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IN VITRO STUDY OF THE EFFECT OF THE SOURCE OF CARBOHYDRATES AND THE INCLUSION OF ADDITIVES IN DIETS FOR ADAPTATION TO INTENSIVE FATTENING OF RUMINANTS

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"In vitro study of the effect of the source of carbohydrates and the inclusion of additives in diets for adaptation to intensive fattening of ruminants"

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ZAHIA AMANZOUGARENE

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Manuel Fondevila Camps, Catedrático del Departamento de Producción Animal y Ciencia de los Alimentos de la Universidad de Zaragoza,

CERTIFICA:

que la presente Memoria titulada "*Estudio in vitro del efecto de la fuente de carbohidratos y la inclusión de aditivos en la dieta sobre la adaptación de rumiantes al cebo intensivo*", presentada por doña Zahia Amanzougarene para optar al título de Doctor en Producción Animal ha sido realizada bajo mi dirección, y una vez finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos oportunos, firmo la presente en Zaragoza, a 13 de marzo de 2020

Dr. Manuel Fondevila Camps

Este trabajo forma parte del Proyecto de Investigación: “Estrategias para mitigar el impacto de la transición de una dieta forrajera a otras altas en concentrado para el cebo de ganado vacuno”; AGL 2013-46820 (MINECO)

This thesis is dedicated to my parents and family for supporting me over all these years.

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ABBREVIATIONS

ADF: acid detergent fibre

ADL: acid detergent lignin

aNDFom: neutral detergent fibre

ARA: acute ruminal acidosis

ATP: adenosine tri-phosphate

B: barley

BCVFA: branched-chain volatile fatty acids

B: biological effect

BP: sugarbeet pulp

C2: acetic acid

C3: propionic acid

C4: butyric acid

CBC: commercial blend CRINA Ruminants

CHT: chestnut

CI: concentrated inoculum,

CIN: cinnamaldehyde

CP: citrus pulp

CPr: crude protein

CTR: control

d: dose

DM: Dry matter

DMd: dry matter disappeared

DNA: deoxyribonucleic acid

EE: ether extract

EO: essential oils

EUG: eugenol

FI: forage inoculum,

GCT: grape

GP: gas production

LIN: linoleic

M: maize

MB: maize:barley

MFA: medium-chain fatty acids

MP: maize:sugarbeet

NDSF: neutral detergent soluble fibre

NS: not significant

OHT: oak

OM: organic matter

OTU: Operational Taxonomic Unit

PAL: palmitic

PCR: polymerase chain reaction

PEG: polyethylene glycol

QCT: quebracho

S: sorghum

SARA: sub-acute ruminal acidosis

SD: standard deviation

SEM: Standard error of means

STd: starch disappearance

TP: Total phenolics

TRF: terminal restriction fragment

tRFLP: terminal restriction fragment length polymorphism

TT: total tannins

VFA: volatile fatty acids

WB: wheat bran

SUMMARY

In intensive ruminant feeding systems, carbohydrates are the main source of energy for both animals and ruminal microorganisms. In contrast to the fibrous carbohydrates that acts as a buffer of rumen environment, the non-fibrous carbohydrates are efficiently used as energy source, as they are rapidly fermented and to a large extent. However, this increases the risk of ruminal acidosis, especially in the case of young ruminants that are not well adapted to this type of diets.

The primary objective of this Memory was to investigate *in vitro* the impact of the transition from a forage diet to another high in concentrate during the early fattening of ruminants by assessing the effect of the source of carbohydrate and additives supplementation on pH and overall microbial fermentation, under *in vitro* conditions.

Two experiments were carried out under the same incubation conditions in the first section of the Thesis, in order to study the fermentation pattern of several carbohydrate sources (Experiment I.1) and of carbohydrate mixtures (Experiment I.2), and their interaction with the nature of microbial inoculum. Both experiments were carried out using an *in vitro* semicontinuous culture system maintaining poorly buffered conditions from 0 to 6 h of incubation and being gradually buffered to 6.5 from 8 to 24 h to simulate rumen pH pattern. The rumen inoculum was obtained from lambs (n=3) fed with either concentrate and barley straw (concentrated inoculum, CI) or alfalfa hay (forage inoculum, FI).

In Experiment I.1, three cereal grains (barley, B; maize, M; and brown sorghum, S) and three agroindustrial by-products (sugarbeet pulp, BP; citrus pulp, CP; and wheat bran, WB) were tested. From 2 to 12 h incubation, medium pH was influenced by the inoculum source, recording lower values with CI than FI ($P<0.05$). Lowest incubation pH was recorded at 6 h with CI (5.96 ± 0.2) and at 8 h with FI (6.22 ± 0.2), thereafter increasing to an average of 6.64 ± 0.02 and 6.63 ± 0.04 at 24 h, respectively. The volume of gas produced and the total volatile fatty acids (VFA) concentration were higher with CI than FI throughout the incubation ($P<0.05$), but the nature of inoculum did not affect dry matter disappeared (DMd). Molar proportions of acetate, propionate, and butyrate did not record inoculum differences ($P>0.05$), whereas valerate was higher and branched-chain volatile fatty acids (BCVFA) were lower for CI at 6 and 10 h. Among incubated

substrates, S, BP and M maintained the highest pH from 4 to 8 h ($P<0.05$), whereas CP recorded the lowest pH from 2 to 12 h with CI and from 4 to 10 h with FI (minimum values of 5.60 and 5.90), but recovered thereafter to 6.63 at 24 h. With CI, the highest gas volume throughout the incubation was recorded by CP, followed by WB and B, then BP and M, and the lowest volume was recorded by S ($P<0.05$). Similar trends were observed with FI except at 4 h, when the highest gas production was recorded with WB ($P<0.05$). At 6 and 10 h, with CI as well as with FI, total VFA concentration was higher with CP than M, S and BP ($P<0.05$), and at 6 h incubation lactic acid with CP was the highest for both inocula ($P<0.05$). The microbial structure was mainly affected by the incubation series (donor animal) than by the substrate type.

In Experiment I.2, three carbohydrate mixtures (1:1 maize:barley, MB, and maize:sugarbeet pulp at either 1:1, MP, or 3:1, 3MP) were evaluated. The inoculum source markedly affected the medium pH, which was lower with CI ($P<0.05$) than FI during the first 8 h of incubation. However, the volume of gas recorded with CI as inoculum was always superior to that with FI ($P<0.05$), and the DMd with CI tended to be higher than that disappeared with FI (0.38 vs. 0.34; $P= 0.077$). At all sampling times, the concentration of total VFA was higher ($P<0.05$) with CI than FI, but this parameter was not affected by the type of mixture ($P>0.05$). At 24 h, the highest proportion of acetate was recorded by MP, whereas MB and 3MP recorded the high proportion of butyrate and valerate ($P<0.05$). At 6 h, the highest and lowest lactic acid concentrations were promoted by 3MP and MP ($P<0.05$). As in Experiment I.1, bacterial diversity was markedly affected by the inoculum type. Similarly, inoculum effects were detected in Shannon index ($P=0.004$) and tended to be significant in Richness index ($P= 0.074$). When CI was used, minimum incubation pH was reached after 6 h, being higher ($P<0.05$) with MP than with MB and 3MP from then (6.06, 5.97 and 5.95 at 6 h, respectively) to 20 h (6.78, 6.67 and 6.67). The gas production (GP) was highest for MB at 2 h and from 6 to 16 h, and lowest with 3MP from 2 to 8 h and with MP from 20 h onwards ($P<0.05$). Higher DMd was recorded by MB and 3MP compared with MP (0.440 and 0.396 vs. 0.305; $P<0.05$). Regarding the microbial diversity, MP and MB clustered together when rumen from lambs 1 and 2 was used; however, with rumen liquid from lamb 3 the two mixtures including sugarbeet pulp clustered together. With FI, pH was lower with MB than with 3MP at 6 h (6.33 vs. 6.39, $P<0.05$), and with MB it was maintained lowest onwards ($P<0.05$). The volume of gas from 3MP was lowest ($P<0.05$) up to 4 h, and it was lower

with 3MP than with MB from 6 h onwards (P<0.05), whereas differences between MB and MP were only recorded after 24 h. Concerning the microbial diversity, when inoculum from lamb 6 was used the mixtures with sugarbeet pulp clustered together. No differences between mixtures were found in Shannon index (P= 0.753); however, mixtures effects were detected in Richness index (P= 0.041), mixtures ranking as follows: 3MP (107.17) followed by MP (102.33) and then MB (96.67; P<0.05, SEM= 2.378). In both concentrate and forage environments, MP maintains a more stable pH pattern while microbial fermentation was not noticeably depressed compared to higher starch proportions mixtures (MB and 3MP). The microbial fermentation with MB and 3MP was depressed at a higher extent than that with MP.

In section II, a first methodological *in vitro* experiment (Experiment II.3) was carried out to evaluate the potential effects of tannin extracts from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT), for reducing rumen fermentation of barley meal under high concentrate feeding. The four tannin sources were included at three levels (10, 20 and 30 mg/g substrate), in three 24 h incubation series. Intensive feeding conditions were simulated by adjusting incubation pH at 6.2 and by using inoculum from beef calves given 0.91 concentrate proportion ad libitum. Incubation pH at 8 and 24 h incubation ranged from 6.14 to 6.18 and from 5.94 to 6.00, respectively. Increasing addition of tannin extracts linearly reduced GP from barley meal alone (control; CTL), for CHT (P< 0.05 up to 6 h; P< 0.10 from 8 to 18 h), OHT (P< 0.05 from 2 to 12 h; P< 0.10 from 18 to 24 h), GCT (P< 0.05 from 2 to 24 h) and QCT (P< 0.10 from 2 to 6 h). However, a quadratic trend (P< 0.10) was also detected on GCT up to 4 h and from 10 h onwards. Among extracts, the effect of GCT was the highest and that of CHT lowest, and the biological effect (BE) of tannins at 24 h tended to differ among tannin sources (P= 0.069), showing higher values with GCT than CHT, irrespective of their level of inclusion (P> 0.10). Similar results were observed on DMd after 24 h, that showed a linear decrease with all sources of tannins (P< 0.05) and was lower with GCT than with both QCT and CHT (P< 0.05). All tannin sources linearly increased (P< 0.05) molar butyrate proportion from barley alone, at the expense of a linear reduction in propionate proportion in GCT (P< 0.01) and CHT (P< 0.10). The four tested tannin sources reduced rumen microbial fermentation of barley grain, with a maximum response being recorded with grape and chestnut extracts. Except for chestnut, all sources already reached their maximum level

of response at their first level of inclusion (10 mg/g). The inclusion of tannins in diets for fattening young ruminants did not negatively affect the microbial fermentation.

In another experiment of this section (Experiment II.4), the effect of increasing levels of either fatty acids or essential oils on fermentation of barley grain was studied in two *in vitro* experiments (Experiment II.4.1 and Experiment II.4.2) under the same incubation conditions than in Experiment II.3. Treatments were: barley alone (CTL), medium-chain fatty acids (MFA; 2, 4 and 6 mg/g), palmitic (PAL) and linoleic (LIN) acids, these included at 15, 30 and 45 mg/g. Compared with non-supplemented barley (CTL), the inclusion of LIN quadratically reduced GP ($P< 0.05$ up to 24 h), whereas such reduction tended to be linear ($P< 0.10$ at 12 and 24 h) with PAL, and a quadratic trend ($P<0.10$ at 24 h) was detected with MFA. DMd and estimated microbial mass in the liquid medium from CTL were quadratically reduced ($P< 0.05$) with MFA and LIN. Total VFA concentration and acetate proportion tended ($P< 0.10$) to increase linearly with LIN, whereas propionate proportion tended to decrease ($P= 0.051$). In Experiment 4.2, cinnamaldehyde (CIN; 30, 60 and 90 mg/g), eugenol (EUG; 60, 120 and 180mg/g), and a commercial blend of essential oils (CBC; 30, 60 and 90 mg/g), were tested in comparison with barley alone (CTL). The GP from CTL decreased linearly with CIN throughout the incubation ($P< 0.001$ up to 24 h) and decreased quadratically with EUG ($P= 0.047$ at 24 h), whereas it increased linearly with CBC ($P< 0.05$ at 12 h, and $P<10$ at 8 and 24 h). Microbial mass decreased quadratically with EUG ($P< 0.001$). The total VFA concentration from CTL decreased both linearly ($P< 0.05$) and quadratically ($P< 0.001$) with CIN and EUG, respectively. Acetate proportion increased quadratically with the inclusion of EUG ($P< 0.05$) at the expense of propionate ($P< 0.001$), and a linear reduction in propionate proportion was also observed with CIN ($P< 0.05$). This study showed that, with different magnitude, both fatty acids and essential oils may reduce barley acidification potential. Among the different additive tested herein, the essential oil CBC can improve the rumen microbial fermentation of barley.

The last Experiment (Experiment II.5) was designed to compare five additives of different nature included at a single dose (grape condensed tannins; GCT 20 mg/g, a mixture of medium-chain fatty acids; MFA 4 mg/g, linoleic acid; LIN 30 mg/g, eugenol; EUG 120 mg/g, cinnamaldehyde; CIN 60 mg/g) in their effect on *in vitro* barley fermentation under simulated intensive feeding conditions for beef diets. Rumen inoculum from beef calves fed ad libitum with a concentrate and straw

(concentrate:forage; 0.91:0.09) was used. Contrarily to both Experiments 3 and 4, this trial was carried out using the semicontinuous system, under a poorly buffered medium from 0 to 6 h, and allowed pH to rise to around 6.5 from 8 to 24 h. On average, the incubation pH reached its minimum after 6 h (6.89 ± 0.07), and thereafter the maximum was reached at the end of incubation (6.41 ± 0.03). From 6 h onwards, the highest pH was recorded by GCT, whereas a lower pH values were recorded by CIN ($P< 0.05$). Throughout all the incubation period, EUG and CIN produced lower volume of gas than CTR ($P< 0.05$); however, GCT produced lower volume than CTR after 8 h ($P< 0.05$). Similar to the results observed with GP, the inclusion of different additives reduced the dry matter disappearance (DMd) of barley, being highest with CTR ($P< 0.05$) and lowest with GCT ($P< 0.05$). High concentration of total VFA was recorded at 8 h ($P< 0.05$), as well as for acetate and propionate proportions ($P< 0.05$). Between treatments, on average, MFA recorded the highest concentration of VFA, while EUG and CIN recorded the lowest concentration of total VFA and propionate proportion. The highest proportion of propionate was promoted by LIN ($P< 0.05$), results that were supported by those showed by bacterial diversity. In contrast to cinnamaldehyde, grape condensed tannins and fatty acids may positively affect barley acidification potential.

Methodologically, the semicontinuous system used in this experiment allowed to detect marked differences in the effect of the different additives on fermentation kinetics of barley, and provides with a useful tool for their relative comparison in non-conventional fermentation conditions.

RESUMEN

En los sistemas de alimentación intensiva de rumiantes, los carbohidratos son la principal fuente de energía tanto para los animales como para los microorganismos ruminales. A diferencia de los carbohidratos fibrosos, que actúan como tampón del ambiente ruminal, los carbohidratos no fibrosos se usan eficientemente como fuente de energía, ya que son fermentados rápida y extensamente. Sin embargo, este hecho aumenta el riesgo de acidosis ruminal, especialmente en el caso de rumiantes jóvenes que no están bien adaptados a dietas altamente concentradas.

El objetivo principal de esta Tesis fue investigar *in vitro* el impacto de la transición de una dieta a base de forraje a otra dieta con alta inclusión de concentrado durante el engorde temprano de rumiantes, mediante la evaluación del efecto de la fuente de carbohidratos y la suplementación de aditivos sobre el pH y la fermentación microbiana.

En la primera sección de la Tesis, se llevaron a cabo dos experimentos sujetos a las mismas condiciones de incubación, para estudiar el patrón de fermentación de varias fuentes de carbohidratos (Experimento I.1) y mezclas de carbohidratos (Experimento I.2), así como su interacción con la fuente de inóculo microbiano. En ambos experimentos se utilizó un sistema de cultivo semicontinuo *in vitro* para mantener un tamponamiento reducido de 0 a 6 h de incubación, aumentando gradualmente a 6,5 de 8 h y hasta 24 h para simular el patrón de pH del rumen. El inóculo ruminal se obtuvo de corderos (n = 3) alimentados con concentrado y paja de cebada (inóculo concentrado, IC) o heno de alfalfa (inóculo forrajero, IF).

En el Experimento I.1, se probaron tres granos de cereal (cebada, C; maíz, M; y sorgo marrón, S) y tres subproductos agroindustriales (pulpa de remolacha azucarera, PR; pulpa de cítricos, PC; y salvado de trigo, ST). De 2 a 12 h de incubación, la fuente del inóculo influenció el pH del medio, registrando con IC valores inferiores a IF ($P < 0,05$). El pH de incubación más bajo se registró a las 6 h con IC (5,96 ± 0,2) y a las 8 h con IF (6,22 ± 0,2), aumentando posteriormente a las 24 h hasta 6,64 ± 0,02 y 6,63 ± 0,04, respectivamente. El volumen de gas producido y la concentración total de ácidos grasos volátiles (AGV) fueron mayores con IC que con IF durante toda la incubación ($P < 0,05$), pero el origen del inóculo no afectó la desaparición de la materia seca (dMS). Las proporciones molares de acetato, propionato y butirato no mostraron diferencias debidas

al inóculo (P >0,05), mientras que el valerato aumentó y los ácidos grasos volátiles ramificados (AGVR) disminuyeron con IC a las 6 y 10 h. Entre los sustratos incubados, S, PR y M mantuvieron un mayor pH a las 4 y 8 h (P <0,05), mientras que con PC se registró el pH más bajo, de 2 a 12 h con IC y de 4 a 10 h con IF (valores mínimos de 5,60 y 5,90), recuperándose a las 24 h (6,63). Con IC, el mayor volumen de gas durante toda la incubación se registró con PC, seguido por ST y C, luego PR y M, y finalmente el volumen más bajo se registró con S (P <0,05). Se observaron tendencias similares con IF excepto a las 4 h, cuando la mayor producción de gas se registró con ST (P <0,05). A las 6 y 10 h, la concentración total de AGV fue superior para PC que M, S y ST (P <0,05) con ambos inóculos, mientras que a las 6 h se observó el pico de mayor producción de ácido láctico con PC resultó para ambos inóculos (P <0,05). En cuanto a la estructura microbiana, ésta se vio afectada en mayor medida por las diferencias entre incubaciones (animal donante) que por el sustrato.

En el Experimento I.2 se evaluaron tres mezclas de carbohidratos (1:1 M:C, MC, y M:PR a 1:1, MP, o M:PR a 3:1, 3MP). La fuente de inóculo afectó notablemente el pH del medio, menor con IC (P <0,05) que con IF durante las primeras 8 h de incubación. Sin embargo, el volumen de gas registrado con IC siempre superó al de IF (P <0,05), y a su vez, con IC la dMS tendió a ser mayor que con IF (0,38 vs. 0,34; P = 0.077). En todos los tiempos de muestreo, la concentración total de AGV fue mayor (P <0,05) con IC que con IF, aunque la mezcla de carbohidratos no incidió en este parámetro (P >0,05). A las 24 h, MP registró la mayor proporción de acetato, mientras que MC y 3MP registraron la mayor proporción de butirato y valerato (P <0,05). A las 6 h, las concentraciones más altas y bajas de ácido láctico se observaron con las mezclas de 3MP y MP (P <0,05), respectivamente. Como en el Experimento I.1, la diversidad bacteriana se vio notablemente afectada por el tipo de inóculo. Del mismo modo, el efecto del inóculo se detectó en el índice de Shannon (P = 0,004) y tendó a ser significativo en el índice de riqueza (P = 0,074). Con IC, el mínimo pH de incubación se alcanzó después de 6 h (6,06, 5,97 y 5,95 a las 6 h con MP, MC y MP), siendo posteriormente más alto (P <0,05) con MP que con MC y 3MP a 20 h (6,78, 6,67 y 6,67, respectivamente). La producción de gas fue mayor con MC a las 2 h y de 6 a 16 h, e inferior con 3MP de 2 a 8 h y con MP a partir de las 20 h (P <0,05). La dMS fue mayor con MC y 3MP en comparación con MP (0,440 y 0,396 vs. 0,305, respectivamente; P <0,05). Con respecto a la diversidad microbiana, MC y MP se agruparon cuando se incubaron con inóculo de los corderos 1 y

2; sin embargo, con el líquido ruminal del cordero 3 las dos mezclas que incluyeron PR se agruparon. Con el uso de IF, a las 6 h se observó un pH más bajo con MC que con 3MP (6,33 vs. 6,39, P <0,05), manteniéndose posteriormente esa diferencia (P <0,05). El volumen de gas producido con 3MP fue el más bajo (P <0,05) hasta las 4 h, y fue menor que con MC a partir de las 6 h (P <0,05), mientras que las diferencias entre MC y MP solo se registraron a las 24 h. Con respecto a la diversidad microbiana, las mezclas con PR se agruparon cuando se usó el inóculo del cordero 6. No se encontraron diferencias entre mezclas en el índice de Shannon (P = 0,753); sin embargo, sí influenciaron el índice de riqueza (P = 0,041), clasificándose de la siguiente manera: 3MP (107,17) seguido de MP (102,33) y finalmente de MC (96,67; P <0,05, EEM = 2,378). Tanto en los ambientes generados por una dieta concentrada como forrajera, la mezcla MP mantuvo un patrón de pH más estable, mientras que la fermentación microbiana no se redujo de forma notable en comparación con las mezclas con mayores proporciones de almidón (MC y 3MP).

En la sección II, se realizó un primer experimento metodológico *in vitro* (Experimento II.3) para evaluar el efecto potencial de los extractos de taninos de quebracho (TQC), uva (TUC), castaño (THC) y roble (THR) en la reducción de la fermentación ruminal de la cebada bajo una alimentación alta en concentrados. Las cuatro fuentes de taninos se incluyeron en tres niveles (10, 20 y 30 mg/g de sustrato), en tres series de incubación de 24 h. Las condiciones de alimentación intensiva se simularon ajustando el pH de incubación a 6,2 y mediante el uso del inóculo de terneros con una proporción de concentrado *ad libitum* de 0,91. El pH de incubación a las 8 y 24 h osciló entre 6,14 y 6,18 y entre 5,94 y 6,00, respectivamente. Con la adición gradual de extractos de taninos se redujo linealmente la producción de gas comparado con la cebada sola (CTL): con THC hasta las 6 h (P <0,05) y de 8 a 18 h (P <0,10), con THR de 2 a 12 h (P <0,05) y de 18 a 24 h (P < 0,10), con TUC de 2 a 24 h (P <0,05) y con TQC de 2 a 6 h (P <0,10). Sin embargo, también se detectó una tendencia cuadrática (P <0,10) con TUC hasta las 4 h y a partir de las 10 h en adelante. Entre los extractos, el mayor efecto se obtuvo con TUC mientras que el menor se obervó con THC, y el efecto biológico (EB) a las 24 h tendió a diferir entre las fuentes de taninos (P = 0.069), mostrando valores más altos con TUC que THC, independientemente de su nivel de inclusión (P >0,10). Resultados similares se observaron sobre la dMS a las 24 h, mostrándose una disminución lineal con todas las fuentes de taninos (P <0,05), aunque fue menor con TUC que con TQC y THC (P <0,05). Todas las fuentes de taninos aumentaron linealmente (P <0,05) la

proporción molar de butirato de la cebada sola, a expensas de una reducción lineal en la proporción de propionato en TUC (P <0.01) y THC (P <0,10). Las cuatro fuentes de taninos analizadas redujeron la fermentación ruminal de la cebada, registrándose una respuesta máxima con los extractos de uva y de castaño. A excepción del castaño, todas las fuentes alcanzaron su nivel máximo de respuesta con su nivel de inclusión más bajo (10 mg/g). La inclusión de taninos en las dietas para engorde de rumiantes jóvenes no afectó negativamente la fermentación microbiana.

En otro experimento de esta sección (Experimento II.4), se estudió el efecto del aumento de los niveles de ácidos grasos o de aceites esenciales en la fermentación del grano de cebada en dos experimentos *in vitro* (Experimento II.4.1 y Experimento II.4.2) bajo las mismas condiciones de incubación que en el Experimento 3. Los tratamientos fueron: cebada sola (CTL), ácidos grasos de cadena media (AGCM; 2, 4 y 6 mg/g), y ácidos palmítico (PAL) y linoleico (LIN), ambos incluidos a 15, 30 y 45 mg/g. En comparación con la cebada no suplementada (CTL), la inclusión de LIN redujo la producción de gas de manera cuadrática hasta las 24 h (P <0,05), mientras que dicha reducción tendió a ser lineal entre las 12 y 24 h con PAL (P <0,10), y a una evolución cuadrática con AGCM a las 24 h (P <0,10). La dMS y la masa microbiana estimada en el medio líquido de CTL se redujeron cuadráticamente con AGCM y LIN (P <0,05). La concentración total de AGV y la proporción de acetato tendieron a aumentar linealmente con LIN (P <0,10), mientras que la proporción de propionato tendió a disminuir (P = 0,051). En el Experimento 4.2, se valoraron cinamaldehído (CIN; 30, 60 y 90 mg/g), eugenol (EUG; 60, 120 y 180 mg/g) y una mezcla comercial de aceites esenciales (CBC; 30, 60 y 90 mg/g) frente la cebada sola (CTL). Con ésta, la producción de gas disminuyó linealmente con CIN durante toda la incubación (P <0,001 hasta 24 h) y cuadráticamente con EUG (P = 0.047 a las 24 h), mientras que aumentó linealmente con CBC (P <0,05 a las 12 h, y P <10 entre las 8 y 24 h). La masa microbiana disminuyó cuadráticamente con EUG (P <0.001). La concentración total de AGV disminuyó tanto linealmente (P <0,05) como cuadráticamente (P <0,001) al suplementar la cebada con CIN y EUG, respectivamente. La proporción de acetato aumentó cuadráticamente con la inclusión de EUG (P <0,05) a expensas del propionato (P <0,001), y también se observó una reducción lineal en la proporción de propionato con la adición de CIN (P <0,05). Este estudio mostró que, en diferente grado, tanto los ácidos grasos como los aceites esenciales pueden reducir

el potencial de acidificación de la cebada. Entre los diferentes aditivos probados aquí, el aceite esencial CBC puede mejorar la fermentación microbiana ruminal de la cebada.

El último experimento (Experimento II.5) fue diseñado para comparar el efecto de cinco aditivos de diferente naturaleza incluidos a dosis única (taninos condensados de uva, TUC 20 mg/g; una mezcla de ácidos grasos de cadena media, AGCM 4 mg/g; ácido linoleico, LIN 30 mg/g; eugenol, EUG 120 mg/g; y cinamaldehído, CIN 60 mg/g) sobre la fermentación de la cebada *in vitro* simulando condiciones de alimentación intensiva para dietas de vacuno de carne. Como inóculo se empleó contenido ruminal de terneros alimentados *ad libitum* con un concentrado y paja (relación concentrado:forraje; 0,91:0,09). Contrariamente a los Experimentos 3 y 4, esta prueba se llevó a cabo utilizando el sistema semicontinuo, bajo un medio con un sistema tampón reducido de 0 a 6 h, y aumentado el pH hasta alrededor de 6,5 de 8 a 24 h. El pH de incubación alcanzó su mínimo después de las 6 h (6,89 ± 0,07), y aumentó posteriormente hasta alcanzar el máximo al final de la incubación (6,41 ± 0,03). A partir de las 6 h en adelante, el pH más alto se registró con TUC, mientras que con CIN los valores fueron más bajos ($P < 0,05$). Durante todo el período de incubación, con EUG y CIN se produjo un volumen de gas inferior al de CTR ($P < 0,05$); sin embargo, con TUC el volumen producido fue menor que el CTR después de 8 h ($P < 0,05$). De manera similar, la inclusión de diferentes aditivos redujo la dMS de la cebada, siendo la mayor con CTR ($P < 0,05$) y la menor con TUC ($P < 0,05$). Se registró una alta concentración de AGV totales a las 8 h ($P < 0,05$), con proporciones de acetato y propionato elevadas ($P < 0,05$). Entre tratamientos, en del medio, con los AGCM se registró la concentración más alta de AGV, mientras que con EUG y CIN se registraron las concentraciones más bajas de AGV totales y de proporción de propionato. En este aspecto, la mayor proporción de propionato se asoció con LIN ($P < 0,05$), respaldado por la diversidad bacteriana. A diferencia del cinamaldehído, los taninos condensados de uva y los ácidos grasos pueden afectar positivamente el potencial de acidificación de la cebada.

Metodológicamente, el sistema semicontinuo utilizado en este experimento permitió detectar diferencias importantes en el efecto de los diferentes aditivos sobre la cinética de fermentación de la cebada. Ello proporciona una herramienta útil para realizar una comparación relativa en condiciones de fermentación no convencionales.

Chapter I. General introduction

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Meat production livestock systems in Europe in general, and in particular in Spain have been suffered radical changes from extensive to intensive systems. Thus, early weaning of the young ruminant is the strategy followed to prepare the animal for fattening. The three pre-stomachs of neonatal ruminants, the rumen in particular is an organ sterile and nonfunctional. Its development starts when animals begin to consume solid feed at two to three weeks of age (Hungate, 1966; Drackley et al., 2008; Govil et al., 2017), but it reaches its full capacity few months later. The transition of monogastric digestion to a polygastric implies anatomical, physiological, and metabolic changes (Quigley at al., 1991; Reynolds et al., 2004; Wang et al., 2009; Jiao et al., 2015). Different microorganisms will colonize the rumen, so it becomes gradually functional (Fonty et al., 1987; Rey et al., 2012). For a good development of the rumen, concentrates are more effective than forages, because the volatile fatty acids produced from the consumption of concentrate, mainly propionic and butyric acids, are responsible for the establishment of a functional rumen (Tamate et al., 1962; Quigley et al., 1997; Heinrichs, 2005). In this way Danielli et al. (1945) observed that the absorption of volatile fatty acids at pH 5.8 was faster than at pH 7.5, and the rate of absorption is higher when the chain of the acid is longer (butyrate > propionate > acetate) owing to their liposolubility, which is highest for butyric acid and lowest for acetic acid. Another hypothesis, proposed by Baldwin (1999) indicates that the presence of butyrate indirectly contributed to an endocrine response, increasing the plasma insulin-like growth factor (IGF-1) concentrations, which stimulated the development of rumen papillae.

In Spain, before weaning, young ruminants are fed with forages or pastures that are occasionally complimented. However, after weaning, the diets used for fattening are based on cereal-rich concentrates (high-energy diets) to reach the objective of slaughter at an early age, particularly for calves and lambs. In general, the former are slaughtered at 8-10 months of age and 280-300 kg carcass weight (García-Rebollar et al., 2008) and the later at 3-4 months and 10-15 kg carcass weight (Sañudo at al., 1998). The abrupt change in diet type without a good management of the transition phase leads to onset of digestive disorders, which would be the second cause responsible of mortality and morbidity of ruminants in feedlots after that of respiratory diseases (Nagaraja and Lechtenberg, 2007). The most common digestive disorder is rumen acidosis, which is

associated to the growth in consumption of high-energy diet with low forage supply. The acidosis processes cause a nonphysiologic accumulation of volatile fatty acids in the rumen, which are produced at a very high rate from microbial fermentation, that cannot be balanced by regulatory factors such as the buffering capacity of saliva produced during rumination, thus leading to reduced rumen pH (Martin et al., 2006; Nagaraja and Titgemeyer, 2007; Nagaraja and Lechtenberg, 2007). It has been known for a long time that the pH fluctuated inversely with the concentration of volatile fatty acids in the rumen (Balch and Rowland, 1957). Normally, the pH of rumen fluid is in the range of 5.8-6.8 (Bergman, 1990), although Mialon et al., (2008) showed that the rumen pH is normally situated above 6.25 to ensure the optimal conditions for majority of microbial species. This pH is typically achieved by forage rich diets. In contrast, high concentration diets that characterised by rapid fermentation can decrease the pH to values below 6.0 or 5.5 or even below 5.0 (Hungate, 1966; Bergman, 1990). The type and composition of diet strongly condition the microbial populations (Jouany and Ushida, 1998). Thus, during rumination, high fibrous diets stimulate saliva production that provides urea and minerals, for maintaining normal rumen microbial growth and development (Govil et al., 2017). However, these diets do not provide sufficient energy for the rapid fattening of the animal. In contrast, the increase of cereals inclusion in diets for intensive fattening provides high-energy, but decreases the saliva and the arrival of bicarbonate ions in the rumen, which leads to drop rumen pH causing both a reduction of cellulolytic population and an increase of organisms that produce both propionate and lactate (Balch and Rowland, 1957). From this point, it is necessary to know in an accurate way the process of microbial fermentation of feeds commonly used in concentrate diets in order to better understand nutritive processes of intensively reared ruminants and to establish strategies to minimise the negative impact of this practice. These strategies must be focused on how to reach a good feeding management preserving the same energy level in diets for the intensive fattening, without involve the appearance of different digestive disorder especially rumen acidosis.

In recent years, many studies have been interested in the addition of different types of products that are defined as additives in diets for ruminants, among them, the zootechnical feed additives like plant extracts (tannin, essential oils), fatty acids, etc. These additives are characterized by their specific capacity to enhance the fermentation of different carbohydrates in the rumen, modifying and modulating the microbial activity. Previous studies (Benchaar et al., 2007; Calsamiglia et al., 2007; Beauchemin et al., 2009;

Rodríguez et al., 2014) supposed that the use of these additives positively affect animal performances and their welfare. Nevertheless, sometimes the results from a study to another are contradictory. In fact, the incompatibility of results between research groups is due to different factors, among them the dose of the dietary inclusion of the additive, the type of active compound of the additive and its concentration, the nature of the diet, the experimental conditions (*in vivo* vs. *in vitro*), and the adaptation time of the ruminal bacteria to the additive. In addition, it is very important to note that major studies were focused on the effect of these additives in diets for beef or dairy cattle, marginalizing the small ruminants production sector. These factors are considered as limitations that make difficult to understand the pure effect of each additive.

In vivo and *in situ* are methods that allow for the study and evaluation of nutritive value of animal feeds. However, these methods have many disadvantages, being expensive, laborious and need more time to obtain results. In contrast, the *in vitro* methods are less expensive and less time consuming. In addition, the *in vitro* methods allow to maintain experimental conditions under control in a more precise way than *in vivo* trials (Raab et al., 1983). The application conditions of the *in vitro* gas measurement technique are suitable to mimic high forage diets, by maintaining an incubation pH over 6.5 using a highly buffered medium (Goering and Van Soest, 1970; Mould et al., 2005a). These conditions, however, are not adapted to the study of fermentative processes in intensive feeding, and therefore estimation of concentrate fermentation is biased. The use of other buffers like citrate or phosphate or the acidification of the medium to get the required pH has been proposed (Grant and Mertens, 1992), but the former is often more expensive and the latter rapidly exhausts the buffering capacity (Mould et al., 2005a). Continuous and semi-continuous incubation systems (Hoover et al., 1976; Czerkawski and Breckenridge, 1977) have been developed for maintaining pH at a low range through systematic infusion of a buffering solution, but this increases the complexity and price of the equipment. Using batch-culture systems and/or a simple semi-continuous incubation system (Fondevila and Pérez-Espés, 2008, modified by Prates et al., 2010), the procedures proposed by Amanzougarene and Fondevila (2018) allow for extending working conditions to the study of effects in intensive feeding systems.

Chapter II. Literature review

Chapter II. Literature review

1. Ruminal acidosis in weaned ruminants

The rumen of young ruminants destined to intensive fattening must be reach its full development prior to weaning because of the strategies followed in this type of production system. To ensure the rumen development, young ruminant should be adapted to digest solid feeds, especially high carbohydrates diets. Church (1988) reported that, in contrast to liquid feeds, solid feeds are directed to the retículo-rumen for digestion, which will stimulate organ development. The lack of rumen development prior to weaning followed by the abrupt weaning and the introduction of high levels of concentrate in the diets for fattening of these animals negatively influences rumen pH, ruminal microbial activity, and rumen volatile fatty acids (VFA) production.

1.1. Rumen pH and fermentation

Rumen fermentation of feedstuffs is the result of coordinated activities of several microorganisms. Rumen pH is an important factor affecting fermentation. It affects the rate and extent of microbial fermentation, as well as the microbial species involved in the process (Russell and Dombrowski, 1980; Hiltner and Dehority, 1983). The pH of the rumen undergoes diurnal fluctuations and reflects the balance of acid production and absorption, as well as the buffering function provided by the bicarbonate ion from the saliva. Ruminal pH varies considerably during the day (Figure 1), and is particularly driven by the amount of fermentable carbohydrates in each meal (Krause and Oetzel, 2006). Dado and Allen, (1993) and Nocek et al. (2002) indicated that from 0.5 to 1.0 units within a 24 h period are common changes in rumen pH. This represents a five to ten-fold change in the hydrogen ion concentration in the rumen (Krause and Oetzel, 2006). After feeding, VFA production from fermentation increases resulting in a drop of rumen pH. As the rate of VFA production decreases while absorption continues in the subsequent hours, the rumen pH will progressively rise again (Crater et al., 2007) until the next feeding. The nature of the diet is a key factor among those that contribute to change rumen pH, although ruminants have a highly developed system to maintain the pH within the physiological limits of 5.5 to 7.0 (Dehority, 2003) based on processes of salivation and absorption through the rumen wall, as well as by inversing rate of passage (Krause and Oetzel, 2006). A drop of pH to 6.00 and below should reduce microbial fermentative

activity (Hungate, 1966; Russell and Dombrowski, 1980), thus contributing to enhancing the negative effect of acidification. Mould et al. (1983) observed that cellulolysis was party inhibited at 6.3 and then almost totally inhibited when pH fell below 6.0. The reduction in the amount of forage in diets, and the increase in the amount of high fermentable carbohydrates can be negatively correlated to rumen pH (Yang et al., 2001; Krause et al., 2002).

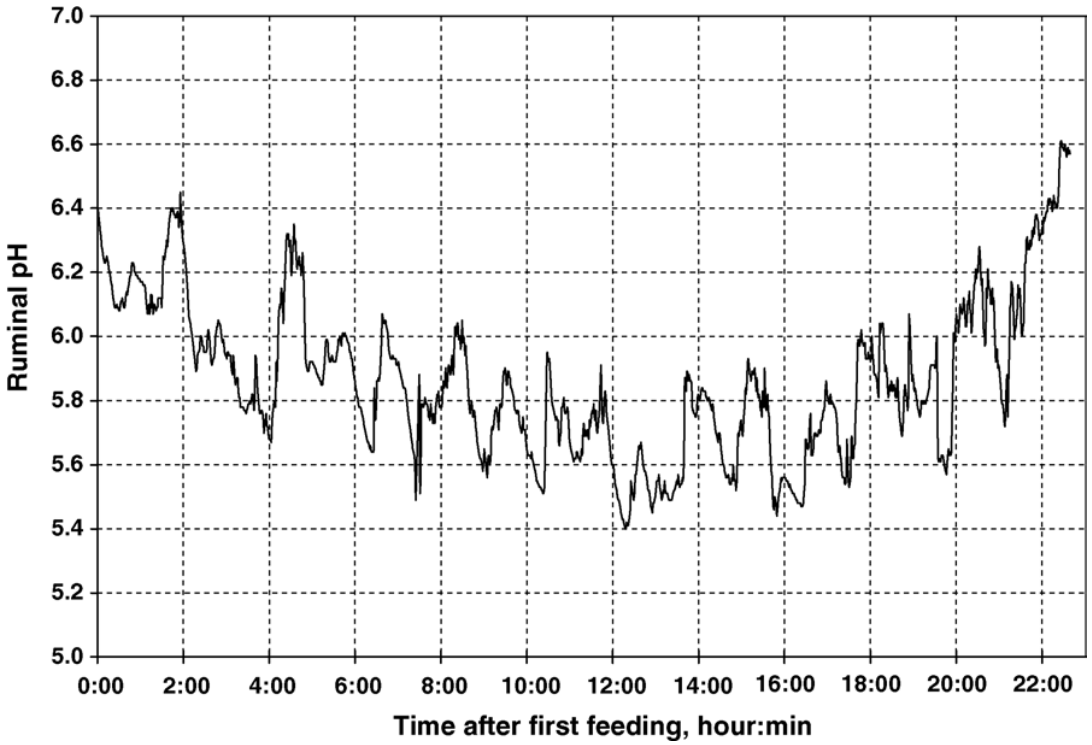

Figure 1. Post-feeding variations in ruminal pH over a period of 24h. The cow was fed dry cracked shelled corn and fine alfalfa silage twice daily (12h interval). Dry matter intake of the current day was 22.7 kg. Average ruminal pH for that day was 5.87 (data from Krause and Combs, 2003).

