comparison to fresh inoculum, decreased microbial activity (−14% VFA, −96% ammonia), anaerobic fungi and protozoa, with holotrichs protozoa being particularly vulnerable. Inoculum lyophilization had a stronger negative effect on microbial activity (−51% VFA) and delayed re-activation of the microbes, leading to lower levels of methanogens and anaerobic fungi, as well as almost complete wipe out of rumen protozoa.

CONCLUSIONS: Fresh rumen fluid sampled at 3 h after feeding from donor animals that were fed concentrate diets should be chosen when the aim is to provide the most diverse and active rumen microbial inoculum.

Keywords: in vitro; microbial activity; preservation method; probiotics; rumen inoculum

INTRODUCTION

There is increasing pressure with respect to the need to develop safe and effective rumen modulators in ruminant production because concerns over the use of antibiotics in livestock production and the requirement for increasing productivity continue to grow.[1] Among the range of options, probiotics offer a source of microorganisms that provide a beneficial microbial balance in the gastrointestinal tract.[2] Although the rumen is home to a highly diverse microbiota, comprising bacteria, protozoa, anaerobic fungi, methanogens and viruses,[3] only a few autochthonous members of this complex community and few allochthonous species (mainly Saccharomyces cerevisae, *Lactobacillus* spp. and Enterococcus spp.) have been explored for their potential as probiotics.[2] The results of using probiotics on rumen function are highly variable and are dependent on various factors, such as the type of probiotic, dose, preservation method, diet and host animal; however, the best results have generally been achieved by generating a multifactorial response through the administration of a combination of various microbial species to maximize their activity.[2,4] Thus, it may be hypothesized that probiotics collected from the rumen may establish more easily into the microbial community than foreign species. Inoculation of rumen fluid from healthy animals to sick animals has often been conducted by producers and veterinarians to re-stablish the rumen balance (especially in animals that have been off feed)[5] and to prevent sickness associated with grain overfeeding;[6] however, there is no such commercial product available based on this concept. Several studies have used microbial factions harvested from the rumen content as probiotics to improve the health and growth of young ruminants before weaning.[7,8] An early study showed that a single inoculation with fresh rumen fluid to growing lambs improved nitrogen balance[9] although the results varied depending on the donor animal diet.[10] Moreover, different types and preservation methods of the rumen inocula have been explored. For example, placing cuds from an adult animals into the mouths of young calves facilitated the establishment of rumen protozoa,[11] whereas the administration of lyophilized[12] or dry stabilized rumen extract[13] showed positive effects on the average weight gain and feed digestibility in young animals. By contrast, two studies[8,14] noted no beneficial effects of inoculating growing lambs with lyophilized rumen microbiota. Thus, the great variability observed across these and other studies suggests that more effort is needed to identify and describe the relevance of the different factors that define the inoculum microbial activity under standardized laboratory conditions before conducting further in vivo studies.

The rumen microbiota has been used extensively as inoculum for in vitro fermentation techniques to evaluate the nutritional value of ruminant feeds and the effectiveness of feed additives.[15] As a result, several recommendations have been provided with respect to the donor animal, microbial adaptation to the diet, type of rumen inoculum, sampling time or preservation method.[16,17] Although there are no standard protocols, fresh rumen liquid sampled before feeding from donor animals adapted to the same (or similar) diets as those used as fermentation substrate represents the inoculum most commonly used when the aim is to minimize inter-animal variation and allow comparisons to be made among in vitro studies.[16,17] However, to date, no studies have assessed how these technical aspects should be addressed when the aim is to achieve the rumen microbiota with the highest possible activity. Therefore, the present study aims to expand knowledge on the factors that maximize and maintain the rumen microbial activity in terms of anaerobic fermentation and an abundance of the main microbial groups, once collected from the animal, thus enabling a description of the most suitable inoculum to be directly inoculated in vivo as probiotics or used for in vitro studies.

MATERIAL AND METHODS

A multifactorial approach was chosen to assess the impact of different factors with respect to maintaining the rumen microbial activity; first, an in vivo monitoring of the diurnal changes in the rumen pH was performed to identify the most suitable sampling time depending on the donoŕs diet. Then, a series of three consecutive in vitro experiments was performed to determine the effect of the type of incubation buffer (Experiment 1), sampling time, rumen fraction (Experiment 2), inoculum adaptation to the diet and preservation method (Experiment 3) on microbial activity. The findings from each trial were implemented on the subsequent experimental design. All incubations were performed using two different diets as substrates to determine whether these findings could be applied across various production systems. Animal procedures were carried out according to the Spanish guidelines (RD 153/2013) and protocols were approved by the Ethical Committee for Animal Research (EEZ-CSIC) regional government (3 September 2017). Eight Murciano-Granadina female goats fitted with permanent rumen fistula were randomly distributed into two groups fed either a forage diet (DF, 50:50 proportions of alfalfa hay and oat hay) or a concentrate diet (DC, ratio 70:30 concentrate to forage ratio).

Chemical composition [g kg^{-1} dry matter (DM)] of the concentrate was 949 organic matter, 36.1 nitrogen, 319 neutral detergent fiber, 87.2 acid detergent fiber, 34 acid detergent lignin and 48.3 ether extract, whereas, for the forage, it was 898 organic matter, 31 nitrogen, 576 neutral detergent fiber, 335 acid detergent fiber, 68 acid detergent lignin and 19 ether extract. Animals were kept in individual pens and diet was offered at 1.2 times the maintenance level divided into two equal meals at 08 00 h and 16.00 h.[18]

Experiment 1: *In vivo* rumen pH monitoring and *in vitro* buffering capacity

This experiment aimed to identify the rumen microbiota with the greatest activity for direct use as inoculum and the most appropriate in vitro conditions to evaluate its activity once removed from the rumen. Rumen pH was monitored in vivo to determine the time after feeding with the greatest fermentation peak (lowest pH) depending on the diet (DC versus DF) consumed by the donor animal. Three incubation solutions with different buffering capacities were used to identify whether the incubation pH could influence the results depending on the substrate considered (interaction Diet × Buffer). After 2 weeks of adaptation to the diets, rumen digesta samples were collected through the cannula at 0, 2, 4 and 6 h after the morning feeding to determine the diurnal changes in rumen pH (**Fig. 1**). Based on previous recommendations,[19] three combinations of salts consisting of decreasing levels of $NaHCO_3$ (35, 10.3 and 3.15 g L^{-1}) and increasing levels of NH_4HCO_3 (4.0, 1.07 and 0.25 g L^{-1}) were used for adjusting the in vitro conditions to the targeted high (6.80), medium (6.25) and low (5.75) pH.

The batch culture incubation[20] used a total of 48 Wheaton bottles in a single run according to a 3 × 2 × 4 × 2 factorial design: three buffer solutions (high versus medium versus low pH) × 2 diets as incubation substrate (DC versus DF) × 4 animal inocula (n = 4) × 2 analytical replicates. Rumen contents were individually collected from donor goats at 3 h after the morning feeding, the time at which the greatest differences in rumen pH among diets were generated (based on the preliminary study), filtered through a double layer of muslin and bubbled with CO_2. This inoculum was immediately diluted with preheated incubation solutions (in a 1:3 ratio) and anaerobically dispensed to 120 mL Wheaton bottles containing 500 mg DM of either DC or DF and 50 mL in total volume. Experimental diets were ground using a hammer mill with a pore size of 1 mm^2 and the incubation of each substrate was performed using rumen inoculum from animals adapted

to the equivalent diet. Bottles were sealed and kept static in an incubator at 39 °C, receiving a gentle mix before each measurement. In vitro incubations lasted for 96 h and microbial activity was determined after 24 h of incubation; a sample representing 7% of the incubation fluid was taken by aspiration using a 14-G needle, the pH was immediately recorded and the sample was divided into two subsamples: the first sample (1.6 mL) was diluted with 0.4 mL of an acid solution (0.5 mol L^{-1} HCl, 200 g L^{-1} metaphosphoric acid containing 0.8 g L^{-1} of crotonic acid as internal standard) and stored at −20 °C until volatile fatty acid (VFA) determination. The second sample (0.8 mL) was diluted with 0.2 mL of trichloroacetate solution (25 g L^{-1}) for ammonia analysis. To determine the fermentation kinetics, gas pressure in the headspace of the bottles was measured and released at 2, 4, 7, 10, 24, 48, 72 and 96 h using a Wide Range Pressure Meter (Sper Scientific LTD, Scottsdale, AZ, USA).

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

The incubation buffer that promoted the highest pH (target 6.80) also promoted the highest microbial fermentation (see below) and thus was chosen for further experiments to prevent a possible limitation of the microbial activity induced by the buffer. Similarly, rumen sampling times (0 and 3 h after feeding) were chosen because they led to the greatest in vivo differences in the rumen pH among diets. A total of 72 Wheaton bottles were used according to a 2 × 2 × 2 × 4 × 2 factorial design distributed into two consecutive incubation runs: 2 inoculum fractions (rumen liquid versus rumen liquid plus solids) × 2 collection times (0 h versus 3h after feeding) × 2 diets as substrate (DC versus DF) × 4 animal inocula (n = 4) × 2 analytical replicates plus eight bottles used as positive controls. Two consecutive incubation runs with a 3-h time difference between them were conducted; thus, positive control bottles containing identical frozen-thawed inocula were added into each incubation run to detect abnormalities between the runs. These positive control bottles comprised rumen inocula from each donor animal [containing dimethyl-sulfoxide (DMSO), as cryopreservant], incubation buffer and the same substrate as that consumed by the donor animal. Before conducting Experiment 2, rumen content (200 g fresh material) was collected from each donor goats, filtrated through double cheesecloth and manually squeezed to separate rumen liquid and solids aiming to: (i) determine the

proportions of liquid and solids in the rumen contents (250 and 750 g kg^{-1} of fresh rumen content, respectively) and (ii) obtain lyophilized and autoclaved rumen solids for subsequent experiments. On the starting day of each incubation run, rumen contents were fractionated into liquid and solids: the liquid fraction (LIQ) was mixed with the incubation buffer placed into incubation bottles containing the experimental diets. The rumen content fraction was artificially reconstituted by mixing rumen liquid and solids (LS) in the proportions described above. Moreover, each LIQ incubation bottle was supplemented with autoclaved rumen solid (113 mg DM) obtained from the same donor animal to compensate for the extra fermentable supply provided by the solids in the LS fraction. Each type of inoculum was incubated with its own related substrate. In vitro incubations and sampling procedures were the same as that described in Experiment 1 in terms of pH, VFA, ammonia and gas production, although three additional samples were taken after 24 h of incubation: a gas sample was collected from the headspace (5 mL into a vacuum tube) after gas pressure excess was released for further methane determination; moreover, one incubation sample (0.2 mL) was frozen in liquid nitrogen for microbial quantification and another sample (0.8 mL) was diluted with 0.8 mL of formaldehyde solution (80 mL L^{-1}) for protozoal counting.

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

Based on the results from Experiment 2 (see below), liquid inoculum was chosen for further experiments because is easier to obtain, homogenize and handle in the laboratory than solid inoculum. Similarly, inoculum collected at 3 h after feeding instead of before feeding was chosen for further experiments given its greater activity, independently of the diet considered (see below). In total, 104 bottles were used according to a 2 × 3 × 2 × 4 × 2 factorial design distributed into two incubation runs: 2 inocula adapted to either forage (AF) or concentrate (AC) × 3 inocula preservation methods [fresh (FRE) versus defrosted (DEF) versus lyophilized (LIO)] × 2 diets used as substrate (DF versus DC) × 4 animal inocula (n = 4) × 2 analytical replicates plus eight bottles used as positive controls. Incubation runs were conducted over two consecutive weeks and positive control bottles were used as described previously. The day of the first incubation, rumen content (500 mL) was extracted from each goat, filtered and divided into two samples:

one was immediate incubated as fresh inocula (FRE), whereas the other sample was distributed into falcon tubes containing DMSO at 5% as cryopreservant (total volume 35 mL). These tubes were gently mixed and placed in a cooling water bath containing ice for 30 min (equilibrium time), before being frozen at −80 °C for 7 days. Half of the tubes remained frozen and the rest were lyophilized to determine the DM content. The day of the second incubation run, frozen inocula were defrosted (DEF) in a water bath at 39 °C for 5 min prior mixing with the incubation buffer under CO_2 bubbling. An equivalent amount of lyophilized rumen fluid (LIO) to 16.67 mL of fresh rumen fluid was dispensed into the incubation bottles along with 16.67 mL of distilled water and incubation buffer (33.3 mL) to five a total volume of 50 mL. Cryopreservant was also spiked into FRE bottles to prevent potential bias across treatments. The incubation procedures were performed as described in Experiment 2.

Sample analysis

Feed composition was measured as described previously.[21] The methane concentration in the headspace samples was determined by gas chromatography (Agilent, Waldbronn, Germany). Concentrations of individual VFA were determined by a gas chromatography system coupled with a flame ionization detector (AutoSystem; Perkin-Elmer Corp., Shelton, CT, USA), whereas the ammonia concentration was measured using a colorimetric method.[22] Protozoal concentration and classification were determined by optical microscopy.[23] DNA was extracted from frozen samples using a commercial kit (FavorPrep Stool DNA Isolation Mini Kit; Favorgen Biotech Corp., Changzhi, Taiwan) and concentrations of total bacteria, methanogenic archaea, protozoa and anaerobic fungi were determined by a quantitative polymerase chain reaction (PCR) as described previously.[24]

Calculations and statistical analysis

Organic matter fermentation of total hexoses (FOM), metabolic hydrogen production and incorporation into fermentation products were calculated stoichiometrically.[25] For gas production (GP), pressure measurements were adjusted for the amount of headspace available and converted to volume units using the ideal gas law. Then, cumulative GP

data were fitted to the predictive equation described by France et al.[26]: $Y = A(1 - e^{-ct})$ where Y (mL) is the cumulative GP at time t (h), A is the asymptotic or potential GP (mL) and c is the GP rate (μL h^{-1}). Data from analytical replicates were averaged and data from quantitative PCR and protozoal counting were log transformed prior to statistical analyses. Data from in vivo rumen pH monitoring were analysed by repeated measurements, whereas data from in vitro experiments were analysed by ANOVA as:

$$\text{Experiment 1 : } Y_{ijk} = \mu + D_i + B_j + G_k + e_{ijk}$$

$$\text{Experiment 2 : } Y_{ilmk} = \mu + D_i + F_l + T_m + G_k + e_{ilmk}$$

$$\text{Experiment 3 : } Y_{inok} = \mu + D_i + A_n + M_o + G_k + e_{inok}$$

where Y is the dependent, continuous variable, μ is the overall population of the mean, D_i is the fix effect of the diet used as substrate (i = DC, DF), B_j is the fix effect of the incubation buffer (j = low, medium, high pH), F_l is the fix effect of the microbial fraction (l = LIQ, LS), Tm is the fix effect of the inoculum collection time (m = 0 h, 3 h after feeding), A_n is the fix effect tof the inoculum adaptation to the diet (n = AF, AC), M_o is the fix effect of the inoculum preservation method (o = FRE, DEF, LIO), G_k is the random effect of the goat used as donor (k = 1, 2, 3, 4) and e is the residual error. When significant effects were detected, means were compared by Fisher's protected least significant difference test using the SPSS, version 21 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered statistically significant, with tendencies to differences at $P < 0.10$.

RESULTS

Experiment 1: In vivo rumen pH monitoring and in vitro buffering capacity

In vivo monitoring of the rumen pH showed a significant effect of the diet ($P = 0.003$) and sampling time ($P = 0.011$). Rumen pH for DC was lower than that observed for DF diets, with the greatest differences among diets observed between 2 and 4 h after feeding (**Fig. 1**). Experiment 1 showed that incubation of DC diet with rumen inoculum from animals adapted to such diet resulted in greater fermentation than those adapted to DF in terms of ammonia and total VFA concentrations, FOM, hydrogen production, asymptotic GP and higher molar proportions of propionate and butyrate (**Table 1**). By contrast, DF

led to higher rumen pH and acetate molar proportion than DC diet. As expected, incubation pH was affected by the buffer used. The buffer that generated the highest pH led to the greatest in vitro fermentation in terms of ammonia, total VFA concentrations, FOM, hydrogen production and asymptotic GP without affecting the VFA profile.

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

No differences in the fermentation parameters were noted between the positive controls used in both incubation runs. Significant interactions were observed between the effect of the diet used as substrate and the sampling time. Thus, in **Table 2**, the fix effect of the rumen fraction and the interaction between diet and sampling time are depicted. The rumen fraction used as inoculum had a minor impact on rumen fermentation: LIQ fraction, in comparison to LS, tended to increase propionate molar proportion and to decrease the levels of anaerobic fungi and methane production per unit of FOM. By contrast, the effects of diet and sampling time were more evident: the use of DC as substrate, in comparison to DF, promoted a greater fermentation extent in terms of higher levels of VFA and FOM, as well as higher asymptotic GP, GP rate, hydrogen production, hydrogen capture and methane emissions (mmol day^{-1}). This DC diet promoted a lower concentration of anaerobic fungi than DF diets but increased the protozoa levels measured either by quantitative PCR or optical counting without affecting the relative proportions of the main protozoal groups. Regarding the rumen sampling time, inocula taken 3 h after feeding, in comparison to 0 h, promoted a greater fermentation activity in terms of ammonia, total VFA, acetate molar proportion, FOM, asymptotic GP, GP rate, methane and hydrogen production. Inocula sampled at 3 h after feeding, in comparison to 0 h, tended to increase levels of bacteria and methanogens but lowered levels of anaerobic fungi and protozoa, promoting the favoured growth of *Diplodiniinae* in detriment to *Entodiniinae*. The differences between DC and DF diets in terms of in vitro pH, propionate and Isotricha proportion were more obvious when the inocula was taken at 3 h after feeding than before feeding, whereas the opposite was true for the molar proportion of isobutyrate, isovalerate and total protozoal counts (interaction D × T).

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

No differences in the fermentation parameters were detected between the positive controls across runs. Only two parameters showed significant interactions between the inoculum adaptation to the diet and the preservation method; thus, the results in **Table 3** are presented for the fix effect of the diet used as substrate and the interaction between the inoculum adaptation and the preservation method. As noted previously, DC diet promoted a greater microbial activity in terms of total VFA, FOM, GP rate, hydrogen production and hydrogen incorporation than DF diet. The microbial adaptation to the diet had a strong effect on the microbial activity in vitro: AC inocula, in comparison to AF, promoted higher levels of total VFA, FOM, asymptotic GP, GP rate, hydrogen production, hydrogen incorporation, propionate and butyrate molar proportions. By contrast, AF inocula led to higher acetate molar proportions than AC inocula. Microbial adaptation to the diet and diet used as substrate did not show significant interactions. Anaerobic fungi reached their highest concentration when the AF inoculum was incubated with DF diets in vitro, whereas subfamily *Diplodiniinae* decreased their proportion when the AF inoculum was incubated with DC diet (D × A).

Inocula preservation method was by far the factor with the greatest impact on the microbial activity, reaching the highest values for FRE inoculum. Microbial activity substantially decreased when using DEF inocula, whereas the LIO inoculum showed the lowest fermentative activity in vitro. This general pattern FRE > DEF > LIO was observed in terms of ammonia, total VFA, FOM, asymptotic GP, methane production, hydrogen production, hydrogen incorporation and in vitro concentration of methanogens, anaerobic fungi and protozoa. Holotrich protozoa were the most negatively affected by the preservation method, whereas the subfamily *Entodiniinae* was the most resilient. The effect of the preservation method was similar across substrates, although it was greatly affected by the donor animal diet (A × M): the negative impact of the preservation was more evident when the inoculum was adapted to forage than to concentrate diets. Thus, the lowest in vitro microbial activity (total VFA, GP, methane, methanogens and protozoa) was observed when AF inocula were lyophilized.

DISCUSSION

Experiment 1: In vivo rumen pH monitoring and in vitro buffering capacity

Rumen pH affects the rate and extent of microbial fermentation of feeds as a result of the balance between the production and absorption of fermentation products and saliva buffering capacity.[27] The in vivo study of the postprandial pH fluctuations showed that the greatest differences among diets occurred between 2 and 4 h after feeding (fermentation peak); thus, an intermediate time (3 h after feeding) was considered as the most appropriate time for rumen sampling.

Different in vitro approaches based on batch cultures have been developed to simulate the pattern of in vivo microbial fermentation in the rumen.[20,28,29] These systems have been designed to maintain the incubation pH relatively high and constant (6.7 – 6.8) by including bicarbonate buffer with a minor proportion of phosphate buffer in the medium. However, most rumen microbes are only metabolically active under a specific pH range,[3] which may limit the in vitro fermentation if the incubation buffering capacity is exceeded. To address this problem, three different bicarbonate concentrations in the incubation buffer were used[19] to cover the pH range observed in vivo. Our results showed that lowering the concentrations of bicarbonate promoted a progressive and concomitant decline in pH and microbial activity. Consequently, a decrease in the VFA concentration (−9%) and in vitro GP (−19%) was noted when the pH dropped from 6.5 to 5.8. Similar further reductions in VFA (−9%) and GP (−21%) were noted when the pH declined from 5.8 to 5.4. These observations indicated that the incubation pH affected the extent of the microbial fermentation across diets but kept similar VFA profile. More research is needed to clarify whether this situation was also associated with minor changes in the microbial community,[3] although it indicated that maximizing the incubation buffering capacity is recommended to prevent a buffer-related limitation of microbial activity in further experiments.

Experiment 2: Effect of microbial fraction, collection time and fermentation substrate

Several studies have demonstrated substantial differences between the rumen microbiota associated with either the liquid or the solid phase in relation to their chemical and microbiological composition.[30–32] Some studies have suggested that liquid-associated bacteria have higher activity as a result of a greater access to soluble and easily digestible

substrates.[33] Nevertheless, a recent study demonstrated that the rumen bacterial community associated with the feed particles had higher levels of fibrolytic microbes (i.e. Firmicutes) and bacterial diversity, and also established more complex microbial networks than liquid-associated bacteria.[34] Theoretically, using rumen content (liquids plus solids) as inoculum for in vitro incubations should increase the microbial fermentation as a result of the combination of a greater supply of fermentable material and solid-associated microbes. To discern between these two confounding factors, the LIQ inoculum was supplemented with autoclaved rumen solids to provide the proportional part of fermentable material but without viable solid-associated microbes. The results obtained showed that LS inoculum led to higher in vitro concentrations of anaerobic fungi (+0.29 log units) and methane production per unit of FOM (+17%), possibly as a result of the anaerobic fungi chemotaxis toward solid material[35] and more active degradation of structural carbohydrates.[36] However, it appears that the absence of viable solid-associated microbes in the LIQ inoculum was compensated for by a greater abundance of liquid-associated bacteria, resulting in microbial numbers and a fermentation pattern similar to that for LS inoculum when incubated with an identical substrate. These findings are in line with a recent study[37] revealing that rumen bacterial distribution in the rumen of Holstein cows determined using next generation sequencing was mainly affected by diet and individual cows rather than by the rumen fractions.

A diurnal variation in the availability of digestible nutrients in the rumen has been linked to the proliferation of rumen microbes, the accumulation of fermentation products and variations in the rumen pH.[38] In a study in which dairy cows were fed diets varying in protein and starch levels, rumen samples taken at 2.5 h after feeding, in contrast to those taken before feeding, had increased levels of fermentation products but lower microbial concentrations per unit of DM as a result of their dilution with feed.[36] In the present study, inocula taken 3 h after feeding also lowered the incubation pH and increased the microbial fermentation in terms of VFA (+12%), ammonia-nitrogen (+17%), asymptotic GP (+24%) and GP rate (+11%) compared to inocula taken before feeding. This fermentative situation was associated with higher levels of bacteria (+0.25 logs) and methanogens (+0.23 logs) in detriment to slow growing microbes such as protozoa (−0.53 logs) and anaerobic fungi (−0.27 logs). The available literature reveals important discrepancies in relation to the ideal rumen sampling time for minimizing diurnal variation in microbial activity,[39] possibly because this microbial activity is determined as much by the time of

rumen sampling relative to feeding as it is by feeding pattern, eating behaviour and other factors. To minimize the inocula variability across different donor animals, rumen sampling before feeding is generally recommended for in vitro feed evaluation studies because microbial activity and diversity of the inoculum are at their lowest.[16] However, this sampling strategy may not capture the 'true potential' that a nutritional strategy (e.g. rumen microbiota as probiotics) may have if a more diverse and active microbiome is sampled after feeding, as was the case in the present study.

Experiment 3: Effect of donor animal diet, fermentation substrate and inoculum preservation method

The results indicated that the diet consumed by the donor animal highly determined the microbial activity in the inoculum, although it did not affect methane emissions or the abundance of the main microbial groups, including protozoa. Thus, the importance of using rumen inoculum adapted to the same diets as those used for substrate in vitro is still debatable (A × D interaction).[16] One study noted a higher fermentation rate of native potato starch in vitro when the rumen inocula were obtained from donor animals adapted to a diet containing potato in contrast to non-diet-adapted rumen inocula.[40] Thus, feeding donor animals a similar diet to the substrate used in vitro may be advantageous to prevent carry-over effects in feed evaluation studies.[16] Nevertheless, the present study revealed a lack of interaction between the inoculum adaptation and the diet used in vitro, suggesting that the effect of the adaptation was substrate-independent. Several studies have noted that in vitro VFA production, bacterial concentration and degradability parameters of various feeds increased by raising the proportion of concentrate in the diet consumed by the donor ruminant.[41,42] Accordingly, it was hypothesized that using an inoculum with high microbial activity can result in a significant proportion of degraded carbohydrates transforming into fermentation products and CO2 gas rather than being incorporated into new microbial matter.[43] This hypothesis was supported by our findings because AC inocula, in contrast to AF inocula, led to a greater yield of fermentation products, in terms of VFA (+19%), asymptotic GP (+16) and GP rate (+26%), as well as a higher propionate production (+16%), although without differences in the microbial numbers after 24 h of incubation. Two studies have shown similar positive effects on productivity when young animals were inoculated with fresh rumen fluids, regardless of the diet consumed by the

donor animal.[7,44] However, this factor had long lasting effects on the structure of the rumen microbial community of the inoculated animals.[10]

There is additional controversy regarding the ideal preservation method for maintaining the microbial activity: Cone et al.,[45] after storing rumen inocula either at 39 °C or −20 °C for different time periods, reported similar in vitro GP values for rumen inocula stored for up to 4 h but decreased values for longer storage times and freezing procedures. In the present study, the greatest values of microbial fermentation and activity also were detected when FRE inoculum was used in vitro, possibly as a result of the main microbial groups (i.e. methanogens, anaerobic fungi and protozoa) having the highest concentrations. The use of DEF inoculum led to moderate decreases in the in vitro microbial activity in terms of VFA (−14%), asymptotic GP (−13%) and hydrogen production (−19%). Moreover, a substantial decrease in the ammonia-nitrogen levels (−96%) resulted in values below 50 mg L^{-1}, which is considered as the minimum concentration required for optimal microbial growth in vitro,[46] and could partially explain the lower microbial numbers observed in DEF inocula. Our findings agree with previous studies noting that freezing the rumen inocula (without cryopreservant) implied a delay in the initiation of the fermentation[47] and negatively affected the in vitro fermentation of fibrous feedstuff (−13%) but not that of starch as substrate.[17,48] Eukaryotic cells and Gram-negative bacteria involved in fiber degradation (i.e. *Fibrobacter succinogenes, Ruminococcus flavefacien*s or *Butyrivibrio fibrisolvens*) are particularly sensitive to mechanical damage caused by ice crystals and disruption of cell membranes; however, cryopreservants can partially overcome this problem.[49] Glycerol is one of the most common cryopreservants used in microbiology; however, it can be rapidly used as an energy source by rumen bacteria once the sample has been thawed, resulting in an increase in VFA production and the propionate molar proportion. Such an indirect artefact may bias the results.[47] To avoid this inconvenience, DMSO was used, despite its toxicity, to freeze the inoculum because it promotes similar in vitro digestibility as that of fresh inocula,[50] and allows a higher protozoal recovery rate (90% after 6 months of storage) than glycerol or ethylene glycol.[51] Because the toxicity of DMSO (i.e. to cells) increases with temperature, a sample equilibration step at 4 °C was used to maximize its effectiveness.[52] Despite these considerations, our freezing-thawing procedure had an important negative impact on the rumen eukaryotic cells such as anaerobic fungi (−0.14 logs) and protozoa cells (−0.70 logs), with holotrich protozoa being particularly

vulnerable to freezing (−86%). These results on protozoal impact should be interpreted carefully because of the intrinsic methodological limitations with respect to studying rumen protozoa as a result of their slow growth in vitro and their variable number of 18S rDNA copies per cell across species.[53]

The use of DEF inocula favoured the proliferation of total bacteria (+24 logs), which may explain the observed increase in the percentage of propionate (+25%), possibly because rumen amylases are more resistant to freezing than cellulases or xylanases.[54] The release of bacteria attached to the feed particles has been considered as justifying the higher values of viable colony counts in rumen fluid stored at 0 ∘C for 8 h compared to fresh rumen fluid.[55] However, the higher bacterial concentration observed when DEF (+0.24 logs) and LIO inocula (+0.08 logs) were incubated in vitro, in comparison to FRE inocula, was most likely a result of: (i) a higher bacterial recovery rate after the preservation in comparison to other microbial groups[49] and (ii) a greater bacterial growth during the in vitro incubation because lower competition with other microbial groups such as rumen protozoa.[53,56] A study that compared different preservation methods of rumen inoculum[47] concluded that cryopreservants are not required if the freezing procedure is carried out quickly using liquid nitrogen and a high container surface to sample volume ratio, resulting in similar in vitro gas production, VFA and bacterial diversity in fresh and defrosted inocula. Unfortunately, this latter study did not assess the impact of the preservation method on the eukaryotic cells, which are considered as the most vulnerable rumen microbes.[52]

Rumen fluid lyophilization has been explored as an alternative, aiming to standardize in vitro studies by decreasing the variation inherent to the technique and also to eliminate the need for constant access to donor animals. The results on the viability of rumen microbes after lyophilization are highly variable across studies depending on the freezing method, use of cryopreservant, storage conditions and microbial group considered.[57–59] The present study showed that lyophilization had a strong negative impact on microbial activity in terms of VFA (−51%), ammonia-nitrogen (−96%), hydrogen production (−51%) and asymptotic GP (−19%) compared to FRE inocula. Moreover, the lower GP rate (−69%) indicated a substantial delay in the re-activation of the rumen microbiome, which led to lower values of methanogens (−1.71 logs) and anaerobic fungi (−0.18 logs), as well as almost the complete wipe out of rumen protozoa (−2.66 logs). Subfamily *Entodiniiane* was the protozoa group that better dealt with the lyophilization process,

possibly as a result of their small size.[55] These findings justify the less positive effects of inoculating lyophilized rumen fluid,[14] in comparison to fresh inoculum,[9] with respect to growing lambs in terms of nitrogen retention and growth. The present study showed a substantial interaction between the rumen microbial adaptation to the diet and the preservation method considered: in general, FRE and LIO procedures had less damaging effects if the rumen microbes were adapted to concentrate rather than to forage diets. This interaction may rely on the different community structure and the lower bacterial and protozoal diversity generally reported in the rumen of animals fed high concentrate diets.[36,60] Further microbial adaptation processes based on the acceleration of the metabolic rate, energy spilling reactions and accumulation of starch particles have been described in the presence of high levels of available energy.[61] These adaptation strategies could help rumen microbes to better prepare their metabolism for further damaging processes, with the protective effect on the microbes being proportional to the severity of the preservation method.

CONCLUSIONS

The present study revealed that fresh rumen fluid sampled at 3 h after feeding from donor animals fed a high concentrate diet led to the greatest microbial numbers and in vitro fermentation rates. Thus, this inoculum is suggested to be the choice if the aim is to provide the most active rumen microbiota. Alternatively, defrosted rumen inoculum could be used, although this should take into consideration that a moderate decrease in microbial activity, but substantial in terms of rumen protozoa and anaerobic fungi, may occur. By contrast, lyophilized rumen inoculum promoted a substantial delay in the microbial reactivation process and a low microbial activity that could compromise its effectiveness as inoculum. The present study also noted that the adaptation of the donor animal to the diet used as fermentation substrate provided no advantages in comparison to non-adapted inocula. Although similar experiments are still needed to confirm these results in different ruminant species and under in vivo conditions, these findings should be considered during the design of further studies.

ACKNOWLEDGMENTS

This study was funded by the Economy and Competitiveness Spanish Ministry (Ref. AGL2017-86938-R) and the CSIC (PIE 201640E045).

REFERENCES

1 Krehbiel C, Rust S, Zhang G and Gilliland S, Bacterial direct-fed microbials in ruminant diets: performance response and mode of action. J Anim Sci 81:120 – 132 (2003).

2 McAllister T, Beauchemin K, Alazzeh A, Baah J, Teather R and Stanford K, The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Can J Anim Sci 91:193 – 211 (2011).

3 Hobson PN and Stewart CS, The Rumen Microbial Ecosystem. Springer Science & Business Media, London, UK (2012).

4 Wallace RJ and Newbold CJ, Probiotics for ruminants, in Probiotics, ed. by Ruller R. Springer, Dordrecht, Netherlands, pp. 317 – 353 (1992).

5 Kung L ed, Direct-fed Microbials for Dairy Cows and Enzymes for Lactating Dairy Cows: New Theories and Applications. Pennsylvania State Dairy Cattle Nutrition Workshop, Grantville, PA (2001).

6 Allison MJ, Bucklin J and Dougherty R, Ruminal changes after over- feeding with wheat and the effect of intraruminal inoculation on adaptation to a ration containing wheat. J Anim Sci 23:1164 – 1171 (1964).

7 Muscato T, Tedeschi L and Russell J, The effect of ruminal fluid preparations on the growth and health of newborn, milk-fed dairy calves. J Dairy Sci 85:648 – 656 (2002).

8 Zhong R, Sun H, Li G, Liu H and Zhou D, Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. Livest Sci 162:154 – 158 (2014).

9 Ewan R, Hatfield E and Garrigus U, The effect of certain inoculations on the utilization of urea or biuret by growing lambs. J Anim Sci 17:298 – 303 (1958).

10 De Barbieri I, Hegarty R, Silveira C, Gulino L, Oddy V, Gilbert R et al., Programming rumen bacterial communities in newborn Merino lambs. Small Rum Res 129:48 – 59 (2015).

11 Pounden W and Hibbs J, The influence of the ration and rumen inoculation on the establishment of certain microorganisms in the rumens of young calves1. J Dairy Sci 31:1041 – 1050 (1948).

12 Abo-Donia F, Ibrahim G, Nadi S and Sayah M, Effect of inoculating new born lambs with fresh or lyophilized rumen fluid on rumen activity and lamb performance. J Anim Sci 7:409 – 422 (2011).

13 Ziolecka A, Osin´ska Z and Ziolecki A, The effect of stabilized rumen extract on growth and development of calves. J Anim Physiol Anim Nutr 51:13 – 20 (1984).

14 Waymack L, Effect of feeding lyophilized rumen contents on adaptation to urea diet by lambs. J Anim Sci 43:712 – 714 (1976).

15 Durmic Z, Ramírez-Restrepo CA, Gardiner C, O'Neill CJ, Hussein E and Vercoe PE, Differences in the nutrient concentrations, in vitro methanogenic potential and other fermentative traits of tropical grasses and legumes for beef production systems in northern Australia. J Sci Food Agric 97:4075 – 4086 (2017).

16 Yáñez-Ruiz DR, Bannink A, Dijkstra J, Kebreab E, Morgavi D, O'Kiely P et al., Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants – a review. Anim Feed Sci Technol 216:1 – 18 (2016).

17 Hervás G, Frutos P, Giráldez FJ, Mora MJ, Fernández B and Mantecón ÁR, Effect of preservation on fermentative activity of rumen fluid inoculum for in vitro gas production techniques. Anim Feed Sci Technol 123:107 – 118 (2005).

18 Agricultural and Food Research Council (AFRC), Energy and protein requirements of ruminant. An advisory manual prepared by the ARC Technical Committee on response to nutrients. CAB International, Wallingford (1993).

19 Amanzougarene Z and Fondevila M, Fitting of pH conditions for the study of concentrate feeds fermentation by the in vitro gas-production technique. Anim Prod Sci (2017). https://doi.org/10.1071/AN16097.

20 Theodorou MK, Williams BA, Dhanoa MS, McAllan AB and France J, A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim Feed Sci Technol 48:185 – 197 (1994).

21 Arco-Pérez A, Ramos-Morales E, Yáñez-Ruiz D, Abecia L and Martín-García A, Nutritive evaluation and milk quality of including of tomato or olive by-products silages with sunflower oil in the diet of dairy goats. Anim Feed Sci Technol 232:57 – 70 (2017).

22 Weatherburn MW, Phenol-hypochlorite reaction for determination of ammonium. Anal Chem 39:971 – 974 (1967).

23 Dehority BA ed, Laboratory Manual for Classification and Morphology of Ruminal Ciliate Protozoa. CRC Press, Inc., Boca Raton, FL (1993).

24 Abecia L, Toral PG, Martin-Garcia AI, Martinez G, Tomkins NW, Molina-Alcaide E et al., 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 (2012).

25 Marty RJ and Demeyer DI, Effect of inhibitors of methane production on fermentation pattern and stoichiometry in vitro using rumen contents from sheep given molasses. Br J Nutr 30:369 – 376 (1973).

26 France J, Dijkstra J, Dhanoa MS, Lopez S and Bannink A, Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations. Br J Nutr 83:143 – 150 (2000).27 Bertipaglia L, Fondevila M, Van Laar H and Castrillo C, Effect of pelleting and pellet size of a concentrate for intensively reared beef cattle on in vitro fermentation by two different approaches. Anim Feed Sci Technol 159:88 – 95 (2010).

28 Menke K, Raab L, Salewski A, Steingass H, Fritz D and Schneider W, The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor in vitro. J Agric Sci 93:217 – 222 (1979).

29 Tilley JM and Terry R, A two-stage technique for the in vitro digestion of forage crops. Grass Forage Sci 18:104 – 111 (1963).

30 Martin C, Bernard L and Michalet-Doreau B, Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. J Anim Sci 74:1157 – 1163 (1996).

31 Vlaeminck B, Fievez V, Demeyer D and Dewhurst RJ, Effect of forage: concentrate ratio on fatty acid composition of rumen bacteria isolated from ruminal and duodenal digesta. J Dairy Sci 89:2668 – 2678 (2006).

32 Belanche A, de la Fuente G, Pinloche E, Newbold CJ and Balcells J, Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J Anim Sci 90:3924 – 3936 (2012).

33 Cecava MJ, Merchen NR, Gay LC and Berger LL, Composition of ruminal bacteria harvested from steers as influenced by dietary energy level, feeding frequency, and isolation techniques. J Dairy Sci 73:2480 – 2488 (1990).

34 Belanche A, Newbold CJ, Lin W, Stevens PR and Kingston-Smith AH, A systems biology approach reveals differences in the dynamics of colonization and degradation of grass vs. hay by rumen microbes with minor effects of vitamin E supplementation. Front Microbiol 8:1 – 18 (2017).

35 Orpin CG and Bountiff L, Zoospore chemotaxis in the rumen phycomycete Neocallimastix frontalis. Microbiol 104:113 – 122 (1978).

36 Belanche A, Doreau M, Edwards JE, Moorby JM, Pinloche E and New- bold CJ, Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. J Nutr 142:1684 – 1692 (2012).

37 Ji SK, Zhang HT, Yan H, Azarfar A, Shi HT, Alugongo G et al., Comparison of rumen bacteria distribution in original rumen digesta, rumen liquid and solid fractions in lactating Holstein cows. J Anim Sci Biotechnol 8:1 – 16 (2017).

38 Gustafsson A and Palmquist D, Diurnal variation of rumen ammonia, serum urea, and milk urea in dairy cows at high and low yields1. J Dairy Sci 76:475 – 484 (1993).39 Menke KH, Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim Res Dev 28:7 – 55 (1988).

40 Cone J and Van Gelder A, Adaptation of the rumen microbial population to native potato starch degradation determined with the gas production technique and the nylon bag technique. J Anim Physiol Anim Nutr 90:511 – 518 (2006).

41 Martínez M, Ranilla M, Tejido M, Saro C and Carro M, The effect of the diet fed to donor sheep on in vitro methane production and ruminal fermentation of diets of variable composition. Anim Feed Sci Technol 158:126 – 135 (2010).

42 Nagadi S, Herrero M and Jessop N, The influence of diet of the donor animal on the initial bacterial concentration of ruminal fluid and in vitro gas production degradability parameters. Anim Feed Sci Technol 87:231 – 239 (2000).

43 Jessop N and Herrero M, Influence of soluble components on parame- ter estimation using the in vitro gas production technique. Anim Sci 62:626 – 627 (1996).

44 De Barbieri I, Hegarty R, Silveira C and Oddy V, Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. Small Rum Res 129:37 – 47 (2015).45 Cone J, Van Gelder A, Bachmann H, (eds). Influence of inoculum source, dilution and storage of rumen fluid on gas production profiles, in Proceedings of the British Society of Animal Science, Cambridge University Press, Edinburgh, UK, (2000).

46 Griswold KE, Apgar GA, BoutonJ and Firkins JL, Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. J Anim Sci 81:329 – 336 (2003).

47 Prates A, De Oliveira J, Abecia L and Fondevila M, Effects of preser- vation procedures of rumen inoculum on in vitro microbial diversity and fermentation. Anim Feed Sci Technol 155:186 – 193 (2010).

48 Mohamed R, Chaudry A, Rowlinson P, (eds). Fresh or frozen rumen contents as sources of inocula to estimate in vitro degradation of ruminant feeds, in Proceedings of the British Society of Animal Science, Cambridge University Press, Edinburgh, UK, (2002).

49 Roger V, Fonty G, Andre C and Gouet P, Effects of glycerol on the growth, adhesion, and cellulolytic activity of rumen cellulolytic bacteria and anaerobic fungi. Curr Microbiol 25:197 – 201 (1992).

50 Denek N, Can A and Avci M, Frozen rumen fluid as microbial inoculum in the two-stage in vitro digestibility assay of ruminant feeds. S Afr J Anim Sci 40:251 – 256 (2010).

51 Abdel-Aziz H, Hassan H, Abd-El-Raof Y, Abou-Zeina HA and Galbt S, Trails for cryopreservation of rumen protozoa in sheep. Global Vet 1:9 – 16 (2007).

52 Nsabimana E, Kišidayová S, Macheboeuf D, Newbold C and Jouany J, Two-step freezing procedure for cryopreservation of rumen ciliates, an effective tool for creation of a frozen rumen protozoa bank. Appl Environ Microbiol 69:3826 – 3832 (2003).

53 Newbold CJ, de la Fuente G, Belanche A, Ramos-Morales E and McEwan N, The role of ciliate protozoa in the rumen. Front Microbiol 6:1 – 14 (2015).54 Hristov AN, McAllister TA, Xu Z and Newbold CJ, Proteolytic activity in ruminal fluid from cattle fed two levels of barley grain: a comparison of three methods of determination. J Sci Food Agric 82:1886 – 1893 (2002).55 Dehority BA and Grubb JA, Effect of short-term chilling of rumen contents on viable bacterial numbers. Appl Environ Microbiol 39:376 – 381 (1980).

56 Belanche A, De la Fuente G, Moorby JM and Newbold CJ, Bacterial protein degradation by different rumen protozoal groups. J Anim Sci 90:4495 – 4504 (2012).

57 Kapp J, Hintz RL and Wagner DJ, Lyophilized rumen fluid for use in in vitro dry matter disappearance studies with grain. Anim Sci Res 1:42 – 44 (1979).

58 Phillips B, Latham M and Sharpe ME, A method for freeze drying rumen bacteria and other strict anaerobes. J Appl Microbiol 38:319 – 322 (1975).

59 Teather RM, Maintenance of laboratory strains of obligately anaerobic rumen bacteria. Appl Environ Microbiol 44:499 – 501 (1982).

60 Fernando SC, Purvis HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG et al., Rumen microbial population dynamics during adaptation to a high-grain diet. Appl Environ Microbiol 76:7482 – 7490 (2010).

61 Denton BL, Diese LE, Firkins JL and Hackmann TJ, Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. Appl Environ Microbiol 81:1832 – 1838 (2015).

Tables and Figures

Table 1. Effect of the diet used as substrate and incubation buffer on the rumen microbial activity *in vitro* (Experiment 1)

Diet	DC			DF				*P*-values		
Buffer	Low pH	Medium pH	High pH	Low pH	Medium pH	High pH	SED[a]	D	B	D × B
Fermentation										
pH	5.19	5.51	6.32	5.68	6.08	6.64	0.131	**	***	NS
Ammonia-N (mg L^{-1})	435	322	661	211	192	276	118.9	*	**	NS
Total VFA (mmol L^{-1})	80.5	91.3	103.1	66.4	70.5	75.3	9.890	†	***	NS
Acetate (mmol mol^{-1})	544	538	538	699	702	699	22.90	***	NS	NS
Propionate (mmol mol^{-1})	230	230	230	160	161	169	20.10	**	NS	NS
Butyrate (mmol mol^{-1})	161	162	154	106	102	95.9	11.50	***	NS	NS
Isobutyrate (mmol mol^{-1})	12.8	14.1	17.1	10.3	10.4	10.8	2.556	†	NS	NS
Valerate (mmol mol^{-1})	32.1	34.3	32.6	11.2	11.0	11.3	4.770	***	NS	NS
Isovalerate (mmol mol^{-1})	20.1	22.8	28.1	13.2	13.6	14.2	4.360	*	NS	NS
FOM[b] (mg)	422	476	529	327	346	367	51.30	*	**	NS
Gas production										
Asymptotic GP (mL)	82.7	106	130	50.2	62.8	77.8	12.55	**	***	NS
GP rate (μL h^{-1})	147	139	139	132	114	94.9	18.38	NS	*	NS
H_2 production[b] (mmol)	8.32	9.41	10.44	6.69	7.08	7.48	1.057	*	*	NS

[a]Standard error of the difference among means (n = 4) for the interaction among diets (D: DC concentrate *versus* DF forage diet) and buffers (B: Low *versus* Medium *versus* High pH). Within a row means without a common superscript differ ($P < 0.05$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.10$. NS,not significant.

[b]Data stoichiometrically calculated based on the VFA production.[25]

Table 2. Effect of the microbial fraction used as inoculum, collection time and diet used as substrate on the rumen microbial activity in vitro (Experiment 2)

	Fraction		Time post-feeding and diet					*P*-values						
			0h		3h									
Treatments	LIQ	LS	DC	DF	DC	DF	SED[a]	D	F	T	D × F	D × T	F × T	D × F × T
Fermentation														
pH	6.38	6.25	6.38 [a]	6.75 [b]	5.63 [c]	6.51 [ab]	0.204	**	NS	***	NS	**	*	NS
Ammonia-N (mgL^{-1})	237	296	296	195	319	256	57.70	NS	NS	†	NS	NS	**	NS
Total VFA ($mmol\ L^{-1}$)	95.9	93.5	98.2	80.8	109	90.8	9.040	**	NS	*	NS	NS	NS	NS
Acetate ($mmolmol^{-1}$)	642	656	578	665	636	719	20.10	**	NS	***	NS	NS	NS	NS
Propionate ($mmolmol^{-1}$)	191	181	197 [b]	187 [b]	197 [b]	165 [a]	16.03	NS	†	†	NS	*	†	NS
Butyrate ($mmolmol^{-1}$)	123	123	151	101	142	98.1	11.91	**	NS	NS	NS	NS	NS	NS
Isobutyrate ($mmolmol^{-1}$)	11.5	9.94	18.9 [b]	14.2 [a]	4.81 [c]	4.88 [c]	1.569	NS	***	***	NS	***	***	NS
Valerate ($mmolmol^{-1}$)	13.1	13.0	22.3	13.4	10.8	5.75	2.079	**	NS	***	NS	†	NS	NS
Isovalerate ($mmolmol^{-1}$)	18.7	16.9	33.5 [b]	20.2 [a]	9.56 [c]	7.94 [c]	3.407	*	NS	***	NS	***	*	NS
FOM (mg)[b]	479	467	496	393	558	446	45.20	**	NS	*	NS	NS	NS	NS
Gas production														
Asymptotic GP (mL)	198	207	206	156	252	197	22.52	*	NS	***	NS	NS	NS	NS
GP rate (μLh^{-1})	58.2	63.2	63.8 [a]	51.3 [c]	76.0 [b]	51.5 [c]	6.780	***	NS	†	NS	NS	*	NS
Methane ($mmol\ L^{-1}$)	5.77	6.04	5.83	5.29	6.51	5.99	1.166	†	NS	**	NS	NS	NS	NS
Methane ($mmol\ day^{-1}$)	0.86	0.95	0.78	0.65	1.18	1.01	0.198	*	NS	***	NS	NS	NS	NS
Methane ($mmol\ g\ FOM^{-1}$)	1.72	2.01	1.56	1.69	2.19	2.30	0.340	†	*	***	NS	NS	NS	NS
H_2 produced (mmol)[b]	9.65	9.47	9.94	7.92	11.25	9.13	0.902	**	NS	*	NS	NS	NS	NS
Hydrogen incorporated (mmol)[b]	4.17	4.07	4.66	3.20	5.12	3.50	0.512	***	NS	NS	NS	NS	NS	NS
Hydrogen incorporation rate ($mmolmol^{-1}$)	424	425	465	405	458	386	20.65	***	NS	NS	NS	NS	NS	NS
Microbes (log copy $g\ DM^{-1}$)														
Bacteria	11.9	11.9	11.8	11.7	12.2	11.9	0.304	NS	NS	†	†	NS	NS	NS
Methanogens	8.78	8.59	8.55	8.59	8.86	8.74	0.241	NS	NS	†	NS	NS	NS	NS
Anaerobic fungi	6.79	7.08	6.84	7.57	6.45	6.90	0.204	**	**	***	†	NS	NS	NS
Protozoa	9.70	9.69	10.2	9.47	9.69	9.43	0.280	*	NS	†	NS	NS	NS	NS
Protozoal counts														
Total ($log\ cells\ mL^{-1}$)	4.73	4.72	5.37 [b]	4.69 [a]	4.43 [c]	4.43 [c]	0.181	†	NS	***	NS	***	NS	NS
Subf. *Entodiniinae* (%)	87.3	86.6	92.6	88.6	80.3	86.3	10.79	NS	NS	*	NS	†	NS	NS
Subf. *Diplodiniinae* (%)	4.28	5.50	1.93	5.28	6.13	6.22	3.351	NS	NS	*	†	NS	NS	NS
Ophryoscolex (%)	2.45	2.86	0.88	0.03	9.65	0.07	6.600	NS	NS	†	NS	NS	NS	NS
Isotricha (%)	0.80	0.52	0.82 [b]	0.70 [b]	0.03 [a]	1.09 [b]	0.518	NS	NS	NS	NS	*	NS	NS
Dasytricha (%)	5.21	4.50	3.80	5.41	3.88	6.32	4.369	NS	NS	NS	NS	NS	NS	NS

[a] Standard error of the difference among means ($n = 4$) for the interaction among diets (D: DC concentrate, DF forage), microbial fraction (F: LIQ liquid *versus* LS liquid + solid) and sampling time (T: 0 h *versus* 3 h after feeding). Within a row, means without a common lowercase letter are significantly different ($P < 0.05$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, † $P < 0.10$. NS, not significant.

[b] Data stoichiometrically calculated based on the VFA production.[25]

Table 3. Effect of the donor animal diet, diet used as substrate and preservation method on the rumen microbial activity *in vitro* (Experiment 3)

	Diet		Inoculum adaptation and preservation													
			FRE		DEF		LIO			*P*-values						
Treatments	DC	DF	AC	AF	AC	AF	AC	AF	SED[a]	D	A	M	D × A	D × M	A x M	D × A × M
Fermentation																
pH	6.34	6.54	6.00	6.56	6.27	6.71	6.37	6.75	0.171	***	*	***	NS	NS	†	NS
Ammonia-nitrogen (mg L^{-1})	125	129	348	359	12.8	12.4	13.4	15.9	59.60	NS	NS	***	NS	NS	†	NS
Total VFA (mmol L^{-1})	109	103	149 ^{b}	122 ^{c}	131 ^{a}	102 ^{d}	88.2 ^{e}	45.2 ^{f}	14.99	*	†	***	NS	NS	*	NS
Acetate (mmol mol^{-1})	607	627	568 ^{c}	660 ^{a}	537 ^{cd}	657 ^{a}	527 ^{d}	754 ^{b}	26.49	†	***	**	NS	NS	***	NS
Propionate (mmol mol^{-1})	216	206	199 ^{a}	186 ^{a}	264 ^{b}	217 ^{a}	265 ^{b}	137 ^{c}	19.85	NS	**	**	NS	NS	***	NS
Butyrate (mmol mol^{-1})	123	114	161	107	130	81.0	152	79.4	27.83	NS	**	*	NS	NS	NS	NS
Isobutyrate (mmol mol^{-1})	9.71	9.90	13.6 ^{b}	11.3 ^{a}	11.8 ^{a}	9.44 ^{c}	5.38 ^{e}	7.38 ^{f}	1.550	NS	NS	***	NS	†	***	NS
Valerate (mmol mol^{-1})	27.3	25.4	31.9 ^{a}	18.3 ^{c}	33.8^{a}	18.6 ^{c}	42.9 ^{b}	12.6 ^{c}	7.960	NS	*	NS	NS	NS	**	NS
Isovalerate (mmol mol^{-1})	16.3	17.5	26.1 ^{b}	17.3 ^{a}	22.9 ^{b}	16.9 ^{a}	8.00 ^{c}	10.1 ^{c}	4.640	NS	NS	***	NS	NS	**	NS
FOM (mg)[b]	554	517	772 ^{b}	603 ^{c}	662 ^{a}	491 ^{d}	467 ^{d}	218 ^{e}	71.49	**	*	***	NS	NS	*	NS
Gas production																
Asymptotic GP (mL)	178	173	235 ^{b}	156 ^{c}	198 ^{a}	143 ^{c}	179 ^{a}	140 ^{c}	26.01	NS	*	***	NS	**	*	**
GP rate (μL h^{-1})	83.1	71.1	117 ^{b}	78.4 ^{a}	125 ^{b}	81.1 ^{d}	37.3 ^{c}	23.3 ^{c}	15.32	*	*	***	NS	NS	*	NS
Methane (mmol L^{-1})	4.04	4.18	6.2	5.75	5.18	5.34	0.83	1.32	1.016	NS	NS	***	NS	NS	NS	NS
Methane (mmol day^{-1})	0.64	0.58	1.26 ^{b}	0.71 ^{bc}	0.91 ^{a}	0.60 ^{c}	0.10 ^{a}	0.06 ^{d}	0.226	NS	NS	***	NS	NS	*	NS
Methane (mmol g FOM^{-1})	1.01	0.94	1.59	1.17	1.35	1.23	0.19	0.30	0.284	NS	NS	***	NS	NS	NS	NS
Hydrogen produced (mmol)[b]	10.9	10.3	15.4 ^{b}	12.2 ^{a}	12.7 ^{a}	9.72 ^{c}	8.95 ^{c}	4.51 ^{d}	1.347	**	*	***	NS	NS	*	NS
H_2 incorporated (mmol)[b]	5.14	4.55	7.54	4.74	6.98	4.01	4.65	1.15	0.857	**	**	***	NS	NS	NS	NS
Hydrogen incorporation rate (mmol mol^{-1})	444	420	489 ^{a}	389 ^{c}	543 ^{b}	412 ^{c}	501 ^{a}	256 ^{d}	33.90	*	***	***	NS	NS	***	NS
Microbes (log copy g DM^{-1})																
Bacteria	12.8	12.8	12.9	12.5	13.1	12.8	12.9	12.6	1.309	NS	NS	**	NS	NS	NS	NS
Methanogens	8.00	7.96	8.61 ^{ab}	8.46 ^{a}	8.51 ^{ab}	8.66 ^{b}	7.15 ^{c}	6.49 ^{d}	0.189	NS	NS	***	NS	NS	***	NS
Anaerobic fungi	7.54	7.57	7.56 ^{a}	7.76 ^{b}	7.54 ^{a}	7.50 ^{bc}	7.46 ^{c}	7.49 ^{bc}	0.083	NS	NS	***	*	NS	**	**
Protozoa	8.52	8.42	9.40	9.50	7.82	7.82	7.58	7.81	0.573	NS	NS	***	NS	NS	†	NS
Protozoal counts																
Total (log cells mL^{-1})	3.62	3.75	4.71 ^{b}	4.90 ^{b}	4.05 ^{a}	4.05 ^{a}	2.68 ^{c}	1.61 ^{d}	0.615	NS	NS	***	NS	*	***	*
Subf. Entodiniinae (%)	91.9	89.4	84.5	88.0	91.5	91.5	90.4	96.9	7.050	†	NS	**	†	NS	NS	NS
Subf. Diplodiniinae (%)	4.37	6.02	4.52 ^{bc}	5.84 ^{ab}	5.38 ^{ab}	5.38 ^{ab}	7.97 ^{b}	1.25 ^{c}	4.505	**	NS	NS	*	NS	***	NS
Ophryoscolex (%)	0.70	0.53	1.58	0.21	1.13	1.13	0.45	0	0.752	NS	†	NS	NS	NS	NS	NS
Isotricha (%)	0.18	0.71	0.45	1.31	0.25	0.25	0.52	0	0.726	*	NS	†	NS	NS	†	NS
Dasytricha (%)	2.83	3.38	8.92	4.65	1.76	1.76	0.63	1.88	3.862	NS	NS	**	NS	NS	NS	NS

[a] Standard error of the difference among means (n = 4) for the interaction among diets (D: DC concentrated *versus* DF forage), inoculum adaptation (A: AC concentrate *versus* AF forage) and preservation method (M: FRE fresh *versus* DEF defrosted *versus* LIO lyophilized). Within a row, means without a common lowercase letter are significantly different ($P < 0.05$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.10$. NS, not significant.

[b] Data stoichiometrically calculated based on the VFA production.[25]

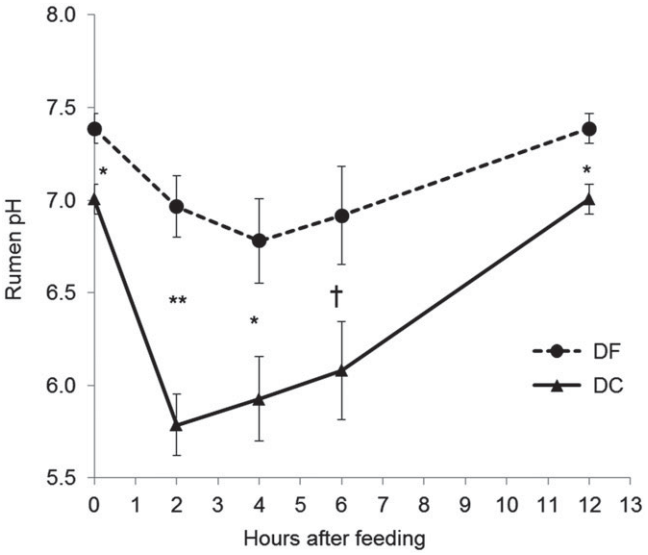

Figure 1. Dynamics of the rumen pH after feeding in goats fed forage (DF) versus concentrate (DC) based diets. $^{**}P < 0.01$, $^{*}P < 0.05$, †$P < 0.10$.

Publication 2

Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems

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Journal of Dairy Science 103 (6): 5047-5060

DOI: https://doi.org/10.3168/jds.2019-18002

ABSTRACT

Ruminants are born with an undeveloped physical, metabolic, and microbial rumen. Rumen development is limited under artificial rearing systems when newborn animals are separated from the dam, fed on milk replacer, and weaned at an early age. This study aims to evaluate the effects of early-life inoculation of young ruminants with rumen fluid from adult animals. Eighty newborn goat kids were randomly allocated to

1 of 4 experimental treatments and inoculated daily from d 1 to wk 11 with autoclaved rumen fluid (AUT), fresh rumen fluid obtained from adult goats fed either a forage diet (RFF) or concentrate-rich diet (RFC), or absence of inoculation (CTL). Goat kids were artificially reared with ad libitum access to milk replacer, starter concentrate, and forage hay. Blood was sampled weekly and rumen microbial fermentation was monitored at 5 (preweaning), 7 (weaning), and 9 wk of age (postweaning). Results indicated that inoculation with fresh rumen fluid accelerated the rumen microbial and fermentative development before weaning. As a result, RFC and RFF animals had higher solid feed intake (+73%), rumen concentrations of ammonia-N (+26%), total volatile fatty acids (+46%), butyrate (+50%), and plasma β-hydroxybutyrate (+48%), and lower milk intake (−6%) than CTL and AUT animals at wk 5. Inoculation with fresh inoculum also promoted early rumen colonization by a complex and abundant protozoal community, whereas CTL animals remained protozoa free. Although all kids experienced moderate growth retardation during 1 wk after weaning, inoculation with fresh rumen fluid favored the weaning process, leading to 2.2 times higher weight gain than CTL and AUT animals during wk 8. Some of these advantages were retained during the postweaning period and RFF and RFC animals showed higher forage intake (up to +44%) than CTL and AUT animals with no detrimental effects on feed digestibility or stress levels.

The superior microbial load of RFC compared with RFF inoculum tended to provide further improvements in terms of forage intake, plasma β-hydroxybutyrate, and rumen protozoa, whereas AUT inoculation provided minor (if any) advantages with respect to CTL animals. Although no differences were noted on animal growth, this study suggests that early life inoculation of goat kids with rumen microbiota can represent an effective strategy to accelerate the rumen development, facilitating a smooth transition from milk to solid feed and to the potential implementation of early weaning strategies.

Key words: artificial rearing, goat kid, microbial inoculation, rumen development, weaning

Introduction

Due to increasing global demand for animal-derived food and mounting pressure over land use, there is an urgent need to make livestock production systems more efficient and sustainable (Kim et al., 2019). The viability of any dairy farm depends, to a large extent, on a successful program of rearing newborns for replacement, which implies keeping low mortality rates and feeding costs but assuring an optimal anatomical and functional development to ensure animal performance in adult life (Khan et al., 2016).

Ruminants are born with a physically and metabolically underdeveloped rumen and function as monogastrics over the first weeks of life. Chronologically the rumen development can be divided in 3 phases (Lane et al., 2000): preruminant phase (0–3 wk) in which animals are fed on milk, which bypasses the rumen through the esophageal groove; transition phase (3–8 wk); and ruminant phase (from 8 wk) in which ruminants are only fed solid diets. Thus, initiation of solid feed consumption, rumen microbial colonization, establishment of rumen fermentation and enzymatic capacity, increment in rumen size, growth and differentiation of papillae, maturation of salivary apparatus, and development of rumination behavior are all needed as the preruminant shifts from milk to solid feed. A smooth transition from a monogastric to ruminant animal is needed to ensure a correct anatomical, microbiological, and physiological development to face the weaning nutritional challenge and ultimately to warrant optimal performances later in life (Heinrichs, 2005). This transition generally occurs with no further problems when newborns are reared with the dam, allowing a natural rumen microbial transfer to the offspring (Belanche et al., 2010; Abecia et al., 2014) and feeding behavior learning from the dam (De Paula Vieira et al., 2012). In contrast, in intensive dairy systems newborns are typically separated from their dams after birth and fed either milk replacer or whole milk. This absence of contact with adult animals has been shown to limit the rumen microbial development with negative effects on feed digestibility and productivity (Belanche et al., 2019c). The magnitude of these detrimental effects increases when artificial rearing is combined with early weaning programs to minimize milk-replacer costs, which may lead to weaning-associated shock (Lu and Potchoiba, 1988). Thus,

future early-life nutritional strategies should be focused on mimicking the physiological events that occur under natural rearing conditions with the dam.

Among the range of options, probiotics offer a source of microorganisms that could accelerate and modulate the microbial colonization at ruminal and intestinal levels (McAllister et al., 2011). It may be hypothesized that probiotics collected from the rumen may establish more easily into the microbial community than "foreign" species. Several studies have explored the concept of direct-fed microbials in preruminants and adult ruminants (McAllister et al., 2011). Inoculation of adult ruminants with fresh (Rodríguez and Rodríguez, 2011) or lyophilized rumen fluid (Waymack, 1976) has shown minor effects on rumen fermentation and animal performances, which can be explained by the difficulty of modifying a mature and well-established rumen microbial community (Weimer, 2015). In contrast, the developing rumen in the newborn may represent a unique opportunity for the manipulation of rumen microbial colonization (Yáñez-Ruiz et al., 2015). Several authors have evaluated the effects of inoculating young lambs with fresh (Ewan et al., 1958; De Barbieri et al., 2015b) or lyophilized rumen fluid (Abo-Donia et al., 2011; Zhong et al., 2014). Others have evaluated with different degrees of success the effect of inoculating young ruminants with cell-free rumen fluid, bacterial polysaccharides (Muscato et al., 2002), and fermentation products (Górka et al., 2018; O'Hara et al., 2018). The great variability observed across these and other studies suggests that more attention must be given to the selection of the microbial inoculum and to the rearing system because most of these studies were performed under natural milk feeding with the dam, which could minimize the effect of the inoculation. Moreover, to date no studies have been reported using goat kids as experimental animals.

The objective of this study was to optimize the artificial rearing systems of goat kids by implementing new nutritional strategies in early life. It was hypothesized that the inoculation of young goat kids with different types of rumen fluid from adult animals could modify or accelerate the rumen microbial colonization pattern toward a desirable anaerobic fermentation during the preweaning period, facilitate the transition to solid diet postweaning, and increase productivity or decrease feeding costs.

Materials and Methods

Preparation and Characterization of the Rumen Microbial Inocula

Animal procedures were conducted by trained personnel according to the Spanish guidelines (RD 53/2013) and protocols were approved by the Ethical Committee for Animal Research (EEZ-CSIC) regional government (09/03/2017). Eight Murciano-Granadina goats fitted with permanent rumen cannula were used as rumen fluid donors (mean ± SD; 46 ± 4 kg of BW). Goats were randomly distributed into 2 groups (n = 4) and fed either a forage diet (50% alfalfa hay and 50% oat hay) or a concentrate-rich diet (70% concentrate and 30% of the same forage mixture as above). Each group of goats was divided into 2 pairs, which were used as rumen fluid donors on alternate days. Forages were chopped to between 4 and 6 cm in length by passing through a garden shredder (Bioline 1000, Atika, Ahlen, Germany). The chemical composition of the forage mixture (in DM) was 90.6% OM, 15.9% CP, 59.4% NDF, 36.6% ADF, 9.5% ADL, and 1.6% ether extract (EE), whereas the pelleted commercial concentrate (Lactación Rumiantes, Macob, Granada, Spain) was 95.1% OM, 20.5% CP, 25.4% NDF, 7.3% ADF, 2.1% ADL, and 4.5% EE. Diets were offered at 1.2 times maintenance level divided into 2 equal meals (0800 and 1600 h) and animals were adapted to the diets during 2 wk before being used as donors.

Rumen fluids from donor animals fed forage (RFF) or concentrate diets (RFC) were collected daily at 3 h after the morning feeding from rumen-cannulated goats fed either the forage diet or a high concentrate-rich diet. A 20-cm-long handle sampling scoop was used to collect rumen contents through the rumen cannula from different parts of the dorsal sac in the rumen (Ramos-Morales et al., 2014). Rumen contents (approximately 100 mL per animal) were strained through double cheesecloth, bubbled with CO2, pooled by diet, maintained at 37°C in a prewarmed thermal flask, and immediately administered as inoculum to young kids. Autoclaved inoculum (AUT) was prepared weekly by mixing equal volumes of RFF and RFC inocula from all donors and autoclaved at 115°C for 30 min to destroy all viable microbes while maintaining fermentation products. A subsample from each pooled inocula was taken every 2 wk (resulting on 4 samples per inocula) to describe their composition in terms of percentage of DM, rumen fermentation (pH, VFA, ammonia, and lactate), and abundance of the main microbial groups by quantitative PCR (qPCR; Table 1).