1.2. Physiology of buffering capacity of the rumen

The intensive livestock feeding systems are based on diets rich in ingredients with highly available nutrients that are digested or fermented at a high rate, in order to get high productive performances. This strategy causes changes on the physiological buffering capacity of the digestive system of the ruminants, especially on the pH ranges that may

result in rumen acidosis. In such scenario, a range of pH from 6.0 to 6.8 in the rumen should be desirable for ensuring an optimum microbial activity (Dehority, 2003).

The buffering capacity is defined as the ability of a solution to resist changes in pH (Giger-Reverdin et al., 2002). This buffering capacity refers to the number of moles of H^+ that must be added to one litre of solution to cause a change in pH (Counotte et al., 1979), or specifically to decrease the pH by one unit (Segel, 1976). This value depends on the buffer system and on the initial pH (Kohn and Dunlap, 1998), and weak acids and bases provide buffering better than strong acids and bases because of the establishment of an equilibrium between the acid and the conjugate base. The buffering capacity of the rumen is mainly defined by the pH value, the partial pressure of CO_2, and the concentration of volatile fatty acid and lactic acid (Counotte et al., 1979). Ruminal pH depends on CO_2 partial pressure, as is shown in the reaction:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

The variations in the amount of CO_2 dissolved in the medium automatically vary the amount of H^+ ions (Marden et al., 2005). Then, the pH of the rumen is defined by:

$$\text{pH rumen} = 7{,}74 + \log (HCO_3^-/pCO_2)$$

The most prevalent ruminal buffers are those originating from saliva (HCO_3, HPO_4). Counotte et al. (1979) and Erdman (1988) reported that the most important is HCO_3. Phosphate is a minor component and is of minor value as buffering agent in rumen fluid, but it is very important in the regulation of rumen pH (Counotte et al., 1979). In bovine, saliva concentrations of HCO_3 are 90 to 120 mM and those of HPO_4 are 20 to 25 mM (Bartley, 1976). Araujo-Febres and Vergara-López (2007) reported that the rumen buffer system is very complex, based on an abundant production of saliva (mainly from secretions of bicarbonate and phosphate), the elimination of VFA by their absorption through the rumen wall, and the mineral salts which react with the organic acids from plants producing CO_2. The saliva secreted by ruminants has a pH of 8.0 to 8.2 and a high concentration of sodium, bicarbonate and phosphate ions, and behaves as lubricant of the feed consumed, characteristics which allows their buffer action on the rumen fluid (Counotte et al., 1979, Krause and Oetzel, 2006). When the saliva pH is 8.0, 86% of the phosphate is in the HPO_4^{2-} form, upon entering the rumen, the following reaction occurs $HPO_4^{2-} + H_3O^+ \leftrightarrow H_2PO_4^- + H_2O$ until 10% is in the form of HPO_4^{2-} and 90% as H_2PO^{4-}. This reaction raises the rumen pH, but reduces the buffering capacity of the phosphate

system (Counotte et al., 1979). Bicarbonate acts similarly, but is more important when the rumen pH is 6.25 or lower because the pH value of the equilibrium reaction (HCO_3^- + H_3O^+ ↔ H_2CO^3 + H_2O ↔ CO_2 + $2H_2O$) is 6.25 (Counotte et al., 1979). The measurement of the buffer capacity of rumen contents is useful to express the ability of this organ to remain more or less stable at certain pH range.

The buffering capacity is influenced by different factors that alter the amount or quality of saliva produced. The high consumption of forage stimulates saliva secretions, and the carbohydrates of these forages are slowly digestible, while the consumption of cereal grains or feeds rich in starch or soluble sugars that are rapidly fermentable generates a high concentration of organic acids (Fischer et al., 1994). Thus, rumen pH drops below the physiological levels when ruminants consume excessive amounts of rapidly fermentable (non-fiber) carbohydrates. Besides, in intensive production systems where the use of concentrates is high, the rate and extent of degradation of the fibrous fraction of feed, which proportion is already reduced, is diminished by the low activity of cellulases of rumen microorganisms at low pH (Araujo-Febres and Vergara-López, 2007). Under these conditions, the rumination decreases as well as the secretion of saliva, and thus the buffering capacity of the rumen fluid decreases.

1.3. Rumen acidosis

Rumen bacteria are considered the most important and diverse microbial group between the different types of microorganisms in the rumen that contribute in process of rumen fermentation and it has been classified in accordance with their main metabolic activity (Belanche et al., 2012). Several types of these bacteria are responsible for carbohydrate fermentation into VFAs, which are the main energy source for ruminants, providing approximately 70% of the total energy requirements (Bergman, 1990). Fernando et al. (2010) reported that the microbial population in the rumen can be modified depending on the type of carbohydrates consumed. *Selenomonas ruminantium* and *Streptococcus bovis* are the species that most contribute in the carbohydrate fermentation. Belanche et al. (2012) added that these two species are considered as lactate producers and *Megasphaera elsdenii* as lactate consumer.

In the intensive fattening systems of ruminants, the increase of intake of highly fermentable organic matter leads to a rapid production and absorption of acids in the rumen (Pan et al., 2016) at the time it causes a reduction in the microbial activity and

ruminal pH (Mao et al., 2016). A frequent consequence of this feeding practice is acidosis, which has become an important problem in ruminant feeding. Two levels of acidosis are distinguished: acute ruminal acidosis (ARA) and subacute ruminal acidosis (SARA).

1.3.1. Acute ruminal acidosis

Acute rumen acidosis occurs with a rapid grain overload offed to non-adapted animals accompanied by clinical signs such as decreased appetite and ruminal motility (Allen, 1997), decreased salivation and redistribution of systemic water (Slyter, 1976), and also accompanied by an increase in rumen lactate concentrations that is caused by proliferation of *Streptococcus bovis* (Russell and Hino, 1985). In addition, ARA may result in severe illness, liver abscesses, and even in death of the animal. Nocek (1997) reported that when ruminal pH is maintained over 5.5, an equilibrium exists between producers and utilizers, and lactic acid does not accumulate in the rumen. In such conditions, Valente et al. (2017) indicated that when lactic acid is produced, it is immediately absorbed and converted into glucose in the liver, or oxidized to provide energy to tissue. Nocek (1997) added that a pH less than 5.5, no cellulolytic and relatively few amylolytic bacteria survive. However, *Streptococcus bovis* multiplies until ruminal a pH is lower than 5.0, a pH that allows an increase in growth of *Lactobacillus sp*. These later are more resistant to low pH than *Streptococcus bovis* (Nagaraja and Lechtenberg, 2007) and finally predominant. Sharpe et al. (1973) showed that the two predominant species of lactobacilli that have been identified and well characterized are *Lactobacillus ruminis* and *Lactobacillus vitulinus*. The former species produces mostly L(+)- and about 5 % D(-) lactic acid, and the latter produces D(-)- lactic acid. Giesecke and Stangassinger (1980) reported that in the rumen, the predominant isomer is L(+) lactate, and the proportion of D(−) lactate increases with lower pH. Bolton and Pass (1988) added that L-lactic acid is metabolised more rapidly than D- lactic acid, thereon the metabolic acidosis is due in large part to the accumulation of D- lactic acid. The sequence of events associated with the induction of acute ruminal lactic acidosis is shown in Figure 2. Schwartzkopf-Genswein et al. (2003) proposed that ARA is defined when the rumen pH ranged from 5.2 to 5.0. However, other studies (Britton and Stock, 1989; Owens et al., 1998; Krause and Oetzel, 2006) reported that ruminal pH of 5.0 or below, approaching 4.5 or lower is considered the benchmark for acute ruminal acidosis. The main reason for pH to reach 4.5 or below is lactic acid accumulation (Nagaraja and Titgemeyer, 2007),

which is the result of its increased production (50 to 120 mmol/L) and decreased fermentation.

Lactic acid is a compound derived from the intermediary metabolism of carbohydrates that is found in very small amounts in normal ruminal conditions (0 to 5 mmol/L; Nagaraja and Titgemeyer, 2007). This acid is stronger than the VFAs (pKa 3.9 vs. ≈ 4.80), and is the first responsible for the alterations observed in cases of ARA. Subsequent absorption of organic acids into the bloodstream might overwhelm the bicarbonate buffering system, the excretion rate of acids and the capacity of tissues and organs to metabolize acids, resulting in systemic acidosis (Brown et al., 2000). The maintaining of the pH drop on time determines the death of a considerable number of rumen bacterial species, among them some lactate utilizing bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, which are not adapted to survive under these conditions. This imbalance in rumen bacteria aggravates the ruminal dysfunction at the time of release endotoxins to the medium, which can be absorbed into the body (Sienra, 2009). The direct action of acids on rumen epithelium determines its inflammation and the destruction of large areas, and finally the accumulation of lactic acid determines a significant increase in the osmotic pressure, so the water can pass from the organism into the rumen (Radostits et al., 1994; Owens et al., 1998; Sienra, 2009). The affected animals show symptoms in two to four hours after grain intake, including complete anorexia, abdominal pain, tachycardia, tachypnea, diarrhoea, lethargy, spasms, and death.

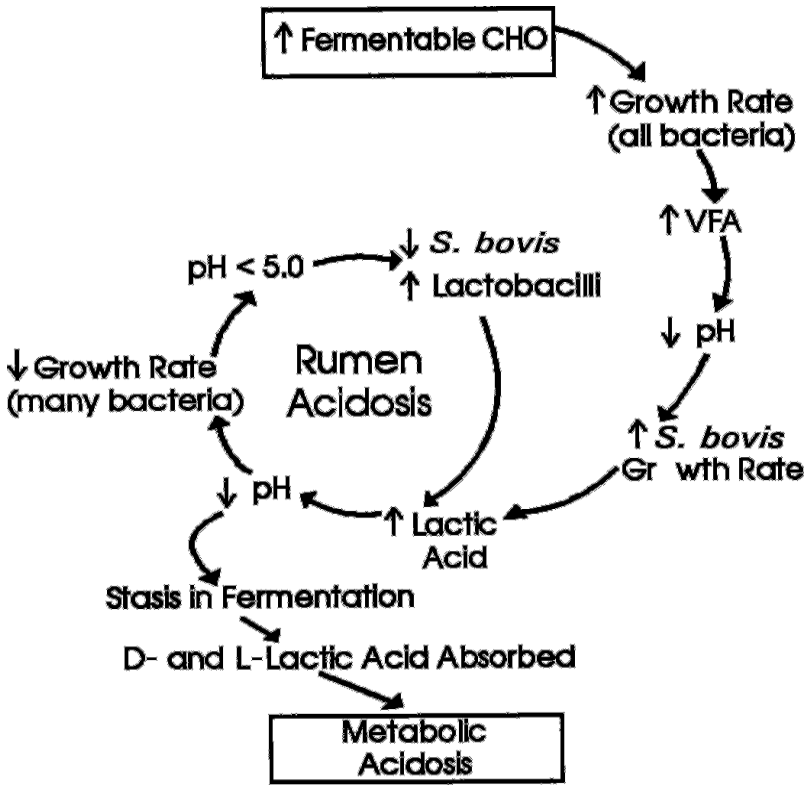

Figure 2. Sequence of events associated with the induction of acute ruminal lactic acidosis (data from Nocek, 1997).

1.3.2. Subacute ruminal acidosis

In contrast to ARA, SARA (chronic or sub-clinical acidosis) habitually is not accompanied with clear clinical signs. Nagaraja and Lechtenberg (2007) reported that the animals experiencing subacute acidosis seldom show any clinical symptoms, the reduction in feed intake being the most important sign of subacute acidosis, although it may observe some other signs like reduction in rumination, mild diarrhoea, foamy faeces containing gas bubbles, appearance of undigested grain (particles larger than 6 mm) in faeces, episodes of laminitis, weight loss and poor body condition and unexplained abscesses. Many studies have been done to investigate the benchmark of pH for subacute acidosis. For Krause and Oetzel (2006) SARA is defined by periods of moderately depressed ruminal pH from about 5.5 to 5.0. Schwartzkopf-Genswein et al. (2003) proposed a threshold of rumen pH ranging from 5.5 to 5.8 to define SARA. However,

Penner et al. (2007) reported that SARA is defined when ruminal pH values ranging between 5.2 and 5.8 for any extended period of time. This drop in ruminal pH is a result of the breakdown of dietary non-fibres carbohydrates (e.g. starch), particularly from cereal grains such as wheat and barley. Grains are high in readily fermentable carbohydrates that are rapidly broken down by ruminal bacteria, leading to the production of VFAs and lactic acid. Under normal feeding conditions, the total produced amount of VFA ranges between 60 and 150 mM depending on the type of diet (Bergman, 1990), these VFA being readily absorbed by papillae from the rumen wall. The same authors added that the concentration of the VFA rises to 200 mM or more when the animals fed starch rich diets. According to Nagaraja and Titgemeyer (2007), SARA results from excessive VFAs production (150-225 mM) that exceeds the ability of the ruminal papillae to absorb them, and therefore they accumulate in the rumen and as a result reduce ruminal pH. Lactic acid does not consistently accumulate in the ruminal fluid of ruminants affected with SARA (Oetzel et al., 1999; Schwartzkopf-Genswein et al., 2003) as it is supported by beef feedlot data (Britton and Stock, 1987). Goad et al. (1998) explained that during subacute acidosis the lactic acid produced does not accumulate because lactate-fermenting bacteria remain active and rapidly metabolize it to VFA. However, transient spikes of ruminal lactate up to 20mM can be discovered if ruminal lactate concentrations are frequently recorded during the day (Kennelly et al., 1999). To measure the duration and the severity of SARA, two important indexes must be known; the time during which the pH remains below the threshold of subacute acidosis, and the area (combination of pH drop and time) under the threshold (Penner et al., 2009; Figure 3). Wales et al. (2004) reported that continuous pH measurements rather than averaging multiple time-point measurements are necessary for diagnosis of rumen acidosis because of the daily variation in rumen pH.

A common cause of SARA occurs at fattening, when animals often undergo acidic challenges in the feedlot when they are transitioned from forage-based to concentrate-based diets as part of normal feedlot management. Nagaraja and Titgemeyer (2007) shown that an abrupt dietary change does not allow ruminal bacteria and ruminal papillae for the adequate time to adapt, thereby leading to a rapid production and accumulation of VFA. Another common cause of SARA is an inadequate balance of mixed rations, such as if the effective fiber content falls below recommended levels or when particle size is too small. This suppresses rumination and reduces stimulation of the production of saliva,

reducing the possibility of buffer changes in ruminal pH. In order to limit the development of acidosis in transition, the young ruminant must adapt their feeding behaviour to very high concentrate rations offered *ad libitum* (Mialon et al., 2008). According to Brugere-Picoux (2004), changes in rumen bacterial populations when exposed to high concentrate rations require from 2 to 3 weeks, and it is recommended that concentrate levels be increased at 5 to 7 day intervals during this period to avoid SARA. To prevent SARA, Brugere-Picoux (2004) recommended that the starch dietary concentration must be less than 30%, and the rate of the concentrate must be below 60% of the total dry matter. Therefore, there is a direct relationship between diet, ruminal ecosystem and the appearance of acidosis (Sauvant and Peyraud, 2010). Fermentation acids production in the rumen needs to be balanced with fermentation acids removal and neutralisation in order to achieve optimal ruminal conditions and optimal production (Krause and Oetzel, 2006).

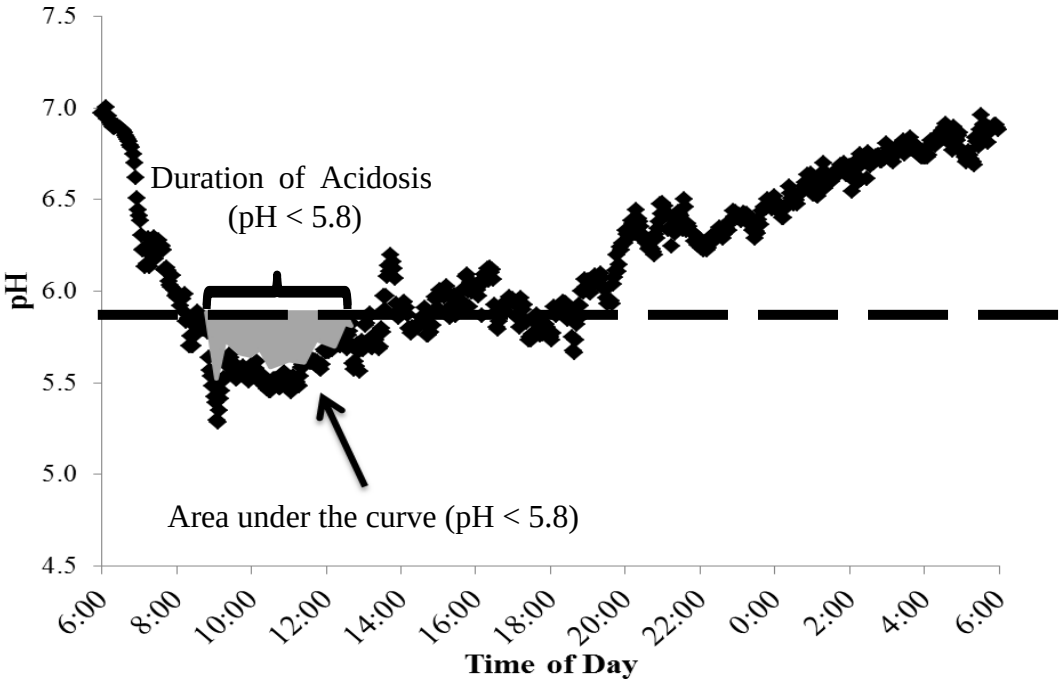

Figure 3. Continuous pH measurements allow for the measurement of duration of sub-acute ruminal acidosis (SARA), where rumen pH < 5.8, as well as the area under pH 5.8, which give an objective measure of SARA severity (data from Penner et al., 2009).

2. Digestion of dietary carbohydrates

Vegetal carbohydrates are classified into two categories: cytoplasmic and structural carbohydrates. The former form two types; the water-soluble carbohydrates such as mono and disaccharides that directly contribute to the metabolism of the plant cell, and the reserve carbohydrates that are stored in different parts of plants (grain, tubers, and roots). Starch, which is composed by amylose and amylopectin is considered as the principal reserve carbohydrate. A second category includes the structural carbohydrates, that are constitute the plants cell wall, includes cellulose, hemicelluloses, lignin and pectins. Although the cellulose is the main component of the plant cell wall, both cellulose and hemicelluloses are systematically related as constitutive of the basal structure of the plant cell wall. Lignin is not a carbohydrate, but a polyphenolic structure, however, due to its importance in the plant cell wall, it is often classified as a vegetable fiber component. Pectins are uronic acid polymers and sugars, and are considered as soluble fiber.

2.1. Biotransformation of dietary carbohydrates in the rumen

The carbohydrates are the major polymers components of ruminant diets. These polymers, except for lignins, are hydrolysed to monomers, which are then metabolised to various fermentation products, mainly acids and gases, depending on the microbial species (Nagaraja, 2016). The biotransformation of carbohydrates (degradation and fermentation, synthesis and conversion) mainly occurs in the rumen. The ruminal biotransformation results from three major driving forces that control ruminal activity: dynamics of microbial populations, thermodynamic, and laws of chemical kinetics (Serment, 2012; Figure 4). According to Hungate (1966) and Bergman (1990), carbohydrates are the main source of energy for the ruminants and for the ruminal microorganisms. In addition to provide energy, carbohydrates present in form of fiber have a physical role in maintaining the optimal functioning of the rumen. The non-fibrous carbohydrates are efficiently used as energy source, as they are rapidly fermented to a high extent, but increase the risk of ruminal acidosis, as previously stated. In contrast, the fibrous carbohydrates are more resistant to degradation, but modulate transit of digesta and stimulate rumination and saliva production that acts as a buffer of rumen contents. Pectins and soluble fiber are also rapidly used by rumen microorganisms.

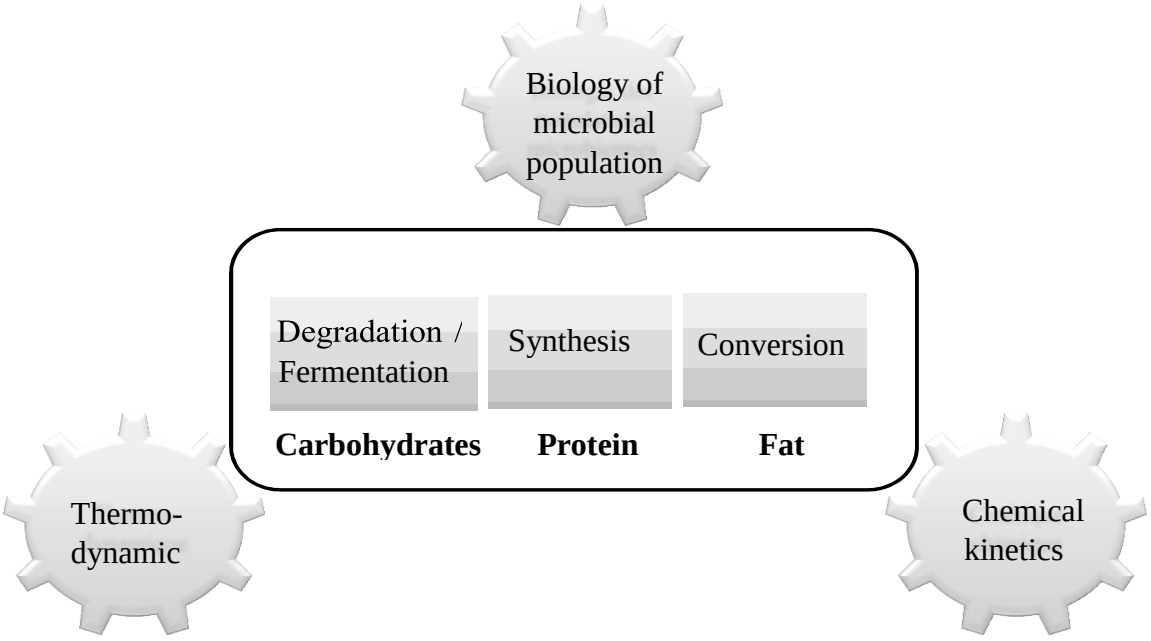

Figure 4. Ruminal biotransformation of carbohydrates in rumen (data from Serment, 2012).

2.1.1. Degradation of dietary carbohydrates in the rumen

The first step in the fermentation of dietary carbohydrates is the breakdown of polysaccharides to oligo, tri, di, and monosaccharides (Baldwin and Allison, 1983). Extracellular enzymes that are secreted by ruminal bacteria achieve the polysaccharides hydrolysis.

Starch (amylose and amylopectin) is hydrolysed by microbial enzymes to maltotriose, maltose and glucose (Baldwin and Allison, 1983; Van Houtert, 1993). The major enzymes involved in this hydrolysis are alpha-amylase, debranching enzyme, and pullulanase (Nagaraja, 2016). The same author shown that the major amylolytic bacteria in the rumen are *Ruminobacter amylophilus*, *Selenomonas ruminantium*, *Streptococcus bovis* and species of Lactobacillus and Bifidobacterium. These bacteria are favoured by a ruminal pH between 5.0 and 6.0 (Belbis, 2007). The amylases produced by these bacteria randomly attack the α-glucosidic bonds, releasing glucose as the final product. The degradation of starch begins with the adhesion of the microorganism to the substrate,

and then continues with the action of several enzymes that degrade the structure of starch to monosaccharides, which can be absorbed and fermented by these and other microorganisms. The differences in starch structure from one plant species to another, or even among varieties, is what influences its fermentation rate and extent (Van Barneveld, 1999), Rooney and Pflugfelder (1986). Colonna et al. (1995) reported that the enzymatic digestibility of starch is in general inversely related to its amylose content, or to their ratio amylose/amylopectin (Offner et al., 2003). This degradability is also influenced by the structure of the endosperm of the cereal grain. Michalet-Doreau and Doreau (1986), Huntington (1997) and Evers et al. (1999) reported that the endosperm is composed of two parts:

- The aleurone layer, peripheral, which mostly contains proteins

- The starchy endosperm, which includes two parts: floury endosperm, and hard endosperm, nested into a protein matrix more or less dense depending on the species and variety.

Since the endosperm of barley is floury, it is more fermentable than that of corn and sorghum, which are vitreous (Chevalier, 2001), results are in agreement with those found by other research groups (Opatpatanakit et al., 1994; Lanzas et al., 2007; Amanzougarene et al., 2018a). Waldo (1973) explained that the fermentation rate of starch depends on the type of starch being degraded and on its physical form. Besides, the presence of phenolic compounds in the grains, such in the case of sorghum which have starch and proteins highly related among them by the effect of tannins (Kristen et al., 2015) that influence their fermentation (Kim et al., 2006, Amanzougarene et al., 2018b). The amount of starch among cereal species is higher in sorghum and corn than in barley varieties. These results have been observed by several authors: 72%, 63%, and 59% respectively (Laurent, 1988); 74% for corn and sorghum, and 59.5% for barley (Chevalier, 2001); 64%, 63%, and 53%, respectively (FEDNA, 2010); 70% for corn, 66% for sorghum, and 65% for barley; (Amanzougarene et al., 2018a). However, as it is shown in Table 1, the rate of starch fermentation is higher for barley (40% to 60%/h depending on the variety) than for corn and sorghum (15% to 30%/h) (Sauvant and Michalet-Doreau, 1988). The fermentation of soluble sugars occurs immediately after the food ingestion, carried out by a very large number of bacteria (Hungate, 1966).

Table 1. Starch degradability (%) of various cereals determined by in situ, in vitro, and in vivo methods (data from Nocek and Tamminga, 1991).

cereal	*In situ, in vitro*	*In vivo* (cows)
	range	range
Oat	89-99	76-91
Barley	83-97	82-93
Corn	53-67	51-93
Sorghum	51-57	42-91
Wheat	96-99	85-91

Chesson and Forsberg (1988) and Orskov and Ryle (1998) showed that the degradation of the fibrous carbohydrates by the rumen microorganisms occurs at a lower and variable extent. Their hydrolysis in the rumen occurs by the secretion of different extracellular enzymes, except for the fibrolytic activity of protozoa, which occurs intracellularly after intake of fibrous particles. Nagaraja (2016) explained that a lot of bacteria, ciliated protozoa, and fungi produce a variety of glycosyl hydrolases that breakdown the glycosidic bonds to produce oligosaccharides first and then the di- and monosaccharides. The action of these microorganisms that are implicated in the degradation of fiber is facilitated by a pH above 6.5 (Dusart, 2014). Cellulose and hemicellulose are the most abundant of plant carbohydrates. The degradation of these carbohydrates occurs as that of the non-structural carbohydrates but at a lower rate. In the case of cellulose, three types of cellulases act in synergy: endo β 1-4 glucanases, cellobiosidases, and β –glucosidases. The first type attacks to the cellulose to form cello-oligosaccharides, the second attacks the non-reducing end of the cellulose to give the cello-biose units, and the third type hydrolyses the cellobiose and cello-oligosaccharides of low degree of polymerization to give glucose (Jouany, 1994). Concerning the hemicelluloses, Nagaraja (2016) reported that the major enzymes involved in hemicelluloses degradations are endoxylanase and several debranching enzymes, with arabinofuranosidase being the most important. A large number of bacterial species secretes endoxylanases. In addition, protozoa and fungi also produce cellulases and hemicellulases. The nonproteolytic bacterial species that are considered to be most abundant in the rumen are *Fibrobacter succinogenes*, *Ruminococcus albus*, and

Ruminococcus flavefaciens (Nagaraja, 2016). Baldwin and Allison (1983) reported that both *Ruminococcus albus* and *Ruminococcus flavefaciens* both have the ability of producing extracellular cellulases in response to the presence of cellulose. Nagaraja (2016) added that the major cellulolytic bacteria can also digest hemicelluloses and pectin. Those that can digest hemicelluloses include Prevotella sp. (P. albensis, P. brevis, P. bryanti, and P. rumincola), *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio xylanivorans*. Although pectin is a structural polysaccharide, it is completely digested in the rumen. It is fermented much more rapidly than cellulose or hemicellulose. For the hydrolysis of the pectin, pectinolytic enzymes have been identified in bacteria and protozoa but not in fungi, microorganism that quickly hydrolyse this polysaccharide (Grenet and Besle, 1991). The predominant pectinolytic enzyme is pectinlyase. The major pectinolytic bacteria include Prevotella sp., *Lachnospira multiparus, Streptococcus bovis*, and Trepnema sp. (T. bryantii and T. saccharophilum) (Nagaraja, 2016). The same author added that although *Streptococcus bovis* is pectinolytic, it does not utilize the products of pectin degradation (d-galacturonic acid). Similarly, ciliate protozoa can breakdown pectin but cannot utilize the products.

The fermentation of the fibrous carbohydrates depends on the lignin content in the feeds, being negatively related to its proportion and the magnitude of linkages with structural carbohydrates (Chesson and Forsberg, 1988). Despite having a considerable fibrous fraction, several agro-industrial by-products used in the nutrition of ruminants, such as sugar beet pulp, citrus pulp, and wheat bran, present the advantage of a low proportion of lignin (1.69%, 0.78%, and 3.4% respectively; FEDNA, 2010). Besides, these feeds have a considerable proportion of easily-fermentable hemicelluloses and pectin (in sugar beet pulp and mainly in citrus pulp), which renders high amount of energy when fermented in the rumen (Nocek and Tamminga, 1991).

2.1.2. Metabolic pathways of carbohydrate fermentation by rumen bacteria

The biochemical pathways involved in carbohydrate fermentation in the rumen have been extensively reviewed (Hungate 1966; Baldwin and Allison, 1983; Czerkawski, 1986; van Houtert, 1993; Dehority, 2003; Nagaraja and Lechtenberg, 2007, Nagaraja, 2016). These authors reported that many of the polymer-fermenting bacteria, ciliated protozoa, and fungi can ferment disaccharides and monosaccharides released from initial hydrolysis. These sugars will be absorbed and then fermented into pyruvate by the

Emden-Meyerhof and pentose phosphate pathways (Figure 5). The pyruvate is metabolised to VFAs like acetic acid (C2), propionic acid (C3), butyric acid (C4) and a minor proportion of valeric acid and branched-chain VFA, and in some cases to lactic acid. Gases (carbon dioxide, hydrogen gas and methane) and energy are released, in form of adenosine tri-phosphate (ATP). Acetate is converted from pyruvate through acetyl CoA, with the ATP yield being 1 ATP /mole. When producing acetate the formate that is a by-product of the conversion of pyruvate to acetyl-CoA is released, and it is converted to CO_2 and H_2 that are converted to methane. Pyruvate is converted to propionate through succinate pathway, and it is converted to butyrate through aceto-acetyl CoA, regenerating NAD^+ from NADH, and therefore resulting in an electron sink product. Lactate is an intermediate product because lactate-utilizing bacteria further metabolize it to acetate, propionate and butyrate, mostly by *Megasphaera elsdenii* and *Selenomonas ruminantium*.

The types of fermentation products produced from pyruvate depend on the substrates available in the rumen, the microorganism and the ruminal conditions like pH and dilution rates (Nagaraja, 2016). A high proportion of fiber fraction causes an increase in the proportion of C2, because the fibrolytic rumen bacteria generally use this metabolic pathway, whereas a high starch content will promote the production of C3. The presence of soluble carbohydrates (sucrose, lactose, inulin) contained in foods such as beets or whey will increase the proportion of C4 (Grenet and Besle, 1991). The total and individual rumen VFA concentration depend on diet type (Bergman, 1990; Jouany et al., 1995; Dusart 2014). Table 2 shows the differences in proportion of VFA according to the nature of diets.

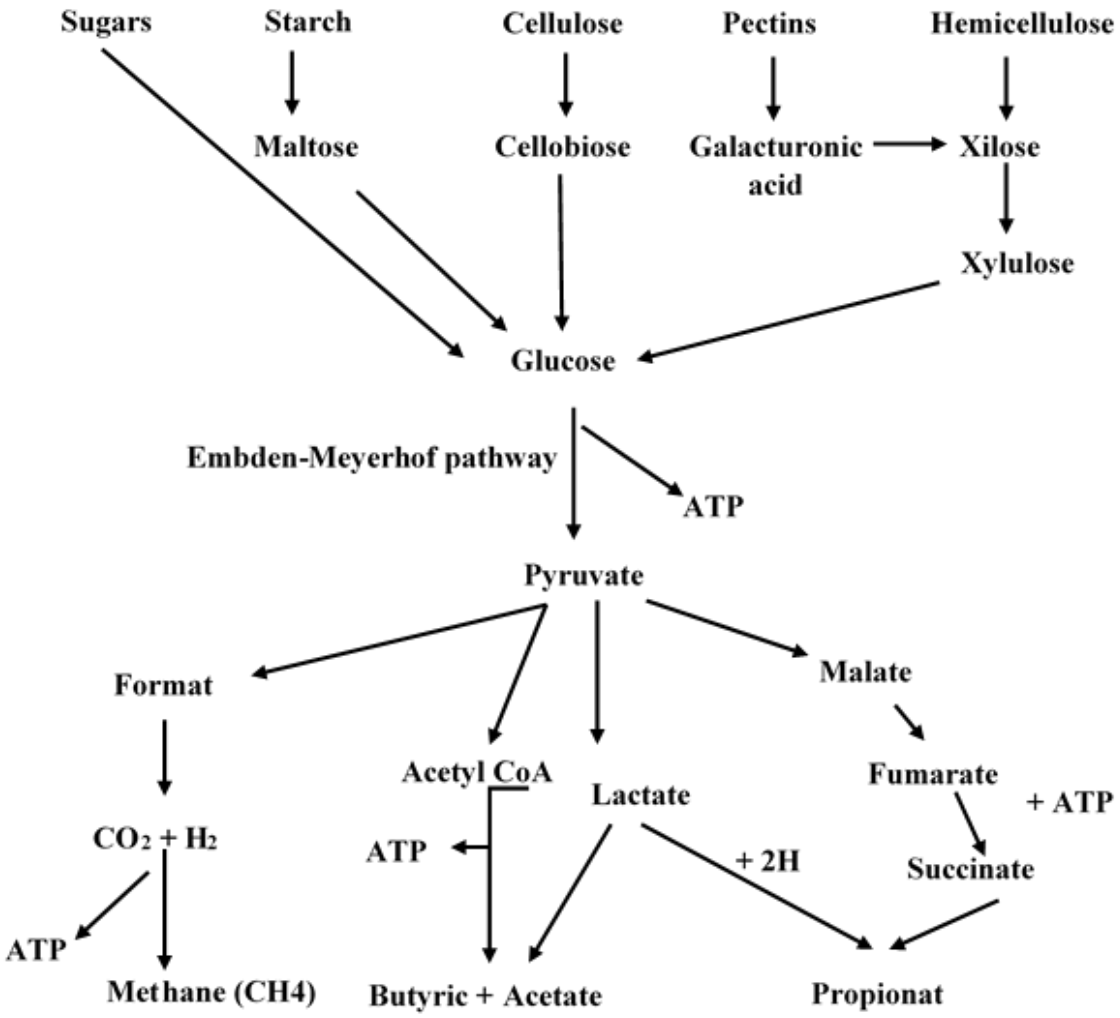

Figure 5. Carbohydrate fermentation in the rumen.

Table 2. Influence of the nature of the diet on the molar proportions of VFA in the rumen (mean values in the 5 hours after the meal; Jouany et al., 1995).

Diet	Total VFA (mmol/L)	Molar proportions (%)			
		C2	C3	C4	Other VFAs*
Grass hay	90.0	72	17	7	4
Hay (44%) +barley (56%)	115.6	61	30	8	1
Hay (18%) + beet (82%)	127.5	56	26	17	1
Hay (48%) + whey (52%)	99.9	59	16	21	4

Other VFAs*: including valeric, isovaleric and isobutyric acids.

Variations in pH promote changes in the microbial population of the rumen: a low pH causes higher proportions of C3 whereas a high pH causes higher proportions of C2 (Blain, 2002). The decrease in the percentage of C2 with starch-rich diets is directly related to the drop in rumen pH, and presumably to the increased concentration of amylolytic bacteria. In such situations, significant disruptions in the rumen fermentations and the onset of increasing amounts of lactic acid are common (Michalet-Doreau and Sauvant, 1989). Blain (2002) reported that the quantity of VFA in the rumen is also dependent on the rate of substrate degradation; the faster this substrate is degraded, the higher the quantity of VFA produced. Thus, high proportions of starch in the diet will cause high ruminal VFA production, promoting a rapid drop in ruminal pH that can cause ruminal acidosis. The fibrous fraction is digested at a lower rate than starch.

The methane production represents losses of about 15% of energy during the ruminal fermentation. This greenhouse gas is produced in greater quantities by the C2 pathway. In intensive feeding systems, the rations consist of higher proportions of starch that promote the pathway of C3, and in such case the amount of methane produced is lower (Jouany, 1994).

3. Strategies of prevention of ruminal acidosis

As cited above, the ruminal pH is the main indicator to evaluate the ruminal acidosis status. This parameter has been directly related to rumen VFA production. Thus, the diet is considered as a main factor that contributes to change these two parameters. For this reason, to minimize the risk of acidosis in ruminants, it is often recommended the knowledge of the buffering capacity of feedstuffs. Several authors (Jasaitis et al., 1987; Wohlt et al., 1987; Erdman, 1988; Le Ruyet et al., 1992; Giger-Reverdin et al., 2002) described that the buffering capacity of roughages is higher than those of cereals, and those of cereals by-products. In addition of its high buffering capacity, roughages stimulate the production of saliva extending the chewing time and buffering the rumen to maintain the pH within the physiological limits of 5.5 to 7.0. So, in diets containing low concentrations of dietary fiber, supplementation of chemical buffers results in increased rumen pH. However, a buffer should reduce the decrease in rumen pH, but without causing a pH increase compared with neutralising agents that elevate pH (Staples and Lough, 1989). Thus, the most used chemical buffers are the sodium bicarbonate and

sodium sesquicarbonate; $NaHCO_3$, Na_2CO_3 (Meschy et al., 2004). In addition to chemical buffers, zootechnical feed additives such as enzymes, yeasts and fungi are used to prevent the chronic acidosis (Chaucheyras-Durand and Durand, 2010; Desnoyers et al., 2009). In this thesis work, a major interest was focused to other zootechnical feed additives such as tannin extracts, essential oils and fatty acids, to evaluate whether its inclusion in diets for fattening of young ruminants can contribute to avoid the risk of acidosis.

3.1. Zootechnical feed additives

Zootechnical feed additives are substances used to favourably affect the performance of animals in good health or used to positively influence the environment. These additives can be classified into four main groups:

1. Digestibility enhancers (for enzymes)
2. Gut flora stabilisers (for microorganisms)
3. Substances that affect the environment
4. Other zootechnical additives

In diets for ruminants, the most zootechnical feed additives included are: plant extracts, fatty acids, probiotics, and organic acids. These categories of additives are used owing to their capacity to involve positive changes on ruminal fermentation and prevention of acidosis.

3.1.1. Effects of zootechnical feed additives on rumen fermentation

3.1.1.1. Plant extracts

Many plants produce important organic compounds derived from their secondary metabolism (Balandrin and Klocke, 1985). These secondary metabolites are not involved in their primary biochemical processes, such as growth, development, or reproduction, but they are important for protection against insect predation and microbial infection (Balandrin and Klocke, 1985; Cobellis et al., 2016a). In addition, they are responsible for the odor and color of plants and spices. When extracted and concentrated often exert antimicrobial activities against a wide variety of microorganisms including bacteria, fungi and viruses (Gershenzon and Croteau, 1991; Chao et al., 2000; Greathead, 2003; Burt,

2004; Benchaar et al., 2008). Cobellis et al. (2016a) reported that, in the past, animal nutritionists considered plant secondary metabolites as antinutritional factors due to adverse effects on nutrient utilization. Nevertheless, the same authors added that many plant extracts have been recently studied for their antimicrobial activity and ability to modify gut function in both ruminant and non-ruminant animals. Previous researches have been conducted to study and to evaluate the effect of plant secondary metabolites including saponins, tannins, and essential oils on rumen fermentation (McAllister et al., 1994; Wang et al., 1996; Hristov et al., 1999; McSweeney et al., 2001; Benchaar et al., 2007; Calsamiglia et al., 2007; Rodríguez et al., 2014). These authors have been concluded that a large number of these compounds have been shown to modulate ruminal fermentation to improve nutrient utilization in ruminants. In this work, we are interested in the study of the effect of inclusion of essential oils (cinnamaldehyde, eugenol, and CRINA® Ruminants) and tannins (hydrolysable and condensed) in diets for fattening ruminant.