Inoculation Experiment

A total of 80 newborn Murciano-Granadina goat kids born within a 2-wk period were used. At birth animals were weighed, separated from their mothers, and fed with natural colostrum via esophageal probe (approximately 200 mL divided in 2 doses). Animals were randomly allocated to 1 of 4 experimental treatments. During this allocation process, sex and initial BW were considered, resulting in similar sex distribution and initial BW across treatments. Although the mother effect was not considered, siblings were always allocated to different treatments. Treatment consisted of oral inoculation from d 1 to wk 11 of age with AUT, RFF, RFC, or absence of inoculation (CTL). Inoculation was conducted daily using a syringe connected to a 10-cm tube to drench the inocula (2.5 mL/animal during wk 1 and 5 mL/animal thereafter), making sure that all volume was swallowed.

Animals from different treatments were physically separated by a 2-m-wide corridor to prevent physical contact. To monitor feed intake, animals within each treatment were distributed in 5 contiguous pens with an equal number of males and females. All 4 animals within the same pen number (1 to 5 according to birth order) had similar age (maximum 2 d difference) and were handled and sampled on the same day across treatments. All animals were raised on milk replacer (declared composition in DM: 92.8% OM, 24.0% CP, and 22.0% EE), which was offered ad libitum and freshly prepared twice per day (Univet Spray, Cargill, Barcelona, Spain). From wk 2, animals had free access to the same forage mixture that has been described for the donor goats and to pelleted starter concentrate (0–14 Rumiantes Transición, Macob, Granada, Spain) with 3 mm diameter. The starter had the following chemical composition (in DM): 94.9% OM, 22.6% CP, 3.19% NDF, 8.72% ADF, 3.38% ADL, 4.83% EE; and ingredient list: wheat bran, corn, sunflower seeds, barley, wheat, soybean flour, CaCO3, NaCl, and vitamin-mineral premix. Animals were weaned at 7 wk of age by progressively decreasing milk powder concentration for 4 d. Intakes were measured daily in each pen and BW was recorded weekly. Feed efficiency was calculated as a ratio between the ADG and ME intake based on their declared composition and the Fundación Española para el Desarrollo de la Nutrición Animal feed tables (de Blas et al., 2010).

Sampling and Analysis

Blood samples (4 mL) were collected at 0900 h from the jugular vein at 1, 3, 5, 7, 9, and 11 wk of age and placed in tubes with anticoagulant (K3-EDTA). Blood was centrifuged at 2,000 × g for 15 min at room temperature and plasma was collected to determine glucose and BHB levels using an auto-analyzer (BA400, BioSystems, Barcelona, Spain). Rumen microbial fermentation was studied at wk 5 (preweaning), 7 (weaning), and 9 (postweaning). Rumen content was withdrawn from each animal by orogastric intubation at 0900 h as previously described (Ramos-Morales et al., 2014): a flexible polyvinyl chloride tube (8 mm internal diameter) with about 20 holes of 5 mm diameter in the 12-cm probe head was warmed up with hot water, externally lubricated with sunflower oil, and inserted to a depth of approximately 60 cm via the esophagus. Rumen samples (approximately 50 mL) were obtained using an electric vacuum pump (down to 9 mbar; Vacuubrand MZ 2C, Wertheim, Germany) connected to a sterile collection container. Samples were filtrated through cheesecloth and solids were discarded given the small and variable proportion of solids in the samples. Then, pH was measured and 4 subsamples were taken for VFA, ammonia, lactate, and protozoal optical counting, respectively (Belanche et al., 2019c). During the postweaning period (10 wk of age), fecal samples were collected from each animal during 3 consecutive days, which together with feed offered and orts were used to determine feed digestibility using the Mn as internal marker (Hidiroglou, 1979). For feed, orts, and feces, chemical composition was determined as previously described (Arco-Pérez et al., 2017), whereas Mn was measured by optical-ICP spectrometry (720-ES ICP-OES spectrometer, Agilent Technologies, Santa Clara, CA). Rumen concentrations of individual VFA were de- termined by a GC system coupled with a Flame Ionization Detector (Auto-System, Perkin Elmer, Waltham, MA), whereas ammonia concentration was measured using a colorimetric method (Weatherburn, 1967). Protozoal concentration and classification were visually determined (Dehority, 1993) using a Sedgewick rafter counting chamber and an optical microscope (Nikon Labophot, Tokyo, Japan). For inocula characterization, freeze-dried samples were bead-beated for 1 min (Mini- BeadBeater, Biospect Products, Bartlesville, OK) and DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain). Concentrations of total bacteria, methanogenic archaea, protozoa, and anaerobic fungi were determined by quantitative PCR as previously described (Belanche et al., 2016). Primer sets used were as follows: 16S rRNA forward GTGSTGCAYGGYTGTCGTCA and reverse ACGTCRTCCMCACCTTCCTC for total bacteria; the mcrA gene forward TTCGGTGGATCDCARAGRGC and reverse GBARGTCGWAWCCGTAGAATCC

for methanogenic archaea; the 18S rRNA forward GAGGAAGTAAAAGTCGTAACAAGGTTTC and reverse CAAATTCACAAAGGGTAGGATGAT for anaerobic fungi; and the 18S rRNA forward GCTTTCGWTGGTAGTGTATT and reverse CTTGCCCTCYAATCGTWCT for protozoa. Cycling conditions were 95 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 30s, and 72 °C for 55 s; and 72 °C for 1 min. The absolute amount of each microbial group, expressed as DNA copies/mL of fresh matter, was determined using serial dilutions of known amounts of standards. The qPCR standards consisted of the plasmid pCR 4-TOPO (Invitrogen, Carlsbad, CA) with inserted 16S, mcrA, or 18S gene fragments from each microbial group, respectively.

To assess the potential effect of the experimental treatments on postweaning stress, cortisol concentration in hair was measured (Moya et al., 2013). Briefly, a surface of 25 cm2 in the dorsal neck was shaved at weaning (wk 7) and the hair grown during the post-weaning was collected at wk 10. Hair samples (250 mg) were washed twice for 3 min with isopropanol, dried, and bead-beated 3 times for 2 min at maximum speed. Hair samples were sonicated 30 min and incubated with isopropanol 18 h at 50°C with constant mixing. Supernatant (0.8 mL) was extracted, evaporated, reconstituted in PBS (0.2 mL), and cortisol concentration was measured using a commercial kit (Cortisol ELISA Saliva, ALPCO, Salem, NH).

Calculation and Statistical Analyses

Two animals per treatment were removed from the study due to deaths and health problems over the course of the study. Protozoa optical counting and qPCR data were log10 transformed to attain normal distributions. Rumen protozoa and fermentation data were analyzed based on a repeated measures mixed-effects model (residual maximum likelihood) as follows:

$$Y_{ijkl} = \mu + I_i + T_j + (I \times T)_{ij} + G_k + A(G)_l + e_{ijkl},$$

where Y_{ijkl} is the dependent, continuous variable, μ is the overall population of the mean, I_i is the fixed effect of the inoculation (i = CTL vs. AUT vs. RFF vs. RFC), T_j is the fixed effect of the sampling time or age (j = 5 vs. 7 vs. 9 wk), $(I \times T)_{ij}$ is the interaction term, G_k is the random effect of the pen considered as a block (k = 1 to 5), $A(G)_l$ is the random

effect of the animal nested to the pen (l = 1 to 80), and e_{ijkl} is the residual error. For BW and ADG data, the sex was also included as a fixed factor. The pen was considered as the experimental unit for feed intake and feed efficiency. When significant effects were detected, means were compared by Fisher's protected least significant difference test using SPSS software (version 21.0, IBM Corp., New York, NY). Significant effects were declared at $P < 0.05$ and tendency to difference at $P < 0.1$.

Results

Description of the Inocula

Substantial differences were detected across the 3 rumen inocula (Table 1), with RFC inoculum being higher in concentrations of DM, lactate, total VFA, propionate, butyrate, bacteria, anaerobic fungi, and protozoa than RFF inoculum. On the contrary, RFF inoculum had higher pH and acetate molar proportion than RFC inoculum. No differences were noted between RFC and RFF inocula on the methanogen concentrations determined by qPCR and on the protozoal group distribution after visual analysis. Autoclaved rumen fluid had intermediate rumen fermentation values in comparison to RFF and RFC given that AUT was generated by mixing equal volumes of both inocula. Autoclaved inocula showed high lactate concentration and undetectable concentrations of bacteria, methanogens, and fungal DNA, as well as the complete disruption of rumen protozoa since no whole protozoal cells were evident after visual inspection.

Feed Intake, Digestibility and Blood Metabolites

Starter feed and forage intakes remained low until weaning and increased exponentially during the post-weaning period (Figure 1), but with differences for forage intake across treatments as revealed by the interaction between inoculation and time ($P < 0.001$). Inoculation of young animals with fresh rumen fluid (RFF and RFC) promoted higher concentrate intake (Figure 1A) during the preweaning period (wk 3, 4, 5, and 6), as well as higher forage intake (Figure 1B) during the postweaning period (wk 5, 9, 10, and 11). On the contrary, animals in treatments CTL and AUT tended to have a higher milk replacer intake (1.35 vs. 1.27 L/d, $P = 0.096$).

The analysis of the blood metabolites (Figure 1) showed a progressive decline of the plasma glucose ($P < 0.001$) and an increase of the BHB concentration as the trial progressed ($P < 0.001$). However, the concentration of BHB showed a significant interaction between inoculation and time ($P < 0.001$), indicating that animals inoculated with fresh rumen fluid had increasingly higher BHB levels at wk 5 ($P = 0.052$), 7 ($P = 0.027$), 9 ($P = 0.004$), and 11 ($P < 0.001$) in comparison to CTL and AUT animals. On the contrary, inoculation with fresh rumen fluid tended to lower blood glucose concentration at wk 7 ($P = 0.10$) and 9 ($P = 0.012$).

To further investigate the effect of the inoculation on the transition from liquid to solid diet, the feed digestibility and cortisol level in hair were measured 3 wk after weaning (Table 2). No differences in total DMI were noted at wk 10 across treatments despite diets being offered ad libitum; however, the forage intake represented a higher proportion of the total DMI in animals inoculated with fresh rumen fluid (mostly RFC) than in CTL animals ($P = 0.012$). Despite these differences in the forage-to-concentrate ratio across treatments, no significant differences in total-tract apparent digestibility were noted for OM, N, NDF, and ADF. The analysis of the cortisol level in hair showed no significant differences across treatments.

Rumen Protozoa and Microbial Fermentation

Control animals remained protozoa free throughout the entire duration of the study (Table 3), whereas for the rest of the treatments a progressive increase in protozoal concentration occurred over time ($P < 0.001$) with increasing numbers of *Isotricha* spp., *Dasytricha* sp., and *Entodiniinae* to the detriment of subfamily *Diplodiniinae*. The significant interaction between inoculation and time observed for the protozoal concentration ($P = 0.025$) indicated that inoculation of young animals with fresh rumen fluid promoted early rumen colonization by an abundant and diverse protozoal population. As a result RFF and RFC had higher protozoal concentration than AUT goat kids across times, with the highest concentration being observed in RFC animals at 9 wk of age. For the AUT treatment, the protozoal community was mostly conformed by subfamily *Entodiniinae* (average 98% across sampling times) and no holotrich protozoa were detected in these animals throughout the experiment. On the contrary, RFF and RFC animals had a lower abundance of *Entodiniinae* (average 79% across sampling times) and higher abundances

of other protozoal groups such as *Diplodiniinae* (9.2%), *Ophryoscolex* spp. (4.7%), *Isotricha* spp. (2.3%), and *Dasytricha* sp. (4.5%) in comparison to AUT animals.

An escalation in the rumen fermentative activity was observed over time as noted by increasing values for total VFA, propionate, butyrate, and valerate along with decreasing values for ammonia, acetate, isobutyrate, and isovalerate molar proportions (Table 4, $P < 0.001$). The significant interaction between inoculation and time observed in most fermentation variables indicated that the inoculation with fresh rumen fluid accelerated the rumen fermentative development. As a result, at 5 wk of age RFF and RFC animals had higher total VFA concentration than CTL and AUT animals (33.9 vs. 23.1 mM, $P < 0.001$); these figures are similar to those observed at wk 7 across treatments (33.1 mM). During the preweaning period, RFF animals had the highest ammonia-N ($P = 0.028$) and butyrate levels ($P = 0.002$), whereas AUT animals had the highest isobutyrate ($P < 0.001$) and isovalerate levels ($P = 0.001$). At weaning (wk 7), AUT animals had the highest levels of valerate and isovalerate across treatments. The largest differences in rumen microbial fermentation profiles were detected during the postweaning period (wk 9) as RFF and RFC animals showed higher butyrate and isobutyrate molar proportions and lower total VFA and propionate levels than AUT and CTL animals.

Animal Performance and Feeding Costs

The animals' sex had a significant effect on ADG (Table 5) as males showed higher BW than females from wk 7, as well as higher ADG from wk 3 (data not shown). No differences were noted in the BW and ADG across treatments during the entire duration of this study; however, for ADG there was a significant interaction between inoculation and time ($P = 0.004$). This interaction suggested that although the weaning process led to an important decrease in the ADG during the first week after weaning (wk 8), the inoculation with fresh rumen fluid helped to ameliorate this decrease, leading to higher ADG values than observed in AUT and CTL animals. Given the different ME density among milk replacer, concentrate, and forage, the feed efficiency was calculated based on total ME intake (Table 5). Feed efficiency decreased over time, but this decrease was modulated by the inoculation as an interaction was noted ($P = 0003$). This interaction suggested that animals inoculated with fresh rumen fluid tended to have higher feed

efficiency than CTL and AUT animals during the following week to weaning (63.5 *vs.* 17.3 kg/Mcal of ME).

The accumulated feed consumption and feeding costs were calculated over the duration of this study (11 wk) to assess the economic feasibility of these interventions (Table 6). During the milk feeding period, RFC animals (followed by RFF) tended to have lower milk intake than CTL and AUT animals (10.1 vs. 11.0 kg of milk powder/animal). No differences were noted on the concentrate intake across treatments (8.83 ± 0.68 kg of DM/animal), whereas RFC (followed by RFF animals) had substantially higher forage intake than CTL and AUT animals (6.35 vs. 4.52 kg of DM/animal). The total feeding cost up to wk 11 was calculated taking in consideration the different purchased feed costs [2.24, 0.34, and 0.17 €/kg (1€ is approximately equal to USD $1.15) for milk replacer, concentrate, and forage, respectively]. Animals within the RFC treatment tended to have lower milk replacer costs than CTL and AUT animals ($P = 0.096$), whereas the opposite was true for forage costs ($P = 0.001$), and no differences were noted for the concentrate feeding cost across animals. As a result, no differences were noted across treatments on the overall feeding cost per animal or per kilogram of BW gained.

Discussion

Selection and Description of the Inocula

In a previous publication we evaluated the microbial activity of different types of rumen inocula, sampling times, and preservation methods, concluding that fresh rumen fluid sampled at 3 h after feeding provides the most diverse and active inoculum based on in vitro incubations (Belanche et al., 2019b). The present in vivo study builds upon this previous observation and indicated that RFC inoculum had higher concentration of total bacteria, anaerobic fungi, protozoa, VFA, propionate, and butyrate than RFF inoculum. This superior microbial activity for RFC than for RFF inocula has also been demonstrated in vitro (higher VFA, ammonia, and gas production) after having been incubated with the same substrate (Belanche et al., 2019b). Although the microbes in the inocula were not taxonomically characterized, the different VFA profile suggests the presence of a different microbial community (Li et al., 2019).

Within the group of probiotics targeting the rumen (e.g., *Saccharomyces*, *Aspergillus*, *Megasphaera elsdenii*, and *Prevotella bryantii*), various modes of action have been suggested including stabilization of ruminal pH, oxygen scavenging, and microbial modulation (Yoon and Stern, 1995). However, there is still controversy whether similar effects could be achieved inoculating microbial extracts (Muscato et al., 2002; Uyeno et al., 2015) or fermentation products such as lactate or butyrate (Górka et al., 2018). To explore this hypothesis, autoclaved rumen fluid was used as inoculum because it would provide a comparable amount of fermentation products to fresh rumen fluid (including VFA, ammonia, peptides, and microbial polysaccharides), but without viable cells, as noted by the undetectable concentration of microbial DNA and intact protozoal cells after optical inspection.

Effect of Inoculation Before Weaning

During the preruminant phase (0–3 wk) there is a substantial increase in the microbial mass in the rumen, this increment being higher in animals with natural rather than artificial rearing (Abecia et al., 2014). However, the inoculation with fresh rumen fluid accelerated the transition phase from liquid to solid diet (3–8 wk), resulting in 68% higher concentrate intake and 12% higher forage intake than CTL and AUT animals. Nevertheless, CTL and AUT animals compensated this situation with a slightly higher milk replacer intake (+6.3%), resulting in similar ME intake across treatments. Several works have indicated that during this transition phase the presence of solid feed in the rumen (preferably as forage) exerts a physical stimuli favoring rumen anatomical development (Beharka et al., 1998), whereas high milk intake favors BW gain but does not prepare the rumen for successful weaning (Meale et al., 2016).

Whereas rumen bacteria and methanogens are early rumen colonizers (Abecia et al., 2014), protozoa colonize the rumen later because they are highly sensitive to oxygen and require direct contact between young and adult animals for an effective transmission (Bird et al., 2010). As a result, CTL animals remained protozoa free during the entire duration of the study, as we have previously described in artificially reared lambs (Belanche et al., 2019c). A natural sequence of rumen colonization has been described for the different protozoal families starting with *Entodiniinae*, followed by *Diplodiniinae* and *Ophryoxcolex* spp. and finishing with holotrichs (Williams and Coleman, 1992). The

visual microscopy examination of the protozoal community confirmed this colonization sequence and showed that inoculation with fresh rumen fluid accelerated this process. The presence of a small protozoal concentration (2.65 log10 cells/mL), mostly composed of *Entodinium*, in AUT animals located in 3 contiguous pens suggested that they may have accidentally been cross-faunated before wk 5. The lack of holotrichs in AUT animals throughout the study indicated an incomplete rumen protozoal colonization given the inherent difficulty of holotrichs to become established in the rumen of young ruminants, even under natural milk feeding conditions (Belanche et al., 2010, 2011).

Fermentation end products can be found in the rumen of goat kids as early as the first week of life (Abecia et al., 2014). The presence of fermentation products (mostly VFA) and low pH act as chemical stimuli for the rumen epithelial development (Sander et al., 1959), with butyrate being the most effective followed by propionate and acetate (Baldwin and McLeod, 2000). Our results indicated that the acquisition of microbial populations and the higher solid feed intake triggered fermentation activity in goat kids during the preweaning period, as previously noted in lambs (Abo-Donia et al., 2011; De Barbieri et al., 2015b). As a result, at wk 5 the animals inoculated with fresh rumen fluid had higher (+47%) total VFA concentrations than their CTL and AUT counterparts and similar to those observed at wk 7 across treatments (33 mM). This inoculation also enhanced the concentration of butyrate (+50%) and ammonia-N (+26%) in comparison to CTL and AUT animals at wk 5. Butyrate is transformed into BHB in the rumen wall during absorption; therefore, the higher plasma BHB level observed in animals inoculated with fresh rumen fluid (+48%) clearly indicates a higher VFA production and absorption than in CLT and AUT animals before weaning. Several factors could explain this enhanced fermentative activity such as the higher solid feed intake (+75%) and the presence of rumen protozoa (Eugène et al., 2004; Khan et al., 2016). A meta-analysis revealed that presence of rumen protozoa, in comparison to defaunated animals, increased rumen OM and NDF digestibility, total VFA, and butyrate concentration as a result of their fibrolytic activity (Newbold et al., 2015). Butyrate has been described as one of the main fermentation products derived from most rumen protozoa (Williams and Coleman, 1992), whereas large protozoa such as Ophryroscolex, Epidinium, Polyplastron, and Eudiplodinium have higher fibrolytic activity (endoglucanase and xylanase) than *Entodinium* spp. and holotrichs (Takenaka et al., 2004). Thus, the presence of an abundant fibrolytic protozoal population at wk 5 in animals inoculated with fresh rumen fluid could

explain the higher rumen fermentation activity than in AUT animals, as previously described in lambs inoculated with fresh (De Barbieri et al., 2015b) or lyophilized rumen fluid (Abo-Donia et al., 2011).

All these observations suggest that during the pre-weaning phase (wk 5) RFF and RFC animals had a more developed rumen (both microbial and fermentative) than their CTL and AUT counterparts. At weaning (wk 7) animals inoculated with RFC showed higher levels of plasma BHB (+40%) and lower levels of glucose (−7%) as indicators of a metabolic transition from pre-ruminant to ruminant (Baldwin et al., 2004); however, most differences in terms of rumen fermentation (e.g., ammonia-N and VFA) disappeared across treatments. The similar acetate-to-propionate ratio observed at wk 7 agrees with the similar forage to concentrate ratio consumption across treatments. Possibly the increased feed intake noted in CTL and AUT animals from wk 5 to 7 as compensatory response, and the increase in protozoal numbers in AUT animals, could explain this lack of differences.

Effect of Inoculation After Weaning

Regarding the third phase of the rumen development (from wk 8), several authors have identified that animals inoculated with fresh rumen fluid had greater DMI than noninoculated animals postweaning (Zhong et al., 2014; De Barbieri et al., 2015b). Our study showed no differences in the postweaning DMI across treatments, but demonstrated that animals inoculated with fresh rumen fluid (particularly with RFC) had increased preference for forage (representing up to 45.5% of the diet), whereas CTL and AUT animals preferred concentrate (67% of the diet). Zhong et al. (2014) reported an increase in the apparent digestibility for DM (+15%) and NDF (+35%) when weaned lambs were inoculated with fresh rumen fluid. Belanche et al. (2019a) found similar results in naturally reared lambs in comparison to those which were artificially reared. This enhanced feed digestibility during postweaning has been associated with higher bacterial (De Barbieri et al., 2015a), protozoal, and anaerobic fungal diversity (Belanche et al., 2019c). Our study showed no differences on the apparent digestibility, possibly because the lower microbial development expected in CTL and AUT animals was compensated by a preferential intake of starter feed as source of easily digestible carbohydrates. This starter preference could also explain their higher levels of total VFA (+21%), propionate

(+29%), and blood glucose (+4.6%). On the contrary, the forage preference and high protozoal levels observed in RFF and RFC animals agreed with their higher rumen butyrate (+35%) and blood BHB concentrations (+22%).

With regard to differences between fresh inocula, De Barbieri et al. (2015b) reported similar short-term increments in rumen protozoa, total VFA, acetate, and bacterial diversity (De Barbieri et al., 2015a) when lambs were inoculated once a week with fresh inocula obtained from donor sheep, which were fed diets rich in either protected fat or coconut oil in comparison with the control group. However, productive responses in this later study were limited given the lack of differences between the 2 types of inocula. Our study showed a similar trend, but the greater concentration of microbes and fermentation products observed in RFC than in RFF inocula would provide further improvements in terms of forage intake, plasma BHB, rumen protozoa, and animal growth during the postweaning period. In this study the daily inoculation was maintained until 4 wk after weaning since it has been reported that although the initial microbial community establishment is affected by early-life interventions, postweaning factors also have a major influence on adult communities and production outcomes (Dill-McFarland et al., 2017). Further taxonomic and functional characterization of the rumen microbiota could elucidate whether RFF or RFC inocula could be used as a rumen microbial programing strategy by favoring the further utilization of forage or concentrate diets later in life (Yáñez-Ruiz et al., 2015). In addition, further studies using a lower inoculation frequency should be performed to make this approach feasible under farm conditions.

With regard to the use of stimulatory compounds in early life, previous studies have shown that inoculation of microbial polysaccharides, cell-free rumen fluid (Muscato et al., 2002), and VFA (mostly butyrate) can stimulate the anatomical (Górka et al., 2018) and microbiological rumen development (O'Hara et al., 2018) with associated positive productive responses. In our study, animals inoculated with autoclaved rumen fluid had higher levels of isobutyrate (+22%) and isovalerate (+41%) at wk 7 in comparison to other treatments. These isoacids have been described to positively affect bacterial growth, microbial protein synthesis, N retention, and fiber digestion in adult ruminants (Muller, 1987; Liu et al., 2008). However, no substantial improvements of AUT inoculation on rumen fermentation and productivity were observed.

Animal performance

In a similar study to this one, De Barbieri et al. (2015a) concluded that rumen fermentation and microbiome composition in lambs can be changed by diet or inoculation with rumen fluid before weaning; however, these changes did not necessarily result in improved performances (De Barbieri et al., 2015b). Our study agrees with the aforementioned because animal performance evaluated up to wk 11 was unaffected by early-life inoculation. The presence of a complex protozoal population in the rumen of RFF and RFC animals positively affected the rumen energy metabolism (i.e., higher VFA production and fiber digestion), but this effect could be in part compensated by the negative effect on rumen protein metabolism through bacterial predation by protozoa and the low protozoal contribution to the microbial protein flow in young ruminants (Belanche et al., 2011b). Both factors could result in similar performances across treatments (Belanche et al., 2011a; Newbold et al., 2015). The higher rumen concentration of protein degradation products such as ammonia-N (+26%) at wk 5 and isobutyrate (+32%) at wk 9 in animals inoculated with fresh rumen fluid support this hypothesis.

Weaning is a critical phase in ruminant production. Abrupt and early weaning may reduce labor and feeding costs but may cause severe weaning shock, stress, and growth retardation (Khan et al., 2016). Two different weaning programs, based on the weight or the age at weaning, have been described (Lu and Potchoiba, 1988). Weaning by weight is considered a low-risk approach because it prevents unhealthy or undernourished kids from being weaned too early. According to Teh et al. (1984), goat kids can be weaned when they reach 3 times their birthweight (8.1 kg in our study). On average this weight was achieved by d 39 of age across treatments (less than 7 wk), suggesting that weaning could have been implemented earlier. This observation may explain the lack of differences in the cortisol concentration during the postweaning period, suggesting similar stress levels across treatments (Moya et al., 2013). Such lack of differences in cortisol levels may also indicate that the daily manipulation of the animals during the inoculation process did not increase the overall stress levels in comparison to absence of inoculation in CTL kids. With regard to weaning by age, Teh et al. (1984) compared the effects of several ages (4, 6, 8, and 10 wk) on animal performance, concluding that 8 wk is the optimum for goat kids. Our study showed that weaning at wk 7 was also successful and did not induce weight loss or health problems. Although all kids experienced moderate growth retardation during 1 wk after weaning, inoculation with fresh rumen

fluid minimized this weaning shock, leading to 2.2 times higher ADG and 3.7 times higher feed efficiency than CTL and AUT animals during wk 8. Independent of the weaning program considered, weaning should be performed when kids have a sufficient rumen anatomical and fermentative development. It has been suggested that animals should consume at least 30 g/d of solid feed before weaning (Lu and Potchoiba, 1988). Kids inoculated with fresh rumen fluid met this requirement by wk 5, whereas 9 extra days were required by their CTL and AUT counterparts. Thus, the higher rumen fermentative development in RFF and RFC kids at wk 5 (e.g., VFA and BHB) suggested that these animals could have succeeded if they went through an early weaning at 5 wk of age.

Cost Analysis

It has been suggested that early weaning could substantially reduce production costs (Khan et al., 2016). The economic profitability of artificial rearing depends essentially on the cost of milk replacer and the availability of specialized labor (Delgado-Pertíñez et al., 2009). Over this 11-wk study no statistical differences in the total feeding cost across treatments were observed, possibly because all animals were weaned at the same age (wk 7) and the higher milk feeding cost noted in CTL and AUT kids (+1.45€) was partially compensated by the higher forage feeding cost in their RFF and RFC counterparts (+0.22€). On average, milk replacer cost was 24€/animal, representing 86% of the total feeding cost across treatments. In this study the labor-associated costs were not assessed, but results indicated that inoculation with fresh rumen fluid could accelerate the rumen development, making it feasible to perform an early weaning at 6 or even 5 wk of age. This could lead to a decrease in the milk feeding cost by 20 or 35%, respectively, and could contribute to the economic viability of intensive dairy goat farms.

Conclusions

This study demonstrated that daily inoculation of young goat kids with fresh rumen fluid from adult animals facilitated early rumen colonization by rumen protozoa, which, together with higher solid feed intake, promoted greater rumen VFA production and absorption during the preweaning period. This intervention minimized the weaning shock

as inoculated animals did not experience growth retardation during the postweaning period, making feasible the potential implementation of early weaning strategies. On the contrary, inoculation of autoclaved rumen fluid resulted in negligible (if any) effects on rumen development. Further studies are needed to describe the effect of these strategies on the rumen microbiome, the persistency of the effects under different dietary situations, and the long-term implications on animal productivity.

Acknowledgements

This study was funded by the Spanish government through the project AGL2017-86938-R and the Training Program for Academics grant, Madrid, Spain (FPU16/01981). The authors thank Isabel Jimenez and Rosa Serrano (Estación Experimental del Zaidín, Granada, Spain) for their assistance with the animal care and sample analyses. The authors have not stated any conflicts of interest.

References

Abecia, L., E. Ramos-Morales, G. Martínez-Fernandez, A. Arco, A. Martín-García, C. Newbold, and D. Yáñez-Ruiz. 2014. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Anim. Prod. Sci. 54:1449–1454. https://doi.org/10.1071/AN14337.

Abo-Donia, F., G. Ibrahim, S. Nadi, and M. Sayah. 2011. Effect of inoculating new born lambs with fresh or lyophilized rumen fluid on rumen activity and lamb performance. J. Am. Sci. 7:409–422.

Arco-Pérez, A., E. Ramos-Morales, D. Yáñez-Ruiz, L. Abecia, and A. Martín-García. 2017. Nutritive evaluation and milk quality of including of tomato or olive by-products silages with sunflower oil in the diet of dairy goats. Anim. Feed Sci. Technol. 232:57–70. https://doi.org/10.1016/j.anifeedsci.2017.08.008.

Baldwin, R. L., and K. R. McLeod. 2000. Effects of diet forage: concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. J. Anim. Sci. 78:771–783. https://doi.org/10.2527/2000.783771x.

Baldwin, R. L., K. R. McLeod, J. L. Klotz, and R. N. Heitmann. 2004. Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J. Dairy Sci. 87:55–65. https://doi.org/10.3168/jds.S0022-0302(04)70061-2.

Beharka, A. A., T. Nagaraja, J. Morrill, G. Kennedy, and R. Klemm. 1998. Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. J. Dairy Sci. 81:1946–1955. https://doi.org/10.3168/jds.S00220302(98)75768-6.

Belanche, A., L. Abecia, G. Holtrop, J. A. Guada, C. Castrillo, G. de la Fuente, and J. Balcells. 2011a. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. J. Anim. Sci. 89:4163–4174. https://doi.org/10.2527/jas.2010-3703.

Belanche, A., J. Balcells, G. de la Fuente, D. R. Yañez-Ruíz, M. Fondevila, and L. Calleja. 2010. Description of development of rumen ecosystem by PCR assay in milk-fed, weaned and finished lambs in an intensive fattening system. J. Anim. Physiol. Anim. Nutr. (Berl.) 94:648–658. https://doi.org/10.1111/j.1439-0396.2009.00952.x.

Belanche, A., J. Cooke, E. Jones, H. Worgan, and C. Newbold. 2019a. Short-and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. Animal 13:740–749. https://doi.org/10.1017/S1751731118002100.

Belanche, A., G. de la Fuente, D. R. Yanez-Ruiz, C. J. Newbold, L. Calleja, and J. Balcells. 2011b. Technical note: The persistence of microbial-specific DNA sequences through gastric digestion in lambs and their potential use as microbial markers. J. Anim. Sci. 89:2812–2816. https://doi.org/10.2527/jas.2010-3193.

Belanche, A., E. Jones, I. Parveen, and C. J. Newbold. 2016. A metagenomics approach to evaluate the impact of dietary supplementation with Ascophyllum nodosum or Laminaria digitata on rumen function in Rusitec fermenters. Front. Microbiol. 7:299. https:// doi.org/10.3389/fmicb.2016.00299.

Belanche, A., J. M. Palma-Hidalgo, I. Nejjam, R. Serrano, E. Jiménez, A. I. Martín-García, and D. R. Yáñez-Ruiz. 2019b. In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. J. Sci. Food Agric. 99:163–172. https://doi.org/10.1002/jsfa.9157.

Belanche, A., D. R. Yáñez-Ruiz, A. P. Detheridge, G. W. Griffith, A. H. Kingston-Smith, and C. J. Newbold. 2019c. Maternal vs artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environ. Microbiol. 21:4360–4377. https://doi.org/10.1111/1462-2920.14801.

Bird, S. H., R. Hegarty, and R. Woodgate. 2010. Modes of transmission of rumen protozoa between mature sheep. Anim. Prod. Sci. 50:414–417. https://doi.org/10.1071/AN09216.

De Barbieri, I., R. Hegarty, C. Silveira, L. Gulino, V. Oddy, R. Gilbert, A. Klieve, and D. Ouwerkerk. 2015a. Programming rumen bacterial communities in newborn Merino lambs. Small Rumin. Res. 129:48–59. https://doi.org/10.1016/j.smallrumres.2015.05.015.

De Barbieri, I., R. Hegarty, C. Silveira, and V. Oddy. 2015b. Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. Small Rumin. Res. 129:37–47. https://doi.org/10.1016/j.smallrumres.2015.05.017.

de Blas, C., G. Mateos, and P. García-Rebollar. 2010. Tablas FEDNA de composición y valor nutritivo de alimentos para la fabricación de piensos compuestos. 3rd ed. FEDNA, Madrid, Spain.

Dehority, B. A. 1993. Laboratory Manual for Classification and Morphology of Ruminal Ciliate Protozoa. CRC Press Inc., Boca Ra- ton, FL.

Delgado-Pertíñez, M., J. Guzmán-Guerrero, Y. Mena, J. Castel, P. González-Redondo, and F. Caravaca. 2009. Influence of kid rearing systems on milk yield, kid growth and cost of Florida dairy goats. Small Rumin. Res. 81:105–111. https://doi.org/10.1016/j.smallrumres.2008.12.007.

De Paula Vieira, A., M. A. G. von Keyserlingk, and D. M. Weary. 2012. Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before and after weaning from milk. J. Dairy Sci. 95:3218–3224. https://doi.org/10.3168/jds.2011-4821.

Dill-McFarland, K. A., J. D. Breaker, and G. Suen. 2017. Microbial succession in the gastrointestinal tract of dairy cows from 2 weeks to first lactation. Sci. Rep. 7:1–12.

Eugène, M., H. Archimède, and D. Sauvant. 2004. Quantitative meta- analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livest. Prod. Sci. 85:81–97. https://doi.org/10.1016/S0301-6226(03)00117-9.

Ewan, R., E. Hatfield, and U. Garrigus. 1958. The effect of certain inoculations on the utilization of urea or biuret by growing lambs. J. Anim. Sci. 17:298–303. https://doi.org/10.2527/jas1958.172298x.

Górka, P., Z. Kowalski, R. Zabielski, and P. Guilloteau. 2018. Invited review: Use of butyrate to promote gastrointestinal tract development in calves. J. Dairy Sci. 101:4785–4800. https://doi.org/10.3168/jds.2017-14086.

Heinrichs, A. 2005. Rumen development in the dairy calf. Adv. Dairy Technol. 17:179–187.

Hidiroglou, M. 1979. Manganese in ruminant nutrition. Can. J. Anim. Sci. 59:217–236. https://doi.org/10.4141/cjas79-028.

Khan, M., A. Bach, D. Weary, and M. von Keyserlingk. 2016. Transitioning from milk to solid feed in dairy heifers. J. Dairy Sci. 99:885–902. https://doi.org/10.3168/jds.2015-9975.

Kim, S. W., J. F. Less, L. Wang, T. Yan, V. Kiron, S. J. Kaushik, and X. G. Lei. 2019. Meeting global feed protein demand: Challenge, opportunity, and strategy. Annu. Rev. Anim. Biosci. 7:221–243. https://doi.org/10.1146/annurev-animal-030117-014838.

Lane, M. A., R. L. Baldwin, and B. W. Jesse. 2000. Sheep rumen metabolic development in response to age and dietary treatments. J. Anim. Sci. 78:1990–1996. https://doi.org/10.2527/2000.7871990x.

Li, R., Z. Teng, C. Lang, H. Zhou, W. Zhong, Z. Ban, X. Yan, H. Yang, M. H. Farouk, and Y. Lou. 2019. Effect of different forage- to-concentrate ratios on ruminal bacterial structure and real-time methane production in sheep. PLoS One 14:e0214777. https://doi.org/10.1371/journal.pone.0214777.

Liu, Q., C. Wang, Y. Huang, K. Dong, H. Wang, and W. Yang. 2008. Effects of isobutyrate on rumen fermentation, urinary excretion of purine derivatives and digestibility in steers. Arch. Anim. Nutr. 62:377–388. https://doi.org/10.1080/17450390802327761.

Lu, C. D., and M. Potchoiba. 1988. Milk feeding and weaning of goat kids—A review. Small Rumin. Res. 1:105–112. https://doi.org/10.1016/0921-4488(88)90025-9.

McAllister, T., K. Beauchemin, A. Alazzeh, J. Baah, R. Teather, and K. Stanford. 2011. The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Can. J. Anim. Sci. 91:193–211. https://doi.org/10.4141/cjas10047.

Meale, S. J., S. Li, P. Azevedo, H. Derakhshani, J. C. Plaizier, E. Khafipour, and M. A. Steele. 2016. Development of ruminal and fecal microbiomes are affected by weaning but not weaning strat- egy in dairy calves. Front. Microbiol. 7:582. https://doi.org/10.3389/fmicb.2016.00582.

Moya, D., K. Schwartzkopf-Genswein, and D. Veira. 2013. Standardization of a non-invasive methodology to measure cortisol in hair of beef cattle. Livest. Sci. 158:138–144. https://doi.org/10.1016/j.livsci.2013.10.007.

Muller, L. D. 1987. Branched chain fatty acids (Isoacids) and valeric acid for ruminants. Prof. Anim. Sci. 3:9–12. https://doi.org/10.15232/S1080-7446(15)32370-6.

Muscato, T., L. Tedeschi, and J. Russell. 2002. The effect of ruminal fluid preparations on the growth and health of newborn, milk-fed dairy calves. J. Dairy Sci. 85:648–656. https://doi.org/10.3168/jds.S0022-0302(02)74119-2.

Newbold, C. J., G. de la Fuente, A. Belanche, E. Ramos-Morales, and N. McEwan. 2015. The role of ciliate protozoa in the rumen. Front. Microbiol. 6:1313. https://doi.org/10.3389/fmicb.2015.01313.

O'Hara, E., A. Kelly, M. S. McCabe, D. A. Kenny, L. L. Guan, and S. M. Waters. 2018. Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Sci. Rep. 8:14901. https://doi.org/10.1038/s41598-018-33122-6.

Ramos-Morales, E., A. Arco-Pérez, A. I. Martín-García, D. R. Yáñez- Ruiz, P. Frutos, and G. Hervás. 2014. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. Anim. Feed Sci. Technol. 198:57–66.

Rodríguez, C., and A. Rodríguez. 2011. Effect on weight gain, rumen bioactivity and rumen pH in weaned sheep using fresh rumen fluid from cattle. Rev. Mvz Cordoba 16:2692–2700.

Sander, E., R. Warner, H. Harrison, and J. Loosli. 1959. The stimulatory effect of sodium butyrate and sodium propionate on the development of rumen mucosa in the young calf. J. Dairy Sci. 42:1600–1605. https://doi.org/10.3168/jds.S0022-0302(59)90772-6.

Takenaka, A., K. Tajima, M. Mitsumori, and H. Kajikawa. 2004. Fiber digestion by rumen ciliate protozoa. Microbes Environ. 19:203– 210. https://doi.org/10.1264/jsme2.19.203.

Teh, T., M. Potchoiba, E. Escobar, and C. Lu. 1984. Weaning methods of goat kids. J. Dairy Sci. 67:137.

Uyeno, Y., S. Shigemori, and T. Shimosato. 2015. Effect of probiotics/ prebiotics on cattle health and productivity. Microbes Environ. 30:126–132. https://doi.org/10.1264/jsme2.ME14176.

Waymack, L. 1976. Effect of feeding lyophilized rumen contents on adaptation to urea diet by lambs. J. Anim. Sci. 43:712–714. https://doi.org/10.2527/jas1976.433712x.

Weatherburn, M. W. 1967. Phenol-hypochlorite reaction for determination of ammonium. Anal. Chem. 39:971–974. https://doi.org/10.1021/ac60252a045.

Weimer, P. J. 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. Front. Microbiol. 6:296. https://doi.org/10.3389/ fmicb.2015.00296.

Williams, A. G., and G. S. Coleman. 1992. The Rumen Protozoa. Springer-Verlag, New York, NY.

Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: A review. Front. Microbiol. 6:1133. https://doi.org/10.3389/fmicb.2015.01133.

Yoon, I., and M. Stern. 1995. Influence of direct-fed microbials on ruminal microbial fermentation and performance of ruminants: A review. Asian-australas. J. Anim. Sci. 8:533–555. https://doi.org/ 10.5713/ajas.1995.553.

Zhong, R., H. Sun, G. Li, H. Liu, and D. Zhou. 2014. Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. Livest. Sci. 162:154–158. https://doi.org/10.1016/j.livsci.2013.12.021.

Tables and Figures

Table 1. Inocula characterization for autoclaved rumen fluid (AUT) and rumen fluid from adult goats fed forage (RFF) or concentrate diets (RFC) in terms of rumen fermentation, microbial concentrations by quantitative PCR, and protozoal visual classification.

Inoculum	AUT	RFF	RFC	SEM	*P*-value
DM, %	4.28^{ab}	2.35^{b}	5.22^{a}	0.620	0.043
pH	6.11^{b}	6.38^{a}	5.79^{c}	0.067	0.002
Lactate, m*M*	1.86^{a}	0.55^{b}	0.85^{b}	0.232	0.017
Ammonia-N, mg/dL	8.64	7.27	10.0	0.774	0.117
Total VFA, m*M*	120^{ab}	103^{b}	134^{a}	7.064	0.055
Acetate, %	63.0^{b}	70.1^{a}	55.5^{c}	0.947	<0.001
Propionate, %	23.6^{b}	18.2^{c}	29.0^{a}	1.210	<0.001
Butyrate, %	10.3^{b}	9.49^{c}	11.8^{a}	0.527	0.004
Microbiota, $\log_{10}$ copies/mL					
Bacteria	—	9.96	11.8	0.625	0.081
Methanogens	—	6.32	7.65	0.858	0.314
Anaerobic fungi	—	6.76^{b}	7.55^{a}	0.088	<0.001
Protozoa	—	6.60^{b}	8.70^{a}	0.349	0.005
Main protozoal group	—				
Subfamily *Entodiniinae*, %	—	80.2	83.3	4.490	0.646
Subfamily *Diplodiniinae*, %	—	6.72	5.30	1.853	0.612
Ophryoscolex spp., %	—	0.32	1.85	0.785	0.226
Isotricha spp., %	—	2.33	1.41	0.993	0.542
Dasytricha sp., %	—	10.4	8.12	2.8072	0.587

$^{a–c}$Means within a row with different superscripts differ ($P < 0.05$).

Table 2. Intake and feed digestibility during postweaning (wk 10) in goats inoculated with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF) or concentrate diets (RFC), and without inoculation (CTL).

Item	CTL	AUT	RFF	RFC	SEM	*P*-value
DMI, kg/d	573	549	582	628	30.90	0.131
Forage, % of DMI	32.0^{c}	35.7^{bc}	40.3^{ab}	45.5^{a}	2.468	0.012
Digestibility, %						
DM	78.4	76.6	78.5	75.2	0.011	0.151
OM	79.7	78.2	80.1	77.0	0.011	0.179
N	76.8	74.5	79.1	76.5	0.013	0.132
NDF	59.2	57.3	61.9	55.8	0.023	0.307
ADF	60.1	56.0	59.4	53.1	0.033	0.431
Cortisol in hair, ng/mg	1.17	1.12	1.19	1.16	0.063	0.878

a–cMeans within a row with different superscripts differ (P < 0.05).

Table 3. Concentration of the main protozoa groups assessed by optical microscopy in goats inoculated with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF) or concentrate diets (RFC), and without inoculation (CTL).

		Inoculation						*P*-value		
Item	Age, wk	CTL	AUT	RFF	RFC	Age mean, wk	SEM	Inoc.[1]	Time	Inoc. x time
Protozoa, log cells/mL	5	0[c]	2.65[b]	4.76[a]	4.61[a]	4.01[Y]	0.310	<0.001	<0.001	0.025
	7	0[c]	4.18[b]	5.10[a]	5.40[a]	4.89[X]				
	9	0[c]	4.19[b]	4.95[b]	6.09[a]	5.08[x]				
Subfamily *Entodiniinae*, %	5	ND[2]	99.9[a]	75.6[b]	79.3[b]	84.9	3.510	<0.001	0.080	0.038
	7	ND	98.9[a]	77.3[b]	72.7[b]	82.9				
	9	ND	93.8[a]	81.7[b]	89.4[ab]	88.3				
Subfamily *Diplodiniinae*, %	5	ND	0.08[b]	17.7[a]	14.5[a]	10.8[X]	2.112	<0.001	<0.001	<0.001
	7	ND	1.14[b]	9.18[a]	10.8[a]	7.04[X]				
	9	ND	5.0	1.9	1.08	2.66[Y]				
Ophryoscolex spp., %	5	ND	0	4.56	4.59	3.05	1.513	0.001	0.187	0.441
	7	ND	0	5.55	7.48	4.34				
	9	ND	1.19	2.97	2.84	2.33				
Isotricha spp., %	5	ND	0	0.70	0.82	0.50	1.144	0.017	0.075	0.382
	7	ND	0	2.17	2.44	1.54				
	9	ND	0	5.09	2.51	2.54				
Dasytricha sp., %	5	ND	0	1.42	0.77	0.73[Y]	1.312	<0.001	0.001	0.057
	7	ND	0	5.80	6.58	4.13[X]				
	9	ND	0	8.36	4.19	4.18[X]				

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

[X,Y]Means within a column with different superscripts differ ($P < 0.05$).

[1]Inoc. = inoculation.

[2]ND = not detected.

Table 4. Rumen microbial fermentation in goats inoculated with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF) or concentrate diets (RFC), and without inoculation (CTL).

		Inoculation						*P*-value		
Item	Age, wk	CTL	AUT	RFF	RFC	Age mean, wk	SEM	Inoc.[1]	Time	Inoc. x time
Rumen pH	5	6.61	6.68	6.70	6.72	6.68^{X}	0.085	0.829	<0.001	0.864
	7	6.40	6.51	6.47	6.51	6.48^{Y}				
	9	6.75	6.66	6.62	6.73	6.69^{X}				
NH_3-N, mg/dL	5	25.4^{b}	24.9^{b}	35.3^{a}	28.4^{ab}	28.5^{X}	2.321	0.276	<0.001	0.028
	7	29.8	33.0	29.7	26.7	29.8^{X}				
	9	6.97	8.69	8.57	11.6	8.95^{Y}				
Total VFA, mM	5	27.7	18.6^{b}	36.5^{a}	31.3^{a}	28.5^{Y}	3.484	0.810	<0.001	<0.001
	7	31.1	30.2	35.4	35.8	33.1^{Y}				
	9	51.3^{ab}	57.4^{a}	43.8^{b}	46.4^{b}	49.7^{X}				
Acetate, %	5	74.0	74.4	73.3	74.0	73.9^{X}	1.407	0.138	<0.001	0.107
	7	70.3	66.3	72.3	70.2	69.8^{X}				
	9	57.7	54.2	57.1	59.4	57.1^{Z}				
Propionate, %	5	13.7	14.9	13.9	15.1	14.4^{Y}	0.967	<0.001	<0.001	<0.001
	7	15.2	16.5	14.7	15.2	15.4^{Y}				
	9	26.1^{b}	30.6^{a}	23.3^{bc}	20.6^{c}	25.2^{X}				
Butyrate, %	5	4.92^{ab}	2.66^{b}	6.44^{a}	4.92^{ab}	4.74^{Z}	0.943	0.074	<0.001	0.002
	7	8.99	10.32	8.00	9.57	9.22^{Y}				
	9	11.4^{b}	11.0^{b}	14.7^{a}	15.5^{a}	13.1^{X}				
Isobutyrate, %	5	2.43^{b}	3.06^{a}	2.06^{b}	2.04^{b}	2.40^{X}	0.133	0.006	<0.001	<0.001
	7	1.69	1.91	1.47	1.52	1.64^{Y}				
	9	0.83^{b}	0.91^{ab}	1.14^{a}	1.14^{a}	1.00^{Z}				
Valerate, %	5	1.57	1.18	1.37	1.31	1.36^{Y}	0.150	0.005	<0.001	<0.001
	7	1.54^{b}	1.85^{a}	1.29^{b}	1.46^{b}	1.54^{Y}				
	9	2.93	2.02	2.11	1.82	2.22^{X}				
Isovalerate, %	5	3.31^{ab}	3.81^{a}	2.89^{bc}	2.66^{c}	3.17^{X}	0.130	0.009	<0.001	0.001
	7	2.33^{b}	3.09^{a}	2.18^{b}	2.08^{b}	2.42^{Y}				
	9	1.07	1.22	1.58	1.63	1.37^{Z}				

[a–c] Means within a row with different superscripts differ (P < 0.05).
[X–Z] Within a column means with different superscripts differ (P < 0.05).
[1]Inoc. = inoculation.

Table 5. Body weight, ADG, and feed efficiency (FE) in goats inoculated with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF) or concentrate diets (RFC), and without inoculation (CTL).

Item	Age, wk	Inoculation				SEM	*P*-value		
		CTL	AUT	RFF	RFC		Inoc.[1]	Time	Inoc. ×time
BW, kg	0	2.65	2.72	2.78	2.69	0.245	0.737	<0.001	0.861
	3	5.31	5.58	5.13	5.10				
	5	6.43	6.62	6.25	6.13				
	7	8.81	8.96	8.62	8.38				
	9	10.3	10.5	10.4	10.3				
	11	14.0	13.9	13.9	14.0				
ADG,[1] g/d	0–3	126	136	112	115	0.011	0.842	<0.001	0.004
	3–5	156	150	157	146				
	5–7	184	184	185	174				
	8	48.7^{bc}	27.9^{c}	75.4^{ab}	97.1^{a}				
	9	178	177	154	165				
	9–11	173	160	172	181				
FE, kg/Mcal of ME	0–3	234	240	214	220	10.40	0.859	<0.001	0.003
	3–5	135	129	126	138				
	5–7	129	128	130	123				
	8	14.2	20.4	71.3	55.7				
	9	63.0	64.0	57.5	58.7				
	9–11	118	114	115	119				

$^{a–c}$Means within a row with different superscripts differ (P < 0.05).
[1]Inoc. = inoculation.

Table 6. Cumulative feed intake during 11 wk (DM) and feeding costs in goats inoculated with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF) or concentrate diets (RFC), and without inoculation (CTL).

Treatment	CTL	AUT	RFF	RFC	SEM	*P*-value
Milk powder intake, kg/animal	11.0	11.1	10.7	10.1	0.271	0.096
Concentrate intake, kg/animal	9.34	8.47	8.73	8.77	0.677	0.830
Forage intake, kg/animal	4.44^{c}	4.61^{bc}	5.27^{b}	6.35^{a}	0.267	0.001
Feeding cost, €						
Milk replacer	24.5^{a}	24.9^{a}	23.9^{ab}	22.6^{b}	0.606	0.096
Concentrate	3.18	2.88	2.97	2.98	0.231	0.830
Forage	0.76^{c}	0.78^{bc}	0.90^{b}	1.08^{a}	0.045	0.001
Total cost	28.5	28.5	27.7	26.7	0.709	0.276
Cost/gain, €/kg	2.50	2.54	2.48	2.40	0.060	0.435

$^{a–c}$Means within a row with different superscripts differ ($P < 0.05$).

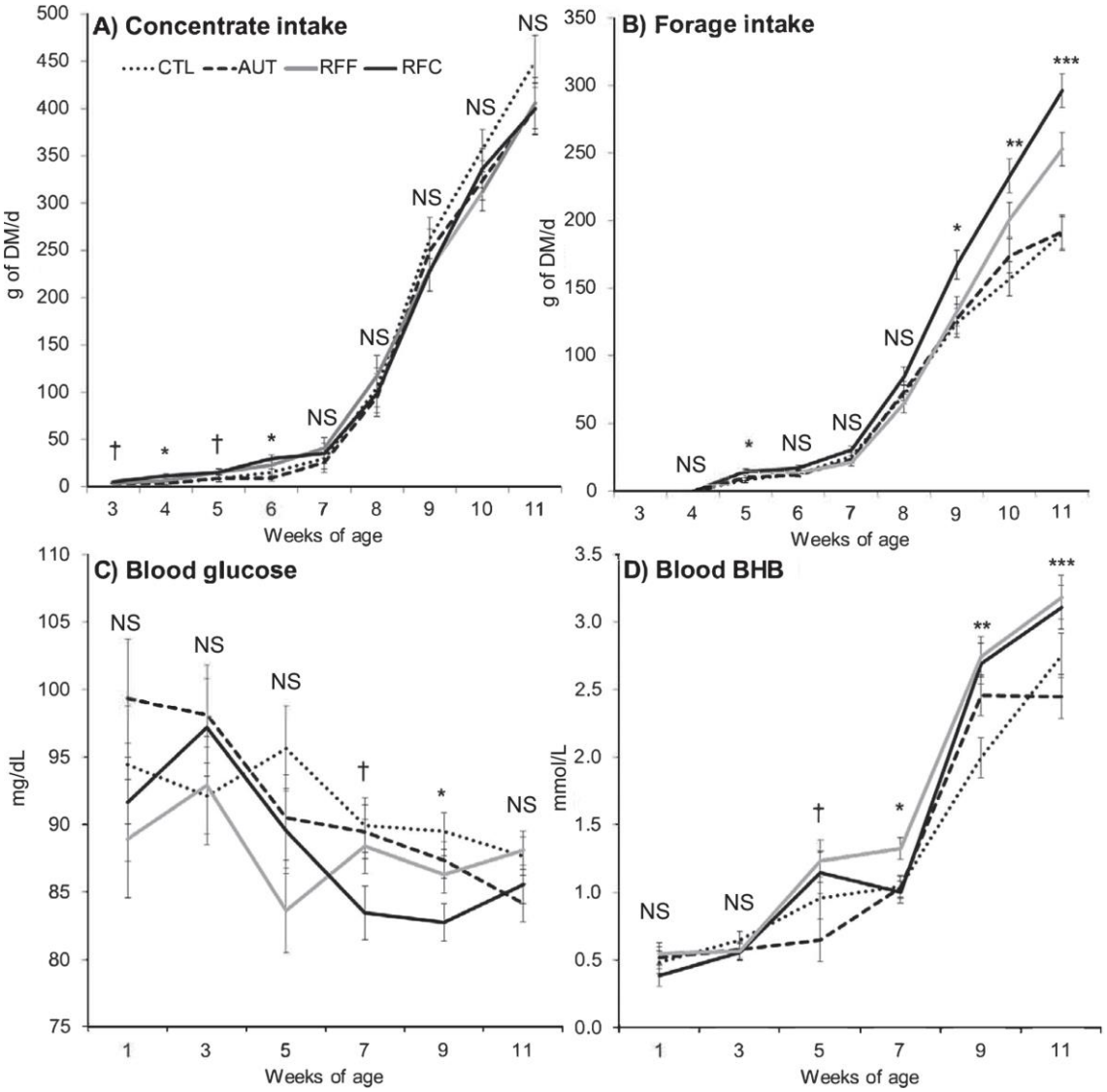

Figure 1. Weekly progression of concentrate intake (A), forage intake (B) and blood concentration of glucose (C) and BHB (D) in goat kids inoculated with autoclaved rumen fluid (AUT, dashed line), rumen fluid from adult animals fed forage (RFF, gray solid line), rumen fluid from adult animals fed forage concentrate diets (RFC, black solid line), or without inoculation (CTL, dotted line). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.1$, NS: $P > 0.1$. Significant interactions between inoculation and time were noted for forage intake and BHB ($P < 0.001$). Error bars indicate SE of the difference.

Publication 3

Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats

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Animal Microbiome 3:11

DOI: https://doi.org/10.1186/s42523-021-00073-9

ABSTRACT

Background: Newborn ruminants possess an underdeveloped rumen which is colonized by microorganisms acquired from adult animals and the surrounding environment. This microbial transfer can be limited in dairy systems in which newborns are separated from their dams at birth. This study explores whether the direct inoculation of fresh or autoclaved rumen fluid from adult goats to newborn kids has a beneficial effect on rumen microbial development and function.

Results: Repetitive inoculation of young kids with fresh rumen fluid from adult goats adapted to forage (RFF) or concentrate diets (RFC) accelerated microbial colonization of the rumen during the pre-weaning period leading to high protozoal numbers, a greater diversity of bacterial (+ 234 OTUs), methanogens (+ 6 OTUs) and protozoal communities (+ 25 OTUs) than observed in control kids (CTL) without inoculation. This inoculation also increased the size of the core bacterial and methanogens community and the abundance of key rumen bacteria *(Ruminococcaceae, Fibrobacteres, Veillonellaceae, Rikenellaceae, Tenericutes*), methanogens (*Methanobrevibacter ruminantium, Methanomicrobium mobile* and *Group 9*), anaerobic fungi (*Piromyces* and *Orpinomyces*) and protozoal taxa (*Enoploplastron, Diplodinium, Polyplastron, Ophryoscolex, Isotricha* and *Dasytricha*) before weaning whereas CTL kids remained protozoa-free through the study. Most of these taxa were positively correlated with indicators of the rumen microbiological and physiological development (higher forage and concentrate intakes and animal growth during the post-weaning period) favoring the weaning process in RFF and RFC kids in comparison to CTL kids.

Some of these microbiological differences tended to decrease during the post-weaning period, although RFF and RFC kids retained a more complex and matured rumen microbial ecosystem than CTL kids. Inoculation with autoclaved rumen fluid promoted lower development of the bacterial and protozoal communities during the pre-weaning period than using fresh inocula, but it favored a more rapid microbial development during the post-weaning than observed for CTL kids.

Conclusions: This study demonstrated that inoculation of young ruminants with fresh rumen fluid from adult animals accelerated the rumen microbial colonization which was associated with an earlier rumen functional development. This strategy facilitated a

smoother transition from milk to solid feed favoring the animal performance during post-weaning and minimizing stress.

Keywords: Core microbial community, Rumen fluid inoculation, Rumen microbial colonization, Weaning

BACKGROUND

Ruminants possess a complex gastric system composed of four chambers of which the rumen is the largest and hosts a vast and diverse microbial community composed of bacteria, methanogenic archaea, protozoa and fungal species which, are adapted to thrive in anaerobic conditions and are responsible for the fermentation of the diet consumed by the animal. At birth, however, the rumen is not fully developed (proto-rumen) and lacks the microbiota present in adult animals [1, 2]. Newborn ruminants rely on milk-based diets that bypass the rumen through the esophageal groove to reach the abomasum where digestion starts [3]. A correct transition from proto-rumen to rumen will later determine the efficiency of the nutrients' digestion and absorption in the gut and other tissues [4, 5]. In this transition, microbial colonization occurring during and after birth plays a pivotal role on the development of the rumen that undergoes dramatic changes through the first weeks of life up to weaning and beyond [1, 6]. Previous studies have shown that early colonization events shape the composition of the rumen microbiome throughout life [7, 8]. The early colonizing microbes may facilitate the establishment of functional gut microbiota by several possible mechanisms. The first colonizers are facultative anaerobes and are thought to render the gut environment suitable to anaerobic rumen microbes [9]. Recent works have reported the unique and potentially important influences of maternal microbiota from the skin, udder, vagina, saliva and colostrum, which each appears to make early contributions to the bio-spatial and longitudinal succession of microbes throughout the early life of the animal [10, 11]. This is particularly critical in the context of modern dairy livestock systems in which the newborn is taken away from the mother after birth, generally fed on artificial milk, and are kept isolated from adult animals, which can limit the rumen microbial development and animal performance [5]. The magnitude of the detrimental effects increases when artificial rearing is combined with early weaning programs to minimize milk-replacer costs, which may lead to weaning-associated health and digestive problems [12].

We have previously shown that the natural rearing of newborns with the dam accelerates the rumen microbial colonization as compared to artificial milk feeding animals [1, 13], having positive effects on feed digestion and animal growth later in life [5]. This early established microbiota seems to facilitate an earlier acquisition of the digestive capacity to ferment solid feed and fiber thanks to a more diverse prokaryotic community, and also to the presence of rumen protozoa that cannot colonize the rumen unless there is direct contact between young and adult animals. Different studies have investigated the potential of inoculation young ruminants with rumen fluid from adult animals to overcome the deficient colonization process occurring under artificial milk feeding with contrasting results [14–16]. However, they have used an intermittent inoculation approach, and the analyses are limited to the most commonly studied bacteria but not to methanogens and eukaryotes (protozoa and fungi) that, despite contributing up to 50% of the total microbial biomass, are usually neglected in rumen microbiome studies [17]. The large variability observed in past studies using rumen fluid inoculation suggests that more attention must be given to the selection of the microbial inoculum and the time window in which the inoculation is applied. From an ecological perspective, the simpler and less diverse gastro-intestinal microbiota of newborn kids is more receptive to exogenous inoculation than in adult animals because it has less colonization resistance [7]. This suggests that divergent rumen microbiotas adapted to different diets could be potentially inoculated into young ruminants to modulate the colonization pattern and the establishment of a desirable rumen microbial activity for a particular production system.

In previous works, we optimized the type of inocula [18] and we showed [19] that early-in-life inoculation of goat kids with rumen fluid from adult animals stimulated feed intake and rumen function in terms of volatile fatty acids (VFA) production during the pre-weaning period. This inoculation also helped the transition from liquid to solid feeding and to minimize the growth retardation following weaning. However, the impact of the inoculation on the rumen microbiome and the identity of the key microbes which promote these physiological advantages are yet unknown. Here, we hypothesize that an early-in-life rumen microbial inoculation would modify its microbial colonization and development processes with potential long-lasting effects in goats. We used a multi-kingdom meta-taxonomic community analysis (including bacteria, methanogens, protozoa and anaerobic fungi) to get a detailed description of the rumen microbiome and how it is modulated by inocula adapted to different diets. We studied the effect of forage-

and concentrate-adapted inocula as well as autoclaved rumen fluid to investigate this hypothesis.

RESULTS

Inocula characterization and effects on rumen fermentation and animal performance

This study investigated the effects of early-in-life inoculation of newborns goat kids with rumen fluid from adult goats adapted to forage (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL). Results indicated that RFF inocula had greater pH and acetate molar proportion, whereas RFC had greater DM content, total VFA concentration and propionate and butyrate molar proportions (Suppl. Table S1). The study of the microbial taxonomy composition also showed differences across inocula: RFF inocula had higher bacterial and methanogens OTUs richness and higher abundances of certain taxa such as *Clostridiales, Rikenellaeae, Methanomassiliicoccaceae, Dasytricha* and *Caecomyces*. On the contrary, RFC inocula had a higher concentration of total bacteria, protozoa and anaerobic fungi, as well as higher abundances of *Lachnospiraceae, Veillonellaceae, Methanobrevibacter, Polyplastron* and *Piromyces* (Suppl. Table S1). AUT inocula had a concentration of fermentation products in between RFF and RFC values, but without viable cells, as noted by the undetectable concentration of microbial DNA and intact protozoal cells after optical inspection.

Goat kids were daily inoculated from birth to 11 weeks of age and reared under artificial milk feeding. Rumen samples were taken at 5, 7 and 9 weeks of age to describe the rumen microbiome before, during and after weaning, respectively. Inoculation with fresh rumen (RFF and RFC) fluid increased solid feed intake (Fig. 1a) and the rumen VFA concentration during the pre-weaning period (Fig. 1b). This inoculation favored the transition to a solid diet as greater average daily gain (ADG) and butyrate molar proportion were observed during the post-weaning period (week 8, Fig. 1c) in comparison with CTL and AUT kids.

Effect of inoculation on the multi-kingdom rumen community

The multi-kingdom analysis included all microbial OTUs from bacterial (88%), methanogens (2.7%), protozoal (2.2%) and anaerobic fungal origin (7.1%) in a combined community representing the entire rumen microbiome. Permutational analysis of variance (PERMANOVA, Table 1) showed that this multi-kingdom community was significantly affected ($P < 0.01$) by the microbial inoculation (explaining 21.8% of the total variance), the age of the animals (16.2%) and their interaction (9.9%). Pair-wise analysis also showed differences be- tween all four inoculation treatments and sampling times being illustrated in the Principal Coordinate Analysis (PCoA, Suppl. Fig. S1). This graphical representation showed that PCO1 discriminated between control (right), AUT (center) and RFF and RFC (left) whereas the PCO2 discriminated between pre-weaning (top) and post-weaning (bottom). Samples from RFF and RFC animals positively correlated with OTUs belonging to *Ruminococcaceae, Christensenellaceae, Clostridiales, Anaerovorax, Mogibacterium, Bacteroidales and Prevotella* at 5 weeks, with *Entodinium* at 7 weeks and with *Prevotellaceae, Selenomonas, Lachnospiraceae* and *Succinivibrio* at 9 weeks of age, indicating a successional colonization process (Suppl. Fig. S1). For a more detailed description of the rumen microbiome, the main microbial groups were analyzed separately.

Effect of inoculation on the rumen bacterial community RFC kids had lower total bacterial abundance per gram of DM than the other three treatments (Fig. 2a). The sequencing analysis generated on average 14,451 ± 716 high quality bacterial sequences per sample. RFF kids, followed by RFC and AUT, showed the highest bacterial diversity in terms of OTUs (Fig. 2b) and Shannon index (Fig. 2c), whereas CTL kids showed the lowest bacterial diversity indexes. Venn diagrams showed that the core bacterial community was composed by 15 OTUs (Fig. 2d). The inoculation with fresh rumen promoted a large core bacterial community in RFF (composed by 202, 231 and 164 OTUs) and RFC kids (139, 190 and 159 OTUs at weeks 5, 7 and 9, respectively). Moreover, many of these OTUs (20–30%) were exclusively shared between these two treatments indicating a similar community structure. AUT kids had a medium size core community (144, 153 and 124 OTUs), whereas CTL kids had much smaller core community (53, 76 and 53 OTUs at week 5, 7 and 9, respectively), being most of these OTUs (up to 64%) common across all treatments.

PERMANOVA analysis showed that inoculation and sampling time had significant impacts on bacterial community structure, explaining 16–17% of the total variance (Table 1). PCoA illustrated these differences in the bacterial community structure (Fig. 2e) in which PCO1 captured a gradient of community development according to the age of the kids (from left to right), whereas PCO2 did so for the CTL kids (from up to down). This PCoA also identified relevant microbes which partially explained these differences. For example, samples taken at 9 weeks of age from AUT, RFF and RFC kids correlated with the presence of *Prevotella* OTUs, whereas those from CTL kids did so with *Succinivibrio* OTUs. Inocula samples clustered together with samples from RFF and RFC kids at 7 weeks of age, indicating a similar bacterial community. The effect of the inoculation differed with the age of the kids (interaction, $P < 0.001$. Table 1), indicating that specific analyses for each sampling time were needed.

During the pre-weaning period (week 5, Fig. 2f), inoculation with fresh rumen fluid showed a unique and different bacterial community as compared to AUT or CTL kids. This bacterial community correlated with indicators of the rumen microbial and functional development such as dry matter intake (DMI), forage intake, protozoal concentration and bacterial and protozoal richness. Most differences among treatments were also detected at weening (week 7, Fig. 2g). Again, clustered samples from these latter groups were related with higher bacterial and protozoal richness, as well as with the average daily gain (ADG-f) and feed efficiency (FE-f) during the following week, suggesting that this community structure minimized the weaning shock. During the post-weaning period (week 9, Fig. 2h) CTL kids retained a different bacterial community than that of other treatments, whereas the bacterial community of AUT kids became closer to those kids inoculated with fresh rumen fluid. Greater bacterial and protozoal richness were again associated with the bacterial community structure of inoculated groups, along with digestible cellulose (DCI) and hemicellulose (DHCI) and forage intake during the following week (Forage –f).

The analysis of the relative abundances of the most predominant bacterial families and genera (Fig. 3a and Suppl. Table S2) indicated that 12 out of the 21 families identified showed significant differences based on the inoculation treatment, regardless of sampling time. Inoculation with fresh rumen fluid promoted the presence of a number of minority bacterial taxa at week 5 which were not present in CTL kids, the first three being also absent in AUT kids. Most of these taxa were not detected at later sampling times in CTL

kids indicating a microbial colonization delay. Inoculation with fresh rumen fluid also increased the abundance of various phyla (e.g. *Firmicutes, Fibrobacteres, Tenericutes, Cyanobacteria* and *Elusimicrobia*) and genera (e.g. *Fibrobacter, Succiniclasticum, Eubacterium or Lachnoclostridium*) in comparison with CTL and AUT kids across sampling times. On the contrary, *Bacteroidales, Alloprevotella* and *Coprococcus* were most abundant in CTL and AUT groups across sampling times. The higher bacterial Shannon index observed in RFF than in RFC inocula was also noted in RFF inoculated animals, however the divergent taxa distribution observed between them was not reflected in the inoculated kids. Moreover, 17 out of the 21 taxa presented significant differences according to sampling time: *Prevotellaceae* and *Succinivibrionaceae* increased over time whereas *Ruminococcaceae* decreased. The interaction between inoculation and sampling time was significant in 10 out of 21 bacterial taxa indicating that the effects were more obvious before than after weaning.

Spearman correlations were performed to assess the potential implications of changes in rumen meta-taxonomic data on animal physiology (Table 2 and Suppl. Table S3). Bacterial richness, a number of bacterial phyla such as *Fibrobacteres*, *Firmicutes*, *Tenericutes*, *Elusimicrobia*, *Cyanobacteria*, *Chloroflexi* and *Lentisphaerae*, and several bacterial families such as *Ruminococcaceae, Veillonellaceae, Rhodocyclaceae* or *Rikenellaceae* positively correlated with various indicators of the rumen physiological development such as forage and solids intake, acetate molar proportion, presence of protozoa and higher bacterial, protozoal and methanogens diversity. Moreover, the abundance of *Firmicutes* and *Veillonellaceae* positively correlated with the ADG during the post-weaning period indicating a better transition from liquid to solid feed during the post-weaning period. On the contrary, the phylum *Bacteroidetes*, the families *Bacteroidaceae*, *Comamonadaceae* and *Neisseriaceae* showed a negative correlation with these indicators of the rumen physiological development as well as with animal performance during the post-weaning period.

Effect of inoculation on the rumen methanogens

The interaction between inoculation treatment and time on the concentration of methanogenic archaea in the rumen was significant (Fig. 4a). Although RFF kids, followed by AUT and RFC, showed the highest concentrations of methanogens at 5

weeks of age (and CTL the lowest) these differences tended to decrease as kids aged. Sequencing analysis generated an average of 14,180 ± 1100 high-quality methanogens sequences per sample and showed that methanogens diversity increased with the age of the kids (Fig. 4b). Moreover, CTL kids showed lower methanogens diversity in terms of OTUs and Shannon index (Fig. 4c) than observed in other treatments across time points. The methanogens core community was composed by only two *Methanobrevibacter* OTUs which were shared across all treatments and time points (Fig. 4d). However, at week 9 new *Methanobrevibacter* and *Methanosphaera* OTUs appeared in this core community. Venn diagrams for individual time points revealed that the methanogens core community remained similar for CTL kids (5, 3 and 5 OTUs at week 5, 7 and 9, respectively) but increased for AUT (6, 12 and 17 OTUs), RFF (7, 9 and 15 OTUs) and RFC kids (7, 11 and 13 OTUs).

Rumen methanogens community structure was greatly affected by inoculation, sampling time and their interaction (Table 1, Fig. 4e), however, PERMANOVA indicated that the proportion of the variance explained by the inoculation treatment was twice more than that explained by sampling time (24.92 vs 11.54%). PCoA showed that PCO1 separated samples from CTL (right) and from the rest of treatments (left), whereas PCO2 disaggregated samples between pre-weaning (bottom) and post-weaning (top). Moreover, samples collected from RFF and RFC at 5 weeks positively correlated with OTUs belonging to *Methanimicrococcus, Methanophanus* and Groups 8, 9 and 10.

The study of the methanogens community structure at 5, 7 and 9 weeks of age using Distance-Based Redundancy Analyses (Fig. 4f) showed a general pattern characterized by a separation through axis 1 between samples from CTL kids (right) and those from RFF and RFC kids (left). At week 5, RFF and RFC samples positively correlated with the presence of higher diversity levels for bacteria, methanogen and protozoa and higher DMI and blood beta-hydroxybutyrate indicating a more microbiological and functional rumen development. At weeks 7 (Fig. 4f) and 9 (Fig. 4h), RFF and RFC samples correlated with higher diversity indexes, protozoal concentration, forage intake and ADG and FE during the following week after weaning whereas CTL samples clustered on opposite direction indicating a more undeveloped methanogens community.

The analysis of the relative abundances of the 15 most predominant methanogen species (Fig. 3b and Suppl. Table S4) showed differences according to the inoculation treatment

(13 species) and sampling time (11 species); however a significant interaction was found for most of them. At 5 weeks of age, nearly the entire methanogens community (99.7%) in CTL kids was formed by *Methanobrevibacter gottschalkii* and *Group8_sp*, however these two species only represented 39.0, 22.9 and 29.1% in AUT, RFF and RFC. On the contrary, RFF and RFF had increased abundances of *Group9_sp* (34.4%), *Methanomicrobium mobile* (9.7%), *Methanobrevibacter ruminantium* (3.8%) and *Methanobassiliicoccaceae* spp. (18.3%), whereas AUT kids were more abundant in *Group10_sp* (14.7%). At week 7, a consistent presence of *Methanimicrococcus blatticola*, *Methanomicrobium mobile*, *Methanosphaera*, *Group12_sp* and *Group10_sp* was detected in AUT, RFF and RFC but were absent in CTL kids which still retained higher numbers of *M. gottschalkii* (39.6%). This over-representation was even bigger at 9 weeks of age (59.9%), whereas inoculated kids were more abundant in a greater number of methanogen species (e.g. *Group8_sp* and *Group_9sp*). The differences in the methanogens community noted between RFF and RFC inocula were not observed in the inoculated kids, although RFF kids had a higher total methanogens concentration.

The abundance of *M. gottschalkii* (and *Group8_sp*) was negatively correlated with forage intake, presence of protozoa and ADG before and after weaning, indicating the presence of an immature methanogens community (Table 2). On the contrary, *M. blatticola*, *M. mobile*, *M. ruminantium*, *Methanosphaera* and *Group9_sp* were positively correlated with indicators of a greater rumen microbiological and physiological development (forage intake and ADG before and after weaning).

Effect of inoculation on the rumen protozoal community

Control kids remained protozoa-free over the entire duration of this study. Inoculation with fresh rumen fluid promoted a higher concentration of rumen protozoa (Fig. 5a) at week 5, but these differences tended to be smaller as time progressed (interaction $P < 0.05$). The 18S amplicon sequencing yielded an average of 18,164 ± 644 sequences per sample and diversity analysis showed that RFF and RFC had higher protozoal diversity in terms of OTUs (Fig. 5b) and Shannon index (Fig. 5c) than AUT kids across sampling times. A total of 14 protozoal OTUs formed the core community shared between AUT, RFF and RFC kids across time points (Fig. 5d). The treatment-specific core community increased over time for RFC (20, 23 and 25 OTUs at week 5, 7 and 9, respectively), and

for RFF kids (25, 27 and 19 OTUs) since most OTUs were shared across these two treatments. On the contrary, the protozoal core community was smaller and remained constant over time for AUT kids.

PERMANOVA revealed that the inoculation and the sampling time greatly modified the protozoal community structure explaining 14.5 and 9.07% of the total variance, respectively (Table 1). Pair-wise comparisons and PCoA analysis (Fig. 5e) showed that inoculation with fresh rumen fluid promoted a protozoal community similar to the observed in the inocula and positively correlated with the presence of 8 different protozoal OTUs, whereas the community in the AUT kids only correlated with *Entodinium* OTUs. The analysis of the protozoal community at different time points showed that RFF and RFC always shared a similar protozoal community which was positively correlated with indicators of a rumen microbiological (higher bacterial and protozoal richness) and functional development (higher intakes, rumen VFA, butyrate and ADG). This protozoal community differed to that observed in AUT kids at 5 (Fig. 5f) and 7 weeks (Fig. 5g) but not at 9 weeks of age (Fig. 5h), indicating a delay in the rumen protozoal colonization in AUT kids.

Analysis of the protozoa relative abundances (Fig. 6a and Suppl. Table S5) showed a progressive decrease over time in the entodiniomorphids (family *Ophryoscolecidae*) and an increase in holotrichs protozoa (family *Buetschliidae*). AUT kids had increased numbers of *Entodinium*, whereas RFF and RFC were more abundant on *Diplodinium*, *Enoploplastron, Isotricha* and *Dasytricha*, these differences being greater before than after weaning. The abundance of most protozoal species was positively correlated with DM intake and bacterial, methanogens and protozoal diversities. Abundances of *Ophryoscolex*, *Isotricha* and *Dasytricha* were also correlated with higher VFA concentration and ADG during the post-weaning period as indicators of rumen development. The higher total protozoal concentration detected in RFC than in RFF inocula, was also observed in RFC kids at 9 weeks of age.

Effect of inoculation and age on the rumen fungal community

Inoculation with fresh rumen fluid increased the anaerobic fungal concentration at week 5 in comparison to CTL and AUT kids (Fig. 7a), some of these differences persisted at weaning but disappeared after weaning (interaction, $P < 0.001$). Fungal sequencing

yielded an average of 6548 ± 618 high quality sequences per sample for those taken at 5 and 7 weeks. However, the number of reads observed at 9 weeks was unexpectedly low and this time point was not further considered. No differences were found in the anaerobic fungal diversity across treatments (Fig. 7b and c).

An absence of a core anaerobic fungal community was observed since no OTUs were shared across treatments and time points (Fig. 7d). Despite that, a treatment-specific core community was observed in CTL (6 and 5 OTUs at week 5 and 7, respectively), RFF (2 and 2 OTUs) and RFC (11 and 3 OTUs) but not in AUT kids. PERMANOVA revealed that the fungal community structure was highly affected by the inoculation ($P < 0.001$) explaining 29.8% of the total variation, whereas no effect was observed for the age of the kids (Table 1). PCoA analysis (Fig. 7e) showed a clear separation between kids inoculated with fresh rumen fluid (right) and those from CTL and AUT (left). A different fungal community was observed between RFF (top) and RFC samples (bottom) being these latter samples correlated with the presence of several *Piromyces* OTUs.

Inoculation with rumen fluid greatly modified the fungal colonization process in terms of taxa abundance (Fig. 6b and Suppl. Table S6). CTL kids had an anaerobic fungal community composed by only 3 taxa: Neocallimastigaceae spp. (76%), *Neocallimastix* (10%) and *Caecomyces* (14%). AUT kids showed lower numbers of *Neocallimastigaceae spp*. and *Neocallimastix* than CTL kids, but higher numbers of *Caecomyces*, *Piromyces*, *Orpinomyces* and *Capnodiales*. Moreover, RFF showed the highest abundance of *Orpinomyces*, whereas RFC did so for *Piromyces*. Correlation analysis showed that *Caecomyces* negatively correlated with the presence of protozoa (Table 2), whereas *Orpinomyces* positively correlated with indicators of the rumen physiological (concentrate and DM intakes) and microbiological development (higher bacterial and protozoal richness). *Piromyces* was correlated with even more indicators of the rumen microbiological development (including methanogens and protozoal concentrations and methanogens richness). The higher abundance of *Pyromices* observed in RFC inocula than in RFF inocula was also reflected in RFC kids.

DISCUSSION

In our study two types of fresh rumen inocula, along with autoclaved inocula, were used to test whether the microbes (or their fermentation products) could affect rumen

microbial colonization in early life as previously suggested [20, 21]. Overall, the inoculation with fresh rumen fluid accelerated the rumen microbial development in goat kids resulting in a more complex and diverse microbiota which facilitate the transition from liquid to solid feeding and minimized the weaning stress. However, this intervention had different impact on the main rumen microbial communities as further discussed.

Rumen bacterial community

The rumen bacterial colonization is a sequential process in which *Proteobacteria* represent the main early colonizers, followed by increasing complexity and abundances of strictly anaerobes such as *Bacteroidetes* and *Firmicutes* [22]. Our study showed that inoculation with fresh rumen fluid accelerated this colonization process during the pre-weaning period leading to a higher diversity (+ 234 OTUs), and a greater core bacterial community shared between RFF and RFC kids (+ 117 OTUs) than reported in CTL kids. Our study showed that CTL kids had higher abundance of several *Proteobacteria* families during the pre-weaning period (e.g. *Comamonadaceae* and *Pasteurellaceae*) indicating delayed microbial colonization. On the contrary, RFF and RFC kids had a higher abundance of members of the *Succinivibrionaceae* family (e.g. *Ruminobacte*r, *Succinomonas* and *Succinivribrio*). This latter family is reported to become more predominant when starter concentrate is consumed [6] due to its ability to degrade starch and simple sugars into propionate [23, 24], as reported here. The positive correlations observed with concentrate intake, propionate, butyrate and ADG confirm the importance of *Succinivibrionaceae* in the transition from milk to solid feed [21] and in the rumen functional development [25]. An increase in the ratio *Firmicutes* to *Bacteroidetes* has been reported when animals consume forage diets [5, 26], and a decrease in the abundance of *Bacteroidetes* has been described when animals start eating solid feed [6], and both aspects are confirmed in our study. In particular, CTL kids, in comparison to those inoculated with fresh rumen fluid, had higher abundance of several *Bacteroidetes* families such as *Bacteroidales* (42% vs 15%) and *Bacteroidaceae* (7.6% vs 0%) during the pre-weaning period. These bacterial taxa were negatively correlated with feed intake, rumen microbial complexity and ADG during the post-weaning period confirming that they are indicators of an underdeveloped rumen bacterial community, as

previously reported [16]. On the contrary, RFF and RFC kids had increased abundances of key *Firmicutes* families such as *Veillonellaceae* (+ 2.78 folds) and *Acidaminococcaceae* (+ 2.46 folds), the plant degrader *Rikenellaceae* (+ 7.28 folds) during the pre-weaning period, as well as the presence of *Fibrobacter* which is considered a highly specialized cellulose and hemicellulose degrader [27]. These microbiological changes are signs of a greater maturity of their bacterial community since all these taxa are strict anaerobes capable of degrading fibrous recalcitrant substrates [28, 29] and showed positive correlation with the forage intake and ADG during the post-weaning period. Several other minor phyla such as *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Spirochaetes* and *Tenericutes* had also higher abundance in kids inoculated with fresh rumen fluid. *Spirochaetes* is considered a xylan degrader, whereas *Cyanobacteria* is most probably *Melainabacteria* which is considered an anaerobic microbe able to act as hydrogen producer [30]. This inoculation with rumen fluid allowed the rumen colonization by *Elusimicrobia*, *Anaerobiospirillum*, *Catenisphaera*, *Clostridiaceae*, *Marvinbryantia*, *Saccharofermentans*, *Quinella*, *Selenomonas*, *Rhodocyclaceae*, *Succinomonas*, *Synergistes*, *Olicosphaeraceae*, *PeH15* and *SP3-e08*, which are mostly strict anaerobes and are considered late rumen colonizers involved in solid feed degradation [16] and were not present in CTL kids during the pre-weaning period. Therefore, their presence in the rumen can explain the greater bacterial diversity, redundancy and adaptability to solid feed observed in RFF and RFC animals [31]. Similar increase in diversity and solid feed intake was reported in artificial-suckling calves with early feeding with solid feed in comparison to those fed milk until weaning [32]. Therefore, our results are in agreement with previous suggestions indicating that rumen bacterial programming through inoculation of rumen fluid is possible [7] with the effects persisting, to some extent, during the post-weaning period since CTL bacterial community remained less developed before and after weaning.

In an earlier in vitro study we evaluated the microbial activity of different types of rumen inocula, sampling time, and preservation methods. It was concluded that fresh rumen fluid sampled at 3 h after feeding provides the most diverse and active inoculum [18]. The present in vivo study builds upon these observations since it has been suggested that the use of microbiologically divergent inocula could modulate the establishment and shape of the rumen bacterial community composition [15, 33]. Our study did not agree with this hypothesis with regards to the bacterial community since most of the differences found

between RFF and RFC inocula were not reflected in the inoculated kids. This observation suggests that the bacterial community structure present in the inocula is not that relevant, since only those microbes able to colonize the newborn animal will get established [34]. As a result, as time progressed the bacterial community structure became more similar between all three groups of inoculated kids, probably because of a complete establishment of an adult-like bacterial community after weaning, when animals fed on solid feeds [32] and as a result of the appearance of rumen protozoa in AUT kids as described below. This observation agrees with most studies in which only the bacterial community was studied [15] concluding that inoculation of young ruminants with rumen fluid is more effective when performed before than after weaning [16].

Rumen methanogens community

Although there are no publications describing the effect of early microbial inoculation on the rumen methanogens and protozoal communities, it has been observed that the natural microbial transfer that occurs by the contact with adult animals promotes substantial changes in the methanogens [1] and protozoal communities [35]. Our study revealed that inoculation with fresh rumen fluid accelerated the rumen colonization by methanogens during the pre-weaning period leading to higher concentrations (+ 2.0 log10 units), richness (+ 5.5 OTUs) and core community, and promoting a more mature methanogens community than observed in CTL kids. This might be caused by the acquisition of protozoa-associated methanogens, which stands for approximately 20% of the methanogens [36], as it has been shown that presence of protozoa increases methanogens diversity [37].

Skillman et al. [2] reported that methanogens colonize the rumen in the first 2 weeks of life. We noted that the most abundant genus across all samples was *Methanobrevibacter* which was identified as a member of the core community as previously described [21, 38]. *M. gottschalkii*, a highly abundant hydrogenotrophic species [39], was predominant in CTL kids before weaning, whereas the inoculation with fresh rumen fluid promoted the proliferation of other methanogens such as *Methanomassiliicocaccaceae* spp., *M. mobile*, *M. ruminantium* and Group9_sp, promoting a more diverse community. These latter taxa showed positive correlations with indicators of the rumen microbiological development (protozoal concentration and richness) and physiological development

(higher forage intake and ADG during the post-weaning period) possibly because some of them (e.g. *Methanobrevibacter* and *Methanom crobium*) have been found inside of rumen protozoa (endosymbionts) and can favour the inter-species H_2 transport and ultimately feed utilization [40]. Similarly, *M. ruminantium* requires the presence of other methanogen species to thrive; therefore its presence can be considered an indicator of rumen microbial maturity [2, 5]. Most of the differences in the methanogens community observed during the pre-weaning period (e.g. higher diversity and abundance of *M. ruminantium*) were maintained during the weaning and the post-weaning stages. As a result, CTL kids still lacked relevant taxa such as *Methanosphaera, M. blatticola* and some *Methanomassilicoccaceae* members, indicating an underdeveloped methanogens community in comparison to RFF and RFC kids. A greater methanogens core community and diversity was also observed at weaning in lambs under natural vs artificial milk feeding [41] indicating that inoculation with fresh rumen fluid can partially mimic the microbial transfer from the dam to the offspring.

The positive effect of the inoculation with fresh rumen fluid was more evident for RFF than for RFC, possibly as a result of the higher methanogens OTU richness in the RFF inoculum. This observation suggests that the concept of "rumen microbial programming" of the methanogens community based on the modification of the colonization process should not be ruled out [7]. The higher methanogens concentration and diversity observed in AUT than CTL kids could be an indirect effect mediated by the presence of protozoa as described below [37].

Rumen protozoal community

Anaerobic protozoa are late rumen colonizers because they are highly sensitive to oxygen and require direct contact between young and adult ruminants for an effective transmission, drinking water being the most likely mode of transfer [42]. This lack of contact with adult ruminants justifies the absence of protozoa in CTL kids during the entire duration of the study, an aspect that we have also described previously in artificially reared lambs [41]. A natural sequence of rumen colonization has been described for the different protozoal families starting by *Entodiniinae,* followed by *Diplodiniinae* and *Ophyoxcolecinae* and finishing with holotrichs [42]. The visual microscopy examination of the protozoal community [19] and sequencing data confirmed this sequence. The

presence of a small concentration of protozoa, mostly composed of *Entodinium*, in AUT kids located in three contiguous pens suggested that they may have accidentally been cross-faunated before week 5. Moreover, the lack of holotrichs and *Diplodinium* at week 5 indicated an incomplete and delayed rumen protozoal colonization in AUT kids given the inherent difficulty of holotrichs to become stabilized in the rumen of young ruminants [43, 44]. This partial faunation of the AUT kids, along with the potential positive effects of some metabolites present in the autoclaved rumen fluid (e.g. VFA, microbial extracts, micro-nutrients) in young calves [20, 21], could explain the moderate but positive impact of this treatment on the rumen microbial and physiological development noted in our study.

Our findings clearly indicated that inoculation with fresh rumen fluid accelerated the rumen protozoal colonization during the pre-weaning period both in concentration and diversity (+ 27 OTUs). As a result, RFF and RFC kids had increased number of fibrolytic protozoa (*Diplodinium*, *Ophryoscolex* and *Enoploplastron*) and holotrichs (*Isotricha* and *Dasytricha*) which are considered late rumen colonizers involved in the H_2 production [45]. The results suggested that these protozoal taxa, along with the protozoal richness, can be considered indicators of the rumen microbial and functional development. The symbiotic relations between rumen protozoa and methanogens in relation to the inter species H_2 transfer [46], and between protozoa and bacteria in relation to cross-feeding processes [45] could partially explain the positive correlation of these protozoal taxa with methanogens and bacterial diversity, as well as with solid feed intake. Moreover, it has been suggested that bacterial predation by protozoa might stimulate the proliferation of different bacterial species occupying similar metabolic niches [47]. After conducting a meta-analysis, Newbold et al. [35] concluded that presence of rumen protozoa (in comparison to defaunated animals) have a positive effect on feed intake (+ 2%), VFA production (+ 5%) and NDF digestibility (+ 11%) but negative on the microbial protein synthesis (− 30%) and ADG (− 9%) being these differences diet-dependent. Our findings suggested that the early colonization of the rumen by a mature protozoal community had positive effects under artificial-milk feeding conditions facilitating the transition from liquid to solid feed.

As it was expected, RFF and RFC kids had a similar protozoal community structure and shared a large core community (up to 23 OTUs), due to the lack of substantial differences in the protozoal community between both types of fresh inocula. However, the protozoal

community structure in AUT kids also converged with that observed in RFF and RFC kids during the post-weaning period. These findings suggest that the rumen protozoal colonization process can be modulated by the type of microbial inocula, but the persistency of the those differences is weak and kids tend to converge into a similar protozoal community, possibly as a result of a cross-faunation between animals [48].

Rumen fungal community

Rumen fungi are considered late rumen colonizers [49]. Like rumen protozoa, anaerobic fungi are high sensitive to oxygen, but their ability to form resistant spores allows them to retain viability in dung, soil and feed, making their transmission easier [50]. Fonty et al. [51] found anaerobic fungi (mostly *Neocallimastix*) in the rumen of flock-reared lambs by 8–10 days after birth, although their presence was intermittent and highly variable until weaning. Orpin [52] also reported that fungi are apparently able to colonize the rumen before the ingestion of large amounts of solid feed. Our findings support this hypothesis since an abundant and diverse anaerobic fungal community was observed before weaning across all treatments. CTL kids were particularly abundant on uncultured *Neocallimastigaceae* genera but lacked some of the most relevant genera such as *Piromyces*, *Orpinomyces* and *Capnodiales*, indicating the presence of an underdeveloped and treatment-specific fungal core community. On the contrary, inoculation with fresh rumen fluid promoted a more abundant and different fungal community than observed in CTL and AUT kids before weaning. In a previous study we showed higher fungal diversity in naturally reared lambs (with their dams) than in their artificially reared counterparts at weaning [41], differences which were not noted in the present study. Instead, inoculation with fresh rumen fluid modulated the fungal community structure during the pre-weaning period leading to increased numbers of *Piromyces* and *Orpinomyces*. *Piromyces* has been described to degrade a wide range of plant structural materials as well as glucose [53, 54] and it has been correlated with ADG during the post-weaning period [41]. *Orpinomyces* is considered a relevant cellulose and xylanase degrader [55] particularly abundant in grazing lambs [5]. Our study showed that *Piromyces* was positively correlated with the bacterial, methanogens and fungal diversities and was the main signature associated to RFC inocula and RFC kids. On the

other hand, whereas *Orpinomyces* correlated with solid feed intake indicating that both are indicators of the rumen development.

It has been suggested that once animals start eating solid feed, the feed composition is the main determining factor of the rumen fungal community [52]. Our study did not allow discerning whether the observed changes in the fungal community were directly determined by the inoculating process or indirectly by the increase in the solid feed intake. Similar co-occurring effects (higher solid feed intake and higher fungal development) were reported in young lambs fed natural milk feeding, in comparison to natural reared, with the peculiarity that these effects persisted during the grazing period leading to higher forage digestibility and ADG during later in life [41]. Our study seems to agree with these findings because RFF and RFC showed higher forage intake than CTL kids up to week 13, but the persistency of the effects on the fungal community needs further research.

Rumen microbiota and animal performance

In terms of productive outcomes, the overall acceleration of the rumen microbial colonization induced by the inoculation with fresh rumen fluid positively correlated with a concomitant acceleration in the rumen functional development during the pre-weaning period. This development implied higher solid feed intake, rumen VFA and blood β-hydroxybutyrate concentrations than in CTL kids [19]. Despite the aforementioned positive indicators, inoculation with fresh rumen fluid did not improve ADG as reported in previous studies [15, 16, 56], possibly because the more complex microbiota is associated with lower feed efficiency when ruminants are fed concentrated diets [31]. However, in our study, this microbial complexity and redundancy provided higher adaptability. As a result, kids inoculated with fresh rumen fluid experienced a higher ADG at week 8 (immediately after weaning), indicating that these kids experienced less growth retardation and weaning shock resulting on positive health and welfare outcomes [19]. Our meta-taxonomic study did not allow assessing causality between this acceleration in the microbial development and changes and its function. The use of metagenomics shotgun sequencing could help to bridge this gap by facilitating a direct inference of the microbial functionality potential.

CONCLUSIONS

This experiment based on a multi-kingdom analysis of the rumen microbiome revealed that an early-in-life repetitive inoculation of young ruminants with fresh rumen fluid from adult animals accelerated the establishment of a more complex and diverse bacterial, methanogenic, protozoal and fungal communities during the pre-weaning period. This microbial complexity facilitated the adaptability of the host ruminant to nutritional challenges favoring the transition from milk to solid feeding during the weaning process. The intensity and persistency of the microbiological effects varied depending on the rumen microbial community considered. The type of diet consumed by the donor animal promoted substantial differences in the inocula, however those microbiological differences were mostly not reflected in the inoculated kids resulting on similar productive outcomes and suggesting that alternative factors such as the availability of nutrients for the rumen microbes or the host-immune system may play a relevant role during the rumen microbial colonization. The inoculation of autoclaved rumen fluid also promoted positive effects on the rumen function but much less evident than using fresh inocula. Further research is needed to evaluate the persistency of these effects in adult animals and their impact on animal productivity.

METHODS

Description of the inocula

Animal procedures were conducted by trained personnel according to the Spanish guidelines (RD 53/2013), and protocols were approved by the Ethical Committee for Animal Research (EEZ-CSIC) regional government (09/ 03/2017). As described in [19], eight adult Murciano-Granadina goats with permanent rumen fistula were distributed into two groups and used as rumen fluid donors. Four received a 100% forage-based diet consisting in 750 g alfalfa hay and 750 g oat hay daily, whereas the other four were fed a concentrate-based diet consisting in 800 g concentrate feed, 125 g alfalfa hay and 125 g oat hay. The forage chemical composition (in g/kg DM) was organic matter 906, nitrogen 25, neutral detergent fiber 594, acid detergent fire 366, acid detergent lignin 95 and ether extract 16, while the pelleted concentrate (Lactación Rumiantes, Macob, Granada, Spain) was 951, 33, 254, 73, 21 and 45, respectively. Diet was offered at 1.2 times maintenance level and divided into two equal meals (8:00, 16:00 h).

After two weeks of adaptation to the diet, rumen fluid from donor goats fed forage (RFF) or concentrate diets (RFC) were collected daily 3 h after the morning feeding (100 ml/donor), pooled by diet, strained through a cheesecloth (approx. 1 mm pore size), bubbled with CO_2, maintained at 37 °C in a pre-warmed thermal flask and immediately administered as fresh inoculum to young kids. Autoclaved inoculum (AUT) was prepared weekly by mixing equal volumes of RFF and RFC inocula from all donors and autoclaved at 115 °C for 30 min to destroy all microbes but maintaining the rumen fermentation products. Four subsamples from each type of inocula were taken at regular intervals for inocula characterization (Suppl. Table S1).

Inoculation experiment

A total of 80 newborn Murciano-Granadina goat kids were randomly distributed in 4 experimental groups: RFF and RFC kids were inoculated with rumen fluid from adult goats fed forage-rich or concentrate-rich diets, respectively. AUT kids were inoculated with the autoclaved rumen fluid whereas CTL kids received no inoculation. Inoculation consisted of an oral and daily drench of rumen fluid (2.5 ml/animal during week 1 and 5 ml/animal thereafter) from day 1 until 11 weeks of age. Kids from different treatments were separated by a 2-m-wide corridor to prevent physical contact. Inoculation was performed by trained personnel following always the same sequence (AUT followed by RFF and RFC kids) and changing all inoculation material (e.g. drench and gloves) to prevent cross-contamination between experimental groups.

After parturition, all kids were separated from their mother and received approximately 200 ml of pooled natural colostrum divided in two doses. To avoid any initial bias, average body weight (BW) and males/females ratio was kept similar in all treatments and siblings were always allocated into different treatments. Kids within each treatment were distributed in 5 contiguous pens with similar age (maximum 2 days difference) and were handled and sampled on the same day across treatments.

All kids were raised on commercial milk replacer (Univet Spray, Cargill, Barcelona, Spain) offered ad libitum. From week 2, kids had free access to the same forage mixture that has been described for the donor goats and to pelleted starter concentrate (0–14 Rumiantes Transición, Macob, Granada, Spain) with the following chemical composition (in g/kg DM): 949 OM, 226 CP, 319 NDF, 87 ADF, 34 ADL, 48 EE. Kids were weaned

at 7 weeks of age by progressively decreasing milk powder concentration during 4 days. Forage and concentrate intakes were daily recorded, BW, blood glucose and β-hydroxybutyrate concentrations were weekly monitored.

Rumen microbial sampling and analyses

Rumen microbiota was studied at weeks 5 (pre-weaning), 7 (weaning) and 9 (post-weaning). Rumen content was withdrawn by orogastric intubation at 09:00 h as previously described [19]. Rumen samples (ca. 50 ml) were filtrated through sterile cheesecloth (approx. 1 mm pore size) and all sampling instruments were changed between animals to prevent cross contamination. A sub-sample of rumen fluid was snap-frozen in liquid N whereas solids were discarded given the small and variable proportion of solids in the samples. Rumen samples were freeze-dried, bead-beated for 1 min (MiniBeadBeater, Biospect Products, Bartlesville, OK, USA) and DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain). DNA was also extracted from the rumen fluids used as inocula (positive controls) and from negative controls (DNA extraction without rumen fluid) and further analyzed. Eluted DNA (2 μl) was used to assess the abundance of the main microbial groups by quantitative PCR (qPCR) an iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA). Specific primers for the 16S bacterial rRNA gene, mcrA gene for archaea and 18S rRNA genes for protozoa and anaerobic fungi were used as reported in Supplementary Table S8. Rumen fermentation characteristics in terms of pH, ammonia and volatile fatty acids (VFA) was also described at the same ages [19]. This metadata (Suppl. Table S7) was used to relate microbial changes in the rumen with animal physiology.

Next generation sequencing

For meta-taxonomic analyses 8 kids from each treatment were selected (all males from pens 1, 2, 3 and 4) and a template of rumen DNA was sent to University of Illinois Biotechnology Center (Urbana, IL, USA) for Fluidgim amplicon sequencing using Miseq V3 (Illumina Inc., San Diego, CA, USA). Primers used for the amplification of the

bacterial 16S (V3-V5 region), methanogens 16S, protozoal 18S and anaerobic fungal ITS3-ITS4 regions were used as described in Supplementary Table S8.

For each of the 4 major microbial groups, primer sorted and demultiplexed paired-end reads were merged and then combined into one file. Downstream processing was performed using QIIME [57] and Mothur [58] for archaea, PIPITS [59] for fungi and IM-Tornado [60] for bacteria and protozoa, where non-overlapping reads are processed while retaining maximal information content. Low-quality reads and bases (PHRED quality score below 25) were trimmed. Minimum length of reads after quality filtering was 350 for bacteria and archaea, 187 for protozoa and 300 for fungi. Chimeras were identified and removed using chimera.vsearch [61]. Operational taxonomic units (OTU) were identified at 97% similarity level and then representative sequences from all OTUs were aligned against Greengenes 13_8 97% [62] for bacteria, RIM-DB [63] for archaea, Silva v. 132 [64] for protozoa and UNITE [65] for fungi. Once alignment was performed, data from each of the 4 major microbial groups were processed separately. The number of sequences per sample for each microbial group was normalized across all the samples and singletons were removed. Only sequences from rumen protozoa *(subclass Trichostomatia*) and anaerobic fungi (class *Neocallimastigomycota*) were further considered to prevent potential bias derived from transient or non-rumen eukaryotes [66]. Raw sequences reads were deposited at European Nucleotide Archive repository (accession: ERP122902).

Calculations and statistical analyses

Statistical analyses were conducted using SPSS software (IBM Corp., Version 21.0, New York, USA). Quantitative PCR data (rDNA copies/mg DM) and taxa abundances (sequences) were tested for normality using the Shapiro–Wilk test and data were log10 transformed to achieve a normal distribution. Data were analysed based on a repeated measures mixed –effects (residual maximum likelihood) as follows:

$$Y_{ijkl} = \mu + I_i + T_j + (I \times T)_{ij} + G_k + A(G)_l + e_{ijkl}$$

where Y_{ijkl} is the dependent, continuous variable, μ is the overall population of the mean, I_i is the fixed effect of the inoculation (i = CTL vs AUT vs RFF vs RFC), T_j is the fixed effect of the sampling time or age (j = 5 vs 7 vs 9 weeks), $(I \times A)_{ij}$ is the interaction term,

G_k is the random effect of the pen considered as a block (j = 1 to 5) $A(G)_l$ is the random effect of the animal nested to the pen (l = 1 to 80 for qPCR and 1 to 32 for sequencing data) and e_{ijkl} is the residual error. For taxonomic data, False Discovery Rate was minimized using the Bonferroni statistical. Significant effects were declared at $P < 0.05$, tendency to difference at $P < 0.1$ and abbreviated as follows: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; † $P < 0.1$; ns, not significant.

Venn diagrams were performed to illustrate the treatment effects on the core microbial community which was defined as the number of OTUs shared across the majority (> 75%) of the individuals in each treatment/ time [8] using a multiple list comparator (www.molbiotools.com). Treatment effects on the rumen multi-kingdom (including all microbial groups) and on the bacterial, methanogens, protozoal and anaerobic fungal communities were assessed based on the Bray-Curtis distance metrics using the UPGMA function of PRIM ER-6 software (PRIMER-E Ltd., Plymouth, UK). Log10- transformed data were analyzed by non-parametric PERMANOVA after 999 random permutations of residuals under the reduced model using the Monte Carlo test [67]. When a significant factor was found in the PERMANOVA, pair-wise comparisons were performed using the same software and settings (999 permutations) to elucidate differences between treatments. Principal Coordinate analysis (PCoA) were performed to illustrate the impact of the treatments on the overall microbial community structure and tripod vectors were included to describe the direction and intensity of the most discriminant OTUs (Spearman's correlation > 0.55). Given that three different nutritional situations were studied, Distance-Based Redundancy Analyses were performed to illustrate the relationship between the community structure of the rumen microbiota and metadata (32 variables reported in Suppl. Table S2) describing the rumen function and animal performance at 5, 7 and 9 weeks of age. Distance based linear models (DistLM) were developed based on the Bray-Curtis similarity and the predictor variables were selected based on a step-wise procedure with 999 random permutations following the Akaike information criterion with corrections for small sample size (AICc) to avoid model overfitting. Only predictor variables which resulted significant ($P < 0.05$) were included in the final model. Spearman correlations (ρ) were calculated to assess the relationships between the microbial taxa abundance (log10 number of sequences) and the metadata. Strong correlations were defined as those with $\rho \geq 0.3$ or ≤ -0.3 and $P < 0.01$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-021-00073-9.

Additional file 1 Table S1. Description of the inocula in terms of rumen fermentation and microbial composition. Table S2. Effect of the early-in-life rumen microbial inoculation and age on the rumen bacteria concentration, diversity and taxonomy. Table S3. Spearman's correlations between the bacterial taxa and the rumen function and animal performance. Table S4. Effect of early-in-life rumen microbial inoculation and age on the rumen methanogens concentration, diversity and taxonomy.

Table S5. Effect of early-in-life rumen microbial inoculation and age on the rumen protozoal concentration, diversity and taxonomy. Table S6. Effect of early-in-life rumen microbial inoculation and age on the rumen anaerobic fungal concentration, diversity and taxonomy. Table S7. Descriptive statistics of the metadata used in the distance-based redundancy analyses and spearman correlations with microbial taxa abundance. Table S8. Primers used for quantitative PCR and Next Generation Sequencing. Fig. S1. Principal coordinates analysis illustrating the inoculation effects on the multi-kingdom rumen microbiome.

Abbreviations

ADG: Average daily gain; AUT: Goat kids inoculated with autoclaved rumen fluid; BHB: Beta-hydroxybutyrate; BW: Body weight; CTL: Control goat kids without inoculation; dbRDA: Distance-based redundancy analysis; DCI: Digestible cellulose intake; DHCI: Digestible hemicellulose intake; PCoA: Principal Coordinate Analysis; PERMANOVA: Permuntational analysis of variance; RFC: Goat kids inoculated with rumen fluid adapted to concentrate diet; RFF: Goat kids inoculated with rumen fluid adapted to forage diet; VFA: Volatile fatty acids

Acknowledgements

The authors thank Isabel Jimenez, Ibtissam Nejjam, Rosa Serrano and Rafael Hueso for their assistance with the animal care and samples analyses. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Author's contributions

AB & DRYR: Conceptualization, validation and supervision. JMPH, AB, & EJ: Methodology, investigation, resources and formal analysis. JMPH, AB & MP: Data curation and software. DRYR & AIMG: funding acquisition. JMPH & AB: Writing

original draft. DRYR, DPM, MP: Writing – review and editing. All authors read and approved the final version.

Funding

This study was funded by the Spanish Research Agency (Ref. AGL2017 86938-R) and the Training Program for Academics grant, Madrid, Spain (Ref. FPU16/01981). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Availability of data and materials

All sequencing data generated in this study are publicly available in the European Nucleotide Archive repository (accession: ERP122902).

Ethics approval and consent to participate

Animal procedures were conducted by trained personnel according to the Spanish guidelines (RD 53/2013), and protocols were approved by the Ethical Committee for Animal Research (EEZ-CSIC) regional government (09/ 03/2017).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Abecia L, Ramos-Morales E, Martínez-Fernandez G, et al. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Anim Prod Sci. 2014;54: 1449–54.

2. Skillman LC, Evans PN, Naylor GE, Morvan B, Jarvis GN, Joblin KN. 16S ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. Anaerobe. 2004;10: 277–85.

3. Davis CL, Drackley JK. The Development, Nutrition, and Management of the Young Calf,. Iowa State.; 1998.

4. Baldwin VIRL, McLeod KR, Klotz JL, Heitmann RN. Rumen development, intestinal growth and hepatic metabolism in the pre- and Postweaning ruminant. J Dairy Sci. 2004;87:E55–65.

5. Belanche A, Kingston-Smith AH, Griffith GW, Newbold CJ. A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. Front Microbiol. 2019;10:1–17.

6. Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O, Monteils V. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. J Appl Microbiol. 2014;116:245–57.

7. Yáñez-Ruiz DR, Abecia L, Newbold CJ. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front Microbiol. 2015;6:1–12.

8. Furman O, Shenhav L, Sasson G, et al. Stochasticity constrained by deterministic effects of diet and age drive rumen microbiome assembly dynamics. Nat Commun. 2020;11:1–13.

9. Nylund L, Satokari R, Salminen S, De Vos WM. Intestinal microbiota during early life - impact on health and disease. Proc Nutr Soc. 2014;73:457–69.

10. Yeoman CJ, Ishaq SL, Bichi E, Olivo SK, Lowe J, Aldridge BM. Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. Sci Rep. 2018;8:1–14.

11. Alipour MJ, Jalanka J, Pessa-Morikawa T, et al. The composition of the perinatal intestinal microbiota in cattle. Sci Rep. 2018;8:1–14.

12. Lu CD, Potchoiba MJ. Milk feeding and weaning of goat kids - a review. Small Rumin Res. 1988;1:105–12.

13. Abecia L, Jiménez E, Martínez-Fernandez G, et al. Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. PLoS One. 2017;12:1–19.

14. De Barbieri I, Hegarty RS, Silveira C, Oddy VH. Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of merino lambs. Small Rumin Res. 2015;129:37–47.

15. De Barbieri I, Hegarty RS, Silveira C, et al. Programming rumen bacterial communities in newborn merino lambs. Small Rumin Res. 2015;129:48–59.

16. Yu S, Zhang G, Liu Z, Wu P, Yu Z, Wang J. Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. BMC Microbiol. 2020;20:1–15.

17. Huws SA, Creevey CJ, Oyama LB, et al. Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. Front Microbiol. 2018;9:1–33.

18. Belanche A, Palma-Hidalgo JM, Nejjam I, et al. In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. J Sci Food Agric. 2019;99:163–72.

19. Belanche A, Palma-Hidalgo JM, Nejjam I, Jiménez E, Martín-García AI, Yáñez-Ruiz DR. Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. J Dairy Sci. 2020;103: 5047–60.

20. Muscato TV, Tedeschi LO, Russell JB. The effect of ruminal fluid preparations on the growth and health of newborn, milk-fed dairy calves. J Dairy Sci. 2002;85:648–56.

21. O'Hara E, Kelly A, McCabe MS, Kenny DA, Guan LL, Waters SM. Effect of a butyrate-fortified milk replacer on gastrointestinal microbiota and products of fermentation in artificially reared dairy calves at weaning. Sci Rep. 2018;8: 1–11.

22. Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 2013;7:1069–79.

23. O'Herrin SM, Kenealy WR. Glucose and carbon dioxide metabolism by Succinivibrio dextrinosolvens. Appl Environ Microbiol. 1993;59:748–55.

24. Danielsson R, Dicksved J, Sun L, et al. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. Front Microbiol. 2017;8:1–15.

25. Li F, Guan LL. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. Appl Environ Microbiol. 2017;83:1–16.

26. Fernando SC, Purvis HT, Najar FZ, et al. Rumen microbial population dynamics during adaptation to a high-grain diet. Appl Environ Microbiol. 2010;76:7482–90.

27. Ransom-Jones E, Jones DL, McCarthy AJ, McDonald JE. The Fibrobacteres: an important phylum of cellulose-degrading Bacteria. Microb Ecol. 2012;63: 267–81.

28. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the genetic basis of fibrolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. Diversity. 2013;5:627–40.

29. Brulc JM, Antonopoulos DA, Berg Miller ME, et al. Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases. Proc Natl Acad Sci U S A. 2009;106: 1948–53.

30. Di Rienzi SC, Sharon I, Wrighton KC, et al. The human gut and groundwater harbor non-photosynthetic bacteria belonging to a new candidate phylum sibling to cyanobacteria. Elife. 2013;2:1–25.

31. Kruger Ben Shabat S, Sasson G, Doron-Faigenboim A, et al. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME J. 2016;10:2958–72.

32. Dias J, Marcondes MI, Noronha MF, et al. Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. Front Microbiol. 2017;8:1–17.

33. Dehority B. Rumen Microbiology: Nottingham University Press; 2003.

34. Friedman N, Jami E, Mizrahi I. Compositional and functional dynamics of the bovine rumen methanogenic community across different developmental stages. Environ Microbiol. 2017;19:3365–73.

35. Newbold CJ, De la Fuente G, Belanche A, Ramos-Morales E, McEwan NR. The role of ciliate protozoa in the rumen. Front Microbiol. 2015;6:1–14.

36. Mackie R, McSweeney C, Klieve A. 4 Microbial Ecology of the Ovine Rumen. Sheep Nutr. Published online 2002:71.

37. Belanche A, De La Fuente G, Newbold CJ. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. FEMS Microbiol Ecol. 2015;91:1–10.

38. Zhou M, Chen Y, Griebel PJ, Guan LL. Methanogen prevalence throughout the gastrointestinal tract of pre-weaned dairy calves. Gut Microbes. 2015;5: 628–38.

39. Tapio I, Snelling TJ, Strozzi F, Wallace RJ. The ruminal microbiome associated with methane emissions from ruminant livestock. J Anim Sci Biotechnol. 2017;8:1–11.

40. Sharp R, Ziemer CJ, Stern MD, Stahl DA. Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiol Ecol. 1998;26:71–8.

41. Belanche A, Yáñez-Ruiz DR, Detheridge AP, Griffith GW, Kingston-Smith AH, Newbold CJ. Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environ Microbiol. 2019; 21:4360–77.

42. Fonty G, Senaud J, Jouany JP, Gouet P. Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. Can J Microbiol. 1988;34:235–41.

43. Belanche A, Balcells J, de la Fuente G, Yañez-Ruíz DR, Fondevila M, Calleja L. Description of development of rumen ecosystem by PCR assay in milk-fed, weaned and finished lambs in an intensive fattening system. J Anim Physiol Anim Nutr. 2010;94:648–58.

44. Belanche A, Abecia L, Holtrop G, et al. Study of the effect of presence or absence of protozoa on rumen fermentation and microbial protein contribution to the chyme. J Anim Sci. 2011;89:4163–74.

45. Williams, A.G., and Coleman GS. The Rumen Protozoa. (Thomas DB, ed.). Springer-Verlag New York Inc.; 1992.

46. Newbold CJ, Lassalas B, Jouany JP. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. Lett Appl Microbiol. 1995;21:230–4.

47. Belanche A, De la Fuente G, Moorby JM, Newbold CJ. Bacterial protein degradation by different rumen protozoal groups. J Anim Sci. 2012;90:4495– 04.

48. Bird SH, Hegarty RS, Woodgate R. Modes of transmission of rumen protozoa between mature sheep. Anim Prod Sci. 2010;50:414–7.

49. Orpin CK, Joblin KN. The rumen anaerobic fungi. In: Hobson PN, Stewart C, eds. The Rumen Microbial Ecosystem. Springer. 1997:140–95.

50. McGranaghan P, Davies JC, Griffith GW, Davies DR, Theodorou MK. The survival of anaerobic fungi in cattle faeces. FEMS Microbiol Ecol. 1999;29: 293–00.

51. Fonty G, Gouet P, Jouany J-P, Senaud J. Establishment of the microflora and anaerobic Fungi in the rumen of lambs. Microbiology. 1987;133:1835–43.

52. Orpin CG. The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim Feed Sci Technol. 1984;10:121–43.

53. de Souza WR. Microbial Degradation of Lignocellulosic Biomass. Sustain Degrad Lignocellul Biomass. Tech Appl Commer. 2013;207–247.

54. Solomon KV, Haitjema CH, Henske JK, et al. Early-branching gut fungi possess large, comprehensive array of biomass-degrading enzymes. Science. 2016;351:1192–5.

55. Li XL, Chen H, Ljungdahl LG. Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. Appl Environ Microbiol. 1997;63:628–35.

56. Zhong RZ, Sun HX, Li GD, Liu HW, Zhou DW. Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. Livest Sci. 2014;162:154–8.

57. Caporaso JG, Kuczynski J, Stombaugh J, et al. Correspondence QIIME allows analysis of high- throughput community sequencing data intensity normalization improves color calling in SOLiD sequencing. Nat Publ Gr. 2010;7:335–6.

58. Schloss PD, Westcott SL, Ryabin T, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537–41.

59. Gweon HS, Oliver A, Taylor J, et al. PIPITS: an automated pipeline for analyses of fungal internal transcribed spacer sequences from the Illumina sequencing platform. Methods Ecol Evol. 2015;6:973–80.

60. Jeraldo P, Kalari K, Chen X, et al. IM-TORNADO: a tool for comparison of 16S reads from paired-end libraries. PLoS One. 2014;9:1–19.

61. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. PeerJ. 2016;2016:1–22.

62. DeSantis TZ, Hugenholtz P, Larsen N, et al. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol. 2006;72:5069–72.

63. Seedorf H, Kittelmann S, Henderson G, Janssen PH. RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea fromthe rumen and other intestinal environments. PeerJ. 2014;2:e494.

64. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013;41:590–6.

65. Nilsson RH, Larsson KH, Taylor AFS, et al. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Res. 2019;47:259–64.

66. Hobson PN, Stewart CS. The Rumen Microbial Ecosystem. 2nd ed. Springer; 1997.

67. Belanche A, Newbold CJ, Lin W, Stevens PR, Kingston-Smith AH. A systems biology approach reveals differences in the dynamics of colonization and degradation of grass vs. Hay by rumen microbes with minor effects of vitamin E supplementation. Front Microbiol. 2017;8:1–18.

Tables and Figures

Table 1. Permutational analysis of variance describing the effects of early-in-life rumen microbial inoculation and time on the rumen community structure.

Community[1]	Inoculation	Time	Interaction
Multi-kingdom			
Variance (%)	21.8	16.2	9.90
Pseudo-F	12.3	13.7	2.79
P-value	<0.001	<0.001	<0.001
Bacteria			
Variance (%)	16.1	17.3	9.26
Pseudo-F	8.20	13.21	2.36
P-value	<0.001	<0.001	<0.001
Methanogens			
Variance (%)	24.9	11.5	9.07
Pseudo-F	12.5	8.70	2.28
P-value	<0.001	<0.001	0.002
Protozoa			
Variance (%)	14.5	9.07	6.28
Pseudo-F	6.79	4.23	1.47
P-value	<0.001	<0.001	0.096
Anaerobic fungi			
Variance (%)	30.4	1.73	4.41
Pseudo-F	8.45	1.44	1.23
P-value	<0.001	0.192	0.193

[1]Microbial data were log10 transformed and only Spearman's correlations coefficients $\rho > 0.3$ (green) or $\rho < -0.3$ (red) and $p < 0.01$ are shown (N = 96). Parameters: milk, concentrate, forage and DM intake (g/d), rumen pH, total volatile fatty acids (mM), acetate (%), propionate, butyrate (%), odd and branched chain fatty acids (%), microbial concentration (log10 copies/mg DM), OTUs richness (−R), plasma β-hydroxybutyrate (mM), blood glucose (mg/dL), average daily gain (g/d) and ADG during the post weaning period (ADG-pw).

Table 2. Correlations among the rumen microbiota and digestive physiology data.

Corelations[1]	Milk	Concentrate	Forage	DMI	pH	Ammonia	VFA	Acetate	Propionate	Butyrate	OBCVFA	Bacteria	Bacterial-R	Methanogens	Methanogens-R	Protozoa	Protozoal-R	Fungi	Fungal-R	BHB	Glucose	ADG	ADG-pw
Total bacterial abundance		-0.32																					
Bacterial richness		0.45	0.51	0.46										0.31	0.55	0.55							
p_Actinobacteria	-0.42																-0.37		-0.38			-0.38	
p_Bacteroidetes		-0.39	-0.4	-0.5						-0.34										-0.33	0.36		-0.46
f_Bacteroidaceae		-0.39	-0.68	-0.44							0.32		-0.44	-0.37	-0.39	-0.53	-0.52						-0.5
f_Bacteroidales			-0.38	-0.34			-0.38				0.35												
f_p-2534-18B5_gut_group	0.39	0.42		0.44															0.43	0.35			
f_PeH15			0.44		0.34								0.52			0.54	0.63						
f_Prevotellaceae		0.39					0.49	-0.33	0.32	0.32									-0.36				
f_Rikenellaceae			0.45					0.35					0.44			0.46	0.58						
p_Chloroflexi			0.37		0.35			0.38	-0.43				0.47	0.46	0.49	0.62	0.56				-0.34		
p_Cyanobacteria		0.49	0.53	0.49							-0.37		0.73			0.52	0.73						
p_Elusimicrobia													0.48		0.38	0.32	0.36						
f_Elusimicrobiaceae			0.41										0.54		0.46	0.59	0.55						
p_Fibrobacteres			0.35										0.56		0.38	0.32	0.43						
p_Firmicutes			0.48															0.37			-0.49		0.39
f_Acidaminococcaceae							0.45						0.52		0.34	0.38	0.57						
f_Christensenellaceae	-0.43							0.42	-0.42	-0.33											-0.36	-0.39	
f_Clostridiaceae	-0.39							0.37	-0.37													-0.44	
f_Defluviitaleaceae			0.38										0.42		0.36	0.64	0.45		-0.38				
f_Family_XIII			0.52						-0.33				0.35	0.46		0.53	0.38		-0.38		-0.32		
f_Lachnospiraceae								-0.4		0.36													
f_Peptococcaceae							-0.5	0.47	-0.38	-0.5				0.34									-0.49
f_Ruminococcaceae			0.36					0.39					0.35			0.34	0.34		-0.32			-0.33	
f_Veillonellaceae		0.54	0.47	0.55			0.35	-0.44		0.58			0.65		0.44	0.34	0.53			0.37			0.47
p_Proteobacteria	0.53	0.34		0.36				-0.43	0.54	0.41												0.5	
f_Campylobacteraceae																							0.35
f_Comamonadaceae			-0.31								0.34			-0.49		-0.36							-0.54
f_Neisseriaceae	-0.53	-0.38		-0.43				0.41	-0.37	-0.39												-0.34	-0.33
f_Pasteurellaceae		-0.4		-0.38				0.45	-0.44	-0.32					-0.33							-0.43	
f_Rhodocyclaceae			0.33										0.46			0.37	0.43						
f_Rhodospirillaceae			0.31										0.49	0.32			0.37						
f_Succinivibrionaceae	0.52	0.35		0.38				-0.48	0.56	0.45												0.53	
p_Spirochaetae		0.39		0.34																			
p_Synergistetes						0.33			-0.33														
p_Tenericutes			0.59						-0.36				0.63	0.31	0.34	0.67	0.69						
p_Lentisphaerae	-0.36		0.37					0.43	-0.38				0.42	0.56		0.48	0.42						
Total methanogens abundance												0.47											
Methanogens richness					0.37											0.48							
f_Methanomassiliicoccaceae						-0.34													0.34				
s_Group10_sp													0.37		0.43			-0.35					
s_Group11_sp									0.33														
s_ Group12_sp															0.34							0.35	
s_Group8_sp			-0.42								0.32		-0.45			-0.58	-0.57		0.31				
s_Group9_sp			0.38								-0.31		0.37		0.33	0.42	0.52						
s_Methanomassiliicoccaceae spp.													0.55		0.36	0.36	0.52						
f_Methanobacteriaceae	-0.31																		-0.35				
g_Methanobrevibacter	-0.32																-0.31		-0.31				
s_Methanobrevibacter_gottschalkii			-0.31								0.35						-0.33					-0.33	-0.33
s_Methanobrevibacter_ruminantium			0.37										0.41	0.39	0.46	0.41	0.35						0.48
s_Methanobrevibacter spp.										0.34								-0.43					0.4
g_Methanosphaera															0.39			-0.39					0.35
s_Methanosphaera_sp_ISO3-F5															0.32					0.39			0.32
s_Methanosphaera_stadtmanae											0.36							-0.33					
s_Methanomicrobium_mobile			0.4										0.58	0.36	0.32	0.43	0.65				-0.43		
s_Methanimicrococcs_blatticola			0.45								-0.54		0.42			0.61	0.56						0.34
Total protozoal abundance			0.46						-0.37					0.38									
Protozoal richness		0.35	0.48	0.34									0.89		0.53	0.65							
f_Ophryoscolecidae													0.42	0.32	0.56	0.49	0.42				-0.33		
g_Entodinium													0.58	0.44	0.59	0.46	0.53						
g_Ophryoscolex		0.44	0.37	0.42			0.34			0.32	-0.32		0.69		0.43	0.65	0.71					0.34	0.34
g_Diplodinium			0.39		0.33								0.63	0.32	0.4	0.55	0.79				-0.38		
g_Polyplastron									-0.33				0.51	0.35	0.49	0.53	0.45						
g_Enoploplastron			0.38					0.38	-0.39				0.63		0.36	0.48	0.69				-0.39		
f_Buetschliidae		0.44	0.5	0.43			0.34				-0.37		0.8		0.49	0.55	0.81						0.42
g_Isotricha		0.45	0.48	0.44							-0.38		0.73		0.47	0.61	0.79			0.32			0.42
g_Dasytricha		0.42	0.53	0.43			0.39				-0.38		0.8		0.45	0.45	0.83						0.33
g_uncultured													0.64		0.45	0.57	0.73						
Anaerobic fungi concentration								0.39															
Anaerobic fungi richness	0.33																						
g_Caecomyces																-0.44	-0.43						
g_Neocallimastix					-0.36														0.45				
g_Orpinomyces	0.36	0.39		0.39									0.55				0.57						
g_Piromyces													0.39	0.31	0.31	0.58	0.45						

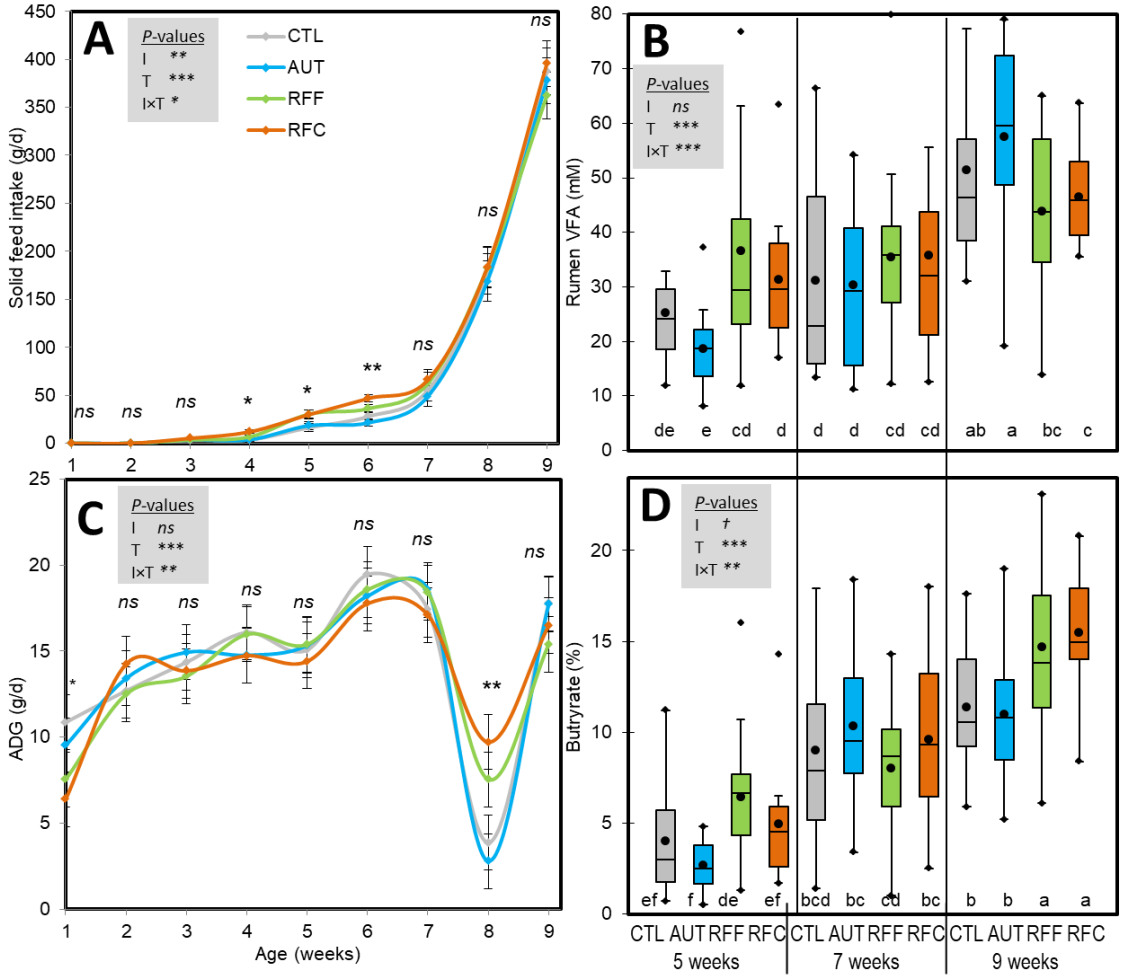


Fig. 1 Summary of the effects of early-in-life rumen microbial inoculation on the solid feed intake (A), rumen VFA concentration (B), average daily gain (C), and butyrate molar proportion (D) in young goats before, during and after weaning. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; †$P < 0.1$; ns, not significant. Means within a row with different letters differ from high to low at $P < 0.05$.

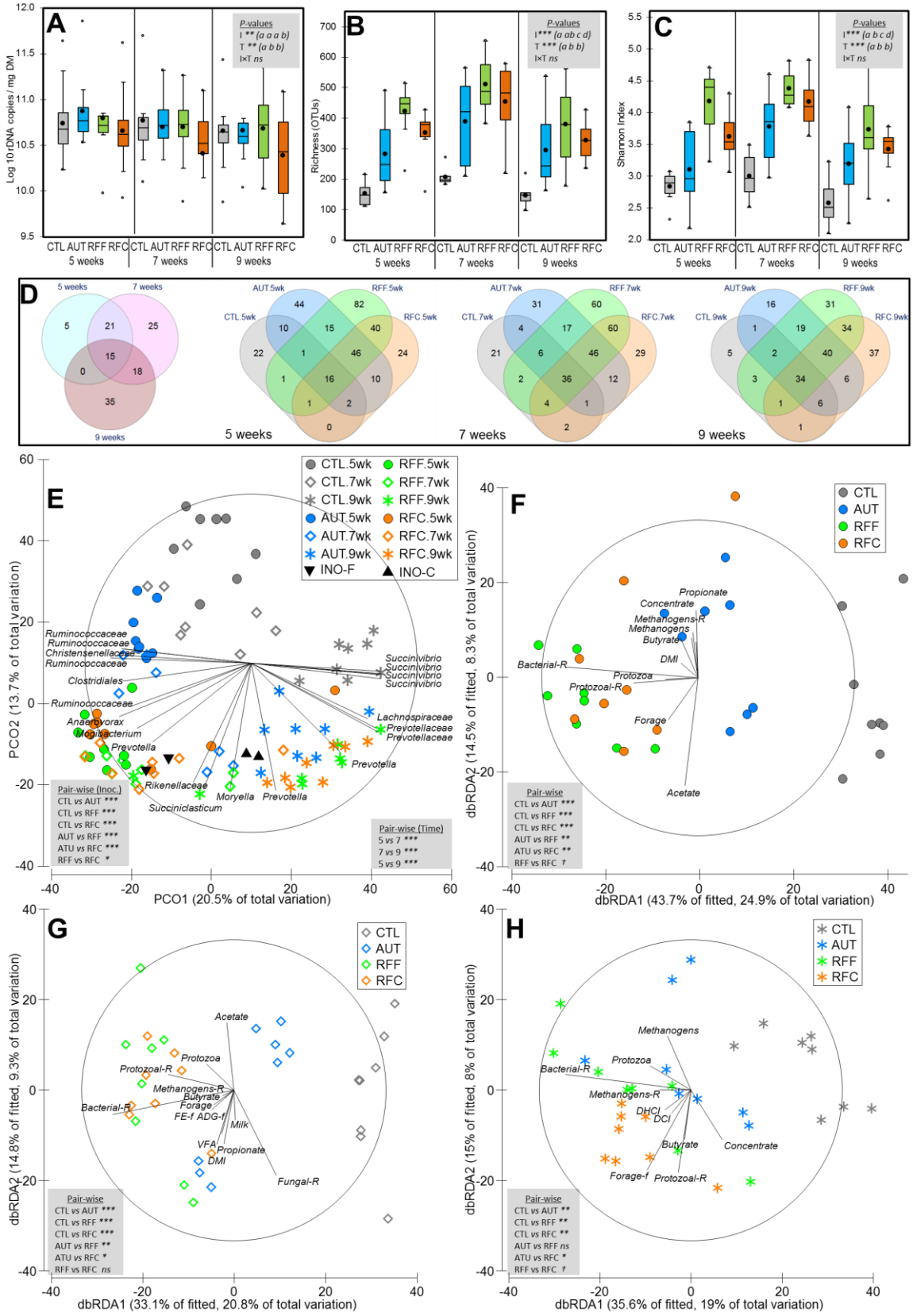


Fig. 2 Effects of early-in-life rumen microbial inoculation on the rumen bacterial community in goats. Boxplots indicating the total bacterial abundance (A) and diversity indexes (B,C). Venn diagrams indicating the number of OTUs in the core community (D). Principal co-ordinates analysis (E) illustrating the treatment effects on the bacterial community showing the most discriminant OTUs ($\rho > 0.75$). Distance-based redundancy

analysis illustrating relationship between the structure of the bacterial community and rumen function indicators before (F), during (G) and after (H) weaning. Pair-wise PERMANOVA values are provided in grey boxes based on the Bray-Curtis dissimilarity. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T). In Figs. A, B and C, treatment means with different letters differ.