The essential oils (EO) are volatile lipophilic secondary metabolites from plants that are obtained by steam and/or water distillation (Losa, 2001). They are characterized by its antimicrobial properties against different types of microorganisms including bacteria, protozoa, and fungi (Dean and Ritchie, 1987; Sivropoulou et al., 1996; Chao et al., 2000; Greathead, 2003). This antimicrobial effect is due to their ability to modify cell permeability in microbes (Helander et al., 1998). The use of EO in diets for ruminants was for their antimicrobial effects (Cardozo et al., 2005; Busquet et al., 2005a; Calsamiglia et al., 2007). McIntosh et al. (2000), Busquet et al. (2005b) and Castillejos et al. (2006) reported that the inclusion of EO in diets might modulate and improve the ruminal fermentation. Wallace (2004) explained that the EO at low dosages may modify of rumen fermentation due to their toxicity to some unfavourable strains of rumen bacteria, such as methanogens. Sivropoulou et al. (1995) indicated that some of these metabolites have antimicrobial activities against Gram-positive and Gram-negative bacteria. Supported these indications, Calsamiglia et al. (2007) explained that, because of their low molecular weight, some of EO have the capacity to inhibit the growth of some Gram-positive and Gram-negative bacteria in the rumen, however their effects on these microorganisms will depend upon essential oils compounds (e.g. phenolic, carbonyl, monoterpenes, etc.).

Cinnamaldehyde (CIN; 3-fenil-2-propenal phenol; C_9H_8O; Figure 6), a non-phenolic phenylpropene with antimicrobial activity, is the main active component of cinnamon (C. cassia) oil, accounting for up to 75% of its composition (Davidson and Naidu, 2000).

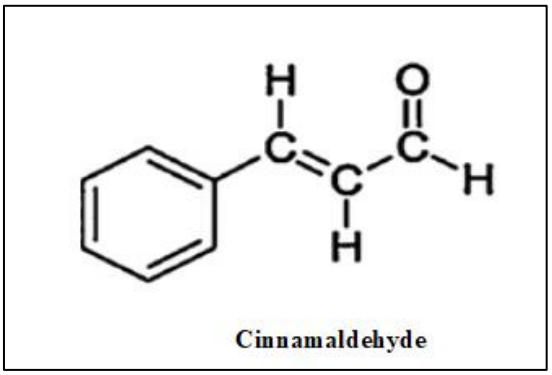

Figure 6. Chemical structures of cinnamaldehyde.

Cinnamaldehyde is characterized by an antimicrobial activity against both Gram-negative and Gram-positive bacteria (Ouattara et al., 1997; Helander et al., 1998; Burt, 2004; Kim et al., 2004), although Smith-Palmer et al. (1998) reported that the Gram-positive bacteria are more sensitive to the antimicrobial proprieties of CIN. Helander et al. (1998) explained that the antimicrobial activity of CIN was similar to that of the phenolics thymol and carvacrol. However, in contrast to them, CIN does not have a hydroxyl or acid group to act as a proton carrier to disrupt the outer membrane or deplete the intracellular ATP pool. As hypothesis, Helander et al. (1998) stated that CIN can access the periplasm and the interior of the cell through the protein absorption pathway (protein porin) and cause its inhibitory effect from inside the cell. Furthermore, Burt (2004) suggested that the antimicrobial properties of CIN might be due to arise through its carbonyl group binding and inactivating microbial enzymes.

The first researches carried out to evaluate the effect of CIN on microbial fermentation were conducted *in vitro*. Cardozo et al. (2004), using a continuous culture system, observed that the inclusion of CIN caused an inhibition of peptidolysis. Using the same system, Ferme et al. (2004) reported that CIN reduced Prevotella spp, that is the main genus of proteolytic bacteria. However, contradictory effects were found on rumen

fermentation using CIN as additive. No effect neither in *in vitro* studies (Benchaar et al., 2007, Tager and Krause 2010) nor in *in vivo* studies (Yang et al., 2010), positively improved the ruminal fermentation (Busquet et al., 2005b), or affected negatively (Mateos et al., 2013, Macheboeuf et al., 2008). For more information about the use of CIN further studies especially *in vivo* are necessary to determine the effectiveness of these extracts on rumen microbial fermentation and animal performance.

Eugenol (EUG; 4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$, Figure 7) is a phenolic monoterpene present in high quantities in clove bud (*S. aromaticum*) oil. It is one of the main active components in this oil, accounting for up to 85% of its composition (Davidson and Naidu, 2000). Eugenol has been shown to have a wide spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria (Davidson and Naidu, 2000; Dorman and Deans, 2000; Walsh et al., 2003).

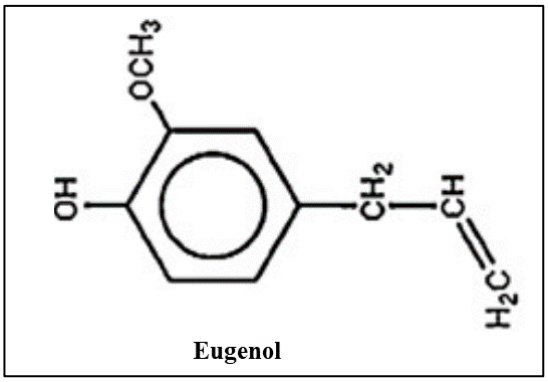

Figure 7. Chemical structures of Eugenol

Some *in vitro* studies that evaluate the effects of EUG on ruminal fermentation have shown negative effects when EUG was added at high doses (Busquet et al., 2006; Castillejos et al., 2006). In terms of medium pH, Bayourthe and Ali-Haimoud-Lekhal (2014) reported that the inclusion of EO belong to phenols group such as eugenol leads to an increase in medium pH, results confirming those found by previous researches (Busquet et al., 2006; Benchaar et al., 2007; Castillejos et al., 2006; Tager and Krause, 2010). Thus, when EUG was included at high doses medium pH increased, although at difference levels according the methods of study. This means that EUG supplementation in diets for ruminants in intensive fattening may lead to maintain ruminal pH within its

physiological limits. In addition, from their results obtained in *in vitro* studies, Busquet et al. (2006) and Castillejos et al. (2006) reported that the inclusion of EUG in diets for dairy cattle may improve energetic and protein metabolism depend on the dose level. In contrast, in *in vivo* studies, Yang et al. (2010) and Benchaar et al. (2012) concluded that the effect of the supplementation of EUG for beef and/ or dairy cattle did not show improvements in animal performances. These researchers suggest that the EUG may have a low potential for being used as feed additive in diets for ruminants (beef as well as dairy cattle).

The number of experiments realized to evaluate and to determine the effectiveness of this essential oil as zootechnical additive is scarce, and more *in vivo* trials must be conducted to specify the real effect of this additive, mainly in diets for fattening of young ruminants.

As additive, the combinations of EO are characterized by its antagonistic and synergistic effects that occur between different components of this mixture. Burt (2004) suggested that combinations of essential oils may enhance efficiency of rumen microbial fermentation although Cobellis et al. (2016a) stated that, because of the complex mixture with a highly variable composition from a combination to another, the mode of action of these combinations against microorganisms or specific cellular targets is often difficult to determine and thus still remains poorly understood. The effects of these mixtures on ruminal fermentation were studied widely *in vitro*, and the results found in different studies were variable. A typical commercial combination that has been widely used is known under the trade name of CRINA® ruminants.

In previous *in vitro* studies (Castillejos et al., 2007; Spanghero et al., 2008; Ahmed et al., 2014) it was observed that the inclusion of essential oil blend in diet for ruminants did not influence the different parameters of rumen fermentation. In contrast, when 0.8 mg/g of five different blends of EO were added, Cobellis et al. (2016b) observed a decrease on *in vitro* gas production and dry matter degradability. Similarly, the production of total VFA and the propionate proportion were significantly decreased. However, the inclusion of these five blends of EO did not affect the proportion of acetate, but an increase in butyrate proportion was recorded. An increase in butyrate proportion was also observed in the *in vivo* studies conducted by Tomkins et al. (2015) and Flores et al. (2013). When Meyer et al. (2007) tested the supplementation of diets for fattening of steers by CRINA® they found that the inclusion of CRINA® did not markedly affect digestibility,

productive performance and carcass characteristics. However, Benchaar et al. (2006a), Spanghero et al. (2008) and Cobellis et al. (2016b), reported that *in vivo* as well as in *in vitro* when the mixtures of essential oils were added at different doses an increase in medium pH was recorded compared with control. In contrast, in an *in vitro* study Ahmed et al. (2014) observed that the addition of EO blend had not significant effect on medium pH.

Dorman and Deans (2000) stated that the effect of EO was related to the chemical structure of the components, the proportion of each of them, and the correlation between them. These conclusions agree with those reported by Busquet et al. (2006), which reported that the effects of blends of EO on ruminal fermentation vary with their main components. Moreover, Cobellis et al. (2016b) explained that the blend of EO containing a phenolic or carbonyl compound demonstrated a stronger antimicrobial activity than other EO that contain monoterpenes, and McIntosh et al. (2003) concluded that the blend of EO inhibit especially Gram-positive bacteria. Calsamiglia et al. (2007) reported that the inclusion of different mixtures of EO at high doses resulted in inhibiting of rumen microbial fermentation, confirming their antimicrobial activity. In their study, Spanghero et al. (2008) suggested that the supplementation of a blend of EO at high dosages caused in depression of ruminal fermentation and added that the use of these blends of EO is acceptable only at dosages that exert a positive effect on microbial population with no adverse effect on rumen fermentation. The interaction between EO blends and their dose is also highlighted by Cobellis et al. (2016b), who reported that supplementation at moderate doses had not adverse effects on rumen microbial fermentation. In addition, Benchaar et al. (2006b) suggested that the mixture of EO at low dose levels have the potential to improve feed efficiency in beef cattle, but at higher dosage this mixture appear to have no beneficial effect on feed efficiency. The effect of blend EO also depends on the type of substrate or diets composition, as well as on medium conditions. Spanghero et al. (2008) suggested that blend of EO might be more effective in an acidic medium (i.e. 5.5), owing to the selective toxicity of these EO that is against gram-negative bacteria. Thus, effectiveness of blend EO may be increase its importance in intensive feeding conditions.

Depending upon results, some authors reported positive effect of blend EO on rumen fermentation, but others suggested a deeper investigation in this topic before giving any recommendation, owing to the broad variability observed between studies.

Generally, the *in vitro* batch system is chosen to study the effect of essential oil because of its friability to detect the effect of these EO on rumen microbial fermentation, and also because this method is not expensive. However, the continuous culture have more advantages. From results recorded in *in vitro* continuous culture Benchaar et al. (2008) could conclude that rumen microbial population may adapt to EO, which explain the lack of effects of the EO on ruminal metabolism and performance in long-term *in vivo* studies.

Tannins are a group of plant secondary compounds, present in a considerable number of vegetables (forage trees, shrubs, legumes, cereals, etc.). Bate-Smith and Swain (1962) defined tannins as water-soluble polyphenolic compounds ranging in molecular weight from 500 to 3000 Daltons that have the ability to precipitate proteins. The term 'tannin' is difficult to define precisely (Mangan, 1988). Harborne (1999) stated that from a chemical point of view it is difficult to define tannins since the term encompasses some very diverse oligomers and polymers. According to McSweeney et al. (2001) and Schofield et al. (2001), these secondary compounds constitute a heterogeneous group of polyphenols with a diverse structure and molecular weight. These compounds have a capacity to bind to proteins, alkaloids, nucleic acids, minerals, and other polymers such as polysaccharides (cellulose, hemicellulose, pectin), to form stable complexes (Mangan, 1988; Chiquette et al., 1988; Mueller-Harvey and McAllan, 1992; Van Soest, 1994; Giner-Chavez, 1996; Schofield et al., 2001).

Tannins are generally divided into two major classes termed condensed and hydrolysable tannins (McMahon et al., 2000) although the classical classification of tannins into hydrolysable and condensed tannins has been criticized because of their variable magnitude of response, irrespective of their chemical composition (Mueller-Harvey, 2006). Kraus et al. (2003) defined these two types as following: Condensed tannins, also referred to as proanthocyanidins, are polymers of three-ring flavanols joined with C-C bonds. The monomer units that make up condensed tannin are distinguished by the number of OH groups on the B-ring: procyanidins have a di-hydroxy B-ring while prodelphinidins have a tri-hydroxy B-ring. The monomer units may also have either cis or trans C2–C3 stereochemistry. Hydrolysable tannins are further grouped into gallotannins and ellagitannins that are composed of gallic acid or hexahydroxydiphenic acid esters, respectively, linked to a sugar moiety. More complex hydrolysable tannins can form by oxidative transformations, which can yield macrocylic ellagitannins. The

structures of the two types are shown in Figure 8. McLeod (1974) indicated that the condensed tannins have a higher molecular weight than the hydrolysable tannins (1000-20000 Daltons vs. 500-3000 Daltons). These polyphenolic compounds are commonly consumed by ruminants (Van Soest 1994).

Hagerman and Butler (1991) explained that tannins can be beneficial or detrimental to ruminants, depending on the quantity consumed, the compound structure and molecular weight, and on the physiology of the consuming species, although the tannins are considered as anti-nutritional compounds due to their adverse effects on intake and animal performance (Kumar and Vaithiyanathan, 1990). However, Patra and Saxena (2011) reported that in the past few years, tannins have been recognised as useful phytochemicals for modulating rumen microbial fermentation beneficially such as reducing protein degradation in the rumen, prevention of bloat, inhibition of methanogenesis and increasing conjugated linoleic acid concentrations in ruminant-derived foods.

The effects of tannins on ruminal fermentation have been studied both *in vivo* and *in vitro*. Tannins bind to proteins and polysaccharides limiting their availability for rumen microbes (McAllister et al., 1994). The nature of binding of tannins with carbohydrates is not entirely clear, Jones and Mangan (1977) indicated that when the condensed tannins bind with the protein in the diet, they form stable and insoluble complexes that dissociate in the abomasum at pH <3.5. Their impact on nutrient fermentation depend not only on substrate protection from microbial attachment, but also on effects on enzyme inhibition and directly on microbial diversity (Barry and Manley 1984; McAllister et al., 1994; McSweeney et al., 2001). Tannins decrease the rate of protein degradation, and the surplus production of ammonia in the rumen, and increase the flow of proteins to the intestine (Al-Dobaib, 2009). Barry and Manley (1984) indicated that high concentrations of tannins in diets may depress fibre digestion by complexing with lignocellulose, thus preventing microbial digestion, and Patra and Saxina (2009) and Bae et al. (1993) added that tannins may directly inhibit cellulolytic microorganisms, and activities of fibrolytic enzymes. Therefore, adverse effects on fiber degradation can be expected (McSweeney et al., 2001, McAllister et al., 2005).

In *in vitro* as well as in *in vivo* studies (Bhatta et al., 2009; Grainger et al., 2009; respectively) diet digestibility is of the diet reduced with the inclusion of tannins. In addition, Beauchemin et al. (2007) and Grainger et al. (2009) observed that the inclusion

of tannins in diets for ruminants decreased the total rumen VFA concentration. However, in other studies, the tannins had not an effect on dray matter digestibility (Carulla et al., 2005; Hariadi and Santoso, 2010). And similarly, Patra et al. (2006) and Animut et al. (2008) found no effect of the inclusion of tannins on VFA concentration. *In vivo* (Carulla et al., 2005), at high doses, tannins decrease the dry matter intake. But in contrast, the phenolic compounds at moderate doses had not effect on this parameter. These differences may be due to the effect of high doses on the palatability (Barry and Manley 1984; Hervás et al., 2003a). Barry and Duncan, (1984) and Barry and Manley, (1984) shown that when the plant species contain more than 50 g/ kg DM of condensed tannins, the voluntary feed intake is significantly reduced, while when the quantity is less than 50 g/kg, feed intake is unaffected. In previous studies, (Wang et al., 1996; Bhatta et al., 2000; Makkar, 2003; Ramírez-Restrepo et al., 2005) positive effects were observed on animal productivity and performance when diets were supplemented with tannins. In contrast, other authors (Priolo et al., 2000; Beauchemin et al., 2007; Grainger et al., 2009) reported that the effects of the inclusion of tannins in diets for ruminants were not beneficial. Thus, Hervás et al. (2003a), McAllister et al. (2005), Hart et al. (2008) and Patra and Saxena (2010) explained that the variation in tannin effects on ruminal fermentation depends on the plant from which they are extracted, the dose of their inclusion in the diet, the type of diet used, and the type of tannins (condensed or hydrolysable). Rodríguez et al. (2014) indicated that the extent of tannins effect depends not only on their concentration, but also on their reactivity, which is associated with their chemical nature. Therefore, the same concentration of tannins from diverse sources may produce effects of different magnitude (Bueno et al., 2008). Bhat et al. (1998) and McSweeney et al. (2001) reported that hydrolysable tannins can be degraded by rumen bacteria, but their impact should be rate-dependent, and affect differently for each tannin source. Commonly, quebracho tannins have been largely used (Frutos et al., 2000; Hervás et al., 2003a) and are usually considered as a reference, but other commercially available tannin extracts have also been assayed, such as those from grape, chestnut and oak (Wischer et al., 2013; Carreño et al., 2015). Whereas tannins from chestnut and oak are considered as hydrolysable tannins, those from grape and quebracho are condensed tannins; however, such generic classification based on their chemical structure is not helpful for predicting animal responses. Hervás et al. (2003a) and Mezzomo et al. (2011) have strategically added tannins to feeds to evaluate their potential protecting effect on certain nutrients, but were mostly used on either fibrous feeds or protein sources as components of forage or mixed

diets rather than on highly fermentable carbohydrates in high concentrate diets, under lower rumen pH. As their direct effect on rumen bacteria and their activity might also affect starch utilisation (Martínez et al., 2006), and depends on environmental pH (McSweeney et al., 2001; Mueller-Harvey, 2006), some tannins might be a suitable alternative to reduce fermentation rate of concentrate feeds, preventing for rumen disturbances associated with intensive feeding in ruminants, such as acidosis. It is worth mentioning that a possible reduction of the extent of rumen fermentation is not necessarily a drawback, as the non-degraded starch that reaches the intestines to be digested there may provide 42% more energy than that digested in the rumen (Owens et al., 1986).

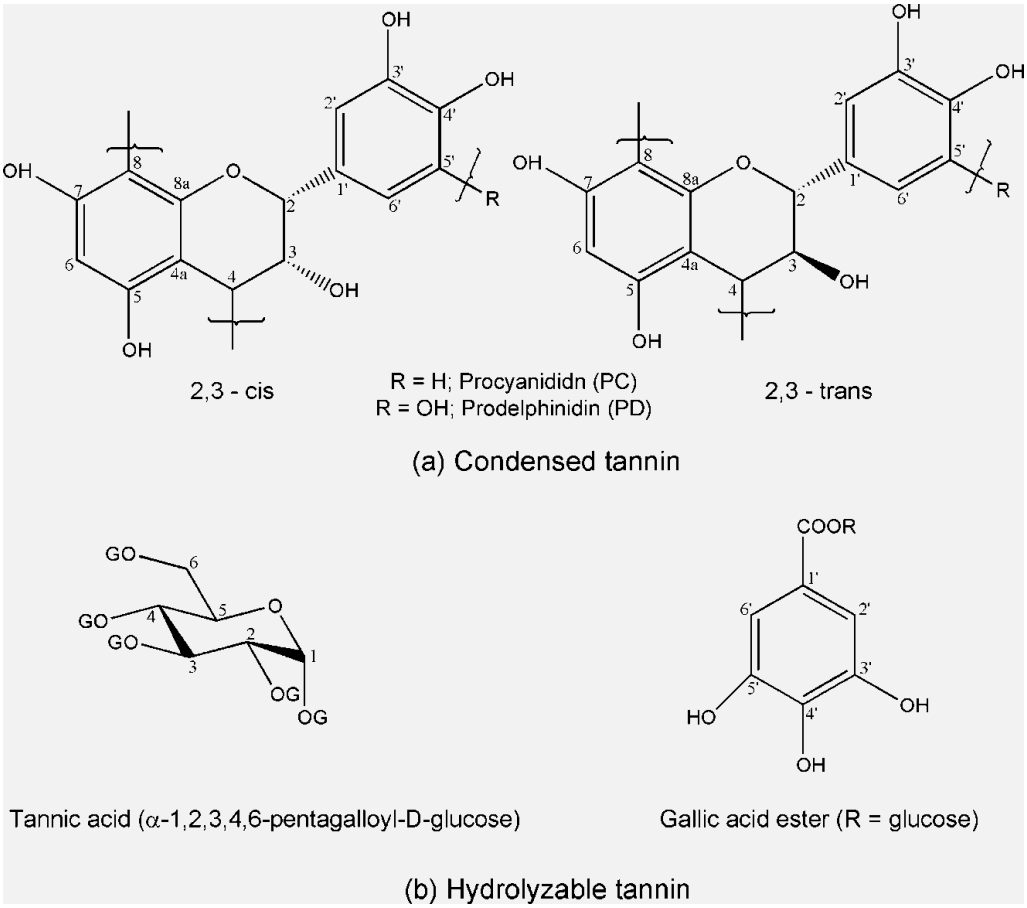

Figure 8. Structures of (a) condensed and (b) hydrolyzable tannins. (Data from Kraus et al., 2003).

3.1.1.2. Fatty acids

Fatty acids that belong to the lipid family, are monocarboxylic acids with an aliphatic hydrophobic chain that are either saturated or unsaturated depending on the presence or the absence of double bonds (C=C), with an even number of carbon atoms from 4 to 36. In fact, different types of fatty acids can be distinguished on the basis of their carbonic chain length.

- Short-chain fatty acids → fatty acids with aliphatic tails of five or fewer carbons.
- Medium-chain fatty acids → fatty acids with aliphatic tails of 6 to 12 carbons.
- Long-chain fatty acids → fatty acids with aliphatic tails of 13 to 21 carbons.
- Very long chain fatty acids → are fatty acids with aliphatic tails of 22 or more carbons.

In diets for ruminants, fatty acids are presented in form of either phospholipids and glycolipids, especially galactolipids, which are the main forage membrane lipids, or in form of triglycerols commonly called triglycerides (Harfoot and hazlewood, 1988). In their review, these authors have reported that these fatty acids are mainly unsaturated with 18 carbons. Doreau and Ferlay (1994) and Chilliard and Ollier (1994) indicated that the percentage range of lipids in diets for ruminants is between 2 and 5%, of which about one-half are fatty acids. However, Palmquist and Jenkins (1980) explained that because of the important role of fats in meeting the animal energy requirements, levels of less than 5% fats are not enough to respond to these requirements that depend on the animal physiological function. The same authors added that, in contrast to non-ruminants, ruminants depend more on non-glucose metabolites for energy metabolism, and for these reasons it is well known that the main objective of the supplementation of ruminant livestock diets with lipids (essentially fatty acids) is to increase the energy density in the ration. An increase in the rate of fatty acids in ruminant diets affects on animal metabolism (Bauman et al., 2011), but it can also manipulate the digestive processes and balance the nutrients absorption, leading to limit ruminal acidosis (Chilliard and Ollier, 1994). However, Palmquist and Jenkins (1980) reported that the addition of fatty acids attenuates the rumen fermentative activity. In their reviews, Boeckaert et al. (2006) and Bayat et al. (2018) indicated that changes occurred in the rumen microbial population when fatty acids were included in ruminant diets, these changes concerning essentially ruminal microbial population responsible for cellulose digestion (Doreau and Ferlay,

1995; Getachew et al., 2001; Vazirigohar et al., 2018). In the same way, Machmüller (2006) demonstrated the decrease on rumen degradation of fibre and organic matter when fatty acids, especially medium chain fatty acids, were used as additive. Huws et al. (2010) also showed that the inclusion of oils in ruminant diets can manipulate the microbial community and fermentation processes in the rumen. However, the effect of the inclusion of fatty acids in ruminant diets depend essentially upon the type of fatty acids, their amount of inclusion, and the basal diet composition (Doreau et al., 2009, Beauchemin et al., 2009, Kubelková et al., 2018). Whereas, the effectiveness of fatty acids depends mainly on the level of its supplementation and the diet type fed to the ruminant (Klevenhusen et al., 2009, Vazirigohar et al., 2018). In fact, Henderson (1973) reported that the medium chain fatty acids have an effect of antimicrobial selectivity, results that were demonstrated in an *in vitro* study conducted by Kleverhusen et al. (2011), showing that monolaurin, which is formed from glycerol and lauric acid (medium chain fatty acid) affected a large number of ruminal microbes involved in carbohydrate degradation. In contrast, Jenkins (1994) explained that long chain fatty acids, especially the unsaturated fatty acids, depressed the microbial activity. To avoid the toxicity of the unsaturated fatty acids on the rumen microbial population, Chilliard and Ollier (1994) explained that from 70 to 90% of the unsaturated fatty acids ingested are hydrogenated in the rumen by bacteria, mainly to stearic acid. From results found by several previous researches (Soliva et al., 2004; Sinclair et al., 2005; Machmüller, 2006; Klevenhusen et al., 2009) it can be concluded that, whatever the type of fatty acids included in the diet, the extent of rumen fermentation is reduced. In addition, Soder et al. (2013) and Costa et al. (2017) recently reported that the inclusion of lipids in diets for ruminants modified the volatile fatty acids profile. In fact, Chalupa et al. (1984) observed a decrease in VFA production caused by medium chain fatty acids (lauric acid), by long-chain fatty acids that contained less than 18 carbons (palmitic acid), and by unsaturated long-chain fatty acids with 18 carbons (linoleic acid). Similarly, Klevenhusen et al. (2009) showed a decrease in total VFA concentration when medium chain fatty acids were added, and they also observed an increase in molar proportion of propionate at the cost of butyrate and acetate. However, in their study, Soliva et al. (2004) reported that no significant effects of medium chain fatty acids treatments were found on the concentration of total VFA and on the molar VFA proportions. In terms of rumen pH, Beauchemin et al. (2009) and Martínez et al. (2010) reported that the addition of oilseeds did not negatively affect the ruminal pH, although Kubelková et al. (2018) observed a reduction of medium pH, but despite

significant treatments variations, the mean pH values remained within the physiological range defined by Krause and Oetzel (2006), which means that these types of fatty acids did not negatively affect medium pH. Note that most of these investigations (*in vivo* or *in vitro*) conducted to study the effect of fatty acids have been focused on dairy cattle diets to reduce methane production, and there is a little information regarding the study of the use of fatty acids in diets for cattle fattening.

Chapter III. Hypothesis, objectives and experimental approach

Chapter III. Hypothesis, objectives and experimental approach

A major part of calves and lambs destined for intensive feeding systems in Southern Europe, especially in Spain, are lactating or newly weaned animals. Calves that have been raised with nursing cows at pasture for 5-6 months or milk-fed lambs for 6 weeks are directly switched from a milk-forage regime to a high concentrate feeding based on cereals. These abrupt changes introduce an important alteration on the rumen environmental conditions. This strategy consists on promoting a high intake of rapidly fermentable carbohydrate sources mostly based on starch which enhances activity of rumen microbiota, and consequently pH drops below levels considered in risk of acute or subacute acidosis, which is at present one of the most common problems during intensive fattening of ruminants in general, and young ruminants in particular. In fact, the transition from weaning to fattening with high concentrate feeds for young ruminants needs some time for the microbial population to be adapted in order to allow for minimizing the risk of this disorder digestive.

In such situation, knowledge about the fermentative behaviour of potential sources of carbohydrates is needed, including the study of their rate and extent of environmental pH reduction, in order to choose the better alternative for an adequate transition to a high concentrate feeding. In addition, another strategy to avoid the onset of acidosis consists in the supplementation of these diets with different types of additives that modulate digestive environment and microbiota. For achieving this in practice, routine *in vitro* nutritive evaluation techniques must be adapted for the study of this type of feeds and additives, with emphasis on the incubation pH, which has a major impact on rumen microbial activity. This PhD thesis aims to investigate *in vitro* the impact of the transition from a forage diet to another high in concentrate for the early fattening of ruminant by assessing the effect of the source of carbohydrate and additives supplementation on pH and overall microbial fermentation, under *in vitro* conditions. We hypothesized that the supplementation of different sources of carbohydrate with additives that have the capacity to modulate the ruminal fermentation can be an alternative to mitigate the risk of acidosis in such fattening systems.

This main objective is divided into the following specific objectives:

1. To evaluate the adequate carbohydrate source for modulating rumen environment in a transition from a ruminal environment based on forage to another rich on concentrate.
2. To study how potential mixtures of dietary components could modulate the characteristics of ruminal fermentation during the transition from a forage to a concentrate diet.
3. To study whether the inclusion of certain additives at various levels in fattening diets for intensive production could affect the rate of microbial fermentation of feeds included in high concentrate diets.
4. To determine how inocula from different feeding conditions can modulate the fermentation response, in order to estimate the magnitude of the importance of adaptation of rumen microbial conditions to diet characteristics.

To achieve these objectives, two experimental sections were carried out, entirely under *in vitro* conditions:

Section I. I*n vitro* study of the magnitude and rate of fermentation of different carbohydrate sources, and their ability to modify fermentation conditions.

Both the chemical characteristics (type of carbohydrate) of the ingredients and the availability of nutrients (accessibility, interaction with other feed components) can modify their fermentation rate, and therefore modulate the microbial population and its fermentative activity (Fondevila et al., 2002). On the other hand, it has been hypothesized that the response to a high level of concentrates in the diet may depend not only on the pH of the ruminal environment caused by the rapid fermentation of highly available carbohydrates but also on substrate effects that are independent of pH (Calsamiglia et al., 2012).

The experiments carried out in this section were established based on previous results of our research group (Amanzougarene et al., 2017a; Amanzougarene et al., 2018a). In these two works, it was studied the *in vitro* acidification potential and the fermentation pattern of different carbohydrate sources with inocula from different nature, in a poorly buffered medium (pH =5.5) using a conventional incubation system (a closed batch *in vitro* system). Thus, two Experiments were scheduled: in a first Experiment (Experiment I.1), six different carbohydrate sources, varying in nature and composition

(three cereals and three by-products) were chosen to evaluate their capacity to modify the microbial fermentation pattern under conditions adjusted to *in vivo* daily rumen fermentation process. Therefore, the experiment was carried out under intensive feeding conditions using a simple semicontinuous system (Fondevila and Pérez-Espés, 2008, modified by Prates et al., 2010) to mimic rumen pH pattern and liquid outflow rates, adapted to modulate incubation pH to daily rumen pH fluctuations following the procedure proposed by Amanzougarene and Fondevila (2018). The incubation substrates were incubated with inoculum of animals given either forage or concentrate-based diets, to compare the fermentative activity of microbiota from both environments. In the second Experiment (Experiment I.2) of this section, the objective was to study the synergistic and antagonistic effects of three mixtures of carbohydrate sources (maize and barley as starch sources, and sugarbeet pulp as high fermentable fibre source), chosen from the results of the first experiment, and was carried out under the same incubation conditions that in the former.

Section II. *In vitro* fermentation of carbohydrate-based substrates added with additives to modulate the ruminal fermentation under intensive fattening conditions.

For an appropriate adaptation of the rumen environment to concentrate-based diets, the use of additives that can reduce the rate and magnitude of fermentation of carbohydrates and provide with a gradual adaptation to the diet, reducing or avoiding the imbalance in rumen fermentation processes. This section aims to assess the effect of different types of additives on the magnitude and rate of fermentation of starch-rich substrates, choosing barley grain (*Hordeum vulgare*, var. Gustav) as a reference incubation substrate for ingredients commonly included in high concentrate diets.

In this section, three Experiments were scheduled. In the two first Experiments (Experiment II.3 and Experiment II.4), different types of feed additives were checked at different levels to evaluate their capacity to modulate the *in vitro* fermentation of barley under intensive ruminant feeding conditions, using a closed batch system following the procedure of Theodorou et al. (1994). The additives were included at three doses (low, medium and high), the medium dose being selected according to the manufacturer recommendations. The additives tested were:

1) Four different commercial sources of tannins (Agrovin SA, Alcázar de San Juan, Spain; Experiment II.3):
   A. Quebracho (2S-profisetinidins from Schinopsis spp., 0.65 w/w of tannins)
   B. Grape condensed tannins (procyanidins from Vitis vinifera, 0.75 w/w tannins),
   C. Chestnut (esters of ellagic acid and sugars from Castanea sativa, 0.65 w/w of tannins).
   D. Oak (ellagitannins from Quercus robur and Q. petraea, 0.77 w/w of tannins).

2) Different types of fatty acids and essential oils (Experiment II.4)
   A. Three types of fatty acids (Experiment II.4.1):
      A.a) Saturated fatty acids (palmitic acid, 0.85 g/mL; MERCK KGaA, Darmstadt, Germany).
      A.b) Polyunsaturated fatty acids (linoleic acid, 0.90 g/mL; Alfa Aesar, Thermo Fisher, Karlsruhe, Germany).
      A.c) A commercial mixture of medium-chain fatty acids (50% C12, 20% C10, 20% C8, and 10% C6; NUTRIKA, Zulte, Belgium).

   B. Three types of essential oils (Experiment II.4.2):
      B.a) Cinnamaldehyde (98% purity, 1.05 g/mL; NOREL Animal Nutrition, Barcelona, Spain).
      B.b) Eugenol (EUG, 99% purity, 1.06 g/mL; NOREL Animal Nutrition, Barcelona, Spain).
      B.c) The commercial blend CRINA Ruminants (DSM Nutritional Products, Basel, Switzerland)

In the third Experiment of this section (Experiment II.5), up to five additives of different nature sources were chosen as treatments, supplementing barley at doses previously established from Experiment II.3 and Experiment II.4. The objective of this experiment was to evaluate the effect of these additives on the adaptation of rumen microbial population to concentrate conditions, and so their capacity to modulate barley fermentation, using an *in vitro* semicontinuous system (Fondevila and Pérez-Espés, 2008, modified by Prates et al., 2010), fitted at such environmental conditions. These additives were:

A. Grape condensed tannins
B. A commercial mixture of medium-chain fatty
C. Polyunsaturated fatty acid (linoleic acid)
D. Eugenol
E. Cinnamaldehyde

Chapter IV. Section I. Experiment 1

Fermentation pattern of several carbohydrate sources incubated in an *in vitro* semicontinuous system with inocula from ruminants given either forage or concentrate-based diets (Animals 2020. 10: 261. doi: 10.3390/ani10020261)

Chapter IV. Fermentation pattern of several carbohydrate sources incubated in an *in vitro* semicontinuous system with inocula from ruminants given either forage or concentrate-based diets.

Simple Summary

A sudden change from a milk/forage diet to a high concentrate diet in young ruminants increases rate and extent of rumen microbial fermentation, leading to digestive problems such as acidosis. The magnitude of this effect depending on the nature of ingredients. Six carbohydrate sources were tested: three cereal grains (barley, maize and brown sorghum) as high starch sources of different availability and three byproducts (sugarbeet pulp, citrus pulp and wheat bran) as sources of either insoluble or soluble fibre. An *in vitro* semicontinuous incubation system was used to compare the fermentation pattern of substrates incubated with inocula simulating concentrate or forage diets, under pH and liquid outflow rate conditions of intensive feeding systems. The magnitude of microbial fermentation was higher with concentrate than forage inoculum, and the drop of pH in the first part of incubation was more profound. Among substrates, citrus pulp had a greater acidification potential and was fermented at a higher extent, followed by wheat bran and barley. In conclusion, the acidification capacity of substrates plays an important role in environmental conditions, depending on the type of diet given to the ruminant. This *in vitro* system used allows to compare substrates under conditions simulating high concentrate feeding.

Abstract

The fermentation pattern of several carbohydrate sources and their interaction with the nature of microbial inoculum was studied. Barley (B), maize (M), sorghum, (S), sugarbeet pulp (BP), citrus pulp (CP) and wheat bran (WB) were tested in an in vitro semicontinuous system maintaining poorly buffered conditions from 0 to 6h and being gradually buffered to 6.5 from 8 to 24h to simulate rumen pH pattern. Rumen fluid inoculum was obtained from lambs fed with either concentrate and barley straw (CI) or alfalfa hay (FI). Extent of fermentation was higher with CI than FI throughout the incubation ($p < 0.05$). Among substrates, S, BP and M maintained the highest pH ($p < 0.05$), whereas CP recorded the lowest pH with both inocula. Similarly, CP recorded the

highest gas volume throughout the incubation, followed by WB and B, and S recorded the lowest volume ($p < 0.05$). On average, total VFA as well as lactic acid concentration were higher with CP than in the other substrates ($p < 0.05$). The microbial structure was more affected by the animal donor of inoculum than by the substrate. The *in vitro* semicontinuous system allows for the study of rumen environment acidification and substrate microbial fermentation under intensive feeding conditions.

1. Introduction

Reaching a high productive performance in the fattening of young ruminants requires high energy diets, that promote a high rate and extent of rumen microbial fermentation, with acidosis as a frequent consequence (Bevans et al., 2005). In practice, ruminants reared at pasture are often abruptly introduced to intensive feeding systems without being previously adapted to high concentrate diets, promoting variable responses in the rate and extent of fermentation (Fernando et al., 2010). Cereals are commonly considered as ingredients of concentrate diets for ruminants. Their energy value depends on starch availability, which differs according to its chemical structure, protein matrix or in some cases the presence of phenolic compounds (O´Brien, 1999; Offner et al., 2003). Fibrous byproducts with either insoluble (cellulose, hemicelluloses) or soluble (mostly pectin) polysaccharides and variable proportions of either starch or sugars (DePeeters et al., 1997; Maes and Delcour, 2001) are also included among the carbohydrate sources currently used. Fitting substrate characteristics to the fermentative ability of rumen microbiota while environmental conditions are maintained at an optimal range is a key factor for maximising efficiency of energy utilisation and the risk of physiological impairment is also reduced. The characteristics of the specific rumen microbial community promoted by a certain diet also affect substrate utilisation (Mould et al., 2005b), as the activity of the bacterial species capable to ferment starch or fibrous polysaccharides depends on environmental characteristics (McAllister et al., 1990; Klieve et al., 2003).

The comparison of these energy sources and their effects in the rumen under *in vivo* conditions is laborious and expensive, and often biased by the feeding pattern and hardly controlled fermentation conditions (Dijkstra et al., 2005). On the other hand, *in vitro* studies are cheaper and faster and allow for a good insight into rumen fermentation processes (Raab et al., 1983). However, most of these *in vitro* methods are designed for

mimicking environment promoted by high forage diets, including the use of inoculum from forage-fed animals (Sari et al., 2015), and it is not easy to adapt main physiological conditions such as pH and rate of passage to conditions promoted by high concentrate diets (Bertipaglia et al., 2010). Amanzougarene and Fondevila (2018) succeed in maintaining a low incubation pH in an *in vitro* closed-batch system by reducing the bicarbonate concentration in the incubation solution, allowing for comparing fermentation of different carbohydrate sources under conditions simulating high concentrate feeding (Amanzougarene et al., 2018a; Amanzougarene et al., 2018b). However, this is not the real physiological situation *in vivo*, where pH changes at a wide range along the day (Krause and Combs, 2003), and besides rumen outflow rate cannot be assessed in this system. In this regard, the semicontinuous incubation system (Fondevila and Pérez-Espés, 2008) modified by Prates et al. (2010), applying the procedure proposed by Amanzougarene and Fondevila (2018) for controlling incubation pH appears as a useful tool to mimic rumen pH pattern and liquid outflow rates under *in vitro* conditions.

Therefore, in a semicontinuous *in vitro* incubation system we compared the acidification potential and the rumen microbial fermentation pattern of several carbohydrate sources of variable composition when a different rumen environment is promoted by either high forage or high concentrate diets, aiming to minimise at possible the risk of acidosis during feeding transition from a fibrous to a high-concentrate diet.

2. Materials and Methods

2.1. Substrates and inocula

Six carbohydrate sources were chosen as substrates: three cereal grains (barley var. Gustav (B), maize var. Dekalb 6667Y (M), and a brown sorghum of unknown variety (S)) and three by-product feeds (sugarbeet pulp (BP), citrus pulp (CP) and wheat bran (WB)). All substrates were ground in a hammer mill (Retsch Gmbh/SK1/417449, Haan, Germany) through a 1 mm sieve. The chemical compositions of the substrates are given in Table 3.

Table 3. Chemical composition (g/kg DM) of feeds used as incubation substrates.