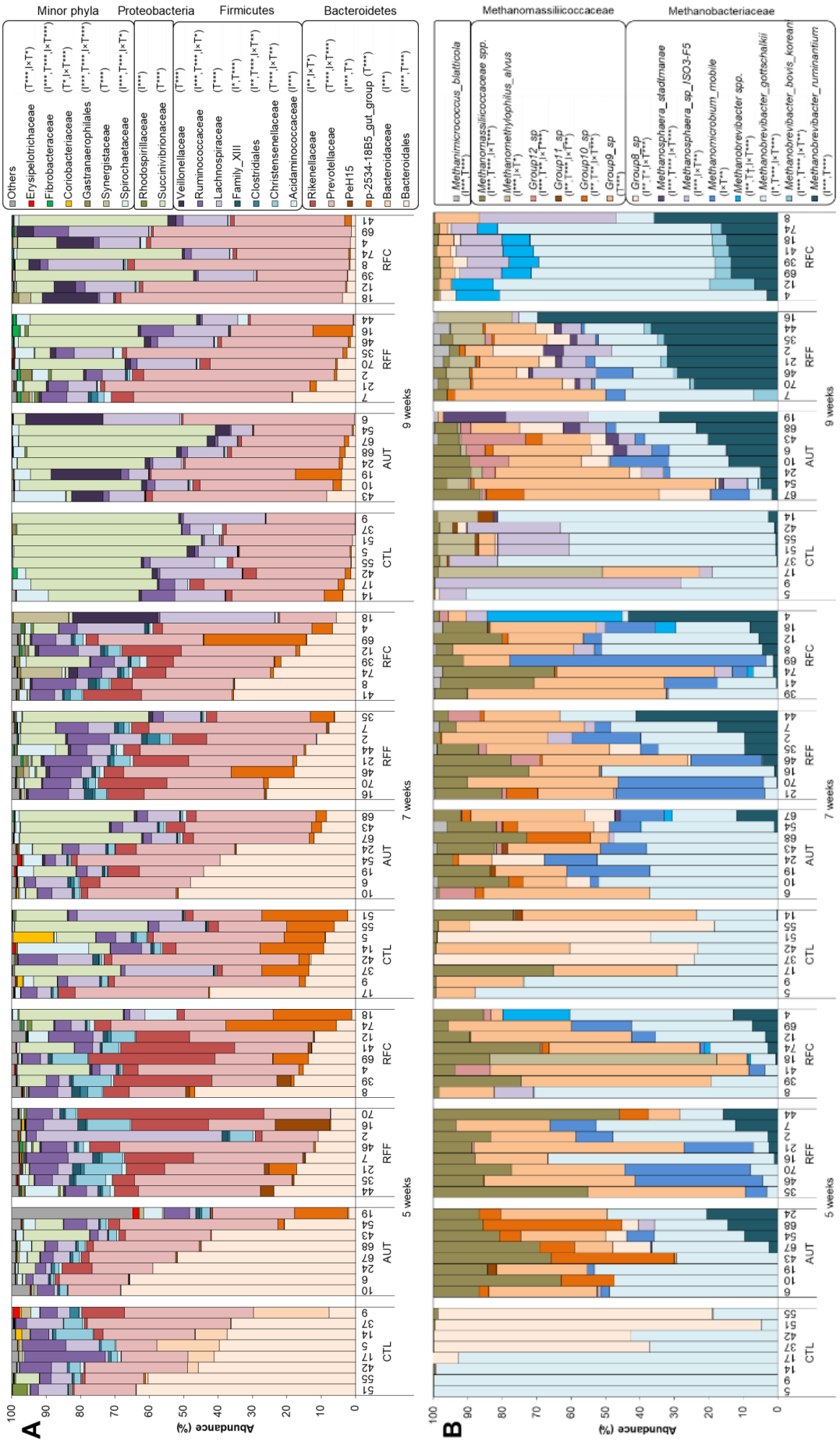


Fig. 3 Effect of the early-in-life rumen microbial inoculation on the rumen prokaryotic taxa distribution. A) Bacterial and (B) methanogens abundances. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T)

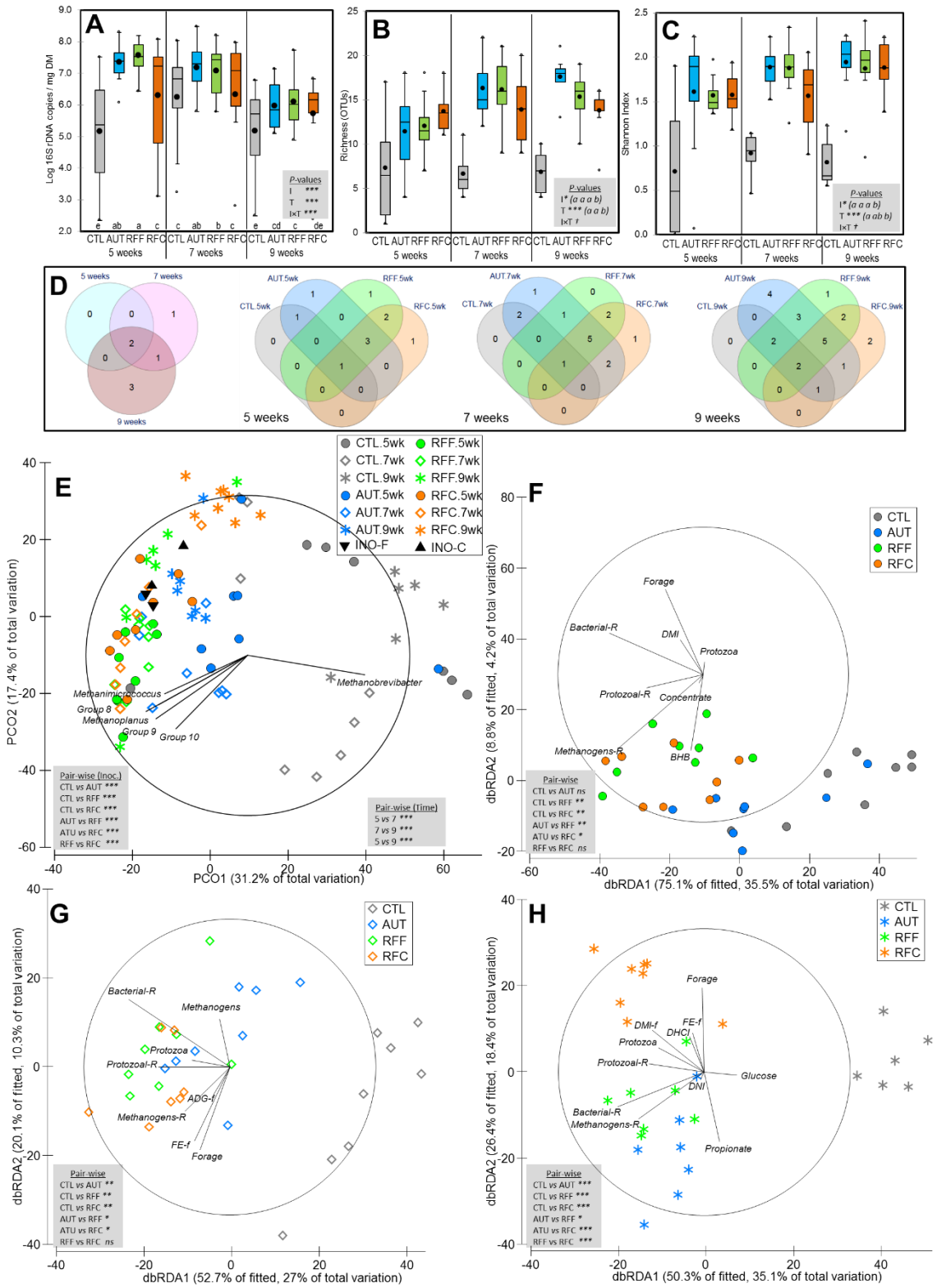


Fig. 4 Effects of early-in-life rumen microbial inoculation on the rumen methanogens community in goats. Boxplots indicating the total methanogens abundance (A) and diversity indexes (B,C). Venn diagrams indicating the number of OTUs in the core community (D). Principal coordinates analysis (E) illustrating the treatment effects on the methanogens community showing the most discriminant OTUs ($\rho > 0.55$). Distance-based redundancy analysis illustrating relationship between the structure of the methanogens community and rumen function indicators before (F), during (G) and after

(H) weaning. Pair-wise PERMANOVA values are provided in grey boxes based on the Bray-Curtis dissimilarity. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T). In Figs. A, B and C, treatment means with different letters differ

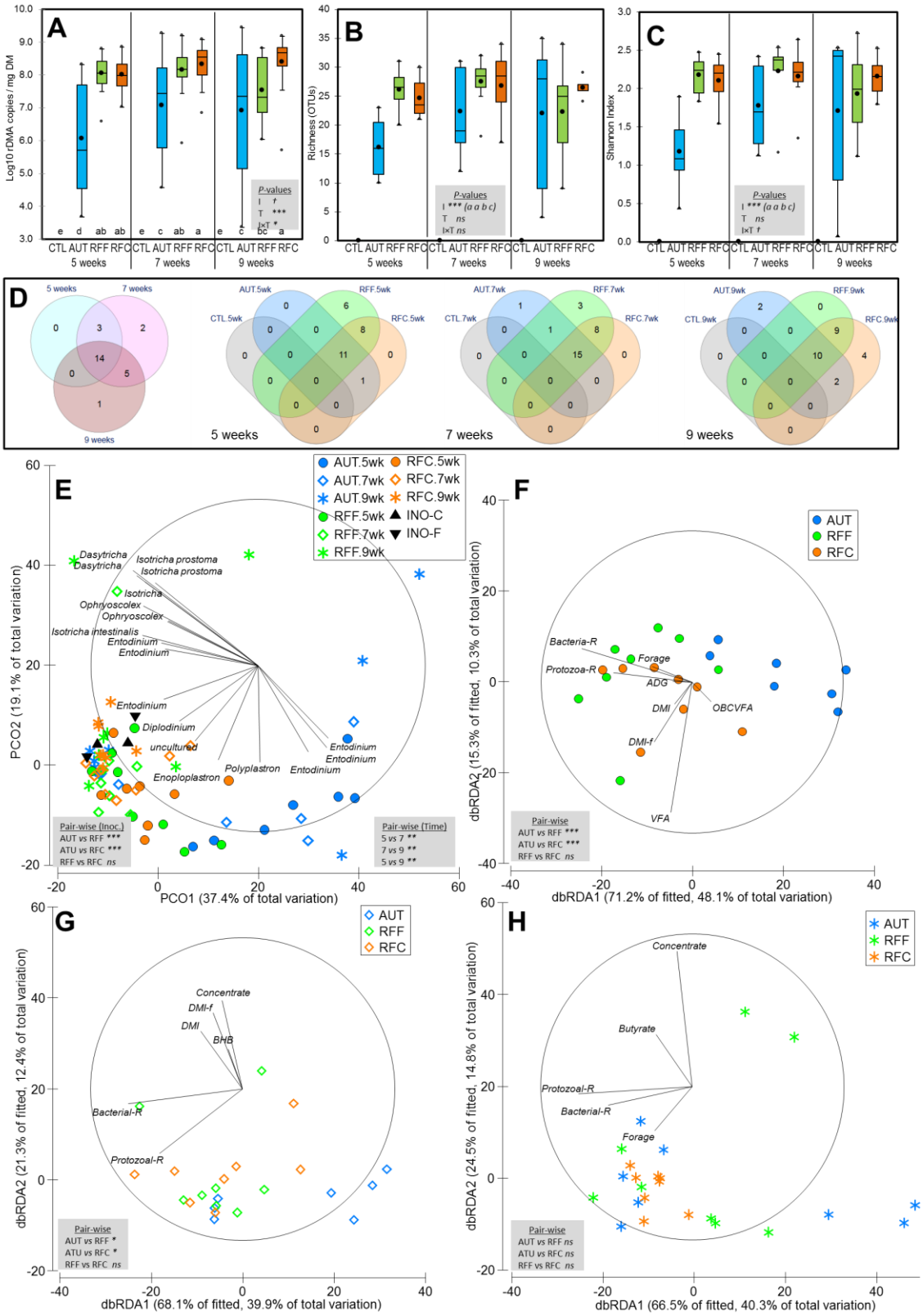


Fig. 5 Effects of early-in-life rumen microbial inoculation on the rumen protozoal community in goats. Boxplots indicating the total protozoal abundance (A) and diversity indexes (B,C). Venn diagrams indicating the number of OTUs in the core community (D). Principal co-ordinates analysis (E) illustrating the treatment effects on the rumen protozoal community showing the most discriminant OTUs ($\rho > 0.55$). Distance-based redundancy analysis illustrating relationship between the structure of the protozoal

community and rumen function indicators before (F), during (G) and after (H) weaning. Pair-wise PERMANOVA values are provided in grey boxes based on the Bray-Curtis dissimilarity. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T). In Figs. A, B and C, treatment means with different letters differ

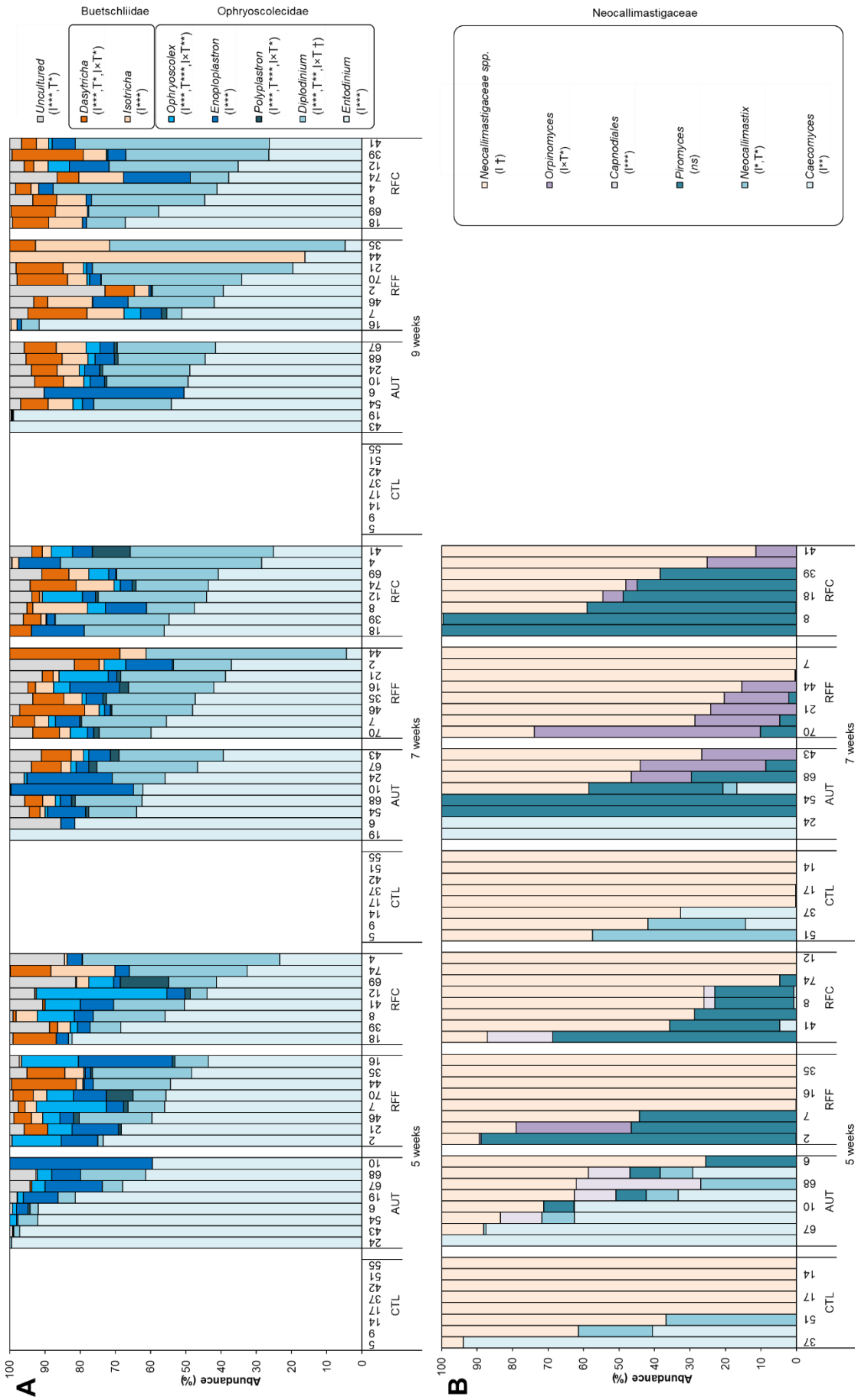


Fig. 6 Effect of the early-in-life rumen microbial inoculation on the rumen eukaryotic taxa distribution. A) Protozoa and (B) anaerobic fungi abundances. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T)

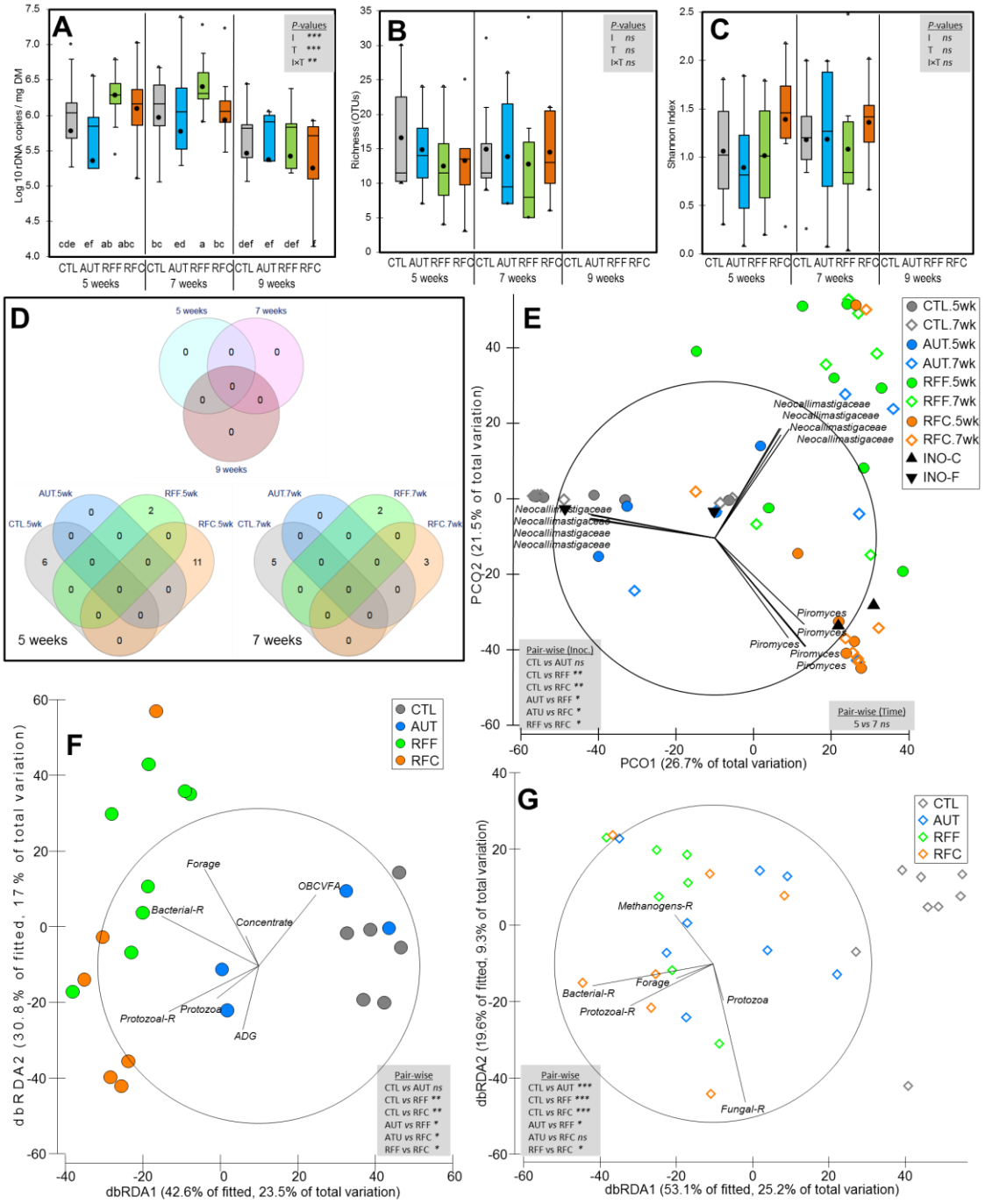


Fig. 7 Effects of early-in-life rumen microbial inoculation on the anaerobic fungal community in goats. Boxplots indicating the total anaerobic fungi abundance (A) and diversity indexes (B,C). Venn diagrams indicating the number of OTUs in the core community (D). Principal co-ordinates analysis (E) illustrating the treatment effects on the anaerobic fungal community showing the most discriminant OTUs ($\rho > 0.75$). Distance-based redundancy analysis illustrating relationship between the structure of the anaerobic fungal community and rumen function indicators before (F), during (G) and after (H) weaning. Pair-wise PERMANOVA values are provided in grey boxes based on the Bray-Curtis dissimilarity. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times (T). In Figs. A, B and C, treatment means with different letters differ

Supplementary Material

Supplementary Table S1. Description of the inocula in terms of rumen fermentation and microbial composition.

Inoculum[1]	AUT	RFF	RFC	s.e.d.	P-value
Fermentation products					
DM (%)	4.28^{ab}	2.35^{b}	5.22^{a}	0.877	0.043
pH	6.11^{b}	6.38^{a}	5.79^{c}	0.095	0.002
Lactate (mM)	1.86^{a}	0.55^{b}	0.85^{b}	0.328	0.017
Ammonia-N (mg/dL)	8.64	7.27	10.0	1.095	0.117
Total VFA (mM)	120^{ab}	103^{b}	134^{a}	9.990	0.055
Acetate (%)	63.0^{b}	70.1^{a}	55.5^{c}	1.339	<0.001
Propionate (%)	23.6^{b}	18.2^{c}	29.0^{a}	1.711	<0.001
Butyrate (%)	10.3^{b}	9.49^{c}	11.8^{a}	0.746	0.004
Bacterial community					
Concentration (log10 copies/l)		9.96	11.8	0.884	0.081
Richness		502	396	30.30	0.025
Shannon index		4.50	4.21	0.170	0.154
Abundance (%)					
f_Acidaminococcaceae		2.28	3.00	0.254	0.397
f_Bacteroidaceae		0.01	0.51	0.528	0.228
f_Bacteroidales		12.5	12.6	0.134	0.943
f_Christensenellaceae		1.58	0.69	0.151	0.129
f_Clostridiaceae		4.15	0.13	0.316	0.020
f_Elusimicrobiaceae		0.11	0.12	0.364	0.506
f_Erysipelotrichaceae		0.39	0.71	0.128	0.194
f_Family_XIII		0.18	0.22	0.068	0.218
f_Fibrobacteraceae		0.74	2.16	0.112	0.019
f_Lachnospiraceae		5.65	15.20	0.134	0.027
f_Porphyromonadaceae		0.05	0.24	0.089	0.003
f_Prevotellaceae		50.1	45.7	0.057	0.638
f_Rhodospirillaceae		0.82	0.12	0.102	0.001
f_Rikenellaceae		8.54	4.91	0.107	0.061
f_Ruminococcaceae		8.43	8.56	0.224	0.586
f_Spirochaetaceae		0.55	2.42	0.277	0.130
f_Succinivibrionaceae		0.25	0.52	0.125	0.088
f_Synergistaceae		0.14	0.10	0.312	0.981
f_Veillonellaceae		0.46	1.28	0.109	0.020
Methanogens community					
Concentration (log10 copies/ml)		6.32	7.65	1.213	0.314
Richness		20.5	14.5	1.683	0.025
Shannon index		1.85	1.75	0.240	0.688
Abundance (%)					
f_Methanobacteriaceae		35.8	55.2	33.30	0.043
f_Methanomassiliicoccaceae		62.6	44.8	38.00	0.079
f_Methanomicrobiaceae		1.61	0.00	4.620	0.158
g_Methanobrevibacter		28.7	55.2	23.10	0.005
g_Methanosphaera		7.12	0.00	15.88	0.089
Protozoal community					
Concentration (log10 copies/ml)		6.60	8.70	0.493	0.005
Richness		25.0	27.0	2.890	0.527
Shannon index		2.24	2.46	0.187	0.297
Abundance (%)					
g_Entodinium		48.8	49.3	0.102	0.863
g_Ophryoscolex		3.19	9.02	0.737	0.126
g_Diplodinium		0.01	0.01	0.123	0.999
g_Polyplastron		0.66	2.84	0.548	0.087
g_Enoploplastron		0.83	0.00	0.101	<0.001
g_Isotricha		18.6	22.7	0.215	0.969
g_Dasytricha		25.5	9.4	0.046	<0.001
*g_*Unclassified		2.31	6.70	0.481	0.121
Anaerobic fungal community					
Concentration (log10 copies/ml)		6.76	7.55	0.125	<0.001
Richness		12.0	15.0	2.380	0.276
Shannon index		1.32	1.63	0.527	0.501
Abundance (%)					
g_Caecomyces		63.6	9.22	0.655	0.064
g_Piromyces		0.00	50.0	0.006	<0.001
g_Neocallimastigaceae		36.4	40.8	0.107	0.621

1Treatments: Autoclaved rumen fluid (AUT), fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diet (RFC). s.e.d.; standard error of the difference and P-values for microbial taxa abundance were calculated based on the log10 transformed number of sequences.

Supplementary Table S2. Effect of the early-in-life rumen microbial inoculation and age on the rumen bacteria concentration, diversity and taxonomy.

Time	5 weeks				7 weeks				9 weeks					*P-value*		
Inoculation[1]	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	s.e.d.	Inoc.	Time	IxT
Diversity																
Concentation (log10 copies/mg DM)	10.7	10.9	10.8	10.7	10.8	10.7	10.7	10.4	10.7	10.7	10.7	10.4	0.11	0.004	0.003	0.543
Richness	151	281	421	350	206	388	510	453	145	294	379	326	48.09	<0.001	<0.001	0.900
Shannon index	2.83	3.10	4.17	3.61	2.99	3.78	4.37	4.16	2.57	3.19	3.73	3.42	0.240	<0.001	<0.001	0.599
Simpson index	0.84	0.83	0.95	0.91	0.87	0.91	0.95	0.94	0.80	0.85	0.90	0.87	0.030	<0.001	<0.001	0.673
Evenness	0.57	0.55	0.69	0.62	0.56	0.64	0.7	0.68	0.52	0.57	0.63	0.59	0.032	<0.001	<0.001	0.379
Chao Index	195^{g}	435^{def}	582^{bcd}	758^{b}	348^{efg}	564^{bcde}	985^{a}	661^{bc}	226^{fg}	453^{cde}	598^{bcd}	545^{bcde}	111.4	<0.001	0.003	0.072
Good´s coverage	0.71^{a}	0.63^{bc}	0.65^{ab}	0.54^{d}	0.63^{bc}	0.63^{bc}	0.57^{cd}	0.63^{bc}	0.65^{ab}	0.59^{bcd}	0.60^{bcd}	0.61^{bcd}	0.036	0.015	0.439	0.014
Abundance (log10 sequences)																
p_Actinobacteria, f_Coriobacteriaceae	0.97	0.66	0.82	0.64	1.02	0.82	0.63	0.7	0.26	0.37	0.42	0.46	0.196	0.642	<0.001	0.243
g_Atopobium	0.75	0.66	0.69	0.41	0.91	0.72	0.45	0.38	0.08	0.22	0.19	0.13	0.202	0.152	<0.001	0.361
g_Olsenella	0.44	0.04	0.22	0.34	0.24	0.19	0.29	0.41	0.2	0.17	0.23	0.35	0.162	0.242	0.818	0.603
p_Bacteroidetes	3.67	3.69	3.59	3.62	3.56	3.61	3.59	3.57	3.35	3.44	3.5	3.49	0.056	0.339	<0.001	0.142
f_Bacteroidaceae, g_Bacteroides	2.15^{a}	0.74^{b}	0.09^{c}	0.04^{c}	0.48^{bc}	0.33^{bc}	0.04^{c}	0.04^{c}	0.04^{c}	0.10^{c}	0^{c}	0.04^{c}	0.183	<0.001	<0.001	<0.001
f_Bacteroidaceae	3.35^{a}	3.29^{a}	2.89^{ab}	2.82^{abc}	2.80^{abc}	3.19^{a}	2.94^{ab}	3.01^{a}	1.69^{d}	2.13^{cd}	2.29^{bcd}	2.05^{d}	0.202	0.110	<0.001	0.017
g_Phocaeicola	0.44^{b}	1.24^{a}	1.30^{c}	0.91^{c}	0.95^{bc}	1.08^{bc}	1.02^{c}	1.11^{c}	0.67^{c}	0.67^{c}	0.93^{c}	0.75^{c}	0.173	0.003	0.005	0.016
f_p-2534-18B5_gut_group	0.29^{c}	1.37^{abc}	0.85^{bc}	2.12^{ab}	2.57^{a}	1.15^{abc}	1.48^{abc}	1.71^{abc}	1.16^{abc}	1.52^{abc}	1.61^{abc}	1.03^{bc}	0.432	0.494	0.039	<0.001
f_PeH15	0^{c}	0.06^{c}	1.54^{a}	0.97^{ab}	0^{c}	0.13^{bc}	0.73^{bc}	0.56^{bc}	0^{c}	0.04^{c}	0.16^{bc}	0.04^{c}	0.232	<0.001	<0.001	<0.001
f_Porphyromonadaceae	0.81^{a}	0.29^{b}	0.11^{b}	0.15^{b}	0.16^{b}	0.15^{b}	0.13^{b}	0.19^{b}	0.11^{b}	0.19^{b}	0.28^{b}	0.11^{b}	0.134	0.058	0.016	<0.001
f_Prevotellaceae	3.16	3.17	3.25	3.19	3.25	3.27	3.33	3.25	3.29	3.38	3.41	3.46	0.077	0.177	<0.001	0.843
g_Alloprevotella	1.90	2.10	1.36	1.36	1.83	1.28	1.15	0.92	0.67	0.70	0.52	0.42	0.304	0.019	<0.001	0.404
g_Prevotella	2.99	2.86	3.00	3.05	2.97	3.19	3.21	3.1	3.22	3.21	3.29	3.34	0.130	0.484	<0.001	0.547
f_Rikenellaceae	1.62^{d}	2.00^{bcd}	2.83^{a}	2.82^{a}	2.19^{abcd}	2.38^{abcd}	2.69^{ab}	2.53^{abc}	1.72^{d}	1.70^{d}	1.95^{bcd}	1.82^{cd}	0.228	<0.001	<0.001	0.019
g_Alistipes	0.78^{a}	0.11^{bc}	0^{c}	0^{c}	0.41^{b}	0.20^{bc}	0.06^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}	0.087	<0.001	<0.001	<0.001
g_SP3-e08	0^{a}	1.23^{bc}	1.69^{c}	1.66^{c}	0.45^{b}	1.83^{bc}	1.17^{c}	1.23^{c}	0^{c}	0.55^{c}	0.47^{c}	0.26^{c}	0.337	<0.001	<0.001	0.021
p_Chloroflexi, f_Anaerolineaceae	0^{d}	0.86^{ab}	1.13^{a}	0.95^{a}	0.08^{d}	0.69^{abc}	0.78^{ab}	0.77^{ab}	0^{d}	0^{d}	0.26^{bcd}	0.16^{cd}	0.169	<0.001	<0.001	0.003
p_Cyanobacteria, f_Gastranaerophilales	0	0	0.23	0.10	0	0.08	0.34	0.44	0	0	0.29	0.06	0.138	<0.001	0.126	0.554
p_Elusimicrobia	0	1.1	0.87	0.98	0.34	1.06	1.01	1.04	0	0.19	0.39	0.06	0.205	<0.001	<0.001	0.063
f_Clostridiaceae	0^{d}	0.92^{a}	0.65^{abc}	0.55^{abcd}	0.21^{bcd}	0.70^{ab}	0.53^{abcd}	0.28^{abcd}	0^{d}	0^{d}	0^{cd}	0^{d}	0.184	<0.001	<0.001	0.05
f_Elusimicrobiaceae, g_Elusimicrobium	0^{b}	0^{b}	0.39^{ab}	0.61^{a}	0^{b}	0.34^{ab}	0.61^{a}	0.46^{ab}	0^{b}	0.19^{ab}	0.33^{ab}	0.04^{b}	0.158	<0.001	0.028	0.027
p_Fibrobacteres, g_Fibrobacter	0	0.75	1.11	0.71	0.44	0.95	1.16	0.86	0.36	0.48	1.53	0.92	0.212	<0.001	0.108	0.106
p_Firmicutes	3.05	2.82	3.18	3.01	3.07	2.92	3.11	3.16	2.99	3.02	3.08	3.15	0.081	<0.001	0.468	0.135
f_Acidaminococcaceae	1.48	1.32	1.73	1.63	1.11	1.31	1.70	1.67	1.42	1.23	1.85	1.55	0.179	<0.001	0.56	0.553
g_Acidaminococcus	0.36	0.12	0	0.22	0.04	0.23	0.13	0.07	0.55	0.51	0.36	0.37	0.171	0.413	<0.001	0.513
g_Phascolarctobacterium	1.00^{a}	0.06^{b}	0^{b}	0^{b}	0.21^{b}	0.10^{b}	0.04^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.110	<0.001	<0.001	<0.001
g_Succiniclasticum	0.61	1.31	1.73	1.63	1.04	1.28	1.69	1.66	1.28	1.11	1.84	1.53	0.207	<0.001	0.489	0.127
f_Christensenellaceae	2.11	2.03	2.50	2.07	1.72	1.81	2.01	1.91	0.41	0.68	0.89	0.92	0.237	0.137	<0.001	0.613
f_Clostridiales	1.4	1.48	1.56	1.2	1.22	1.25	1.51	1.36	0.16	0.43	0.69	0.30	0.224	0.263	<0.001	0.607
f_Defluviitaleaceae	0.04^{c}	0.06^{c}	0.36^{abc}	0.51^{ab}	0.06^{c}	0.10^{bc}	0.70^{a}	0.66^{a}	0.04^{c}	0.11^{bc}	0.16^{bc}	0.21^{bc}	0.123	<0.001	<0.001	0.007
f_Erysipelotrichaceae	0.66	1.11	1.14	0.93	0.58	1.08	0.86	0.98	0.4	0.5	0.99	0.71	0.195	0.019	0.002	0.152
g_Catenisphaera	0	0.46	0.08	0.19	0	0.22	0	0.06	0	0	0	0.11	0.131	0.096	0.028	0.131
g_Sharpea	0.60	0.11	0	0.06	0.52	0.07	0	0	0.35	0.14	0.15	0.04	0.158	<0.001	0.824	0.633
g_Eubacterium	1.11	1.61	1.64	1.57	1.08	1.57	1.52	1.51	1.13	1.64	1.76	1.52	0.205	0.005	0.565	0.984
f_Family_XIII	0.63^{efg}	1.25^{bcde}	1.95^{a}	1.51^{abc}	1.40^{abc}	1.37^{abcd}	1.78^{ab}	1.65^{ab}	0.15^{g}	0.56^{fg}	0.93^{cdef}	0.70^{defg}	0.19	<0.001	<0.001	0.022
g_Anaerovorax	0.08^{e}	0.70^{bcd}	1.38^{a}	0.94^{abc}	0.75^{bc}	0.78^{bc}	1.15^{ab}	1.10^{ab}	0.04^{e}	0.20^{de}	0.43^{cde}	0.17^{de}	0.151	<0.001	<0.001	<0.001
g_Mogibacterium	0.21^{cd}	0.34^{cd}	1.15^{a}	0.99^{ab}	0.29^{cd}	0.77^{abc}	1.09^{a}	0.98^{ab}	0.08^{d}	0.22^{d}	0.39^{bcd}	0.36^{cd}	0.171	<0.001	<0.001	0.007
f_Lachnospiraceae	2.41	2.22	2.55	2.31	2.75	2.62	2.55	2.6	2.83	2.74	2.82	2.90	0.145	0.420	<0.001	0.407
g_Acetitomaculum	0.45	0.70	1.28	1.21	0.63	1.11	1.20	1.13	0.71	0.67	0.83	1.00	0.244	0.008	0.180	0.301
g_Anaerosporobacter	0.48^{ab}	0.41^{ab}	0.38^{b}	0^{b}	0.52^{ab}	1.02^{a}	0.19^{b}	0.08^{b}	0^{b}	0.15^{b}	0.06^{b}	0.06^{b}	0.178	<0.001	<0.001	0.011
g_Butyrivibrio	1.30^{b}	1.47^{ab}	1.33^{ab}	1.16^{b}	2.01^{a}	1.53^{ab}	1.25^{b}	1.14^{b}	0.86^{b}	1.14^{b}	1.35^{ab}	1.27^{b}	0.194	0.238	0.008	<0.001
g_Coprococcus	1.00	0.64	0.51	0.39	0.60	0.89	0.43	0.39	0.15	0.42	0.18	0.15	0.245	0.032	0.005	0.515

g_Lachnoclostridium	0.04	0.50	1.02	0.61	0.17	0.64	1.08	1.08	0.72	1.15	1.41	1.72	0.223	<0.001	<0.001	0.369
g_Lachnospira	0.58^{a}	0.24^{bcde}	0^{e}	0.04^{cde}	0.04^{cde}	0.37^{abc}	0.06^{cde}	0^{de}	0.44^{ab}	0.22^{bcde}	0.33^{abcd}	0.21^{bcde}	0.169	0.077	0.085	0.03
g_Lachnospiraceae	1.80	1.71	2.23	1.87	2.39	2.26	2.22	2.18	2.1	2.26	2.39	2.54	0.232	0.622	<0.001	0.151
g_Marvinbryantia	0	0.16	0.34	0.18	0.08	0.23	0.17	0.36	0.14	0.04	0.26	0.32	0.117	0.06	0.781	0.123
g_Moryella	0.43	0.79	1.21	1.14	0.68	1.01	1.56	1.77	0.53	1.13	1.38	1.62	0.188	<0.001	<0.001	0.552
g_Oribacterium	0.46	0.59	0.72	0.58	0.57	0.8	0.89	0.81	0.83	1.25	1.20	1.59	0.233	0.006	<0.001	0.63
g_Pseudobutyrivibrio	0.60	0.80	0.83	0.50	0.78	0.98	0.79	0.89	1.01	1.04	0.95	1.02	0.234	0.597	0.037	0.896
g_Roseburia	0.77	0.52	0.53	0.58	0.74	1.44	1.17	1.00	1.80	1.73	1.52	1.48	0.267	0.647	<0.001	0.18
g_Syntrophococcus	0.27	0.13	0.34	0.19	0.16	0.35	0.13	0.34	0.28	0.15	0.29	0.33	0.122	0.792	0.893	0.136
g_Tyzzerella	0.55	0.39	0.20	0.06	0.30	0.25	0.13	0.07	0	0	0.12	0	0.14	0.032	0.001	0.192
f_Peptococcaceae	0.36	0.56	0.62	0.33	0.25	0.19	0.34	0.34	0	0	0	0	0.152	0.684	<0.001	0.49
f_Ruminococcaceae	2.57	2.38	2.59	2.45	2.4	2.31	2.70	2.55	2.02	1.82	2.35	2.07	0.152	0.037	<0.001	0.457
g_Anaerotruncus	0.14	0	0.51	0.26	0.25	0.15	0.55	0.47	0	0.10	0.15	0.08	0.109	<0.001	<0.001	0.127
g_Oscillibacter	0.69	0.52	0.10	0.08	0.59	0.44	0.13	0.21	0.25	0.16	0.15	0.04	0.119	<0.001	0.002	0.06
g_Papillibacter	0.17	0.07	0.76	0.45	0.29	0.47	0.55	0.45	0	0.04	0.23	0	0.148	0.003	<0.001	0.083
g_Ruminiclostridium	0.80^{a}	0.75^{a}	0.5^{abc}	0.31^{abc}	0.67^{a}	0.70^{a}	0.63^{ab}	0.64^{ab}	0.04^{e}	0.07^{bc}	0.23^{abc}	0.41^{abc}	0.171	0.871	<0.001	0.033
g_Ruminococcus	1.63^{ab}	1.04^{ed}	1.41^{abc}	1.12^{ed}	1.25^{abcd}	1.40^{abc}	1.65^{ab}	1.30^{abcd}	0.87^{d}	1.18^{bcd}	1.69^{a}	1.60^{ab}	0.243	0.090	0.675	0.011
g_Saccharofermentans	0	0.06	0.27	0.08	0	0.1	0.48	0.43	0	0.06	0.50	0.63	0.144	<0.001	0.022	0.133
g_Streptococcus	0.22^{a}	0^{b}	0.08^{ab}	0^{b}	0^{b}	0.04^{ab}	0.04^{ab}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.057	0.114	0.037	0.038
f_Veillonellaceae	0.73^{f}	0.70^{f}	1.16^{cdef}	1.02^{def}	0.92^{ef}	1.28^{cdef}	1.6^{cde}	1.72^{abc}	1.62^{bcd}	2.42^{a}	1.81^{abc}	2.34^{ab}	0.205	0.002	<0.001	0.002
g_Anaerovibrio	0.43^{c}	0.16^{c}	0.16^{c}	0.19^{c}	0.34^{c}	0.47^{bc}	0.47^{bc}	0.49^{bc}	0.62^{bc}	1.26^{a}	0.58^{bc}	1.10^{ab}	0.179	0.12	<0.001	0.008
g_Megasphaera	0.25	0.08	0	0.11	0.14	0.07	0.04	0.07	0.51	0.32	0.31	0.28	0.129	0.201	<0.001	0.938
g_Quinella	0^{d}	0^{d}	0.50^{bcd}	0.16^{cd}	0^{d}	0.16^{cd}	0.97^{abc}	1.15^{ab}	0^{d}	1.15^{ab}	0.73^{abcd}	1.45^{a}	0.247	<0.001	<0.001	<0.001
g_Selenomonas	0^{e}	0.10^{de}	0.35^{de}	0.43^{de}	0.37^{de}	0.51^{cde}	0.67^{cde}	0.89^{bcd}	0.79^{bcde}	1.96^{a}	1.33^{abc}	1.62^{ab}	0.243	0.004	<0.001	0.033
p_Proteobacteria	1.82	2.40	1.95	2.36	2.57	2.63	2.50	2.15	3.41	3.02	2.91	2.78	0.285	0.45	<0.001	0.065
f_Campylobacteraceae, g_Campylobacter	0.32	0.04	0.19	0.08	0	0	0.10	0.09	0.08	0.04	0.04	0	0.112	0.333	0.069	0.423
f_Comamonadaceae, g_Comamonas	0.75^{a}	0.04^{b}	0^{b}	0^{b}	0.23^{b}	0.18^{b}	0.07^{b}	0.04^{b}	0^{b}	0.04^{b}	0^{b}	0.04^{b}	0.125	0.004	0.012	<0.001
f_Neisseriaceae	0.74	0.96	0.63	0.55	0.38	0.33	0.42	0.32	0.04	0	0	0.06	0.146	0.679	<0.001	0.189
f_Pasteurellaceae	0.80^{a}	0.35^{bcd}	0.64^{ab}	0.26^{bcd}	0.34^{bcd}	0.22^{bcd}	0.22^{cd}	0.46^{abc}	0^{d}	0.06^{cd}	0.04^{cd}	0^{d}	0.12	0.006	<0.001	0.001
f_Pasteurellaceae, g_Bibersteinia	0.62^{a}	0.25^{abc}	0.39^{ab}	0.23^{bc}	0.27^{bc}	0.16^{bc}	0.19^{bc}	0.34^{abc}	0^{c}	0.06^{bc}	0^{c}	0^{c}	0.105	0.223	<0.001	0.012
f_Rhodocyclaceae	0^{d}	0.62^{ab}	0.72^{a}	0.36^{abcd}	0.11^{cd}	0.29^{abcd}	0.21^{bcd}	0.52^{abc}	0^{d}	0.04^{cd}	0^{d}	0^{d}	0.138	0.006	<0.001	0.001
f_Rhodospirillaceae	0.48	0.56	1	0.68	0.35	0.5	0.94	0.9	0.16	0.74	1.27	0.34	0.250	0.001	0.890	0.127
f_Succinivibrionaceae	0.78^{d}	2.21^{abc}	1.59^{cd}	2.11^{abc}	2.43^{abc}	2.59^{abc}	2.41^{abc}	1.91^{bcd}	3.41^{a}	3.01^{ab}	2.81^{abc}	2.77^{abc}	0.372	0.349	<0.001	0.002
g_Anaerobiospirillum	0	0	0.27	0.04	0	0.12	0.19	0	0	0.28	0.5	0.32	0.115	<0.001	0.002	0.403
g_Ruminobacter	0.08^{c}	2.07^{a}	0.97^{abc}	1.5^{ab}	0.78^{bc}	1.17^{abc}	1.46^{ab}	0.74^{bc}	0.29^{bc}	0.81^{bc}	0.31^{bc}	0.70^{bc}	0.344	0.001	<0.001	0.003
g_Succinimonas	0	0.11	0.5	0.35	0	0.36	0.27	0.28	0	0.04	0.25	0	0.215	0.055	0.244	0.684
g_Succinivibrio	0.71	0.88	0.79	1.23	2.29	1.92	1.56	1.45	3.40	2.5	2.62	2.66	0.483	0.475	<0.001	0.345
p_Spirochaetae, f_Spirochaetaceae	0.95^{b}	2.04^{a}	1.68^{ab}	1.72^{ab}	1.80^{ab}	2.02^{a}	1.84^{ab}	1.67^{ab}	1.42^{ab}	2.09^{a}	2.08^{a}	1.25^{ab}	0.277	0.005	0.206	0.046
g_Sphaerochaeta	0.82	1.69	0.82	1.26	0.63	1.31	0.99	1.12	0.2	0.63	0.66	0.21	0.278	0.007	<0.001	0.249
g_Treponema	0.16^{b}	1.72^{a}	1.49^{a}	1.24^{a}	1.60^{a}	1.85^{a}	1.63^{a}	1.19^{a}	1.41^{a}	2.06^{a}	2.04^{a}	1.22^{a}	0.291	<0.001	<0.001	0.005
p_Synergistetes, f_Synergistaceae	1.48^{ab}	1.36^{ab}	1.10^{abc}	0.82^{abcd}	1.07^{abcd}	1.07^{abcd}	1.32^{abc}	1.59^{a}	0.25^{d}	0.49^{cd}	0.68^{bcd}	1.08^{abcd}	0.240	0.411	<0.001	0.001
g_Fretibacterium	0.04^{c}	0.04^{c}	0.65^{abc}	0.34^{bc}	0.14^{c}	0.42^{bc}	1.17^{ab}	1.47^{a}	0.08^{c}	0.36^{bc}	0.42^{bc}	0.90^{abc}	0.256	<0.001	<0.001	0.032
g_Pyramidobacter	1.47^{a}	1.03^{ab}	0.76^{bcd}	0.68^{bcde}	0.80^{bc}	0.76^{bcd}	0.59^{bcde}	0.68^{bcde}	0.19^{e}	0.25^{de}	0.30^{cde}	0.37^{cde}	0.151	0.007	<0.001	0.003
g_Synergistes	0^{b}	0.95^{a}	0.18^{b}	0.08^{b}	0.54^{ab}	0.34^{b}	0.10^{b}	0.10^{b}	0^{b}	0^{b}	0^{b}	0.08^{b}	0.163	0.005	0.002	<0.001
p_Tenericutes	0.37	0.77	1.27	1.16	0.29	0.66	1.07	1.09	0.29	0.17	0.73	0.42	0.19	<0.001	<0.001	0.132
f_Erysipelotrichaceae	0.66	1.11	1.14	0.93	0.58	1.08	0.86	0.98	0.40	0.5	0.99	0.71	0.195	0.019	0.002	0.152
f_Mollicutes_RF9_uncultured	0.14	0.15	0.53	0.39	0.10	0.14	0.44	0.47	0.13	0.13	0.31	0.04	0.141	0.002	0.077	0.28
f_NB1-n_uncultured	0	0.13	0.15	0.54	0	0.21	0.37	0.29	0	0	0.11	0.04	0.139	0.01	0.018	0.092
p_Minor phyla	0.22^{cde}	0.58^{bcd}	1.44^{a}	0.97^{ab}	0.22^{cde}	0.34^{cde}	0.76^{bc}	0.97^{ab}	0^{e}	0.13^{de}	0.21^{de}	0.22^{cde}	0.158	<0.001	<0.001	<0.001
f_Oligosphaeraceae	0^{c}	0.26^{bc}	0.86^{a}	0.50^{ab}	0.04^{c}	0.21^{bc}	0.30^{bc}	0.36^{bc}	0^{c}	0^{c}	0.06^{c}	0^{c}	0.105	<0.001	<0.001	<0.001

[1]Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at different times. $^{a-f}$Means within a row with different superscript differ ($P<0.05$) based on the Bonferroni test.

Supplementary Table S3. Spearman's correlations between the bacterial taxa and the rumen function and animal performance.

Correlations[1]	Milk	Concentrate	Forage	DMI	pH	Ammonia	VFA	OBCVFA	Acetate	Propionate	Butyrate	Methanogens	Methanogens-R	Protozoa	Protozoal-R	Fungi	Fungal-R	BHB	Glucose	ADG	ADG-pw
Concentation		-0.32																			
Richness		0.45	0.51	0.46								0.31	0.55	0.55							
p_Actinobacteria, f_Coriobacteriaceae	-0.42														-0.37		-0.38			-0.38	
g_Atopobium	-0.37																			-0.37	-0.38
p_Bacteroidetes		-0.39	-0.40	-0.50							-0.34							-0.33	0.36		-0.46
f_Bacteroidaceae, g_Bacteroides		-0.39	-0.68	-0.44				0.32				-0.37	-0.39	-0.53	-0.52						-0.50
f_Bacteroidaceae			-0.38	-0.34			-0.38	0.35													
g_Phocaeicola						0.32															
f_p-2534-18B5_gut_group	0.39	0.42		0.44													0.43	0.35			
f_PeH15			0.44		0.34									0.54	0.63						
f_Prevotellaceae		0.39					0.49		-0.33	0.32	0.32						-0.36				
g_Alloprevotella		-0.42	-0.42	-0.45							-0.43		-0.38	-0.46							-0.53
g_Prevotella		0.39					0.63		-0.55	0.38	0.59										
f_Rikenellaceae			0.45						0.35					0.46	0.58						
g_Alistipes			-0.54									-0.38	-0.41	-0.61	-0.65						
g_SP3-e08							-0.32		0.39				0.35	0.42	0.52						
p_Chloroflexi, f_Anaerolineaceae			0.37		0.35				0.38	-0.43		0.46	0.49	0.62	0.56				-0.34		
p_Cyanobacteria, f_Gastranaerophilales		0.49	0.53	0.49				-0.37						0.52	0.73						
p_Elusimicrobia													0.38	0.32	0.36						
f_Elusimicrobiaceae, g_Elusimicrobium			0.41										0.46	0.59	0.55						
p_Fibrobacteres, g_Fibrobacter			0.35										0.38	0.32	0.43						
p_Firmicutes			0.48													0.37			-0.49		0.39
f_Acidaminococcaceae							0.45						0.34	0.38	0.57						
g_Acidaminococcus																	0.34			0.35	
g_Phascolarctobacterium			-0.40					0.32				-0.45	-0.33	-0.37	-0.47						-0.31
g_Succiniclasticum			0.41				0.43	-0.37					0.35	0.46	0.65						
f_Christensenellaceae	-0.43								0.42	-0.42	-0.33								-0.36	-0.39	
f_Clostridiaceae	-0.39								0.37	-0.37										-0.44	
f_Defluviitaleaceae			0.38										0.36	0.64	0.45		-0.38				
f_Erysipelotrichaceae																					
g_Sharpea			-0.52										-0.52	-0.56	-0.60				0.41		
g_Eubacterium													0.34		0.57						
f_Family_XIII			0.52							-0.33		0.46		0.53	0.38		-0.38		-0.32		
g_Anaerovorax		0.34	0.55	0.32								0.38		0.59	0.68						
g_Mogibacterium	-0.34		0.37						0.39	-0.39		0.39	0.39	0.56	0.37		-0.43		-0.37		
f_Lachnospiraceae									-0.40		0.36										
g_Acetitomaculum			0.36									0.36	0.43	0.47							
g_Anaerosporobacter									-0.39	0.31				-0.31							
g_Butyrivibrio														-0.57	-0.45						-0.36
g_Coprococcus																					-0.44
g_Lachnoclostridium			0.41								0.32	0.45	0.56	0.53	0.68						
g_Lachnospira			-0.37					0.31						-0.33			0.36				
g_Marvinbryantia													0.32				-0.37				
g_Moryella		0.53	0.55	0.53				-0.43					0.36	0.47	0.65			0.31			0.40
g_Oribacterium			0.40	0.31											0.46						
g_Pseudobutyrivibrio													0.37						-0.33		
g_Roseburia	0.42								-0.57	0.38	0.53									0.45	
g_Syntrophococcus								0.44													
g_Tyzzerella		-0.39		-0.39				0.34							-0.35						-0.33
f_Peptococcaceae							-0.50		0.47	-0.38	-0.50	0.34									-0.49
f_Ruminococcaceae			0.36						0.39					0.34	0.34		-0.32			-0.33	
g_Anaerotruncus		0.36	0.42	0.37											0.33						
g_Oscillibacter			-0.44		-0.33								-0.36	-0.57	-0.39		0.43		0.32		
g_Papillibacter			0.44						0.39			0.43		0.32	0.34						
g_Ruminiclostridium																					-0.35
g_Ruminococcus							0.38										-0.35	0.35			
g_Saccharofermentans		0.39	0.35	0.40										0.46	0.45			0.36			0.58
g_Streptococcus	-0.32	-0.41		-0.33																	
f_Veillonellaceae		0.54	0.47	0.55			0.35		-0.44		0.58		0.44	0.34	0.53			0.37			0.47
g_Anaerovibrio						0.37			-0.50		0.48									0.33	
g_Megasphaera			-0.32									-0.33		-0.32			0.32				
g_Quinella			0.51	0.37									0.52	0.54	0.66			0.34			0.31
g_Selenomonas	0.32	0.54	0.53	0.58					-0.37		0.45		0.36		0.45			0.38			0.41
p_Proteobacteria	0.53	0.34		0.36					-0.43	0.54	0.41									0.50	
f_Campylobacteraceae, g_Campylobacter	-0.36		0.37						0.43	-0.38		0.56		0.48	0.42						
f_Comamonadaceae, g_Comamonas																					0.35
f_Neisseriaceae			-0.31					0.34				-0.49		-0.36							-0.54
f_Pasteurellaceae	-0.53	-0.38		-0.43					0.41	-0.37	-0.39									-0.34	-0.33
f_Pasteurellaceae, g_Bibersteinia		-0.40		-0.38					0.45	-0.44	-0.32		-0.33							-0.43	
f_Rhodocyclaceae			0.33											0.37	0.43						
f_Rhodospirillaceae			0.31									0.32			0.37						
f_Succinivibrionaceae	0.52	0.35		0.38					-0.48	0.56	0.45									0.53	
g_Anaerobiospirillum															0.44						
g_Ruminobacter													0.34		0.35						
g_Succinimonas											0.31				0.47						
g_Succinivibrio	0.49	0.37		0.33					-0.49	0.39	0.45						0.38			0.43	
p_Spirochaetae, f_Spirochaetaceae		0.39		0.34																	
g_Sphaerochaeta							-0.35				-0.33										
g_Treponema	0.33	0.43		0.39					-0.40	0.46	0.33										
p_Synergistetes, f_Synergistaceae						0.33				-0.33											
g_Fretibacterium		0.42	0.55	0.45				-0.37				0.35	0.36	0.62	0.66			0.32			0.33
g_Pyramidobacter		-0.37	-0.45	-0.48				0.36						-0.34	-0.46			-0.37			
p_Tenericutes			0.59							-0.36		0.31	0.34	0.67	0.69						
f_Mollicutes_RF9_uncultured																0.43			0.40		
f_NB1-n_uncultured																					
p_Lentisphaerae, f_Oligosphaeraceae					0.37											0.40			0.48		
p_Minor phyla	-0.46		0.49						0.46	-0.54		0.46		0.56	0.46						

[1]Parameters: pH, ammonia-N (mg/dl), total volatile fatty acids (mM), acetate (%), propionate (%), butyrate (%), lactate (mM) and average daily gain (kg/d) during the post-weaning (ADG-pw, weeks 6–13) and finishing periods (ADG-f, weeks 13–23). Microbial data were log10 transformed and Spearman's correlations coefficients ≥ 0.4 (green) or ≤ −0.4 (red) and $P<0.001$ were shown (n=72).

Supplementary Table S4. Effect of early-in-life rumen microbial inoculation and age on the rumen methanogens concentration, diversity and taxonomy.

Time	5 weeks				7 weeks				9 weeks					*P*-value		
Inoculation[1]	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	s.e.d.	Inoc.	Time	IxT
Diversity																
Concentration (log10 copies/mg DM)	5.15^{e}	7.35^{ab}	7.56^{a}	6.29^{c}	6.24^{c}	7.18^{ab}	7.08^{b}	6.32^{c}	5.18^{e}	5.96^{cd}	6.1^{c}	5.73^{de}	0.292	<0.001	<0.001	<0.001
Richness	7.25^{d}	11.4^{c}	12^{c}	13.6^{bc}	6.63^{d}	16.3^{ab}	16.1^{ab}	13.9^{bc}	6.83^{d}	17.6^{a}	15.3^{ab}	13.8^{bc}	1.856	<0.001	0.012	0.094
Shannon index	0.71	1.6	1.56	1.57	0.91	1.88	1.87	1.56	0.81	1.94	1.87	1.88	0.216	<0.001	0.023	0.839
Simpson index	0.33	0.69	0.72	0.7	0.51	0.8	0.79	0.68	0.42	0.77	0.76	0.79	0.085	<0.001	0.062	0.633
Evenness	0.14^{c}	0.28^{ab}	0.3^{ab}	0.27^{ab}	0.29^{ab}	0.29^{ab}	0.29^{ab}	0.26^{ab}	0.23^{b}	0.27^{ab}	0.28^{ab}	0.31^{a}	0.037	0.042	0.105	0.014
Chao Index	10.7	12.2	15.9	15.8	10.7	23	24.2	16.7	7.5	20.7	17.4	15.6	4.385	0.03	0.036	0.26
Good´s coverage	0.66	0.78	0.75	0.78	0.67	0.7	0.77	0.77	0.83	0.8	0.81	0.82	0.083	0.518	0.097	0.862
Abundance (log10 sequences)																
f_Methanomassiliicoccaceae	1.48^{cd}	2.43^{a}	2.42^{a}	2.38^{ab}	2.41^{a}	2.35^{ab}	2.36^{ab}	2.24^{abc}	1.34^{bcd}	2.22^{abcd}	2.22^{abcd}	1.48^{d}	0.236	0.007	<0.001	0.001
s_Methanomethylophilus_alvus	0^{c}	0^{c}	0^{c}	0.31^{bc}	0.06^{c}	0.07^{c}	0^{c}	0^{c}	1.09^{ab}	0.65^{abc}	1.21^{a}	0^{c}	0.224	0.049	<0.001	<0.001
s_Group10_sp	0^{a}	1.55^{c}	0.25^{bc}	0.14^{c}	0^{ab}	0.98^{c}	0.46^{bc}	0.21^{bc}	0^{bc}	0.59^{c}	0.67^{abc}	0^{c}	0.238	<0.001	0.383	0.005
s_Group11_sp	0	0.15	0	0	0.13	0.29	0	0	0.48	0.04	0	0	0.127	0.109	0.316	0.009
s_ Group12_sp	0^{b}	0^{b}	0.08^{ab}	0.45^{a}	0^{b}	0.50^{a}	0.66^{a}	0.19^{ab}	0^{b}	1.10^{a}	0.15^{ab}	0^{b}	0.219	0.009	0.094	<0.001
s_Group8_sp	1.48^{c}	0.63^{a}	0^{bc}	0^{c}	1.33^{c}	0.81^{ab}	0.3^{bc}	0^{bc}	0.32^{c}	1.24^{bc}	1.06^{abc}	0.81^{c}	0.329	0.003	0.026	<0.001
s_Group9_sp	0^{c}	1.37^{a}	2.17^{a}	2.1^{a}	1.56^{a}	1.97^{a}	2.19^{a}	2.09^{a}	0.36^{bc}	1.97^{a}	1.66^{a}	1.24^{ab}	0.301	<0.001	<0.001	<0.001
s_Methanomassiliicoccaceae spp.	0.04^{d}	1.98^{a}	1.91^{ab}	1.5^{ab}	0.54^{cd}	1.62^{ab}	1.51^{ab}	1.47^{ab}	0.05^{d}	1.19^{abc}	1.03^{bc}	0.04^{d}	0.267	<0.001	<0.001	0.006
f_Methanobacteriaceae	2.34^{ab}	2.34^{ab}	2.27^{ab}	2.17^{b}	2.23^{ab}	2.41^{ab}	2.38^{ab}	2.38^{ab}	2.57^{ab}	2.31^{ab}	2.5^{ab}	2.66^{a}	0.118	0.955	0.001	0.037
g_Methanobrevibacter	2.34^{ab}	2.32^{ab}	1.9^{b}	2.06^{ab}	2.23^{ab}	2.31^{ab}	1.98^{ab}	2.04^{ab}	2.42^{ab}	2.11^{ab}	2.42^{ab}	2.55^{a}	0.159	0.053	0.009	0.017
s_Methanobrevibacter_bovis_koreani	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0.11^{b}	0.76^{a}	1.03^{a}	0.137	<0.001	<0.001	<0.001
s_Methanobrevibacter_gottschalkii	2.34^{a}	2.24^{a}	1.82^{c}	2.02^{abc}	2.23^{ab}	2.29^{a}	1.73^{c}	1.81^{c}	2.41^{a}	1.83^{c}	1.92^{bc}	2.37^{a}	0.191	<0.001	0.447	0.039
s_Methanobrevibacter_oralis	0	0	0	0	0	0	0	0	0	0	0	0.09	0.032	0.416	0.31	0.358
s_Methanobrevibacter_ruminantium	0	0.84	0.85	0.8	0.04	0.32	1.25	1.12	0.36	1.7	1.93	1.77	0.343	<0.001	<0.001	0.27
s_Methanobrevibacter spp.	0^{b}	0^{b}	0^{b}	0.45^{b}	0^{b}	0.19^{b}	0^{b}	0.62^{b}	0^{b}	0^{b}	0^{b}	1.43^{a}	0.203	<0.001	0.005	<0.001
g_Methanosphaera	0.1	0.31	0	0.22	0	0.24	0.48	0.76	1.72	1.42	1.36	1.33	0.290	0.69	<0.001	0.139
s_Methanosphaera_sp_ISO3-F5	0.1	0.26	0	0.22	0	0.12	0.48	0.76	1.58	1.31	1.28	1.33	0.291	0.534	<0.001	0.187
s_Methanosphaera_stadtmanae	0^{b}	0.05^{b}	0^{b}	0^{b}	0^{b}	0.12^{b}	0^{b}	0^{b}	0.2^{b}	0.78^{a}	0.75^{a}	0^{b}	0.158	0.003	<0.001	0.007
s_Methanomicrobium_mobile	0^{b}	0.47^{ab}	1.45^{a}	0.8^{ab}	0^{b}	1.30^{a}	1.48^{a}	1.35^{a}	0^{b}	1.06^{ab}	0.76^{ab}	0^{b}	0.317	<0.001	0.001	0.006
f_Methanosarcinaceae	0^{c}	0^{c}	0.38^{abc}	0.32^{bc}	0^{c}	0^{c}	0.59^{ab}	0.91^{a}	0^{c}	0.42^{abc}	0^{c}	0^{c}	0.165	0.002	0.002	<0.001
s_Methanimicrococcus_blatticola	0^{c}	0^{c}	0.38^{abc}	0.32^{bc}	0^{c}	0^{c}	0.59^{ab}	0.91^{a}	0^{c}	0.42^{abc}	0^{c}	0^{c}	0.165	0.002	0.002	<0.001

[1]Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at 5, 7 and 9 weeks of age.[a-f]Means within a row with different superscript differ (P<0.05) based on the Bonferroni test.

Supplementary Table S5. Effect of early-in-life rumen microbial inoculation and age on the rumen protozoal concentration, diversity and taxonomy.

Time	5 weeks				7 weeks				9 weeks					*P-value*		
Inoculation[1]	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	s.e.d.	Inoc.	Time	AxT
Diversity																
Concentation (log10 copies/mg DM)	0^{e}	6.07^{d}	8.05^{ab}	8.00^{ab}	0^{e}	7.07^{c}	8.15^{ab}	8.33^{a}	0^{e}	6.91^{c}	7.53^{bc}	8.40^{a}	0.3387	<0.000	0.076	0.062
Richness	0	16.1	26.1	24.6	0	22.3	27.5	26.8	0	22.0	22.3	26.4	2.821	<0.001	0.194	0.180
Shannon index	0^{d}	1.17^{c}	2.17^{ab}	2.10^{ab}	0^{d}	1.77^{ab}	2.22^{a}	2.16^{ab}	0^{d}	1.70^{b}	1.93^{ab}	2.16^{ab}	0.230	<0.001	0.185	0.050
Simpson index	0^{d}	0.52^{c}	0.84^{a}	0.82^{a}	0^{d}	0.75^{a}	0.84^{a}	0.82^{a}	0^{d}	0.63^{b}	0.75^{ab}	0.81^{a}	0.071	<0.001	0.124	0.029
Evenness	0	0.42	0.67	0.66	0	0.57	0.67	0.66	0	0.52	0.63	0.66	0.060	<0.001	0.311	0.185
Chao Index	0	16.9	28.8	25.3	0	25.5	31.7	32.9	0	23.7	23.9	27.4	3.203	<0.001	0.012	0.133
Good´s coverage	0	0.89	0.86	0.92	0	0.85	0.87	0.83	0	0.86	0.87	0.90	0.034	<0.001	0.128	0.252
Abundance (log 10 sequences)																
f_Ophryoscolecidae	0^{c}	3.73^{a}	3.69^{a}	3.67^{ab}	0^{c}	3.7^{a}	3.64^{ab}	3.67^{a}	0^{c}	3.68^{a}	3.54^{b}	3.65^{ab}	0.038	<0.001	0.054	0.042
g_Entodinium	0	3.64	3.50	3.41	0	3.53	3.28	3.36	0	3.50	3.19	3.35	0.090	<0.001	0.033	0.286
g_Ophryoscolex	0	1.93	2.43	2.82	0	2.08	3.14	3.20	0	2.13	2.71	3.18	0.393	<0.001	0.229	0.892
g_Diplodinium	0^{c}	0.22^{bc}	1.39^{ab}	0.70^{abc}	0^{c}	0.90^{abc}	1.47^{a}	1.05^{abc}	0^{c}	0.83^{abc}	0.53^{abc}	0^{c}	0.367	<0.001	0.021	0.048
g_Polyplastron	0	1.98	2.55	2.37	0	2.26	2.11	2.47	0	1.90	1.64	2.27	0.351	<0.001	0.165	0.196
g_Enoploplastron	0^{c}	1.32^{abc}	2.40^{a}	1.78^{ab}	0^{c}	1.21^{abc}	2.00^{ab}	1.71^{ab}	0^{c}	1.46^{abc}	0.94^{bc}	0.70^{bc}	0.422	<0.001	<0.001	0.003
f_Buetschlidae	0^{d}	0.44^{cd}	2.24^{ab}	2.28^{ab}	0^{d}	1.23^{bc}	2.82^{a}	2.6^{a}	0^{d}	1.95^{ab}	2.99^{a}	2.88^{a}	0.331	<0.001	<0.001	0.037
g_Isotricha	0^{c}	0.19^{c}	1.65^{ab}	1.67^{ab}	0^{c}	1.01^{bc}	2.29^{a}	1.98^{ab}	0^{c}	1.73^{ab}	2.71^{a}	2.53^{a}	0.338	<0.001	<0.001	0.045
g_Dasytricha	0	0.24	1.92	1.69	0	1.12	2.63	2.17	0	1.67	2.05	2.59	0.403	<0.001	0.003	0.057
g_uncultured	0	1.27	1.96	2.31	0	2.01	2.14	2.11	0	2.03	1.74	2.06	0.378	<0.001	0.635	0.326

[1]Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at 5, 7 and 9 weeks of age.[a-f]Means within a row with different superscript differ (P<0.05) based on the Bonferroni test.

Supplementary Table S6. Effect of early-in-life rumen microbial inoculation and age on the rumen anaerobic fungal concentration, diversity and taxonomy.

Time	5 weeks				7 weeks				9 weeks					*P*-value		
Inoculation[1]	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	CTL	AUT	RFF	RFC	SED1	Inoc.	Time	AxT
Diversity																
Concentation (log10 copies/mg DM)	5.77^{cde}	5.35^{ef}	6.28^{ab}	6.09^{abc}	5.96^{bc}	5.77^{cd}	6.40^{a}	5.93^{bc}	5.46^{def}	5.37^{ef}	5.42^{def}	5.24^{f}	0.203	0.006	<0.001	0.006
Richness	16.5	14.8	12.4	13.2	14.9	13.8	12.7	14.4	n.m.	n.m.	n.m.	n.m.	3.885	0.765	0.989	0.942
Shannon index	1.06	0.89	1.01	1.38	1.17	1.18	1.08	1.35	n.m.	n.m.	n.m.	n.m.	0.305	0.554	0.395	0.889
Simpson index	0.46	0.40	0.47	0.61	0.56	0.53	0.48	0.64	n.m.	n.m.	n.m.	n.m.	0.126	0.515	0.159	0.742
Evenness	0.38	0.31	0.40	0.54	0.44	0.44	0.43	0.51	n.m.	n.m.	n.m.	n.m.	0.091	0.324	0.194	0.554
Chao Index	20.5	34.4	17.3	14.5	18.0	19.7	18.7	18.8	n.m.	n.m.	n.m.	n.m.	7.753	0.523	0.686	0.308
Good´s coverage	0.74	0.61	0.71	0.84	0.74	0.76	0.67	0.71	n.m.	n.m.	n.m.	n.m.	0.090	0.672	0.718	0.085
Abundance (log10 sequences)																
g_Caecomyces	0.93	1.49	0	0.28	0.70	1.03	0.04	0	n.m.	n.m.	n.m.	n.m.	0.503	0.062	0.247	0.433
g_Capnodiales	0	0.64	0	0.38	0	0	0	0	n.m.	n.m.	n.m.	n.m.	0.165	0.325	0.113	0.237
g_Neocallimastix	0.81^{a}	0.82^{a}	0.04^{b}	0^{b}	0.65^{a}	0.20^{ab}	0.04^{b}	0.04^{b}	n.m.	n.m.	n.m.	n.m.	0.342	0.187	0.247	0.037
g_Orpinomyces	0	0	0.42	0	0	0.90	1.39	0.85	n.m.	n.m.	n.m.	n.m.	0.292	0.030	0.023	0.496
g_Piromyces	0	0.60	1.07	1.58	0	1.62	0.57	2.00	n.m.	n.m.	n.m.	n.m.	0.537	<0.001	0.524	0.303
g_Neocallimastigaceae spp.	2.70	1.88	2.76	2.79	2.90	1.37	2.87	2.09	n.m.	n.m.	n.m.	n.m.	0.362	0.007	0.195	0.388

[1]Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at 5, 7 and 9 weeks of age.[a-f]Means within a row with different superscript differ (P<0.05) based on the Bonferroni test; n.m., not measured.

Supplementary Table S7. Descriptive statistics of the metadata used in the distance-based redundancy analyses and spearman correlations with microbial taxa abundance.

		5 weeks				7 weeks				9 weeks			
Abreviation[1]	**Units**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	**Mean**	**SD**
ADG	kg/d	0.04	0.22	0.16	0.05	0.08	0.25	0.19	0.04	0.08	0.29	0.19	0.05
ADG-f	kg/d	0.12	0.29	0.20	0.04	0.00	0.26	0.14	0.06	0.11	0.37	0.23	0.06
FE	kg/Mcal ME	0.05	0.29	0.19	0.06	0.08	0.26	0.18	0.04	0.10	0.34	0.21	0.06
FE-f	kg/Mcal ME	0.14	0.32	0.22	0.05	0.01	0.62	0.37	0.15	0.09	0.33	0.18	0.05
Milk	L/d	1.09	1.58	1.38	0.13	1.49	1.88	1.65	0.11	0.00	0.00	0.00	0.00
Concentrate	g/d	3.34	24.18	9.75	5.64	8.14	62.22	25.71	13.96	117.9	274.2	171.2	44.67
Concentrate-f	g/d	8.14	62.22	25.71	13.96	117.9	274.2	171.2	44.67	299.3	488.9	373.6	54.40
Forage	g/d	4.68	20.16	11.56	4.82	9.75	23.61	16.19	4.01	68.62	139.99	98.45	20.25
Forage-f	g/d	9.75	23.61	16.19	4.01	68.62	140.0	98.45	20.25	143.4	285.5	209.4	36.99
DMI	g/d	6.94	33.58	15.53	7.34	22.54	75.90	41.91	14.50	202.4	344.8	269.7	45.53
DMI-f	g/d	22.54	75.90	41.91	14.50	202.4	344.8	269.7	45.53	493.5	714.8	583.0	71.90
pH	pH units	5.76	7.13	6.71	0.24	5.48	6.92	6.57	0.29	5.79	7.40	6.68	0.45
Ammonia-N	mg/dL	11.82	49.71	27.49	8.65	12.22	50.48	29.18	9.49	0.03	24.15	8.80	6.65
VFA	mM	10.58	76.72	29.45	15.01	12.58	52.01	30.81	13.09	17.79	79.03	51.28	14.90
Acetate	%	54.30	81.30	73.87	5.75	58.80	84.90	69.58	6.81	42.80	67.30	56.93	6.58
Propionate	%	9.40	32.70	14.43	3.86	9.80	25.90	15.81	3.61	16.30	39.70	24.94	6.47
Butyrate	%	0.70	18.70	4.87	4.11	1.00	18.00	8.67	4.62	5.90	23.10	13.80	4.65
OBCVFA	%	3.80	14.00	6.83	1.93	3.90	11.00	5.94	1.69	2.20	7.30	4.29	1.12
Bacteria	log copies/mg DM	9.92	12.03	10.66	0.35	8.97	11.32	10.47	0.45	9.64	11.03	10.47	0.35
Bacterial-R	OTUs	111.0	514.0	300.8	130.6	183.0	653.0	389.1	154.9	97.0	561.0	285.7	130.2
Methanogens	log copies/mg DM	2.64	9.02	6.46	1.77	2.83	8.47	6.59	1.37	2.38	7.73	5.84	1.20
Methanogens-R	OTUs	1.00	18.00	11.06	4.76	4.00	22.00	13.21	5.20	4.00	21.00	13.36	4.75
Protozoa	log copies/mg DM	2.98	8.85	6.63	1.99	3.46	9.27	6.92	1.87	2.96	9.19	6.84	2.04
Protozoal-R	OTUs	0.00	31.00	16.72	11.05	0.00	34.00	19.13	12.46	0.00	35.00	17.66	12.76
Fungi	log copies/mg DM	3.48	7.00	5.89	0.81	3.48	7.39	6.25	0.67	3.40	7.25	5.42	0.98
Fungal-R	OTUs	3.00	30.00	14.20	6.26	5.00	34.00	13.92	7.63				
BHB	mM	0.95	5.25	2.57	1.13	1.31	4.98	3.07	0.76	1.31	3.95	2.49	0.73
Glucose	mg/dL	44.65	130.1	95.83	20.75	70.79	104.32	90.29	8.71	76.04	99.17	87.84	5.95
DOMI	g/d									342.58	498.73	435.04	45.00
DNI	g/d									14.22	20.42	17.42	1.94
DHCI	g/d									43.71	70.31	58.07	6.84
DCI	g/d									25.33	61.12	41.50	10.34

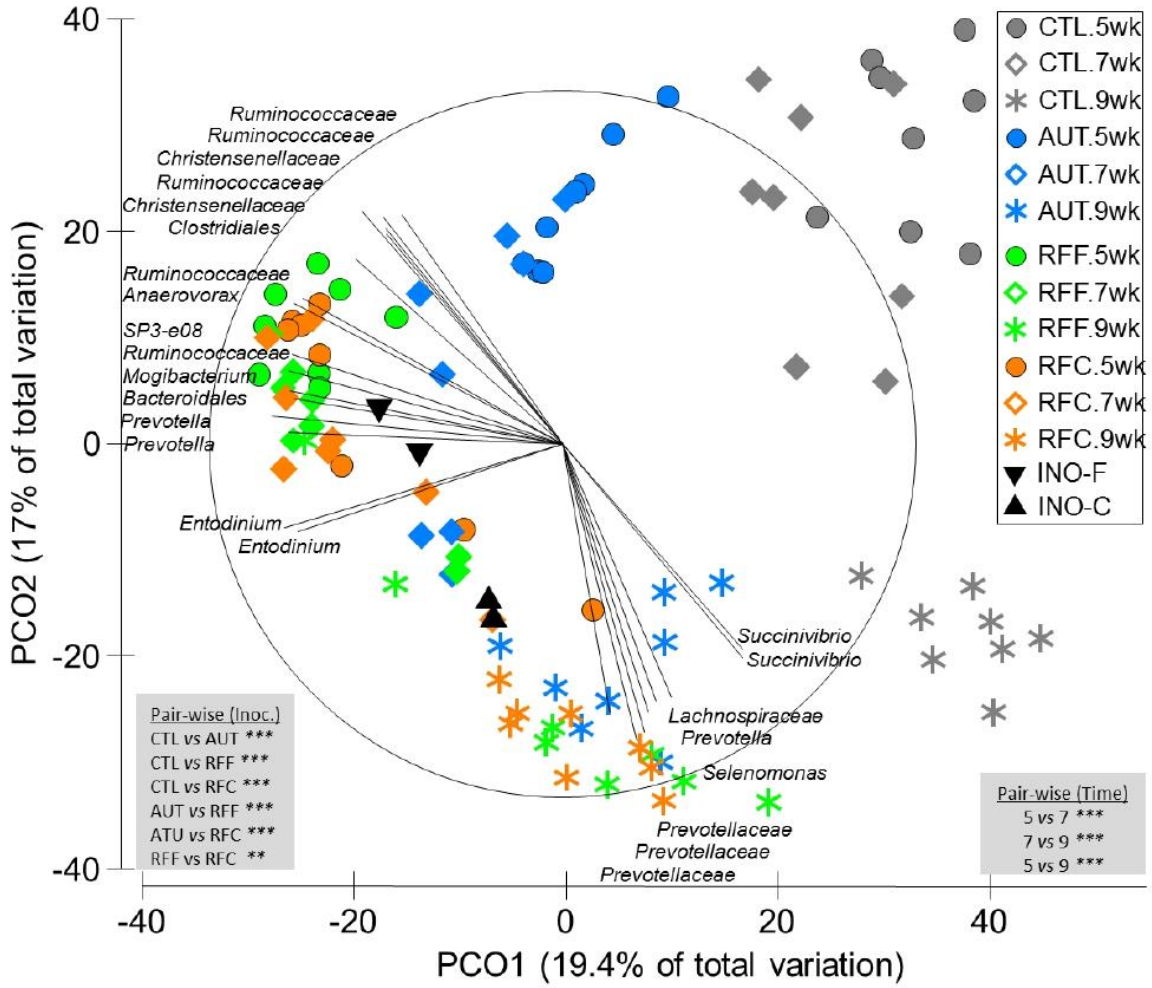
[1]ADG, average daily gain; -f, following week; FE, feed efficiency; Concentrate, concentrate-feed intake; Forage, forage-feed intake; DMI, dry mater intake; NH_3, rumen ammonia; VFA, volatile fatty acids; OBCVFA, odd and branched chain VFA; -R, richness; BHB, plasma β-hydroxybutyrate; Glucose, plasma glucose; DOMI, digestible organic matter intake; DNI; digestible nitrogen intake; DHCI, digestible hemicellulose intake; DCI, digestible cellulose intake.

Supplementary Table S8. Primers used for quantitative PCR and Next Generation Sequencing.

Target	Ref.	Name Primer	Forward Primer	Name	Reverse Primer	Amplicon (bp)
Quantitative PCR						
Total bacteria	[1]	1048F	GTGSTGCAYGGYTGTCGTCA	1175R	ACGTCRTCCMCACCTTCCTC	150
Methanogens	[2]	qmcrA-F	TTCGGTGGATCDCARAGRGC	qmcrA-R	GBARGTCGWAWCCGTAGAATCC	140
Protozoa	[3]	P-SSU-316f	GCTTTCGWTGGTAGTGTATT	PS-SU-539r	CTTGCCCTCYAATCGTWCT	223
Anaerobic fungi	[4]	qPCR fungi-F	GAGGAAGTAAAAGTCGTAACAAGGTTTC	qPCR fungi-R	CAAATTCACAAAGGGTAGGATGATT	120
Sequencing						
Bacteria	[5]	V3_F357	CCTACGGGAGGCAGCAG	V5_926	CCGTCAATTCMTTTRAGT	570
Methanogens	[6]	Arch349F	GYGCASCAGKCGMGAAW	Arch806R	GGACTACVSGGGTATCTAAT	457
Protozoa	[7]	F566Euk	CAGCAGCCGCGGTAATTCC	R1200Euk	CCCGTGTTGAGTCAAATTAAGC	660+var.
Anaerobic fungi	[8]	ITS3	GCATCGATGAAGAACGCAGC	ITS4	TCCTCCGCTTATTGATATGC	356+var.

References

1. Maeda H, Fujimoto C, Haruki Y, et al. Quantitative real-time PCR using TaqMan and SYBR Green for *Actinobacillus actinomycetemcomitans* , *Porphyromonas gingivalis* , *Prevotella intermedia* , tetQ gene and total bacteria. *FEMS Immunol Med Microbiol* 2003;39:81–86.
2. Denman, S.E., N. Tomkins, C.S. McSweeney, *Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane.* Fems Microbiol Ecol 2007;**62**:313-22.
3. Sylvester JT, Karnati SKR, Yu ZT et al. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J Nutr* 2004;**134**: 3378-84.
4. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol* 2006;**58**: 572-82.
5. Sim K, Cox MJ, Wopereis H, et al. Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. *PloS one* 2012;**7**:e32543.
6. Gantner S, Andersson AF, Alonso-Sáez L, et al. Novel primers for 16S rRNA-based archaeal community analyses in environmental samples. *J Microbiol Meth* 2011;**84**: 12-18.
7. Hadziavdic K, Lekang K, Lanzen A, et al. Characterization of the 18S rRNA gene for designing universal eukaryote specific primers. *PloS one* 2014;**9**: e87624.
8. De Beeck MO, Lievens B, Busschaert P, et al. Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. *PloS one* 2014;**9**: e97629.



Supplementary Figure S1. Principal co-ordinates analysis illustrating the inoculation effects on the multi-kingdom rumen microbioma. Analysis included bacteria, methanogens, protozoa and anaerobic fungi data along with the most discriminant OTUs ($\rho > 0.75$). Pair-wise PERMANOVA values are provided in grey boxes based on the Bray-Curtis dissimilarity. ***$P < 0.001$; **$P < 0.01$. Goat kids inoculated (I) with fresh rumen fluid from adult goats adapted to forage-rich (RFF) or concentrate-rich diets (RFC), autoclaved rumen fluid (AUT) or absence of inoculation as control (CTL) and sampled at 5, 7 and 9 weeks of age.

Publication 4

Presence of Adult Companion Goats Favors the Rumen Microbial and Functional Development in Artificially Reared Kids

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Frontiers in Veterinary Science 8:706592

DOI: https://doi.org/10.3389/fvets.2021.706592

ABSTRACT

Newborn dairy ruminants are usually separated from their dams after birth and fed on milk replacer. This lack of contact with adult animals may hinder the rumen microbiological and physiological development. This study evaluates the effects of rearing newborn goat kids in contact with adult companions on the rumen development. Thirty-two newborn goat kids were randomly allocated to two experimental groups which were reared either in the absence (CTL) or in the presence of non-lactating adult goats (CMP) and weaned at 7 weeks of age. Blood and rumen samples were taken at 5, 7, and 9 weeks of age to evaluate blood metabolites and rumen microbial fermentation. Next-generation sequencing was carried out on rumen samples collected at 7 weeks of age. Results showed that CTL kids lacked rumen protozoa, whereas CMP kids had an abundant and complex protozoal community as well as higher methanogen abundance ç which positively correlated with the body weight and blood β-hydroxybutyrate as indicators of the physiological development. CMP kids also had a more diverse bacterial community (+132 ASVs) and a different structure of the bacterial and methanogen communities than CTL kids. The core rumen bacterial community in CMP animals had 53 more ASVs than that of CTL animals. Furthermore, the number of ASVs shared with the adult companions was over 4-fold higher in CMP kids than in CTL kids. Greater levels of early rumen colonizers Proteobacteria and Spirochaetes were found in CTL kids, while CMP kids had higher levels of Bacteroidetes and other less abundant taxa (Veillonellaceae, Cyanobacteria, and Selenomonas). These findings suggest that the presence of adult companions facilitated the rumen microbial development prior to weaning. This accelerated microbial development had no effect on the animal growth, but CMP animals presented higher rumen pH and butyrate (+45%) and ammonia concentrations than CTL kids, suggesting higher fibrolytic and proteolytic activities. CMP kids also had higher blood β-hydroxybutyrate (+79%) and lower blood glucose concentrations (-23%) at weaning, indicating an earlier metabolic development which could favor the transition from pre-ruminant to ruminant after the weaning process. Further research is needed to determine the effects of this intervention in more challenging farm conditions.

Keywords: bacteria, methanogens, protozoa, rumen colonization, weaning

INTRODUCTION

Weaning of ruminants in natural conditions is a progressive process that occurs between 6 and 9 months of age, and it is characterized by a decrease in the frequency of suckling, with an increase in the frequency and amount of solid feed intake and development of more complex social interactions (1). However, in the current dairy production systems ruminants are weaned much earlier and newborns are typically separated from their dams immediately or during the first hours after birth, and they have no contact with adult ruminants until they are weaned or later. This practice has been shown to be stressful for both the newborn and the dam (2).

Artificial milk represents a substantial feeding cost in the current production systems, but weaning is not recommended until the rumen has a sufficient anatomical, physiological, and microbiological development (3), particularly when early weaning (conventional) is performed at 2 months of age. Several nutritional strategies have been proposed to maximize the solid feed intake prior to weaning such as decreasing milk allowance (step-down weaning) or optimizing feeder location and type of solid feed (1, 4). Nevertheless, these strategies are unlikely to favor the rumen microbial development.

Microbiota present in several maternal-associated sources, such as colostrum, vagina, udder skin, and saliva, has been reported to be able to colonize the gastrointestinal tract (GIT) of newborn ruminants within the first days of life (5). However, ruminants are born protozoa-free (afaunated) and rumen protozoa only get established after a direct and continuous nose–nose contact with adult animals (6). Therefore, under the isolation farm conditions described above, an optimal microbial GIT colonization in the newborns may be jeopardized (7, 8), having negative consequences on the transition from milk to solid feed diet throughout the weaning process (9, 10). The use of pre- and probiotics has been gaining importance in the livestock sector in the twenty first century (11) to improve animal performance and to prevent the growth of pathogenic microorganisms, promoting a more favorable microbial community in the GIT (12). Previous works have evaluated the effects of providing individual or a simple mix of microorganisms, specially *Saccharomyces cerevisiae* yeast, that are not autochthonous from the rumen or the GIT altogether (13, 14). As a result, in recent years, several authors have explored the inoculation of GIT autochthonous microbial strains (e.g., *Lactobacillus*, *Enterococcus*, *Megasphaera*, etc.) and rumen microbiota from adult animals to young ruminants (9, 15–

18). A repetitive oral inoculation of a mature rumen microbiota has shown to accelerate the rumen microbial colonization and increase microbial diversity in the rumen of goat kids (10). This strategy resulted in a less severe weaning process in terms of average daily gain (ADG) mainly because inoculated animals started consuming solid feed earlier than those without inoculation (9). However, this microbial inoculation may not be feasible in commercial farms due to veterinary regulations, animals' health concerns and well-being issues, and/or the inherent difficulty to obtain fresh rumen fluid to be used as inoculum to maximize its efficacy (19). A potential approach to circumvent this limitation is to house young animals with older weaned companions that would serve as main sources of microbiota. De Paula Vieira et al. (20) demonstrated that the presence of adult companions stimulated the feeding social learning of feeding and reported improved concentrate feed intake and growth rate before and after weaning in calves. However, the impact of such intervention on the rumen function and microbial development using next-generation sequencing has not been investigated under modern dairy management practices.

The present study builds upon this existing knowledge and aims to further explore whether the presence of non-lactating adult goats (as companions) could act as natural source of microbes favoring the rumen microbial colonization in young ruminants. We hypothesized that the sole contact between adult and young animals would allow a microbial transfer, leading to an acceleration in the rumen microbial development and its function with potential positive effects during the weaning process to be further implemented under on farm conditions.

MATHERIALS AND METHODS

Experimental Design

Animal procedures conducted in this work were approved by the Ethical Committee for Animal Research (EEZ-CSIC) and carried out by trained personnel according to the Spanish guidelines (RD 53/2013). A total of 32 Murciano-Granadina newborn goats were used in this experiment. After parturition, goat kids were separated from their dams, weighed, and fed with ~250 ml of natural colostrum in two separate doses as previously described (9). Goat kids were randomly allocated to two experimental groups (16 animals each), keeping a similar average body weight (BW) and male/female ratio in both groups.

One group was used as control (CTL) and kept isolated from any contact with adult animals throughout the whole experiment (conventional practice), while the other group was in continuous contact with two non-lactating goats as companion (CMP). These adult goats belonged to a healthy experimental herd and were dewormed (Ivomec® Oral, Boehringer Ingelheim, Barcelona, Spain) 1 week before the beginning of the trial. Both groups had free access to water (CMP kids shared water through with the adults) and a milk replacer at 170 g/ml (Univet® Spray, NutralSCA, Colmenar Viejo, Spain). From day 14, all animals had *ad libitum* access to starter concentrate (0–18 Granulado Arranque Pequeños Rumiantes Pulmorex, Macob, Granada, Spain) and oat hay until week 9. The chemical composition of these feeds is reported in **Supplementary Table 1**. The amount of starter concentrate offered to each group was recorded throughout the experiment. In the CMP group, a fence only traversable by goat kids was placed to prevent adult goats to have access to milk replacer and starter concentrate. Adult goats were fed with oat hay *ad libitum* and a limited amount of the starter compound feed (300 g DM/day). Milk feeding of the kids was stopped abruptly at 7 weeks of age. Body weight was monitored weekly from birth until the end of the experiment at week 9.

Sampling and Analyses

Blood and rumen samples were collected before the morning feeding (09:00 h) at 5, 7, and 9 weeks of age, representing the pre-weaning, weaning, and post-weaning stages, respectively.

Blood samples were centrifuged at 2,000 × g for 15 min, and supernatant was stored at −80°C for β-hydroxybutyrate (BHB), glucose, urea, and total protein determination as previously reported (9). Rumen content from each animal was collected by orogastric intubation (∼30 ml) as previously described (21). Rumen content was filtrated through a double layer of cheesecloth to discard solid debris, and pH was immediately recorded. Similarly to how it is described in Belanche et al. (9), three subsamples of 0.8 ml were taken: the first sample was diluted with 0.2 ml of trichloroacetate solution (25 g/l) for ammonia determination. The second sample was diluted with 0.8 ml of an acid solution (0.5 mol/l HCl, 200 g/l metaphosphoric acid and 0.8 g/l crotonic acid as internal standard) for volatile fatty acid (VFA) determination. The third sample was diluted with 0.8 ml of formaldehyde solution (8% v/v) for protozoal counting. The ammonia concentration was

determined by a colorimetric method (22). Individual and total VFA were measured using a gas chromatograph with a flame ionization detector (Auto-System, Perkin Elmer, Waltham, MA, USA), and protozoal counting and classification were visually determined in 15 µl of rumen liquid (23) using an optical microscope (Nikon Labophot, Tokyo, Japan). An additional subsample of rumen fluid was snap-frozen in liquid N and stored at −80°C for DNA extraction. These subsamples were then freeze-dried and bead-beat for 1 min (Mini-BeadBeater, BioSpec Products, Bartlesville, OK, USA), and DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain). Rumen content from the two adult companions was also sampled by orogastric intubation at the beginning of the experiment for protozoal counting and DNA extraction using the same procedures. Negative controls for the DNA extraction and sequencing were also included.

Eluted DNA (2 µl) was used to assess the abundance of the main microbial groups by quantitative PCR (qPCR) using an iQ5 multicolor Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) as described by Abecia et al. (24). Specific primers for the 16S bacterial rRNA gene, mcrA gene for methanogenic archaea, and 18S rRNA genes for protozoa and anaerobic fungi were used as previously described and validated [(25–28); respectively]. Cycling conditions were

95°C for 5 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 55 s; and 72°C for 1 min. The absolute amount of each microbial group, expressed as the corresponding gene copies/g of dry matter, was determined using serial dilutions of standards. The standards consisted of the plasmid PCR 4-TOPO (Invitrogen, Carlsbad, CA, USA), with an inserted 16S, mcrA, or 18S rRNA gene fragment from each microbial group, respectively.

Next-Generation Sequencing

The structure of the rumen prokaryotic community was explored at weaning (week 7) using a meta-taxonomic approach. Eight kids from each treatment were randomly selected, and a template of the extracted DNA was sent to the Genomics Service at Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain) for amplicon sequencing using the MiSeq V3 (600 cycles) kit (Illumina Inc., San Diego, CA, USA). The prokaryotic universal primers used for the amplification were Pro341F

5′-CCTACGGGAGGCAGCAG-3′ and Pro805R 5′-GACTACNVGGGTATCTAATCC-3′ targeting the V3_V4 hypervariable region of the 16S rRNA gene including bacteria and methanogenic archaea (29). Primer-sorted and demultiplexed paired-end reads were used separately for bacteria and methanogens, and downstream processing was performed using QIIME 2 (30). Low-quality reads and bases (PHRED quality score below 25) were trimmed. Chimeras were identified and removed using chimera.vsearch. Amplicon sequence variants (ASVs) were identified, and then representative sequences from all ASVs were aligned against Greengenes 13_8 97% for bacteria (31) and RIM-DB for archaea (32). Once alignment was performed, data from each of the two major microbial groups were processed separately. The number of sequences per sample for each microbial group was normalized across all the samples, and singletons were removed. Raw sequence reads were deposited at the European Nucleotide Archive repository (accession: ERP126589) (33).

Statistical Analyses and Calculations

Statistical analyses were conducted using SPSS software (IBM Corp., Version 26.0, New York, NY, USA). Rumen fermentation parameters, blood metabolites, quantitative PCR, and BW data were analyzed based on a repeated-measure mixed-effect ANOVA as follows:

$$Y_{ijklm} = \mu + C_i + T_j + CT_{ij} + A_k + e_{ijkl}$$

where Y_{ijkm} is the dependent, continuous variable, μ is the overall population of the mean, C_i is the fixed effect of the presence of companion goats (i = CTL vs. CMP), T_j is the fixed effect of the time (j = 5 vs. 7 vs. 9 weeks of age), CT_{ij} is the interaction term, A_k is the random effect of the goat kids (k = 1 to 32), and e_{ijkl} is the residual error. When significant effects were detected, means were compared by Fisher's protected LSD test. Quantitative PCR data and protozoal optical count data were log10 transformed before the analysis to achieve a normal distribution. Bacterial and methanogen diversity indexes at week 7 were analyzed using an ANOVA test with the treatment (CTL vs. CMP) as fixed effect. Microbial data were analyzed following the overall approach described by Belanche et al. (34). Microbial taxa abundances were analyzed with the Kruskal–Wallis non-parametric test, given that data did not have a normal distribution after performing

the Shapiro–Wilk test. Only taxa with relative abundance > 0.05% were shown. In all analyses, significant effects were declared at $p < 0.05$, tendency to difference at $p < 0.1$.

Venn diagrams were performed to illustrate the treatment effects on the core microbial community, defined as the number of ASVs shared across the majority (>75%) of the individuals within each group. Venn diagrams also included the microbial community found in the two adult companion goats which were considered as a potential source of rumen microbiota. To illustrate the treatment impact on the rumen prokaryotic community, a permutation-based analysis of variance (PERMANOVA) including *p*-values and similarity was calculated based on the Bray–Curtis distance matrix. To achieve this, the log10 transformed bacteria and methanogen sequencing data were submitted to 999 random permutations under the reduced model and the Monte Carlo method (34) using PRIMER-6 software (PRIMER-E Ltd., Plymouth, UK). Pair-wise comparisons were performed to compare the microbial composition across treatments. Principal coordinate analyses (PCoA) were carried out to show the effects on the bacterial and methanogen rumen community structure. Tripod vectors were included in the PCoA to identify the most discriminant ASVs (based on Spearman correlation > 0.8). Additional Spearman correlations (ρ) were calculated to assess the relationships between the microbial taxa abundance (log10 number of sequences) and the rumen fermentation and blood parameters. Strong correlations were defined as those with $\rho \geq 0.4$ or ≤ -0.4 and $p < 0.01$.

RESULTS

Animals' Growth, Rumen Fermentation, and Blood Metabolites

The presence of adult companions had no effect on the animal performance in terms of BW and ADG from birth to 9 weeks of age (**Table 1**). Similarly, both experimental groups had similar starter DMI until week 9 (average 6.25 kg per goat kid). The rumen fermentation pattern was highly affected by the age of the kids leading to a progressive increase in VFA concentration and propionate and butyrate molar proportions, whereas rumen ammonia and acetate molar proportions decreased over time ($p < 0.01$). CMP animals also had higher rumen pH ($p = 0.046$) and branched-chain VFA (i.e., iso-valerate and iso-butyrate, $p = 0.033$) than CTL kids. The butyrate molar proportion showed an interaction ($p < 0.001$) since the higher values observed in CMP than in CTL kids

increased with age, leading to substantial increments at 7 (+23%) and 9 weeks of age (+83%). The concentrations of all measured blood metabolites presented significant differences according to the sampling time (**Table 1**). The concentration of BHB and urea increased ($p < 0.05$) while glucose concentration decreased over time ($p = 0.013$). The blood BHB concentration in CMP goat kids was higher than in CTL across all sampling times ($p = 0.048$). The ratio BHB/glucose also increased with age ($p = 0.013$) and tended to be higher in CMP than in CTL kids ($p = 0.088$).

Rumen Microbial Community

Quantitative PCR analysis showed that the ruminal concentrations of bacteria and fungi were unaffected by the experimental treatment (**Table 2**). The methanogen concentration was higher in CMP than in CTL kids at 5 weeks of age, but differences tended to become smaller over time (interaction, $p = 0.039$). The largest differences promoted by the presence of adult companions were related to the protozoal community: CTL kids were protozoa-free (afaunated) since no protozoal cells were detected with optical microscope examination, and qPCR showed negligible concentrations of protozoal DNA (1,000 folds lower than CMP kids). On the contrary, CMP kids showed an abundant (from 4.96 to 5.21 log10 cells/ml) and diverse protozoal community dominated by the subfamily *Entodiniinae* but also with other protozoal groups such as *Diplodiniinae*, *Ophryoscolex*, *Isotricha*, and *Dasytricha*. These protozoal groups were also present in both of the adult companions, *Entodiniinae* being the most abundant group (**Supplementary Table 2**). The rumen protozoal community in the CMP animals also developed over time, promoting an increase in the protozoal concentration, both in DNA gene copies ($p < 0.001$) and in cells ($p = 0.012$), and a substantial increase in the proportions of *Ophryoscolex* ($p < 0.001$), *Isotricha*, and *Dasytricha* in detriment of the subfamily *Entodiniinae*.

Sequencing Analysis

The sequencing analysis generated 30,151 ± 9,600 high-quality prokaryotic sequences per sample. The number of sequences was normalized to 20,551 for further processing and analyses. The Good's coverage for the bacterial and methanogen communities was

99 and 70%, respectively. The rumen bacterial diversity in terms of observed ASVs, Shannon index, and evenness was much higher in CMP than in CTL goat kids at weaning ($p < 0.001$, $p < 0.001$, and $p < 0.05$; respectively) (**Figures 1A–C**; **Table 3**). CMP kids also tended to have a more diverse methanogen community in terms of evenness and Simpson index ($p < 0.1$), but no differences were observed for number of ASVs or Shannon index (**Figures 1D–F**; **Table 2**).

The Venn's diagram (**Figure 1G**) showed that the number of ASVs that made the core rumen bacterial community in each group of animals (ASVs present in >75% animals within each group) was greater in CMP than in CTL (107 vs. 54 ASVs). Among those ASVs, only 20 of them were shared between CMP and CTL kids. The two adult goats had the largest rumen bacterial core community (228 ASVs), and although most of them were adult goats-specific, a larger proportion of them was specifically shared with CMP than with the CTL kids (29 vs. 7 ASVs). Out of the 29 ASVs shared between CMP kids and the adults, 15 belonged to the *Bacteroidales* order and 8 to *Clostridiales* order. Only 10 bacterial ASVs were shared across CTL, CMP, and adult goats. A similar core community pattern, but with much lower numbers, was found regarding the core rumen methanogens ASVs (**Figure 1H**).

PERMANOVA analysis showed a clear effect of the treatment on the rumen prokaryotic community structure (**Figure 2**). Pair-wise comparisons identified a substantial difference between the CMP and CTL rumen bacterial ($p = 0.002$) and methanogen communities ($p = 0.001$), indicating a relatively low level of similarity among these treatments (21.33 and 17.65%, respectively). Both CMP and CTL kids differed in the bacterial and methanogen community structure with the adult goats ($p < 0.05$); however, the level of similarity was higher between CMP kids and adult goats (27.0 and 24.8% for bacteria and methanogens) than between CTL kids and adult goats (17.1 and 17.0%, respectively). These differences were also noted in the subsequent PCoA analysis for the bacterial community (**Figure 2A**), in which the PCO1 axis (which explained 25.9% of the total variation) sorted the samples by the treatment factor. A similar pattern was depicted in the PCoA for the methanogen community, where the most discriminant axis (PCO1 explaining 36.6% of the total variation) separated CTL kids from CMP kids and adults. Several bacterial groups such as *Prevotella*, *Fibrobacter*, CF231, *Paraprevotellaceae*, *Ruminococcaceae*, RFP12, and some *Bacteroidales* ASVs positively correlated with the rumen community structure of CMP kids, whereas others such as *Treponema*, *Clostridiales*, or

Lachnospiraceae ASVs negatively correlated with the CMP bacterial community (**Figure 2A**). Within the methanogen community, the most discriminant ASVs were *Methanomicrobium mobile* and Group 9 sp for the CMP kids, Group 12 sp for the CTL kids, and *Methanobrevigacter gottschalkii* for the adult goats (**Figure 2B**).

The assessment of the relative abundance of the rumen prokaryotic taxa showed that 7 out of the 20 most abundant bacterial families (**Table 3**; **Supplementary Table 3**) and 5 out of 10 methanogens species (**Table 4**) denoted differences between CMP and CTL kids. *Prevotella* was by far the most abundant genus in the rumen of goat kids regardless of treatment (37% of the total sequences). At phylum level, *Bacteroidetes* tended to be more abundant in CMP kids in contrast with *Firmicutes*, whose abundance was higher in CTL kids. CTL kids also harbored a higher presence of *Proteobacteria* and *Spirochaetes* (10.8% between the two phyla in CTL kids, in comparison with 4.32% in CMP kids). Other than comparisons between phyla, CMP kids had higher abundance of several rumen bacterial taxa including *Bacteroidaceae*, *Veillonellaceae*, *Erysipelotrichaceae*, *F16*, *RFP12*, *Selenomonas*, *Succinispira*, *Bulleidia*, *Spirochaeta*, and *Dethiosulfovibrio*. On the contrary, *Rhodocyclaceae*, *Desulfovibrionaceae*, *Spirochaetaceae*, R4_41B family, *Georgfuchsia*, and *Treponema* showed a greater abundance in CTL kids. With regard to methanogens, the species *Group9_ISO4_G1*, *M. mobile*, and *Group9_sp_CH1270* were more abundant in CMP kids, while *Group12_ISO4_H5* was more prevalent in CTL kids. Most of the taxa which were significantly more abundant in CMP than in CTL kids were also numerically more abundant in the rumen of the adult companions than in the CTL kids.

Spearman correlations were performed to assess the potential implications of changes in rumen meta-taxonomic data on animal physiology and rumen fermentation parameters (**Supplementary Table 5**). Bacterial, methanogen, and anaerobic fungal abundance, as well as various microbial taxa such as *Fibrobacteres*, *SR1*, *TM7 F16*, and *Methanomicrobium* and various protozoa, positively correlated with physiological indicators such as total rumen VFA, blood BHB, and BHB/glucose ratio. The abundance of the phyla SR1, TM7, bacterial diversity, and anaerobic fungi concentration also had a positive correlation with the BHB/Glucose ratio, whereas this ratio negatively correlated with the taxa Rhodocyclaceae, Spirochaetaceae, and Methanobrevibacter. The rumen concentration of protozoa, anaerobic fungi, methanogens, and *Fibrobacteres* positively correlated with BW. Rumen protozoal concentration and abundances of most protozoal

groups had a positive correlation with the concentrations of rumen butyrate and blood urea and proteins.

DISCUSSION

Previous studies with calves have demonstrated that decreasing the milk allowance during the artificial milk feeding period or optimizing the type of solid feed (e.g., presentation form and taste) and feeder location prior to weaning can encourage young ruminants to increase the solid feed intake (1, 4). However, artificial milk feeding in conjunction with early weaning strategies can limit the rumen microbiological colonization by a microbial consortium able to perform the major fermentative and metabolic functions required at weaning and beyond, which may have negative effects on the weaning process (3). Previous works have demonstrated that lambs (35) and goat kids (36) reared with their dams experienced an earlier rumen microbial and physiological development than those artificially reared in the absence of adults. Moreover, we have confirmed that this microbial transfer can be mimicked by inoculating young ruminants with rumen fluid from adult animals (9, 10), promoting an acceleration of the rumen microbiological colonization and favoring the solid feed intake prior to weaning. The present study builds upon these previous findings and investigates an alternative strategy based on the presence of a low number of adult non-lactating companions to be practical under farm conditions, but potentially maintaining similar positive effects.

Rumen Fermentation

Although individual DMI was not measured in our study, CMP kids showed a higher rumen pH than CTL kids which could be due to a greater forage intake as a result of the presence of rumen protozoa (9, 37). However, there is still controversy on whether the higher solid feed intake could rely on a direct effect derived from a social feeding learning from the adult goats (20) or on an indirect effect driven by the microbial transfer which allows a better digestion of fibrous feeds (38). Even though no major differences were found in the total VFA and ammonia concentrations between CTL and CMP goat kids, a notably higher production of rumen butyrate (+45%) was observed in CMP kids across sampling times. Butyrate has been described as a key fermentation product involved in

the rumen and intestinal epithelial development (39, 40) and in the overall health status of young ruminant (41). Butyrate production is a result of starch and cellulose degradation, a process which is greatly associated with the fibrolytic protozoal activity (42, 43), which explains its positive correlation with rumen protozoal abundance in this study. A recent study also reported a similar increase (+52%) in the butyrate molar proportion in naturally reared in comparison with artificially reared lambs (35). Therefore, the increasingly greater butyrate concentration in CMP than in CTL kids over time (from +25% at week 5 to +83% at week 9) could rely on a greater solid feed intake which could favor a smooth transition from liquid to solid feeding as previously reported (9). Similarly, the greater levels of branched-chain VFA (+20.4%) and twice higher ammonia concentration noted in CMP than in CTL kids across sampling times suggest a higher rumen proteolysis (44). As already reported in previous works where either young (9) or adult (45) ruminants were inoculated with rumen fluid, the higher concentration of ammonia and branched-chain VFA was associated with a higher breakdown of dietary and bacterial protein by rumen protozoa (46). The higher plasma BHB concentration (+41%) noted in CMP than in CTL kids across all sampling times can be justified by a greater ruminal butyrate concentration. This increase in BHB concentration has been associated with greater fermentative activity, VFA absorption, and bio-transformation in the rumen wall, as it was also reported when direct rumen microbiota inoculation was performed on young ruminants (9, 15). On the contrary, blood glucose levels in young ruminants decrease with age and can be considered as an indicator of insufficient GIT physiological development (9). In our study, the lower blood glucose concentrations observed in CMP compared with CTL kids at 5 (−10%) and 7 weeks of age (−23%) ascertain that the transition from proto-rumen to matured rumen could have been accelerated in CMP kids (47), possibly as a result of a more developed rumen microbiota and/or feeding behavior.

Rumen Eukaryotes

This study showed that the presence of adult goats as companions of CMP kids, in contrast to CTL kids, accelerated the rumen microbial development, leading to the presence of a more complex microbial community characterized by an abundant and diverse protozoal community. Unlike rumen prokaryotes, rumen protozoa are strict

anaerobes considered late rumen colonizers which can only be transferred by direct contact with adult animals (48, 49), with the common drinking water area being one of the main sources (6). As a result, CTL kids lacked rumen protozoa throughout the whole experiment. On the contrary, CMP kids harbored a relevant protozoal community at 5 weeks of age, which became more complex over time and therefore more closely resembling the protozoal community observed in the adult companions. The predatory activity on rumen bacteria by the protozoa could explain the similar total bacteria abundance between CTL and CMP kids (46). Despite the protozoal abundance not changing between sampling times, *Entodiniinae* was by far the predominant rumen protozoa in CMP kids at 5 weeks of age followed by *Diplodiniinae*. However, *Entodiniinae* abundance decreased over time in favor of *Ophryoscolex* and holotrichs (*Isotricha* and *Dasytricha*) resulting in a more diverse protozoal community. The concentration of rumen protozoa and the abundance of some protozoal groups positively correlated with the BW and blood BHB, indicating a positive effect on the rumen function which could favor the fiber degradation in CMP kids after weaning (37). In relation to the rumen anaerobic fungal population, previous studies have shown that young ruminants natural reared on the dam (35) or inoculated with rumen fresh rumen fluid from adult animals (10) had higher rumen fungal diversity (or concentration) and a different community structure than those artificially reared on milk replacer. In our study, the rumen anaerobic fungal concentration positively correlated with rumen acetate and certain indicators of physiological development (i.e., BW and blood BHB); however, no differences between treatments were noted between treatments, possibly because fungi can develop resistant spores that facilitate their transmission without the need of physical contact across animals (50).

Rumen Prokaryotes

The rumen colonization by prokaryotes has been described as a sequential process that occurs earlier than for eukaryotes (51). The rumen is primarily colonized by facultative anaerobes (mostly *Proteobacteria*) (52, 53), but shortly after the first days of life a great microbial shift takes place as strict anaerobes (i.e., *Firmicutes* and *Bacteroidetes*) get established, making the bacterial community more diverse. This process is particularly evident when animals start ingesting solid diet, and cellulolytic bacteria such as

Fibrobacteres and Firmicutes start occupying relevant niches (54). Similarly, rumen methanogens (e.g., *Metanobacteriales* and *Methanomicrobiales*) have also been reported to colonize the rumen from the first week of age with substantial taxonomic changes thereafter (55, 56). In our study, a higher rumen bacterial diversity (+132 ASVs) was observed in CMP kids at 7 weeks of age compared to CTL kids. Similar increments in the bacterial diversity have been found in naturally reared lambs compared with artificially reared lambs (35, 36) and in young ruminants inoculated with rumen fluid (10, 57).

Although the primers used in our study have been validated to simultaneously study the rumen bacterial and methanogen communities (29, 58), the lower coverage observed for the latter community may limit the identification of low-abundance methanogens. A small but consistent number of methanogen sequences were detected in our study, representing ~0.6% of the total sequences and being in agreement with the proportion expected in the rumen (59). Despite this methodological limitation, our study showed that the CMP kids tended to have higher methanogen diversity (Evenness and Simpson index) than the CTL kids. The presence of rumen protozoa has been associated with greater bacterial and methanogen diversities due to the presence of an important epi- and endo-symbiotic prokaryotic community associated with rumen protozoa (37).

The study of the core rumen community has been proposed as a useful approach to describe the colonization pattern (52, 60). Our study showed that a greater proportion of the bacterial and methanogen core communities were shared between the adult companion goats and CMP kids than with CTL kids. Moreover, a great number of bacterial ASVs remained present only in the adult animals, indicating that the rumen prokaryotic colonization process is still far from being completed at weaning. This observation is in line with previous observations (52), which suggested that the rumen colonization is a long-lasting and continuous process which takes several years to be fully accomplished. Similarly, a substantial number of prokaryotic ASVs were only present in either CMP or CTL kids, indicating that the shared environment can determine and homogenize the rumen microbial composition as a result of the cross-contamination between animals. The greater treatment-specific core community observed in CMP kids was most likely a result of a microbiological enriched environment due to the presence of several microbial sources from the adult goats such as feces, udder or skin (5).

Regarding the community structure of the rumen microbiota, the higher level of similarity observed between the adult animals and CMP kids, in comparison to CTL kids, reinforces the existence of a microbial transmission. Moreover, some of the most discriminant ASVs between CMP and CTL kids (e.g., *Prevotella*, *Ruminococcaceae*, and *Fibrobacter*) have been identified as part of the adult core rumen microbiome (61) and/or indicators of the rumen microbial development (10). This study also noted differences in the relative abundance of some microbes, which may indicate the presence of a more mature rumen microbiota in CMP than in CTL kids (10, 17). Most of the bacterial taxa with higher abundance in CTL kids belonged to *Proteobacteria* and *Spirochaetes* (mainly sugar-degrading or sulfate-reducing bacteria), indicating that an important number of early colonizers were still present in the rumen of CTL kids at 7 weeks of age. On the contrary,

CMP kids had higher abundances of *Bacteroidetes* (+11%) which has been reported as an indicator of a rumen microbiota suited to digest grain diets (59) as well as being regarded as a phylum with a high lignocellulolytic and hemicellulolytic activity (62). Furthermore, other relevant taxa such as *Tenericutes*, *Cyanobacteria*, and *Veillonellaceae* were also increased in CMP kids and have been recently correlated with indicators of rumen functional development such as forage and solid intake, presence of protozoa, and higher bacterial, protozoal, and methanogen diversity (10). In this sense, the greater presence of late colonizer *Selenomonas* in CMP kids (52) could contribute to enhance fiber digestion when partnered with *F. succinogenes* (63). The present study did not find a clear transmission pattern of individual methanogen species from adult companions to CMP goat kids. However, the increased levels of *M. mobile* and *Group9* in CMP kid are consistent with the changes found in goat kids inoculated with fresh rumen fluid, being both methanogen species positively correlated with bacterial diversity and rumen development indicators (10). This observation, along with the greater total methanogen abundance (which positively correlated with BW and BHB) and a tendency to greater diversity in this group, indicates that CMP kids may host a more mature rumen methanogen community at weaning, possibly shaped by the presence of a complex protozoal community as previously described (64–66).

These observations demonstrated that rearing young ruminants in the presence of adult goats accelerated the rumen microbial and functional development with potential benefits in the transition from the liquid to solid feed. A previous study with artificially reared kids pointed out a milk replacer cost of 24e per kid when animals are weaned at 7 weeks

of age, but with the possibility of decreasing this cost by 20 or 35% if kids are weaned at 5 or 6 weeks of age, respectively (9). The feeding practices used in this study and based on *ad libitum* milk feeding and late weaning did not help to visualize the full potential of this strategy in terms of animal performance, as noted in similar studies (9, 20). However, the implementation of more challenging farm conditions such as low milk allowance or early weaning practices (1) could potentially enhance the positive effects derived from a greater rumen microbial and physiological development.

CONCLUSIONS

Overall, these findings revealed that the presence of adult goats as companions facilitated the rumen microbial transfer of protozoa and specific methanogens and bacterial taxa to young ruminants accelerating the rumen microbial and functional development. This strategy could facilitate the transition from liquid to solid feed with potential positive effects on the weaning process. Further studies are needed to investigate the short- and long-term effects of young ruminants reared in the presence of non-lactating adults on the rumen microbiota and animal performance under more challenging farm conditions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee for Animal Research (EEZ-CSIC).

AUTHOR CONTRIBUTIONS

DY-R, AB, and AM-G: conceptualization and funding acquisition. JP-H, AB, and EJ: methodology and data acquisition. JP-H and AB: data curation and software. JP-H:

writing original draft. AB and DY-R: writing, review, editing, and project administration. All authors have read and approved the final manuscript.

FUNDING

This work was supported by the European Union's Horizon 2020 Research and Innovation program under grant agreement no. 818368 (MASTER) and by the Spanish Government through the project AGL2017-86938-R. JP-H has a grant from the Training Program for Academics, Madrid, Spain (FPU16/01981) and AB is a Ramón y Cajal Fellow [RYC2019- 027764-I/AEI/10.13039/501100011033] from the Spanish Research Agency.

ACKNOWLEDGMENTS

The authors thank Isabel Jiménez (Estación Experimental del Zaidín, Granada, Spain) for her assistance with the sample analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets. 2021.706592/full#supplementary-material

REFERENCES

1. Mikuš T, Marzel R, Mikuš O. Early weaning: new insights on an ever- persistent problem in the dairy industry. *J Dairy Res*. (2020) 87:88– 92. doi: 10.1017/S0022029920000503
2. Mandel R, Wenker ML, van Reenen K, Keil NM, Hillmann E. Can access to an automated grooming brush and/or a mirror reduce stress of dairy cows kept in social isolation? *Appl Anim Behav Sci*. (2019) 211:1– 8. doi: 10.1016/j.applanim.2018.12.007

3. Meale SJ, Li S, Azevedo P, Derakhshani H, DeVries T, Plaizier J, et al. Weaning age influences the severity of gastrointestinal microbiome shifts in dairy calves. *Sci Rep*. (2017) 7:1–13. doi: 10.1038/s41598-017-00223-7

4. Parsons SD, Steele MA, Leslie KE, Renaud DL, DeVries TJ. Investigation of weaning strategy and solid feed location for dairy calves individually fed with an automated milk feeding system. *J Dairy Sci*. (2020) 103:6533– 56. doi: 10.3168/jds.2019-18023

5. Yeoman CJ, Ishaq SL, Bichi E, Olivo SK, Lowe J, Aldridge BM. Biogeographical differences in the influence of maternal microbial sources on the early successional development of the bovine neonatal gastrointestinal tract. *Sci Rep*. (2018) 8:1–14. doi: 10.1038/s41598-018-21440-8

6. Bird SH, Hegarty RS, Woodgate R. Modes of transmission of rumen protozoa between mature sheep. *Anim Prod Sci*. (2010) 50:414–7. doi: 10.1071/AN09216

7. Abecia L, Ramos-Morales E, Martínez-Fernandez G, Arco A, Martín-García AI, Newbold CJ, et al. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. *Anim Prod Sci*. (2014) 54:1449–54. doi: 10.1071/AN14337

8. Belanche A, Cooke J, Jones E, Worgan HJ, Newbold CJ. Short- and long-term effects of conventional and artificial rearing strategies on the health and performance of growing lambs. *Animal*. (2018) 13:740– 9. doi: 10.1017/S1751731118002100

9. Belanche A, Palma-Hidalgo JM, Nejjam I, Jiménez E, Martín-García AI, Yáñez-Ruiz DR. Inoculation with rumen fluid in early life as a strategy to optimize the weaning process in intensive dairy goat systems. *J Dairy Sci*. (2020) 103:5047–60. doi: 10.3168/jds.2019-18002

10. Palma-Hidalgo JM, Jiménez E, Popova M, Morgavi DP, Martín-García AI, Yáñez-Ruiz DR, et al. Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. *Anim Microb*. (2021) 3. doi: 10.1186/s42523-021-00073-9

11. Buntyn JO, Schmidt TB, Nisbet DJ, Callaway TR. The role of direct-fed microbials in conventional livestock production. *Ann Rev Anim Biosci*. (2016) 4:335–55. doi: 10.1146/annurev-animal-022114-111123

12. Apás AL, Dupraz J, Ross R, González SN, Arena ME. Probiotic administration effect on fecal mutagenicity and microflora in the goat's gut. *J Biosci Bioeng*. (2010) 110:537–40. doi: 10.1016/j.jbiosc.2010.06.005

13. Chaucheyras-Durand F, Walker ND, Bach A. Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. *Anim Feed Sci Technol*. (2008) 145:5–26. doi: 10.1016/j.anifeedsci.2007.04.019

14. Desnoyers M, Giger-Reverdin S, Bertin G, Duvaux-Ponter C, Sauvant D. Meta-analysis of the influence of saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants. *J Dairy Sci*. (2009) 92:1620–32. doi: 10.3168/jds.2008-1414

15. De Barbieri I, Hegarty RS, Silveira C, Oddy VH. Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of merino lambs. *Small Ruminant Research*. (2015) 129:37–47. doi: 10.1016/j.smallrumres.2015.05.017

16. Li R, Teng Z, Lang C, Zhou H, Zhong W, Ban Z, et al. Effect of different forage-to-concentrate ratios on ruminal bacterial structure and real-time methane production in sheep. *PLoS ONE*. (2019) 14:1– 20. doi: 10.1371/journal.pone.0214777

17. Yu S, Zhang G, Liu Z, Wu P, Yu Z, Wang J. Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. *BMC Microbiol*. (2020) 20:1–15. doi: 10.1186/s12866-020-1716-z

18. Zhong RZ, Sun HX, Li GD, Liu HW, Zhou DW. Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. *Livest Sci*. (2014) 162:154– 8. doi: 10.1016/j.livsci.2013.12.021

19. Belanche A, Palma-Hidalgo JM, Nejjam I, Serrano R, Jiménez E, Martín- García AI, et al. In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. *J Sci Food Agric*. (2019) 99:163–72. doi: 10.1002/jsfa.9157

20. De Paula Vieira A, von Keyserlingk MAG, Weary DM. Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before

and after weaning from milk. *J Dairy Sci*. (2012) 95:3218–24. doi: 10.3168/jds.2011-4821

21. Ramos-Morales E, Arco-Perez A, Martin-Garcia AI, Yanez-Ruiz DR, Frutos P, Hervas G. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Anim Feed Sci Technol*. (2014) 198:57–66. doi: 10.1016/j.anifeedsci.2014.09.016

22. Weatherburn MW. Phenol-hypochlorite reaction for determination of ammonia. *Anal Chem*. (1967) 39:971–4. doi: 10.1021/ac60252a045

23. Dehority BA. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. *Appl Environ Microbiol*. (1984) 48:182– 5. doi: 10.1128/aem.48.1.182-185.1984

24. Abecia L, Toral PG, Martín-García AI, Martínez G, Tomkins NW, Molina- Alcaide E, et al. Effect of the bromocholoromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. *J Dairy Sci*. (2012) 95:2027–36. doi: 10.3168/jds.2011-4831

25. Maeda H, Fujimoto C, Haruki Y, Maeda T, Kokeguchi S, Petelin M, et al. Quantitative real-time PCR using TaqMan and SYBR green for actinobacillus actinomycetemcomitans, porphyromonas gingivalis, prevotella intermedia, TetQ gene and total bacteria. *FEMS Immunol Med Microbiol*. (2003) 39:81– 6. doi: 10.1016/S0928-8244(03)00224-4

26. Denman SE, Tomkins NW, McSweeney CS. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol Ecol*. (2007) 62:313–22. doi: 10.1111/j.1574-6941.2007.00394.x

27. Sylvester JT, Karnati SKR, Yu Z, Morrison M, Firkins JL. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J Nutr*. (2004) 134:3378–84. doi: 10.1093/jn/134.12.3378

28. Denman SE, McSweeney CS. Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiol Ecol*. (2006) 58:572–82. doi: 10.1111/j.1574-6941.2006.00190.x

29. Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. *PLoS ONE*. (2014) 9:e0105592. doi: 10.1371/journal.pone.0105592

30. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al- Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. (2019) 37:852– 7. doi: 10.1038/s41587-019-0209-9

31. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, et al. Greengenes, a chimera-checked 16S RRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*. (2006) 72:5069– 72. doi: 10.1128/AEM.03006-05

32. Seedorf H, Kittelmann S, Henderson G, Janssen PH. RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea fromthe rumen and other intestinal environments. *PeerJ*. (2014) 2:e494. doi: 10.7717/peerj.494

33. Palma-Hidalgo JM, Jiménez E, Martín-García AI, Yáñez-Ruiz DR, Belanche A. Data from: presence of adult companion goats favors the rumen microbial and functional development in artificially reared kids. *Eur Nucleotide Arch*. (2021).

34. Belanche A, Newbold CJ, Lin W, Stevens PR, Kingston-Smith AH. A systems biology approach reveals differences in the dynamics of colonization and degradation of grass vs. Hay by rumen microbes with minor effects of vitamin E supplementation. *Front Microbiol*. (2017) 8:1– 18. doi: 10.3389/fmicb.2017.01456

35. Belanche A, Yáñez-Ruiz DR, Detheridge AP, Griffith GW, Kingston-Smith AH, Newbold CJ. Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. *Environ Microbiol*. (2019) 21:4360–77. doi: 10.1111/1462-2920.14801

36. Abecia L, Jiménez E, Martínez-Fernandez G, Martín-García AI, Ramos- Morales E, Pinloche E, et al. Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. *PLoS ONE*. (2017) 12:1–19. doi: 10.1371/journal.pone.0182235

37. Newbold CJ, de la Fuente G, Belanche A, Ramos-Morales E, McEwan NR. The role of ciliate protozoa in the rumen. *Front Microbiol*. (2015) 6:1– 14. doi: 10.3389/fmicb.2015.01313

38. Belanche A, de La Fuente G, Newbold CJ. Effect of progressive inoculation of fauna-free sheep with holotrich protozoa and total-fauna on rumen fermentation, microbial diversity and methane emissions. *FEMS Microbiol Ecol*. (2015) 91:fiu026. doi: 10.1093/femsec/fiu026

39. Baldwin RL, VI, McLeod KR. Effects of diet forage:concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. *J Anim Sci*. (2000) 78:771–83. doi: 10.2527/2000.783771x

40. Bedford A, Gong J. Implications of butyrate and its derivatives for gut health and animal production. *Anim Nutr*. (2018) 4:151–9. doi: 10.1016/j.aninu.2017.08.010

41. Górka P, Kowalski ZM, Pietrzak P, Kotunia A, Kiljanczyk R, Flaga J, et al. Effect of sodium butyrate supplementation in milk replacer and starter diet on rumen development in calves. *J Physiol Pharmacol*. (2009) 60(Suppl. 3):47–53.

42. Demeyer DI, Van Nevel CJ. Effect of defaunation on the metabolism of rumen micro-organisms. *Br J Nutr*. (1979) 42:515–24. doi: 10.1079/bjn19790143

43. Eugène M, Archimède H, Sauvant D. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livestock Prod Sci*. (2004) 85:81–97. doi: 10.1016/S0301-6226(03)00117-9

44. Belanche A, Doreau M, Edwards JE, Moorby JM, Pinloche E, Newbold CJ. Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. *J Nutr*. (2012) 142:1684–92. doi: 10.3945/jn.112.159574

45. Ribeiro GO, Oss DB, He Z, Gruninger RJ, Elekwachi C, Forster RJ, et al. Repeated inoculation of cattle rumen with bison rumen contents alters the rumen microbiome and improves nitrogen digestibility in cattle. *Sci Rep*. (2017) 7:1–16. doi: 10.1038/s41598-017-01269-3

46. Belanche A, de la Fuente G, MoorbyJM, Newbold CJ. Bacterial protein degradation by different rumen protozoal groups. *J Anim Sci*. (2012) 90:4495– 504. doi: 10.2527/jas.2012-5118

47. Baldwin RL, VI, McLeod KR, Klotz JL, Heitmann RN. Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. *J Dairy Sci*. (2004) 87(Suppl. 13):E55–65. doi: 10.3168/jds.S0022-0302(04)70061-2

48. Becker ER, Hsiung TS. The method by which ruminants acquire their fauna of infusoria and remarks concerning experiments on host specificity of these protozoa. *Proc Natl Acad Sci USA*. (1929) 15:684– 90. doi: 10.1073/pnas.15.8.684

49. Fonty G, Senaud J, Jouany JP, Gouet P. Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. *Can J Microbiol*. (1988) 34:235– 41. doi: 10.1139/m88-044

50. McGranaghan P, Davies JC, Griffith GW, Davies DR, Theodorou MK. The survival of anaerobic fungi in cattle faeces. *FEMS Microbiol Ecol*. (1999) 29:293–300. doi: 10.1016/S0168-6496(99)00024-0

51. Williams AG, Coleman GS. *The Rumen Protozoa*. New York, NY: Springer- V (1992).

52. Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. *ISME J*. (2013) 7:1069– 79. doi: 10.1038/ismej.2013.2

53. Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O, Monteils

V. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *J Appl Microbiol*. (2014) 116:245–57. doi: 10.1111/jam.12405

54. Ransom-Jones E, Jones DL, McCarthy AJ, McDonald JE. The fibrobacteres: an important phylum of cellulose-degrading bacteria. *Microb Ecol*. (2012) 63:267–81. doi: 10.1007/s00248-011-9998-1

55. Skillman LC, Evans PN, Naylor GE, Morvan B, Jarvis GN, Joblin KN. 16S Ribosomal DNA-directed PCR primers for ruminal methanogens and identification of methanogens colonising young lambs. *Anaerobe*. (2004) 10:277–85. doi: 10.1016/j.anaerobe.2004.05.003

56. Guzman CE, Bereza-Malcolm LT, De Groef B, Franks AE. Presence of selected methanogens, fibrolytic bacteria, and proteobacteria in the gastrointestinal tract of neonatal dairy calves from birth to 72 hours. *PLoS ONE*. (2015) 10:e0133048. doi: 10.1371/journal.pone.0133048

57. De Barbieri I, Hegarty RS, Silveira C, Gulino LM, Oddy VH, Gilbert RA, et al. Programming rumen bacterial communities in newborn merino lambs. *Small Ruminant Res*. (2015) 129:48–59. doi: 10.1016/j.smallrumres.2015.05.015

58. McGovern E, Waters SM, Blackshields G, McCabe MS. Evaluating established methods for rumen 16S rRNA amplicon sequencing with mock microbial populations. *Front Microbiol*. (2018) 9:1365. doi: 10.3389/fmicb.2018. 01365

59. Fernando SC, Purvis HT, Najar FZ, Sukharnikov LO, Krehbiel CR, Nagaraja TG, et al. Rumen microbial population dynamics during adaptation to a high-grain diet. *Appl Environ Microbiol*. (2010) 76:7482– 90. doi: 10.1128/AEM.00388-10

60. Zhang K, Li B, Guo M, Liu G, Yang Y, Wang X, et al. Maturation of the goat rumen microbiota involves three stages of microbial colonization. *Animals*. (2019) 9:1–17. doi: 10.3390/ani9121028

61. Li F, Guan LL. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl Environ Microbiol*. (2017) 83:1–16. doi: 10.1128/AEM.00061-17

62. Do TH, Dao TK, Nguyen KHV, Le NG, Nguyen TMP, Le TL, et al. Metagenomic analysis of bacterial community structure and diversity of lignocellulolytic bacteria in vietnamese native goat rumen. *Asian Aust J Anim Sci*. (2018) 31:738–47. doi: 10.5713/ajas.17.0174

63. Sawanon S, Koike S, Kobayashi Y. Evidence for the possible involvement of selenomonas ruminantium in rumen fiber digestion. *FEMS Microbiol Lett*. (2011) 325:170–9. doi: 10.1111/j.1574-6968.2011.02427.x

64. Newbold CJ, Lassalas B, Jouany JP. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. *Lett Appl Microbiol*. (1995) 21:230–4. doi: 10.1111/j.1472-765X.1995.tb0 1048.x

65. Belanche A, de la Fuente G, Newbold CJ. Study of methanogen communities associated with different rumen protozoal populations. *FEMS Microbiol Ecol*. (2014) 90:663–77. doi: 10.1111/1574-6941.12423

66. Levy B, Jami E. Exploring the prokaryotic community associated with the rumen ciliate protozoa population. *Front Microbiol*. (2018) 9:e02526. doi: 10.3389/fmicb.2018.02526

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Tables and Figures

Table 1. Rumen fermentation and blood parameters of goat kids reared in the absence (CTL) or presence of adult companions (CMP) at 5, 7, and 9 weeks of age (n =16).

Time	Week 5		Week 7		Week 9			*p*-value		
Treatments	CTL	CMP	CTL	CMP	CTL	CMP	SEM	Treat.	Time	T×T
BW, kg	6.53	6.66	8.33	8.39	9.49	9.15	0.208	0.980	<0.001	0.124
Rumen pH	6.53	6.65	6.42	6.55	7.00	7.08	0.036	0.046	<0.001	0.775
NH_3-N, mg/dL	17.1	18.1	14.8	13.7	2.74	7.93	0.883	0.279	<0.001	0.158
VFA, mM	25.0	23.9	31.8	32.4	30.6	36.4	1.160	0.452	0.002	0.193
Acetate, %	74.7	72.5	71.4	69.0	64.0	63.4	0.723	0.158	<0.001	0.576
Propionate, %	13.5	13.7	16.1	16.6	19.0	17.1	0.374	0.515	<0.001	0.229
Butyrate, %	4.83^{d}	6.06^{cd}	7.04^{c}	8.68^{b}	6.28^{c}	11.5^{a}	0.298	<0.001	<0.001	<0.001
Valerate, %	2.29	2.22	2.09	2.02	2.04	2.02	0.051	0.955	0.039	0.534
Isobutyrate, %	2.04	2.69	1.50	1.59	2.29	2.38	0.101	0.187	0.920	0.263
Isovalerate, %	2.61^{bc}	2.91^{ab}	1.92^{d}	2.12^{cd}	2.21^{cd}	3.40^{a}	0.103	0.009	0.831	0.049
BCVFA[1], % %	4.64	5.59	3.36	3.68	4.50	5.78	0.187	0.033	0.957	0.685
Blood metabolites										
BHB, mM	0.97	1.49	1.34	2.40	2.69	3.18	0.198	0.048	0.015	0.935
Glucose, mg/dL	105	94.0	101	78.1	73.8	82.6	3.020	0.052	0.008	0.082
Ratio BHB/Glucose	0.18	0.38	0.28	0.72	0.72	0.70	0.069	0.088	0.013	0.354
Urea, mg/dL	16.1	21.1	24.0	17.9	35.0	40.3	1.660	0.479	0.001	0.705
Total proteins, g/L	53.1	53.4	61.0	59.4	57.1	59.6	0.746	0.554	0.052	0.297

[a-d]Means within a row with different superscripts differ ($p < 0.05$).
[1]Branched-chain VFA= iso-valerate + iso-butyrate.

Table 2. Abundance of the major rumen microbial groups in goat kids reared in the absence (CTL) or presence of adult companions (CMP) at 5, 7, and 9 weeks of age (n = 16).

Time	Week 5		Week 7		Week 9			*p*-value		
Treatments	CTL	CMP	CTL	CMP	CTL	CMP	SEM	Treat.	Time	TxT
Microbes, log10 copy/g DM										
Bacteria	9.14	9.34	9.26	9.31	9.27	9.33	0.036	0.149	0.28	0.251
Methanogens	6.18^{c}	6.77^{a}	6.35^{bc}	6.65^{ab}	6.22^{c}	6.37^{bc}	0.051	0.020	0.467	0.039
Protozoa	<4.0^{c}	6.87^{b}	<4.0^{c}	7.25^{ab}	<4.0^{c}	7.62^{a}	0.168	<0.001	<0.001	0.077
Anaerobic fungi	4.96	4.77	5.16	5.10	4.42	4.08	0.088	0.955	0.279	0.180
Protozoal counts, log10 cells/mL	ND	4.96	ND	5.12	ND	5.21	0.260		0.125	
Subf. *Entodiniinae*, %	ND	95.3	ND	94.2	ND	89.8	4.740		0.598	
Subf. *Diplodiniinae*, %	ND	4.37	ND	4.65	ND	6.28	0.403		0.315	
Ophryoscolex spp., %	ND	0.30^{b}	ND	0.93^{b}	ND	3.20^{a}	0.161		<0.001	
Isotricha spp., %	ND	0^{b}	ND	0.19^{ab}	ND	0.54^{a}	0.051		0.056	
Dasytricha spp., %	ND	0^{b}	ND	0.05^{ab}	ND	0.18^{a}	0.017		0.062	

$^{a-c}$Means within a row with different superscripts differ ($p < 0.05$). ND, not detected.

Table 3. Abundance of rumen bacterial taxa in the rumen of goat kids reared in absence (CTL) or presence of adult companions (CMP) at 7 weeks of age (n = 8).