Code	**B**	**M**	**S**	**BP**	**CP**	**WB**
OM	978	986	979	953	940	944
CPr	105	75	113	107	59	161
EE	24	34	11	5	14	31
Starch	672	706	647	-	-	245
aNDFom	173	91	97	437	207	499
ADF	56	25	60	272	192	145
ADL	18	2	5	75	21	37
NDSF	4	77	110	457	423	155
Sugars	1.6	13	1.3	9	243	31
TP	-	-	2.6	-	-	-
TT	-	-	1.3	-	-	-

Barley (B); maize (M); sorghum (S); sugar beet pulp (BP); citrus pulp (CP); wheat bran (WB). Dry matter (DM); organic matter (OM); crude protein (CPr); ether extract (EE); neutral detergent fibre (aNDFom); acid detergent fibre (ADF); acid detergent lignin (ADL); neutral detergent soluble fibre (NDSF). Total phenolics (TP); total tannins (TT).

Rumen fluid was obtained from six lambs housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the Universidad de Zaragoza. Animal care and procedures for extraction of rumen inoculum were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Lambs were weaned at 49 ± 8 days (average weight 13.6 ± 0.78 kg), and thereafter three lambs (1, 2 and 3) were fed *ad libitum* with a concentrate mixture (composed by barley, maize, wheat, and soybean meal) and barley straw (88:12 concentrate to straw ratio) for 35 days, and then slaughtered (average weight 20.6 ± 1.85 kg) for obtaining concentrated inoculum (CI). The other three lambs (4, 5 and 6) were fed *ad libitum* with alfalfa hay and slaughtered after 45 days (average weight 16.5 ± 0.33 kg) for forage inoculum (FI). The rumen contents of each animal were individually filtered through a cheesecloth and dispensed in 16 mL aliquots into 110 x 16 mm tubes, that were immediately frozen in liquid nitrogen and preserved at -80 ºC until use (Prates et al., 2010). Immediately before incubation, rumen inoculum was thawed in a water bath at 39ºC (about 2 minutes).

2.2. Experimental conditions

The *in vitro* semicontinuous system of Fondevila and Pérez-Espés (2008), modified by Prates et al. (2010), was used. Substrate samples (800 mg) were dispensed into 4 x 4 cm nylon bags (45 µm pore size) that were sealed and introduced into duplicated bottles (123 mL total volume). Bottles were filled under CO_2 flux with 80 ml of incubation solution including 16 mL (0.20 of total volume) thawed rumen inoculum, without resazurin and microminerals (Mould et al., 2005a), and were incubated in a water bath at 39ºC for 24 h in three incubation series, each corresponding to a different donor animal, for each type of inoculum. Buffer solution was modified to include 0.006 M bicarbonate ion in order to get a poorly buffered medium (Amanzougarene and Fondevila 2018).

Pressure produced on each bottle was measured every 2 (from 0 to 12 h incubation) or 4 h (from 12 to 24 h) with a HD8804 manometer provided with a TP804 pressure gauge (DELTA OHM, Caselle di Selvazzano, Italy). Readings corrected for the atmospheric pressure were converted to volume (ml) using a pre-established linear regression recorded in this type of bottles (n=48, R^2 =0.993), and expressed per unit of incubated organic matter (OM). Along the incubation, an aliquot volume of medium was extracted immediately after each gas measurement and replaced anaerobically by the same volume of incubation solution (without microbial inoculum) to simulate an approximate liquid turnover rate of 0.08/h. In order to simulate daily rumen pH fluctuations, from 0 to 6 h the incubation solution was poorly buffered, as explained above, to allow incubation pH to drop as fermentation proceeds, whereas from 8 h onwards the replacing incubation solution was made up with 0.058 M bicarbonate ion for allowing pH to increase to around 6.5.

Incubation pH was recorded on every extraction. Besides, medium was sampled at 6 and 10 h for determination of volatile fatty acids concentration (VFA; 2 mL on a 0.5 mL solution of 0.5M phosphoric acid with 1 mg 4-methyl-valeric acid as internal standard) and at 6 h for determination of lactic acid concentration (2 mL). Samples were stored at -20ºC until analysis. Besides, another samples (6 mL) was also taken at 8 h and immediately frozen (-80ºC) for determination of microbial biodiversity by terminal restriction fragment length polymorphism (tRFLP). At the end of incubation, substrate bags were removed from the bottles, rinsed and dried at 60ºC for 48 h for determination of dry matter disappearance (DMd).

2.3. Chemical and microbiological analyses

The dry matter (DM) and OM content in the substrates and the incubation residues were analysed following the AOAC (2005) procedures (methods ref. 934.01 and 942.05). The substrates were also analysed for crude protein (CPr) and ether extract (EE) (ref. 976.05 and 2003.05) (AOAC, 2005), and their concentration of neutral detergent fibre (aNDFom) was analysed as described by Mertens (2002) in an Ankom 200 Fibre Analyser (Ankom Technology, New York, NY, USA), using α–amylase and sodium sulphite, with results being expressed exclusive of residual ashes. The acid detergent fibre (ADF) (ref. 973.18) and acid detergent lignin (ADL) were determined as described by AOAC (2005) and Robertson and Van Soest (1981), respectively. Neutral detergent soluble fibre (NDSF) was estimated following Hall et al. (1997), discounting the aNDFom and the ethanol insoluble EE, CPr and starch fractions from the insoluble OM. The total starch content in B, M, S and WB substrates was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). The total phenolic (TP) content in S was analysed following the colourimetric method of Makkar et al. (1993) using the Folin–Ciocalteau reagent and with tannic acid (MERCK Chemicals, Madrid, Spain) as the reference standard. The total tannins (TT) were estimated as the difference between TP before and after treatment with polyvinyl polypyrrolidone.

The frozen samples of incubation medium were thawed and centrifuged at 13,000 g for 15 minutes at 4 ºC for their analysis of lactic acid and VFA. The VFA were determined by gas chromatography on an Agilent 6890 apparatus equipped with a flame detector and a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm id). The lactic acid concentration was determined by the colorimetric method proposed by Barker and Summerson (1941). For the microbial diversity analysis, frozen microbial samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The deoxyribonucleic acid (DNA) was extracted using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer recommendations, except that samples were initially heated at 95ºC for 5 min to maximise the lysis of bacterial cells. Concentration of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). PCR was performed using a 16S rRNA bacteria specific primer (cyanine-labelled forward 27F, 5´-AGA GTT TGA TCC TGG CTCAG-3´ and unlabelled reverse 1389R, 5´-AGG GGG

GGT GTG TAG AAG-3´; Hongoh et al., 2003) using a DNAEngine® Gradient Cycler (Bio-Rad, Spain). The polymerase chain reaction (PCR) product was purified using a Purelink PCR purification kit (ref. K3100-01; Invitrogen) and diluted to 10 µL. The DNA concentration of each amplified and purified sample was obtained by spectrophotometry (Nanodrop® ND-1000 spectrophotometer) to enable a standardised quantity of 50 ng DNA to be used per restriction enzyme digest reaction. Digestion of samples was carried out using HhaI, HaeIII and MspI (Promega, Spain), following the manufacturer recommendations except for HhaI, where the recommended addition of bovine serum albumin was omitted. Restriction digests were purified by ethanol precipitation (de la Fuente et al., 2014) in 35 µL sample loading solution buffer including a 600 bp size standard (Beckman Coulter Inc., Fullerton) before being applied to a 3500xL Genetic Analyzer (Applied Biosystems). Once getting the size and height of every peak, 1% of the second highest peak was used as criteria for the lower threshold for peaks, to detect and eliminate smaller, broader peaks that would not be indicative of single true Operational Taxonomic Units (OTUs).

2.4. Calculations and statistical analyses

The TRFLP results were analysed from a matrix generated for each data list obtained, and results were presented in the form of relative abundance. The three matrices resulting from each series and enzyme were concatenated and analysed with R statistical software (https://cran.r-project.org/bin/windows/base/, version 3.5.0). FactoMineR, Factoextra, MixOmics, Vegan, MASS, and Ggplot2 packages were used to carry out the analysis of hierarchical classification on principal components for obtaining the cluster dendrogram.

The results were analysed statistically by ANOVA using the Statistix 10 package (2010). On each sampling time, the effect of the incubation series (equivalent to the donor animal; interaction inoculum x incubation series, random effect), the type of inoculum, the type of substrate, and the interaction of both factors on pH, gas production, total VFA and lactic acid concentration, and VFA profile were studied as factors. The treatment differences among the means with $p < 0.05$ and $0.05 < p < 0.10$ were accepted as representing statistically significant differences and a trend to the differences, respectively. When significant, the differences were contrasted by the Tukey *t*-test.

Simple and multiple linear regressions were established to study the relationships among the different parameters studied.

3. Results

3.1. Pattern of incubation pH

The mean inoculum pH at the start of the incubation series was 6.45 ± 0.15 and 6.87 ± 0.02 for CI and FI, respectively (n = 3). The average minimum pH was recorded at 6 h incubation (5.96) for CI, and at 8 h (6.22) for FI. Thereafter, the pH increased to reach its maximum (6.64 for both inocula) at 24 and 20 h for CI and FI. The pH differences in the incubation medium among inocula ($p < 0.05$) were ± 0.3 units from 2 to 6 h, decreasing gradually to ± 0.1 at 12 h. A significant interaction inoculum × substrate ($p < 0.05$) observed on pH at 4, 8, 10, 12, 16 and 20 h and a tendency ($p = 0.052$) at 2 h incubation indicates the different behaviour of the substrates depending on the inoculum. Therefore, a comparison of the pH pattern among the incubated substrates is presented in Figure 9 separately for each inoculum. With CI (Fig. 9a), the lowest incubation pH from 2 to 12 h was recorded with CP ($p < 0.05$), reaching its minimum at 6 h (5.60), although recovered thereafter to 6.63 at 24 h incubation. In ascending order, WB and B reached their minimum pH at 8 h (5.89 and 5.97, respectively), whereas BP, M, and S maintained a higher medium pH from 4 to 8 h ($p < 0.05$). The differences among M, S, BP, and B disappeared from 10 to 16 h ($p > 0.05$), and no differences were detected among the substrates ($p > 0.05$) at the end of incubation. When the substrates were incubated using FI (Fig. 9b), CP recorded the lowest pH from 4 to 10 h incubation ($P < 0.05$), and its minimum value was 5.90, whereas S, M and BP maintained the highest medium pH during this period (6.30 to 6.46), and B and WB were grouped at intermediate values ($p < 0.05$). At 16 and 20 h incubation only, B recorded a lower value (6.44 and 6.57; $p < 0.05$) and, again, no differences were detected among the substrates at the end of incubation ($p > 0.05$).

Fig. 9a)

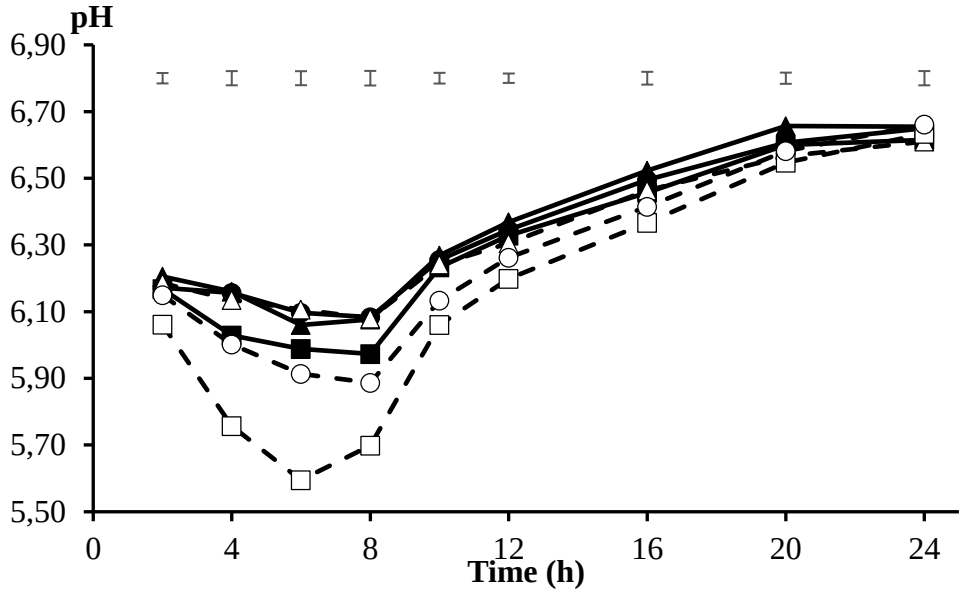

Fig. 9b)

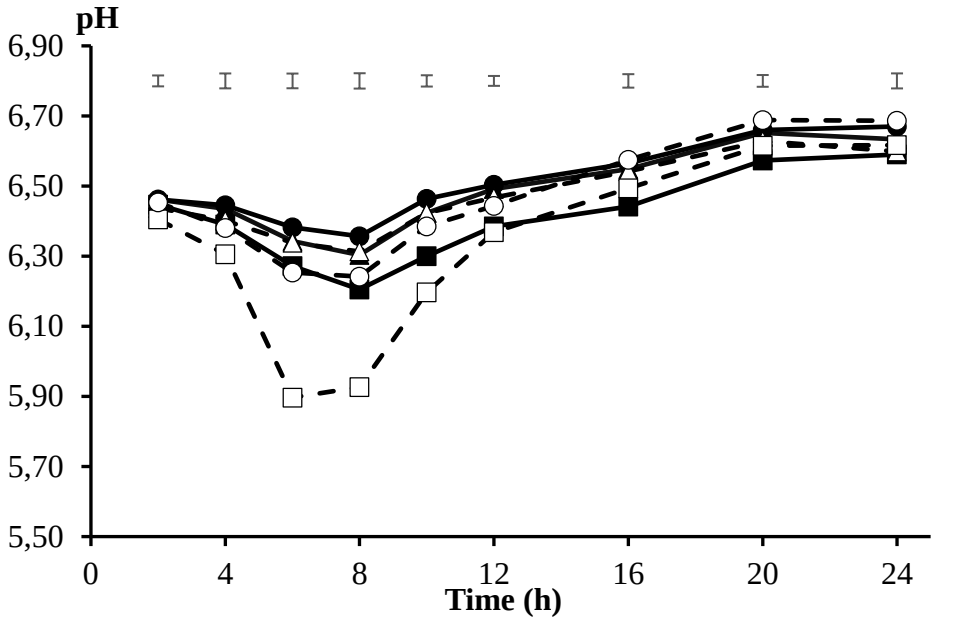

Figure 9. Pattern of medium pH of carbohydrate substrates (barley, B ■, maize, M ▲, sorghum, S ●; solid lines, citrus pulp, CP □, sugar beet pulp, BP △, wheat bran, WB ○; dashed lines) incubated with inoculum from concentrate (CI, Fig. 1a) or forage (FI, Fig. 1b) diets. Initial pH was 6.45 (Fig 1a) and 6.87 (Fig. 1b). The upper bars show the standard error of the means (n=3).

3.2. Pattern of in vitro gas production

The volume of gas produced with the CI inoculum was higher than that obtained with FI at all incubation times ($p < 0.05$). Because of the interaction inoculum x substrate at 4 h and from 8 to 24 h ($p < 0.05$), for an easier understanding, the gas production is presented separately for CI and FI (Figure 10). The major difference among substrate fermentative behaviour between the inocula is manifested in the magnitude of differences among them. Thus, with CI (Fig. 10a), CP recorded the highest gas volume from 4 h onwards, at 12 h being on average 0.42 times higher than the other substrates, while also recorded differences at 2 h with BP and S ($p < 0.05$). The gas volume with WB was higher than BP and S from 4 h onwards, and higher than M from 6 h and B from 8 to 20 h ($p < 0.05$). Differences were also recorded between B and S from 8 to 16 h and at 24 h ($p < 0.05$). A similar pattern was observed with FI (Fig. 10b), but the magnitude of differences was lower. Thus, CP was higher than B, M, BP and S from 6 to 24 h ($p < 0.05$), with differences at 12 h reaching 0.59 of their average, but did not differ from WB, which was higher than BP and S in that period and also higher than M from 6 to 10 h ($p < 0.05$). Differences between B and M respect to S were also detected from 16 and 20 h onwards, respectively ($p < 0.05$).

Fig. 10a)

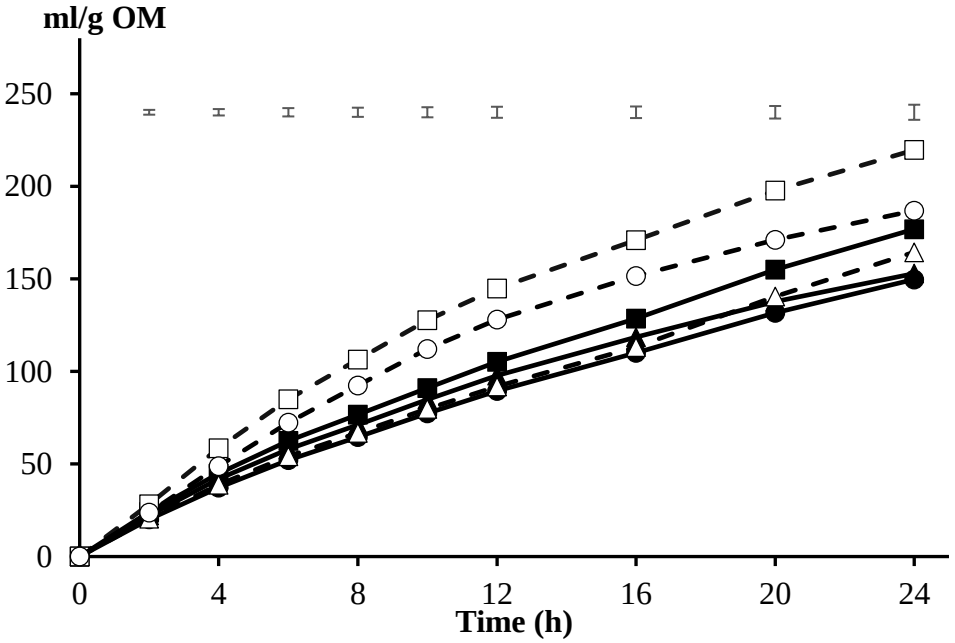

Fig. 10b)

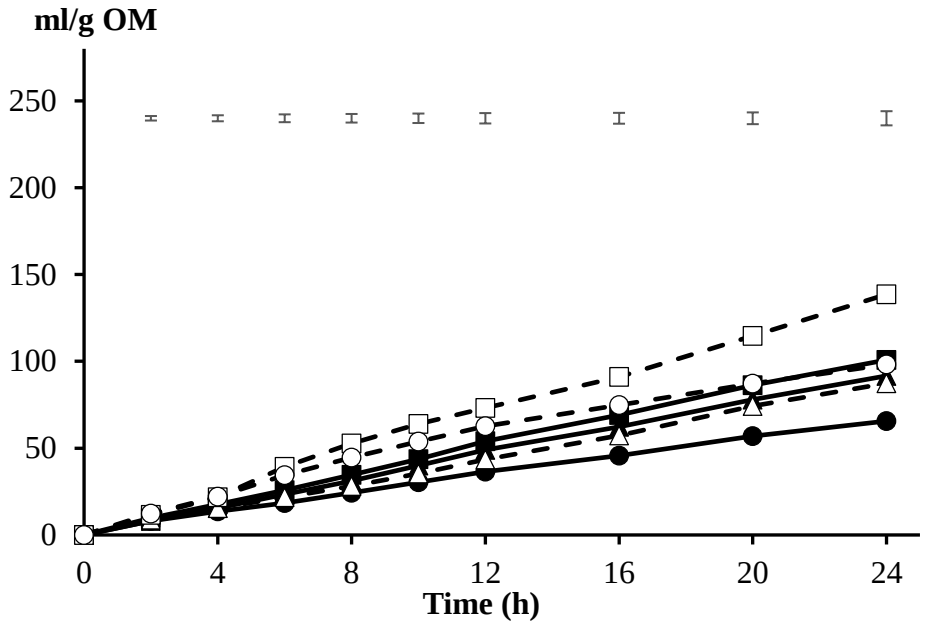

Figure 10. Pattern of gas production from the carbohydrate substrates (barley, B ■, maize, M ▲, sorghum, S ●; solid lines, citrus pulp, CP □, sugar beet pulp, BP △, wheat bran, WB ○; dashed lines) incubated with inoculum from concentrate (Fig. 2a) or forage (Fig. 2b) diets. The upper bars show the standard error of the means (n=3).

3. 3. Dry matter disappearance (DMd)

Inoculum differences in DMd after 24 h of incubation were not detected, although CI was numerically higher than FI (proportions of 0.382 *vs*. 0.339 from the substrate weight; *P*>0.05). Substrates ranked according to the proportion of DMd as follows: CP, 0.502 > B, 0.449 > WB, 0.360, M, 0.343 > BP, 0.265, S, 0.243 (*P*<0.001; SEM=0.0120). The interaction inoculum x substrate was not significant (*P*=0.21), indicating that substrates behaved similarly with both inocula.

3.4. Volatile fatty acids and lactic acid production

Tables 4 and 5 show that CI promoted a higher ($p < 0.05$) concentration of total VFA than FI at both 6 (23.2 *vs.* 9.8 mM) and 10 (22.2 *vs.* 9.3 mM) h. Molar proportions of acetate, propionate, and butyrate did not manifest differences between inocula ($p > 0.05$), whereas with CI valerate was higher and branc hed-chain volatile fatty acids (BCVFA, sum of isobutyrate and isovalerate) lower than with FI at both incubation times ($p < 0.05$).

Among substrates, at 6 h (Table 4) CP recorded a higher total VFA concentration than BP, M and S (average values of 19.3, 15.6, 14.5 and 15.0 mM, respectively), whereas WB (15.9 mM) was also higher than M and S ($p < 0.05$). Differences in the molar VFA profile were only recorded for BCVFA, with the highest proportions in S and M and the lowest with CP ($p < 0.05$); however, the interaction inoculum x substrate ($p < 0.001$) indicates that differences in BCVFA proportion were only observed with FI. Regarding the concentration of lactic acid at 6 h among substrates, CP recorded the highest concentration and BP and S the lowest (8.7 *vs.* 1.3 and 1.4 mM; $p < 0.05$). Similar trends were observed at 10 h on total VFA concentration (Table 5), with CP rendering a higher concentration than BP, M and S, but tending to be significant only with CI interaction inoculum x substrate, ($p = 0.058$). The interaction inoculum x substrate in the proportion of propionate ($p = 0.017$) indicates that values recorded with WB and CP were higher to those with BP and S, but differences were only manifested with FI, whereas no differences ($p > 0.05$) among substrates were recorded on acetate, butyrate and valerate proportions. The highest proportion of BCVFA was promoted by S and the lowest by CP ($p < 0.05$).

3.5. Bacterial biodiversity

Bacterial biodiversity after 8 h of incubation was markedly affected by the source of rumen inoculum. Thus, the substrates incubated with rumen inoculum from lambs fed high concentrate diet clustered together, except for WB in the first incubation run, as well as substrates incubated with FI (Figure 11). Bacterial biodiversity was also markedly affected by the incubation series, that is, the donor animal, for both inocula.

Table 4. Average of total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol), together with lactate concentration (mM) recorded at 6 h of the different carbohydrate sources incubated as substrates with concentrate (CI) or forage (FI) inoculum.

Substrates	VFA	Acetate	Propionate	Butyrate	Valerate	BCVFA	Lactic acid
with CI							
B	22.34^{ab}	0.570	0.240	0.153	0.022	0.014	3.83^{b}
M	20.18^{bc}	0.578	0.235	0.149	0.023	0.016	2.35^{c}
S	21.72^{abc}	0.588	0.245	0.131	0.022	0.015	1.93^{c}
BP	21.66^{abc}	0.593	0.235	0.135	0.021	0.016	0.90^{c}
CP	26.55^{a}	0.595	0.238	0.135	0.020	0.012	8.70^{a}
WB	26.94^{a}	0.590	0.245	0.132	0.020	0.013	2.95^{c}
with FI							
B	10.31^{xyz}	0.633	0.226	0.102	0.009	0.030^{y}	3.05^{y}
M	8.83^{yz}	0.632	0.229	0.095	0.010	0.034^{xy}	2.69^{y}
S	8.31^{z}	0.642	0.216	0.094	0.009	0.039^{x}	0.84^{y}
BP	9.52^{yz}	0.665	0.207	0.088	0.008	0.032^{y}	1.64^{y}
CP	12.12^{x}	0.686	0.209	0.075	0.008	0.023^{z}	8.67^{x}
WB	9.83^{yz}	0.653	0.225	0.083	0.009	0.030^{y}	2.97^{y}
SEM	1.065	0.0156	0.0076	0.0085	0.0010	0.0009	0.588
P-value							
Inoculum	0.002	0.077	NS	NS	0.005	<0.001	NS
Substrate	<0.001	NS	NS	NS	NS	<0.001	<0.001
Inoc. x Subs.	NS	NS	NS	NS	NS	<0.001	NS

Means within a column with different superscripts for CI (^{a,b,c}) or FI (^{x,y,z}) differ ($p < 0.05$). Standard error of the means (SEM). Branched-chain volatile fatty acids (BCVFA) (sum of isobutyrate + isovalerate). NS: $p > 0.10$.

Table 5. Average of total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol), recorded at 10 h of the different carbohydrate sources incubated as substrates with concentrate (CI) or forage (FI) inoculum.

Substrates	VFA	Acetate	Propionate	Butyrate	Valerate	BCVFA
with CI						
B	21.16^{b}	0.561	0.225	0.172	0.028	0.014
M	19.42^{b}	0.548	0.227	0.178	0.031	0.017
S	19.87^{b}	0.557	0.249	0.152	0.026	0.017
BP	20.25^{b}	0.604	0.229	0.130	0.022	0.016
CP	27.32^{a}	0.553	0.240	0.164	0.031	0.012
WB	25.10^{ab}	0.537	0.261	0.158	0.029	0.015
with FI						
B	10.46	0.597	0.256^{xy}	0.117	0.009	0.022
M	8.51	0.620	0.236^{xy}	0.109	0.009	0.026
S	8.48	0.620	0.222^{y}	0.120	0.009	0.029
BP	8.34	0.611	0.227^{y}	0.126	0.009	0.027
CP	10.47	0.579	0.267^{x}	0.124	0.010	0.019
WB	9.76	0.575	0.274^{x}	0.117	0.010	0.025
SEM	1.137	0.0233	0.0078	0.0169	0.0019	0.0024
P-value						
Inoculum	0.011	NS	NS	NS	0.008	0.008
Substrate	0.001	NS	<0.001	NS	NS	0.046
Inoc. x Subs.	0.058	NS	0.017	NS	NS	NS

Means within a column with different superscripts for CI (^{a,b,c}) or FI (^{x,y,z}) differ ($p < 0.05$). SEM: standard error of the means. Branched-chain volatile fatty acids (BCVFA) (sum of isobutyrate + isovalerate).

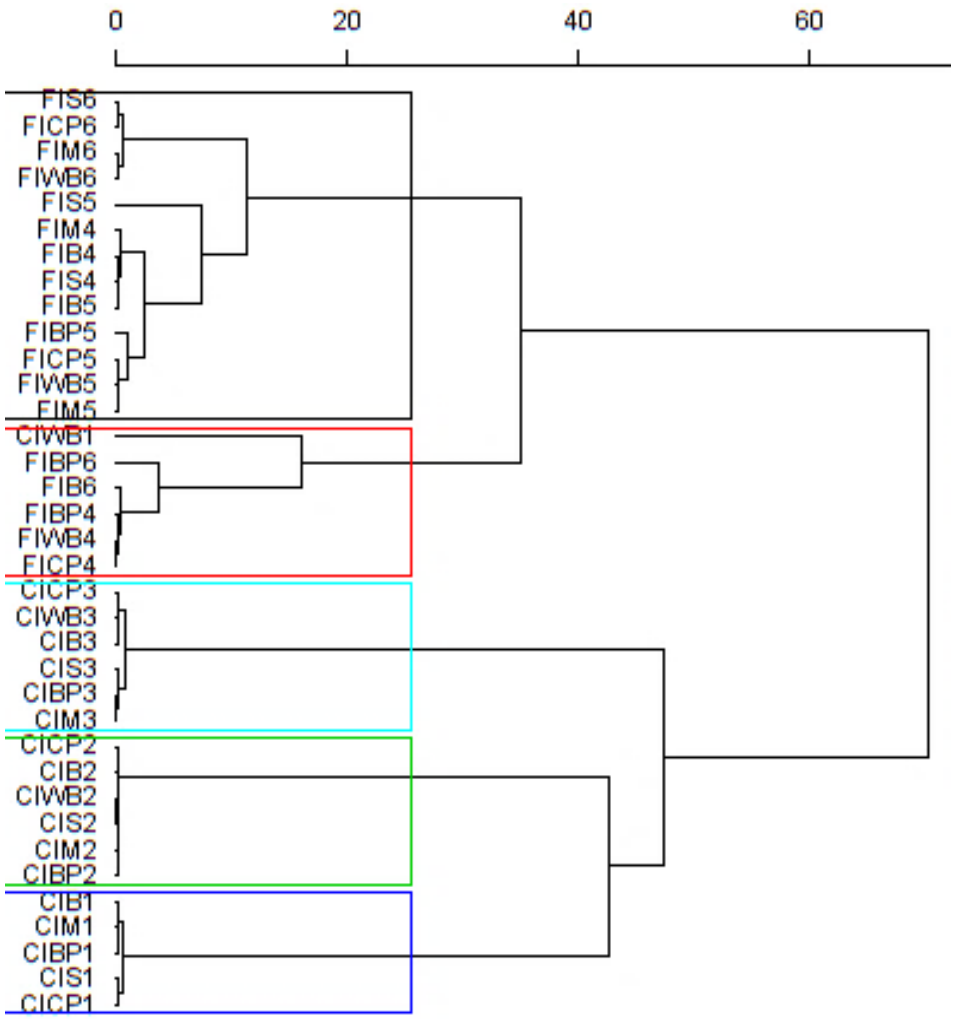

Figure 11. Dendrogram of bacteria diversity from terminal restriction fragment length polymorphism (tRFLP) data generated by enzyme digestion (HhaI, MspI, and HaeIII) for the carbohydrate substrates (B, M, S, BP, CP, and WB) incubated for 8 h with inoculum from concentrate (CI) or forage (FI) diets. Scale bar shows Euclidean distances, "ward method".

4. Discussion

Conventional *in vitro* closed batch systems are adapted for the study of microbial fermentation under conditions mimicking high forage diets, which is not applicable to evaluate diets given in intensive ruminants fattening systems. An *in vitro* semicontinuous incubation system (Fondevila and Pérez-Espés, 2008; Prates et al., 2010), adapted to control of pH by modifying bicarbonate ion concentration (Amanzougarene and Fondevila, 2018) allows for approaching the ruminal fermentation pattern of the different

carbohydrate sources to the rumen physiological conditions that occur in intensive feeding systems, either during a transition process to high concentrate diets (i.e., when ruminants are still on rumen conditions promoted by a forage diet) or when animals are adapted to such feeding conditions (as promoted by a concentrate diet). The pH pattern obtained along the *in vitro* incubation with CI and FI, reaching a minimum value at 6-8 h after substrate availability and then progressively increasing to final pH values of around 6.4-6.5, fitted well with the circadian evolution of rumen pH observed with practical forage or concentrate feeding of ruminants (Nagaraja and Titgemeyer, 2007). Thus, we can assume it allows to the different substrates to express their acidification potential, at the time their fermentation is compared under more realistic conditions.

4.1. Effect of the inoculum source on the in vitro fermentation pattern

The source of rumen fluid has an important role on the pattern of *in vitro* fermentation (Broudiscou et al., 20014; Amanzougarene et al., 2018a; Kim et al., 2018), an inoculum promoted by a concentrate diet having a higher fermentative potential than another from a forage diet. In our experiment, the lower buffering of the incubation medium during the first 6-8 hours allowed for a clear expression of the acidification potential of incubated substrates, which was expressed at a higher extent with CI than FI (average pH along the 24 h incubation from 6.45 to 5.96 *vs.* 6.87 to 6.22) as pH dropped to values close to those considered as a threshold for microbial activity (Hiltner and Dehority, 1983), whereas pH was maintained higher with FI. Despite of this, substrates incubated with CI rendered almost two-fold gas volume than with FI, irrespective the chemical nature (starch- or fibre-rich) of those substrates. Despite the more pronounced drop of pH with CI, the incubation environment promoted by a concentrate diet given to the donor animals was more favourable for fermentation of non-fibrous carbohydrates than that induced by a fibrous diet (Menke and Steingass, 1988; Mould et al., 2005a), probably because the lack of adaptation of microbiota to ferment starch and sugar substrates with a forage inoculum (Nagadi et al., 2000; Amanzougarene et al., 2018a) and the inherent buffering capacity of forage legumes such as alfalfa. However, assuming that a part of gas produced comes from the activity of bicarbonate ion in buffering of fermentation acids produced, such differences in gas production could be partly associated with the lower pH promoted by CI inoculum, although the contribution of this indirect gas is hard to quantify (Amanzougarene and Fondevila, 2018). In the case of the byproducts, characterised by their richness in rapidly fermentable fibre, microbiota might

easily counterbalance the lack of adaptation for their degradation (Hatfield and Weimer, 1995; Barrios-Urdaneta et al., 2003). In contrast, the low pH occurring during the initial part of incubation may affect at a higher extent the activity of bacterial species adapted to fibre degradation, causing a lower magnitude of fermentation of structural polysaccharides like cellulose and hemicelluloses (Mould and Ørskov, 1983; Grant and Mertens, 1992; Sari et al., 2015).

Contrary to what could be expected, the results of gas production were not supported by those of DMd. This parameter was especially low compared to the extent of rumen degradation of starch-rich sources (around 0.70-0.80, Cerneau and Michalet-Doreau, 1991) or fibrous sources (ranging from 0.40 to 0.70, Demarquilly and Andrieu, 1988). This is difficult to explain, but we have also observed this low response in previous *in vitro* experiments (Amanzougarene et al., 2017a), partly associated to a low pH (Bertipaglia et al., 2010). Calsamiglia et al. (2008) justified similar results by the differences between rumen and *in vitro* microbial ecosystems, partly because the dilution of inoculum in the latter reduces extent of degradation. In contrast to DMd, the concentration of total VFA followed a similar trend than that of gas production, being higher for CI at both sampling times as it has been observed by others (Calsamiglia et al., 2008; Amanzougarene et al., 2018a; Kim et al., 2018). As in our study, Calsamiglia et al. (2008) did not observe any inoculum effect on acetate and butyrate proportions, and propionate proportion was higher with concentrate inoculum as here at 10 h incubation. However, differences in the proportion of BCVFA, which results from fermentation of protein and branched-chain amino acids (Saro et al., 2014) were higher with FI at 6 at 10 h, probably because of the fermentation of protein from the alfalfa hay fed to the donor lambs. The effect of inoculum source was also observed on microbial diversity, reassuring so the recent findings reported by Tapio et al. (2017) and Nagata et al. (2018) who showed the difference in rumen microbial population when bulls were fed with forage or concentrate diets.

4.2. Effect of different substrates on the in vitro fermentation kinetics

Despite the marked differences on the magnitude of fermentation between CI and FI, the fermentation pattern among substrates was almost the same between both inocula. The results of the measured parameters showed a strong correlation between gas production and the other parameters (pH, VFA and lactic acid concentrations) at 6 h (n =

36; adjusted R^2 = 0.90; $p < 0.001$). Similarly, at 10 h incubation the volume of gas produced strongly correlated with pH and VFA (n = 36; adjusted R^2= 0.84; $p < 0.001$) incubation. These results confirm that the gas production and so the concentration of total VFA and lactic acid are the main factors indicating the acidification potential of the incubated substrates (Russell and Hino, 1985; Sauvant et al., 2006; Broudiscou et al., 2014). Citrus pulp had a higher acidification capacity than the other substrates, associated with a higher magnitude of fermentation that is manifested in high gas production as well as VFA and lactic acid concentration. Despite the high concentration of lactic acid with CP at 6 h (Table 4), it did not achieve the range considered as a risk of acidosis *in vivo* (Nagaraja and Titgemeyer, 2007) and in fact did not promoted values of incubation pH below 5.5, that is considered as a threshold for the onset of subacute acidosis (Krause and Oetzel, 2006). These results were in agreement with those found by Amanzougarene et al. (2017a) in a batch culture with a minimum buffer concentration, and could be associated to its richness in soluble sugars (Hall et al., 1998; Ariza et al., 2001), estimated as 0.24 g/kg DM (Table 3), that are fermented at a very fast rate. Although CP has also a high proportion of soluble fibre (0.42, Table 3), this response cannot be directly associated to the fast fermentation of pectin (Barrios-Urdaneta et al., 2003; Bampidis and Robinson, 2006) since BP includes a similar NDSF proportion (0.46) and it was fermented at a slower rate and magnitude. In fact, Strobel and Russel (1986) reported that at a pH of 6.00 the extent of pectin fermentation was reduced respect to higher pH. The lower fermentation rate of BP and thus its lower acidification potential can be also related to its high NDF content, which does not ensure its maximum fermentation in the 24 h incubation period (Sauvant et al., 1986). Considering the mentioned characteristics of BP composition, mainly its high NDF and NDSF proportions as well as its low sugar content, its lower concentration of lactic acid produced respect to the other incubated substrates could be expected. Others (Strobel and Russel, 1986; Bampidis and Robinson, 2006) have also stated that the yield of lactic acid production from pectins fermentation is very low.

The extent of fermentation of WB and B was lower than that of CP, but higher than those of the remaining substrates, probably linked to the high proportion of rapidly fermentable starch in these substrates, compared with those of M and S. Nocek and Tamminga (1991) indicated that 0.80 to 0.90 of barley or wheat starch is digested in the rumen, compared to only 0.55 to 0.70 of that of corn and sorghum. In addition, WB have a considerable amount of NDSF and highly fermentable NDF (Table 3). The structure of

the starch endosperm of maize and sorghum together with their different proportions of amylose (Offner et al., 2003), as well as the protein matrix in the endosperm in these cereal species (McAllister et al., 1993) and the presence of phenolic compounds in the brown sorghum (Amanzougarene et al., 2018b) explain why the fermentation of starch of barley and wheat bran by ruminal bacteria was higher (Overton et al., 1995; Firkins et al., 2001; Offner et al., 2003). Consequently, the differences in starch characteristics and fermentation rate promote the response in medium pH (Amanzougarene et al., 2018a). Incubating several grains in a well buffered medium, Lanzas et al. (2007) observed a higher fractional rate of 48h gas production with barley than maize and sorghum varieties (on average, 0.24, 0.15 and 0.06/h). Opatpatanakit et al. (1994), modified incubation pH similarly to the present work, and also observed a highest gas production with barley, intermediate with maize and lowest with sorghum (on average, 222, 138 and 104 mL/g DM, respectively), under pH values at 7 h incubation ranging from 5.7 to 6.1 for barley, 6.5 to 6.9 for maize and 6.5 to 6.8 for sorghum.

From our findings, it can be indicated that citrus pulp and, to a lower extent wheat bran, had an acidic capacity of even higher magnitude than cereal sources, including barley. Despite of the differences on the magnitude and extent of fermentation between the different incubated substrates, results of microbial diversity with both inocula showed a major effect of the donor animal on this parameter, partly because of aspects related to *in vitro* methodology, such as the short period of incubation. These results were in accordance with those reported by Taxis et al. (2015) and Söllinger et al. (2018) explaining the differences in microbial diversity from one animal to another. However, within each series (donor animal), our results did not demonstrate differences between substrates.

5. Conclusions

Under fermentation conditions of high-concentrate feeding, some sources of highly fermentable fibre, such as citrus pulp and at a lower extent wheat bran may create a more acidic environment than cereals. Among these, barley promotes a lower pH than maize or sorghum, associated to a higher rate and extent of fermentation. Rumen environment promoted by high forage/fibre diets are not adapted for non-fibrous carbohydrates, and fermentation of soluble fibre is not differentially enhanced, producing a lower extent of

substrate fermentation than concentrate diets. Therefore, choosing of ingredients is important when ruminants are changed from a forage to a high-concentrate diet, although this cannot be inferred from this study. In any case, in this experiment acidification levels did not reach those that may change fermentation pattern., Care must be taken on substrate comparison in terms of gas production, since buffering of medium under low pH conditions may overestimate fermentation differences by increasing indirect gas production. The *in vitro* semicontinuous system adapted to a variable medium pH has proven to be useful for the study of rumen microbial fermentation under intensive feeding conditions.

Chapter V. Section I. Experiment 2

Rumen microbial fermentation pattern of cereal grains and sugar beet pulp mixtures in an *in vitro* semicontinuous system with inocula from concentrate or forage-based diets. (Under review; Animal Feed Science and Technology).