	Treatments				
	CTL	CMP	Adults[a]	SEM	*p*-value[b]
p_Actinobacteria, f_Coriobacteriaceae	0.12	0.04	0.82	0.025	0.079
p_Bacteroidetes	64.2	71.5	62.6	1.960	0.093
f_Paraprevotellaceae	3.23	5.77	2.40	0.704	0.059
f_Bacteroidaceae, g_BF311	0.18	0.62	1.57	0.108	0.027
p_Cyanobacteria	0.02	0.08	0.05	0.164	0.021
p_Firmicutes	16.6	13.7	21.4	0.837	0.059
f_Ruminococcaceae	7.31	5.09	5.71	0.705	0.093
f_Veillonellaceae	0.68	1.40	1.21	0.184	0.027
g_Selenomonas	0.14	0.33	0.15	0.068	0.035
g_Succinispira	0.01	0.69	0.85	0.142	0.001
f_Erysipelotrichaceae	1.03	1.47	0.75	0.269	0.046
g_Bulleidia	0.00	0.12	0.05	0.036	0.004
p_Proteobacteria	4.24	2.23	0.86	1.040	0.338
f_Rhodocyclaceae	0.21	0.05	0.02	0.048	0.059
g_Georgfuchsia	0.21	0.05	0.00	0.048	0.046
f_Desulfovibrionaceae	0.44	0.24	0.44	0.057	0.036
p_Spirochaetes	6.57	2.09	0.70	1.220	0.021
f_Spirochaetaceae	5.05	1.25	0.41	1.020	0.021
g_Spirochaeta	0.02	0.55	0.13	0.012	0.062
g_Treponema	2.04	0.58	0.15	0.389	0.036
p_SR1	0.00	0.11	0.26	0.038	0.001
p_Synergistetes	0.07	0.19	0.18	0.034	0.059
g_Dethiosulfovibrio	0.01	1.20	0.72	0.035	0.004
p_Tenericutes	0.04	0.10	0.11	0.022	0.088
p_TM7, f_F16	0.08	0.23	0.04	0.037	0.021
p_Verrucomicrobia	1.28	3.35	0.76	0.620	0.074
f_RFP12	0.59	2.95	0.60	0.577	0.016

[a]Description of the rumen bacterial community in the adult companions.

[b]*p*-values for the differences between CTL and CMP kids. Only taxa with an average abundance > 0.05% are shown. p, phylum; f, family; g, genus.

Table 4. Abundance of rumen methanogens in the rumen of goat kids reared in the absence (CTL) or presence of adult companions (CMP) at 7 weeks of age (n = 8).

	Treatments				
	CTL	CMP	Adults[a]	SEM	*p*-value[b]
Methanobrevibacter gottschalkii	12.2	9.60	44.9	1.91	0.442
Group12_ISO4_H5	48.2	0.76	0.00	7.370	<0.001
Group8_WGK1	2.48	0.00	2.04	0.703	0.234
Group9_ISO4_G1	11.4	37.1	13.3	4.650	0.002
Group10_sp	1.61	1.51	6.12	0.489	0.878
Group9_sp_CH1270	0.89	18.4	1.53	3.080	0.002
Methanomicrobium mobile	5.48	26.5	0.00	5.040	0.007
Group11_sp_ISO4_G11	9.26	3.72	14.3	3.180	0.878
Unidentified	8.54	2.21	6.12	1.350	0.007

[a]Description of the rumen bacterial community in the adult companions.
[b]*p*-values for the differences between CTL and CMP kids.

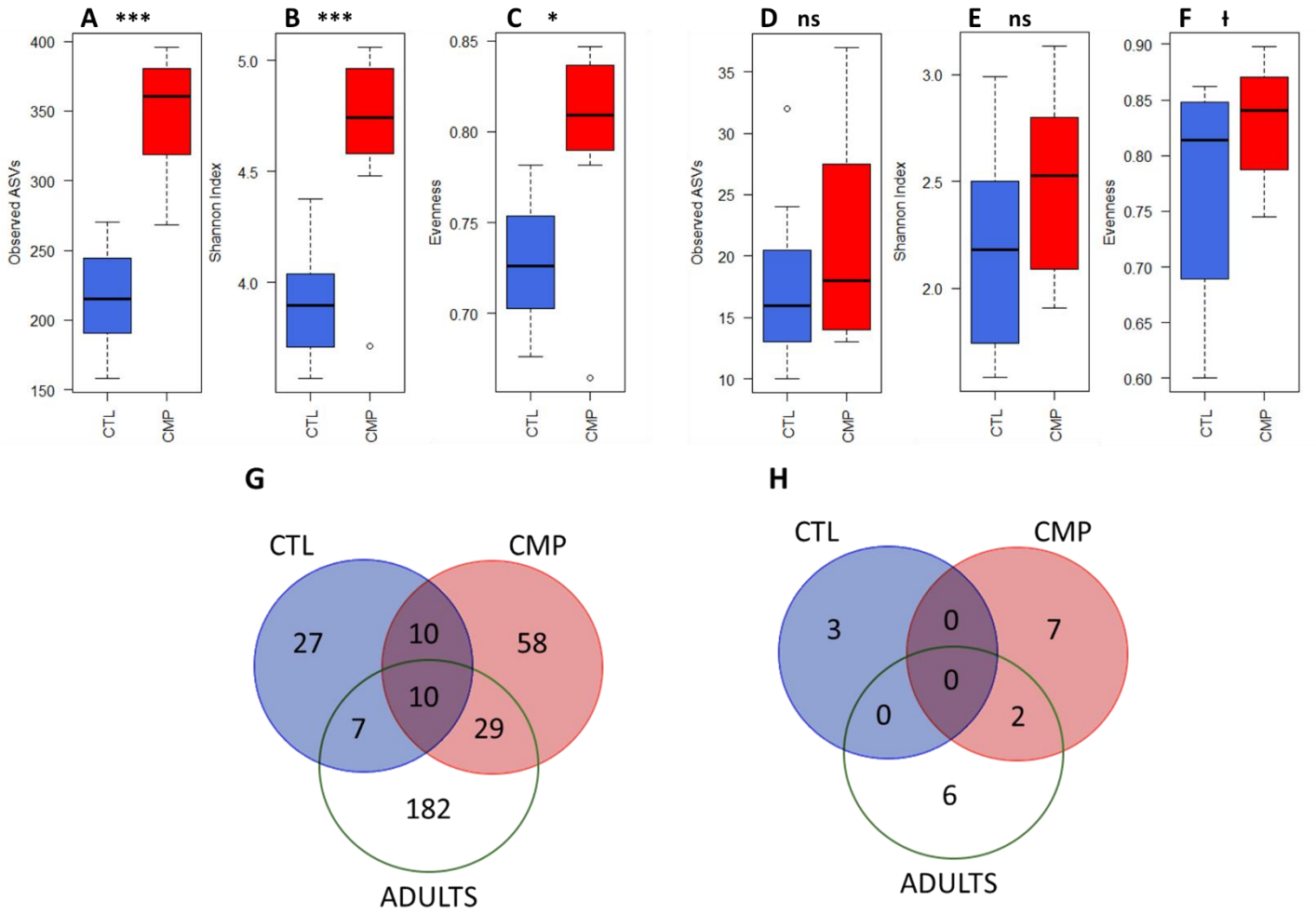

Figure 1. Boxplots indicating the rumen bacterial diversity in terms of observed ASVs (A), Shannon index (B), and evenness (C) and the rumen methanogen diversity (D–F; respectively) in goat kids (n = 8) reared in absence (CTL) or presence of adult companions (CMP) at 7 weeks of age. Venn diagrams of the core rumen bacterial (G) and methanogen (H) communities (present in >75% of animals in each group) at ASV level in CTL and CMP goat kids at 7 weeks of age, along with the two adult companions (Adults).

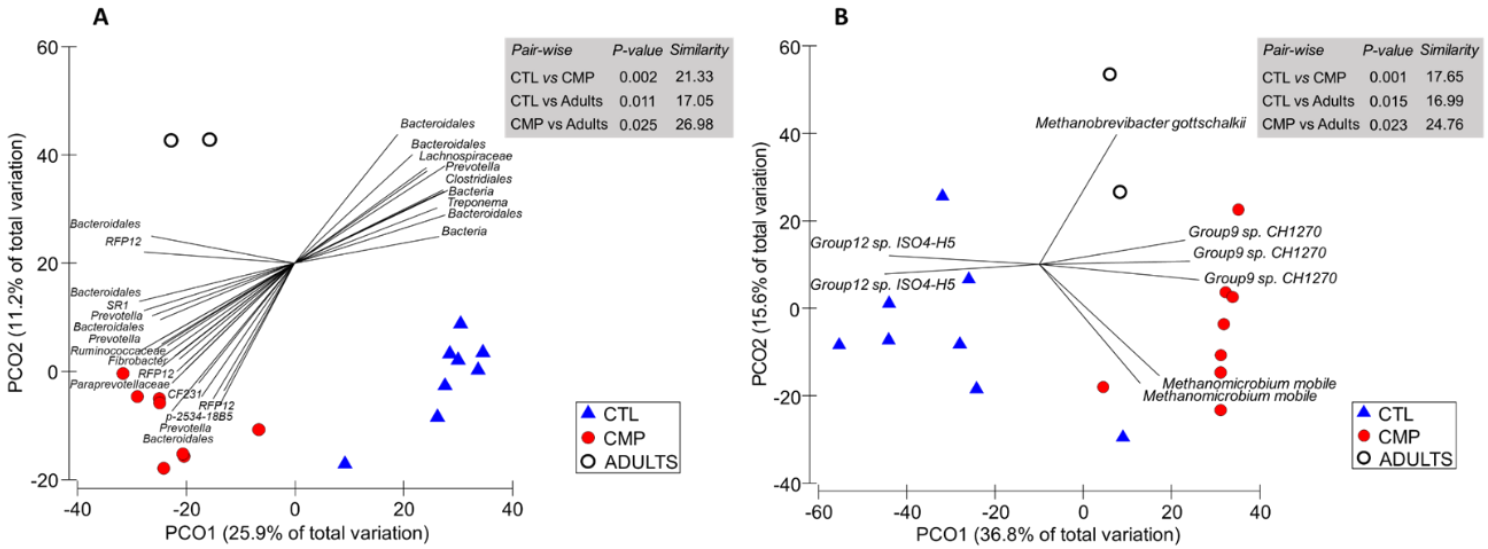

Figure 2. Principal coordinate analysis of the rumen bacterial (A) and methanogen (B) communities in goat kids (n = 8) reared in the absence (CTL) or presence of adult companions (CMP) at 7 weeks of age. Tripod vectors are included to describe the direction and intensity of the most discriminant ASVs (based on Spearman's correlation > 0.8). Samples from adult companions are also shown (Adults). Pair-wise PERMANOVA values are provided in gray boxes based on the Bray–Curtis dissimilarity.

Supplementary Material

Supplementary Table S1. Chemical composition (in % DM) of the feeds.

	Milk replacer[1]	Starter concentrate[2]	Oats hay
Organic matter	92.8	93.1	90.2
Crude protein	24.0	12.9	8.1
Ether extract	22.0	3.2	2.2
Neutral-detergent fiber		22.9	50.9
Acid-detergent fiber		9.12	32.6

[1] Declared composition

[2] Ingredient list: wheat bran, barley, corn, wheat, sunflower seed flour, soybean flour, $CaCO_3$, NaCl, vitamin mineral premix and $NaCO_3$.

Supplementary Table S2. Description of the of the rumen protozoal community in the two adult companions (*n*=8).

	Adults[1]
Protozoal counts, log10 cells/mL	5.81
Subf. *Entodiniinae*, %	75.2
Subf. *Diplodiniinae*, %	5.06
Ophryoscolex spp., %	10.6
Isotricha spp., %	2.33
Dasytricha spp., %	6.80

[1]Average between the two adult companions.

Supplementary Table S3. Diversity indexes and abundances of rumen bacterial taxa in the rumen of goat kids reared in absence (CTL) or presence of adult companions (CMP) at 7 weeks of age (n =8).

	Treatments				
	CTL	CMP	Adults[1]	SEM	*P*-value[2]
Richness	216	348	446	20.3	<0.001
Shannon	3.910	4.670	5.290	0.135	<0.001
Evenness	0.728	0.798	0.871	0.015	0.016
Simpson	0.949	0.973	0.999	0.006	0.051
Good's coverage	0.991	0.994	0.999	0.002	0.502
Abundance (%)					
p_Actinobacteria, f_Coriobacteriaceae, g_Olsenella	0.124	0.036	0.823	0.025	0.079
p_Bacteroidetes	64.20	71.50	62.60	1.960	0.093
f_Barnesiellaceae, g_Barnesiella	3.960	0.579	0.639	1.040	0.115
f_Paraprevotellaceae	3.230	5.770	2.400	0.704	0.059
g_CF231	0.346	0.621	1.290	0.090	0.195
g_YRC22	0.136	0.029	0.064	0.058	0.643
f_Bacteroidaceae, g_BF311	0.179	0.615	1.57	0.108	0.027
f_Porphyromonadaceae	0.117	0.016	0.113	0.031	0.130
f_Prevotellaceae, g_Prevotella	35.30	38.70	19.90	2.370	0.753
f_RF16	0.034	0.282	0.049	0.107	0.125
f_Sphingobacteriaceae, g_Pedobacter	0.349	0.154	0.049	0.119	0.746
p_Chloroflexi	0.010	0.021	0.042	0.120	0.334
p_Cyanobacteria	0.015	0.081	0.054	0.164	0.021
p_Elusimicrobia	0.162	0.393	0.108	0.078	0.290
f_Elusimicrobiaceae	0.154	0.205	0.049	0.054	0.742
p_Fibrobacteres, g_Fibrobacter	2.180	2.230	8.590	0.451	0.916
p_Firmicutes	16.60	13.70	21.40	0.837	0.059
f_Clostridiaceae	0.167	0.121	0.196	0.037	0.916
g_Clostridium	0.383	0.245	0.771	0.052	0.248
f_Lachnospiraceae	3.240	1.640	4.340	0.537	0.345
g_Lactonifactor	1.160	0.232	0.277	0.403	0.318
f_Ruminococcaceae	7.310	5.090	5.710	0.705	0.093
g_Ruminococcus	0.390	0.432	0.766	0.073	0.753
g_Papillibacter	0.299	0.057	0.044	0.0667	0.125
g_Sporobacter	0.301	0.086	0.213	0.075	0.267
f_Veillonellaceae	0.677	1.398	1.210	0.184	0.027
g_Selenomonas	0.139	0.325	0.147	0.068	0.035
g_Succinispira	0.009	0.685	0.850	0.142	0.001
f_Erysipelotrichaceae	1.030	1.470	0.747	0.269	0.046
g_Bulleidia	0	0.121	0.054	0.036	0.004
p_Lentisphaerae	0.207	0.242	0.306	0.044	0.834
f_Victivallaceae	0.204	0.242	0.267	0.044	0.834
g_Victivallis	0.063	0.065	0.054	0.016	0.832
p_Proteobacteria	4.240	2.230	0.857	1.040	0.338
f_Neisseriaceae	0.052	0.045	0	0.009	0.431
f_Rhodocyclaceae	0.214	0.051	0.024	0.048	0.059
g_Georgfuchsia	0.209	0.046	0	0.048	0.046
f_Desulfovibrionaceae	0.439	0.237	0.443	0.057	0.036
f_Succinivibrionaceae	0.581	0.480	0.323	0.196	0.529
g_Succinivibrio	0.277	0.040	0.147	0.108	0.324
p_Spirochaetes	6.570	2.090	0.695	1.220	0.021
f_Sphaerochaetaceae, g_Sphaerochaeta	0.026	0.059	0	0.019	0.666
f_Spirochaetaceae	5.050	1.250	0.409	1.020	0.021

g_Spirochaeta	0.020	0.551	0.127	0.012	0.062
g_Treponema	2.040	0.582	0.152	0.389	0.036
p_SR1	0	0.114	0.260	0.038	0.001
p_Synergistetes	0.072	0.187	0.176	0.034	0.059
f_Dethiosulfovibrionaceae	0.067	0.181	0.166	0.035	0.093
g_Dethiosulfovibrio	0.007	1.200	0.716	0.035	0.004
p_Tenericutes	0.042	0.097	0.105	0.022	0.088
p_TM7, f_F16	0.080	0.231	0.037	0.037	0.021
p_Verrucomicrobia	1.280	3.350	0.764	0.620	0.074
f_R4_41B	0.683	0.395	0.071	0.145	0.207
f_RFP12	0.585	2.95	0.597	0.577	0.016

[1]Description of the rumen bacterial community in the two adult companions.

[2]*P*-values for the differences between CTL and CMP kids. Only taxa with an average abundance > 0.05% are shown. *p*=phylum; *f*=family; *g*=genus

Supplementary Table S4. Diversity indexes of the methanogen community in the rumen of goat kids reared in absence (CTL) or presence of adult companions (CMP) at 7 weeks of age (n=8).

	Treatments				
	CTL	CMP	Adults[1]	SEM	*P*-value[2]
Richness	17.6	21.1	18.5	2.080	0.439
Shannon	2.18	2.48	2.38	0.124	0.190
Evenness	0.77	0.83	0.82	0.021	0.064
Simpson	0.80	0.87	0.87	0.023	0.103
Good's coverage	0.741	0.642	24.4	0.0292	0.237

[1]Description of the rumen bacterial community in the adult companions.
[2]*P*-values for the differences between CTL and CMP kids. Only taxa with an average.

Supplementary Table S5. Correlations between the rumen microbiota and rumen fermentation and physiological parameters (only Spearman correlations with $\rho > 0.4$ and $P < 0.01$ are shown).

Correlations	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	VFA	Bacteria	Methanogens	Protozoa	Anaerobic fungi	BW	ADG-pre[1]	ADG-post[2]	BHB	Glucose	Urea	Blood proteins	BHB/Glucose	Bacterial ASV	Methanogens ASV
Bacterial abundance				0,45	-0,40		0,45			0,51	0,67				0,85	0,57		0,43	0,65		0,85
Bacterial ASV			-0,40			-0,45	0,41									-0,49	0,55		0,42		
p__Actinobacteria									-0,40												
p__Cyanobacteria																	0,45			0,70	0,60
p__Elusimicrobia																					0,76
p__Fibrobacteres												0,47	0,47		0,40						0,94
p__Firmicutes											0,43							-0,41			0,80
p__Proteobacteria																					0,60
p__Spirochaetes													-0,44		-0,40	0,50	-0,67		-0,50	-0,52	
p__SR1						-0,40									0,53	-0,59	0,62		0,61	0,77	
p__Synergistetes			-0,41																	0,59	0,56
p__Tenericutes																				0,48	
p__TM7		0,43	-0,44													-0,47			0,52	0,61	0,58
p__Verrucomicrobia																	0,42			0,59	
f__Coriobacteriaceae									-0,40												
f__[Barnesiellaceae]																	-0,42				
f__[Paraprevotellaceae]																				0,64	
f__Bacteroidaceae																				0,73	
f__Porphyromonadaceae																				-0,52	
f__RF16																				0,41	
f__Ruminococcaceae																	-0,58				0,63
f__Veillonellaceae																					
f__Erysipelotrichaceae																	0,50			0,48	0,76
f__Rhodocyclaceae															-0,43	0,43			-0,50		
f__Desulfovibrionaceae																				-0,53	
f__Succinivibrionaceae																	0,40				0,58
f__Spirochaetaceae													-0,41		-0,43	0,56	-0,67		-0,53	-0,52	
f__Dethiosulfovibrionaceae																				0,48	0,51
f__F16		0,43	-0,44												0,53	-0,47			0,52	0,61	0,58
f__R4-41B		-0,41																			
f__RFP12																				0,73	
f__WCHB1-25																					0,98
Methanogens abundance	-0,56	0,52		0,53			0,40	0,71			0,85	0,93			0,85	0,48	0,69	0,69	0,63		0,95
g__Methanobrevibacter															-0,74	0,74	-0,45	0,40	-0,79		
g__Group12			0,44			0,46	-0,45										-0,58			-0,85	
g__Group9																	0,67			0,74	0,46
g__Methanomicrobium														-0,52	0,40		0,50			0,61	
Protozoal abundance	-0,60			0,55					0,47		0,72	0,57					0,78	0,99		0,90	
Protozoa (optical count)	-0,55			0,43				0,94			0,63	0,64					0,57	0,81			0,76
Entodinium	-0,44							0,50	0,47		0,87	0,54			0,45		0,75	0,95		0,65	0,96
Diplodiniinae	-0,40							0,83			0,54	0,68				0,46	0,91	0,99		0,87	1,00
Ophryoscolex											0,84	0,65						0,51			
Isotricha								0,68			0,62				0,42	0,59	0,71		0,40	0,61	
Dasytricha										0,51		0,87			0,60						0,59
Anaerobic fungi abundance	0,43											0,40			0,90	0,77			0,85	0,81	

[1]ADG-pre: Average daily gain from 5 to 7 weeks of age. [2]ADG-post: Average daily gain from 7 to 9 weeks of age

Publication 5

Short communication: Saliva and salivary components affect goat rumen fermentation in short-term batch incubations

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Animal 15(7): 100267

DOI: https://doi.org/10.1016/j.animal.2021.100267

ABSTRACT

The research about the role of saliva in ruminants has been mainly focused on its buffering capacity together with facilitation of the rumination process. However, the role of salivary bioactive components on modulating the activity of the rumen microbiota has been neglected until recently. This study developed an in vitro approach to assess the impact of different components in saliva on rumen microbial fermentation. Four different salivary fractions were prepared from four goats: (i) non-filtrated saliva (NFS), (ii) filtrated through 0.25 mm to remove microorganisms and large particles (FS1), (iii) centrifuged through a 30 kDa filter to remove large proteins, (FS2), and (iv) autoclaved saliva (AS) to keep only the minerals. Two experiments were conducted in 24 h batch culture incubations with 6 ml of total volume consisting of 2 ml of rumen fluid and 4 ml of saliva/buffer mix. In Experiment 1, the effect of increasing the proportion of saliva (either NFS or FS1) in the solution (0%, 16%, 33% and 50% of the total volume) was evaluated. Treatment FS1 promoted greater total volatile fatty acids (VFA) (+8.4%) and butyrate molar proportion (+2.8%) but lower NH_3-N concentrations than NFS fraction. Replacing the bicarbonate buffer solution by increasing proportions of saliva resulted in higher NH_3-N, total VFA (+8.0%) and propionate molar proportion (+11%). Experiment 2 addressed the effect of the different fractions of saliva (NFS, FS1, FS2 and AS). Saliva fractions led to higher total VFA and NH_3-N concentrations than non-saliva incubations, which suggests that the presence of some salivary elements enhanced rumen microbial activity. Fraction FS1 promoted a higher concentration of total VFA (+7.8%) than the other three fractions, and higher propionate (+26%) than NFS and AS. This agrees with findings from Experiment 1 and supports that 'microbe-free saliva', in which large salivary proteins are maintained, boosts rumen fermentation. Our results show the usefulness of this in vitro approach and suggest that different salivary components can modulate rumen microbial fermentation, although the specific metabolites and effects they cause need further research.

Keywords: Immunoglobulin, *In vitro* fermentation, Microbial immune modulation, Rumen microbiome, Salivary proteins

IMPLICATIONS

An effective modulation of the rumen microbiome requires comprehensive understanding of the factors that drive its composition and activity. The vast amount of saliva that enters the rumen together with the presence of a range of metabolites suggest that some salivary components may play a relevant role in shaping rumen microbial activity. These initial *in vitro* tests have shown that using saliva (instead of buffer) enhances microbial fermentation and that the effect relies on the presence of large salivary proteins. This represents an important step toward identifying the specific salivary components and mechanisms involved in rumen.

INTRODUCTION

Saliva, together with drinking water, represents the main liquid input into the rumen and it has been reported that total daily saliva secretion may reach from 117 to 183 kg in cattle (Meyer et al., 1964) and from 1.2 to 10.2 L in sheep (Somers, 1957; Tomas, 1973; Piccione et al., 2006). In all domestic animals the main function of saliva is to assist mastication and deglutition. In ruminants, however, given the lack of secretions through the rumen wall, saliva also has other important roles, including facilitating the deglutition and regurgitation of the feed during the rumination process, allowing nitrogen (as urea) to be recycled into the rumen and providing a buffered medium in which the rumen microbial activity can take place. The composition and volume of saliva that goes into the gastrointestinal tract depends on a number of variables such as the type of diet, water intake, the physiological stage of the animal, the frequency of mastication and the environmental temperature (Humphrey & Williamson, 2001). An essential component of ruminant saliva are the ions (mainly bicarbonates and phosphates), whose variable secretion rates are responsible for the buffering effect that helps balance rumen pH (Nørgaard, 1993), and counters volatile fatty acids (VFA) accumulation. Early studies (Mcdougall, 1948; Somers, 1957) focused on the saliva mineral composition and the factors affecting the rate of secretion. Despite the potential effect that such a great amount of fluid could have on rumen physiology, in addition to the numerous studies on the mineral composition of saliva, research on the bioactive components is relatively scarce. More recent studies have shown that the function of saliva may go beyond lubrication and pH buffering, as the composition might change in response to the presence of dietary components (i.e., tannins) in the diet (Salem et al., 2013) and suggested potential

interaction between some salivary components, specially proteins, and rumen fluid inoculum (i.e., microbiota) in in vitro incubations in sheep and goats (Salem et al., 2013). The protein components of saliva have been suggested to play a major role in the rumen activity, both buffering pH (i.e. albumin) and regulating the microbiota (i.e. lysozyme, cytokines, immunoglobulins, Fouhse et al., 2017). Salivary immunoglobulins, especially secretory immunoglobulin A (IgA), are of particular importance due to their abundance and ability to modify symbiotic microbiota proliferation (Donaldson et al., 2018) and activity in the rumen (Fouhse et al., 2017), which might help explain the individual host specificity associated with the rumen microbiome in individual animals (Weimer, 2015). Nonetheless, the extent to which the saliva exerts such modulatory control over the rumen microbiota still remains unknown. This study was designed to develop an in vitro approach to assess the impact of different components in saliva on rumen microbial fermentation. Preliminary results of this study have already been published in an abstract form (Palma-Hidalgo et al., 2018).

MATERIAL AND METHODS

Animal procedures were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013) and protocols were approved by the Ethical Committee for Animal Research at EEZ-CSIC. Four non-lactating 3 years-old Murciano-Granadina goats were used as saliva donors. Saliva was sampled prior to providing the diet (80% oat hay, 20% commercial concentrate) for five consecutive days by swabbing the mouth with absorbent sponges (approximately 7 x 7 cm) inserted in the mouth of the animals for 5 min. Declared composition (g/kg) of the commercial concentrate (Granulado Cabras Lactación, Macob, Granada, Spain) was 220 CP, 115 crude fiber, 85 ash, 60 fat, 5 lysine, 5 phosphorus, 3 sodium and 2 methionine; and the main ingredients were wheat bran, distillates from corn fermentation, sunflower cake and wheat. Saliva was extracted from the sponges by centrifugation at 190g for 10 min. Saliva from each animal was separated into four fractions: (i) Non-filtrated saliva (NFS) that was not processed further, (ii) Filtrated saliva 1, (FS1) that was centrifuged at 16,300g for 5 min and filtrated through 0.25 mm pore size to remove microorganisms and large particles, (iii) Filtrated saliva 2, (FS2) that was initially processed as fraction FS1 and then centrifuged through a 30 kDa filter (Amicon@Ultra-15 Centrifugal Filter Devices) at 2

000g for 20 min to remove large proteins, including immunoglobulins, and (iv) Autoclaved saliva (AS) that consisted of NFS which was autoclaved for 30 min at 121 °C to denature active metabolites in the saliva but keeping the minerals. Each of the four fractions of saliva samples were pooled separately for each animal and stored at 80 °C until further use. Total protein concentration was determined in each saliva fraction by spectrophotometry (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA), and polyacrilamide gel electrophoresis at 4–12% gradient was performed (Invitrogen NuPAGETM Bis-Tris Mini Protein Gels, Thermo Fisher Scientific, Waltham, MA) to assess the effectiveness of the filtering and autoclaving processes on salivary proteins (Fig. 1). Twenty mg of each fraction was loaded in wells and the electrophoresis was run for 40 min at 180 V. Bands were stained using the Colloidal blue staining kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and observed with a Gel Doc XR + System (BioRad Laboratories Inc., Hercules, CA, USA).

Two in vitro experiments were conducted to assess: (i) the effect of replacement of bicarbonate buffer solution with saliva (Experiment 1) and (ii) the impact of different saliva fractions (Experiment 2) on the rumen microbial fermentation. Both experiments used 24 h in vitro batch culture incubations in anaerobic conditions at 39 °C in Hungate tubes with 6 ml of total volume consisting of 2 ml of rumen fluid and 4 ml of a saliva/buffer mix.

In Experiment 1, the tubes contained 2 ml fresh rumen fluid obtained from a single goat (rumen cannulated) before the morning feeding and adapted during two weeks to a diet consisting of 50:50 commercial concentrate:oat hay. Rumen fluid was then filtrated through double layer of cheese cloth, and mixed with 4 ml of saliva and/or bicarbonate buffer (3.5 g NaHCO3 + 0.4 g (NH4) HCO3 in 100 ml dH2O). A total of 32 incubation tubes were used following a 2 4 factorial design including the incubation of two saliva fractions (NFS and FS1) and four increasing doses of saliva (0%, 16%, 33% and 50% of the total volume) as a replacement of bicarbonate buffer solution. These saliva samples were obtained from four donors (n = 4) which were considered as experimental units. Incubation substrate consisted of 75 mg of the aforementioned commercial concentrate and 75 mg oat hay.

In Experiment 2, 54 incubation tubes were used to analyze the effect of four saliva fractions (NFS, AS, FS1 and FS2). These saliva fractions obtained from four saliva

donors were incubated with rumen fluid obtained from three goats, different from saliva donors. Saliva fractions represented 66% of the total incubation volume and six control tubes (two for each rumen fluid) were used with the aforementioned bicarbonate buffer solution instead of saliva as negative control. In this incubation the amount of substrate was 30 mg commercial concentrate and 30 mg oat hay.

In both experiments, gas pressure in the headspace of tubes was measured at 2, 4, 7, 10 and 24 h using a Wide Range Pressure Meter (SperScientific LTD, Scottsdale, AZ, USA), which then was transformed into volume units by the ideal gas law. Incubation pH was measured at the beginning and end of incubations. At 24 h, incubation was stopped by opening the bottles, then samples were taken to determine the concentration of NH_3-N by spectrophotometry (Victor X microplate reader, Perkin Elmer, Waltham, MA) and volatile fatty acids by gas chromatography (AutoSystem gas chromatograph, Perkin Elmer, Waltham, MA). In Experiment 1, results were statistically analyzed as a 2 x 4 factorial ANOVA: the effect of the saliva fraction (NFS vs FS1), the saliva proportion (0% vs 16% vs 33% vs 50%) and their interaction were considered as fixed effects, whereas the saliva donor (animal 1–4) was considered as a random effect. In Experiment 2, a one-way ANOVA was used with the saliva fraction (NFS vs FS1 vs FS2 vs AS) as the only fixed effect whereas the saliva donor (animals 1–4) and the rumen liquid donor (animals 5–7) were considered as random blocking factors. When significant effects were detected, means were compared by Fisher's protected LSD-test using the SPSS software (IBM Corp., Version 21.0, New York, USA). Effects were considered significant at $P < 0.05$ and tendency to difference at $P < 0.1$.

RESULTS AND DISCUSSION

The determination of the protein concentration using a commercial kit resulted in significant differences between the FS2 saliva (mean = 212 mg/ml) and the NFS, FS1 and AS saliva samples ($P < 0.001$). No differences were found between these three saliva samples (908, 882, 992 mg/ml; respectively). The protein concentration did not decrease in the AS saliva because the kit's first chemical reaction can be prompted with peptides comprising as few as three amino acid residues. Polyacrilamide gradient gel electrophoresis (Fig. 1) illustrated the effect of the two filtration treatments (FS1 and FS2) and autoclaving on the protein bands visualized in each saliva fraction. Saliva obtained

from NFS and FS1 filtrations presented similar band patterns, given that only microbial and epithelial cells and large size feed particles were removed from FS1. In both cases, bands corresponded to proteins both larger and smaller than 30 kDa. It was assumed that these bands included transporting proteins, especially serum albumin, which comprises over 50% of the total salivary proteins and whose precursor have been previously identified within the 70 kDa and 28 kDa regions (Lamy et al., 2009). Some of those bands may also correspond to large proteins involved in immune response such as IgA and immunoglobulin G (Dietzen, 2018; Janeway et al., 2001). Indeed, Immunoglobulin heavy chain C region and Immunoglobulin gamma 2 heavy chain C region (a component of immunoglobulin G) have been previously identified in saliva from sheep and goat, respectively, around the 50 kDa region (Lamy et al., 2009) and may correspond to the bands found in the samples close to 55.4 kDa. Saliva obtained from FS2 only presented bands corresponding to proteins smaller than 25 kDa, therefore all the aforementioned high molecular weight proteins were not present. Small proteins and polypeptides such as lysozyme and most cytokines and antimicrobial peptides could pass through FS2 filtration (Stenken & Poschenrieder, 2015). Lysozyme molecular weight (14.3 kDa, Canfield, 1963) matches the profuse bands just below 14.4 kDa, whereas the rest of the bands below 30 kDa are compatible with cathelicidin antimicrobial peptides and hemoglobin subunits (Lamy et al., 2009). Despite the significant role that most of these molecules could play in the control of microorganisms entering the GIT (Fouhse et al., 2017), very few studies have explored their expression and activity in saliva from any animal other than humans. Interestingly, Lamy et al., (2009) reported that the region between 25 kDa and 35 kDa is the most discriminant for the salivary proteome across individuals and ruminant species. Unlike the other saliva fractions, no bands were detected in the AS fraction as a result of effective protein removal.

Experiment 1 (Table 1) showed that FS1 saliva promoted greater (+8.4%) total VFA and lower (17%) NH_3-N concentrations ($P < 0.01$) than NFS fraction. More VFAs produced when incubating with FS1 saliva may be a consequence of the absence of salivary microorganisms which could potentially compete with the autochthonous rumen microbiota, hindering its fermentative activity. The removal of microbial cells in FS1 may explain the lower NH_3-N concentrations due to provision of live or dead microbial cells to extensive proteolysis of their proteins (Belanche et al., 2012). The molar proportion of both branched short-chain fatty acids (isobutyrate and isovalerate) and valerate was

higher in NFS than FS1 containing incubations ($P < 0.01$), indicating greater proteolysis in the incubations containing NFS. Gas production and the concentration of the two major rumen VFA (acetate and propionate) were unchanged as a result of saliva filtration.

Replacing the bicarbonate buffer solution by increasing proportions of saliva had a substantial effect on most fermentation parameters (Table 1), such as higher NH_3-N (up to + 23%), total VFA (+8.0%) and propionate molar proportion (+11%), whereas other parameters decreased (pH, gas production and acetate molar proportion). Unlike the bicarbonate buffer, both NFS and FS1 salivary fractions contain a range of proteins that have been suggested to enhance microbial activity in the gut (Fouhse et al., 2017). NH_3-N was increased and the acetate:propionate ratio decreased as a consequence of the increasing proportion of saliva ($P < 0.05$). Butyrate molar proportion was significantly different among the four proportions of saliva used ($P < 0.01$). The increase in total VFA and N-NH_3 concentrations suggest that some salivary components might foster microbial hydrolysis activity, which may be related to the drop in the incubation pH. Indeed, a previous study in which tannins-rich substrates were pre-incubated with saliva from sheep or goats adapted to different diets and then incubated with rumen fluid showed a greater substrate degradation than when these substrates were pre-incubated with artificial saliva (Ammar et al., 2013). In order to avoid the low values in the incubation pH and potential impairment of microbial fermentation, it was decided to decrease the amount of substrate to be used in experiment 2.

Experiment 2 (Table 2) addressed the effect of the different fractions of saliva on rumen fermentation pattern. Bicarbonate buffer solution (used as control) promoted a similar pH in all four saliva fractions, suggesting that both (buffer and saliva fractions) are effective in maintaining appropriate rumen pH; however, saliva fractions led to greater total VFA and NH_3-N concentrations, which suggests that the presence of some salivary elements enhanced rumen microbial activity. Despite the initial pH being similar in all treatments, final pH after 24 h significantly diverged across salivary fractions (lower values with FS1 and FS2 compared to NFS and AS; $P = 0.001$). These pH differences partially concur with the higher NH_3-N concentrations (+35%; $P < 0.001$) found with NFS and AS saliva in comparison to the other two. On the contrary, no significant differences were found in total VFA concentration between NFS, FS2 and AS, while they were significantly higher with FS1 (+7% on average; $P < 0.001$). This agrees with findings from Experiment 1 and supports that 'microbe-free saliva', in which large salivary proteins are maintained,

boosts rumen fermentation. Incubation with FS1 also resulted in the highest gas production of all treatments (+5% on average, $P < 0.001$). The fermentation patterns, represented by the relative abundances of each VFA, differed across fractions of saliva. NFS and AS both resulted in similar acetate and propionate proportions. As for FS1 vs. FS2, no differences were observed regarding acetate and propionate molar proportions. However, FS1 and FS2 promoted notably higher propionate (+23%) and lower (-8%) acetate molar proportions compared to NFS ($P < 0.001$).

Experiment 2 showed similar effects with the NFS and AS fractions (Table 2). This could be seen as contradictory, given that they only share the mineral component of saliva, while NFS and filtrated fractions also share the protein components. Differences in rumen fermentation when incubating with filtrated or non-filtrated saliva suggest a modulatory role of salivary microbiota and proteins on the rumen fermentation. This is supported by the significantly higher gas production (+5.1%), total VFA concentration (+7.8%) and propionate molar proportion (+26%) observed in FS1 than in AS, suggesting proteins with molecular weight over 30 kDa (including immunoglobulins) may play a key role in modulating rumen microbiota activity. Fouhse et al. (2017) and Tsuruta et al. (2012), demonstrated that salivary secretory IgA from cattle could bind to symbiotic rumen bacteria, here this observation is expanded to show that this can affect the pattern of fermentation in the rumen. Ammar et al. (2013) suggested possible interactions between saliva and rumen inoculum from sheep and goats. Given the high specificity of Ig, it would be interesting to assess their role between species or across individuals in longer term incubations periods in which salivary components and rumen microbiota can interact in a fully stabilized ecosystem.

CONCLUSION

Our results show that the *in vitro* model in which rumen fluid is incubated with saliva collected from animals is a useful research tool and indicates that some salivary components modulate rumen microbial fermentation. In particular, microbe-free filtrated saliva and its bioactive components have shown to increase rumen fermentative activity compared with incubation with non-filtrated or autoclaved saliva. The individual role of specific salivary metabolites and the impact across different individual animals require further research.

Ethics approval

Animal procedures were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013) and protocols were approved by the Ethical Committee for Animal Research at EEZ-CSIC.

Data and model availability statement

None of the data were deposited in an official repository. The data that support the study findings are available upon request.

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JMPH, AB: Data curation and software.

DRYR & AIMG: funding acquisition.

JMPH: Writing original draft.

DRYR, AB & CJN: Writing – review and editing. All authors read and approved the final version.

Declaration of competing interest

None.

Acknowledgements

The authors thank Isabel Jiménez and Pablo González for their assistance in the sample analyses.

Financial support statement

This work was financially supported by the Spanish Research Agency (AEI) through the project AGL2017-86938-R and the Training Program for Academics Grant, Madrid, Spain (FPU16/01981).

References

Ammar, H., Bodas, R., González, J.S., Salem, A.Z.M., Giráldez, F.J., Andrés, S., López, S., 2013. Effects of pre-incubation in sheep and goat saliva on in vitro rumen digestion of tanniferous browse foliage. Journal of Agricultural Science 151, 898–906. https://doi.org/10.1017/S002185961300018X.

Belanche, A., De la Fuente, G., Moorby, J.M., Newbold, C.J., 2012. Bacterial protein degradation by different rumen protozoal groups. Journal of Animal Science 90, 4495–4504. https://doi.org/10.2527/jas.2012-5118.

Canfield, R.E., 1963. The amino acid sequence of egg white lysozyme. The Journal of Biological Chemistry 238, 2698–2707. https://doi.org/10.1016/S0021-9258(18)67888-3.

Dietzen, D.J., 2018. Amino acids, peptides, and proteins. In: Horvath, A.R., Wittwer,

C.T. (Eds.), Principles and Applications of Molecular Diagnostics. Elsevier Inc., Amsterdam, Netherlands, pp. 345–380. https://doi.org/10.1016/B978-0-12-816061-9.00013-8.

Donaldson, G.P., Ladinsky, M.S., Yu, K.B., Sanders, J.G., Yoo, B.B., Chou, W.C., Conner, M.E., Earl, A.M., Knight, R., Bjorkman, P.J., Mazmanian, S.K., 2018. Gut microbiota utilize immunoglobulin A for mucosal colonization. Science 360 (6390), 795– 800. https://doi.org/10.1126/science.aaq0926.

Fouhse, J.M., Smiegielski, L., Tuplin, M., Guan, L.L., Willing, B.P., 2017. Host immune selection of rumen bacteria through salivary secretory IgA. Frontiers in Microbiology 8, 1–9. https://doi.org/10.3389/fmicb.2017.00848.

Humphrey, S.P., Williamson, R.T., 2001. A review of saliva Normal composition, flow, and function. Humphrey, Williamson. 2001. Journal of Prosthetic Dentistry.pdf. The Journal of Prosthetic Dentistry 85, 162–169.

Janeway, C.A., Travers, P., Walport, M., Shlomchik, M., 2001. Immunobiology: The Immune System in Health and Disease. Garland Publishing, New York City, NY, USA.

Lamy, E., da Costa, G., Santos, R., Capela e Silva, F., Potes, J., Pereira, A., Coelho, A.V., Sales Baptista, E., 2009. Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research? Physiology and Behavior 98, 393–401. https:// doi.org/10.1016/j.physbeh.2009.07.002.

Mcdougall, E.I., 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43, 99–109.

Meyer, R.M., Bartley, E.E., Morrill, J.L., Stewart, W.E., 1964. Salivation in cattle. I. Feed and animal factors affecting salivation and its relation to bloat. Journal of Dairy Science 47, 1339–1345. https://doi.org/10.3168/jds.S0022-0302(64)88915-3.

Nørgaard, P., 1993. Saliva secretion and acid-base status of ruminants. A review. Acta Veterinaria Scandinavica 89, 93–100.

Palma-Hidalgo, J.M., Belanche, A., Martín-García, A.I., Yáñez-Ruiz, D.R., 2018. In vitro assessment of goat's saliva on rumen fermentation. In: Proceedings of the 10th International Symposium on the Nutrition of Herbivores. Advances in Animal Biosciences, 9, p. 396. https://doi.org/10.1017/S2040470018000146.

Piccione, G., Foà, A., Bertolucci, C., Caola, G., 2006. Daily rhythm of salivary and serum urea concentration in sheep. Journal of Circadian Rhythms 4, 2–5. https://doi.org/10.1186/1740-3391-4-16.

Salem, A.Z.M., López, S., Ranilla, M.J., González, J.S., 2013. Short- to medium-term effects of consumption of quebracho tannins on saliva production and composition in sheep and goats. Journal of Animal Science 91, 1341–1349. https://doi.org/10.2527/jas.2010-3811.

Somers, B.M., 1957. Salivary secretion and its functions in ruminants. The Australian Veterinary Journal 33, 297–301.

Stenken, J.A., Poschenrieder, A.J., 2015. Bioanalytical chemistry of cytokines – A review. Analytica Chimica Acta 853, 95–115. https://doi.org/10.1016/j. aca.2014.10.009.

Tomas, F.M., 1973. Parotid salivary secretion in sheep: Its measurement. Quarterly Journal of Experimental Physiology 58, 131–138.

Tsuruta, T., Inoue, R., Tsukahara, T., Nakamoto, M., Hara, H., Ushida, K., Yajima, T., 2012. Commensal bacteria coated by secretory immunoglobulin A and immunoglobulin G in the gastrointestinal tract of pigs and calves. Animal Science Journal 83, 799–804. https://doi.org/10.1111/j.1740-0929.2012.01026.x.

Weimer, P.J., 2015. Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. Frontiers in Microbiology 6, 1–16. https://doi.org/10.3389/fmicb.2015.00296.

Tables and Figures

Table 1. Effect of increasing the proportions of two goat saliva fractions to replace an artificial buffer on in vitro rumen fermentation (Experiment 1).

	Saliva fraction		Saliva proportion					P-value		
	NFS	FS1	0%	16%	33%	50%	SEM	Fraction	Proportion	Interaction
Initial pH	6.94	6.89	6.85	6.90	6.98	6.94	0.052	0.189	0.109	0.647
Final pH (after 24h)	5.50	5.50	5.63^{a}	5.51^{b}	5.47^{b}	5.40^{c}	0.022	0.748	<0.001	0.071
Gas Production (ml)	22.4	22.8	23.9^{a}	23.9^{a}	22.3^{b}	20.2^{c}	0.423	0.217	<0.001	0.455
NH_3-N (mg/dl)	35.1	29.1	28.7^{c}	29.8^{bc}	34.3^{ab}	35.3^{a}	2.337	0.001	0.023	0.176
Total VFA (mM)	214	232	212^{b}	221^{a}	228^{a}	229^{a}	4.734	<0.001	0.001	0.882
Acetate (%)	61.4	61.0	62.6^{a}	61.9^{a}	60.8^{b}	59.7^{c}	0.495	0.303	<0.001	0.120
Propionate (%)	20.9	21.1	19.9^{c}	20.7^{bc}	21.4^{ab}	22.0^{a}	0.418	0.331	<0.001	0.750
Isobutyrate (%)	0.68	0.63	0.61	0.66	0.68	0.65	0.024	0.007	0.080	0.002
Butyrate (%)	14.3	14.7	14.4^{bc}	14.1^{c}	14.5^{b}	15.0^{a}	0.200	0.034	0.003	<0.001
Isovalerate (%)	1.09	0.97	0.96^{b}	1.05^{a}	1.08^{a}	1.03^{ab}	0.033	<0.001	0.017	0.028
Valerate (%)	1.62	1.54	1.50^{b}	1.54^{b}	1.63^{a}	1.65^{a}	0.036	0.004	0.001	0.330
Ac/Prop	2.95	2.90	3.14^{a}	2.99^{b}	2.85^{c}	2.72^{c}	0.071	0.354	<0.001	0.514

NFS, non-filtered saliva; FS1, filtered saliva through 0.25 mm pore size filter; VFA: volatile fatty acids; Ac/Pr: acetate/propionate ratio.

$^{a–c}$Within a row means with different superscripts differ.

Table 2. Effect of using different fractions of goat saliva on *in vitro* rumen fermentation (Experiment 2).

	Saliva Fraction					
	NFS	FS1	FS2	AS	SEM	*P*-Value
Initial pH	7.03	7.02	7.02	6.99	0.014	0.176
Final pH (after 24h)	6.51^{a}	6.39^{b}	6.41^{b}	6.48^{a}	0.021	0.001
Gas Production (ml)	11.5^{c}	12.3^{a}	12.1^{b}	11.7^{bc}	0.139	<0.001
NH_3-N (mg/dl)	35.4^{a}	30.7^{b}	26.2^{c}	31.8^{ab}	1.388	<0.001
Total VFA (mM)	103^{b}	111^{a}	105^{b}	103^{b}	1.095	<0.001
Acetate (%)	61.3^{a}	56.2^{b}	57.2^{b}	61.4^{a}	0.439	<0.001
Propionate (%)	21.4^{b}	26.3^{a}	25.7^{a}	20.9^{b}	0.434	<0.001
Isobutyrate (%)	1.31^{a}	1.16^{b}	1.04^{c}	1.25^{a}	0.020	<0.001
Butyrate (%)	12.1	12.7	12.8	12.5	0.232	0.114
Isovalerate (%)	2.19^{a}	1.94^{b}	1.70^{c}	2.15^{a}	0.050	<0.001
Valerate (%)	1.70	1.71	1.59	1.71	0.044	0.202
Ac/Prop	2.98^{a}	2.25^{b}	2.35^{b}	3.06^{a}	0.052	<0.001

NFS, non-filtered saliva; FS1, filtered saliva through 0.25 mm filter; FS2 filtered saliva through 30 kDa filter; AS, autoclaved saliva; VFA: volatile fatty acids; Ac/Pr: acetate/propionate ratio.

$^{a–c}$Within a row means with different superscripts differ. The buffer column was used as control but not included in the statistical analysis.

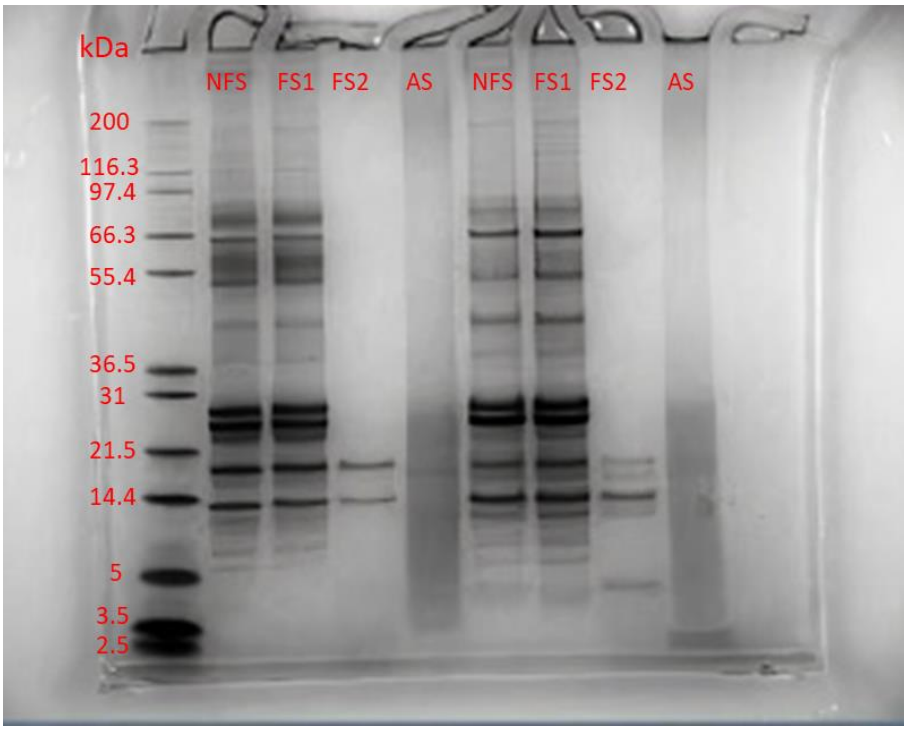

Fig. 1. Polyacrilamide gel illustrating the presence of different goat saliva metabolites. Lane 1 corresponds to Mark12TM molecular weight marker. Lanes 2–5 and 6–9 correspond to saliva fractions from the first and second saliva donor, respectively. NFS, non-filtered saliva; FS1, filtered saliva through 0.25 mm filter; FS2 filtered saliva through 30 kDa filter; AS, autoclaved saliva.

Publication 6

Multi-omics study of the salivary modulation of the rumen microbiome

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Scientific Reports (under review)

SCIENTIFIC REPORTS

ABSTRACT

Ruminants are able to produce large quantities of saliva which enter into the rumen. Although previous research has indicated that salivary immunoglobulins can partially modulate the rumen microbial activity, the role of the salivary components other than ions on the rumen microbial ecosystem has not been thoroughly investigated in ruminants. A total of 16 semi-continuous *in vitro* cultures were used to incubate rumen fluid from 4 donor goats inoculated with autoclaved saliva (AUT) as negative control, saliva from the same rumen fluid donor (OWN) as positive control, and either GOAT or SHEEP saliva as experimental interventions. Fermentation was monitored throughout the 7 days of incubation and the prokaryotic communities and metabolome were analysed at day 7 of incubation. Characterization of the salivas used prior to incubation showed a high degree of individual variability in terms of the salivary metabolites and proteins, including immunoglobulins. The prokaryotic community composition in AUT incubators was the most divergent across treatments, suggesting a modulatory effect of active salivary components, which were not affected in the other treatments (OWN, GOAT and SHEEP). The differences across treatments in microbial diversity were mostly caused by a greater abundance of *Proteobacteria* and *Rikenellacea* and lower of *Prevotellaceae*, a key rumen bacterium with greater abundance in GOAT and SHEEP treatments. These results suggest that specific salivary components contribute to host-associated role in selecting the rumen commensal microbiota and its activity.

Keywords: saliva, incubation, rumen, modulation, omics

INTRODUCTION

The rumen of ruminant animals contains a great diversity of prokaryotic (bacteria, archaea, virus) and eukaryotic (protozoa and fungi) microorganisms that together breakdown and ferment the feed ingested by the host animal to convert complex plant carbohydrates into short-chain volatile fatty acids (Dehority, 2003). The rumen microbial diversity and function largely influence many animal traits such as the efficiency of utilization of feeds and the environmental impact through methane emissions (Jami et al., 2014). The digestive microbiomes in most mammals are controlled by host genetic variation (Koskella & Bergelson, 2020) through multiple processes, one of the most crucial being immune modulation, by secreting many substances from epithelial cells (i.e.

antimicrobial peptides, immunoglobulins,..) and germline-encoded pattern recognition receptors (Zheng et al., 2020). However, in the rumen no organized lymphoid tissue exists in the epithelium (Sharpe et al., 1977), which includes up to a 15 cell layer, that limit the permeability of large molecules. Saliva has been suggested as main vehicle of introducing immune active metabolites in to the rumen (Yáñez-Ruiz et al., 2015).

Ruminants' saliva is secreted in large amounts and assists the animal in the process of feed lubrication, deglutition and regurgitation. Saliva constituents include a significant amount of ions (mainly bicarbonate and phosphate), that help maintain rumen osmotic pressure and pH within physiological range (Warner & Stacy, 1977) providing a buffered medium to allow rumen microorganisms to thrive (Faniyi et al., 2019). The protein fraction of saliva comprises a number of proteins involved in transportation and pH buffering (Cheaib & Lussi, 2013), from which albumin is found in greater amounts (Lamy et al., 2009). However, the ruminant salivary proteome also includes a complex mix of other proteins with a wide range of physiological and enzymatic functions (Ang et al., 2011). Immunoglobulins, especially secretory immunoglobulin A (IgA), modulate the proliferation of symbiotic microbiota (Fouhse et al., 2017), either inhibiting or stimulating their growth (Donaldson et al., 2018). Smaller salivary proteins, which includes a variety of cytokines (Stenken & Poschenrieder, 2015) and antimicrobial peptides (Fábián et al., 2012), have been shown to be the most discriminant in the salivary proteome across individuals and animal species (Lamy et al., 2009). In a recent *in vitro* batch culture study, we have shown that some specific protein components have the ability to modulate rumen fermentation in goats (Palma-Hidalgo et al., 2021a). However, due to such specificity, the mechanisms behind the complex and modulatory interaction that takes place between salivary components and host rumen microbiota are still largely unknown.

This work aimed to assess the role of saliva on modulating the rumen fermentation and microbial diversity using a 7-days semi-continuous *in vitro* trial. A detailed characterization of the protein and metabolites composition of saliva from different individual animals was conducted and rumen fluid from goats was incubated with different types of saliva (own animal-saliva, goat-saliva, sheep-saliva and autoclaved-saliva) to elucidate the potential modulatory effect on the rumen microbial ecosystem.

RESULTS

Immunological, proteomic and metabolomics profiling of the individual salivas

Saliva from Goats 1-4 (used in OWN treatment), Goat 5 (used in GOAT treatment) and the sheep (used in SHEEP treatment) showed distinct immunological, proteomic and metabolomic profiles. Average protein concentration across salivas was 908±146 µg/ml. IgA Elisa resulted in an IgA concentration of 36.6, 30.0, 26.3 and 57.3 µg/ml in Goats 1-4, respectively, in comparison with 44.6 µg/ml (+19%) in Goat 5's saliva and 29.1 µg/ml (-22%) in the sheep's saliva. In the case of salivary IgG quantification, the concentration in Goats 1-4 was 9.98, 9.72, 11.7 and 11.5 µg/ml, respectively; similar to that in Goat 5's saliva (10.48 µg/ml), but much higher (+24%) than that of the sheep's saliva (8.12 µg/ml).

The proteomic MS/MS analysis of the saliva samples resulted in the identification of 195 proteins/polypeptides across the 6 samples. The average number of proteins per sample was 59, with Goat 5's saliva having the lowest count (46), and the sheep's saliva having the highest (68). The heatmap of the 30 most abundant proteins across the saliva samples showed a very variable proteomic profile based on emPAI values (**Figure 1**). On average, the protein with the greatest abundance was Thymosin beta, but its emPAI values were very variable, ranging from 0 (Goat 2) to 153 (Goat 3). Even after saliva samples were processed for albumin depletion, albumin (fragment) was still the second most abundant protein in the saliva samples. The 3rd (Beta A globin chain), 4th (II alpha globin) and 5th (I alpha globin) most abundant proteins were all hemoglobin subunits, and their abundance pattern was similar in each saliva sample. The variability in the abundance of the rest of the proteins helped determine, to some extent, the clustering pattern between the 6 samples. Interestingly, not the sheep's but Goat 3's saliva turned out to be, compared with the others, the most different sample with regards to the proteomic profile. However, unlike all the goats' salivas, sheep's saliva did not contain goat-specific Capra hircus Akirin 2 mRNA but it did have high values of other proteins such as Glutathione S-transferase and Insulin-like growth factor 1, which were almost not present in goat salivas.

The metabolomic MS/MS analysis identified 39 metabolites present in all the saliva samples. The heatmap of the 39 compounds based on mTIC values showed very unique metabolic profiles in each saliva sample (Figure 2). The peak heights of most of the compounds detected in the MS/MS spectra were very variable across the 6 samples, hence the high degree of variability observed in the constructed heatmap. Similar to what was found in the proteomic analysis, the Goat 3's saliva was, again, the one with the most

different metabolomic profile. On average, the 1st (Tetraethylene glycol), 4th (Hexaethylene glycol) and 5th (Deithylene glycol monoethyl ether) most abundant metabolites were ethylene glycol derivatives. Other abundant detected compounds include the aminoacids L-Isoleucine (2nd) and L-Phenylalanine (8th), as well as the choline cation (3rd) and the Tri(3-chloropropyl) phosphate (6th), all of them with up to 1000 fold mTIC value variability between at least two of the saliva samples. In comparison to GOAT saliva, the SHEEP saliva had lower concentrations of urea and higher concentrations of creatinine and nucleic acids derivatives such as guanine, guanosine or hypoxantine.

In vitro fermentation and microbial abundances

The fermentative activity peak was reached in the first 12 hours of incubation, as shown by the lowest pH values and greatest gas production and VFA concentration ($P < 0.001$ according to sampling time). From the first day of incubation a stable fermentative activity was observed in terms of pH and gas production, with only a slight gradual decrease in total VFA concentration as incubation time progressed (**Table 1**). The incubation of rumen fluid from 4 different goats with AUT, OWN, GOAT and SHEEP salivas led to substantial differences in the fermentation pattern (**Table 2**). Incubation with AUT led to the lowest gas production (-9.4 %) and highest pH and butyrate molar proportion (+6.4 %), while GOAT samples produced the lowest pH, butyrate molar proportion and acetate: propionate ratio ($P < 0.001$, $P = 0.018$ and $P = 0.005$; respectively). Bottles with SHEEP saliva generated the highest gas production and highest acetate: propionate ratio (+4% and + 6%; respectively) compared with the rest.

Results from qPCR analyses showed significantly higher concentration of bacteria in bottles incubated with SHEEP and OWN salivas, whereas the lowest bacterial concentration was found when bottles were incubated with AUT and GOAT saliva ($P = 0.013$) (**Table 2**). Likewise, the abundance of rumen protozoa was the highest in SHEEP and the lowest in GOAT bottles ($P = 0.046$). No significant effects were noted on the methanogenic archaea or anaerobic fungi concentrations according to the different type of saliva.

Microbial diversity

The sequencing analysis performed on incubation samples generated 41,514 ± 13,383 high quality prokaryotic sequences per sample. The number of sequences was normalized to 28,131 for further processing and analyses. Good's coverage index was 98.8 % on average and similar for the 4 saliva treatments, hence a good level of sequencing depth was achieved. The primers used for sequencing mostly targeted bacterial amplicons, however, ~0.75 % of the detected reads were identified as archaeal sequences. The prokaryotic alpha-diversity in terms of observed ASVs, Chao1, Shannon and Simpson indexes within the incubation bottles was not affected as a consequence of the incubation with the different types of saliva (**Figure 3**).

The Venn diagram (**Figure 4**) showed that a majority of the detected ASVs (959) were shared across the 4 saliva treatments. AUT was the treatment with the least overlapping ASVs with the rest of the treatments (1468 vs. 1549 vs. 1517 vs. 1494 in AUT, OWN, GOAT and SHEEP treatments, respectively).

PERMANOVA analysis showed that the differences in the prokaryotic community structure across saliva treatments were significant in specific pair-wise comparisons (**Figure 5**). The clearest difference was that observed between the communities in the AUT and the rest of the treatments. The level of dissimilarity between the AUT community structure and the other three treatments (mainly SHEEP and GOAT) was also very apparent in the subsequent sPLS-DA. The component 1 axis in the sPLS-DA (explaining 5% of the total variation) sorted the AUT samples apart from the rest, whereas the component 2 (explaining 4% of the total variation) disaggregated the OWN from the other two treatments with fresh saliva (GOAT and SHEEP). PERMANOVA analysis showed no significant differences in the prokaryotic community structure between OWN, GOAT and SHEEP treatments.

The relative abundance of the identified prokaryotic taxa was moderately variable according to the saliva treatment (**Table 3**). At phyla level, 4 out of 18 had significantly different abundances across saliva treatments. *Actinobacteria* (2.73 % average relative abundance) was more predominant in bottles incubated with GOAT and SHEEP saliva (P = 0.0385). On the contrary, *Proteobacteria* (6.88 % average relative abundance) was more predominant in bottles incubated with AUT and OWN saliva. Thirteen out of the 32 most abundant prokaryotic families (**Figure 6**) and twelve out of the 33 most abundant

genera denoted differences across the saliva treatments. *Prevotella 1*, the most abundant genus (22.4 % sequences), was 22.3 % more abundant in GOAT and SHEEP compared with AUT. Several relevant taxa including *Atopobium, Olsenella, Lachnospiraceae XPB1014 group* and *Streptococcus* also showed a greater abundance in GOAT samples, while Elusimicrobia and *Saccharimonadaceae* were more abundant in SHEEP. On the contrary, AUT samples had higher levels of *Bacteroides, Prevotellaceae UCG-003, Rikenellaceae, Family XIII, [Eubacterium] oxidoreducens group, Butyrivibrio, Succinivibrionaceae UCG-002* and *Veillonellaceae*. In OWN samples only *F082, Prevotellaceae UCG-001, Quinella* and *Succinivibrionaceae* were more abundant compared to other treatments.

Effects of the type of saliva on the metabolomic composition

The MS-MS metabolomics analysis on in vitro incubation samples identified 19 compounds after processing and filtration (**Figure 7, Table 4**). On average, the most abundant metabolite was 15-Ketoprostaglandin E1, followed by ethyldiethanolamine and N-Methyl-2-pyrrolidone. Eight metabolites presented significantly different abundances according to saliva treatment. The heatmap based on mTIC values (**Figure 7**) clustered samples from AUT treatment separately and then separated samples from SHEEP to those from GOAT/OWN saliva treatments. This was further demonstrated, in agreement with microbial diversity results, by the significantly distinct metabolomic profile in AUT treatment compared with the other three treatments and that the SHEEP metabolome was different ($P < 0.005$) from that of the GOAT and OWN samples.

DISCUSSION

In our study, a thorough description of the protein and metabolite components of the animals´ saliva was achieved prior to incubation with rumen fluid. The immunological profiling of fresh saliva from the five goats and one sheep revealed relatively low concentrations of IgA compared to the 5.95 mg/ml recently reported in bovine saliva (Fouhse et al., 2017). Even though some previous works where ELISA was not used for quantification (Porter & Noakes, 1970) had difficulties at detecting IgA even after a 20 fold concentration using dialysis, others such as Mach & Pahud (1971) and Lascelles &

McDowell (1974) reported much higher IgA saliva concentrations (560 µg/ml, 157 µg/ml; respectively) than what we detected in our study (37.6 µg/ml). As expected, the average concentration of IgA, which is the major immunoglobulin in ruminants' saliva (Lascelles & McDowell, 1974), was ~4 fold higher than that of IgG. Interestingly, even though this IgA:IgG ratio was maintained in the sheep's saliva, both concentrations were notably lower in comparison with the other salivas.

The total identified proteins across the salivas used in our study (195) was much greater than the 33 and 13 proteins annotated in sheep and goat saliva following a two-dimensional gel electrophoresis (2D PAGE) approach with two different spectrometry methods (Lamy et al., 2009, 2011; respectively). Despite the number of annotated proteins was much higher in our study, we hypothesize that this difference could partially be caused by the utilization of a protein database such as TrEMBL which, unlike SwissProt, contains computationally annotated protein features instead of manually reviewed annotated proteins. A comprehensive study of the bovine salivary proteome where similar nontargeted MS-MS approaches were used (Ang et al., 2011) identified an average of 179 proteins across different sample preparation methods, which is similar to our figure and slightly closer to the hundreds of proteins identified in human saliva studies (Loo et al., 2010). Like in our study, variability based on different methodologies used and/or animal specificity in previous works played a significant role in this high rate of detected proteins. Such a wide array of salivary proteins are involved in numerous physiological functions across the animal kingdom (Mandel, 1987). Despite the inter- and intraspecies variability with regards to salivary protein components in ruminants, these proteins seem to be involved in similar physiological functions (Ang et al., 2011). Indeed, the functional profile of the salivary proteins detected in cows (Ang et al., 2011) was pretty consistent with that found in goat and sheep proteins identified in our study, most of which are involved in nutrient-binding, transport, enzymatic activity and, to a less extent, immune response.

A previous *in vitro* study revealed that pre-incubation of specific diets (such as tannins-rich forages) with either sheep or goat saliva had a positive effect on diet degradation when incubated with rumen fluid (Ammar et al., 2013). On the other hand, other works have reported that the diet provided to ruminants and their saliva composition (including its protein fraction) have only minor effects on the rumen microbial activity (Ammar et al., 2011) and *vice-versa* (Salem et al., 2013). The lack of substantial effects found in

these studies could be caused by the relatively short time of incubation (48 h) but also due to a missing exploration of the salivary proteome and metabolome, which we addressed in our study. In this context, the use of different diets or the inoculation with unique microbial strains have been suggested to induce a number of immunological mechanisms in the GIT (Yáñez-Ruiz et al., 2015). This has been reported to be of particular importance with regards to immunological proteins (mainly Ig), given that their concentration varies significantly depending on their rate of secretion through saliva (Subharat et al., 2016), which greatly depends on the presence of specific microorganisms in the rumen (Sharpe et al., 1977).

The metabolomic profile of the ruminants' saliva has not been thoroughly explored to date. In general, research on the saliva metabolome has been focused on the identification and characterization of salivary biomarkers that could be used as indicators for the detection of a number of diseases (Yoshizawa et al., 2013). Other studies have attempted to better assess the metabolome composition throughout the gut, and the cross-effects that might take place between this and the host microbiota (Gardner et al., 2019; Nicholson et al., 2012). In our study, substantial amounts of polyethylene glycol derivatives were detected, which could come from the use of commercial sponges for collection. Overall, individual specificity on the saliva metabolome observed across our samples could most likely be driven by the unique microbiota present in each animal (Gardner et al., 2019), that altogether could be shaped by salivary proteins with immunological function (Palma-Hidalgo et al., 2021a). Moreover, the substantial differences between GOAT and SHEEP saliva observed in the proteome and metabolome indicated a species-specificity in the abundance of salivary compounds which could partially explain the rumen microbial differences observed between these two species in previous works (Henderson et al., 2015; Langda et al., 2020).

Our semi-continuous incubation system reached a peak of microbial activity in the first hours of incubation and then remained stable in terms of pH and gas production from 36 hours and thereafter. Through the last days of incubation, gas production was very low in AUT bottles compared with the rest, indicating that untreated saliva from goats or sheep contain bioactive components that enhance fermentative activity. At this stage of incubation, the saliva donor species was the most influential factor *in vitro* fermentation as the SHEEP saliva promoted the highest levels of fermentative activity (+4% gas production) as well as the greatest bacterial and protozoal concentrations (+2% and +7%,

respectively). The high butyrate molar proportion and acetate: propionate ratio in SHEEP samples also suggest that a greater fibrolytic activity could have taken place by the more abundant rumen protozoa present in this treatment (Belanche et al., 2019; Eugène et al., 2004). These differences in *in vitro* rumen fermentation when incubating with saliva of the two small ruminants species were also reported by Ammar et al. (2013) when using tannins-rich substrates, which again suggests that the unique salivary composition of each species or even individuals may modulate microbial activity differently.

Incubation with AUT saliva led to the most divergent rumen microbial community in terms of overlapping ASVs with other treatments and general microbial composition. At phyla level, the relative abundance of the two main bacteria phyla across all treatments was 53 % for *Bacteroidetes* and 30 % for *Firmicutes*, a ratio (1.76) which is almost half (3.25) of what has been previously described in the rumen of goats (Palma-Hidalgo et al., 2021b). We hypothesize that the salivary proteins promoted the growth of *Firmicutes* bacteria, which have been demonstrated to be more abundant in the proximal GIT or the oral cavity (Fouhse et al., 2017; Yeoman et al., 2018). The salivary components of GOAT and SHEEP salivas also increased the proliferation of saliva-abundant *Actinobacteria* (Fouhse et al., 2017) which includes numerous species known for their ability to degrade complex compounds like fiber (Barka et al., 2016).

The three microbial taxa that contributed the most to make the AUT prokaryotic composition differ from the rest (particularly that from SHEEP), were *Proteobacteria* phylum and *Prevotellaceae and Rikenellaceae* families. With the exception of the AUT-abundant *Succinivibrionaceae* family, which has been recently correlated with animal growth and VFA production (Palma-Hidalgo et al., 2021b), *Proteobacteria* are commonly categorized as early rumen colonizers (Jami et al., 2013) and have been often associated with a suboptimal rumen microbial development. The greater abundance of this phylum in AUT treatment may suggest a deficient regulation by the lack of salivary bioactive components with immunological function, which were most likely denatured by autoclaving (Palma-Hidalgo et al., 2021a). This explanation would also be in line with the lower abundances in AUT samples of *Prevotella 1* and *Prevotellaceae* (-22.3%), which is a cornerstone bacterial genus in the rumen and ruminant's oral cavity (Rey et al., 2014; Tapio et al., 2016) and plays a pivotal role in the rumen metabolism (Precup & Vodnar, 2019). Given the harmless commensal nature of most *Prevotella* species in the rumen, it might be possible that its growth could be (directly or indirectly) stimulated

when incubating with untreated salivas by modulation of salivary protein components, namely immunoglobulins, as it has been demonstrated with other commensal bacteria in mice (Donaldson et al., 2018; Peterson et al., 2007). Indeed, IgA and IgG and its different isoforms have been shown to modulate bacterial populations throughout the GIT (Tsuruta et al., 2012) to maintain mucosal homeostasis (Mantis et al., 2011). However, IgA tagged bovine oral or rumen microbiota have been reported to include significant lower abundance of *Prevotellaceae* compared to regular rumen microbiota (Fouhse et al., 2017). The high variability in the Ig concentrations in our study, and particularly the low concentrations (-34 % IgA) in the sheep saliva coupled with the high abundances of *Prevotellaceae* in the SHEEP treatment, suggest that other immunological mechanisms driven by different proteins or molecules (*e.g*. cytokines, defensins, cathelicidins, miRNA; Yáñez-Ruiz et al., 2015) could also be involved in the stimulation or inhibition of the rumen microbes and their fermentative activity (Palma-Hidalgo et al., 2021a). The specificity of these modulatory mechanisms, which seems to vary moderately across species and individuals, may be partially responsible of the resilience and individual host specificity of the ruminal microbiota reported through complete rumen exchange experiments (Weimer, 2015). In line with this, our results suggest that the bioactive components of saliva, have a positive effect on the proliferation of crucial goat rumen bacteria as well as on the microbiota capable of degrading fibrous feeds. However, these positive effects on rumen microbial composition and activity are not as clear when goat rumen fluid is incubated with the specific salivary components of the same animal (OWN), indicating that the influx of new exogenous salivary elements could have synergistic effects on the rumen microbiome and fermentation. The different effects on the rumen microbial composition and activity seemed to be more notable across different species, which supports the fact that the specificity of the goat's or sheep's saliva composition (Lamy et al., 2009) observed in our study leads to the development of distinct microbial communities under similar dietary conditions (Langda et al., 2020).

Previous research on the rumen metabolome (Artegoitia et al., 2017; de Almeida et al., 2018) also resulted in the detection of thousands of distinct 'raw' metabolic features. The fact that, after quality filtering, the vast majority of them could not be reliably annotated using different search engines and libraries speaks for the great complexity of the rumen metabolome and how it could be a reservoir of novel compounds. The amount of annotated metabolites (19) compared with the 67 identified by de Almeida et al. (2018)

made it difficult to discern clear effects of the incubation with different salivas on the rumen metabolomics profile. Despite this, the variability in the level of detection of the different metabolites indicate how its presence and abundance are most likely driven by salivary components *per se* or by the distinct microbial community (Gardner et al., 2019) modulated by saliva from different donors. The distinct salivary components of different species seem to have a strongest influence on the rumen content as they shape the rumen metabolome differently depending on whether they are constituents of goat's or sheep's saliva. Our data indicate that saliva components may partly responsible for the host species-specific rumen microbiota and related metabolites, potentially due to the co-evolution of the microbiome and host (Koskella & Bergelson, 2020).

The characterization of sheep and goats saliva showed distinct metabolomic and proteomic profiles across individuals and animal species, though the general functions (enzymatic, transport, immune response) remain consistent. Inactivating these compounds (i.e. autoclaving) exhibit an important change in the function of saliva in shaping rumen fermentation and the microbial community. This finding together with the differences observed between species suggest that the cross-talk mechanisms between salivary components and rumen microbiota can be specific for individuals and/or species and that may contribute to the host selection of the commensal microbiota and its function.

METHODS

Saliva collection

Experimental protocols were approved by the Ethical Committee for Animal Research at EEZ-CSIC and animal procedures were conducted by trained personnel according to the Spanish Animal Experimentation guidelines (RD 53/2013). Five adult rumen-cannulated goats (Goat 1 to Goat 5) and one adult sheep, all housed in different pens, were used as saliva donors. During the study, all animals were fed at maintenance level with a diet consisting on 80% oats hay and 20% commercial concentrate. Saliva collection was conducted before the morning feeding by swabbing the base of the cheek on both sides of the mouth of the animals with absorbent sponges for 5 min. Saliva was collected from the sponges by centrifuging at 190×*g* for 10 min, then filtrated through 0.25 µm pore size to remove microorganisms and large particles, pooled per animal and stored in aliquots

at -80 °C until the start of the *in vitro* incubation. Additionally, equal volumes of saliva from goats 1-4 were mixed, autoclaved at 121 °C for 30 min and stored at -80 °C (AUT). Four aliquots from each saliva (goats 1-5 and sheep) were used for immunoglobulins, proteome and metabolome analyses.

Experimental design and *in vitro* incubation

An *in vitro* semi-continuous incubation was conducted during 7 days using rumen fluid from goats 1-4 sampled before the morning feeding and filtrated through a double layer of cheesecloth. Sixteen Wheaton bottles with 30 ml capacity were used in the incubation. Each rumen fluid was incubated with 4 different types of saliva (*n*=4): saliva from the same rumen fluid donor (Goats 1-4) (OWN) as positive control, saliva from goat 5 (GOAT), saliva from sheep (SHEEP) and pooled autoclaved saliva to denature active metabolites in the saliva but keeping the minerals, (AUT) from goats 1-4, as negative control. Incubations consisted in a total volume of 20 ml per bottle composed of 6.67 mL of rumen fluid, 6.67 mL of saliva and 6.67 mL of bicarbonate buffer (3.5 g $NaHCO_3$ + 0.4 g $(NH_4)HCO_3$ in 100 ml dH_2O). The same oats hay and commercial concentrate that were offered to the animals were grinded to 1mm size particles and used as incubation substrate (100 mg each).

In order to maintain an active *in vitro* system, every 12 hours (9.00 and 21.00), gas pressure in the headspace of the bottles was measured using a Wide Range Pressure Meter (SperScientific LTD, Scottsdale, AZ, USA), which then was transformed into volume units by the ideal gas law. After gas measurement, bottles were opened, the content was homogenized by a gentle horizontal movement, and 1/3 of the incubation volume (6.67 ml) was removed with a syringe and used to measure the pH. The removed incubation volume was replaced by 3.33 ml of the aforementioned bicarbonate buffer, 3.33 ml of the same saliva used in each treatment, and 1/3 of diet (33 mg oats hay and 33 mg commercial concentrate). A continuous flow of CO_2 was applied to each bottle through this process to maintain the anaerobic conditions. One sub-sample (800 µl) of the removed incubation content was taken at 12, 36, 60, 84, 108, 132 and 156 hours, mixed with 800 µL of an acid solution (0.5 mol/l HCl, 200 g/l metaphosphoric acid and 0.8 g/l crotonic acid as internal standard) for volatile fatty acids (VFA) determination by gas chromatography (AutoSystem gas chromatograph, Perkin Elmer, Waltham, MA). A second sub-sample

(200 µl) of the removed incubation content at day 7 was used for metabolome analysis following a similar procedure to that described for saliva samples. A third sub-sample (200 µl) taken at days 2, 4, 6 and 7 was used for DNA extraction using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen Ltd., Barcelona, Spain).

Characterization of proteins and metabolites in saliva

One aliquot of each saliva was thawed to measure the protein content by spectrophotometry using a commercial assay kit (PierceTM BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA). For salivary immunoglobulins A and G (IgG) quantification, aliquots of each saliva were thawed and centrifuged at 3,000×g for 10 min. IgA and IgG concentrations were measured using the Goat Immunoglobulin A and Goat Immunoglobulin G ELISA kits (MyBioSource, San Diego, CA, USA), respectively.

Before conducting saliva proteome analysis, albumin depletion was performed on thawed aliquots of each saliva, using the Pierce™ Albumin Depletion Kit (Thermo Fisher Scientific, Waltham, MA, USA) in order to reduce the high concentrations of albumin in saliva. After that, albumin-depleted saliva samples were sent to Proteomics Service at Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain) for non-targeted proteomic analysis using a nLC (Easy nano Liquid Chromatograph, Proxeon, Odensee, Denmark) coupled with an Amazon Speed ETD ion trap mass spectrometer fitted with CaptiveSpray ion source (Bruker, Bremen, Germany). Saliva samples were processed and analysed as described by Mancera-Arteu et al., (2020). Identified spectra were searched against the TrEMBL database (Bateman et al., 2021). Exponentially modified protein abundance index (emPAI), which is proportional to protein content in a protein mixture (Ishihama et al., 2005), was used for estimation of absolute protein amount in the saliva samples (Arike & Peil, 2014).

Saliva aliquots for non-targeted metabolomics analysis were sent to the Metabolomic Platform at Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, Madrid, Spain). Metabolites were extracted and injected in a Ultrahigh-pressure liquid chromatograph (Agilent 1290 Infinity UHPLC, Santa Clara, CA, USA) coupled with a Quadrupole Time-Of-Flight Mass Spectrometer (Agilent 6540 UHD Q-TOF MS, Santa Clara, CA, USA) in a similar manner as described by Gómez et al., (2016). MS/MS

spectra were processed and filtrated using MS-DIAL v 4.12 software (http://prime.psc.riken.jp/compms/msdial/main.html) and identified by searching against NIST (https://www.nist.gov/pml/atomic-spectra-database), MoNA (https://mona.fiehnlab.ucdavis.edu/) and LipidBlast (https://fiehnlab.ucdavis.edu/projects/lipidblast) databases. The sum peak height of all structurally annotated compounds (mTIC) score (Fiehn, 2017) was normalized for each sample to allow comparisons across salivas.

qPCR and next generation sequencing

After extraction, DNA concentration and purity were assessed at A260 and A280nm on a NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Eluted DNA (2 µl) were used to assess the abundance of the main microbial groups by quantitative PCR (qPCR) using a iQ5 multicolor Real-Time PCR Detection System (BioRad Laboratories Inc., Hercules, CA, USA). Specific primers for the 16S bacterial rRNA gene (Maeda et al., 2003), mcrA gene for methanogenic archaea (Denman et al., 2007) and 18S rRNA genes for protozoa and anaerobic fungi (Sylvester et al., 2004 and Denman & McSweeney, 2006; respectively) were used. Quantitative PCR standards consisted of the plasmid PCR 4-TOP (Invitrogen, Carlsbad, CA, USA), with an inserted 16S, mcrA or 18S rRNA gene fragment from each microbial group, respectively.

Extracted DNA from incubation samples taken at days 6 and 7 were also used for meta-taxonomic analysis of the prokaryotic community. DNA samples were sent to the Genomics Service at Instituto de Parasitología y Biomedicina López Neyra (IPBLN-CSIC, Granada, Spain) for amplicon sequencing using Miseq V3 (600 cycles) kit (Illumina Inc., San Diego, CA, USA). Primers used for the amplification were 5'-CCTACGGGNBGCASCAG-3' and reverse: 5'-GACTACNVGGGTATCTAATCC-3' targeting the V3-V5 hypervariable region of the prokaryotic 16S rRNA gene (Takahashi et al., 2014). Paired-end reads were demultiplexed and had primer sequences removed using QIIME 2 (Bolyen et al., 2019). Reads were merged, denoised and chimera checked using the DADA2 plugin (Callahan et al. 2016). Amplicon sequence variants (ASV) were identified and then taxonomy was assigned to ASVs using the q2-feature-classifier (Bokulich et al. 2018) classify-sklearn naïve Bayes taxonomy classifier against the Silva

132 99% reference sequences (Quast et al, 2013). Once alignment was performed, the number of sequences per sample for each microbial group was normalized across all the samples and singletons were removed. Raw sequences reads were deposited at European Nucleotide Archive repository (accession: PRJEB45956).

Calculations and statistical analysis

Statistical analyses were carried out using SPSS software (IBM Corp., Version 26.0, New York, USA). To assess the effect of time on the fermentative activity throughout the incubation, rumen fermentation parameters were analysed based on a repeated measures mixed effects ANOVA as follows:

$$Yijlkm = \mu + Si + Tj + STij + R_k + eijlk$$

where *Yijlm* is the dependent, continuous variable, μ is the overall population of the mean, *Si* is the fixed effect the type of saliva (i= AUT vs OWN vs GOAT vs SHEEP), *Tj* is the fixed effect of the time (j= 12h vs 36h vs 60h vs 84h vs 108h vs 132h vs 156h sampling times), *STij* is the interaction term, R_k is the random effect of the rumen fluid and *eijlk* is the residual error. To assess only the effect of the type of saliva used in the incubation when this became stable, rumen fermentation parameters, quantitative PCR data and microbial taxa abundances at days 6 and 7 and incubation metabolites at day 7 were analysed using an ANOVA test with the saliva treatment (AUT vs OWN vs GOAT vs SHEEP) as fixed effect and the sampling times as a block. When significant effects were detected, means were compared by Fisher's protected LSD-test. Quantitative PCR data and microbial relative abundances were log10 transformed before the analysis to achieve a normal distribution. Only prokaryotic families & genera with relative abundance > 0.1% across saliva treatments were further considered for taxonomic analyses (in % of sequences). In all analyses, significant effects were declared at $P < 0.05$ and tendency to difference at $P < 0.1$.

Proteomic and metabolomic heatmaps based on emPAI and mTIC values, respectively, were constructed using RStudio (R Foundation for Statistical Computing, Vienna, Austria) to characterize the salivas before incubation and the effect of saliva on the rumen metabolome at 156h of *in vitro* incubation. A Permutation based Analysis of Variance (PERMANOVA) with 999 random permutations based on the Bray Curtis Dissimilarity

Matrix was performed based on the mTIC values to compare the metabolomes across treatments using PAST software (Hammer et al., 2001). A Venn diagram was performed to illustrate the saliva treatment effects on the microbial community using a multiple list comparator (www.molbiotools.com). To illustrate the treatment impact on the *in vitro* rumen prokaryotic community, a PERMANOVA based on the Bray Curtis Dissimilarity Matrix was performed on log10 transformed sequencing data with 999 random permutations. Pair-wise comparisons were performed to compare the microbial composition across treatments. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was carried out on center log ratio transformed sequencing data to show the effect of the treatment on the prokaryotic communities' structure.

REFERENCES

Ammar, H., Bodas, R., González, J. S., Salem, A. Z. M., Giráldez, F. J., Andrés, S., & López, S. (2013). Effects of pre-incubation in sheep and goat saliva on in vitro rumen digestion of tanniferous browse foliage. *Journal of Agricultural Science*, *151*(6), 898–906. https://doi.org/10.1017/S002185961300018X

Ammar, H., López, S., Salem, A. Z. M., Bodas, R., & González, J. S. (2011). Effect of saliva from sheep that have ingested quebracho tannins on the in vitro rumen fermentation activity to digest tannin-containing shrubs. *Animal Feed Science and Technology*, *163*(2–4), 77–83. https://doi.org/10.1016/j.anifeedsci.2010.10.007

Ang, C. S., Binos, S., Knight, M. I., Moate, P. J., Cocks, B. G., & McDonagh, M. B. (2011). Global survey of the bovine salivary proteome: Integrating multidimensional prefractionation, targeted, and glycocapture strategies. *Journal of Proteome Research*, *10*(11), 5059–5069. https://doi.org/10.1021/pr200516d

Arike, L., & Peil, L. (2014). Spectral Counting Label-Free Proteomics. In *Methods in Molecular Biology* (Vol. 1156, pp. 213–222). https://doi.org/10.1007/978-1-4939-0685-7

Artegoitia, V. M., Foote, A. P., Lewis, R. M., & Freetly, H. C. (2017). Rumen fluid metabolomics analysis associated with feed efficiency on crossbred steers. *Scientific Reports*, *7*(1), 1–14. https://doi.org/10.1038/s41598-017-02856-0

Bailey, C. B., & Balch, C. C. (1961). Saliva secretion and its relation to feeding in cattle. *British Journal of Nutrition*, *15*(4), 489–498. https://doi.org/10.1079/bjn19610062

Barka, E. A., Vatsa, P., Sanchez, L., Nathalie Gaveau-Vaillant, C. J., Klenk, H.-P., Clément, C., Ouhdouch, Y., & P. van Wezeld, G. (2016). Taxonomy, Physiology, and Natural Products of Actinobacteria. *American Society for Microbiology*, *80*(1), 1–43. https://doi.org/10.1128/MMBR.00019-15.Address

Bateman, A., Martin, M. J., Orchard, S., Magrane, M., Agivetova, R., Ahmad, S., Alpi, E., Bowler-Barnett, E. H., Britto, R., Bursteinas, B., Bye-A-Jee, H., Coetzee, R., Cukura, A., Silva, A. Da, Denny, P., Dogan, T., Ebenezer, T. G., Fan, J., Castro, L. G., … Zhang, J. (2021). UniProt: The universal protein knowledgebase in 2021. *Nucleic Acids Research*, *49*(D1), D480–D489. https://doi.org/10.1093/nar/gkaa1100

Belanche, A., Palma-Hidalgo, J. M., Nejjam, I., Serrano, R., Jiménez, E., Martín-García, I., & Yáñez-Ruiz, D. R. (2019). In vitro assessment of the factors that determine the activity of the rumen microbiota for further applications as inoculum. *Journal of the Science of Food and Agriculture*, *99*(1), 163–172. https://doi.org/10.1002/jsfa.9157

Bokulich, N. A., Kaehler, B. D., Rideout, J. R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., & Gregory Caporaso, J. (2018). Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome*, *6*(1), 1–17. https://doi.org/10.1186/s40168-018-0470-z

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cheaib, Z., & Lussi, A. (2013). Role of amylase, mucin, IgA and albumin on salivary protein buffering capacity: A pilot study. *Journal of Biosciences*, *38*(2), 259–265. https://doi.org/10.1007/s12038-013-9311-1

de Almeida, R. T. R., do Prado, R. M., Porto, C., dos Santos, G. T., Huws, S. A., & Pilau, E. J. (2018). Exploring the rumen fluid metabolome using liquid chromatography-high-resolution mass spectrometry and Molecular Networking. *Scientific Reports*, *8*(1), 4–11. https://doi.org/10.1038/s41598-018-36196-4

Dehority, B. A. (2003). Rumen Microbiology. Nottingham: Nottingham University Press. ISBN: 1-897676-99-9

Denman, S. E., & McSweeney, C. S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology Ecology*, *58*(3), 572–582. https://doi.org/10.1111/j.1574-6941.2006.00190.x

Denman, S. E., Tomkins, N. W., & McSweeney, C. S. (2007). Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology*, *62*(3), 313–322. https://doi.org/10.1111/j.1574-6941.2007.00394.x

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W. C., Conner, M. E., Earl, A. M., Knight, R., Bjorkman, P. J., & Mazmanian, S. K. (2018). Gut microbiota utilize immunoglobulin a for mucosal colonization. *Science*, *360*(6390), 795–800. https://doi.org/10.1126/science.aaq0926

Eugène, M., Archimède, H., & Sauvant, D. (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. https://doi.org/10.1016/S0301-6226(03)00117-9

Fábián, T. K., Hermann, P., Beck, A., Fejérdy, P., & Fábián, G. (2012). Salivary defense proteins: Their network and role in innate and acquired oral immunity. *International Journal of Molecular Sciences*, *13*(4), 4295–4320. https://doi.org/10.3390/ijms13044295

Faniyi, T. O., Adegbeye, M. J., Elghandour, M. M. M. Y., Pilego, A. B., Salem, A. Z. M., Olaniyi, T. A., Adediran, O., & Adewumi, M. K. (2019). Role of diverse fermentative factors towards microbial community shift in ruminants. *Journal of Applied Microbiology*, *127*(1), 2–11. https://doi.org/10.1111/jam.14212

Fouhse, J. M., Smiegielski, L., Tuplin, M., Guan, L. L., & Willing, B. P. (2017). Host immune selection of rumen bacteria through salivary secretory IgA. *Frontiers in Microbiology*, *8*(MAY), 1–9. https://doi.org/10.3389/fmicb.2017.00848

Gardner, A., Carpenter, G., & So, P. W. (2020). Salivary metabolomics: From diagnostic biomarker discovery to investigating biological function. *Metabolites*, *10*(2). https://doi.org/10.3390/metabo10020047

Gardner, A., Parkes, H. G., So, P. W., & Carpenter, G. H. (2019). Determining bacterial and host contributions to the human salivary metabolome. *Journal of Oral Microbiology*, *11*(1). https://doi.org/10.1080/20002297.2019.1617014

Gómez, E., Muñoz, M., Simó, C., Ibáñez, C., Carrocera, S., Martín-González, D., & Cifuentes, A. (2016). Non-invasive metabolomics for improved determination of embryonic sex markers in chemically defined culture medium. *Journal of Chromatography* A, 1474, 138–144. https://doi.org/10.1016/j.chroma.2016.10.080

Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., & Janssen, P.J. (2015). Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports*, 5(1), 1-15. https://doi.org/10.1038/srep14567

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J., & Mann, M. (2005). Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Molecular and Cellular Proteomics*, *4*(9), 1265–1272. https://doi.org/10.1074/mcp.M500061-MCP200

Jami, E., Israel, A., Kotser, A., & Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. *ISME Journal*, *7*(6), 1069–1079. https://doi.org/10.1038/ismej.2013.2

Jami, E., White, B. A. & Mizrahi, I. (2014). Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. PLoS ONE 9, e85423. https://doi.org/10.1371/journal.pone.0085423.

Koskella, B., & Bergelson, J. (2020). The study of host-microbiome (co)evolution across levels of selection. Philos. Trans. R. Soc. B Biol. Sci. 375, 8. https://doi.org/10.1098/rstb.2019.0604

Lamy, E., da Costa, G., Santos, R., Capela e Silva, F., Potes, J., Pereira, A., Coelho, A. V., & Sales Baptista, E. (2009). Sheep and goat saliva proteome analysis: A useful tool for

ingestive behavior research? *Physiology and Behavior*, *98*(4), 393–401. https://doi.org/10.1016/j.physbeh.2009.07.002

Lamy, E., Da Costa, G., Santos, R., Capelae Silva, F., Potes, J., Pereira, A., Coelho, A. V., & Sales Baptista, E. (2011). Effect of condensed tannin ingestion in sheep and goat parotid saliva proteome. *Journal of Animal Physiology and Animal Nutrition*, *95*(3), 304–312. https://doi.org/10.1111/j.1439-0396.2010.01055.x

Langda, S., Zhang, C., Zhang, K., Gui, B., Ji, D., Deji, C., Cuoji, A., Wang, X., & Wu, Y. (2020). Diversity and composition of rumen bacteria, fungi, and protozoa in goats and sheep living in the same high-altitude pasture. *Animals*, *10*(2). https://doi.org/10.3390/ani10020186

Lascelles, A. K., & McDowell, G. H. (1974). Localized Humoral Immunity with Particular Reference to Ruminants. *Immunological Reviews*, *19*(1), 170–208. https://doi.org/10.1111/j.1600-065X.1974.tb00132.x

Loo, J. A., Yan, W., Ramachandran, P., & Wong, D. T. (2010). Comparative human salivary and plasma proteomes. *Journal of Dental Research*, *89*(10), 1016–1023. https://doi.org/10.1177/0022034510380414

Mach, J. P., & Pahud, J. J. (1971). Secretory IgA, a major immunoglobulin in most bovine external secretions. *The Journal of Immunology*, *106*(2), 552–563.

Maeda, H., Fujimoto, C., Haruki, Y., Maeda, T., Kokeguchi, S., Petelin, M., Arai, H., Tanimoto, I., Nishimura, F., & Takashiba, S. (2003). Quantitative real-time PCR using TaqMan and SYBR Green for Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, tetQ gene and total bacteria. *FEMS Immunology and Medical Microbiology*, *39*(1), 81–86. https://doi.org/10.1016/S0928-8244(03)00224-4

Mancera-Arteu, M., Giménez, E., Sancho, J., & Sanz-Nebot, V. (2020). Alterations in the Glycan Profile of Mouse Transferrin: New Insights in Collagen-Induced Arthritis. Journal of Proteome Research, 19(4), 1750–1759. https://doi.org/10.1021/acs.jproteome.0c00016

Mandel, I. D. (1987). The functions of saliva. *Journal of Dental Research*, *66*, 623–627. https://doi.org/10.1007/BF01354542

Mantis, N. J., Rol, N., & Corthésy, B. (2011). Secretory IgA's Complex Roles in Immunity and Mucosal Homeostasis in the Gut. *Mucosa Immunology*, *4*(6), 603–611. https://doi.org/10.1038/mi.2011.41.Secretory

Mcdougall, E. I. (1948). Studies on ruminant saliva. 1. The composition and output of sheep's saliva. *Biochemical Journal*, *43*, 99–109.

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-gut microbiota metabolic interactions. *Science*, *336*(6086), 1262–1267. https://doi.org/10.1126/science.1223813

Fiehn, O. (2017). Metabolomics by Gas Chromatography-Mass Spectrometry: the combination of targeted and untargeted profiling. In *Curr Protoc Mol Biol.* (Vol. 7, Issue 3). https://doi.org/10.1002/0471142727.mb3004s114.Metabolomics

Palma-Hidalgo, J. M., Belanche, A., Jiménez, E., Martín-García, A. I., Newbold, C. J., & Yáñez-Ruiz, D. R. (2021). Short communication: Saliva and salivary components affect goat rumen fermentation in short-term batch incubations. *Animal*, *15*(7), 100267. https://doi.org/10.1016/j.animal.2021.100267

Palma-Hidalgo, J. M., Jiménez, E., Popova, M., Morgavi, D. P., Martín-García, A. I., Yáñez-Ruiz, D. R., & Belanche, A. (2021). Inoculation with rumen fluid in early life accelerates the rumen microbial development and favours the weaning process in goats. *Animal Microbiome*, *3*(1). https://doi.org/10.1186/s42523-021-00073-9

Peterson, D. A., McNulty, N. P., Guruge, J. L., & Gordon, J. I. (2007). IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis. *Cell Host and Microbe*, *2*(5), 328–339. https://doi.org/10.1016/j.chom.2007.09.013

Porter, P., & Noakes, D. E. (1970). Immunoglobulin IgA in bovine serum and external secretions. *BBA - Protein Structure*, *214*(1), 107–116. https://doi.org/10.1016/0005-2795(70)90074-7

Precup, G., & Vodnar, D. C. (2019). Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: A comprehensive literature review. *British Journal of Nutrition*, *122*(2), 131–140. https://doi.org/10.1017/S0007114519000680

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: Improved data

processing and web-based tools. *Nucleic Acids Research*, *41*(D1), 590–596. https://doi.org/10.1093/nar/gks1219

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., & Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. *Journal of Applied Microbiology*, *116*(2), 245–257. https://doi.org/10.1111/jam.12405

Salem, A. Z. M., López, S., Ranilla, M. J., & González, J. S. (2013). Short- to medium-term effects of consumption of quebracho tannins on saliva production and composition in sheep and goats. *Journal of Animal Science*, *91*(3), 1341–1349. https://doi.org/10.2527/jas.2010-3811

Sharpe, M. E., Latham, M. J., & Reiter, B. (1977). The immune response of the hosts animal to bacteria in the rumen and caecum. In A. C. I. I. W. McDonald (Ed.), *Proceedings of the IV International Symposium on Ruminant Physiology: Digestion and Metabolism in the Ruminant*. University of New England Publishing Unit.

Skillman, L. C., Toovey, A. F., Williams, A. J., & Wright, A. D. G. (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. https://doi.org/10.1128/AEM.72.1.200-206.2006

Somers, B. M. (1957). Salivary secretion and its functions in ruminants. *The Australian Veterinary Journal*, *33*(11), 297–301.

Stenken, J. A., & Poschenrieder, A. J. (2015). Bioanalytical Chemistry of Cytokines-A Review. *Analytica Chimica Acta*, *853*(1), 95–115. https://doi.org/https://doi.org/10.1016/j.aca.2014.10.009

Subharat, S., Shu, D., Zheng, T., Buddle, B. M., Kaneko, K., Hook, S., Janssen, P. H., & Wedlock, D. N. (2016). Vaccination of sheep with a methanogen protein provides insight into levels of antibody in saliva needed to target ruminal methanogens. *PLoS ONE*, *11*(7), 1–14. https://doi.org/10.1371/journal.pone.0159861

Sylvester, J. T., Karnati, S. K. R., Yu, Z., Morrison, M., & Firkins, J. L. (2004). Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *The Journal of Nutrition*, *134*(12), 3378–3384. https://doi.org/134/12/3378 [pii]

Takahashi, S., Tomita, J., Nishioka, K., Hisada, T., & Nishijima, M. (2014). Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0105592

Tapio, I., Shingfield, K. J., McKain, N., Bonin, A., Fischer, D., Bayat, A. R., Vilkki, J., Taberlet, P., Snelling, T. J., & Wallace, R. J. (2016). Oral samples as non-invasive proxies for assessing the composition of the rumen microbial community. *PLoS ONE*, *11*(3), 1–15. https://doi.org/10.1371/journal.pone.0151220

Tsuruta, T., Inoue, R., Tsukahara, T., Nakamoto, M., Hara, H., Ushida, K., & Yajima, T. (2012). Commensal bacteria coated by secretory immunoglobulin A and immunoglobulin G in the gastrointestinal tract of pigs and calves. *Animal Science Journal*, *83*(12), 799–804. https://doi.org/10.1111/j.1740-0929.2012.01026.x

Warner, A. C. I., & Stacy, B. D. (1977). Influence of ruminal and plasma osmotic pressure on salivary secretion in sheep. *Quarterly Journal of Experimental Physiology*, *62*, 133–142.

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. *Frontiers in Microbiology*, *6*(APR), 1–16. https://doi.org/10.3389/fmicb.2015.00296

Williams, A.G., and Coleman, G. S. (1992). *The Rumen Protozoa* (Vol. 1, Issue). Springer-Verlag New York Inc. https://doi.org/10.1007/978-1-4612-2776-2

Yáñez-Ruiz, D. R., Abecia, L., & Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: A review. *Frontiers in Microbiology*, *6*(OCT), 1–12. https://doi.org/10.3389/fmicb.2015.01133

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., & Aldridge, B. M. (2018). Biogeographical Differences in the Influence of Maternal Microbial Sources on the Early Successional Development of the Bovine Neonatal Gastrointestinal tract. *Scientific Reports*, *8*(1), 1–14. https://doi.org/10.1038/s41598-018-21440-8

Yoshizawa, J. M., Schafer, C. A., Schafer, J. J., Farrell, J. J., Paster, B. J., & Wong, D. T. W. (2013). Salivary biomarkers: Toward future clinical and diagnostic utilities. *Clinical Microbiology Reviews*, *26*(4), 781–791. https://doi.org/10.1128/CMR.00021-13

Zheng, D., Liwinski, T. & Elinav, E. (2020). Interaction between microbiota and immunity in health and disease. *Cell Resesearch*, 30, 492–506. https://doi.org/10.1038/s41422-020-0332-7

ACKNOWLEDGEMENTS

This study was funded by the Spanish Research Agency (Ref. AGL2017 86938-R). JMPH had a Training Program for Academics grant, Madrid, Spain (Ref. FPU16/01981) and AB is a Ramón y Cajal fellow [RYC2019-027764-I/AEI/10.13039/501100011033] from the Spanish Research Agency. The authors thank Isabel Jiménez, Eugenia Guillén and Manuel Fondevila for their help in laboratory procedures and advice.

AUTHOR CONTRIBUTIONS

DRYR, CJN & AB: Conceptualization, validation and supervision.

JMPH, AB, JMPH, EJ & AIMG: Methodology, investigation, resources and formal analysis.

JMPH, AB, SED: Data curation and software.

DRYR: funding acquisition.

JMPH: Writing original draft.

DRYR, AB, SED & CJN: Writing – review and editing.

COMPETING INTERESTS

The authors declare no competing interests.

Tables and Figures

Table 1. Effect of the sampling time on the *in vitro* rumen fermentation.

	Sampling time							SEM	*P*-value		
	12h	36h	60h	84h	108h	132h	156h		*Saliva*	*Time*	*SxT*
pH	6.44	6.62	6.63	6.68	6.69	6.68	6.62	0.00549	0.432	<0.001	0.106
Gas volume, mL/12h	14.3a	6.86c	8.03b	8.41b	6.59c	8.08b	8.19b	0.155	0.203	<0.001	0.038
Total VFA, mM	70.8a	69.6ab	67.6bc	68.7abc	66.1c	63.2d	58.5e	0.599	0.017	<0.001	0.013
Acetate, %	60.3	59.3	60.4	62.1	63.7	64.6	64.4	0.242	0.409	<0.001	0.066
Propionate, %	27.4a	26.8a	25.4b	23.8c	22.8cd	22.3d	22.9cd	0.280	0.012	<0.001	0.030
Isobutyrate, %	1.18	1.35	1.32	1.31	1.53	1.36	1.24	0.0219	0.598	0.162	0.469
Butyrate, %	9.02ab	9.44a	9.44a	9.19a	8.58bc	8.19c	8.17c	0.105	0.264	<0.001	0.016
Isovalerate, %	1.13d	1.51c	1.69ab	1.81a	1.74a	1.82a	1.67bc	0.0257	0.103	<0.001	0.025
Valerate, %	0.963d	1.56c	1.69b	1.81a	1.74ab	1.82a	1.67bc	0.0321	0.217	<0.001	0.022
Ac/Pro	2.26d	2.26d	2.41c	2.63b	2.81a	2.91a	2.82a	0.368	0.061	<0.001	0.047

Within a row, means with different letters differ ($P < 0.05$).

Table 2. Effect of the incubation with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP) on in vitro rumen fermentation and the abundance of the major rumen microbial groups.

	Saliva				SEM	*P-value*
	AUT	OWN	GOAT	SHEEP		
pH	6.65^{a}	6.62^{a}	6.56^{b}	6.64^{a}	0.0087	<0.001
Gas Volume, ml/12h	5.97^{b}	6.55^{a}	6.60^{a}	6.61^{a}	0.118	0.003
Total VFA, mM	58.8	60.1	61.3	62.4	0.786	0.287
Acetate, %	63.9	64.7	64.5	65.3	0.195	0.061
Propionate, %	22.7^{ab}	22.3^{b}	23.6^{a}	21.8^{b}	0.200	0.003
Isobutyrate, %	1.32	1.34	1.24	1.26	0.0389	0.766
Butyrate, %	8.52^{a}	8.06^{ab}	7.58^{b}	8.39^{a}	0.128	0.018
Isovalerate, %	1.67^{ab}	1.78^{a}	1.49^{c}	1.61^{bc}	0.0345	0.005
Valerate, %	1.62	1.66	1.54	1.60	0.0365	0.089
Ac/Pro	2.82^{b}	2.91^{ab}	2.74^{bc}	3.00^{a}	0.305	0.005
Microbes, log10 copy/ml						
Bacteria	10.2^{bc}	10.4^{ab}	10.2^{c}	10.4^{a}	0.0341	0.013
Archaea	7.09	7.11	7.00	7.23	0.0367	0.156
Protozoa	6.01^{ab}	6.17^{ab}	5.75^{b}	6.38^{a}	0.0924	0.046
Fungi	5.89	6.11	5.90	5.97	0.0697	0.297

Within a row, means with different letters differ ($P < 0.05$)

Table 3. Abundance of the rumen prokaryotic taxa when incubated incubated with autoclaved (AUT), own, goat or sheep saliva.

Prokaryotic taxa, % sequences	AUT	OWN	GOAT	SHEEP	SEM	*P*-Value
p_Actinobacteria	2.03^{b}	2.41^{ab}	3.44^{a}	3.04^{a}	0.196	0.0385
f_Atopobiaceae, g_Atopobium	0.499^{b}	0.761^{ab}	0.948^{a}	0.724^{ab}	0.0559	0.023
f_Coriobacteriaceae, g_Olsenella	1.81^{b}	1.99^{ab}	2.96^{a}	2.81^{a}	0.187	0.013
f_Eggerthellaceae	0.139	0.142	0.155	0.123	0.00962	0.463
p_Bacteroidetes	51.7	52.7	52.1	54.0	0.865	0.837
f_Bacteroidaceae, g_Bacteroides	3.53^{a}	3.01^{a}	1.58^{b}	1.82^{b}	0.192	0.001
f_Bacteroidales BS11 gut group	0.421	0.395	0.442	0.449	0.0652	0.413
f_Bacteroidales RF16 group	0.182^{a}	0.0767^{c}	0.101^{bc}	0.134^{ab}	0.0127	0.013
f_Bacteroidales UCG-001	0.364	0.543	0.431	0.468	0.0346	0.210
f_F082	4.14^{b}	6.77^{a}	5.85^{a}	6.01^{a}	0.397	0.031
g_Bacteroidales bacterium Bact_22	1.09	1.88	1.22	0.333	0.311	0.461
f_Muribaculaceae	0.115	0.356	0.185	0.207	0.0503	0.127
f_p-251-o5	0.137	0.177	0.260	0.156	0.0245	0.381
f_Prevotellaceae	22.8^{b}	27.0^{a}	27.9^{a}	27.7^{a}	0.571	<0.001
g_Prevotella 1	19.5^{b}	22.3^{a}	23.9^{a}	23.8^{a}	0.489	0.001
g_Prevotellaceae Ga6A1 group	0.577	0.761	0.844	0.631	0.0483	0.226
g_Prevotellaceae UCG-001	0.738^{b}	1.164^{a}	1.138^{a}	0.999^{ab}	0.0525	0.022
g_Prevotellaceae UCG-003	4.85^{a}	3.80^{bc}	3.36^{c}	4.36^{ab}	0.147	0.001
f_Rikenellaceae	19.8^{a}	14.9^{b}	15.3^{b}	17.1^{ab}	0.618	0.003
g_Rikenellaceae RC9 gut group	19.2	15.5	15.5	16.8	0.719	0.193
p_Chloroflexi, f_Anaerolineaceae	0.118	0.184	0.135	0.184	0.0126	0.121
p_Cyanobacteria	0.124	0.0458	0.0733	0.137	0.0209	0.630
f_Endomicrobiaceae	0.0315^{b}	0.0187^{b}	0.0447^{b}	0.434^{a}	0.0519	<0.001
p_Elusimicrobia	0.244^{a}	0.0920^{b}	0.0475^{b}	0.426^{a}	0.0523	<0.001
p_Euryarchaeota	0.699	0.672	0.753	0.884	0.0607	0.801
f_Methanobacteriaceae, g_Methanobrevibacter	0.780	0.698	0.818	0.978	0.0627	0.916
p_Fibrobacteres	0.644	0.964	0.886	0.695	0.0683	0.403
f_Fibrobacteraceae, g_Fibrobacter	0.794	1.21	1.09	0.866	0.0683	0.060
p_Firmicutes	31.7	29.4	30.9	28.2	0.818	0.434
f_Acidaminococcaceae, g_Succiniclasticum	1.98	2.17	2.09	2.11	0.0792	0.974
f_Christenellaceae, g_Christensenellaceae R-7 group	8.41	7.27	7.45	6.96	0.275	0.307
f_Clostridiales vadimbb60 group	0.271	0.342	0.260	0.260	0.0150	0.229
f_Erysipelotrichaceae	0.889	0.784	1.01	0.831	0.0490	0.223
f_Family XIII	1.51^{a}	1.32^{ab}	1.11^{b}	1.12^{b}	0.0526	0.035
g_Family XIII AD3011 group	0.592	0.746	0.629	0.523	0.0383	0.639
f_Lachnospiraceae	8.75	7.43	6.70	7.68	0.497	0.063
g_[Eubacterium] oxidoreducens group	1.51^{a}	0.997^{c}	0.817^{bc}	1.25^{ab}	0.159	0.004
g_Butyrivibrio 2	1.15^{a}	0.954^{ab}	0.725^{b}	0.873^{b}	0.0567	0.021
g_Lachnospiraceae XPB1014 group	0.784^{ab}	0.694^{b}	0.954^{a}	0.751^{b}	0.0333	0.044
f_Ruminococcaceae	6.70	6.23	5.79	6.24	0.125	0.093
g_Ruminococcaceae NK4A214 group	1.47	1.49	1.50	1.70	0.0608	0.213
g_Ruminococcaceae UCG-010	0.783	0.871	0.727	0.769	0.0250	0.593

(continues on the next page)

Prokaryotic taxa, % sequences	AUT	OWN	GOAT	SHEEP	SEM	*P*-Value
g_Ruminococcaceae UCG-014	1.18	1.13	0.858	1.22	0.107	0.056
g_Ruminococcus 1	0.773	0.737	0.765	0.605	0.0424	0.416
f_Streptococcaceae, g_Streptococcus	1.43^{b}	1.95^{b}	5.07^{a}	2.11^{ab}	0.424	0.020
f_Veillonellaceae	3.75^{a}	3.60^{a}	3.68^{a}	2.76^{b}	0.134	0.002
g_Anaerovibrio	1.38	1.23	1.45	1.51	0.0829	0.644
g_Quinella	0.920^{a}	1.01^{a}	0.792^{a}	0.0898^{b}	0.100	<0.001
g_Veillonellaceae UCG-001	1.55	1.68	1.75	1.38	0.0661	0.060
p_Kiritimatiellaeota	0.201^{c}	0.668^{a}	0.397^{ab}	0.330^{bc}	0.0472	0.001
p_Lentisphaerae	0.0742	0.0755	0.0475	0.0587	0.00565	0.435
p_Patescibacteria	0.792	0.752	0.956	1.522	0.130	0.127
f_Saccharimonadaceae, g_Candidatus Saccharimonas	0.881^{b}	0.926^{b}	1.16^{b}	1.87^{a}	0.124	0.009
p_Planctomycetes	0.0409	0.0782	0.0502	0.0547	0.00681	0.300
p_Proteobacteria	7.47^{ab}	8.08^{a}	6.03^{b}	5.95^{b}	0.321	0.036
f_Desulfovibrionaceae, g_Desulfovibrio	0.695	0.784	0.856	0.751	0.0353	0.596
f_Succinivibrionaceae	6.96^{ab}	7.37^{a}	5.18^{bc}	5.12^{c}	0.331	0.031
g_Ruminobacter	4.29	5.35	3.14	4.31	0.332	0.213
g_Succinivibrio	1.36	1.27	1.15	0.961	0.0938	0.158
g_Succinivibrionaceae UCG-002	2.18	2.04	1.72	0.796	0.232	0.075
p_Spirochaetes, f_Spirochaetaceae	1.42	1.36	1.36	1.54	0.117	0.749
g_Treponema 2	1.29	1.32	1.30	1.44	0.112	0.729
p_Synergistetes, f_Synergistaceae	1.71	1.39	1.59	1.75	0.119	0.065
g_Fretibacterium	1.18	1.29	1.49	1.50	0.0969	0.429
p_Tenericutes	0.931	0.966	1.16	1.21	0.0973	0.639
f_Anaeroplasmataceae, g_Anaeroplasma	0.684	0.766	1.21	1.06	0.0845	0.193
p_Verrucomicrobia	0.0809	0.109	0.0893	0.107	0.0139	0.971
p_unidentified	0.0364	0.0458	0.0293	0.0071	0.00618	0.136

Within a row, means with different letters differ ($P < 0.05$).

Table 4. Detected metabolites when goat rumen fluid is incubated with autoclaved (AUT), own, goat or sheep saliva.

Metabolites, mTIC	AUT	OWN	GOAT	SHEEP	SEM	*P*-Value
(10R)-2,8-dihydroxy-[…]10H-anthracen-9-one	74	8556	31769	130	5664	0.395
(3-Carboxypropyl)trimethylammonium cation	12703	23513	15115	26276	2287	0.054
15-Ketoprostaglandin E1	55802	60253	69154	56449	3029	0.392
1-Phenylethanol	8140	11334	13970	12088	1517	0.431
2,2'-Dithiobis(benzothiazole)	26^{b}	542^{ab}	1085^{a}	367^{ab}	146	0.042
2-Mercaptobenzothiazole	61	8217	16321	2189	2513	0.037
Dibutyl sebacate	1457	1799	2053	1940	115	0.137
Dimethyl sulfoxide	1191	1681	2643	1848	228	0.163
Ethyldiethanolamine	84738^{a}	51507^{ab}	45690^{ab}	3647^{b}	9902	0.012
Gabapentin related compound D	10807	13399	15949	14736	819	0.089
Monoethyl phthalate	887	461	602	548	95.6	0.423
Nicotinamide	1086^{b}	1709^{ab}	1661^{ab}	2185^{a}	150	0.038
N-Methyl-2-pyrrolidone	37945	36997	62211	42600	4480	0.205
Norsufentanil	11620^{a}	640a^{b}	56^{b}	80^{b}	1474	0.010
Spermidine	11023	19473	19498	18089	1448	0.084
Stearamide	730^{b}	7142^{ab}	21626^{a}	1235^{ab}	3152	0.011
Tri(3-chloropropyl) phosphate	23630^{ab}	12505^{ab}	35530^{a}	6972^{b}	3270	0.010
Tryptamine	14953	20035	23434	23087	1700	0.229
Tyramine	3624^{b}	6475^{ab}	6136^{ab}	9718^{a}	743	0.020

Within a row, means with different letters differ ($P < 0.05$).

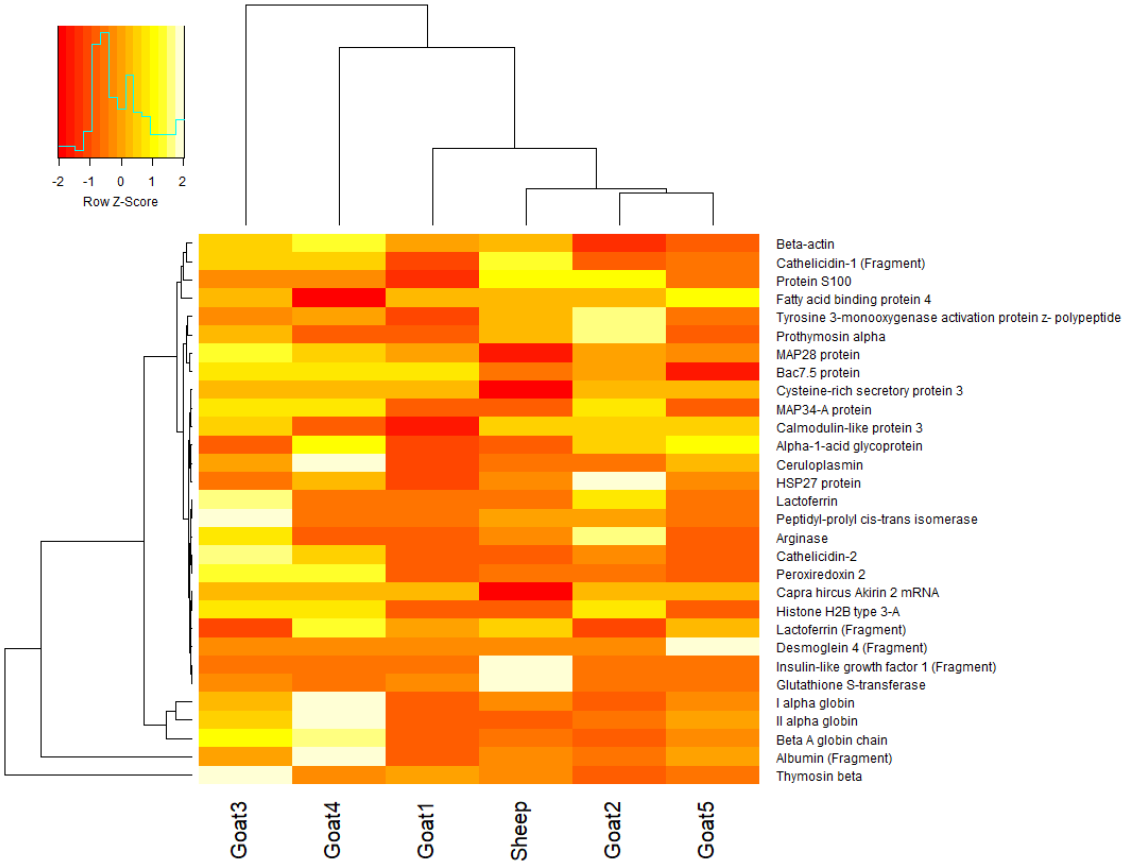


Figure 1. Heatmap showing the abundance based on emPAI values of the 30 most abundant proteins/polypeptides found across the salivas used for the in vitro incubation.

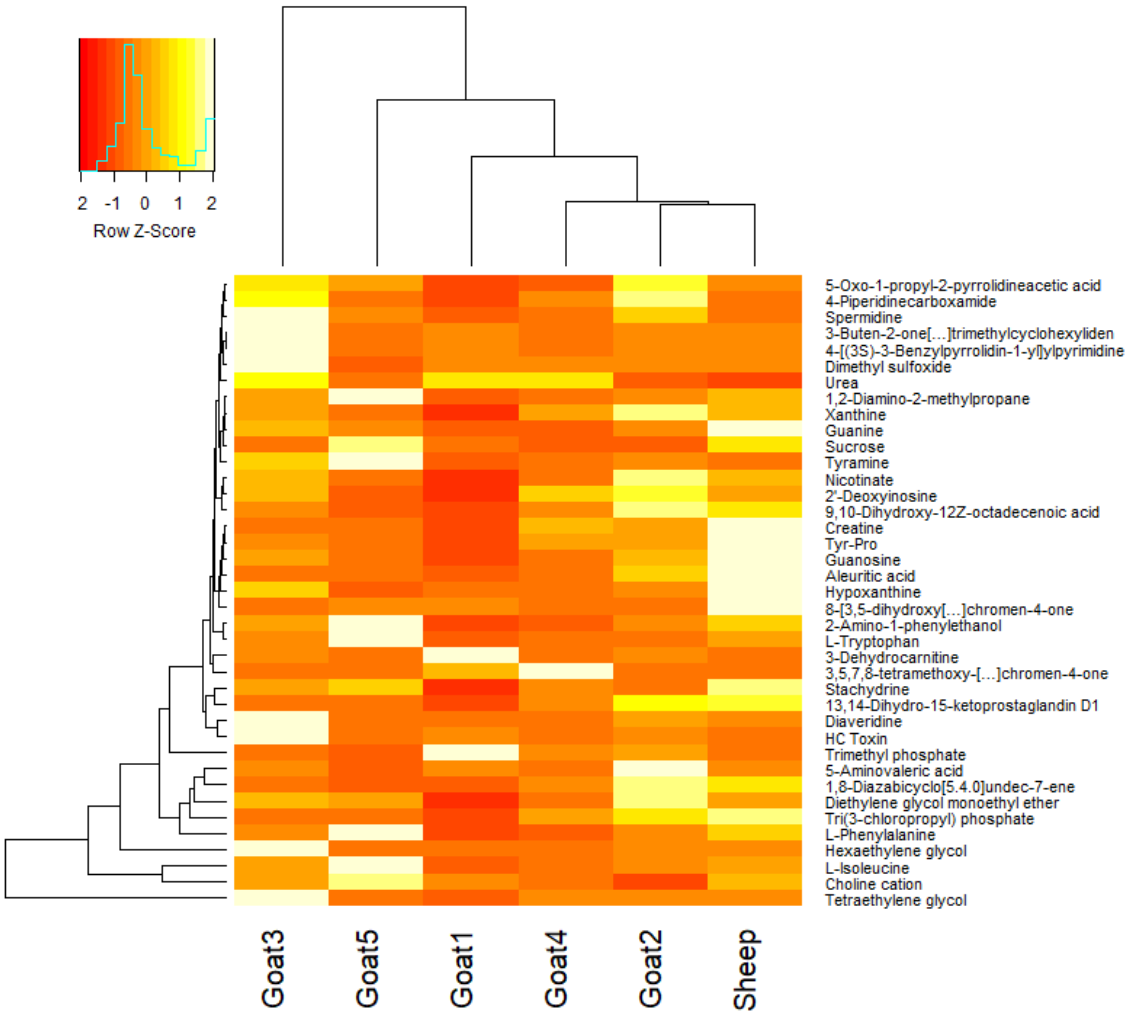


Figure 2. Heatmap showing the abundance based on mTIC scores of the 39 detected metabolites found across all saliva samples used in the *in vitro* incubation.

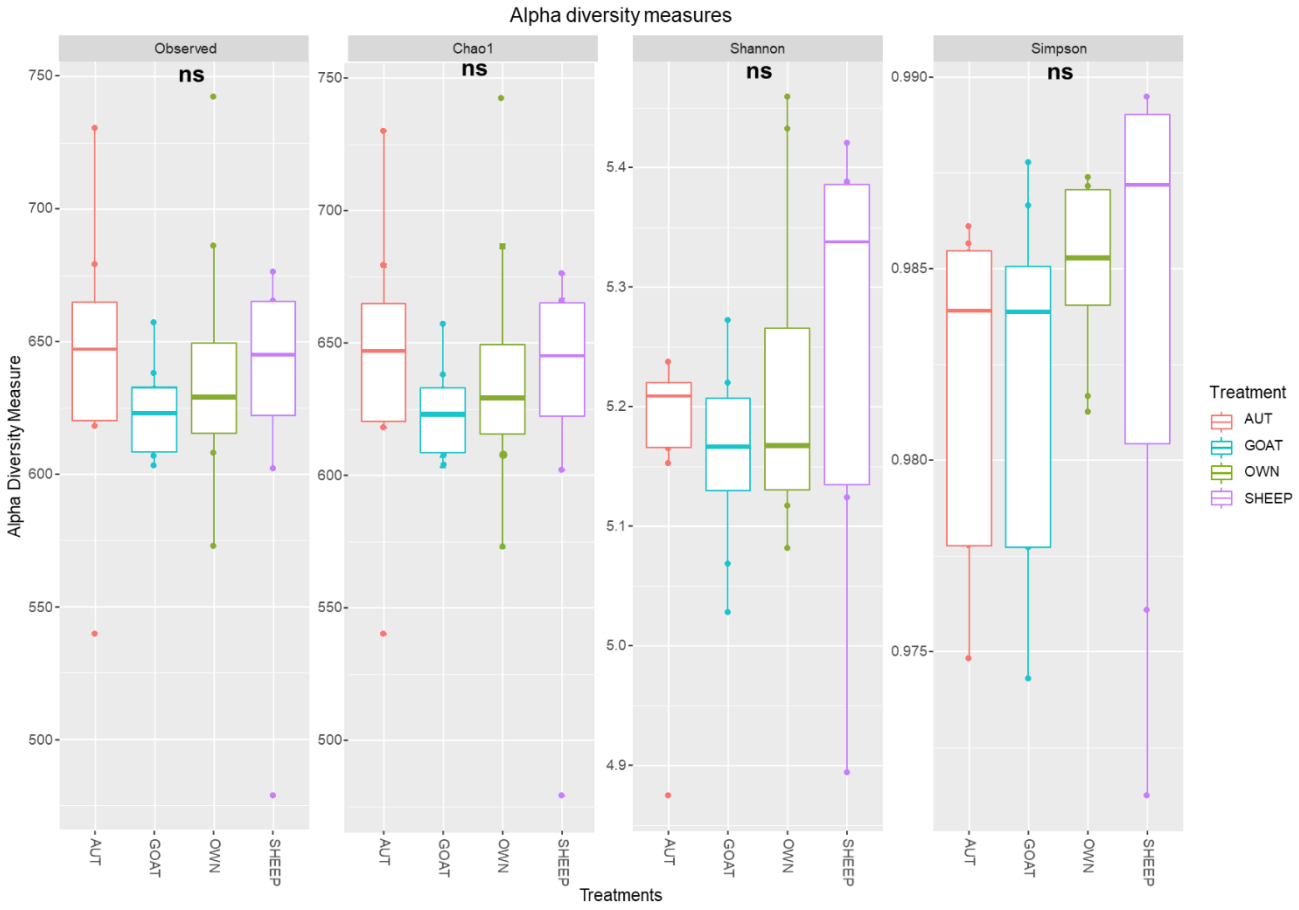


Figure 3. Boxplots indicating the rumen prokaryotic alpha-diversity in terms of observed ASVs, Chao1, Shannon and Simpson indexes.

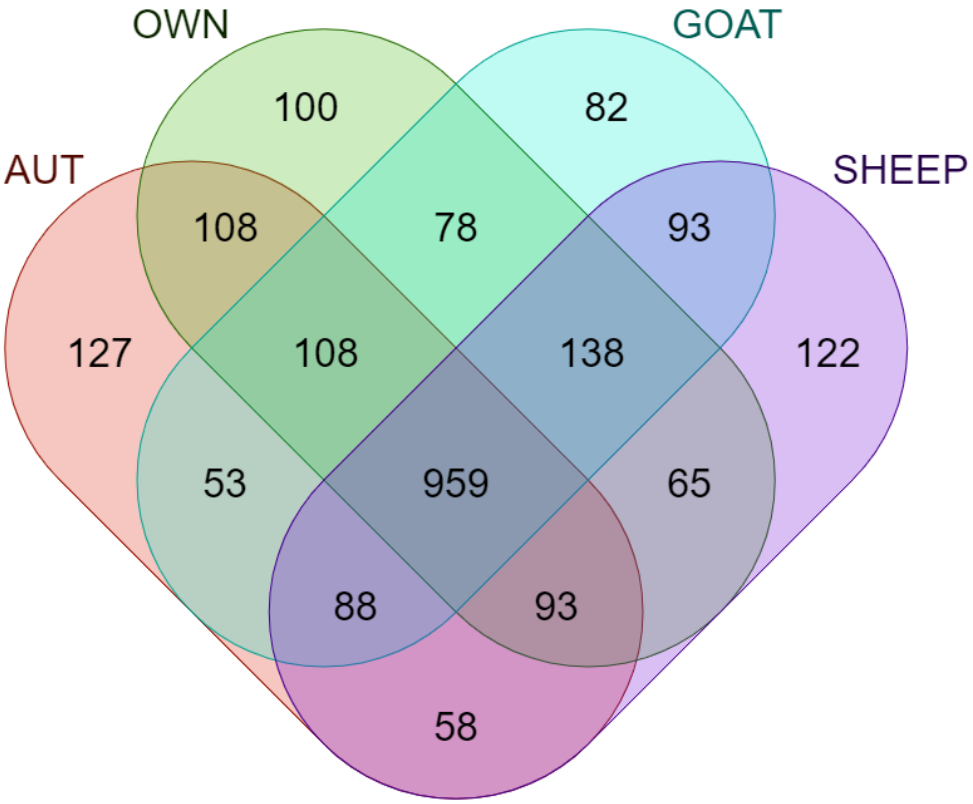


Figure 4. Venn diagram showing the unique and overlapping prokaryotic ASVs across the 4 saliva treatments used in the rumen incubation: autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP).

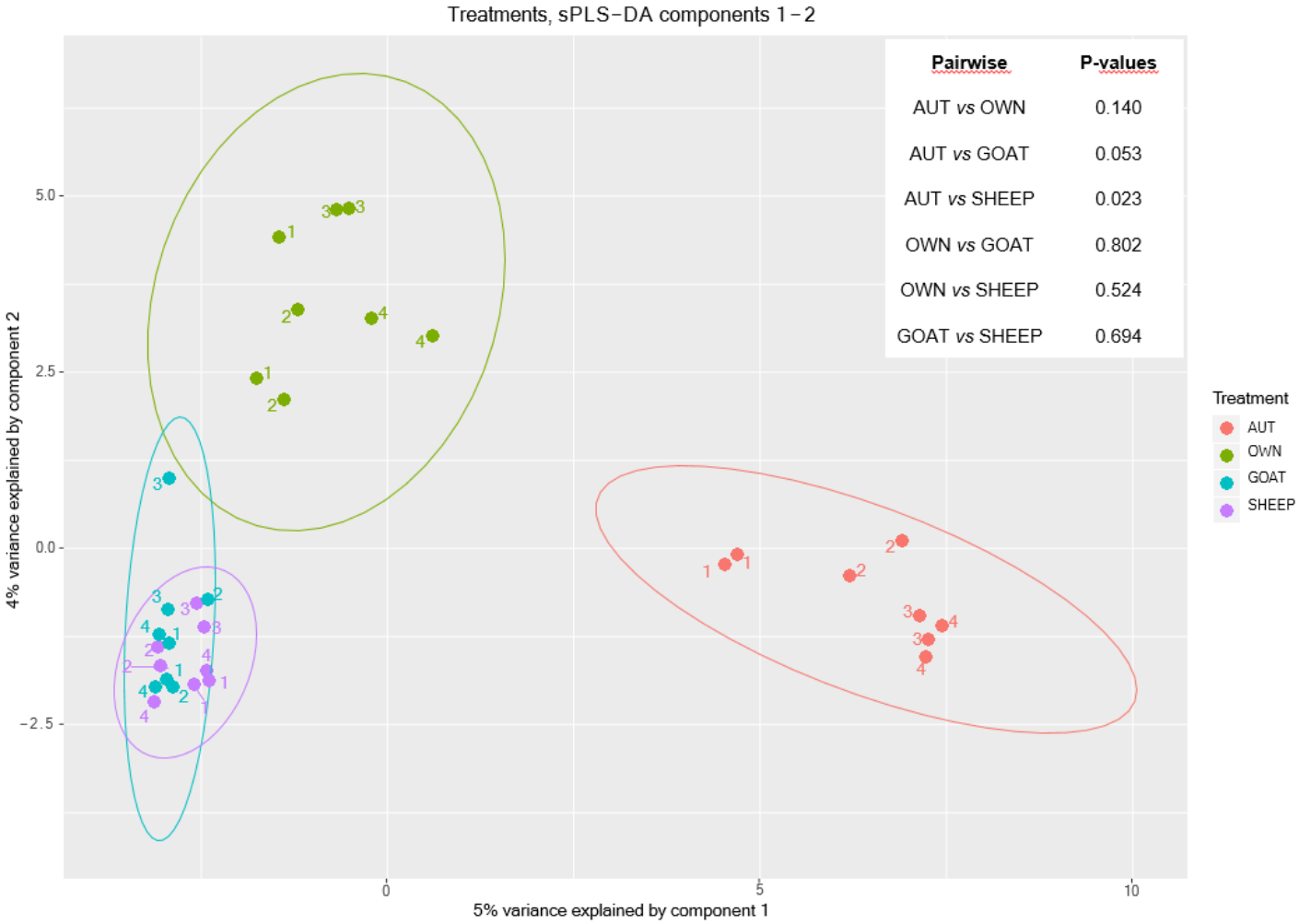


Figure 5. Sparse partial least squares discriminant analysis of the prokaryotic communities in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT), and sheep saliva (SHEEP).

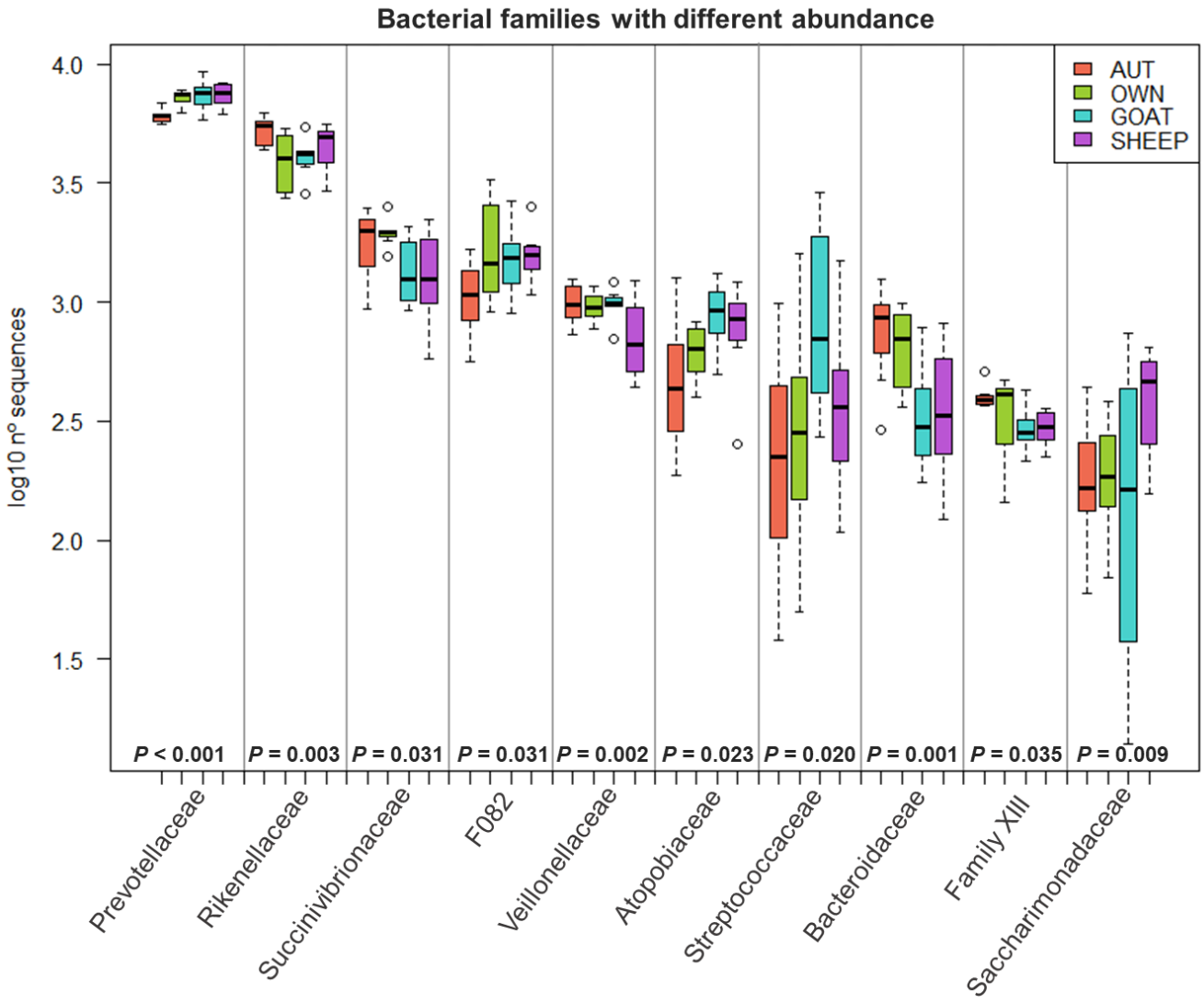
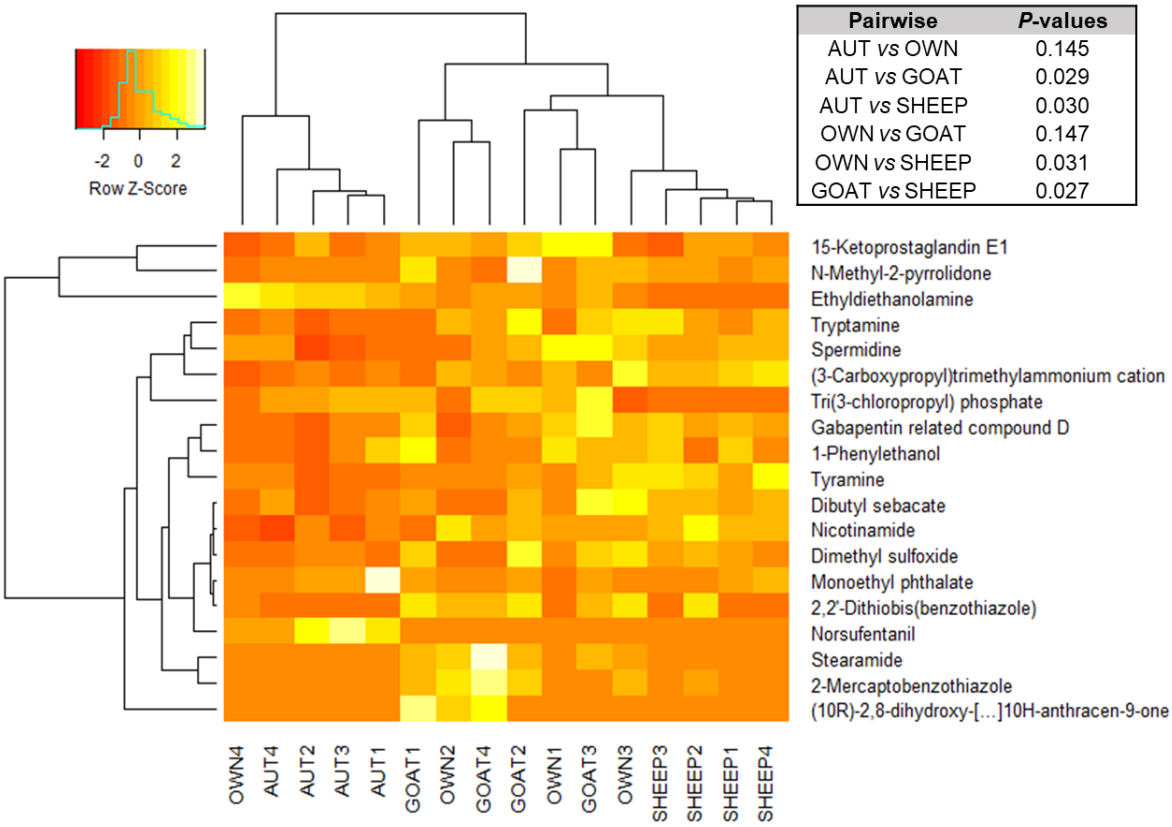


Figure 6. Relative abundance of the ten most abundant prokaryotic families in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT) or sheep saliva (SHEEP).