Chapter V. Rumen microbial fermentation pattern of cereal grains and sugar beet pulp mixtures in an in vitro semicontinuous system with inocula from concentrate or forage-based diets.

Abstract.

Microbial fermentation of three feed mixtures (1:1 maize:barley, MB, and maize:sugarbeet pulp at either 1:1, MP, or 3:1, 3MP) were studied in an *in vitro* semicontinuous culture system, using inoculum from lambs receiving either a concentrate (CI) or a forage (FI) diet. Medium pH was poorly buffered from 0 to 6 h and allowed to rise to around 6.5 from 8 h onwards. Medium pH was lower with CI ($P<0.05$) for 0 to 8 h, whereas the volume of gas recorded with CI was always superior to that with FI ($P<0.05$), as well as 24 h dry matter disappearance (DMd, 0.38 *vs.* 0.34; $P=0.077$) and total volatile fatty acids concentration (VFA). At 6 and 24 h ($P<0.05$). At 24 h, the highest proportion of acetate was recorded by MP, that also recorded the lowest proportions of butyrate and valerate ($P<0.05$). Highest and lowest lactic acid concentration at 6 h were observed in 3MP and MP ($P<0.05$). Bacterial diversity was markedly affected by the inoculum type, as it was also observed on Shannon ($P<0.01$), and Richness ($P= 0.074$) indexes. With CI, incubation pH was higher ($P<0.05$) with MP than MB and 3MP from 6 to 20h. Gas production (GP) was highest for MB at 2 h and from 6 to 16 h, and lowest with 3MP from 2 to 8 h, and with MP from 20 h onwards ($P<0.05$). Higher DMd was recorded by MB and 3MP than MP (0.440 and 0.396 *vs.* 0.305; $P<0.05$). Regarding microbial diversity, MP and MB clustered together when CI from lambs 1 and 2 was used, but MP and 3MP were similar with inoculum from lamb 3. With FI, pH was lower with MB than with 3MP at 6 h ($P<0.05$), and it was the lowest onwards ($P<0.05$). Gas production from 3MP was lowest ($P<0.05$) up to 4 h, and it was also lower than with MB from 6 h onwards ($P<0.05$). Similarly to CI, the highest DMd was recorded by MB (0.423, 0.316 and 0.283 for MB, 3MP and MP, respectively; $P<0.05$). Mixtures ranked as follows on the Richness index: 3MP (107.2), MP (102.3) and then MB (96.7; $P<0.05$, SEM= 2.38). In both concentrate and forage environments, MP maintained a more stable pH pattern while microbial fermentation was not noticeably depressed compared to higher starch proportions mixtures (MB and 3MP).

1. Introduction

Intensive fattening diets fed to young ruminants are characterised by a high rate of cereal grains as the main carbohydrate source. However, an excessive rate and extent of starch fermentation, which depends on the nature of dietary starch source (Van Barneveld., 1999), promotes the risk of ruminal acidosis (Svihus et al., 2005; Zebeli et al., 2008), especially in young ruminants abruptly changed from milk or forage to a high concentrate diet. Despite the amount of starch is higher in corn than barley (FEDNA, 2010), its availability is restricted by a protein matrix (McAllister et al., 1993) and the rate of starch fermentation is higher with the latter (Sauvant and Michalet-Doreau, 1988; Amanzougarene et al., 2018a). On the other hand, Calsamiglia et al. (2012) proposed that acidosis may be caused by the combined effects of pH and changes in the microbial profile related to the type of diet.

The risk of acidosis can be minimized giving adequate amounts of structural carbohydrates, which may avoid a ruminal overload of volatile fatty acids and lactic acid, at the time increasing chewing activity and the flow of salivary buffers. Sauvant et al. (1999) conclude that to avoid the risk of acidosis, ruminant diets should contain no more than 0.25 starch and 0.30 to 0.40 neutral detergent fibre (NDF), on dry matter (DM) basis. However, this practice may dilute the energy concentration of diet and thus negatively affect energy intake of animal. In this regard, several agro-industrial byproducts, such as sugarbeet pulp, which have a considerable proportion of easily fermentable hemicelluloses and pectin are used in ruminants nutrition, rendering a high amount of energy when fermented in the rumen (Nocek and Tamminga, 1991). Marounek et al. (1985) and Münnich et al. (2017) highlighted the beneficial effect of sugarbeet pulp in maintaining rumen pH, especially when high proportions of this byproduct are included in diet. However, the objective of intensively rearing feedlots is to achieve maximum energy intake and efficiency at the time a healthy rumen environment is preserved, and high levels of sugarbeet pulp may reduce energy intake. In any case, there is not much information about the effect of combining different levels of sugarbeet pulp with cereal grains on rumen fermentation environment.

This experiment aims to assess the synergistic and antagonistic effects of different combinations of carbohydrate sources consisting of a mixture of cereal grains (maize and barley) as sources of starch, and maize and two levels of sugarbeet pulp as a source of

highly fermentable fibre, on rumen fermentation parameters. This will be approached *in vitro* using a semicontinuous system fitted to intensive feeding conditions.

2. Material and methods

2.1. Substrates

Three carbohydrate-rich feeds were studied as substrates of incubation, two cereal grains (barley, var. Gustav, and maize, Dekalb 6667YG), and an agro-industrial byproduct (sugarbeet pulp). Substrates were milled through a sieve of 1 mm and used as components of three mixtures, 1:1 maize:barley (MB) and 1:1 and 3:1 maize:sugarbeet pulp (MP and 3MP). Chemical composition of substrates is given in Table 6.

Table 6. Chemical composition (g/kg DM) of feeds used as incubation substrates.

	OM	**CPr**	**EE**	**Starch**	**aNDFom**	**ADF**	**ADL**	**NDSF**	**Sugars**
M	986	75	34	706	91	25	2.0	77	13
B	978	105	24	672	173	56	17.5	4	1.6
P	953	107	5	---	437	272	75	457	9

DM: dry matter; OM: organic matter; CPr: crude protein; EE: ether extract; aNDFom: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; NDSF: neutral detergent soluble fibre.

2.2. Microbial inoculum sources

Rumen fluid used as inoculum was obtained from six donor lambs housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the Universidad de Zaragoza. Animal care and procedures for extraction of rumen inoculum were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Lambs were abruptly weaned at 49 ± 8 days (average weight 13.6 ± 0.78 kg), and then were fed *ad libitum* in groups of three with a commercial concentrate composed essentially by barley, maize and soybean meal, plus barley straw (CI; lambs 1, 2, 3; average final weight 20.63 ± 1.85 kg) for 35 days or with alfalfa hay (FI; lambs 4, 5, 6; average final weight 16.52 ± 0.33 kg) for 45 days. Then, lambs were slaughtered, rumen contents were sampled and individually filtered through a cheesecloth and

dispensed in 18 mL aliquots into 110 x 16 mm polypropylene tubes that were immediately frozen in nitrogen liquid and stored thereafter at -80 ºC (Prates et al., 2010). Before incubation, frozen inocula were thawed in a 39◦C water bath for 1-2 min.

2.3. Incubation procedures

The fermentation kinetics of experimental feeds were determined by the *in vitro* incubation system of Fondevila and Pérez-Espés (2008), modified by Prates et al. (2010). For each inoculum, three *in vitro* incubation series of 24 h were carried out, each one corresponding to a different donor lamb, with two flasks per treatment in each series. The incubation solution was prepared under a CO_2 atmosphere, and flasks were maintained at 39 ºC in a water bath throughout the incubation.

Sealed nylon bags (45 µm pore size) containing 800 mg of substrate mixtures were incubated in each of 123 mL Erlenmeyer flasks. Each flask was filled with 80 mL of incubation solution (Theodorou et al., 1994) including 0.20 of rumen inoculum. Buffer composition was modified to include 0.006 M bicarbonate ion to get a poorly buffered medium (Amanzougarene and Fondevila, 2018) during the first 6 h of incubation. Every 2 h from 0 to 12 h and every 4 h from 12 to 24 h, fixed volumes of incubation media were extracted by suction through the filter port, and the exact volume was replaced with incubation solution (without rumen inoculum) to adjust liquid phase turnover to approximately 0.08/h. From 8 h onwards, concentration of bicarbonate ion in the buffer of incubation solution was corrected to 0.058 M for allowing pH to increase to around 6.7. Replacement incubation solution was maintained anaerobically at 39 ºC until used.
The pressure of gas produced in each flask was recorded before liquid replacement at 2, 4, 6, 8, 10, 12, 16, 20, and 24 h, with a HD8804 manometer provided with a TP804 pressure gauge (DELTA OHM, Caselle di Selvazzano, Italy). Pressure readings were converted to volume (mL) using a pre-established linear regression between pressures recorded in this type of flasks and known inoculated air volumes at the same incubation temperature (n=48, R^2 =0.993). Gas volume was expressed per unit of incubated organic matter (OM).

Immediately after gas recording, one part of the extracted media was used for pH measurement. At 6 and 24 h, the extracted incubation media was sampled for determination of total volatile fatty acids concentration (VFA; 2 mL over a 0.5 mL solution of 0.5M phosphoric acid with 1 mg 4-methyl-valeric acid as internal standard).

Samples were also taken at 6 h for lactic acid (2 mL) concentration, and at 8 h for microbial biodiversity (6 mL). Samples were stored frozen (-80ºC) until their analysis. At the end of the 24 h incubation, substrate bags were removed, rinsed and dried at 60ºC for 48 h for determination of dry matter disappearance (DMd).

2.4. Chemical and microbiological analyses

DM and OM content in substrates and incubation residues were analysed following the AOAC (2005) procedures (methods ref. 934.01 and 942.05). Substrates were also analysed following the procedures of AOAC (2005) for CPr (ref. 976.05) and EE (ref. 2003.05) analysis. Concentration of aNDFom was analysed as described by Mertens (2002) in an Ankom 200 Fibre Analyser (Ankom Technology, New York), using α–amylase and sodium sulphite, results being expressed exclusive of residual ashes. The acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined by the 973.18 procedure of AOAC (2005) and as described by Robertson and Van Soest (1981), respectively. Neutral detergent soluble fibre (NDSF) was estimated following Hall et al. (1997), discounting the aNDFom and the ethanol insoluble EE, CPr and starch fractions from the insoluble OM. The solubilized OM fraction was considered as representative of soluble sugars content once corrected for soluble CPr and starch. Total starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland).

After thawing, frozen samples of incubation medium were centrifuged at 13,000 g for 15 minutes at 4 ºC for their analysis of VFA and lactic acid. The VFA were determined by gas chromatography on an Agilent 6890, apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm id), and the lactic acid concentration was determined by the colorimetric method proposed by Barker and Summerson (1941).

For the microbial diversity analysis, frozen microbial samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The DNA was extracted using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer recommendations, except that samples were initially heated at 95ºC for 5 min to maximise the lysis of bacterial cells. Concentration of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). PCR was performed using a 16S rRNA bacteria specific primer (cyanine-labelled forward 27F, 5´-AGA GTT TGA TCC TGG CTCAG-3´ and unlabelled reverse 1389R, 5´-AGG GGG GGT GTG TAG AAG-3´;

Hongoh et al., 2003) using a DNAEngine® Gradient Cycler (Bio-Rad, Spain). The PCR product was purified using a Purelink PCR purification kit (ref. K3100-01; Invitrogen) and diluted to 10 µL. The DNA concentration of each amplified and purified sample was obtained by spectrophotometry (Nanodrop® ND-1000 spectrophotometer) to enable a standardised quantity of 50 ng DNA to be used per restriction enzyme digest reaction. Digestion of samples was carried out using HhaI, HaeIII and MspI (Promega, Spain) following the manufacturers recommendations exception for HhaI, where the recommended addition of bovine serum albumin was omitted. Restriction digests were purified by ethanol precipitation (de la Fuente et al., 2014) in 35 µL sample loading solution buffer including a 600 bp size standard (Beckman Coulter Inc., Fullerton) before being applied to a 3500xL Genetic Analyzer (Applied Biosystems). Once getting the size and height of every peak, 1% of the second highest peak was used as criteria for the lower threshold for peaks, to detect and eliminate smaller, broader peaks that would not be indicative of single true OTUs.

2.5. Calculations and statistical analyses

The TRFLP results were analysed from a matrix generated for each data list obtained, and results were presented in the form of relative abundance. The three matrices resulting from each series and enzyme were concatenated and analysed with R statistical software (https://cran.r-project.org/bin/windows/base/, version 3.5.0). FactoMineR, Factoextra, MixOmics, Vegan, MASS, and Ggplot2 packages were used to carry out the analysis of hierarchical classification on principal components for obtaining the cluster dendrogram. Diversity was estimated by the calculation of Shannon and Richness diversity indexes for terminal restriction fragment (TRFs; Dunbar et al., 2000) as well as total number of TRFs obtained from amalgamation of three restriction enzymes.

Results were subjected to ANOVA using the Statistix 10 package (Analytical Software, 2010), considering the interaction (inoculum x incubation series) as a block. Each flask was considered as the experimental unit, because of the individual processing of each of them. The effect of the type of inoculum, the type of mixture, and the interaction of both factors on the pH, gas production, total VFA concentration, VFA profile and lactic acid were studied as factors for each time of sampling. Treatment differences among means with $P<0.05$ and $0.05<P<0.10$ were accepted as representing

statistically significant differences and trends to differences, respectively. When significant, differences were contrasted by the Tukey *t*-test.

3. Results

At the start of incubation series, the mean inoculum pH for CI and FI was 6.44 ± 0.12 and 7.01 ± 0.20, respectively (n=3). From this value, pH dropped in about 0.5 and 0.7 units to reach its minimum during the first 6 and 8 h of incubation for CI and FI, respectively, and then increased to reach its maximum at 20 h. During the first 8 h of incubation pH with FI was higher than that of CI ($P<0.05$), but from 10 h onwards inoculum differences gradually disappeared ($P>0.05$). Since an interaction inoculum x mixture was detected at 6, 8, 10, 12, 20 and 24 h ($P<0.05$) as well as at 16 h incubation ($P=0.057$), to facilitate the interpretation of results in Figure 12 the comparison of the pH pattern among the incubated mixture is presented separately for each inoculum. With CI (Fig. 12a), the pH at 6 h was lower with MB and 3MP than MP (5.97 and 5.94 *vs.* 6.06; $P<0.05$). As planned, medium pH increased thereafter ($P<0.01$) until 6.78 for MP and 6.67 for both MB and 3MP at 20 h. A similar pattern was observed when mixtures were incubated with FI (Fig. 12b), being lower at 6 h with MB than 3MP (6.33 *vs.* 6.39; $P<0.05$), and from 8 h onwards higher with MP and 3MP than MB ($P<0.05$), not existing differences between the treatments including sugarbeet pulp.

Fig. 12a)

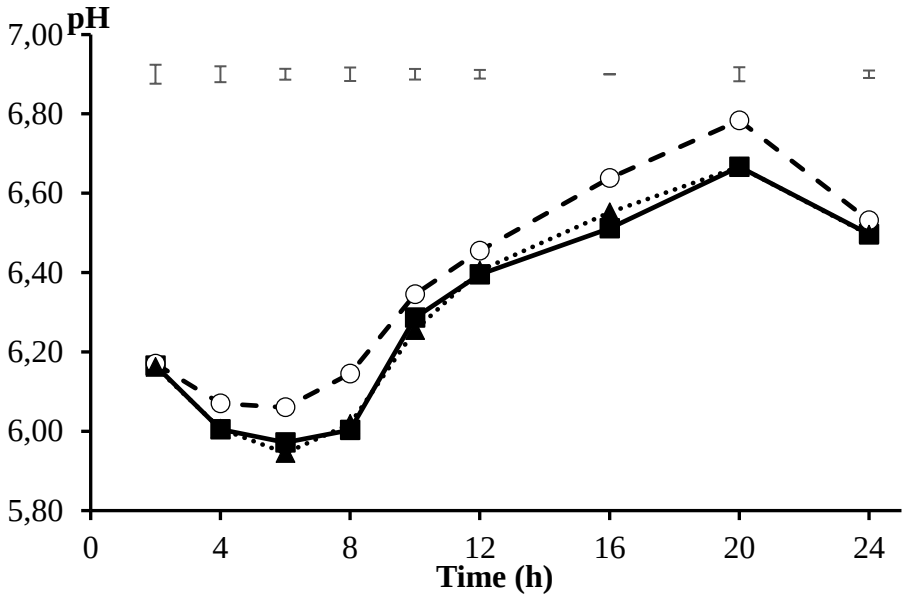

Fig. 12b)

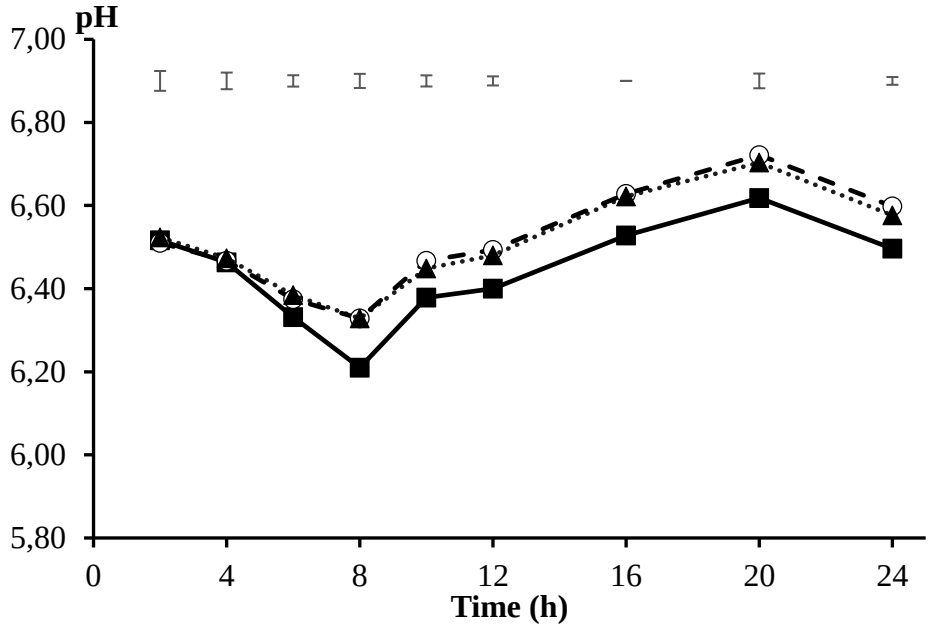

Figure 12. Pattern of incubation pH from MB (■), MP (○) and 3MP (▲) when incubated in vitro with inoculum from a concentrate diet (CI, Fig. a) or from a forage diet (FI, Fig. b). Initial pH were 6.44 ± 0.12 and 7.01 ± 0.20 for CI and FI. Upper bars show standard error of means (n=3).

The comparison of gas production between inocula along the incubation period shows higher volumes with CI than FI (P<0.05), with differences increasing from 8 to 66 ml/g OM from 2 to 24 h, and resulting 0.56 higher with CI at the end of incubation. At 2, 4, 16, 20, and 24 h incubation a significant interaction (inoculum x mixture) was recorded (P<0.05), and this interaction tended to be significant at 6 h (P=0.076) indicating that mixtures behaved differently between inocula. Therefore, the evolution of gas production of the different mixtures is also reported separately for each inoculum (Figure 13). With CI (Fig. 13a), the volume of gas recorded was highest with MB at 2 h and from 6 to 16 h (P<0.05), whereas it was lowest with 3MP from 2 to 8 h and with MP from 20 and 24 h (P<0.05). No differences (P>0.05) were recorded between MP and 3MP from 10 to 16 h, nor between MB and 3MP at 20 and 24 h. With FI (Fig. 13b), the volume of gas recorded by 3MP was lowest (P<0.05) up to 4 h and from 6 h onwards it was lower with 3MP than with MB (P<0.05), whereas differences between MB and MP were only recorded after 24 h.

Fig. 13a)

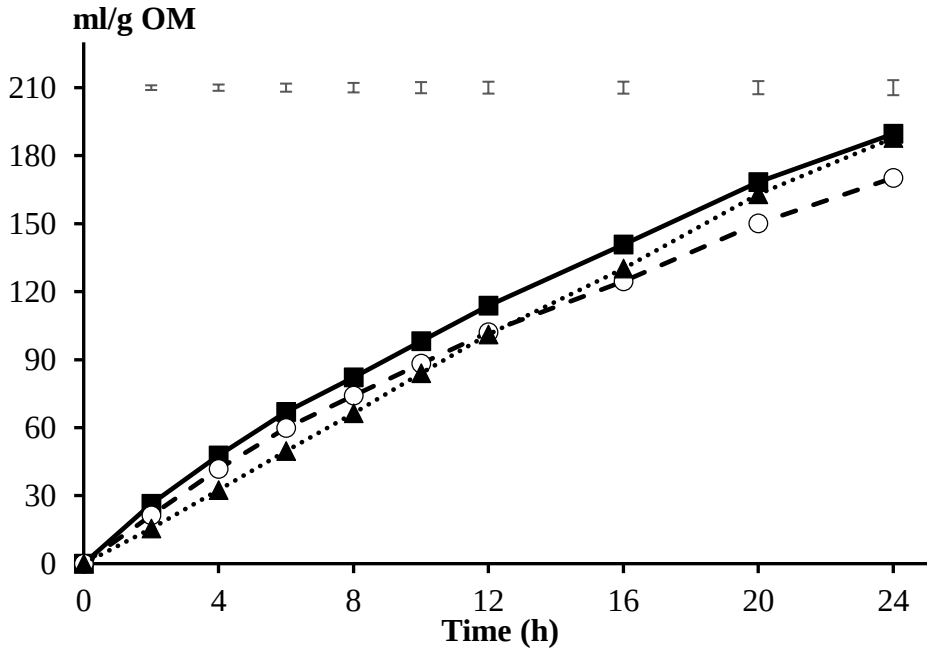

Fig. 13b)

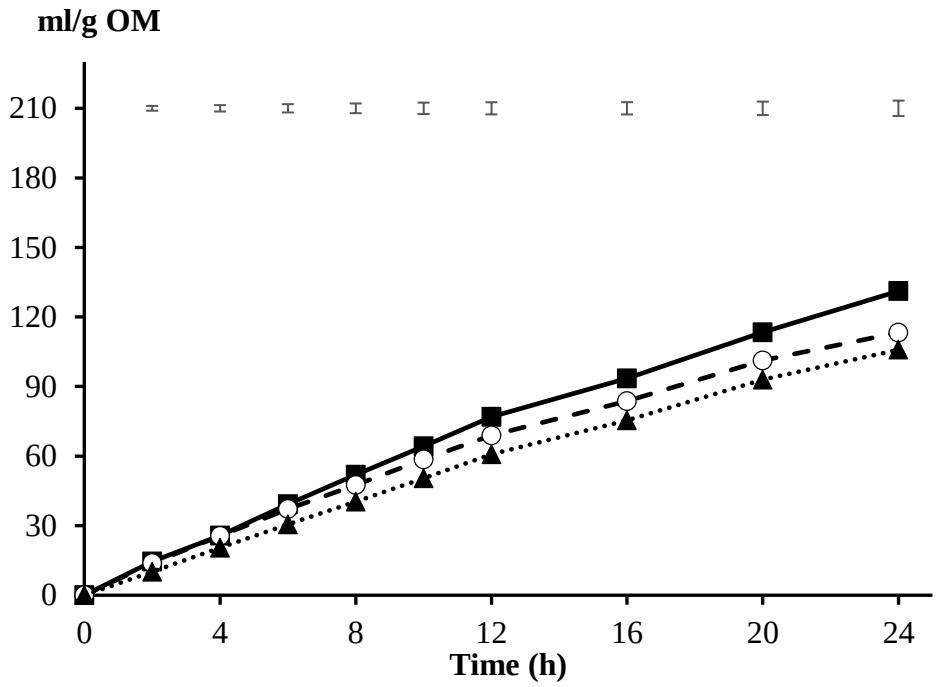

Figure 13. Pattern of gas production from MB (■), MP (○) and 3MP (▲) when incubated in vitro with inoculum from a concentrate diet (CI, Fig. 4a) or from a forage diet (FI, Fig. 4b). Upper bars show standard error of means (n=3).

After 24 h of incubation, the proportion of DMd with CI tended to be higher than with FI (0.38 vs. 0.34; *P*= 0.077). The recorded interaction between inocula and mixtures (*P*<0.05) indicates that MB and 3MP promoted a higher DMd than MP with CI (0.440 and 0.396 *vs.* 0.305; *P*<0.05), whereas MB recorded the highest DMd and no differences were recorded between 3MP and MP when FI was used (0.423, 0.316 and 0.283, respectively; *P*<0.05).

At 6 and 24 h, higher total VFA concentration was recorded by CI (*P*<0.05, Tables 7 and 8). At 6 h of incubation, the molar proportion of valerate was higher (*P*<0.05) and that of branched chain fatty acids (sum of isobutyrate and isovalerate, BCVFA) lower for CI than FI; however, at 24 h CI promoted higher proportions for both. The molar proportions of acetate, propionate and butyrate did not differ among inocula at any sampling time (*P*>0.05). On average, neither the VFA concentration nor molar VFA proportions were affected by the type of mixture at 6 h (*P*>0.05), but at 24 h MP promoted the highest proportion of acetate at the expense of butyrate and valerate (*P*<0.05). The interaction inoculum x mixture observed on butyrate proportion at 6 (*P*<0.05) and 24 (*P*=0.093) h indicates that, despite the effects of inoculum and mixture did not reach significance, differences between 3MP and MP were only manifested with FI. An interaction was also observed at 24 h in acetate and propionate proportions (*P*=0.051 and *P*<0.05, respectively), which highlights the different fermentation pattern of MP and 3MP in FI and CI, respectively.

The concentration of lactic acid at 6 h was not affected by the type of inoculum (*P*>0.05, Table 7). Among mixtures, 3MP promoted a higher concentration of lactic acid than MP (*P*<0.05, Table 7), but the interaction inoculum x mixture at this time (*P*<0.05) indicates that, although no differences were resulted among the three mixtures with FI (MB; 4.22 mM, 3MP; 3.00 mM and MP; 2.77mM), when CI was used 3MP recorded the highest lactic acid concentration (7.08 mM) and MP recorded the lowest concentration (2.3 mM, P<0.05), MB recording an intermediate value (3.69 mM).

Table 7. Average total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol), together with lactic acid concentration (mM) recorded at 6 h of incubation of a 1:1 mixture of maize and barley (MB) and 1:1 (MP) and 3:1 (3MP) mixtures of maize and sugarbeet pulp, with inocula from concentrate (CI) or forage (FI) diets.

	VFA	**C2**	**C3**	**C4**	**Valerate**	**BCVFA***	**Lactic acid**
Inoculum							
CI	20.23[a]	0.584	0.239	0.140	0.022[a]	0.016[b]	4.36
FI	9.27[b]	0.662	0.199	0.098	0.009[b]	0.032[a]	3.33
SEM	1.690	0.0248	0.0242	0.0201	0.0015	0.0020	0.749
Mixtures							
MB	13.74	0.614	0.224	0.123	0.016	0.024	3.96[ab]
MP	14.53	0.633	0.209	0.119	0.015	0.023	2.55[b]
3MP	15.98	0.622	0.224	0.115	0.015	0.025	5.04[a]
SEM	1.3568	0.0107	0.0060	0.0041	0.0005	0.0012	0.560
P-value							
Inoculum	<0.05	0.090	NS	NS	<0,01	<0,01	NS
Mixture	NS	NS	NS	NS	NS	NS	<0.05
Inoc. x Mixt.	NS	NS	NS	<0.05	NS	NS	<0.05

[a,b] Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means. C2; Acetate, C3; propionate, C4; Butyrate. * BCVFA: branched-chain fatty acids (sum of isobutyrate + isovalerate).

Table 8. Average total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol), together with lactic acid concentration (mM) recorded at 24 h of incubation of a 1:1 mixture of maize and barley (MB) and 1:1 (MP) and 3:1 (3MP) mixtures of maize and sugarbeet pulp, with inocula from concentrate (CI) or forage (FI) diets.

	VFA	**C2**	**C3**	**C4**	**Valerate**	**BCVFA***
Inoculum						
CI	17.85[a]	0.507	0.264	0.152	0.052[a]	0.026[a]
FI	8.51[b]	0.445	0.259	0.269	0.018[b]	0.009[b]
SEM	1.532	0.043	0.0351	0.0561	0.0049	0.0037
Mixtures						
MB	13.16	0.458[b]	0.268	0.217[a]	0.039[a]	0.018
MP	13.06	0.527[a]	0.261	0.172[b]	0.026[b]	0.014
3MP	13.33	0.442[b]	0.256	0.242[a]	0.040[a]	0.020
SEM	0.960	0.0120	0.0061	0.0080	0.0033	0.0020
P-value						
Inoculum	<0.05	NS	NS	NS	<0,05	<0,05
Mixture	NS	<0.05	NS	<0.001	<0.05	NS
Inoc. x Mixt.	NS	0.051	<0.05	0.093	NS	NS

[a,b] Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means. C2; Acetate, C3; propionate, C4; Butyrate. * BCVFA: branched-chain fatty acids (sum of isobutyrate + isovalerate)

The results from tRFLP after 8 h of incubation (Figure 14) showed that the bacterial diversity was markedly affected by the type of inoculum. Further, within inoculum, the results indicated that the donor animal influenced the bacterial diversity. This effect was more noticeable for CI, and thus bacterial population in MP and MB clustered together in lambs 1 and 2, whereas in lamb 3 the two mixtures including sugarbeet pulp (MP and 3MP) grouped together. When substrates were incubated with FI, lambs 4 and 5 clustered together with minor differences between incubated mixtures, but in lamb 6 MP and 3MP

clustered apart. In both cases (lamb 3 for CI and lamb 6 for FI) differences between MB and the sugarbeet pulp mixtures were of minor magnitude than those observed in lambs 1 and 2 (CI) with 3MP. Inoculum effects were detected on the Shannon index (P=0.004) and tended to be significant on Richness index (P= 0.074), recording with CI higher values for both indexes (3.91 *vs.* 3.35; SEM= 0.064, and 109.3 vs. 94.8; SEM= 4.28, respectively). No differences among substrate mixtures were found on Shannon index (P= 0.75), but Richness index (P= 0.041; SEM= 2.38) ranked mixtures as follows: 3MP (107.2), MP (102.3) and MB (96.7).

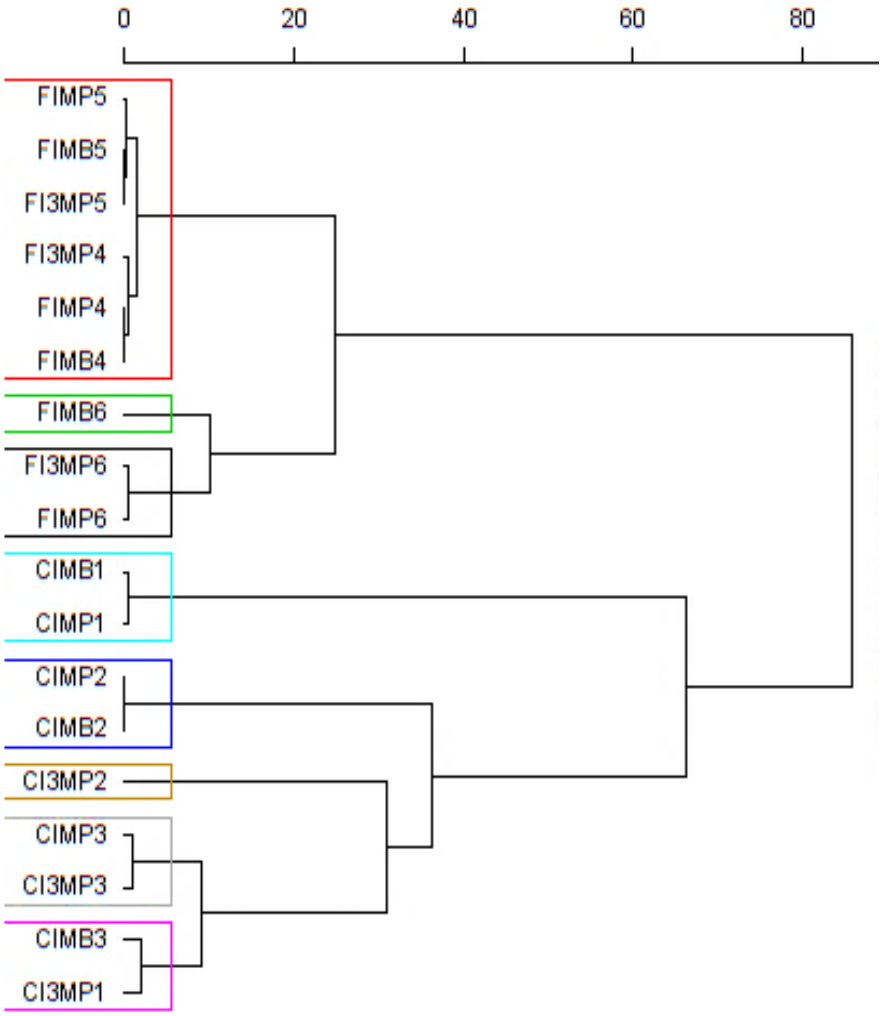

Figure 14. Dendrogram of bacteria diversity from terminal restriction fragment length polymorphism (tRFLP) data generated by enzyme digestion (HhaI, MspI, and HaeIII) at 8 h in vitro incubation of a 1:1 mixture of maize and barley (MB) and 1:1 (MP) and 3:1 (3MP) mixtures of maize and sugarbeet pulp, with inocula from concentrate (CI) or forage (FI) diets. Scale bar shows Euclidean distances, "ward method".

4. Discussion

In young, non-adapted ruminants subjected to an intensive fattening system the use of a high proportion of rapidly fermentable carbohydrates often causes digestive disorders. However, the introduction of fibrous sources such as sugarbeet pulp in these diets helps to maintain the rumen functions within physiological values (Faleiro et al., 2011). Nevertheless, some studies reported that diet is not the only factor that affect rumen microbial fermentation, but also the actual pH pattern at which the process occurs (Calsamiglia et al., 2008; Sari et al., 2015). Thus, in the current *in vitro* experiment we aimed to study the interaction of both factors on microbial fermentation to determine the adequate ingredient mixture that allows to provide the required level of energy to the animals at the same time the negative effects on their health are avoided.

4.1. Effect of the inoculum source on the in vitro fermentation kinetics

Buffer concentration in the incubation medium was adjusted to mimic the actual pH pattern occurring in ruminant animals in practical feeding situations. Such conditions were manifested in a minimum pH reached at 6 and 8 h for CI and FI, respectively, and a maximum pH reached at 20 h incubation, with differences between extremes ranging between 0.8 and 0.5 pH units. The comparison between both inocula shows that the medium pH with CI reached lower minimum values than those achieved with FI (5.99 at 6 h *vs.* 6.36 at 8 h, respectively), indicating that the extent of fermentation was higher with CI, as expected (Broudiscou et al., 2014; Amanzougarene et al., 2018a; Kim et al., 2018). These results were supported by those from gas production and total VFA concentration, which showed a greater magnitude of gas production with CI, that is explained by the fact that the microbiota promoted by such diet should be better adapted than that from a forage diet for the fermentation of this kind of mixtures (Amanzougarene et al., 2018a). Thus, at 6 h incubation, despite the higher fermentation extent with CI, the microbiota adaptation was reflected in a medium pH maintained within the rumen physiological limits (Hiltner and Dehority, 1983) and by a lactic acid concentration (Table 7) also within the rumen normal range (0 to 5 mmol/L; Nagaraja and Titgemeyer, 2007). In the case of FI, a negative effect of a low pH on fermentative microbiota during the first 6 h cannot be inferred, even for fibrolytic species, since values were over the threshold for fermentative activity (Hiltner and Dehority, 1983). Our results from microbial diversity were in agreement with those found by Nagata et al. (2018) who showed

differences in rumen microbial population when bulls fed with forage or concentrate diets. Further, Richness and Shannon indexes showed higher values with CI than FI, indicating that microbiota was more diverse with the former. However, differences in bacterial diversity were not manifested in changes in the fermentation pattern, since proportions of acetate, propionate, and butyrate were not affected by inocula. Similarly, Calsamiglia et al. (2008) did not record any inoculum effect on acetate or butyrate proportions.

In contrast to inoculum differences in gas production and VFA, DMd with CI only tended to be higher than FI (0.38 *vs.* 0.34; $P = 0.077$). This may be partly due to the low DMd values observed, considering that barley and/or maize are starch rich ingredients which expected rumen degradation in a range from 0.70 to 0.80 (Cerneau and Michalet-Doreau, 1991). *In vitro* degradation values in a similar range have also been observed by Devant et al. (2001) and Calsamiglia et al. (2008), and these attributed such low values to the differences in microbial ecosystems between *in vivo* and *in vitro*, where a high dilution rate reduces the magnitude of fermentation respect to the rumen.

4.2. Effect of different mixtures on the in vitro fermentation kinetics

It is assumed that the inclusion of high levels of cereal based concentrates decreases rumen pH (Fondevila et al., 1994; Carro et al., 2000). Treatment MB, including a mixture of cereal grains (maize and barley) showed lower incubation pH than those including maize and sugarbeet pulp (MP and 3MP), mainly with CI, because of the higher acidification capacity of barley than maize (Khorasani et al., 2001), which depends on the structure of the starch endosperm and the proportion of amylose (O´Brien, 1999). Besides, the high starch proportion in 3MP rendered a more acidic environment compared with MP when incubated with CI, despite the buffering capacity of maize and sugarbeet pulp does not greatly differ (Amanzougarene et al., 2017a) This was not the case with FI, probably because of the above mentioned adaptation of microbiota induced by CI to starch fermentation. The major positive effect of sugarbeet pulp on fermentation can be driven through maintaining rumen pH (Münnich et al., 2017). Thus, previous studies (Marounek et al., 1985; Hall and Herejk, 2001) indicated that, despite the rapid degradation of the soluble fibre of sugarbeet pulp, fermentation of this byproduct renders a low lactic acid concentration and a high acetate proportion at the expense of propionate, thus preventing the pH drop. Moreover, Maktabi et al. (2016) stated that when grain

sources such as barley and maize are substituted by sugarbeet pulp, the ruminal pH increased.

Among cereal grains, the starch in barley grain is fermented faster than the starch in maize (Firkins et al., 2001; Offner et al., 2003; Amanzougarene et al., 2018a), and Khorasani et al. (2001) concluded that when the animals fed a 50:50 mixture of barley and maize their performance is improved respect to that of animals fed either barley or maize. In this work, MB recorded a higher gas production than MP and 3MP with both inocula. Hence, differences between MB and 3MP with CI are determined by the faster rate of fermentation of barley respect to maize, effect that is balanced at later stages of fermentation (from 20 h onwards). In addition, the lower medium pH with CI up to 8 h incubation may negatively affect fibre fermentation of sugarbeet pulp from MP and 3MP (Sari et al., 2015). However, the gas production in 3MP when FI was used can be attributed to the low capacity of microbiota induced by a forage diet for fermenting the vitreous starch of maize (Amanzougarene et al., 2017a). In any case, differences in gas production among MP, MB and 3MP were of minor magnitude, and even disappear at later stages of incubation mainly with FI, probably due to scarce differences between utilisation of slowly fermentable maize starch and rapidly fermentable fibre of sugarbeet pulp.

Among mixtures, the gas production results were supported by those observed on DMd. However, no differences among mixtures were detected on total VFA concentration, and an effect was only observed on proportions of acetate, butyrate and valerate at 24 h incubation. At the initial stages of fermentation (first 6 h), the lack of differences may be due to the low fermentation rate of both maize and sugarbeet pulp, that did not allow for manifesting clear effects. After 24 h incubation, the higher proportion of sugarbeet pulp in the MP mixture promoted an increased proportion of acetate, since soluble and insoluble fibre fermentation yields more acetate at the expense of propionate and butyrate (Marounek et al., 1985; Khan et al., 2016; Nagata et al., 2018). However, the starch fermentation has a propensity for propionate and butyrate (Marounek et al., 1985; Maktabi et al., 2016; Kim et al., 2018), as it is shown by the higher butyrate proportion in MB and 3MP compared to MP. In a similar way, lactic acid concentration was lower with MP than MB and 3MP, which can also be attributed to the fermentation of soluble fibre from sugarbeet pulp (Strobel and Russel, 1986; Marounek et al., 1985; Bampidis and Robinson, 2006), as commented above. In any case, in the present work, except for 3MP that recorded 7.08 mM with CI, observed lactic acid concentrations were

within the normal range observed in the rumen (0 to 5 mmol/L; Nagaraja and Titgemeyer, 2007). Differences in VFA molar pattern among incubated mixtures, although being of minor magnitude, are not supported by changes in bacterial biodiversity (Figure 14), that showed a close relationship between substrates within inocula. In addition, the volume of gas produced at 6 h incubation was significantly correlated with the decrease in pH (adjusted R^2 = 0.75; P< 0.01), as expected, highlighting the major relationship between pH and gas production (Amanzougarene and Fondevila, 2018). Similarly, a strong correlation was also observed at 24 h incubation between the volume of gas produced and total VFA concentration (adjusted R^2 = 0.93; P< 0.001). These relationships confirm the important combination between the incubation environment conditions and diet type on microbial fermentation.