Pairwise	*P*-values
AUT *vs* OWN	0.145
AUT *vs* GOAT	0.029
AUT *vs* SHEEP	0.030
OWN *vs* GOAT	0.147
OWN *vs* SHEEP	0.031
GOAT *vs* SHEEP	0.027

Figure 7. Heatmap showing the abundance based on mTIC scores of the 19 detected metabolites found in rumen content incubated with autoclaved saliva (AUT), own saliva (OWN), goat saliva (GOAT) and sheep saliva (SHEEP).

CAPÍTULO IV

DISCUSIÓN GENERAL

Contexto

El desarrollo del rumen y todos los factores que influyen en el mismo han sido objeto de estudio durante décadas. Gracias al avance y consolidación de las técnicas de biología molecular basadas en el material genético microbiano, la investigación pormenorizada del desarrollo microbiológico del rumen y la microbiota en el rumen adulto ha experimentado un auge considerable en los últimos años. La microbiota del rumen puede ser variable y específica en función de la especie animal, edad, dieta, ubicación geográfica, o del propio animal, pero en general posee un alto grado de resiliencia y por tanto es poco susceptible de ser modulada con efectos permanentes una vez está plenamente establecida en el animal adulto (Weimer, 2015). No obstante, en la fase en la que el rumen aún está en desarrollo existe una mayor plasticidad, lo que hace posible llevar a cabo intervenciones para modular la microbiota ruminal que puedan tener un efecto en el animal tanto a corto como, potencialmente, a largo plazo (Yáñez-Ruiz et al., 2015). Numerosas intervenciones nutricionales y microbiológicas se han llevado a cabo en rumiantes en edad temprana con resultados variables sobre la función y el desarrollo ruminal (Kelly & Waghorn, 2020). El empleo de la microbiota del propio rumen adulto como para optimizar el desarrollo del rumen ha sido escaso y con resultados variables dependiendo de la dosis o la dieta del donante (Ewan et al., 1958; De Barbieri et al., 2015a). En este sentido, los primeros ensayos de esta tesis estaban destinados a identificar, mediante cultivo *in vitro*, la microbiota con mayor potencial para alcanzar unos mayores niveles de actividad fermentativa en función de distintos factores como la dieta del donante, el tiempo de muestreo, la fracción empleada o su conservación (**Publicación 1**). En base a los resultados obtenidos, se procedió a escoger las fuentes de microbiota ruminal con una mayor aptitud para promover la colonización y el desarrollo del rumen en cabritos recién nacidos (Publicaciones 2 y 3). El primer ensayo *in vivo* de la tesis ha permitido ver cómo el inóculo de contenido ruminal adulto (especialmente el fresco adaptado a dieta forrajera o concentrada) tenía un efecto positivo sobre el desarrollo y fermentación ruminal de los recién nacidos (**Publicación 2**), así como sobre la colonización microbiana del rumen (**Publicación 3**). Esta estrategia, pese a sus prometedores resultados, tiene una difícil aplicación a nivel de granja, principalmente por la dificultad de disponer de animales donantes a diario y sus implicaciones sanitarias. Por ello, el siguiente ensayo *in vivo* consistió en promover la inoculación o transmisión de

microbiota del animal adulto en recién nacidos pero de forma indirecta, mediante la mera presencia de adultos co-habitando en el mismo parque que los cabritos (**Publicación 4**).

Un elemento importante que puede influir en el establecimiento de distintas poblaciones microbianas en el rumen, especialmente en edad temprana, es el sistema inmune del hospedador (Malmuthuge & Guan, 2017). La saliva contiene un gran número de componentes capaces de interaccionar con el microbioma (Fábián et al., 2012), pero la investigación acerca de cómo estos pueden modular la proliferación y actividad de los microorganismos del rumen es escasa. En las **Publicaciones 5** y **6** de esta tesis se ha profundizado sobre la composición de la saliva del rumiante y, sobre todo, cómo la saliva puede modular a la microbiota ruminal de forma distinta en función de la fracción considerada o del animal donante.

Identificación de los factores que determinan la microbiota ruminal más prometedora como inóculo para promover la colonización y desarrollo del rumen

El primer experimento de esta tesis se centró en evaluar varios factores que potencialmente modulan la actividad de la microbiota ruminal para identificar las condiciones más favorables de muestreo y preservación de la microbiota para maximizar la concentración microbiana y su actividad fermentativa con el objetivo de ser utilizada como inóculo en los sucesivos experimentos. El pH del rumen se puede ver alterado en función de la dieta ya que depende en gran medida de la producción y absorción de productos de fermentación (Rustomo et al., 2006; Li et al., 2020). En esta tesis (Publicación 1) se monitorizó la evolución de los valores de pH ruminal en cabras adultas tras la administración de dietas distintas (forraje vs. concentrado), lo que reveló que las mayores diferencias de pH entre una dieta y otra se alcanzaban en torno a las 3 horas post-ingesta. En el subsiguiente ensayo *in vitro* se emplearon tres concentraciones distintas de bicarbonato en el tampón de incubación (Amanzougarene & Fondevila, 2017) para simular los distintos pH alcanzados en el rumen *in vivo* y así evaluar con qué tampón se alcanzaba una mayor actividad microbiana. Independientemente de la dieta empleada, la incubación con más bicarbonato (pH alto) resultó ser la más propicia para mantener el pH en niveles fisiológicos (~6.5) y aumentar considerablemente la producción de AGV y gas (+9 % y +19 % respectivamente) con respecto a la incubación con pH medio y aún más

con respecto a la incubación con pH bajo. Estos resultados demostraron que el sistema *in vitro* con mayor capacidad tamponadora era el más óptimo para que la microbiota desarrollase su actividad (Hobson & Stewart, 1997), especialmente en dietas ricas en concentrado, y por ello fue el empleado en los siguientes ensayos.

El rumen puede albergar comunidades microbianas distintas en función de si estas están asociadas al epitelio o se encuentran en el propio contenido ruminal (digesta) (Malmuthuge et al., 2011). Incluso dentro del propio contenido, también es posible diferenciar entre las poblaciones presentes en la fase líquida o asociadas a las partículas de alimento (Belanche et al., 2012a), estando estas últimas fuertemente relacionadas con la degradación de fibra (Belanche et al., 2017). Al comparar la incubación con la fracción líquida del rumen *vs*. la fracción líquida+sólida como inóculo, se observó que se alcanzaban unos niveles de concentración microbiana y un patrón de fermentación similares en ambos casos. Estos resultados coinciden con las pocas diferencias encontradas a nivel microbiológico entre estas fracciones de contenido ruminal en un ensayo *in vivo* (Ji et al., 2017), si bien otros estudios apuntan a que sí existen diferencias entre ambas fracciones (De Mulder et al., 2017). La fracción líquida además promovió unas concentraciones más altas de hongos anaerobios (+0.29 log) que, siendo importantes degradadores de carbohidratos complejos, posiblemente se adhirieron a las partículas de sólido autoclavado suplementadas en este tratamiento en ausencia de otros microorganismos asociados a ellas (Orpin & Bountiff, 1978). Fruto de estos resultados se decidió utilizar líquido ruminal (en vez de sólido) para los sucesivos ensayos dada su mayor facilidad de manejo durante el proceso de inoculación.

El momento de muestreo de contenido ruminal también determina en gran medida el pH y el grado de actividad fermentativa que se puede obtener en incubaciones *in vitro*, siendo práctica habitual el muestreo en ayuno para minimizar el posible impacto de las diferencias entre individuos (Yáñez-Ruiz et al., 2016). El estudio del efecto del tiempo de muestreo (Publicación 1) mostró que el inóculo de contenido ruminal muestreado a las 3 horas disminuyó el pH y aumentó las concentraciones de AGV, nitrógeno amoniacal y la producción de gas (+12 %, +17 % y +24 %; respectivamente) comparado con el inóculo muestreado en ayuno. El mayor nivel de actividad microbiana y productos de fermentación observado unas pocas horas tras la ingesta también se ha observado en estudios *in vivo* (Belanche et al., 2012b). La mayor disponibilidad de nutrientes y sobre

todo la mayor capacidad fermentativa del inóculo muestreado 3 horas post-ingesta hizo que se escogiese para los siguientes ensayos por su mayor potencial de cara a su inoculación en cabritos recién nacidos.

La disponibilidad de empleo de animales canulados en rumen inherente a la utilización de un inóculo de contenido ruminal fresco para promover el desarrollo ruminal conlleva cierta dificultad. Por ello, se planteó la necesidad de llevar a cabo un ensayo que sirviese para constatar el grado de eficacia de algunos métodos de conservación para mantener al inóculo en condiciones óptimas. La bibliografía muestra que la congelación de la microbiota ruminal ralentiza la fermentación especialmente de la fibra (Prates et al., 2010) como consecuencia del descenso de la viabilidad de los microorganismos fibrolíticos, especialmente los protozoos ruminales (Abdel-Aziz et al., 2007). Además, se ha comprobado que cuanto más tiempo está congelado el inóculo de microbiota ruminal, más baja es la actividad microbiana a posteriori (Cone et al., 2000). Para evitar estos posibles efectos negativos, se empleó dimetilsulfóxido (DMSO) como criopreservante, ya que en incubaciones *in vitro* hace que se mantenga una digestibilidad similar a la del inóculo fresco, pese a su toxicidad (Denek et al., 2010). Pese a ello, nuestro ensayo mostró que la congelación del inóculo y posterior descongelación e incubación supuso una notable reducción en los niveles de los productos de fermentación (-13 % gas, -14 % AGV, -96 % nitrógeno amoniacal) en comparación con el inóculo fresco (Prates et al., 2010), además de unas concentraciones más bajas de hongos y protozoos ruminales, especialmente los holotricos (-86 %). La menor presencia de estos microorganismos y la mejor capacidad de las bacterias (y sus enzimas amilasas) para aguantar la congelación pudo favorecer una ligera mayor concentración de bacterias (+0.24 log) y el mayor porcentaje de propionato (+25 %) empleando el inóculo congelado (Hristov et al., 2002; Belanche et al., 2012c).

La liofilización del contenido ruminal es otra alternativa interesante ya que facilitaría la obtención y almacenamiento del inóculo. Los efectos de emplear contenido ruminal liofilizado en incubaciones *in vitro* son variables en función del método de congelación empleado o los parámetros o microorganismos evaluados (Phillips et al., 1975; Kapp et al., 1979). En nuestro estudio, la incubación con contenido ruminal liofilizado tuvo un impacto muy negativo en la fermentación ruminal (-51 % AGV) y las concentraciones de arqueas, hongos y especialmente protozoos (-2.66 log), indicando un considerable

descenso de la viabilidad y actividad microbianas. En lo referente a bacterias ruminales, es posible que los mecanismos de resistencia de estas frente a la congelación o liofilización evitaran un descenso en su concentración tras el liofilizado del contenido ruminal. No obstante, algunos autores apuntan a que este método de conservación afecta a la comunidad bacteriana del rumen alterando su composición y disminuyendo la diversidad (Granja-Salcedo et al., 2017). En la misma incubación, en la que se emplearon contenidos ruminales de animales con distinta dieta (forrajera vs. concentrada), se observó que la liofilización afectaba bastante menos al contenido ruminal adaptado a dieta concentrada, posiblemente debido a la menor diversidad de la comunidad microbiana de ese inóculo (Fernando et al., 2010) o incluso a diversos procesos adaptativos derivados del consumo de ese tipo de dieta (Denton et al., 2015). El mayor grado de actividad del inóculo adaptado a dieta concentrada con respecto al adaptado a dieta forrajera se observó también en el resto de tratamientos (+19 % AGV, +26 % producción de gas), lo cual también queda reflejado en la bibliografía (Martínez et al., 2010). No obstante, no existió una interacción positiva entre la dieta y el sustrato de incubación, lo que sugiere que la adaptación del inóculo era similar independientemente del sustrato empleado.

Todas estas consideraciones se tuvieron en cuenta para el siguiente ensayo *in vivo*, de forma que se escogió el inóculo líquido fresco muestreado 3 horas post-ingesta por su mayor potencial en términos de concentración y actividad microbiana. Por otro lado, este ensayo y los resultados obtenidos a raíz del mismo podrían ser de gran utilidad para futuros ensayos de cultivos *in vitro* no renovados de contenido ruminal. En este sentido, estas incubaciones describen las condiciones óptimas para maximizar la fermentación ruminal *in vitro* en base a diversos factores. Cómo cada factor afecta a la fermentación ruminal de forma distinta puede ser tenido en cuenta de cara a estandarizar protocolos de incubación *in vitro* (Yáñez-Ruiz et al., 2016) donde se evalúen diferentes dietas, aditivos pre o probióticos, etc.

Efectos de la inoculación de líquido ruminal sobre el desarrollo y colonización microbiana de cabritos durante la lactancia

En el primer ensayo *in vivo* de la tesis (Publicaciones 2 y 3) se empleó, en base a los resultados anteriores, líquido ruminal fresco muestreado 3 horas tras ingesta para su inoculación diaria a cabritos en las primeras semanas de vida. Además del grupo de

cabritos sin inocular (CTL) y los grupos de cabritos inoculados con líquido ruminal fresco adaptado a dieta concentrada o forrajera (RFC o RFF, respectivamente), también se incluyó un grupo inoculado con líquido ruminal autoclavado (AUT), que contuviese los mismos nutrientes que los anteriores pero sin células viables.

Los cabritos de los grupos RFC y RFF presentaron una mayor ingesta de concentrado (+68 %) y forraje (+12 %) antes del destete (5 semanas) con respecto a los otros dos grupos, de forma que estos animales podrían estar más preparados para el consumo de alimento sólido en el momento del destete (Meale et al., 2017a). Esta mayor ingesta de alimento sólido fue acompañada por un aumento de la actividad fermentativa reflejada por la mayor concentración de AGV (+47 %) y nitrógeno amoniacal (+26 %), estimulándose así el desarrollo fisiológico del rumen (Baldwin et al., 2004). Además, la concentración relativa de butirato ruminal, que es el producto de fermentación con un mayor impacto en el desarrollo del epitelio del rumen (Baldwin & McLeod, 2000), en los cabritos RFC y RFF fue un 50 % más alta que en AUT y CTL. La concentración sanguínea de BHB, que es el metabolito fruto de la absorción de butirato, fue un 48 % más alto en los grupos inoculados con líquido ruminal fresco. Esto reafirma el efecto beneficioso de la inoculación sobre la actividad fermentativa y absorción de nutrientes, como ya se había descrito en estudios de inoculación en corderos (Abo-Donia et al., 2011; De Barbieri et al., 2015a). En el momento del destete (7 semanas), los cabritos RFC siguieron mostrando unos mejores indicadores de desarrollo ruminal (+40 % concentración de BHB y -7% concentración de glucosa en sangre) (Baldwin et al., 2004) pero las diferencias en otros parámetros como concentración de AGV y nitrógeno amoniacal desaparecieron con respecto a AUT y CTL, posiblemente debido a un mayor desarrollo compensatorio de estos entre las semanas 5 y 7.

Algunos trabajos apuntan a que la ingesta de alimento sólido tras el destete sigue siendo más alta en grupos de animales inoculados con líquido ruminal (Zhong et al., 2014; De Barbieri et al., 2015a), pero en nuestro estudio las diferencias en la ingesta de alimento sólido se fueron disipando entre los grupos. Sí que se observó, especialmente en el grupo RFC, una mayor preferencia por la ingesta de forraje (45.5 % de la ingesta total) en comparación con los grupos AUT y CTL (33 % de la ingesta total), posiblemente fruto de la inoculación de microbiota mejor adaptada a la degradación de este sustrato. Esto conllevó que los niveles de producción y absorción de butirato y BHB, respectivamente,

siguiesen siendo más altos en RFC y RFF, pero la mayor ingesta de concentrado en CTL y AUT llevó a estos grupos a producir una mayor concentración AGV y concentración relativa de propionato (Fernando et al., 2010). La dieta a la que estaba adaptado cada inóculo fresco tuvo un efecto tras el destete ya que el grupo RFC presentó unos mayores niveles de BHB en sangre, productos de fermentación ruminal e ingesta de forraje. Esto último sugiere que la administración de este inóculo podría tener mayor potencial como intervención para "programar" la microbiota en edad temprana (Yáñez-Ruiz et al., 2015) independientemente de la dieta que reciban los animales a medio-largo plazo.

A nivel microbiológico, la inoculación en los grupos RFC y RFF supuso una notable aceleración de la colonización del rumen por parte de los protozoos, que son indicadores de un rumen mejor preparado para la degradación de alimento sólido (Newbold et al., 2015). Los protozoos conformaban una comunidad mucho más abundante y diversa (+2 log; +25 OTUs) y con representación de protozoos fibrolíticos (eg. *Diplodinium*) y holotricos (*Isotricha* y *Dasytricha*) en estos dos grupos en comparación con el grupo CTL y, en menor medida, AUT. El examen microscópico de las comunidades protozoarias mostró que el grupo AUT presentaba concentraciones bajas de tan sólo el grupo protozoario que primero coloniza el rumen (*Entodinium* sp.), mientras que el grupo CTL careció de protozoos ruminales durante todo el experimento, ya que son microorganismos que se transmiten por contacto directo con animales portadores (Bird et al., 2010). La mayor presencia de protozoos en RFC y RFF puede contribuir a explicar los mayores niveles de AGV, nitrógeno amoniacal y sobre todo butirato en el rumen de estos cabritos, debido a su elevada actividad fibrolítica y proteolítica (Williams & Coleman, 1992; Newbold et al., 2015). Además, la concentración de muchos de los protozoos presentes tanto en RFC como en RFF tuvieron una correlación positiva con la ingesta de alimento sólido y la diversidad de metanógenos y de bacterias, debido a la relación simbiótica descrita con las primeras (Williams & Coleman, 1992) y a que la depredación de las segundas promueve la proliferación de distintas bacterias con los mismos nichos metabólicos (Belanche et al., 2012c). Tras el destete, los cabritos RFF y RFC albergaron comunidades protozoarias semejantes e incluso los AUT empezaron a albergar una comunidad similar a las anteriores, perdiéndose parcialmente la persistencia de los efectos observados antes del destete.

A diferencia de los protozoos, la abundancia de bacterias ruminales apenas se vio afectada por la inoculación, pero sí que hubo un efecto importante en la diversidad y estructura de la comunidad. A las 5 semanas, los grupos RFC y RFF presentaban 234 OTUs más que los grupos AUT y CTL, muchos de ellos compartidos. Cabe destacar que, además, en el grupo CTL había una mayor abundancia de bacterias caracterizadas por ser colonizadoras primarias del rumen (*Proteobacteria*) (Jami et al., 2013) o asociadas a un desarrollo sub-óptimo del rumen (*Bacteroidales*) (Yu et al., 2020). En cambio, los cabritos RFC y RFF presentaron mayor abundancia relativa de *Succinivibrionaceae* (asociado al desarrollo funcional del rumen (Li & Guan, 2017)) y otras bacterias pertenecientes a *Firmicutes*, algunas de las cuales tienen un papel importante en la degradación de fibra (Ransom-Jones et al., 2012; Biddle et al., 2013). Muchas otras bacterias consideradas colonizadores tardíos del rumen y que mostraron correlaciones positivas con la ingesta de alimento sólido y la producción de diversos productos de fermentación no estaban presentes en el grupo CTL antes del destete. Pese a que la comunidad bacteriana del grupo AUT se asimiló a la de RFC y RFF tras el destete, la del grupo CTL siguió estando menos desarrollada, indicando que la intervención antes del destete permitió que los cabritos inoculados albergasen una comunidad bacteriana ruminal más desarrollada y adaptada al consumo de dieta sólida (De Barbieri et al., 2015b; Dias et al., 2017).

En cuanto a las arqueas metanogénicas, la inoculación con líquido ruminal fresco aceleró la colonización del rumen por parte de estas antes del destete en base a la mayor concentración (+2 log) y diversidad (+5.5 OTUs) de estas. Mientras que en el grupo CTL sólo hubo una especie mayoritaria (*Methanobrevibacter gottschalkii*), en los grupos RFC y RFF se alcanzó una mayor diversidad gracias a la proliferación de varias especies del género *Methanobrevibacter* y familia *Methanomassilicocacceae*, las cuales se caracterizan por desarrollarse en presencia de un ecosistema ruminal maduro (Belanche et al., 2019a). Estas diferencias se pudieron deber en parte a que en torno a un 20 % de los metanógenos viven en simbiosis con protozoos (Sharp et al., 1998). Tras el destete, la comunidad de metanógenos del rumen del grupo CTL seguía siendo menos diversa y desarrollada que la de los otros grupos, tal y como sucede en animales criados con lactoreemplazante, lo que sugiere que la inoculación pudo en cierta medida simular la transmisión microbiológica que ocurre entre las madres y las crías con lactancia natural (Belanche et al., 2019b). Algo similar se observó en lo referente a los hongos ruminales, ya que estos microorganismos, que se consideran colonizadores tardíos del rumen (Orpin

& Joblin, 1997), presentaron una concentración mayor en los grupos inoculados antes del destete. El grupo CTL no presentó una comunidad fúngica menos diversa pero esta sí que era significativamente distinta a la de los grupos RFC y RFF, y además carecía de géneros relevantes en la degradación de material vegetal en el rumen como *Piromyces* y *Orpminomyces* (Solomon et al., 2016; Belanche et al., 2019a), los cuales eran abundantes en RFC y RFF y se correlacionaron con la diversidad microbiana del rumen y la ingesta de alimento sólido, respectivamente.

Estos resultados indicaron que los cabritos inoculados con líquido ruminal fresco experimentaron una aceleración de la colonización microbiana del rumen, lo cual les llevó a consumir mayores cantidades de alimento sólido y a poseer una mayor actividad fermentativa con respecto a los no inoculados. Además, las concentraciones de numerosos microorganismos con mayor abundancia en los grupos inoculados se correlacionaron positivamente con diversos indicadores de desarrollo funcional y microbiológico del rumen, e incluso con la ganancia de peso. Sin embargo, la inoculación, como se ha visto en otros trabajos, no mejoró la ganancia de peso en el total del ensayo (De Barbieri et al., 2015a; Yu et al., 2020), posiblemente porque un microbioma más diverso no implica necesariamente siempre una mejor eficiencia digestiva (Kruger Ben Shabat et al., 2016). Pese a ello, la mayor ganancia de peso observada inmediatamente después del destete (8 semanas) y la presencia de una microbiota más adaptada al consumo de alimento sólido sugieren que el proceso de destete fue menos severo en los cabritos inoculados (Meale et al., 2017b). Esto no quedó reflejado en indicadores de estrés sanguíneo (niveles de cortisol similares entre tratamientos) pero la más pronta toma de alimento sólido por parte de los cabritos inoculados podría plantear la implementación de un destete precoz con el consiguiente ahorro en lactoreemplazante. El destete precoz suele ser una práctica que conlleva un riesgo importante para el animal, especialmente cuando se realiza de forma abrupta y cuando el animal carece de un desarrollo ruminal óptimo (Mikus et al., 2020). En nuestro ensayo, los animales inoculados estaban más preparados para la ingesta y aprovechamiento de alimento sólido (comunidad microbiana más compleja), por lo que quizá las consecuencias de un destete precoz no serían tan severas.

La inoculación de microbiota ruminal en edad temprana también podría en cierta medida "programar" la comunidad microbiana del rumen en estos animales, con efectos a medio-largo plazo (Yáñez-Ruiz et al., 2015). Pese a que en los ensayos *in vitro* no se vieron

interacciones claras entre el inóculo empleado y la dieta, la inoculación en animales jóvenes sí que podría resultar en una mayor eficiencia digestiva en determinadas situaciones alimenticias en edad adulta, ya sea basadas en dieta concentrada (sistemas intensivos) o especialmente en dieta forrajera (sistemas de pastoreo) (Belanche et al., 2019b), con un potencial impacto positivo sobre parámetros productivos. La inoculación del conjunto del microbioma ruminal adulto puede ser dificultosa por la necesidad continua de disponer de animales donantes y por las posibles implicaciones sanitarias. Por ello, también sería interesante identificar microorganismos concretos del rumen adulto que puedan estimular el desarrollo ruminal y promover una mejor adaptación para la ingesta de alimento sólido de cara a facilitar su aplicación a modo de mezcla de microorganismos (Buntyn et al., 2016) en las primeras semanas de edad.

Efectos de la presencia de animales adultos sobre el desarrollo y colonización microbiana de cabritos durante la lactancia

En base a los resultados obtenidos en el anterior ensayo *in vivo* y a los estudios en los que se demostraba que se producía una aceleración del desarrollo microbiológico y fisiológico del rumen en cabritos o corderos con lactancia natural (Abecia et al., 2017; Belanche et al., 2019b), se procedió a llevar a cabo un ensayo que evaluase si estos efectos también ocurrían en un sistema de lactancia artificial por la mera presencia de animales adultos distintos de las madres (Publicación 4).

La actividad fermentativa en términos de AGV totales fue similar entre los cabritos acompañados de adultos (CMP) y los no acompañados (CTL). Sin embargo, el aumento del pH y las mayores concentraciones de ácidos grasos ramificados (+20.4 %) y nitrógeno amoniacal en el grupo CMP, especialmente tras el destete, se ha relacionado con una mayor degradación de proteína dietética y bacteriana por parte de los protozoos (Belanche et al., 2012b,c), lo cual también podría ser consecuencia de una mayor ingesta de forraje (Newbold et al., 2015). Esta hipótesis se ve reforzada por la mayor producción de butirato en el rumen de los cabritos CMP (+45 % de media antes y después del destete), que es más abundante en crías con lactancia natural (Belanche et al., 2019b) y se asocia a la actividad fermentativa de los protozoos ruminales (Eugène et al., 2004). La concentración relativa de butirato en CMP en comparación con CTL fue ascendiendo de forma paulatina hasta ser un 83 % mayor tras el destete (9 semanas), indicando que los cabritos CMP probablemente estaban mejor preparados para la transición de dieta líquida a sólida, como

ocurría con los cabritos inoculados con contenido ruminal en el trabajo previo. Además, esto se tradujo en una mayor concentración de BHB (+41 %) y menor concentración de glucosa (-23 % a las 7 semanas) en los cabritos CMP, lo que sugiere un mayor desarrollo fisiológico del rumen en estos cabritos (De Barbieri et al., 2015a).

Compartir espacio con animales adultos propició una inoculación indirecta de microbiota ruminal a los cabritos, especialmente en lo referente a los protozoos ruminales debido a la naturaleza anaerobia estricta de estos (Fonty et al., 1988). Los cabritos CTL no albergaron protozoos durante todo el ensayo, mientras que los CMP ya tenían una comunidad protozoaria bien establecida a las 5 semanas, que aumentó en complejidad con el paso del tiempo. Esto quedó reflejado por el descenso en la abundancia del protozoo ruminal mayoritario (*Entodiniinae*) y el aumento de otros grupos, lo que originó una comunidad más diversa y capacitada para la degradación de fibra (Newbold et al., 2015), que además se relacionó positivamente con el peso vivo y la concentración de BHB en sangre. Se ha comprobado que la inoculación con líquido ruminal fresco en edad temprana (Publicación 3) o la lactancia natural (Belanche et al., 2019b) estimulan el crecimiento de una comunidad fúngica diversa y distinta a los animales criados en lactancia artificial. Sin embargo, estas observaciones discrepan con la ausencia de diferencias observadas en nuestro estudio, probablemente por la capacidad de los hongos ruminales de generar esporas resistentes (McGranaghan et al., 1999).

La presencia de adultos también promovió que los cabritos CMP albergasen una comunidad bacteriana mucho más diversa (+132 ASVs) con respecto a los cabritos CTL en el momento de destete (7 semanas), que es el momento en el que bacterias anaerobias celulolíticas (*Fibrobacteres*, *Firmicutes*), empiezan a adquirir más importancia (Jami et al., 2013). La menor profundidad de secuenciación de la comunidad de metanógenos no permitió identificar especies poco abundantes pero aun así representó en torno al 0.6 % del total de secuencias, fue más abundante en el grupo CMP (+1 log) y tendió a ser más diversa en este grupo, probablemente gracias a la presencia de protozoos en estos cabritos (Newbold et al., 2015). En base a todos los ASVs identificados, se observó que la comunidad microbiana de los adultos acompañantes seguía estando más desarrollada que en los cabritos CTL y CMP a las 7 semanas, por lo que se deduce que la colonización microbiana aún no se había completado totalmente en ninguno de los grupos a esa edad (Jami et al., 2013). No obstante, la comunidad ruminal de CMP fue notablemente distinta

a la de CTL y guardó un mayor grado de similitud con los adultos, ya que estos (a través de la saliva, heces, bolo ruminal, piel, etc.) pudieron ejercer de transmisores de un amplio rango de microorganismos (Yeoman et al., 2018). La distinta estructura de la comunidad procariota entre CMP y CTL vino determinada por las diferentes abundancias relativas de algunos microorganismos. En concreto, gran parte de los taxones más abundantes en CTL pertenecieron a *Proteobacteria* y *Spirochaetes*, que son principalmente colonizadores tempranos. En el grupo CMP, hubo una mayor abundancia relativa de *Bacteroidetes*, *Veillonellaceae* o *Selenomonas*, que se han descrito como microorganismos relacionados con la degradación de alimento sólido, la diversidad microbiana del rumen y la degradación de fibra, respectivamente (Fernando et al., 2010; Sawanon et al., 2011). Otros microorganismos importantes en los cabritos CMP (*Prevotellaceae, Fibrobacteraceae* y los metanógenos *M. mobile* y *Group9*) han sido identificados como miembros de la comunidad adulta del rumen e indicadores de desarrollo del rumen (Li & Guan, 2017).

Los resultados obtenidos sugieren que el ecosistema ruminal de los cabritos acompañados (CMP) era más maduro y diverso en el momento de destete, lo que podría implicar una mejor adaptación del mismo para la degradación de alimento sólido, especialmente fibra. Los efectos de la presencia de adultos fueron algo más atenuados a los observados en el ensayo de inoculación directa si bien, de forma similar a lo que ocurrió en este, eso no se tradujo en diferencias en el peso de los animales con respecto al grupo CTL. No obstante, la relativamente fácil implementación de una intervención de este tipo a nivel de granja invita a estudiar esta estrategia en combinación con prácticas que limiten la ingesta (que fue *ad libitum* en este ensayo) o el destete precoz, donde sí se podría ver un impacto más claro a nivel de rendimiento del animal (De Paula Vieira et al., 2012). A diferencia de otras intervenciones que requieren el uso de microorganismos (como en el primer ensayo *in vivo*) o aditivos, la disposición de un número limitado de adultos con lotes de animales lactantes resulta más factible a nivel de manejo, especialmente en pequeños rumiantes donde los animales suelen estar agrupados durante el destete, y no requeriría gastos extra relevantes. Esta práctica podría emular parcialmente los beneficios de la lactancia natural (Abecia et al., 2014) en términos de transmisión de microbiota y aprendizaje de hábitos de alimentación, atenuando los posibles efectos negativos de la separación madre-cría que se da en los sistemas lecheros (Mandel & Nicol, 2017), a la vez que se sigue aprovechando la totalidad de la producción lechera de las madres. La presencia de un

microbioma ruminal más diverso y capacitado para la degradación de sustratos sólidos complejos como la fibra podría además tener implicaciones en estos animales a medio plazo y en edad productiva, mejorando su eficiencia digestiva especialmente si se proporciona dieta a base de forraje o pasto (Belanche et al., 2019b).

Modulación de la microbiota y actividad ruminal por parte de la saliva y sus componentes

Durante muchos años, la mayoría de los estudios sobre el papel de la saliva del rumiante se han centrado en su capacidad tamponadora o en las fluctuaciones en su producción y composición derivadas de la toma de alimento (Duric et al., 1994). En esta tesis se procedió a abordar el papel modulador de los componentes bioactivos de la saliva sobre la microbiota ruminal en base al empleo, en incubaciones *in vitro* con líquido ruminal, de distintas cantidades y fracciones de saliva (Publicación 5) y a los distintos animales (individuos) o especies animales utilizados como donantes de saliva (Publicación 6).

La utilización de proporciones crecientes de saliva en lugar de tampón artificial de incubación dio lugar a un aumento de la fermentación ruminal en términos de nitrógeno amoniacal (+23 %) y AGV (+8 %), al mismo tiempo que se produjo una bajada de pH y del ratio acetato:propionato. Estos mayores niveles fermentativos a mayor cantidad de saliva empleada pueden responder a la presencia de componentes salivales, como ciertas proteínas, que además de mantener el pH en niveles fisiológicos, promuevan la actividad microbiana del rumen (Fouhse et al., 2017), y la mejora de la hidrólisis y degradación del sustrato, tal y como se ha observado al pre-incubar dietas con saliva que posteriormente se han sometido a digestión *in vitro* (Ammar et al., 2013).

En el siguiente ensayo *in vitro* se incubó líquido ruminal con salivas sin filtrar (NFS), saliva filtrada (FS1) sin microorganismos, saliva ultrafiltrada (FS2) carente de proteínas de elevado peso molecular (p.e. inmunoglobulinas) o saliva autoclavada (AS) sin proteínas ni microorganismos. El pH tras 24 h de incubación fue significativamente más bajo en FS1 y FS2 en comparación con NFS y AS, lo cual puede explicarse por las mayores concentraciones de nitrógeno amoniacal (+35 %) en estos últimos, posiblemente fruto de una mayor proteólisis derivada de la depredación de bacterias (o sus restos) presentes en estas salivas por parte de los protozoos (Belanche et al., 2012c). Además, la saliva FS1 estimuló la producción de AGV y gas con respecto a las otras (+7 % y +5 %,

respectivamente), lo cual puede deberse a la carencia de microbiota salival que pueda competir con la ruminal. La presencia en FS1 de proteínas de gran tamaño que pudo interaccionar positivamente con la microbiota como las inmunoglobulinas (Lamy et al., 2009; Tsuruta et al., 2012), también pudo contribuir a una mayor actividad fermentativa.

Se ha comprobado que existen diversas isoformas de inmunoglobulinas y que estas actúan de forma específica inhibiendo o estimulando el crecimiento de distintos microorganismos (Donaldson et al., 2018). En base a esto y a las posibles interacciones que se pueden establecer entre saliva y contenido ruminal entre especies de rumiantes (Ammar et al., 2013), se desarrolló un ensayo *in vitro* semi-continuo de mayor duración (7 días) donde se evaluaron los efectos sobre la microbiota y fermentación ruminal de caprino del uso de saliva autoclavada (AUT), saliva propia del animal donante de contenido ruminal (OWN), saliva de cabra ajena (GOAT) y saliva de oveja (SHEEP).

Previamente a la incubación, se caracterizaron las salivas empleadas en cuanto a su contenido en inmunoglobulinas y perfiles proteómico y metabolómico. La concentración de IgA fue en torno a 4 veces mayor que la de IgG de media en todas las salivas, ya que IgA es mayoritaria en la saliva del rumiante (Lascelles & McDowell, 1974); aunque la concentración de ambas fue ligeramente inferior en la saliva de oveja. El número de proteínas identificadas fue 195 entre todas las salivas, un número superior a lo mostrado en otros trabajos (Lamy et al., 2009; Lamy et al., 2011). Pese a la variabilidad individual y entre especies, se observó un perfil de proteínas con funciones fisiológicas similares a lo descrito anteriormente (Ang et al., 2011), como el transporte y la actividad enzimática. También se observó un alto grado de especificidad individual en el metaboloma de las salivas que podría estar motivado por la microbiota específica presente en cada animal (Gardner et al., 2019), que asimismo está modulada por las proteínas salivales con función inmune (Fouhse et al., 2017).

La incubación semi-continua con saliva alcanzó estabilidad a las 36 horas y a partir de ahí la especie donante de saliva fue el elemento que más influyó en la actividad fermentativa. El autoclavado de la saliva resultó ser el factor más determinante a la hora de que se estableciesen unas poblaciones microbianas u otras en el sistema de fermentación, ya que la comunidad microbiana en el tratamiento AUT fue la más divergente con respecto al resto de tratamientos. Ello pone en evidencia la presencia de compuestos bioactivos en las salivas sin autoclavar. La incubación con AUT resultó en

una mayor abundancia de proteobacterias, que se suelen asociar con una microbiota ruminal poco desarrollada ya que en gran medida son colonizadores primarios del rumen con carácter oportunista y degradadores de sustratos simples (Jami et al., 2013; Auffret et al., 2017). Todas las salivas en general presentaron un ratio *Bacteroidetes:Firmicutes* casi la mitad de bajo de lo que es habitual en el rumen. Una mayor abundancia de *Firmicutes* ha sido previamente detectada en la cavidad oral (Fouhse et al., 2017; Yeoman et al., 2018), motivada quizá por la acción de los componentes de la saliva. *Prevotellaceae*, que es la familia bacteriana más abundante en el rumen, tuvo una abundancia relativa mucho mayor con el uso de salivas distintas de AUT, especialmente SHEEP. El hecho de que *Prevotella* sea una bacteria comensal con un papel fundamental en el rumen (Precup & Vodnar, 2019), y que la IgA salival (algo menos abundante en SHEEP) parece no estimular su crecimiento (Fouhse et al., 2017), puede sugerir que existen otros componentes inmunológicos (citoquinas, miRNA, etc.) implicados en la modulación de la microbiota ruminal (Yáñez-Ruiz et al., 2015). El factor especie del animal donante de saliva también tuvo cierta importancia ya que la saliva SHEEP, en comparación con las otras, aumentó la producción de gas (+4%), las concentraciones de bacterias (+2 %) y protozoos (+7 %) y las proporciones de butirato y acetato:propionato, debido posiblemente a una mayor actividad fibrolítica por parte de protozoos (Belanche e tal., 2019a).

El estudio del metaboloma al final de la incubación con saliva mostró, en línea con lo observado en la diversidad microbiana, que la inactivación de los componentes bioactivos de la saliva (autoclavado) fue el factor más determinante a la hora de separar muestras en base a los metabolitos presentes en ellas. Tras este factor, las diferencias entre especies (SHEEP vs. OWN/GOAT) también fueron determinantes a la hora de discriminar el metaboloma ruminal. Estos resultados muestran que no solo los componentes de la saliva son relevantes en cuanto a la diversidad microbiana que existe en el rumen, sino también en cuanto a su actividad metabólica. Además, refuerza que la especificidad de los componentes o mecanismos modulatorios de la saliva podrían ser parcialmente responsables de la resiliencia y la especificidad individual de la microbiota ruminal para establecer la relación hospedador-microbiota (Weimer, 2015), así como de las diferencias entre especies (Langda et al., 2020).

La saliva del rumiante, con la participación directa de los diversos actores antes descritos, parece modular el crecimiento de los microorganismos del TGI para mantener así un equilibrio adecuado microbiota-huésped en base a lo 'aprendido' por el sistema inmune. Esto queda patente en estudios donde la incubación *in vitro* de microbiota ruminal con saliva incluso promueve cierta mejoría en la actividad microbiana y en la digestibilidad del alimento (Ammar et al., 2013). La modulación de la microbiota del rumen por parte de la saliva podría ser consecuencia de un proceso evolutivo que ha permitido no sólo la tolerancia entre la microbiota y el huésped sino el beneficio mutuo (Kolodny & Schulenburg, 2020). En ese proceso, parece que los componentes bioactivos de la saliva intervienen de forma fundamental en la regulación del crecimiento microbiano y, posiblemente, en la adaptación de la microbiota a circunstancias eventuales como la entrada de un patógeno o de una dieta distinta (Kolodny & Schulenburg, 2020; Moeller & Sanders, 2020). En ese sentido, es importante recalcar que un número considerable de componentes del sistema inmune presentes en la saliva (anticuerpos, lisozima, citoquinas, microRNA), pueden inhibir la proliferación de microorganismos pero también promover el crecimiento de otros, tal y como se ha visto en este estudio, impulsando así el establecimiento de una microbiota autóctona mutualista-comensalista (Peterson et al., 2007; Li et al., 2020). Esto puede tener especial relevancia a la hora de evaluar el papel de la saliva en el establecimiento de la microbiota autóctona del TGI en edad temprana. Junto con el calostro, la saliva constituye una de las fuentes de componentes bioactivos y microbiota de la madre o de animales adultos que pueden transmitirse a las crías en las primeras semanas de vida (Yeoman et al., 2018). No obstante, aún queda mucho camino por recorrer a la hora de establecer los mecanismos de acción concretos de cada componente salival y de qué manera afectan de forma distinta a microorganismos específicos del rumen, así como sus posibles implicaciones productivas.

Referencias

Abecia, L., Ramos-Morales, E., Martínez-Fernandez, G., Arco, A., Martín-García, A. I., Newbold, C. J., & Yáñez-Ruiz, D. R. (2014). Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Animal Production Science, 54(9), 1449–1454. https://doi.org/10.1071/AN14337

Abecia, L., Jiménez, E., Martínez-Fernandez, G., Martín-García, A. I., Ramos-Morales, E., Pinloche, E., Denman, S. E., Newbold, C. J., & Yáñez-Ruiz, D. R. (2017). Natural and artificial feeding management before weaning promote different rumen microbial colonization but not differences in gene expression levels at the rumen epithelium of newborn goats. PLoS ONE, 12(8), 1–19. https://doi.org/10.1371/journal.pone.0182235

Abo-Donia, F. M., Ghada, S. I., Nadi, S., & Sayah, M. S. (2011). Effect of Inoculating New Born Lambs with Fresh or Lyophilized Rumen Fluid on Rumen Activity and Lamb Performance. Journal of American Science, 7(9), 409–422. http://www.americanscience.orghttp//www.americanscience.org.

Amanzougarene, Z., & Fondevila, M. (2017). Fitting of pH conditions for the study of concentrate feeds fermentation by the in vitro gas-production technique. Animal Production Science, 58(9), 1751–1757. https://doi.org/10.15421/021027

Ammar, H., Bodas, R., González, J. S., Salem, A. Z. M., Giráldez, F. J., Andrés, S., & López, S. (2013). Effects of pre-incubation in sheep and goat saliva on in vitro rumen digestion of tanniferous browse foliage. Journal of Agricultural Science, 151(6), 898–906. https://doi.org/10.1017/S002185961300018X

Ang, C. S., Binos, S., Knight, M. I., Moate, P. J., Cocks, B. G., & McDonagh, M. B. (2011). Global survey of the bovine salivary proteome: Integrating multidimensional prefractionation, targeted, and glycocapture strategies. Journal of Proteome Research, 10(11), 5059–5069. https://doi.org/10.1021/pr200516d

Auffret, M. D., Dewhurst, R. J., Duthie, C. A., Rooke, J. A., John Wallace, R., Freeman, T. C., Stewart, R., Watson, M., & Roehe, R. (2017). The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. Microbiome, 5(1), 1–11. https://doi.org/10.1186/s40168-017-0378-z

Baldwin VI, R. L., McLeod, K. R., Klotz, J. L., & Heitmann, R. N. (2004). Rumen Development, Intestinal Growth and Hepatic Metabolism In The Pre- and Postweaning Ruminant. J. Dairy Sci., 87(13_suppl), E55-65. https://doi.org/10.3168/jds.S0022-0302(04)70061-2

Baldwin VI, R. L., & McLeod, K. R. (2000). Effects of diet forage:Concentrate ratio and metabolizable energy intake on isolated rumen epithelial cell metabolism in vitro. Journal of Animal Science, 78(3), 771–783.

Belanche, A., De la Fuente, G., Moorby, J. M., & Newbold, C. J. (2012). Bacterial protein degradation by different rumen protozoal groups. Journal of Animal Science, 90C(12), 4495–4504. https://doi.org/10.2527/jas.2012-5118

Belanche, A., de la Fuente, G., Pinloche, E., Newbold, C. J., & Balcells, J. (2012). Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. Journal of Animal Science, 90A(11), 3924–3936. https://doi.org/10.2527/jas.2011-4802

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M., Pinloche, E., & Newbold, C. J. (2012). Shifts in the rumen microbiota due to the type of carbohydrate and level of protein ingested by dairy cattle are associated with changes in rumen fermentation. Journal of Nutrition, 142B(9), 1684–1692. https://doi.org/10.3945/jn.112.159574

Belanche, A., Kingston-Smith, A. H., Griffith, G. W., & Newbold, C. J. (2019). A multi-kingdom study reveals the plasticity of the rumen microbiota in response to a shift from non-grazing to grazing diets in sheep. Frontiers in Microbiology, 10(FEB). https://doi.org/10.3389/fmicb.2019.00122

Belanche, A., Newbold, C. J., Lin, W., Stevens, P. R., & Kingston-Smith, A. H. (2017). A systems biology approach reveals differences in the dynamics of colonization and degradation of grass vs. Hay by rumen microbes with minor effects of vitamin E supplementation. Frontiers in Microbiology, 8(AUG), 1–18. https://doi.org/10.3389/fmicb.2017.01456

Belanche, A., Yáñez-Ruiz, D. R., Detheridge, A. P., Griffith, G. W., Kingston-Smith, A. H., & Newbold, C. J. (2019). Maternal versus artificial rearing shapes the rumen microbiome having minor long-term physiological implications. Environmental Microbiology, 21(11), 4360–4377. https://doi.org/10.1111/1462-2920.14801

Biddle, A., Stewart, L., Blanchard, J., & Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. Diversity, 5(3), 627–640. https://doi.org/10.3390/d5030627

Bird, S. H., Hegarty, R. S., & Woodgate, R. (2010). Modes of transmission of rumen protozoa between mature sheep. Animal Production Science, 50(6), 414–417. https://doi.org/10.1071/AN09216

Buntyn, J. O., Schmidt, T. B., Nisbet, D. J., & Callaway, T. R. (2016). The role of direct-fed microbials in conventional livestock production. Annual Review of Animal Biosciences, 4, 335–355. https://doi.org/10.1146/annurev-animal-022114-111123

Chen, H., Wang, C., Huasai, S., & Chen, A. (2021). Effects of dietary forage to concentrate ratio on nutrient digestibility, ruminal fermentation and rumen bacterial composition in Angus cows. Scientific Reports, 11(1), 1–11. https://doi.org/10.1038/s41598-021-96580-5

Cone, J., Van Gelder, A., & Bachmann, H. (2000). Influence of inoculum source, dilution and storage of rumen fluid on gas production profiles. Proceedings of the Brittish Society of Animal Science.

De Barbieri, I., Hegarty, R. S., Silveira, C., Gulino, L. M., Oddy, V. H., Gilbert, R. A., Klieve, A. V., & Ouwerkerk, D. (2015). Programming rumen bacterial communities in newborn Merino lambs. Small Ruminant Research, 129, 48–59. https://doi.org/10.1016/j.smallrumres.2015.05.015

De Barbieri, I., Hegarty, R. S., Silveira, C., & Oddy, V. H. (2015). Positive consequences of maternal diet and post-natal rumen inoculation on rumen function and animal performance of Merino lambs. Small Ruminant Research, 129, 37–47. https://doi.org/10.1016/j.smallrumres.2015.05.017

De Mulder, T., Goossens, K., Peiren, N., Vandaele, L., Haegeman, A., De Tender, C., Ruttink, T., De Wiele, T. Van, & De Campeneere, S. (2017). Exploring the methanogen and bacterial communities of rumen environments: Solid adherent, fluid and epimural. FEMS Microbiology Ecology, 93(3), 1–12. https://doi.org/10.1093/femsec/fiw251

De Paula Vieira, a, von Keyserlingk, M. a G., & Weary, D. M. (2012). Presence of an older weaned companion influences feeding behavior and improves performance of dairy calves before and after weaning from milk. Journal of Dairy Science, 95(6), 3218–3224. https://doi.org/10.3168/jds.2011-4821

Denek, N., Can, A., & Avci, M. (2010). Frozen rumen fluid as microbial inoculum in the two-stage in vitro digestibility assay of ruminant feeds. South African Journal of Animal Sciences, 40(3), 251–256. https://doi.org/10.4314/sajas.v40i3.9

Denton, B. L., Diese, L. E., Firkins, J. L., & Hackmann, T. J. (2015). Accumulation of reserve carbohydrate by rumen protozoa and bacteria in competition for glucose. Applied and Environmental Microbiology, 81(5), 1832–1838. https://doi.org/10.1128/AEM.03736-14

Dias, J., Marcondes, M. I., Noronha, M. F., Resende, R. T., Machado, F. S., Mantovani, H. C., Dill-McFarland, K. A., & Suen, G. (2017). Effect of pre-weaning diet on the ruminal archaeal, bacterial, and fungal communities of dairy calves. Frontiers in Microbiology, 8(AUG). https://doi.org/10.3389/fmicb.2017.01553

Donaldson, G. P., Ladinsky, M. S., Yu, K. B., Sanders, J. G., Yoo, B. B., Chou, W. C., Conner, M. E., Earl, A. M., Knight, R., Bjorkman, P. J., & Mazmanian, S. K. (2018). Gut microbiota utilize immunoglobulin a for mucosal colonization. Science, 360(6390), 795–800. https://doi.org/10.1126/science.aaq0926

Duric, M., Zhao, G., Orskov, E., & Chen, X. (1994). Indirect measurement of saliva secretion in sheep fed diets of different structures and the effect of such diets on ruminal fluid kinetics and fermentation pattern. Experimental Physiology, 79(5), 823–830. https://doi.org/10.1113/expphysiol.1994.sp003810

Eugène, M., Archimède, H., & Sauvant, D. (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. https://doi.org/10.1016/S0301-6226(03)00117-9

Ewan, R. C., Hatfield, E. E., & Garrigus, U. S. (1958). Effect of Certain Inoculations on the Utilization of Urea or Biuret by Growing Lambs. Journal of the Animal Science, 17(2), 198–303.

Fábián, T. K., Hermann, P., Beck, A., Fejérdy, P., & Fábián, G. (2012). Salivary defense proteins: Their network and role in innate and acquired oral immunity. International Journal of Molecular Sciences, 13(4), 4295–4320. https://doi.org/10.3390/ijms13044295

Fonty, G., Senaud, J., Jouany, J. P., & Gouet, P. (1988). Establishment of ciliate protozoa in the rumen of conventional and conventionalized lambs: influence of diet and management conditions. Canadian Journal of Microbiology, 34(3), 235–241. https://doi.org/10.1139/m88-044

Fouhse, J. M., Smiegielski, L., Tuplin, M., Guan, L. L., & Willing, B. P. (2017). Host immune selection of rumen bacteria through salivary secretory IgA. Frontiers in Microbiology, 8(MAY), 1–9. https://doi.org/10.3389/fmicb.2017.00848

Hobson, P. N., & Stewart, C. S. (1997). The Rumen Microbial Ecosystem. In The Rumen Microbial Ecosystem. https://doi.org/10.1007/978-94-009-1453-7

Jami, E., Israel, A., Kotser, A., & Mizrahi, I. (2013). Exploring the bovine rumen bacterial community from birth to adulthood. ISME Journal, 7(6), 1069–1079. https://doi.org/10.1038/ismej.2013.2

Ji, S., Zhang, H., Yan, H., Azarfar, A., Shi, H., Alugongo, G., Li, S., Cao, Z., & Wang, Y. (2017). Comparison of rumen bacteria distribution in original rumen digesta, rumen liquid and solid fractions in lactating Holstein cows. Journal of Animal Science and Biotechnology, 8(1), 1–7. https://doi.org/10.1186/s40104-017-0142-z

Kapp, J., Hintz, R. L., & Wagner, D. G. (1979). Lyophilized Rumen Fluid for Use in In Vitro Dry Matter Disappearance Studies with Grain. Animal Science Research, 1, 42–44.

Kelly, B. W., & Waghorn, G. (2020). Early life interventions and possible mechanisms affecting methane emissions from ruminants. A summary of key findings from the

literature. NZAGRC Report Early Life Interventions By William Kelly and Garry Waghorn. February, 1–40.

Kolodny, O., & Schulenburg, H. (2020). Microbiome-mediated plasticity directs host evolution along several distinct time scales: Microbiome influence on host evolution. Philosophical Transactions of the Royal Society B: Biological Sciences, 375(1808). https://doi.org/10.1098/rstb.2019.0589rstb20190589

Kruger Ben Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., White, B. A., Shterzer, N., & Mizrahi, I. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. ISME Journal, 10(12), 2958–2972. https://doi.org/10.1038/ismej.2016.62

Lamy, E., Da Costa, G., Santos, R., Capelae Silva, F., Potes, J., Pereira, A., Coelho, A. V., & Sales Baptista, E. (2011). Effect of condensed tannin ingestion in sheep and goat parotid saliva proteome. Journal of Animal Physiology and Animal Nutrition, 95(3), 304–312. https://doi.org/10.1111/j.1439-0396.2010.01055.x

Langda, S., Zhang, C., Zhang, K., Gui, B., Ji, D., Deji, C., Cuoji, A., Wang, X., & Wu, Y. (2020). Diversity and composition of rumen bacteria, fungi, and protozoa in goats and sheep living in the same high-altitude pasture. Animals, 10(2). https://doi.org/10.3390/ani10020186

Lascelles, A. K., & McDowell, G. H. (1974). Localized Humoral Immunity with Particular Reference to Ruminants. Immunological Reviews, 19(1), 170–208. https://doi.org/10.1111/j.1600-065X.1974.tb00132.x

Li, C., Beauchemin, K. A., & Yang, W. (2020). Feeding diets varying in forage proportion and particle length to lactating dairy cows: I. Effects on ruminal pH and fermentation, microbial protein synthesis, digestibility, and milk production. Journal of Dairy Science, 103(5), 4340–4354. https://doi.org/10.3168/jds.2019-17606

Li, F., & Guan, L. L. (2017). Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. Applied and Environmental Microbiology, 83(9), 1–16. https://doi.org/10.1128/AEM.00061-17

Li, M., Chen, W. D., & Wang, Y. D. (2020). The roles of the gut microbiota–miRNA interaction in the host pathophysiology. Molecular Medicine, 26(1). https://doi.org/10.1186/s10020-020-00234-7

Malmuthuge, N., & Guan, L. L. (2017). Understanding host-microbial interactions in rumen: Searching the best opportunity for microbiota manipulation. Journal of Animal Science and Biotechnology, 8(1), 1–7. https://doi.org/10.1186/s40104-016-0135-3

Malmuthuge, N., Li, M., Chen, Y., Fries, P., Griebel, P. J., Baurhoo, B., Zhao, X., & Guan, L. L. (2012). Distinct commensal bacteria associated with ingesta and mucosal epithelium in the gastrointestinal tracts of calves and chickens. FEMS Microbiology Ecology, 79(2), 337–347. https://doi.org/10.1111/j.1574-6941.2011.01220.x

Mandel, R., & Nicol, C. J. (2017). Re-direction of maternal behaviour in dairy cows. Applied Animal Behaviour Science, 195(June 2018), 24–31. https://doi.org/10.1016/j.applanim.2017.06.001

Martínez, M. E., Ranilla, M. J., Tejido, M. L., Saro, C., & Carro, M. D. (2010). The effect of the diet fed to donor sheep on in vitro methane production and ruminal fermentation of diets of variable composition. Animal Feed Science and Technology, 158(3–4), 126–135. https://doi.org/10.1016/j.anifeedsci.2010.04.005

McGranaghan, P., Davies, J. C., Griffith, G. W., Davies, D. R., & Theodorou, M. K. (1999). The survival of anaerobic fungi in cattle faeces. FEMS Microbiology Ecology, 29(3), 293–300. https://doi.org/10.1016/S0168-6496(99)00024-0

Meale, S. J., Chaucheyras-Durand, F., Berends, H., Guan, L. L., & Steele, M. A. (2017). From pre- to postweaning: Transformation of the young calf's gastrointestinal tract. Journal of Dairy Science, 100(7), 5984–5995. https://doi.org/10.3168/jds.2016-12474

Mikuš, T., Marzel, R., & Mikuš, O. (2020). Early weaning: New insights on an ever-persistent problem in the dairy industry. Journal of Dairy Research, 87(S1), 88–92. https://doi.org/10.1017/S0022029920000503

Moeller, A. H., & Sanders, J. G. (2020). Roles of the gut microbiota in the adaptive evolution of mammalian species: Microbiotas alter mammalian adaptation.

Philosophical Transactions of the Royal Society B: Biological Sciences, 375(1808), 1–10. https://doi.org/10.1098/rstb.2019.0597rstb20190597

Newbold, C. J., De la Fuente, G., Belanche, A., Ramos-Morales, E., & McEwan, N. R. (2015). The role of ciliate protozoa in the rumen. Frontiers in Microbiology, 6(NOV), 1–14. https://doi.org/10.3389/fmicb.2015.01313

Orpin, C. G., & Bountiff, L. (1978). Zoospore chemotaxis in the rumen phycomycete Neocallimastix frontalis. Journal of General Microbiology, 104(1), 113–122. https://doi.org/10.1099/00221287-104-1-113

Orpin, C. K., & Joblin, K. N. (1997). The rumen anaerobic fungi. In P. N. Hobson & C. Stewart (Eds.), The rumen microbial ecosystem (Springer, pp. 140–195).

Peterson, D. A., McNulty, N. P., Guruge, J. L., & Gordon, J. I. (2007). IgA Response to Symbiotic Bacteria as a Mediator of Gut Homeostasis. Cell Host and Microbe, 2(5), 328–339. https://doi.org/10.1016/j.chom.2007.09.013

Phillips, B., Latham, M., & Sharpe, M. E. (1975). A method for freeze drying rumen bacteria and other strict anaerobes. Journal of Applied Microbiology, 38, 319–322.

Prates, A., de Oliveira, J. A., Abecia, L., & Fondevila, M. (2010). Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation. Animal Feed Science and Technology, 155(2–4), 186–193. https://doi.org/10.1016/j.anifeedsci.2009.12.005

Precup, G., & Vodnar, D. C. (2019). Gut Prevotella as a possible biomarker of diet and its eubiotic versus dysbiotic roles: A comprehensive literature review. British Journal of Nutrition, 122(2), 131–140. https://doi.org/10.1017/S0007114519000680

Ransom-Jones, E., Jones, D. L., McCarthy, A. J., & McDonald, J. E. (2012). The Fibrobacteres: An Important Phylum of Cellulose-Degrading Bacteria. Microbial Ecology, 63(2), 267–281. https://doi.org/10.1007/s00248-011-9998-1

Rey, M., Enjalbert, F., Combes, S., Cauquil, L., Bouchez, O., & Monteils, V. (2014). Establishment of ruminal bacterial community in dairy calves from birth to weaning

is sequential. Journal of Applied Microbiology, 116(2), 245–257. https://doi.org/10.1111/jam.12405

Roger, V., Grard, F., Andre, C., & Gouet, P. (1992). Microbiology Effects of Glycerol on the Growth , Adhesion , and Cellulolytic Activity of Rumen Cellulolytic Bacteria and Anaerobic Fungi. 25, 197–201.

Rustomo, B., Alzahal, O., Odongo, N. E., Duffield, T. F., & McBride, B. W. (2006). Effects of rumen acid load from feed and forage particle size on ruminal pH and dry matter intake in the lactating dairy cow. Journal of Dairy Science, 89(12), 4758–4768. https://doi.org/10.3168/jds.S0022-0302(06)72525-5

Sawanon, S., Koike, S., & Kobayashi, Y. (2011). Evidence for the possible involvement of Selenomonas ruminantium in rumen fiber digestion. FEMS Microbiology Letters, 325(2), 170–179. https://doi.org/10.1111/j.1574-6968.2011.02427.x

Sharp, R., Ziemer, C. J., Stern, M. D., & Stahl, D. A. (1998). Taxon-specific associations between protozoal and methanogen populations in the rumen and a model rumen system. FEMS Microbiology Ecology, 26(1), 71–78. https://doi.org/10.1016/S0168-6496(98)00024-5

Solomon, K. V., Haitjema, C. H., Henske, J. K., Gilmore, S. P., Borges-Rivera, D., Lipzen, A., Brewer, H. M., Purvine, S. O., Wright, A. T., Theodorou, M. K., Grigoriev, I. V., Regev, A., Thompson, D. A., & O'Malley, M. A. (2016). Early-branching gut fungi possess large, comprehensive array of biomass-degrading enzymes. Science, 351(6278), 1192–1195. https://doi.org/10.1126/science.aad1431

Tsuruta, T., Inoue, R., Tsukahara, T., Nakamoto, M., Hara, H., Ushida, K., & Yajima, T. (2012). Commensal bacteria coated by secretory immunoglobulin A and immunoglobulin G in the gastrointestinal tract of pigs and calves. Animal Science Journal, 83(12), 799–804. https://doi.org/10.1111/j.1740-0929.2012.01026.x

Weimer, P. J. (2015). Redundancy, resilience, and host specificity of the ruminal microbiota: Implications for engineering improved ruminal fermentations. Frontiers in Microbiology, 6(APR), 1–16. https://doi.org/10.3389/fmicb.2015.00296

Williams, A.G., and Coleman, G. S. (1992). The Rumen Protozoa (Vol. 1, Issue). Springer-Verlag New York Inc. https://doi.org/10.1007/978-1-4612-2776-2

Yáñez-Ruiz, D. R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D. P., O'Kiely, P., Reynolds, C. K., Schwarm, A., Shingfield, K. J., Yu, Z., & Hristov, A. N. (2016). Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants-a review. Animal Feed Science and Technology, 216, 1–18.

Yáñez-Ruiz, D. R., Abecia, L., & Newbold, C. J. (2015). Manipulating rumen microbiome and fermentation through interventions during early life: A review. Frontiers in Microbiology, 6(OCT), 1–12. https://doi.org/10.3389/fmicb.2015.01133

Yeoman, C. J., Ishaq, S. L., Bichi, E., Olivo, S. K., Lowe, J., & Aldridge, B. M. (2018). Biogeographical Differences in the Influence of Maternal Microbial Sources on the Early Successional Development of the Bovine Neonatal Gastrointestinal tract. Scientific Reports, 8(1), 1–14. https://doi.org/10.1038/s41598-018-21440-8

Yu, S., Zhang, G., Liu, Z., Wu, P., Yu, Z., & Wang, J. (2020). Repeated inoculation with fresh rumen fluid before or during weaning modulates the microbiota composition and co-occurrence of the rumen and colon of lambs. BMC Microbiology, 20(1), 1–15. https://doi.org/10.1186/s12866-020-1716-z

Zhong, R. Z., Sun, H. X., Li, G. D., Liu, H. W., & Zhou, D. W. (2014). Effects of inoculation with rumen fluid on nutrient digestibility, growth performance and rumen fermentation of early weaned lambs. Livestock Science, 162(1), 154–158. https://doi.org/10.1016/j.livsci.2013.12.021

CAPÍTULO V

CONCLUSIONES

Conclusiones

- El líquido ruminal fresco muestreado 3 horas tras ingesta proveniente de animales adaptados a dietas a base de concentrado representa la fuente de microbiota con más actividad *in vitro* y con mayor potencial como inóculo de animales jóvenes.

- La inoculación diaria con líquido ruminal fresco de animales adultos a cabritos durante la lactancia promueve una mayor ingesta de alimento sólido y unos mejores indicadores de desarrollo fisiológico del rumen pero los efectos se disipan con el tiempo.

- Esta inoculación en edad temprana favorece la colonización microbiana del rumen y el establecimiento de una comunidad microbiana diversa, con presencia de protozoos y mejor adaptada a la transición hacia la ingesta de alimento sólido.

- La presencia de animales adultos estimula el desarrollo de la comunidad microbioana del rumen de cabritos en edad temprana criados en lactancia artificial y su adaptación al consumo de forraje.

- Las intervenciones anteriores apenas suponen mejoras en el rendimiento del animal, aunque podrían observarse efectos mayores con la práctica de un destete precoz o retos alimenticios a medio-largo plazo.

- La saliva y particularmente sus componentes bioactivos, donde se incluyen proteínas con función inmune, modulan la microbiota del rumen y su actividad de manera que pueden contribuir a la especificidad de la relación microbiota-animal hospedador.

Conclusions

- Fresh rumen fluid collected at 3 hours post-feeding from animals adapted to concentrate-based diet represents the source of microbiota with greatest activity *in vitro* and potential to be used as inoculum *in vivo*.

- Daily inoculation of fresh rumen fluid from adult goats to goat kids during the milk-feeding period promotes a higher solid feed intake and greater indicators of physiological development although the effects dissipate as time goes by.

- This early-life inoculation favours the rumen microbial colonization and the establishment of a more diverse microbial community with the presence of protozoa and a greater adaptation for the transition to solid feed intake.

- The presence of adult companions stimulates the development of the rumen microbial community in goat kids during early-life reared in artificial systems as well as the adaptation to forage intake.

- The previous interventions promote minor improvements on animal performance but greater effects could be observed under early weaning practices or nutritional challenges in the mid-long term.

- Saliva and particularly its bioactive components, including proteins involved in immune function, modulate the rumen microbiota and its activity in such a way that can contribute to the specificity between animal host and microbiota.