5. Conclusions

The type of diet defines rumen environment characteristics that determine the rate and extent of fermentation. Despite a potential risk of acidosis can occur during transition from a forage to a concentrate rich feeding, the fermentation pattern is not necessarily harmful if animals are adapted to a high-concentrate diet. In both concentrate and forage environments, inclusion of a fibrous source such as sugarbeet pulp at a level of 0.50 (MP) maintained a more stable pH pattern, whereas microbial fermentation was not noticeably affected compared to higher starch proportion mixtures like 3MP (0.25 sugarbeet pulp), or the high starch maize-barley substrate (MB). From the results obtained, it can be concluded that combining maize as a cereal grain with fibrous byproducts such as sugarbeet pulp can promote positive effects on rumen environment without negatively affecting the magnitude of fermentation.

Chapter VI. Section II. Experiment 3

Addition of several tannin extracts to modulate fermentation of barley meal under intensive ruminant feeding conditions simulated *in vitro* by incubating at pH 6.0–6.2 (Animal Production Science. 2019. 59: 1081-1089. Doi: 10.1071/AN17741)

Chapter VI. Addition of several tannin extracts to modulate fermentation of barley meal under intensive ruminant feeding conditions simulated *in vitro* by incubating at pH 6.0–6.2.

Abstract.

The potential use of tannin extracts from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT) included at 10, 20 or 30 mg/g to modulate rumen fermentation of concentrates was studied in three 24-h *in vitro* incubation runs, with barley grain as reference substrate and simulating high concentrate feeding conditions by adjusting pH at 6.2. Incubation pH at 8 and 24 h ranged from 6.14 to 6.18 and from 5.94 to 6.00, respectively. Gas production from barley alone (CTL) was linearly reduced with CHT ($P < 0.05$ up to 6 h; $P < 0.10$ from 8 to 18 h), OHT ($P < 0.05$ up to 12 h; $P < 0.10$ from 18 h), GCT ($P < 0.05$ from 2 to 24 h) and QCT ($P < 0.10$ up to 6 h), but a quadratic trend ($P < 0.10$) was also detected on GCT. The effect of GCT was highest and that of CHT lowest. Similarly, dry matter disappearance after 24 h showed a linear decrease with all tannin sources ($P < 0.05$), being lower with GCT than with QCT and CHT ($P < 0.05$). All tannin sources linearly increased ($P < 0.05$) molar butyrate proportion from CTL, at the expense of propionate proportion in GCT ($P < 0.01$) and CHT ($P < 0.10$). Except for the linear effect of chestnut on barley fermentation, all sources already reached their maximum level of response at their first level of inclusion (10 mg/g), especially with GCT. Qualitatively, tannins did not largely affect pH or other environmental parameters, except for an increase in butyrate proportion.

1. Introduction

Rumen acidosis is a common problem in intensive feeding systems from Southern Europe, especially when weaned beef calves that have been raised with nursing cows at pasture for 5-6 months are switched from a milk-forage regime to a high concentrate feeding based on cereals. The change of feeding regime implies a high intake of rapidly fermentable carbohydrate sources, mostly based on starch, which promotes an enhanced activity of rumen microbiota, and pH drops below levels considered in risk of acute (below pH 5.0) or subacute (pH 5.0-5.6) acidosis (Owens et al., 1998; Krause and Oetzel, 2006). In such a scenario, a viable strategy may be the reduction of the rate of fermentation, that would potentially maintain rumen pH above the mentioned threshold.

This may shorten fermentable energy from the rumen, but it can be balanced by an increased arrival of non-fermented starch to lower sites of the gastrointestinal tract (Owens et al., 1986).

Tannins interact with rumen microbiota, reducing the rate and extent of nutrient fermentation. Tannins are polyphenols with a diverse structure and molecular weight (McSweeney et al., 2001; Schofield et al., 2001) that bind to proteins and polysaccharides limiting their availability for rumen microbes (McAllister et al., 1994). Impact of tannins on nutrient fermentation depend not only on substrate protection from microbial attachment, but also on effects on enzyme inhibition and directly on microbial diversity (Barry and Manley, 1984; McAllister et al., 1994; McSweeney et al., 2001). The extent of their effect depends not only on their concentration, but also on their reactivity, which is associated with their chemical nature (Rodríguez et al., 2014). Therefore, the same concentration of tannins from diverse sources may produce effects of different magnitude (Bueno et al., 2008). Commonly, quebracho tannins have been largely used (Frutos et al., 2000; Hervás et al., 2003b) and are usually considered as a reference, but other commercially available tannin extracts have also been assayed, such as those from grape, chestnut and oak (Wischer et al., 2013; Carreño et al., 2015). Whereas tannins from chestnut and oak are considered as hydrolysable tannins, those from grape and quebracho are condensed tannins; however, despite different responses have been attributed to the chemical nature of tannin sources, as reviewed by Mueller-Harvey (2006) such generic classification based on their chemical structure is not helpful for predicting animal responses.

Tannins have been strategically added to feeds to evaluate their potential protecting effect on certain nutrients (Hervás et al., 2003b; Mezzomo et al., 2011), but mostly used on either fibrous feeds or protein sources as components of forage or mixed diets, rather than on highly fermentable carbohydrates in high concentrate diets, under lower rumen pH. As their direct effect on rumen bacteria and their activity might also affect starch utilisation (Martínez et al., 2006) and depends on environmental pH (Mueller-Harvey, 2006), the objective of this work was to assess the potential effect of different sources of condensed and hydrolysable tannin extracts, included at various levels, on *in vitro* microbial fermentation of a cereal substrate, under conditions simulating those occurring in intensive beef cattle feeding systems. This was achieved by using inoculum from high-concentrate fed calves and also by reducing incubation pH to 6.20. As a reference incubation substrate, barley grain was chosen as a major dietary component in this feeding

system, where its highly fermentable starch content is related with a high acidifying capacity (Opatpatanakit et al., 1994; Lanzas et al., 2007).

2. Material and methods

Experimental treatments consisted of four different commercial sources of tannins (Agrovin SA, Alcázar de San Juan, Spain), from quebracho (2S-profisetinidins from *Schinopsis* spp., 0.65 w/w of tannins; QCT), grape (procyanidins from *Vitis vinifera*, 0.75 w/w tannins; GCT), chestnut (esters of ellagic acid and sugars from *Castanea sativa*, 0.65 w/w of tannins; CHT) and oak (ellagitannins from *Quercus robur* and *Q. petraea*, 0.77 w/w of tannins; OHT). Despite the differences in concentration of tannins, for a clearer comparison among treatments these tannin sources were added at three doses of 10, 20 and 30 mg/ g substrate and contrasted to the non-supplemented substrate, used as Control (CTL).

2.1. Incubation procedures

A closed batch *in vitro* system was used for the experiment, following the procedures of Theodorou et al. (1994) but without adding microminerals and resazurin (Mould et al., 2005a). Besides, concentration of bicarbonate ion in the buffer solution was reduced to 0.029 M to adjust incubation pH to 6.20 (Kohn and Dunlap, 1998). Three consecutive incubation series were arranged in different days, each one with triplicated glass bottles (116-mL total volume) containing 80 mL of incubation solution with 0.10 proportion of rumen inoculum. Barley meal (Hordeum vulgare, var. Gustav, 500 mg approximate air-dry matter per bottle) ground to 1-mm particle size was used as substrate. To facilitate its recovery for subsequent determinations, barley was introduced into 4 × 4 cm nylon bags (45-mm pore size, Sefar Maissa, Barcelona, Spain), proven to ensure a free flow of medium through the bag pores. Three additional bottles with rumen inoculum but without substrate were also incubated on each series as blanks, resulting in a total of 42 bottles on each incubation series. Rumen contents from three Limousine crossbred beef male calves (8 months of age, around 300 kg live weight) provided with a 2-cm i.d. cannula fitted in the dorsal sac of the rumen were used as inoculum, where a different donor animal was sampled on each incubation series.

Calves were housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the Universidad de Zaragoza, and were daily fed ad libitum with a concentrate

(0.59 barley, 0.15 maize, 0.17 soybean meal, 0.06 maize gluten and 0.03 minerals and vitamins) and straw, at an actual 0.91:0.09 proportion in total ration. Management and extraction procedures of rumen inoculum from donor animals were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Rumen contents were sampled and filtered through cheesecloth, then dispensed in 16-mL aliquots and immediately frozen in liquid nitrogen following the Prates et al. (2010) procedures and maintained at 80°C until utilisation. Bottles were filled with the incubation solution under a CO_2 stream, sealed and incubated for 24 h in a water bath at 39°C. During the experiment, pressure from two out of three incubated bottles was recorded at 2, 4, 6, 8, 10, 12, 18 and 24 h by means of a HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). Readings were converted into volume (mL) by a pre-established linear regression equation between the pressure (mbar) recorded in the same bottles under the same conditions and known air volumes (volume = (pressure – 10.348)/24.030; n = 103; R^2 = 0.996), and expressed per unit of incubated organic matter (OM). The average of the two bottles for each treatment on each incubation series was considered as the experimental unit. After 8 h of incubation, the remaining bottle from each treatment was opened, the pH recorded (CRISON micropH 2001, Barcelona, Spain) and samples of the incubation medium were taken for volatile fatty acids (VFA) analysis (2 mL, collected over 0.5 mL of a deproteinising mixture of 0.5M PO_4H_3 with 2 mg/mL 4-methyl valeric acid) and immediately frozen and stored at 20°C until subsequent analysis. At the end of the incubation, pH was recorded and the incubation medium sampled for estimation of microbial mass (10-mL sample) in the two bottles per treatment maintained for 24 h. Then, bags of substrate were removed from each bottle, squeezed and dried (60°C, 48 h) to estimate by difference dry matter and starch disappearance after 24 h of incubation (DMd and STd, respectively).

2.2. Calculations and chemical analyses

The biological effect of tannins (BE; Makkar et al., 1995) was calculated as the ratio of gas production results recorded at 24 h with barley alone and with added tannins, assuming that barley grain is free of tannins. Dry matter in the barley substrate and incubation residues and OM content in the substrate were analysed following the AOAC

(2005) procedures (methods ref. 934.01 and 942.05). Total starch content was determined enzymatically from samples and incubation residues after grinding to 0.5 mm, by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). Frozen samples from the incubation medium were thawed and centrifuged at 20 000g for 15 min at room temperature for their analysis of VFA. The VFA were determined by gas chromatography on an Agilent 6890 apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30-m × 530-mm id). Microbial mass in the liquid medium was approached by centrifuging samples at 10 000g for 20 min at 4°C and weighing the washed lyophilized residue, based on Hsu and Fahey (1990).

2.3. Statistical analyses

Results were analysed by ANOVA using the Statistix 10 software package (Analytical Software, 2010), considering the incubation series as a block and the experimental treatments (CTL and the four tannin sources included at three levels, n = 13) as a factorial effect. Polynomial (lineal and quadratic) contrasts were planned to estimate the trend in the response of each single additive (CTL and the three levels of inclusion, n = 4), and orthogonal contrasts were established to compare the four tannin sources among them. For the comparison of results of biological effect, the effects of the tannin source and level were considered as factors, and the incubation series as a block. In all cases, differences were considered significant when $P < 0.05$, and a trend for significance was considered when $0.05 \leq P < 0.10$.

3. Results

Inoculum pH averaged 6.29 ± 0.20. No effects of experimental treatments were detected on incubation pH at either 8 or 24 h, except for a linear decrease with OHT at 8 h (from 6.18 with CTL to 6.14 with the highest level of OHT; $P = 0.048$). However, this response may be attributed to the low magnitude of the error term, considering the minimal differences between extreme pH values at 8 and 24 h, which ranged from 6.14 to 6.18 and from 5.94 to 6.00, respectively.

3.1. Pattern of response at diverse levels of tannin sources

For an easier understanding, results of gas production for the three addition levels of each additive compared with non-supplemented barley (CTL) are presented separately in Figures 15-18. In the first stages of fermentation, addition of increasing levels of QCT tended to reduce linearly gas production from barley alone ($P < 0.10$ from 2 to 6 h incubation; Figure 15), but this effect was not significant afterwards. In GCT (Figure 16), the reduction of the volume of gas compared with CTL followed a linear pattern throughout all the incubation period ($P < 0.05$), whereas a trend ($P < 0.10$) for a quadratic drop was also observed at 4 and 6 h, and from 10 to 24 h. This quadratic trend in the gas production pattern with GCT (Figure 16) could be explained by a similar magnitude of reduction with all inclusion levels of GCT. Addition of CHT also promoted a linear decline in the volume of gas generated from CTL ($P < 0.05$ from 2 to 6 h, and $P < 0.10$ from 8 to 18 h; Figure 17). Similarly, gas production was linearly reduced when increasing levels of OHT were added ($P < 0.05$ from 2 to 12 h, and $P < 0.10$ from 18 to 24 h; Figure 18).

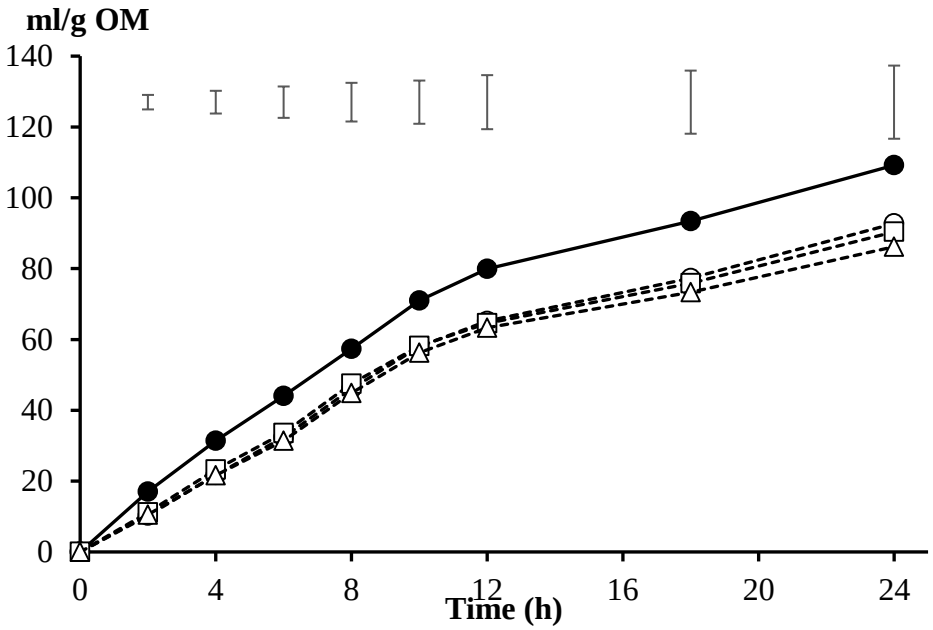

Figure 15. Gas production pattern from barley, as the only substrate (●) or supplemented with 10 (○), 20 (□) or 30 (△) mg/g of the tannin extract from quebracho (QCT). Upper bars show standard error of means.

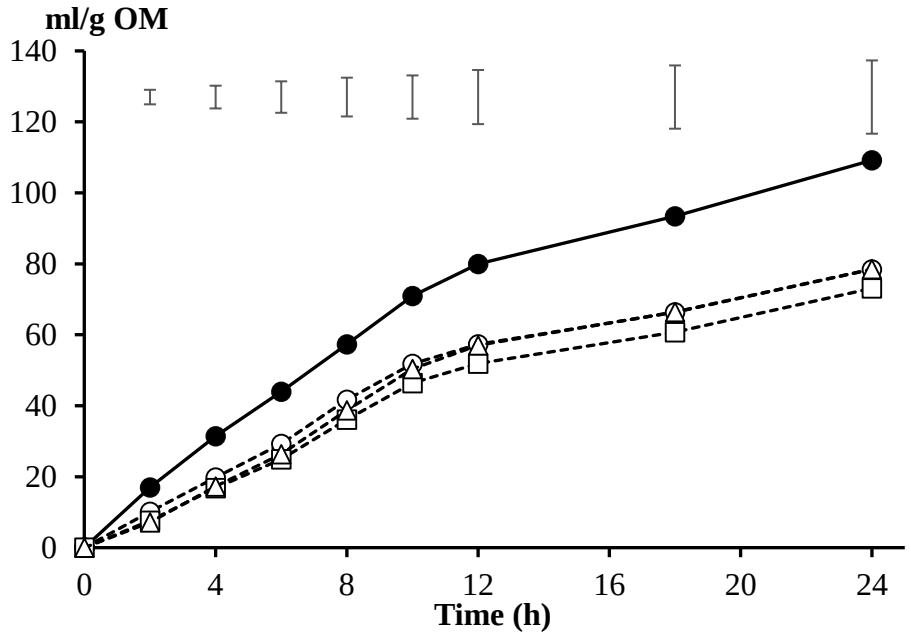

Figure 16. Gas production pattern from barley, as the only substrate (●) or supplemented with 10 (○), 20 (□) or 30 (△) mg/g of the tannin extract from grape (GCT). Upper bars show standard error of means.

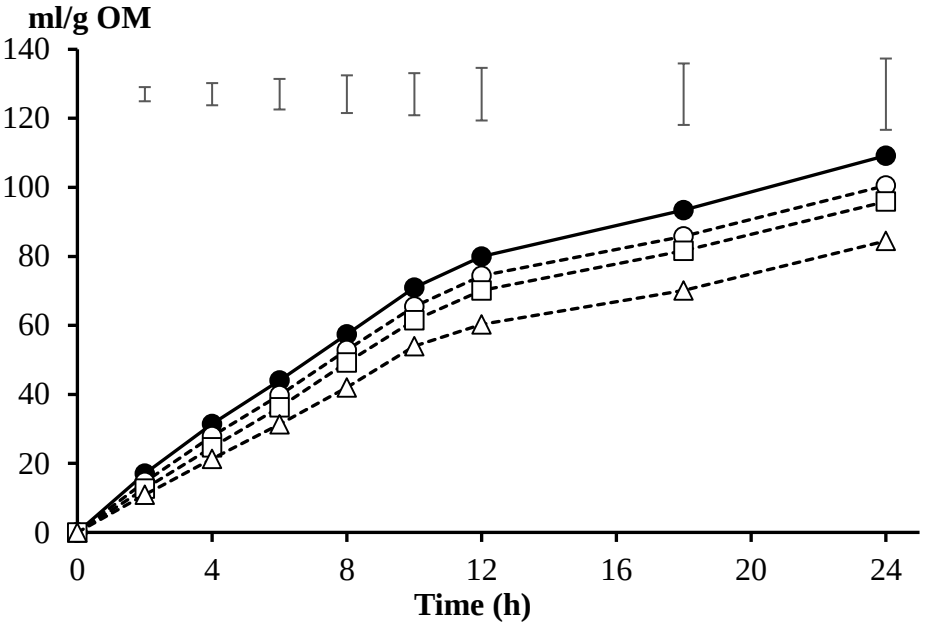

Figure 17. Gas production pattern from barley, as the only substrate (●) or supplemented with 10 (○), 20 (□) or 30 (△) mg/g of the tannin extract from chestnut (CHT). Upper bars show standard error of means.

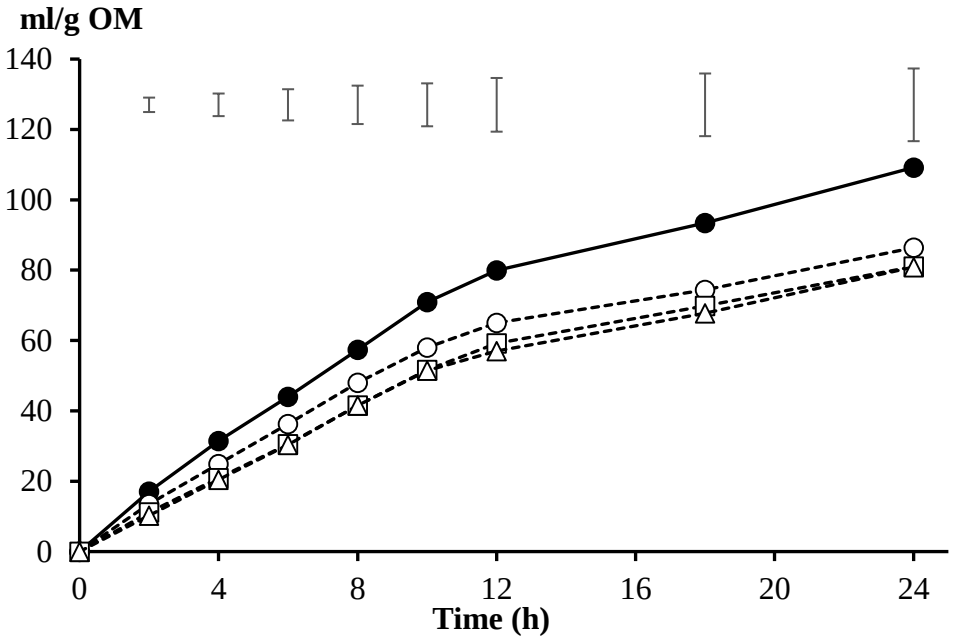

Figure 18. Gas production pattern from barley, as the only substrate (●) or supplemented with 10 (○), 20 (□) or 30 (△) mg/g of the tannin extract from oak (OHT). Upper bars show standard error of means.

The pattern of DMd and STd at 24 h when increasing levels of tannins were added is shown in Table 9. A linear decrease on DMd was observed with all sources of tannins ($P < 0.05$), whereas the quadratic trend with OHT ($P = 0.066$) shows the lack of response from the first level of inclusion. In contrast, no polynomial responses to the inclusion of increasing levels of the different tannins were detected on STd ($P > 0.10$), despite this parameter numerically decreased, on average, in 0.016, 0.028, 0.033 and 0.057 units with CHT, QCT, OHT and GCT, respectively, in a similar way to DMd. A linear drop ($P < 0.05$) in microbial mass in the liquid fraction was observed with all tannin sources, but a subsequent peak in concentration with the higher level of tannins from QCT and OHT promoted a quadratic response ($P < 0.05$).

Table 9. Average values and pattern of response (P-value of linear, L, or quadratic, Q, trend) of dry matter (DMd) and starch (STd) disappearance, and microbial mass (mg/mL) after 24 h of in vitro incubation of barley as the only substrate (Control) or supplemented with 10, 20 or 30 mg/g of the tannin extract from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT).

	CTL	Level	QCT	GCT	CHT	OHT	s.e.m.
DMd, 24 h	0.345	10	0.314	0.289	0.325	0.295	
		20	0.315	0.282	0.306	0.289	
		30	0.274	0.260	0.279	0.290	
		Pattern	L(**)	L(***)	L(**)	L(**),Q(T)	0.0132
STd, 24 h	0.481	10	0.464	0.415	0.486	0.458	
		20	0.467	0.440	0.461	0.444	
		30	0.428	0.416	0.447	0.442	
		Pattern					0.0282
Microb. mass	2.460	10	2.314	2.242	2.217	2.311	
		20	2.303	2.311	2.233	2.265	
		30	2.340	2.285	2.330	2.306	
		Pattern	L(T),Q(*)	L(*),Q(*)	L(T),Q(**)	L(*),Q(*)	0.0434

*, P < 0.05; **, P < 0.01; ***, P < 0.001; T, P < 0.10. s.e.m., standard error of the means

Total VFA concentration after 8 h of incubation linearly decreased with the inclusion of QCT ($P < 0.001$), but the rest of tannin sources did not promote differences compared with CTL (Table 10). In terms of molar VFA proportions, all tannin sources linearly increased ($P < 0.05$) butyrate proportion from barley alone, but QCT reached its maximum with the intermediate level of inclusion (20 mg/g). The increase in butyrate was at the expense of propionate, which proportion was linearly reduced in GCT ($P < 0.01$) and CHT ($P < 0.10$). No effects on the other VFA were detected.

3.2. Comparison among tannin sources

No differences among tannin sources were detected on gas production (Table 11), except for a lower volume with GCT compared with CHT ($P < 0.05$ from 2 to 12 h, and $P < 0.10$ at 18 and 24 h). The BE of tannins estimated at 24 h of incubation was independent of their level of inclusion ($P > 0.10$), but tended to differ among tannin sources, showing higher values with GCT than CHT ($P = 0.069$). When comparing among tannin sources, DMd after 24 h was lower with GCT than with QCT and CHT ($P < 0.05$), whereas no differences were detected on STd, except for a trend ($P = 0.085$) to a lower value in GCT compared with OHT. There were no differences in microbial mass among tannin sources, although with QCT it was numerically higher than with CHT ($P = 0.11$). Among tannin sources, on average CHT recorded lower total VFA concentration (Table 12) than QCT ($P < 0.01$), GCT ($P < 0.10$) and OCT ($P < 0.05$). No major differences were recorded on the molar VFA proportions, except for a higher BCVFA proportion with GCT than with OHT ($P < 0.05$) and CHT ($P < 0.10$).

Table 10. Total volatile fatty acid (VFA) concentration (mM) and molar proportions of the main VFA after 8 h of in vitro incubation of barley as the only substrate (CTL) or supplemented with 10, 20 or 30 mg/g of the tannin extract from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT).

	CTL	Level	QCT	GCT	CHT	OHT	s.e.m.
VFA	30.3	10	34.2	31.6	30.0	31.1	0.96
		20	32.7	31.4	28.9	32.0	
		30	29.8	30.1	30.1	31.0	
		Pattern	L(***)				
Acetate	0.433	10	0.436	0.431	0.433	0.438	0.0047
		20	0.426	0.433	0.432	0.429	
		30	0.435	0.439	0.433	0.432	
		Pattern					
Propionate	0.215	10	0.207	0.213	0.213	0.207	0.0037
		20	0.209	0.208	0.208	0.213	
		30	0.206	0.200	0.208	0.209	
		Pattern		L(**)	L(T)		
Butyrate	0.118	10	0.124	0.122	0.121	0.122	0.0023
		20	0.131	0.125	0.127	0.125	
		30	0.125	0.126	0.126	0.127	
		Pattern	L(*),Q(*)	L(*)	L(**)	L(**)	
Valerate	0.012	10	0.011	0.012	0.012	0.012	0.0003
		20	0.012	0.012	0.012	0.012	
		30	0.012	0.012	0.011	0.011	
		Pattern					
BCVFA	0.022	10	0.021	0.022	0.022	0.022	0.0006
		20	0.023	0.023	0.022	0.021	
		30	0.022	0.023	0.022	0.021	
		Pattern					

*, P < 0.05; **, P < 0.01; ***, P < 0.001; T, P < 0.10. s.e.m., standard error of the means

Table 11. Average means of gas production (mL/g OM) at different incubation times, together with estimated biological effect (BE), dry matter and starch disappearance (DMd and STd) and microbial mass in the liquid fraction (mg/mL) after 24 h of in vitro incubation, from barley supplemented with different levels of tannins from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT). s.e.m., standard error of the means.

	QCT	GCT	CHT	OHT	s.e.m.
Gas production					
4 h	22.2	18.0	24.6	21.9	1.61[A]
8 h	46.1	38.9	48.0	42.2	2.71[A]
12 h	64.4	55.4	68.3	63.1	3.65[A]
24 h	89.8	76.7	93.6	87.2	4.99
BE	1.25	1.43	1.17	1.26	0.069
DMd	0.301	0.277	0.304	0.291	0.0076[A,B]
STd	0.453	0.424	0.465	0.448	0.0163[C]
Microbial mass	2.319	2.294	2.279	2.260	0.0250

[A]GCT versus CHT ($P < 0.05$).
[B]QCT versus GCT ($P < 0.05$).
[C]GCT versus CHT ($P < 0.10$).

Table 12. Average means of total volatile fatty acid (VFA) concentration (mM) and molar proportions of the main VFA after 8 h of in vitro incubation, from barley supplemented with different levels of tannins from quebracho (QCT), grape (GCT), chestnut (CHT) and oak (OHT). s.e.m., standard error of the means.

	QCT	GCT	CHT	OHT	s.e.m.
VFA	32.2	31.0	29.6	31.4	0.55 [A,B,C]
Acetate	0.432	0.434	0.433	0.433	0.0027
Propionate	0.207	0.207	0.209	0.210	0.0021
Butyrate	0.127	0.125	0.125	0.124	0.0004
Valerate	0.012	0.012	0.011	0.012	0.0002
BCVFA	0.022	0.023	0.022	0.021	0.0004 [D,E]

[A]QCT versus CHT ($P < 0.01$).
[B]OHT versus CHT ($P < 0.05$).
[C]GCT versus CHT ($P < 0.10$).
[D]GCT versus OHT ($P < 0.05$).
[E]GCT versus CHT ($P < 0.10$).

4. Discussion

Conventional *in vitro* batch systems maintain pH ~ 6.7-6.9 (Mould et al., 2005a), being far from mimicking rumen environmental conditions induced by intensive feeding. A previous study (Amanzougarene and Fondevila, 2018) succeeded in maintaining *in vitro* pH at 6.25 by reducing the bicarbonate concentration included as buffer in the incubation solution. However, microbial fermentation at lower pH could only be maintained for 10 h, because of the fast exhaustion of buffering capacity thereafter. Thus, in this experiment the bicarbonate concentration in the buffering solution was reduced for adjusting pH to 6.2. The fermentation pattern of barley could thus be monitored at mid-term by gas production, and concomitantly it allowed to study the acidification properties of treatments once bicarbonate has been consumed in the first part of incubation (after 10-12 h). Moreover, using rumen inoculum from beef calves fed on a high concentrate diet contributes to maintain the desired incubation conditions, with both an expected pH value (Amanzougarene et al., 2018a) and a microbiota adapted to ferment concentrate diets. In any case, minor effects of tannin sources on pH were detected in this experiment, either at first stages (up to 8 h) or complete (24 h) incubation. Observed pH values throughout the experiment were within the range considered favorable for starch fermentation, and well above the pH 5.6 assumed as threshold for subacute acidosis (Owens et al., 1998; Krause and Oetzel, 2006); therefore, no limitation in fermentation related with this parameter could be attributed to the experimental treatments.

It has been widely shown that tannins as secondary plant compounds can be selectively toxic to ruminal bacteria (Krause et al., 2005), reducing both growth and activity of bacterial species implicated in carbohydrate fermentation, such as *Butyrivibrio fibrisolvens, Streptococcus bovis* and *Ruminobacter amylophilus* (Jones et al., 1994). Besides, tannins affect microbial attachment to substrate (McAllister et al., 1994; Guimarães-Beelen et al., 2006) and enzymatic activity (Kumar and Vaithiyanathan, 1990; Scalbert, 1991), thus reducing nutrient digestion by rumen microbes. This feature has been mainly documented for proteins (Hagerman et al., 1992; Frutos et al., 2000) and for the fibrous fraction of feeds (Chiquette et al., 1988; Rodríguez et al., 2011, 2014). Although less studied, this aspect might also be suggested for microbial activity over starch, although the effect of tannins over amylase activity is unclear, and depends on their chemical structure and dose (Mueller-Harvey, 2006). Therefore, some tannins might be a suitable alternative to reduce fermentation rate of concentrate feeds, preventing for

rumen disturbances associated with intensive feeding in ruminants, such as acidosis. It is worth mentioning that a possible reduction of the extent of rumen fermentation is not necessarily a drawback, as the non-degraded starch that reaches the intestines to be digested there provides 42% more energy than that digested in the rumen (Owens et al., 1986). Taking into account that tannin binding to substrate is pH-dependent (McSweeney et al., 2001; Mueller-Harvey, 2006), some association may be re-established at the lower gut, although that is out of the scope of this study.

Several attempts have aimed to reduce rumen fermentation of substrates by adding tannins. Martínez et al. (2006) reported a reduction on fermentation of wheat and corn grains in terms of *in vitro* gas production, VFA concentration and DM disappearance, by adding either tannic acid or quebracho tannins, but hypothesized that such effect was exerted mostly on the protein matrix of starch granules. Therefore, a reduced magnitude of their effect on barley grain should be expected. In a similar study, Hervás et al. (2000) observed a reduction on *in situ* rapid degradable fraction of soybean meal with increasing levels of tannic acid. Furthermore, Barros et al. (2012) reported interactions between condensed tannins and starch molecules, these being higher with amylose than amylopectin. The response seems to be less clear under *in vivo* conditions, where a lack of effect on rumen carbohydrate fermentation was observed with concentrate diets by Krueger et al. (2010) and Mezzomo et al. (2011). Anyway, a reduction in gas production was observed in our experiment, especially with GCT and OHT, although with minor qualitative effects on fermentation (pH, VFA), despite a higher response would have been be expected with maize as substrate. It is worth mentioning that recorded disappearance values were lower than expected, considering the type of substrate (barley grain). However, previous *in vitro* incubation results of concentrate mixtures from our group showed a reduction in DMd from 0.74 to 0.39, as well as in gas production volume (a 0.26 lower volume) when medium pH was reduced to 5.80 (Bertipaglia et al., 2010).

The classical classification of tannins into hydrolysable and condensed tannins has been criticised because of their variable magnitude of response, irrespective of their chemical composition (Mueller-Harvey, 2006). Rather, differences in the procyanidin to prodelphinidin ratio, the molecular weight or the degree of esterification with gallic acid of tannin sources may explain the variable responses, and therefore different tannins sources were chosen for this study. It has been shown that hydrolysable tannins can be degraded by rumen bacteria (Bhat et al., 1998; McSweeney et al., 2001), but their impact should be rate-dependent, and affect differently to each tannin source. This should explain

the observed differences between both hydrolysable sources (OHT and CHT) throughout the incubation period. Among the four tannin extracts studied here, the reduction in gas production was higher with GCT, already manifested in the first stages of fermentation, followed by OHT and QCT, with CHT showing the lowest (33, 26, 19 and 16 mL/g OM lower than the CTL after 24 h incubation, respectively). Consequently, the biological effect of tannins at 24 h as an index of reduction of substrate fermentation tended to be (P = 0.069) higher for GCT than for CHT, being intermediate for the other two sources (Table 11). Just for comparative purposes, previous studies in our laboratory (Rodríguez et al., 2014) gave a BE value at 24 h of 1.44 for the tropical tanniferous legume *Leucaena leucocephala* (61 g/kg total tannins, expressed as tannic acid equivalents), within the range of GCT obtained here. All tannin sources linearly increased butyrate molar proportion, in agreement with *in vivo* results from Krueger et al. (2010) after 42 days of adding tannins to a concentrate feed, but in contrasts with the lack of effect at 12 h reported by Rodríguez et al. (2011) with forages. This different effect can be related to variations in pH, as well as the nature of substrates, as molar butyrate proportion estimated at 8 h of incubation increased with the addition of tannins with wheat but not maize as substrate (Martínez et al., 2006).

In any case, it has to be considered that declared chemical analyses from the manufacturers showed that concentration of tannins expressed per weight unit of extract differ to some extent, being OHT and GCT higher than QCT and CHT. Although this might affect the response as extracts were dosed on a weight basis, the response to extracts does not directly correspond to their tannins concentration. Moreover, the constraints in accuracy of chemical analysis of tannins (Álvarez del Pino et al., 2005) and the mismatch between chemical analysis of tannins and their biological effect (Schofield et al., 2001; Rodríguez et al., 2014) precluded a bias in the response in this sense.

It is generally assumed that less than 50 mg condensed tannins/g feed is beneficial for ruminants, but this largely depends on the chemical nature of the tannin source (Mueller-Harvey, 2006). Experiments supplementing concentrate diets with added tannins have applied several levels for the study of rumen fermentation processes, like single doses of 15 (Krueger et al., 2010), 20 (Carreño et al., 2015) or 50 mg/g substrate (Martínez et al., 2006) or concentration ranges from 1 to 25 (Hervás et al., 2000). Krueger et al. (2010) did not observe any rumen effect by adding mimosa (condensed tannins) or chestnut (hydrolysable tannins) extracts *in vivo*, nor Carreño et al. (2015) with oak extract on a 50:50 forage:concentrate substrate *in vitro*. In contrast, Martínez et al. (2006) showed

a reduced *in vitro* gas production from cereal grains with supplementation of both quebracho and tannic acid, and Hervás et al. (2000) reported a reduction on *in situ* DM degradation of soybean meal with 10 mg tannic acid/g substrate. With grass silage, Wischer et al. (2013) reported significant reductions in gas production when adding oak or chestnut tannins from 3 to 10 or 8 to 10 mg/g, respectively, but inconsistent results were observed with quebracho or grape seed tannins dosed up to 13 to 17 mg/g. These results support the importance of considering both source and dose of tannins in this type of studies. In our case, the response in gas production was only dose-dependent for CHT, whereas the first level of inclusion of the other tannin extracts already promoted a maximum reduction in comparison with the non-supplemented substrate. A similar pattern was also observed for all tannin sources in DMd, and the linear decrease ($P < 0.001$) of total VFA concentration, only showed by QCT, was in fact caused by the increase in relation to CTL observed with the lowest level of inclusion (Table 10). Also, a general linear increase in butyrate proportion was detected with the inclusion of tannins, which in the case of GCT and CHT was at the expense of propionate.

Despite the relative low specificity of the method used to determine microbial mass, a reduction compared with the CTL was already observed with the lower level of all studied extracts, without recording differences among them. In any case, according to literature the effect of the presence of tannins on this parameter is unclear. Makkar et al. (1995) and Getachew et al. (2008) suggest that partition of nutrients utilisation in tannin-rich substrates is preferably focused on fermentation (i.e. VFA and gas production) than microbial mass production, and McSweeney et al. (2001) support that secondary compounds inhibit microbial growth and enzyme activity. In contrast, in a meta-analysis, Jayanegara et al. (2012) observed a positive relationship between bacterial counts and tannin level in vitro ($r = 0.513$, $n = 48$), and Salem et al. (2007) and Guerrero et al. (2012), using purine bases as microbial marker, observed a reduction in microbial protein yield when tannins were inactivated by adding PEG.

5. Conclusions

Results show that the four tested tannin sources (from quebracho, grape, chestnut and oak) reduced rumen microbial fermentation of barley grain, with maximum and minimum responses being recorded with grape and chestnut tannins, respectively. Except

for chestnut, which showed a linear negative response in fermentation with inclusion levels from 10 to 30 mg/g, all the other sources reached their maximum level of response at their first level of inclusion (10 mg/g substrate). In fact, the magnitude of the response in gas production to the lowest level of both QCT and OHT was only reached with a 3-fold dose of CHT inclusion. The reduction in substrate fermentation in the rumen should imply a higher proportion of feed that may reach lower sites of the gastrointestinal tract, with a subsequent improvement in the potential feed digestive utilisation. Qualitatively, addition of tannins did not largely affect medium pH or other environmental parameters, except for an increase in butyrate proportion.

Chapter VII. Section II. Experiment 4

Fermentation of barley added with increasing levels of fatty acids or essential oils by the *in vitro* gas production technique under conditions simulating intensive beef production (Under review; Animal Production Science).

Chapter VII. Fermentation of barley added with increasing levels of fatty acids or essential oils by the *in vitro* gas production technique under conditions simulating intensive beef production.

Abstract.

Intensive beef production based on high-energy diets increases the risk of rumen acidosis. *In vivo* studies increase costs and reducing comparisons. To check if additives can modulate rate of microbial fermentation in beef cattle fed concentrates. Level of fatty acids or essential oils on barley fermentation were studied *in vitro*, using rumen inoculum from intensively fed beef calves, adjusting medium pH around 6.2. In Experiment 4.1 treatments were: barley alone (CTL), medium-chain fatty acids (MFA; 2, 4 and 6 mg/g) and palmitic (PAL) and linoleic (LIN) acids, included at 15, 30 and 45 mg/g. In Experiment 4.2, cinnamaldehyde (CIN; 30, 60 and 90 mg/g), eugenol (EUG; 60, 120 and 180 mg/g) and a commercial blend of essential oils (CBC; 30, 60 and 90 mg/g) were compared with CTL. Respect to CTL, gas production with LIN was quadratically reduced ($P<0.05$ up to 24 h), and such reduction tended to be linear ($P<0.10$ at 12 and 24 h) with PAL, and quadratic ($P<0.10$ at 24 h) with MFA. Dry matter disappearance (DMd) and estimated microbial mass in the liquid medium were quadratically reduced ($P<0.05$) with MFA and LIN. Total volatile fatty acids (VFA) concentration and acetate proportion tended ($P<0.10$) to increase linearly with LIN, whereas propionate proportion tended to decrease ($P=0.051$). Gas production from CTL decreased linearly with CIN ($P<0.001$ up to 24 h) and quadratically with EUG ($P=0.047$ at 24 h), whereas it increased linearly with CBC ($P<0.05$ at 12 h, and $P<0.10$ at 8 and 24 h). A similar pattern of response was observed with the three additives on DMd. Microbial mass decreased quadratically with EUG ($P<0.001$). Total VFA concentration from CTL decreased linearly ($P<0.05$) and quadratically ($P<0.001$) with CIN and EUG. Acetate proportion increased quadratically with the inclusion of EUG ($P<0.05$) at the expense of propionate ($P<0.001$), and a linear reduction in propionate proportion was observed with CIN ($P<0.05$). Although depending on their dose and composition, both fatty acids and essential oils may reduce barley acidification potential. The rumen microbial fermentation of barley may also be improved with the essential oil CBC.

1. Introduction

Beef production is often intensified for improving livestock performance potentials and reducing production time. However, feeding high-energy diets that are rich in rapidly fermentable carbohydrates implies the risk of appearance of rumen acidosis (Nagaraja and Titgemeyer, 2007), especially during the abrupt change from forage to concentrate diets. The increasing intake of highly fermentable organic matter leads to a rapid production and absorption of acids in the rumen (Pan et al., 2016), that consequently causes a reduction in the ruminal pH and microbial activity (Mao et al., 2016). A way to minimize such effect in ruminants is using feed additives, such as fatty acids (Doreau et al., 2009; Beauchemin et al., 2009), and essential oils (Castillejos et al., 2006; Benchaar et al., 2007, Calsamiglia et al., 2007).

Supplementation of ruminant diets with lipids is largely practised for increasing the energy density in the ration (Vargas et al., 2017), but also because of their effect attenuating the rumen fermentative activity (Palmquist and Jenkins, 1980) and modifying the volatile fatty acids profile (Costa et al., 2017). Their effect depends upon their nature (saturated or unsaturated fatty acids), with long chain unsaturated fatty acids tending to depress microbial activity (Jenkins, 1993), and their dose (Doreau et al., 2009; Beauchemin et al., 2009). The medium chain fatty acids have a selective antimicrobial effect (Henderson, 1973) that remains through time (Sheu and Freese, 1972; Desbois and Smith, 2010), which gives them a prevalent role as additives in animal nutrition.

The essential oils are volatile lipophilic secondary metabolites from plants, characterized by their antimicrobial properties against different types of microorganisms including bacteria, protozoa and fungi (Greathead, 2003). Their capacity for inhibit growth of different types of bacteria (Calsamiglia et al., 2007) depends upon their composition (presence of phenolics, carbonyl groups, monoterpenes, etc.), and some may improve the ruminal fermentation (Busquet et al., 2005b; Castillejos et al., 2006). Among them, cinnamaldehyde and eugenol are widely used to modify the pattern of microbial fermentation, especially the volatile fatty acids profile (Cardozo et al., 2006). In practice, blends of essential oils with specific antibacterial selectivity are commonly used for a broad spectrum of effects (Wallace, 2004).

Most studies conducted with these zootechnical feed additives have been focused on dairy cattle given mixed diets to reduce methane production and to improve milk production and quality, but there is limited information about their role in intensive beef

fattening systems. Most previous studies have been carried out *in vivo* (Firkins et al., 1990; Castillejos et al., 2007) or with continuous/semicontinuous *in vitro* systems (Castillejos et al., 2006; Cardozo et al., 2006), both being laborious, cost expensive and limited to a reduced number of tested treatments. Limitation of closed batch *in vitro* systems to the use of a well-buffered medium limits the extrapolation of results to production conditions, further considering the pH-dependent effect of essential oils (Juven et al., 1994; Cardozo et al., 2005). The adaptation of the gas production procedure proposed by Amanzougarene and Fondevila (2018) allows for incubation at a range of pH closer to intensive feeding conditions. This *in vitro* work aimed to study whether the inclusion of fatty acids or essential oils at various levels under intensive beef production conditions could affect the rate of microbial fermentation of concentrate feeds, estimated by the gas production technique.

2. Material and methods

Two experiments were carried out testing fatty acids and essential oils in Experiments 1 and 2, respectively. Additives were included at three doses, considered as low, medium and high (d1, d2 and d3). In Experiment 1, the additives were chosen as representatives of saturated (palmitic acid, PAL, 0.85 g/mL; MERCK KGaA, Darmstadt, Germany) and polyunsaturated (linoleic acid, LIN, 0.90 g/mL; Alfa Aesar, Thermo Fisher, Karlsruhe, Germany) fatty acids, and a commercial mixture of medium chain fatty acids (MFA; 50% C12, 20% C10, 20% C8, and 10% C6; NUTRIKA, Zulte, Belgium). Inclusion levels of PAL and LIN were 15, 30 and 45 mg/g substrate, whereas the dose of MFA was 2, 4 and 6 mg/g substrate, considering the dose recommended by the manufacturer as d2. In Experiment 2, the sources of essential oils were cinnamaldehyde (CIN, 98% purity, 1.05 g/mL) included at 30, 60 and 90 mg/g substrate and eugenol (EUG, 99% purity, 1.06 g/mL) included at 60, 120 and 180 mg/g substrate, both provided by NOREL Animal Nutrition (Barcelona, Spain), and the commercial blend CRINA Ruminants (CBC; DSM Nutritional Products, Basel, Switzerland), that was included at 30, 60 and 90 mg/g substrate. The doses of essential oils were chosen considering the manufacturer recommendation as d2. In both experiments, treatments were contrasted with the unsupplemented barley substrate, that was used as control (CTL).

2.1. Inoculum source and incubation procedures

Three Limousine crossbred beef male calves (8 months of age, around 300 kg live weight) provided with a 2 cm i.d. cannula fitted in the dorsal sac of the rumen and housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the University of Zaragoza, were used as donors of inoculum. Calves were daily fed *ad libitum* with a concentrate mixture (in proportions of 0.59 barley, 0.15 maize, 0.17 soybean meal, 0.06 maize gluten and 0.03 mineral-vitamin mixture) and straw, at a concentrate:forage ratio of 0.91:0.09 of total feed intake. Management and extraction procedures of rumen inoculum from donor animals were approved by the Ethics Committee for Animal Experimentation. Care and management of animals agreed with the Spanish Policy for Animal Protection RD 53/2013, which complies with EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes. Rumen contents were sampled, filtered through cheesecloth, dispensed in 16 mL aliquots and immediately frozen in liquid nitrogen and maintained at -80ºC until utilisation (Prates et al., 2010).

The *in vitro* fermentation process was carried out in a closed batch system following the procedure of Theodorou et al. (1994). Triplicate bottles (116 mL total volume) were filled with 80 mL of incubation solution made up with 0.10 rumen inoculum and without micro-minerals and resazurin (Mould et al. 2005a). Buffer composition was modified to adjust the incubation pH around of 6.2 (Amanzougarene and Fondevila 2018). An amount of 500 mg air-dry barley meal (*Hordeum vulgare*, var. Gustav; 105 g/kg DM crude protein, 24 g/kg DM ether extract, 171 g/kg neutral detergent fiber, 672 g/kg DM starch), ground to 1 mm particle size, was included on each bottle as basal substrate.

Three *in vitro* incubation series were carried out, with rumen contents from a different calf as donor for each series, thus considering animal as a block. On every series, triplicate glass bottles for each treatment were incubated. Three additional bottles without substrate were also incubated on each series as blanks of inoculum. To facilitate substrate recovery for subsequent determinations, barley was introduced into 4 x 4 cm nylon bags (45 µm pore size, Sefar Maissa, Barcelona, Spain), proven to ensure a free flow of medium through the bag pores. Bottles were filled with the incubation solution under a CO_2 stream, sealed and incubated for 24 h in a water bath at 39 ºC. During the experiment, internal pressure of two out of the three incubated bottles was recorded at 2, 4, 6, 8, 10, 12, 18 and 24 h by means of a HD 2124.02 manometer fitted with a TP804 pressure gauge

(Delta Ohm, Caselle di Selvazzano, Italy). Readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same conditions and known air volumes, and expressed as either accumulated volume (total mL) or as rate of gas produced (mL produced per hour at each incubation interval) per unit of incubated organic matter (OM). The average of the two bottles for each treatment on each incubation series was considered as the experimental unit. After 8 h of incubation, the third bottle of each treatment was opened, its pH recorded (CRISON micropH 2001, Barcelona, Spain) and samples of the incubation medium were taken and immediately frozen and stored at -20 ºC until volatile fatty acids (VFA) analysis (2 mL, collected over 0.5 mL of 0.5M PO_4H_3 with 2 mg/mL 4-methyl valeric acid). At the end of the 24 h incubation, pH was also measured, and the incubation medium sampled for estimation of microbial mass (10 mL sample). Bags of substrate were removed from each bottle, squeezed and dried (60 ºC, 48 h) to estimate dry matter disappearance (DMd).

2.2. Chemical analysis

The barley substrate was analysed following the procedures of AOAC (2005) for dry matter (DM; ref. 934.01), organic matter (OM; ref. 942.05), crude protein (CPr; ref. 976.05) and ether extract (EE; ref. 2003.05). Concentration of neutral detergent fibre (aNDFom) was analysed as described by Mertens (2002) in an Ankom 200 Fibre Analyser (Ankom Technology, New York), using α–amylase and sodium sulphite, and results are expressed exclusive of residual ashes. The acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as described by AOAC (2005) and by Robertson and Van Soest (1981), respectively. Total starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). VFA and the concentration of individual MCFA in the additive were determined by gas chromatography on an Agilent 6890, apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm id). Microbial mass in the liquid fraction was approached according to Hsu and Fahey (1990), by centrifuging samples at 13000 x g for 20 min and weighing the washed lyophilised residue.

2.3. Statistical analysis

Results were analysed by ANOVA using the Statistix 10 software package (Analytical Software, 2010), considering the incubation series as a block and the experimental treatments in each experiment (CTL and the three additives included at three doses; n=10) as a factorial effect. Each given value is mean of the three incubation series. In each experiment, Polynomial (lineal and quadratic) contrasts were planned to estimate the trend in the response of each single additive (control and the three levels of inclusion, n=4), and orthogonal contrasts were established to compare the three additives among them. In all cases, differences were considered significant when $P<0.05$, and a trend for significance was considered when $0.05 \leq P < 0.10$.

3. Results

The pH of rumen fluid inoculum before starting the three incubation series of Experiments 1 and 2 was 6.29 ± 0.20 and 6.16 ± 0.04, respectively. Incubation pH at 8 and 24 h averaged 6.11 and 5.90 in Experiment 1, and 6.17 and 5.98 in Experiment 2. Treatment differences in each time of measurement were non- significant and in all cases below 0.13 pH units.

Experiment 1: fatty acids

Table 13 summarises the accumulated gas production from the different levels of each at certain incubation times (4, 8, 12 and 24 h), chosen to be representative of the whole fermentation pattern. The inclusion of MFA and PAL did not affect the volume of gas produced from barley alone, except for a trend for a quadratic decrease ($P=0.071$) in gas production at 24 h with increasing doses of MFA and for a linear decrease at 12 ($P=0.088$) and 24 h ($P=0.052$) with PAL. In contrast, the inclusion of LIN promoted a quadratic decrease ($P<0.05$) in the volume of gas produced throughout all the incubation period, with differences detected between d2 and d3 respect to CTL from 12 h onwards ($P<0.05$).

When comparing among the different fatty acids, the volume of gas produced after 24 h tended ($P=0.074$) to be higher with MFA than LIN, but no other differences were recorded at any incubation time ($P>0.05$). In order to better compare the effect of the

different additives on microbial fermentation of barley, the average rate (mL/g OM per h) of gas production was estimated and contrasted in Figure 19. Although it was not considered in the statistical comparison, the rate of gas production from barley alone (CTL) is also shown in the figure for comparative purposes. At 10 h LIN showed the lowest rate ($P<0.01$), and LIN was also lower than MFA at 18 h ($P=0.010$) and tended to be lower than PAL at 24 h ($P=0.051$). The numerically higher rate with MFA at 2 h respect to LIN and PAL was not significant ($P=0.13$ and $P=0.15$, respectively) because of the high magnitude of the error term (variation coefficient 0.46).

The DMd and the estimated microbial mass in the liquid fraction at the end of the 24 h incubation period is also shown in Table 13. A quadratic decrease in DMd was detected with the inclusion of increasing levels of MFA and LIN ($P<0.01$), but the inclusion of PAL did not affect this parameter. Similarly, a quadratic reduction of the microbial mass in the liquid fraction was observed with the inclusion of MFA ($P=0.008$) and LIN ($P=0.020$), and a trend for the same effect was observed with PAL ($P=0.090$). When comparing among additives, the lowest ($P<0.05$) DMd and microbial mass were recorded with MFA, but no differences were manifested among PAL and LIN ($P>0.10$).

Table 13. Accumulated volume of gas produced (mL/g OM) at different incubation times, dry matter disappearance (DMd) and microbial mass in the liquid fraction (mg/mL) from barley as the only substrate (CTL) or supplemented with increasing doses (d1, d2 and d3; n=3) of medium-chain fatty acids (MFA), palmitic acid (PAL) or linoleic acid (LIN). Mean values for each additive (n=9) and probability of the response pattern (linear, L or quadratic, Q) and additives comparison are also shown.

		Gas volume					Microbial
	Dose	**4 h**	**8 h**	**12 h**	**24 h**	**DMd**	**mass**
CTL	0	56.0	100.9	132.5	175.6	0.434	2.64
MFA	d1	49.6	86.6	116.2	157.0	0.387	2.45
	d2	47.1	83.2	111.2	149.9	0.372	2.40
	d3	44.4	79.9	107.2	141.9	0.339	2.26
	mean	47.1	83.2	111.5	149.6	0.366	2.37
Pattern					*Q(T)*	*Q(**)*	*Q(**)*
PAL	d1	39.7	77.1	104.2	142.7	0.403	2.64
	d2	38.6	75.0	105.4	140.4	0.395	2.53
	d3	37.2	73.0	99.8	140.0	0.396	2.49
	mean	38.5	75.0	103.1	141.0	0.398	2.55
Pattern				*L(T)*	*L(T)*		Q(T)
LIN	d1	48.2	84.5	109.9	144.5	0.420	2.62
	d2	35.1	71.3	96.6	132.4	0.385	2.50
	d3	33.8	67.8	90.0	122.7	0.378	2.40
	mean	39.0	74.5	98.9	133.2	0.394	2.51
Pattern		*Q(*)*	*Q(*)*	*Q(*)*	*Q(**)*	Q(**)	Q(*)
MFA *vs.* PAL						*	**
MFA *vs.* LIN					*T*	*	*
PAL *vs.* LIN							
SD		13.07	15.83	17.00	18.38	0.0251	0.124

*: $P<0.05$; **: $P<0.01$; T: $P<0.10$; SD: standard deviation

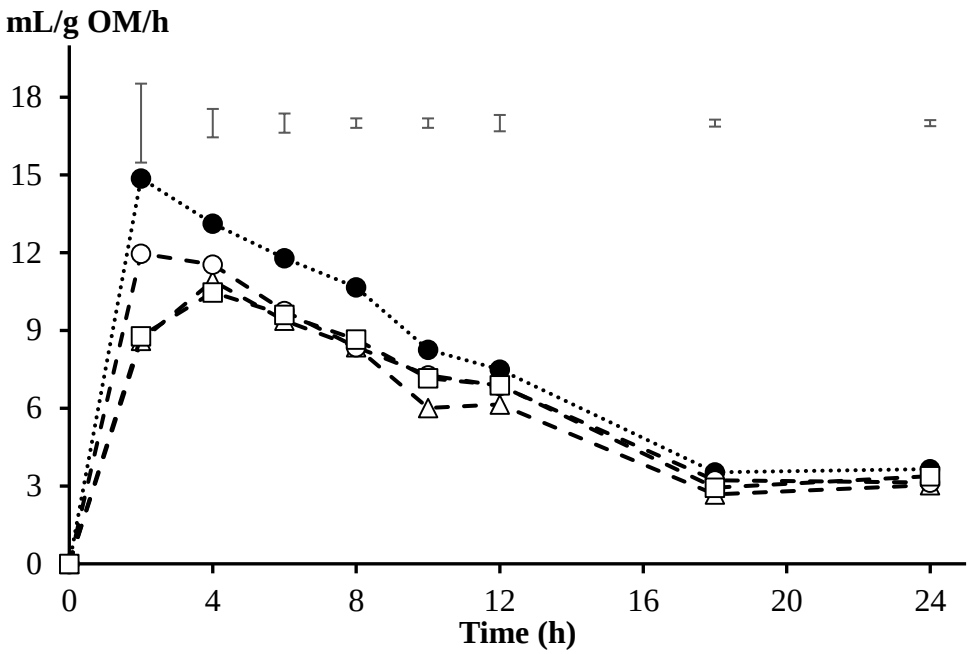

Figure 19. Rate of gas production (mL/g OM per h) at the different time intervals from barley supplemented with MFA (○), PAL (□), and LIN (△). Each value from each fatty acid is the average of three doses (n=9). Upper bars show standard error of means. For comparative purposes, unsupplemented barley (CTL,●) is also included in the graph.

Total VFA concentration after 8 h of incubation (Table 14) tended (P=0.066) to increase linearly with the inclusion of LIN. Regarding the molar proportions of the different VFA, the inclusion of LIN tended to promote a linear increase of acetate proportion (P=0.060), also tending to decrease that of propionate (P=0.051). No effect on total VFA concentration or on molar VFA proportions was detected with the inclusion of MFA or PAL. No differences (P>0.10) among fatty acid sources were recorded on total VFA concentration, but a higher molar proportion of acetate and lower of butyrate were observed with LIN with respect to both MFA and PAL (P<0.05), whereas the molar proportion of propionate and branched-chain fatty acids (sum of isobutyrate and isovalerate, BCVFA) tended to be lower with LIN than with MFA (P=0.083) and PAL (P=0.084), respectively.

Table 14. Total volatile fatty acid (VFA) concentration (mM) and molar VFA proportions after 8 h incubation of barley as the only substrate (CTL) or supplemented with increasing doses (d1, d2 and d3; n=3) of medium-chain fatty acids (MFA), palmitic acid (PAL) or linoleic acid (LIN). Mean values for each additive (n=9) and probability of the response pattern (linear, L or quadratic, Q) and additives comparison are also shown.

	Dose	Total VFA	Acetate	Propionate	Butyrate	Valerate	BCVFA
CTR	0	30.9	0.541	0.265	0.153	0.021	0.021
MFA	d1	33.3	0.544	0.266	0.151	0.020	0.020
	d2	33.7	0.536	0.268	0.156	0.021	0.019
	d3	32.8	0.525	0.272	0.160	0.022	0.021
	mean	33.3	0.535	0.269	0.156	0.021	0.020
Pattern							
PAL	d1	35.7	0.536	0.268	0.155	0.021	0.020
	d2	32.6	0.530	0.269	0.159	0.021	0.022
	d3	33.3	0.533	0.266	0.158	0.023	0.021
	mean	33.9	0.533	0.267	0.157	0.022	0.021
Pattern							
LIN	d1	35.2	0.548	0.267	0.144	0.022	0.019
	d2	35	0.561	0.251	0.145	0.023	0.020
	d3	35.5	0.593	0.231	0.137	0.020	0.019
	mean	35.2	0.567	0.250	0.142	0.021	0.019
Pattern		*L(T)*	*L(T)*	*L(T)*			
MFA *vs.* PAL							
MFA *vs.* LIN			*	*T*	*		
PAL *vs.* LIN			*		*		*T*
SD		2.7	0.0263	0.0177	0.0082	0.0016	0.0016

*: $P<0.05$; T: $P<0.10$; SD: standard deviation

Experiment 2: essential oils

Table 15 shows the volume of gas produced at different time intervals from barley as the only substrate or added with the different levels of essential oils. A linear decrease in gas production was observed with CIN ($P<0.001$) throughout all the incubation period, whereas the inclusion of CBC promoted a linear increase in gas at 12 h of incubation ($P<0.05$) and an increasing trend for this at 8 ($P=0.091$) and 24 h ($P=0.064$). In contrast, the addition of different levels of EUG did not affect gas production from barley, except for a quadratic decrease at 24 h ($P=0.047$). When the average rate of gas production was monitored (Figure 20), it was lower with CIN than EUG from 2 to 8 h ($P<0.05$) and CBC from 2 to 12 h ($P<0.001$), whereas with CBC it was higher than EUG at 2 ($P<0.05$) and

at 8, 10 and 12 h ($P<0.01$). Although not considered in the statistical comparison, the rate of gas production in CTL, also included in the figure, was similar to the mean rate with EUG from 4 to 8 h, and numerically lower than CBC along the mid part of fermentation period.

The DMd from barley (Table 15) decreased linearly with the addition of CIN ($P<0.001$), and quadratically with the addition of EUG ($P< 0.001$), whereas the inclusion of CBC linearly increased DMd ($P<0.001$), thus resulting the highest DMd of the three additives. EUG decreased quadratically ($P<0.01$) the microbial mass from the liquid fraction, but no effect of CIN or CBC was detected on this parameter.

Total VFA concentration after 8 h incubation linearly decreased ($P=0.036$) with the level of inclusion of CIN (Table 16), whereas such effect followed a quadratic trend with EUG ($P<0.001$). Molar propionate proportion decreased linearly with CIN ($P=0.010$), whereas with EUG acetate quadratically increased at the expense of propionate ($P<0.001$). Inclusion of CBC did not affect total VFA concentration, and resulted higher than both CIN and EUG ($P<0.05$). Similarly, molar propionate proportion was highest, and that of acetate lowest, with CBC ($P<0.05$) compared with the other additives, whereas butyrate proportion was highest with EUG ($P<0.05$).

Table 15. Accumulated volume of gas produced (mL/g OM) at different incubation times, dry matter disappearance (DMd) and microbial mass in the liquid fraction (mg/mL) from barley as the only substrate (CTL) or supplemented with increasing doses (d1, d2 and d3; n=3) of cinnamaldehyde (CIN), eugenol (EUG) or a commercial blend (CBC). Mean values for each additive (n=9) and probability of the response pattern (linear, L or quadratic, Q) and additives comparison are also shown.

		Gas volume					**Microbial**
	Dose	**4 h**	**8 h**	**12 h**	**24 h**	**DMd**	**mass**
CTL	0	30.8	58.2	82.9	117.9	0.424	1.99
CIN	d1	21.6	46.2	70.4	100.2	0.398	2.05
	d2	12.7	25.4	43.3	72.7	0.374	2.10
	d3	8.8	21.1	34.8	63.5	0.347	2.19
	mean	14.4	30.9	49.5	78.8	0.373	2.12
Pattern		*L(***)*	*L(***)*	*L(***)*	*L(***)*	*L(***)*	*L(T)*
EUG	d1	29.5	57.8	80.3	111.6	0.404	1.65
	d2	27.6	53.4	75.6	103.4	0.369	1.52
	d3	24.9	51.6	71.0	100.2	0.355	1.51
	mean	27.3	54.3	75.6	105.0	0.376	1.56
Pattern					*Q(*)*	*Q(***)*	*Q(***)*
CBC	d1	31.2	58.9	83.0	116.8	0.433	1.99
	d2	32.4	62.7	90.6	128.1	0.489	1.99
	d3	34.6	68.7	99.6	132.1	0.487	2.03
	mean	32.7	63.4	91.1	125.7	0.470	2.02
Pattern			*L(T)*	*L(*)*	*L(T)*	L(***)	
CIN *vs.* EUG		***	***	***	***		***
CIN *vs.* CBC		***	***	***	***	***	
EUG *vs.* CBC		*	*	**	***	***	***
SD		1.93	0.0134	0.0096	0.0076	0.0015	0.0010

*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; T: $P<0.10$; SD: standard deviation.

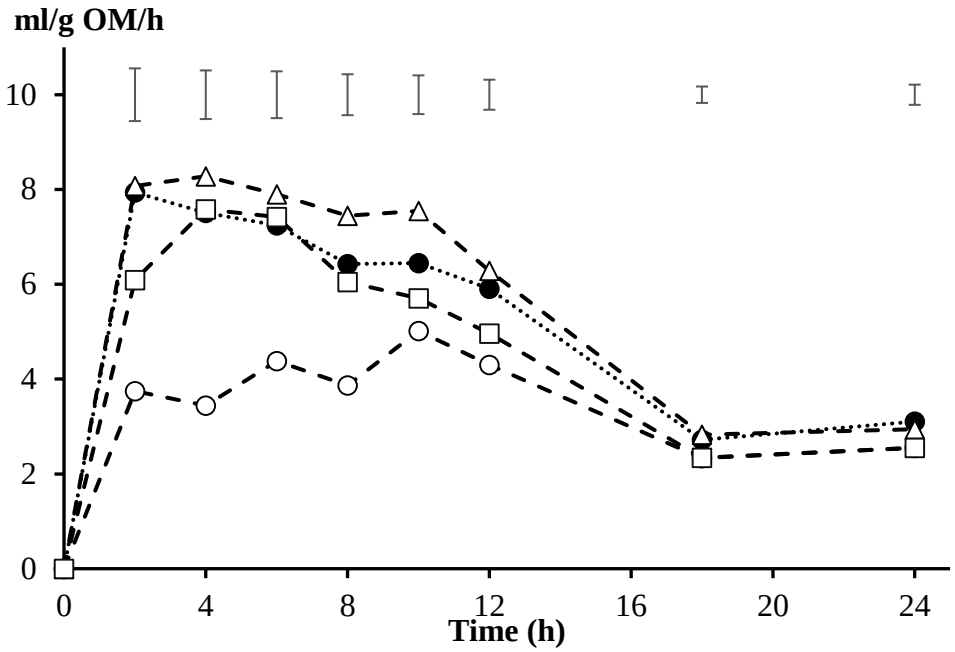

Figure 20. Rate of gas production (mL/g OM per h) at the different time intervals from barley supplemented with CIN (○), EUG (□) and CBC (△). Each value from each fatty acid is the average of three doses (n=9). Upper bars show standard error of means. For comparative purposes, unsupplemented barley (CTL,●) is also included in the graph.

Table 16. Total volatile fatty acid (VFA) concentration (mM) and molar VFA proportions after 8 h incubation of barley as the only substrate (CTL) or supplemented with increasing doses (d1, d2 and d3; n=3) of cinnamaldehyde (CIN), eugenol (EUG) or a commercial blend (CBC). Mean values for each additive (n=9) and probability of the response pattern (linear, L or quadratic, Q) and additives comparison are also shown.

	Dose	**Total VFA**	**Acetate**	**Propionate**	**Butyrate**	**Valerate**	**BCVFA**
CTR	0	31.2	0.512	0.278	0.166	0.023	0.021
CIN	d1	27.0	0.558	0.241	0.153	0.025	0.023
	d2	27.0	0.562	0.230	0.164	0.023	0.022
	d3	24.7	0.550	0.230	0.172	0.025	0.023
	mean	26.2	0.557	0.234	0.163	0.024	0.023
Pattern		L(*)		L(*)			
EUG	d1	29.8	0.535	0.240	0.177	0.027	0.022
	d2	22.9	0.549	0.217	0.188	0.025	0.022
	d3	22.1	0.624	0.161	0.175	0.023	0.018
	mean	25.0	0.569	0.206	0.180	0.025	0.020
Pattern		Q(***)	Q(**)	Q(***)			
CBC	d1	30.7	0.505	0.280	0.169	0.025	0.021
	d2	31.1	0.513	0.269	0.174	0.024	0.021
	d3	30.2	0.507	0.269	0.178	0.024	0.022
	mean	30.7	0.508	0.273	0.174	0.024	0.021
Pattern							
CIN *vs.* EUG				*	*		*
CIN *vs.* CBC		*	**	***			
EUG *vs.* CBC		**	***	***			
SD		3.34	0.0289	0.0208	0.0164	0.0032	0.0022

*: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; T: $P<0.10$; SD: standard deviation.

4. Discussion

Incubation pH was maintained between 5.9 and 6.2 throughout the whole incubation period, as it was previously approached for simulating high concentrate feeding conditions (Amanzougarene and Fondevila, 2018). In any case, pH values were recorded only for purposes of validation of incubation conditions, since the inclusion of certain level of buffer prevents for any treatment comparison.

4.1. Effect of fatty acids

Inclusion of lipids in ruminant diets has been commonly assayed for reducing microbial fermentative activity (Palmquist and Jenkins, 1980), thus preventing the risk of acidosis in dairy cows fed on mixed diets (Soliva et al., 2004; Beauchemin et al., 2009) but can also be extended to intensive feeding beef cattle. The noticeable variability of obtained results depends on factors such as the amount and fatty acid profile of dietary fats, as well as presentation form and composition of basal diets. It is generally assumed that levels of dietary inclusion of fat over 50 mg/g affect rumen fermentation in high concentrate diets (Jenkins, 1993; Doreau and Chilliard, 1997), but a higher effect can be expected from free fatty acids. In our case, the level of inclusion of LIN and PAL was chosen according to these statements and, from a common level of 30 mg free fatty acid/g, doubling or dividing this proportion. Instead, in the case of medium-chain fatty acids, the level recommended for practical use is fixed according to their selective antimicrobial effect (Henderson, 1973), and therefore their dosage was considerably lower (4 mg/g), according to manufacturer recommendations.

The effect of lipids on rumen environment is related with a decrease in bacterial growth (Maczulak et al., 1981). In terms of fermentation products, addition of fats is also associated with an increase in rumen propionate proportion through a decrease in that of acetate (Doreau et al., 1991), although this has not always been detected (Pantoja et al., 1995). It is also assumed that long-chain polyunsaturated fatty acids, such as C18:2 linoleic acid, exert a greater inhibitory effect on microbial fermentative activity than saturated fatty acids (Palmquist and Jenkins, 1980; Jenkins, 1993), such as C16:0 palmitic fatty acid. Toxic effects of polyunsaturated fatty acids sources on cellulolytic bacteria have been reported by Vazirigohar et al. (2018), and Sinclair et al. (2005) reported that diets rich in polyunsaturated fatty acids reduced the gas production profiles. However, in our experiment, no major effect of PAL at any inclusion level was observed on barley

fermentation, and only a quadratic reduction of gas production was observed throughout the incubation period with the inclusion of LIN because of the response to d3 of LIN (Table 13). This was associated to a linear reduction in propionate proportion that may be associated to a depressed fermentation of barley starch. In the same way, no differences were observed among both additives.

Certain medium-chain fatty acids have been assessed for their potential to interact with rumen microbiota to modulate ruminal fermentation and improve nutrient utilization (Hristov et al., 2004). Medium chain fatty acids have a stronger effect on bacteria and thus on carbohydrate fermentation than long-chain fatty acids. Klevenhusen et al. (2011) showed that addition of lauric acid in form of monolaurin affected many ruminal microbes involved in carbohydrate degradation, and Soliva et al. (2004) and Machmüller (2006) reported that the addition of medium chain fatty acids to ruminant diets reduce nutrient disappearance. In our experiment, the effect of MFA was manifested in a quadratic sense towards a trend (P=0.071) to depress 24 h gas production and a decrease (P<0.01) in DMd and estimated microbial mass (Table 13), although the accuracy of the latter approach is relative because of the non-specificity of the method of analysis. The fact that the response to this additive followed a quadratic trend indicates that a significant effect was not attained until d3, suggesting that the initial dose was to a certain extent undervalued, and a level of 8 mg per gram of substrate should be applied. In any case, no major effect on VFA profile were detected, supporting that the effect of MFA was of minor magnitude and not related to a different fermentation profile.

4.2. Effect of essential oils

The essential oils used here were chosen looking for a potential stabilisation in the rumen environment that should prevent, or at least reduce, the risk of acidosis. However, testing essential oils have often been conducted under forage or mixed diets conditions, whereas their effect on rumen fermentation may be affected by medium pH that can influence the dissociated or undissociated status of their molecules (Cardozo et al., 2005). In fact, Cardozo et al. (2006) stated that cinnamaldehyde may be interesting for intensively reared beef steers because of its selective activity over some Gram-positive bacteria by acting over the cell membrane, and because of its positive effects on rumen bacterial fermentation under low-pH conditions for reducing the rate of rumen fermentation and increase the acetate to propionate ratio (Busquet et al., 2006). In

contrast, eugenol, which has a wide spectrum of antimicrobial activity (Cobellis et al., 2016b), decreased total VFA concentration and propionate proportion in an *in vitro* system with a 90:10 concentrate to forage diet (Castillejos et al., 2006), thus suggesting a limited interest in intensively reared beef steers diets. In any case, a combination of different compounds is the common practice in practical feeding conditions looking for a synergistic effect, and thus a commercial mixture of essential oils was also tested in this study.

A moderate fermentative response was observed with EUG, that was manifested in a numerically lower accumulated gas volume with levels d2 and d3 at 24 h respect to CTL (Table 15), but this effect was more apparent on DMd and total VFA concentration (Table 16). These results agree with those found *in vitro* by Benchaar et al. (2007), who reported a decrease in both gas production and DMd with 40 mg EUG/g over a 55:45 forage to concentrate mixture. However, Tager and Krause (2010) did not observed any effect on DMd at doses of 6 mg/g. The lower total VFA concentration and the reduction in propionate proportion with EUG agree with studies by Busquet et al. (2006) and Benchaar et al. (2007) using higher and lower dose levels than here (300 and 40 mg/g), respectively. Besides, the lower concentration of VFA was associated with a quadratic decrease of microbial mass (Table 3) that might be explained by the strong effect of EUG on ruminal bacteria (Dorman and Deans, 2000).

In contrast to that observed with EUG, the inclusion of CIN resulted in a linear decrease of substrate fermentation. As expected, the results of gas production were supported by those of DMd. In contrast, no significant differences were observed on microbial mass between CIN and CTL, maybe because of the low specificity of the method used to determine the microbial mass. With similar doses than those used here (39 and 65 mg/g) Macheboeuf et al. (2008) observed a decrease in 16 h gas production and in total VFA concentration. However, others did not find any effect on *in vitro* fermentation at doses of 30 mg/g or lower (Busquet et al., 2006; Benchaar et al., 2007; Tager and Krause, 2010). Regarding to individual VFA proportions, our results showed a linear decrease on propionate (Table 16), without affecting others molar VFA proportions, results supported by those observed by Macheboeuf et al. (2008). In the present study, the response of CIN on *in vitro* patterns of microbial fermentation was lower than expected because the pH of the incubation solution (≈6.2) might consider slightly high according to Juven et al. (1994).

The effect of CIN on fermentation and microbiota seem to be related to environmental pH (Juven et al., 1994), and Cardozo et al. (2005) suggested that cinnamaldehyde can be potentially useful at the low ruminal pH expected in high-concentrate diets for intensive beef production. Besides, despite the doses used were around those recommended by the manufacturer, they were lower to other in literature (Benchaar et al., 2011). However, Busquet et al. (2005b) suggested that at low doses, CIN might be an active modulator of rumen microbial activity.

A lack of effect of essential oils blends has been reported *in vivo* (Flores et al., 2013; Tomkins et al., 2015) and variable responses have been observed *in vitro* (Ahmed et al., 2014; Cobellis et al., 2016a). These different responses can be attributed mostly to their composition, the compounds proportions and the dose of additive (Dorman and Deans, 2000; Busquet et al., 2006), as well as possible synergistic or antagonistic effects among compounds (Burt, 2004). In many cases blends are commercial products and their composition and proportion of ingredients is not published. In our case, we used a commercial, widely used mixture, choosing the dose recommended by the manufacturer as d2, with the aim to have a standard response to compare with CIN and EUG. With a 50% concentrate substrate, Cobellis et al. (2016a) reported an increase in pH with 80 mg/g of five different blends of EO, at a rate differing from one blend to another. Similarly, using corn silage as basal substrate with 64 mg/g of essential oils blend, Spanghero et al. (2008) observed an increase in pH. In any case, in contrast to the negative effect of CIN and the minor response to EUG, the supplementation of barley with CBC promoted a linear increase on gas production at 12 h ($P<0.05$) and a trend to such effect at 8 and 24 h ($P=0.091$, $P=0.064$; respectively), in addition to a linear increase in DMd (Table 15), but did not affect total VFA concentration. As commented regarding CIN, Spanghero et al. (2008) suggested that a blend of essential oils might be more effective in an acidic medium, and thus the effectiveness may be higher under intensive feeding conditions. In contrast to CIN and EUG, CBC increased the measured fermentative parameters respect to unsupplemented barley. This might be explained by the broad and often nonspecific antimicrobial activity of CIN and EUG (Cobellis et al., 2016a), in contrast to CBC which has been characterized by potential synergistic effects among its components (Burt, 2004).

5. Conclusions

The use of fatty acids and essential oils as additives depends on the objective, since their effect *on in vitro* barley fermentation largely differ among the nature of the compounds used. The three studied fatty acids reduced the magnitude and rate of *in vitro* barley fermentation, but a lower rumen activity can be associated with a less challenging pH, that should be positive if it is balanced by the intestinal digestion of bypass feed. Such effect was dose dependent in the case of LIN, since the response was already maximised with d2. Similarly, a linear depression was observed with CIN, and a negative response although of a lower magnitude was also observed up to d2 of EUG. In contrast, CBC linearly enhanced the magnitude and rate of barley fermentation, thus increasing rumen substrate disappearance but without affecting fermentation pattern. Both fatty acids and essential oils did not negatively affect medium pH throughout the incubation, suggesting that both types of additives may modulate barley acidification potential. The reduction in the extent of barley fermentation with some of these additives does not mean that their effects were not nutritionally beneficial to beef cattle, since the increased proportion of feed that should reach the lower sites of the gastrointestinal tract could be digested there, providing nutrients to the animals.

Chapter VIII. Section II. Experiment 5

Estimation of rumen fermentation pattern of barley supplemented with additives of different nature in an *in vitro* semicontinuous incubation system (under review; Journal of Animal Physiology and Animal Nutrition)

Chapter VIII. Estimation of rumen fermentation pattern of barley supplemented with additives of different nature in an *in vitro* semicontinuous incubation system.

Abstract.

Grape condensed tannins (GCT, 20 mg/g), a mixture of medium-chain fatty acids (MFA, 4 mg/g), linoleic acid (LIN, 30 mg/g), eugenol (EUG, 120 mg/g), and cinnamaldehyde (CIN, 60 mg/g), were used as additives to evaluate their effect on barley fermentation. A semicontinuous *in vitro* system inoculated with rumen contents from beef calves fed *ad libitum* with concentrate and straw was used, with a poorly buffered medium from 0 to 6 h, and gradually buffered to 6.5 from 8 to 24 h the medium. Gas production and pH were recorded from 2 to 24 h. Total volatile fatty acids (VFA) concentration was determined after 8 and 24h. Microbial diversity was characterised at 8 h by terminal restriction fragment length polymorphism (tRFLP). Incubation pH reached its minimum after 6 h (6.89 ± 0.07), and from then maximum pH was reached at the end of incubation (6.41 ± 0.03). From 6 h onwards, GCT recorded the highest pH ($p < 0.05$), while CIN recorded lower pH values ($p < 0.05$). Throughout the incubation EUG and CIN produced lower volumes of gas ($p < 0.05$) than unsupplemented barley (CTR). The inclusion of different additives reduced 24h dry matter disappearance of barley, which was highest with CTR ($p < 0.05$) and lowest with GCT ($p < 0.05$). High total VFA concentration and higher acetate and propionate proportions were recorded at 8h ($p < 0.05$). Among treatments, MFA recorded the highest VFA concentration, while EUG and CIN recorded the lowest VFA concentration and propionate proportion, and the highest proportion of propionate was promoted by LIN ($p < 0.05$). Results were supported by those showed by bacterial diversity. Essential oils as additives, especially cinnamaldehyde, reduced barley fermentation at a higher extent, and negatively affected environmental conditions. In contrast, grape condensed tannins and fatty acids may reduce barley acidification potential.

1. Introduction

In intensive ruminants fattening systems, the transition phase from milk and/ or forage-feeding to high concentrate diets is considered as the critical moment for animal health and productivity. Acidosis is the main frequent digestive disorder that occurs during this period, as a consequence of the intensive fermentation of readily available carbohydrates in non-adapted animals. However, it has been demonstrated that the risk of acidosis might be reduced by the use of feed additives that are able to modulate rumen fermentation, thus maximizing the efficiency of feed utilization and consequently increasing ruminant productivity (Greathead, 2003). It has to be considered that non-degraded nutrients may reach the intestines to be digested there, providing to the animal more energy than that fermented in the rumen (Owens et al., 1986). Thus, the effects on ruminal microbial fermentation of additives such as tannins (Rodríguez at al., 2011), fatty acids (Beauchemin et al., 2009), and essential oils (Cardozo et al., 2005) have already been studied, but in most cases they have been compared within the same type of them. Because of this, a global comparison of their effects is needed for addressing a potential ranking of practical interest among them.

Tannins are polyphenolic compounds that are able to bind to feed proteins and polysaccharides, limiting their degradability by rumen microbes (McAllister et al., 1994) but favourably modulating the rumen microbial fermentation (Patra and Saxena, 2011). The extent of their effect depends not only on their concentration, but also on their reactivity, which is associated with their chemical nature (Rodríguez et al., 2011). Condensed tannins from grape can significantly reduce fermentation of a fibrous substrate (Wischer et al., 2013), and a notable reduction of microbial fermentation has also been reported on barley grain (Amanzougarene et al., 2019).

Medium chain fatty acids and long chain polyunsaturated fatty acids are often added to diets for fattening ruminants to improve their energy density, promoting changes in rumen microbial population and in fermentation processes (Huws et al., 2010; Bayat et al., 2018). Chilliard and Ollier (1994) suggested a positive effect of these lipids on the digestive process balancing the nutrients absorption and limiting rumen acidosis. However, Jenkins (1994) reported that supplementation of ruminant diets with unsaturated fatty acids tended to depress microbial activity. On the other hand, Henderson (1973) indicated that medium chain fatty acids have an antimicrobial selective effect, especially affecting those species involved in carbohydrate degradation (Klevenhusen et

al., 2011). In any case, Machmüller (2006) and Klevenhusen et al. (2009) concluded that, whatever the type of fatty acids added, the extent of rumen fermentation is reduced.

Essential oils are natural feed additives characterised by their antimicrobial properties against different types of rumen microorganisms (Greathead, 2003). Furthermore, Busquet et al. (2005b) and Castillejos et al. (2006) reported that the inclusion of essential oils as additives in diets for ruminants might modulate and improve the ruminal fermentation. Cinnamaldehyde and eugenol are commonly used plants extracts because of their positive effects on the ruminal fermentation (Busquet et al., 2006) and their capacity to maintain ruminal pH within its physiological limits (Benchaar et al., 2007; Tager and Krause, 2010).

This work studies whether the inclusion of additives of different characteristics in concentrate-rich diets for ruminant fattening can reduce microbial fermentation, avoiding an excessive decrease in ruminal pH and thus contributing to prevention of acidosis. For doing so, an *in vitro* semicontinuous system adapted to mimic intensive feeding conditions was used.

2. Material and methods

Six experimental treatments, consisting of five additives from different nature sources included at doses previously established (Amanzougarene et al., 2019; Amanzougarene et al., 2017b; Amanzougarene et al., 2017c) were used supplementing barley, chosen as a reference feed commonly included in intensive feeding diets. These additives were: grape condensed tannins (GCT; procyanidins from *Vitis vinifera*, 0.75 w/w tannins; Agrovin SA, Alcázar de San Juan, Spain) added at 20 mg/g; a commercial mixture of medium-chain fatty acids (MFA; 0.50 C12, 0.20 C10, 0.20 C8, and 0.10 C6; NUTRIKA, Zulte, Belgium) that was included at 4 mg/g; linoleic acid as a source of polyunsaturated fatty acid (LIN, 0.90 g/mL; Alfa Aesar, Thermo Fisher, Karlsruhe, Germany), included at 30 mg/g; and eugenol (EUG, 99% purity, 1.06 g/mL) and cinnamaldehyde (CIN, 98% purity, 1.05 g/mL), both provided by NOREL Animal Nutrition (Barcelona, Spain), included at 120 and 60 mg/g, respectively. Unsupplemented barley was also incubated as control (CTR).

2.1. Inoculum source and incubation procedures

Rumen contents from three Limousine crossbred beef male calves (8 months of age, around 300 kg live weight), housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the University of Zaragoza, were used as inoculum. Calves were provided with a 2 cm i.d. cannula fitted in the dorsal sac of the rumen, and were daily fed *ad libitum* with a concentrate mixture (main proportions of 0.59 barley, 0.15 maize, 0.17 soybean meal, 0.06 maize gluten and 0.03 mineral-vitamin mixture) and straw, at a concentrate:forage ratio of 0.91:0.09 of total feed intake. Rumen contents were sampled, filtered through cheesecloth, dispensed in 16 mL aliquots, immediately frozen in liquid nitrogen and maintained at -80ºC until utilisation (Prates et al., 2010). Before incubation, rumen inocula were thawed in a water bath at 39ºC.

Fermentation kinetics were determined *in vitro* in a semicontinuous system (Fondevila and Pérez-Espés, 2008, modified by Prates et al., 2010). In order to simulate daily rumen pH fluctuations, two buffered solutions were used: the first one consisted of a poorly buffered solution that was made up with 0.006 M bicarbonate ion and used for allowing pH to drop from the first 6 h incubation, and the second one was made up with 0.058 M bicarbonate ion and was used for allowing pH to recover around 6.5 from 8 to 24 h. Barley grain (*Hordeum vulgare*, var. Gustav; composition in g/kg dry matter, DM: crude protein, CPr, 105, ether extract, EE, 24, neutral detergent fiber, aNDFom , 173, and starch, 672), ground to 1 mm particle size, was used as basal substrate. An approximate amount of 800 mg of barley was dispensed into 4 x 4 cm nylon bags (45µm pore size) that were sealed and introduced in duplicated bottles. Bottles were filled under CO_2 flux with 80 mL of incubation solution including 16 mL inoculum (0.20 of total volume) and incubated in a water bath at 39ºC for 24 h in three incubation series, each corresponding to a different donor animal inoculum.

Gas production was recorded from 2 to 24 h (every 2 h from 0 to 12 h incubation, or every 4 h from 12 to 24 h) with a HD8804 manometer provided with a TP804 pressure gauge (DELTA OHM, Caselle di Selvazzano, Italy). Readings were corrected for atmospheric pressure and then converted to volume (mL) using a pre-established linear regression (n=48, R^2 =0.993), and expressed per unit of incubated organic matter (OM). Every time of reading, a volume of incubation medium was extracted immediately after gas measurement and replaced anaerobically by incubation solution (without microbial inoculum) to simulate an approximate liquid turnover rate of 0.08/h. Extracted liquid

medium was used for recording pH at every time of reading. Besides, samples of liquid medium were taken at 8 and 24 h to determine the concentration of volatile fatty acids (VFA; 2 mL on a 0.5 mL solution of 0.5M phosphoric acid with 1 mg 4-methyl-valeric acid as internal standard) and at 8 h for determination of microbial biodiversity (6 mL), being stored at -20 and -80 ºC, respectively, until analysis. At the end of incubation, substrate bags were removed from the bottles, rinsed in distilled water and dried at 60ºC for 48 h for determination of dry matter disappearance (DMd).

2.2. Chemical and microbial analysis

The barley substrate was analysed following the procedures of AOAC (2005) for DM (method ref. 934.01, organic matter (OM, ref. 942.05), CPr (ref. 976.05) and EE (ref. 2003.05). Concentration of aNDFom was analysed as described by Mertens (2002) in an Ankom 200 Fibre Analyser (Ankom Technology, New York), using α–amylase and sodium sulphite, and results are expressed exclusive of residual ashes. Total starch content was determined enzymatically from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland).

Frozen samples of incubation medium were thawed and centrifuged at 13,000 g for 15 min, 4 ºC for their analysis of VFA. The concentration of individual VFA in incubated medium and of MFA in the additive were determined by gas chromatography on an Agilent 6890 apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm id). For the microbial diversity analysis, frozen microbial samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The DNA was extracted using the Qiagen QIAmp DNA Stool Mini Kit (Qiagen Ltd., West Sussex, UK) following the manufacturer recommendations, except that samples were initially heated at 95ºC for 5 min to maximise the lysis of bacterial cells. Concentration of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). PCR was performed using a 16S rRNA bacteria specific primer (cyanine-labelled forward 27F, 5´-AGA GTT TGA TCC TGG CTCAG-3´ and unlabelled reverse 1389R, 5´-AGG GGG GGT GTG TAG AAG-3´; Hongoh et al., 2003) using a DNAEngine® Gradient Cycler (Bio-Rad, Spain). The PCR product was purified using a Purelink PCR purification kit (ref. K3100-01; Invitrogen) and diluted to 10 µL. The DNA concentration of each amplified and purified sample was obtained by spectrophotometry (Nanodrop® ND-1000 spectrophotometer) to enable a

standardised quantity of 50 ng DNA to be used per restriction enzyme digest reaction. Digestion of samples was carried out using HhaI, HaeIII and MspI (Promega, Spain), following the manufacturer recommendations except for HhaI, where the recommended addition of bovine serum albumin was omitted. Restriction digests were purified by ethanol precipitation (de la Fuente et al., 2014) in 35 µL sample loading solution buffer including a 600 bp size standard (Beckman Coulter Inc., Fullerton) before being applied to a 3500xL Genetic Analyzer (Applied Biosystems). Once getting the size and height of every peak, 1% of the second highest peak was used as criteria for the lower threshold for peaks to detect and eliminate smaller, broader peaks that would not be indicative of single true OTUs.

2.3. Calculations and statistical analyses

The TRFLP results were analysed from a matrix generated for each data list obtained, and results were presented in the form of relative abundance. The three matrices resulting from each series and enzyme were concatenated and analysed with R statistical software (https://cran.r-project.org/bin/windows/base/, version 3.5.0). FactoMineR, Factoextra, MixOmics, Vegan, MASS, and Ggplot2 packages were used to carry out the analysis of hierarchical classification on principal components for obtaining the cluster dendrogram.

Results were statistically analysed by ANOVA, using the Statistix 10 package (Analytical Software, 2010), with the incubation series (equivalent to donor animal, n=3) as block. Having into account that responses may be affected by the individual handling of each bottle for sampling along the incubation, bottles were considered as the experimental unit. Differences were considered significant when $P<0.05$, and a trend for significance was considered when $0.05 \leq p < 0.10$. The Tukey t-test was applied at a $p < 0.05$ for multiple mean comparisons among treatments.

3. Results

The mean incubation pH at the start of incubation was 6.43 ± 0.16. This pH dropped to values below 6.0 at 4, 6 and 8 h (5.96 ± 0.05, 5.89 ± 0.07 as minimum average value, and 5.93 ± 0.10, respectively). Thereafter, pH was allowed to recover, reaching its maximum at 20-24 h (6.41). Among treatments (Figure 21), no pH differences were

recorded in the first 4 h ($p > 0.05$), but at 6 h it was lowest with CIN and highest with GCT (5.80 and 5.99; $p < 0.05$). Lower pH values were recorded with CIN than CTR at 12 and 16 h ($p < 0.05$), than LIN at 6 and 8 h, and than MFA, LIN and EUG at 10, 12, 16, and 20 h ($P<0.05$). From 6 h onwards incubation pH was the highest with GCT, recording differences between this treatment and CTR higher than 0.2 pH units.

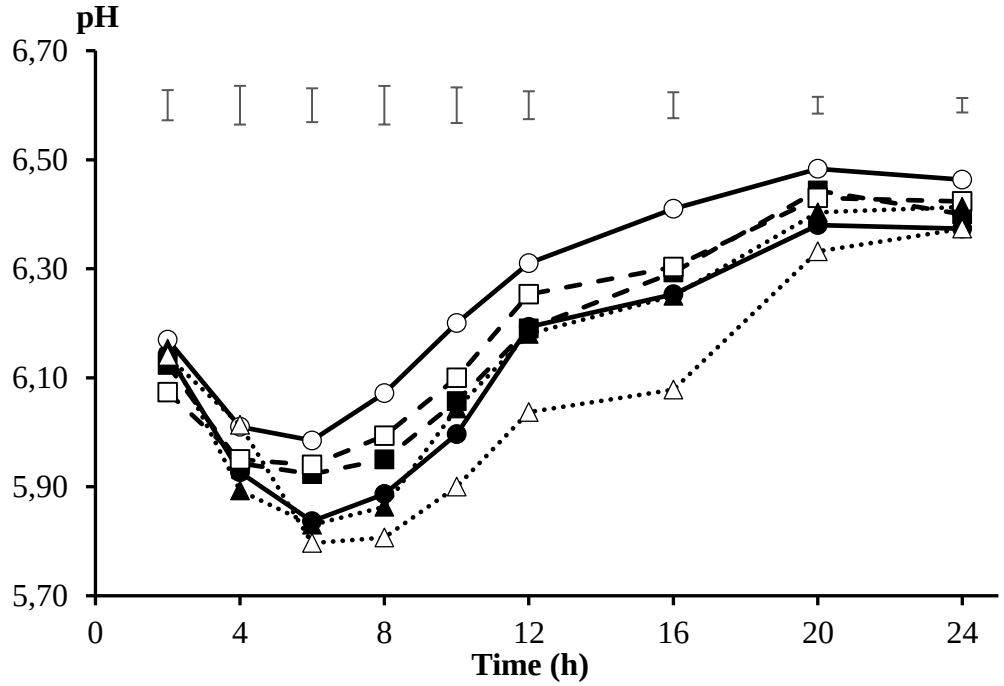

Figure 21. Pattern of *in vitro* incubation pH of barley, alone or supplemented with additives of different nature (CTR ●, GCT ○; solid line, MFA ■, LIN □ ; dashed line, EUG ▲, CIN △; dotted line). Upper bars show standard error of means (n=3).

From 2 to 12 h the lowest volume of gas produced (Figure 22) was recorded by CIN ($p < 0.05$), whereas at 20 and 24 h differences between GCT, CIN, and EUG were not detected ($p > 0.05$). During the whole incubation period, the two essential oils treatments (EUG and CIN) promoted lower volumes than CTR ($p < 0.05$), whereas the volume of gas produced with GCT was lower than CTR after 8 h. Lower volumes were produced when barley was supplemented with MFA and LIN than CTR at 2, 4 and 24 h for MFA, and at 2 and 4 h for LIN; however, no differences were recorded between these treatments and CTR at any other time of measurement.

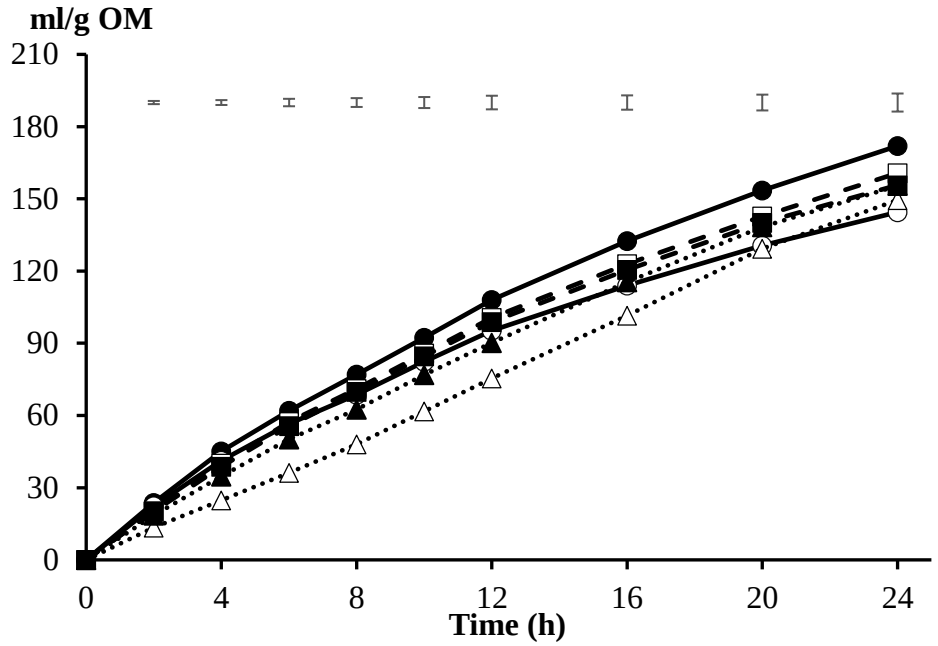

Figure 22. Pattern of *in vitro* gas production from barley alone or supplemented with additives of different nature (CTR ●, GCT ○; solid line, MFA ■, LIN □ ; dashed line, EUG ▲, CIN △; dotted line). Upper bars show standard error of means (n=3).

Following gas production results, DMd showed that the inclusion of different additives reduced barley degradability ($p < 0.001$). Thus, the proportion of DMd after 24 h incubation was the highest ($p < 0.05$, SEM=0.010) with CTR (0.404), followed by CIN, MFA, EUG and LIN (0.356, 0.355, 0,352 and 0.350), and it was lowest with GCT (0.299).

The concentration of total VFA was reduced along the incubation time, being higher ($p < 0.05$) at 8 h than 24 h (Table 17). Besides, molar proportions of acetate and propionate were higher ($p < 0.05$) at 8 h, whereas those of butyrate and valerate recorded higher values at 24 h ($p < 0.05$). On average, the highest concentration of total VFA was recorded by MFA, and the lowest by CIN ($p < 0.05$). Propionate proportion was highest ($p < 0.05$) for LIN, and lowest for EUG ($p < 0.05$), whereas no differences were recorded in acetate, butyrate, valerate and BCVFA proportions ($p > 0.05$). Respect to CTR, both treatments EUG and CIN promoted lower proportion of propionate ($p < 0.05$). Apart for butyrate, where the interaction time x treatment resulted significant ($p = 0.043$), this interaction did not reach significance for any other studied parameter ($p > 0.05$).

Table 17. Main effects means of total volatile fatty acids concentration (VFA, mM) and molar VFA proportions (mmol/mmol) recorded with the different treatments at 8 and 24 h of incubation of barley alone or supplemented with additives of different nature.

		VFA	Acetate	Propionate	Butyrate	Valerate	BCVFA*
Time							
	8	24.1^{a}	0.582^{a}	0.228^{a}	0.146^{b}	0.020^{b}	0.025
	24	16.7^{b}	0.489^{b}	0.181^{b}	0.229^{a}	0.075^{a}	0.027
SEM		0.51	0.0136	0.0063	0.0093	0.0052	0.0011
Treatment							
CTR		21.3^{abc}	0.523	0.222^{ab}	0.179	0.050	0.027
GCT		21.2^{abc}	0.542	0.212^{ab}	0.176	0.045	0.025
MFA		22.1^{a}	0.532	0.225^{ab}	0.168	0.048	0.027
LIN		21.8^{ab}	0.519	0.234^{a}	0.173	0.047	0.028
EUG		18.1^{bc}	0.585	0.148^{c}	0.205	0.039	0.023
CIN		17.7^{c}	0.509	0.185^{bc}	0.222	0.057	0.026
SEM		0.88	0.0235	0.0110	0.0161	0.0091	0.0019
Probability							
Time		<0.001	<0.001	<0.001	<0.001	<0.001	NS
Treatment		0.004	NS	<0.001	NS	NS	NS
Time x Treat.		NS	NS	NS	0.043	NS	NS

^{a,b,c} Means within a column with different superscripts differ (P<0.05). SEM: standard error of means. *BCVFA: branched-chain volatile fatty acids (sum of isobutyrate + isovalerate).

Bacterial biodiversity after 8 h of incubation (Figure 23) were markedly affected by the incubation series (which is equivalent to the donor animal). Apart of this, within each incubation series most treatments clustered together with CTR except for EUG and CIN (especially in series 2 and 3).

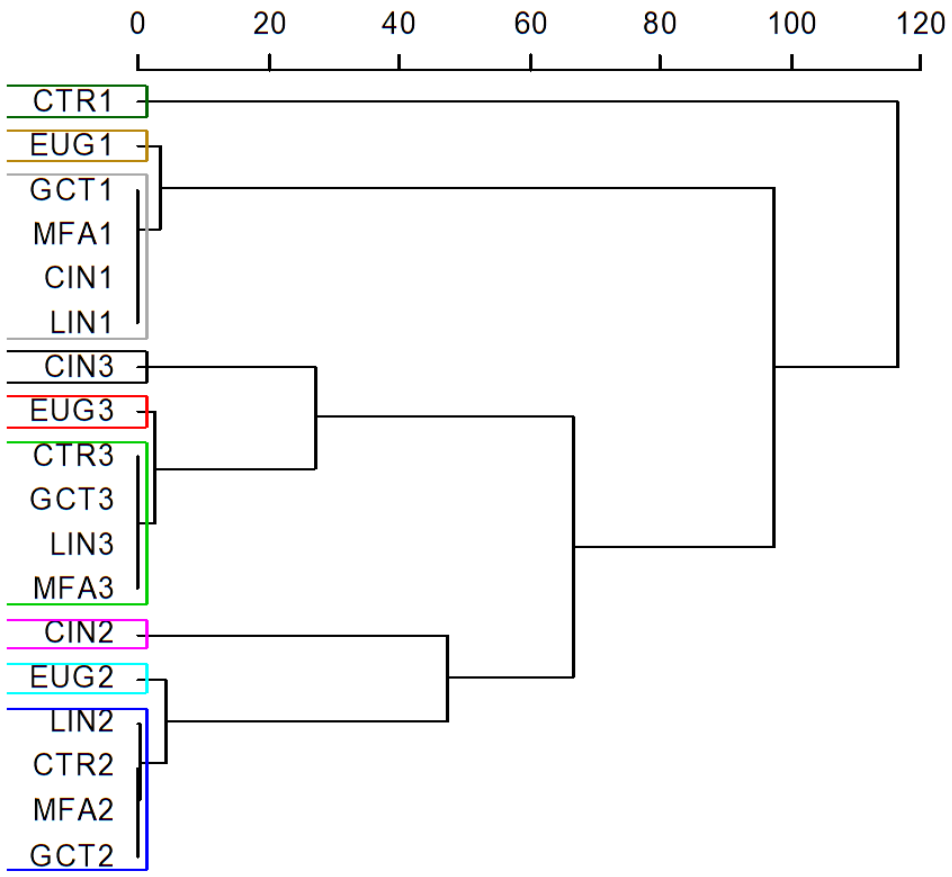

Figure 23. Dendrogram of bacteria diversity from terminal restriction fragment length polymorphism (tRFLP) data generated by enzyme digestion (HhaI, MspI, and HaeIII) at 8 h *in vitro* incubation of barley, alone or supplemented with additives of different nature (CTR, GCT, MFA, LIN, EUG, CIN). Scale bar shows Euclidean distances, "ward method".

4. Discussion

The pattern of incubation pH showed that using a poorly buffered solution from 0 to 6 h and another solution buffered to pH 6.5 from 8 h onwards allowed to mimic the daily *in vivo* rumen pH fluctuation in high concentrate-fed ruminants, reaching a minimum below pH 6.0 from 4 to 8 h incubation, and subsequently increasing to maximum values of 6.4 at 20-24 h. Either in the first 8 h or from 8 h onwards, the pH values recorded were above 5.6, that is considered as a threshold for subacute acidosis (Krause and Oetzel, 2006). However, differences were observed in the response depending on the type of additive. Thus, tannins promoted more favourable medium pH than the non-supplemented barley, and moderate responses were also observed with

medium-chain fatty acids and linoleic acid, confirming the potential effect of these additives for modulating rumen fermentation (Hristov et al., 2004; Amanzougarene at al., 2019; Amanzougarene et al., 2017b). Jones et al. (1994) reported that tannins may inhibit the activity of certain species involved in carbohydrate fermentation, such as *Butyrivibrio fibrisolvens, Streptococcus bovis and Ruminobacter amylophilus*, that are considered as lactate producers, and may negatively affect rumen pH. Similarly, Vazirigohar et al. (2018) suggested that polyunsaturated fatty acids may have some degree of toxicity on carbohydrate digesting bacteria, and Kleverhusen et al. (2011) indicated that the response to medium-chain fatty acids is based on their effect over a large scope of ruminal microbes involved in carbohydrate degradation, thus explaining that fats may depress overall microbial activity (Jenkins, 1994). In our experiment, these hypotheses may justify the increase of medium pH with GCT, and the moderate positive response with LIN and MFA compared to CTR. In contrast, the lowest values of medium pH were observed with both essential oils, especially with CIN. According to Cardozo et al. (2005), medium pH may influence the dissociated or undissociated status of essential oils molecules affecting the rumen fermentation in a different sense, so maintaining a wide range of pH by using different buffer solutions may express the trends in microbial fermentation in response to CIN and EUG compared to the other treatments.

The supplementation of barley with the different additives showed a reduction in the volume of gas produced respect to non-supplemented barley, recording a lower magnitude of fermentation with both essential oils, especially with CIN. The reduction in gas production was supported by the decrease in DMd with all additives respect to CTR. Tannins have a direct effect on microbial diversity (McAllister et al., 1994; McSweeney et al., 2001), but their response on microbial rumen fermentation can be either beneficial or detrimental, depending on their nature and dose (Hagerman and Butler, 1991). Krause et al. (2005) indicated that tannins can be selectively toxic to ruminal bacteria; however, Patra and Saxena (2011) stated that the inclusion of tannins in ruminant diets has generally been beneficial to rumen fermentation. Thus, although the limited availability of proteins and polysaccharides for rumen microbes may reduce the extent of degradation of these nutrients in the rumen (McAllister et al., 1994; Theodoridou et al., 2012; Orlandi et al., 2015), Jones and Mangan (1977) indicated that stable and insoluble complexes formed by bound condensed tannins can dissociate in the abomasum at pH < 3.5, thus increasing nutrient availability in the small intestine for being digested there. In this

regard, Owens et al. (1986) estimated that the non-degraded starch that reaches the intestines to be digested there provides 0.42 more energy than that digested in the rumen.

In terms of VFA, the reduction in total concentration along the incubation period, being highest at 8 h and lowest at 24 h should be explained by the major extent of fermentation occurred in the first part of incubation, but also in part because of the dilution of the incubation system. The interaction time x treatment ($p = 0.043$) observed in butyrate proportion indicates the different behavior of additives between 8 and 24 h incubation. These results may indicate that the potential effect of the different treatments on butyrate-producing bacteria like *Butyrivibrio fibrisolvens, Megasphaera elsdenii* or *Clostridium lochheadii* at 8 h was different than that at 24 h. On average, no differences were detected with GCT respect to control neither on total VFA concentration nor on VFA profile, in agreement with results observed by Patra et al. (2006) and Animut et al. (2008). However, a moderate response was observed with the inclusion of LIN and MFA on VFA concentration and propionate proportion, effect already stated by Doreau et al. (1991). This response can be partly explained by the antibacterial effect of these lipids on fibrolytic rumen bacteria (Kleverhusen et al., 2011; Vazirigohar et al., 2018), that promote a more acetogenic fermentation.

Results with tannins and fats show an effect of minor magnitude on barley fermentation measured as gas production compared to both essential oils tested, which in addition to a decrease in gas production also reduced the VFA concentration and the proportion of propionate compared to the other treatments. In fact, Cardozo et al. (2006) indicated that these two essential oils can modify the microbial fermentation pattern, especially that of VFA. The antimicrobial characteristic of eugenol has also been reported by Dorman and Deans (2000) and Cobellis et al. (2016a), and Castillejos et al. (2006) suggested that the use of this additive in diets for intensively reared beef steers cannot be recommended. In our case, the effect with CIN over all fermentation aspects was more pronounced than expected, especially on incubation pH. Busquet et al. (2005b) suggested that cinnamaldehyde might be an active modulator of rumen microbial activity, and Cardozo et al. (2005) added that this essential oil might be used in high-concentrate diets for intensive beef production because of its potential effects to modulate rumen fermentation and microbiota.

Results of bacterial biodiversity highlight the strong effect of both EUG and CIN on rumen microbiota, confirming the results observed on other parameters (pH, gas production, VFA pattern) with both essential oils. In contrast, the inclusion of MFA and

LIN, which are considered that depress microbial activity (Jenkins, 1994) especially those polyunsaturated, presented a lower effect on bacterial diversity and *in vitro* microbial fermentation of barley. On the other hand, the inclusion of GCT reduced the fermentation of barley but did not modify the pattern of VFA and bacterial biodiversity, and also promoted a higher fermentation pH than the other treatments. The strong effect of the donor animal on microbial diversity observed in this experiment agrees with our results in other studies (Amanzougarene et al., unpublished), but also are in agreement with findings reported by others (Taxis et al., 2015; Söllinger et al., 2018). In any case, it is very important to take into account that experimental factors such as the dose of the inclusion, the type of active compound, the nature of the diet or the experimental conditions (*in vivo* vs. *in vitro*) modulate the comparison of our results with those from other studies.

5. Conclusions

The comparison among additives by means of this semicontinuous *in vitro* incubation system allows for clearly differentiate their effect on rumen fermentation. Additives such as grape condensed tannins, linoleic acid and medium-chain fatty acids moderately reduce *in vitro* ruminal fermentation of barley, at the time they positively affect ruminal environment by maintaining a higher pH than barley alone, especially tannins. In contrast, essential oils, especially cinnamaldehyde, negatively affected environmental conditions and notably reduced barley fermentation to an extent that cannot be expected to compensate at the small gut.

Chapter IX. General discussion

Chapter IX. General discussion

The intensive fattening of early weaned young ruminants requires good feeding management to prevent the onset of digestive disorder processes like acidosis. The *in vitro* evaluation of the ruminal fermentation pattern of different carbohydrate sources used as energy sources in intensive feeding systems helps to know the acidification potential of each carbohydrate type. Knowing the acidification potential of these nutrients is certainly the key factor contributing to acidosis prevention. However, standard *in vitro* conditions, though giving a good approach, are not the best to give an accurate image in the evaluation of the ruminal fermentation pattern of carbohydrates, as it is set up in a pH range between 6.5 and 6.9, well over the common rumen pH occurring in high concentrate diets. Therefore, used *in vitro* methods should be adapted for mimicking high concentrate diets, simulating some of the main physiological conditions such as pH and rate of passage. The interaction of these different parameters is the main factor to evaluate each carbohydrate type, and to obtain a good insight into rumen fermentation processes.

As far as we know, the heterogeneous chemical nature of carbohydrate sources in terms of proportion and nature of structural and/or non-structural carbohydrates is reflected through the rate and magnitude of their fermentation, that differently affect the rumen environment leading to important shifts in bacterial diversity. Thus, the experiments included in section I were carried out using carbohydrate sources as incubation substrates chosen because of their marked differences in chemical structure (starch structure, presence or absence of phenolics compounds, protein matrix, soluble or insoluble fibre polysaccharides, etc.).

Based on the results observed in the experiments of the first section and considering that barley grain is a major component in diets for intensive feeding systems, this cereal grain was chosen as the incubation substrate of reference in the experiments carried out in section II. Several feed additives were used to modulate barley fermentation, as fermentation enhancers/depressors that selectively affect microbial rumen population that are commonly used in diets for fattening ruminants in the last decades. Therefore, a major interest in this doctoral thesis was not only to evaluate and to compare the fermentation pattern of different types of carbohydrate, incubated alone (Experiment 1 of section I) or combined (Experiment 2 of the same section) under conditions simulating high concentrate feeding, but also to demonstrate the potential of certain feed additives to

modulate the microbial fermentation of readily fermentable carbohydrates (Experiments 3 to 5 of section II).

Buffering the pH of incubation medium for intensive feeding conditions

The adjustment of pH of incubation medium within a certain range (5.5-6.5) can be an important tool to study the fermentation kinetics of diets destined to intensive feeding for fattening ruminants. Thus, contrary to the conventional *in vitro* batch system where the medium pH was maintained between 6.7-6.9 (Goering and van Soest, 1970, Mould et al., 2005a), Amanzougarene and Fondevila (2018) demonstrated that the incubation medium pH in such system can be maintained at low levels for some time by reducing the concentration of bicarbonate ion (HCO_3^-) in the incubation solution. The maintenance of a low incubation pH might inform us about the buffering capacity of the incubation substrate and the fermentative capacity of microbiota, as it has been previously applied in our lab (Amanzougarene et al., 2017a; Amanzougarene et al., 2018a), but not about the effects of different shifts of the main rumen physiological factors such as the fluctuations of rumen pH along the day *in vivo.* Besides, with the conventional *in vitro* closed batch system we can study the nutritive value of incubation substrates, but we cannot estimate the effect of the rumen outflow rate for studying the fermentation pattern of high concentrate diets.

Research based on adjustments of *in vitro* systems for the study of the fermentation pattern under conditions mimicking actual rumen conditions in high concentrate diets is scarce. For this reason, in this thesis we hypothesized that fitting of *in vitro* techniques to such conditions can give a real screening about the rumen microbial fermentation of these diets. Therefore, the two *in vitro* fermentation experiments of the first section were carried out using a simple semicontinuous incubation system (Fondevila and Pérez-Espés, 2008, modified by Prates et al., 2010), adapted for controlling incubation pH by applying the procedure proposed by Amanzougarene and Fondevila (2018).

In Experiment 1 the objectives were to evaluate and to compare the rumen fermentation pattern of six carbohydrate sources with varying composition, adapting the rumen pH and the liquid outflow rate to conditions of intensive feeding systems. With the same incubation conditions, the synergistic and antagonistic effects of three mixtures of carbohydrate sources were studied in Experiment 2.

The results obtained in both experiments showed that the rumen fermentation pattern can be simulated *in vitro* by changing the buffer concentration, as well as by maintaining a high liquid outflow rate (0.08/h). Knowing that the incubation medium pH largely depends on the concentration of bicarbonate ion in the incubation solution (Amanzougarene and Fondevila, 2018), during the first six or eight hours of incubation a fraction of incubation solution was replaced at each time by a poorly buffered solution (0.006 M bicarbonate ion), and consequently the medium pH was allowed to abruptly decrease. From 8 h incubation onwards, the incubation solution inoculated for maintaining dilution rate was high in bicarbonate ion concentration (0.058M), and thereafter the medium pH gradually increased to reach its maximum at 20-24 h. With this *in vitro* method, we could reproduce the daily *in vivo* rumen pH fluctuation in high concentrate-fed ruminants, as planned in our hypothesis, showing that the *in vitro* semicontinuous method can be used for the study of microbial fermentation under intensive feeding conditions. Thus, Experiment 3 of section II was carried out using the same methodology, and again the conditions of this experiment reassured those recorded in both Experiments 1 and 2 of section I.

Effect of inoculum source on in vitro fermentation kinetics

According to the results obtained in both experiments carried out in section I, the inoculum source seems to have a major influence on *in vitro* fermentation kinetics of the incubation substrates, as already described (Broudiscou et al., 2014; Amanzougarene et al., 2018a; Kim et al., 2018). In this aspect, using rumen inoculum sources with different characteristics is another tool helping to explain the *in vitro* extent of adaptation of young ruminants abruptly introduced to high carbohydrate diets used in intensive feeding systems.

The results recorded in both Experiments of section I showed that rumen inoculum from forage-based diets maintained a much more stable medium pH than concentrate-based diets, which confirms the potential effect of concentrate inoculum in decreasing the incubation pH (Amanzougarene et al., 2018a). Thus, in Experiment 1 as well as in Experiment 2, it was observed that during the first six hours of incubation, the medium pH remained higher with the forage diet inoculum. The poorly buffering capacity of incubation solution during the first six hours of incubation allow to demonstrate the high

effect of concentrate inoculum causing such decrease in medium incubation pH (Sauvant et al., 2006).

For both Experiments of section I, results showed a greater extent of gas production with concentrate inoculum, results that were reflected in a high concentration of total VFA. However, among inocula, at the studied incubation times the proportions of the main VFA (acetate, propionate and butyrate) did not reach significance in Experiment 1 nor in Experiment 2, indicating that the production of these VFA followed the same trend whatever the source of inoculum. No effect was also detected on the concentration of lactic acid in Experiment 1 (CI; 3.45, FI; 3.31 mM, $P>0.05$), nor in Experiment 2 (CI; 4.36, FI; 3.33 mM, $P>0.05$). These observed concentrations cannot be assumed as a factor for the onset of acidosis, because they are inferior to 5 mmol/L (Nagaraja and Titgemeyer, 2007). The short incubation time (24 h) and the dilution of inoculum (0.20 of total incubation solution), enhanced by the periodic replacement of incubation medium by artificial saliva (every 2 h from 0 to 12 h, and every 4 h from 12 to 24 h) may partly explain the minor magnitude of differences.

Although in both experiments the effect of inoculum sources resulted significant on several parameters studied, no differences were observed in DM disappearance. This response was also found by Calsamiglia et al. (2008), who explained it through a limited OM degradation by microbial activity with the concentrate inoculum, that may result from a limited bacterial growth, where an additional supply of energy does not improve growth or activity. Under these circumstances, bacteria reduce growth efficiency and then degradation of nutrients becomes limited by either concentration or activity of bacteria. Despite of it, the extent of DM disappearance was not expected to be so low (CI; 0.38, FI; 0.34 for both experiments), and in fact starch disappearance recorded in Experiment 1 was also low (CI; 0.48, FI; 0.39). In this regard, comparing *in vitro* results with other from different *in vivo* studies (Demarquilly and Andrieu, 1988; Cerneau and Michalet-Doreau, 1991) where the extent of degradation of substrates was estimated around 0.4-0.8 depending on their richness in fiber and starch, Calsamiglia et al. (2008) concluded that these results were due to differences in the microbial ecosystem between the rumen *in vivo* and the *in vitro* medium.

Comparing the two incubation environments (concentrate inoculum *vs.* forage inoculum) in carbohydrate fermentation, the use of rumen liquid from animals fed with the concentrate diet was more favourable for these incubated substrates than that from

animals given the fibrous diet in both experiments (Menke and Steingass 1988; Mould et al. 2005b). Thus, the three experiments of section II were carried out using rumen liquid from calves given concentrate diets.

Effect of substrates on in vitro fermentation kinetics

The six substrates used in Experiment 1 as carbohydrate sources present clear differences in chemical composition, which were reflected through their magnitude of fermentation during the whole incubation period. Thus, in this experiment we confirmed that among cereal grains, barley ferments at higher extent and promotes a higher drop of medium pH than maize and sorghum (Herrera-Saldana et al., 1990; Amanzougarene et al., 2018a). Similarly, based on results already found by Amanzougarene et al. (2017a), the high drop of pH with CP (Experiment 1) could be expected because of its higher proportion of soluble sugars (Hall et al., 1998; Ariza et al., 2001; Barrios-Urdaneta et al., 2003). In addition, the medium pH also resulted low with WB, which can be attributed to its higher proportion of starch and soluble fibre. Moreover, the high drop in medium pH with CP and WB (although at a lower extent with the latter; Experiment 1) due to the fast fermentation of both substrates was reflected through a higher extent of total VFA concentration, especially with CP. In addition, the lactic acid concentration with CP at 6 h was also the highest (8.68 mmol/l). Therefore, contrary to CP and WB, BP as a byproduct is characterised by a low sugar content, and high NDF and NDSF proportions, that are reflected in a positive effect on rumen pH (Marounek et al., 1985; Münnich et al., 2017; Amanzougarene et al., 2017a). In addition, no marked differences were recorded in Experiment 1 between the fermentation extent of maize and sugarbeet pulp (Amanzougarene et al., 2017a), reasons why we chose both ingredients as components, at different levels, of the different mixtures evaluated in Experiment 2.

Thus, based on results of Experiment 1, in Experiment 2 it was confirmed that the mixture MB, including 0.50 of barley, presented a higher acidification capacity that was reflected through its higher extent of fermentation. These results were clearly due to the faster rate of fermentation of barley starch compared to mixtures including maize and sugarbeet pulp (3MP and MP). Therefore, as initially hypothesized, the partial or total replacement of barley by maize and/or sugarbeet pulp may modulate the rate and extent of ruminal fermentation and thus prevent the onset of acidosis in these animals, especially during the transition period.

Concerning sorghum, despite its positive effect on microbial fermentation (Experiment 1) we preferred barley and maize as ingredients in Experiment 2 because of the shortage of this cereal in European cereal markets, and because its cost is expensive compared to barley and maize that are considered as major ingredients in ruminants diets in Europe.

As expected, the different screenings of *in vitro* fermentation pattern of the incubation substrates obtained in both Experiments 1 and 2 showed positive responses confirming our hypotheses, especially that of the methodology used, in the sense that the *in vitro* semicontinuous system was adapted to study the microbial fermentation in intensive feeding conditions. Thus, our results showed that the adaptation of the *in vitro* systems to conditions based on the study objectives is a key to obtain clear and concrete results.

Inclusion of feed additives to mitigate barley fermentation under in vitro conditions simulating intensive feed production.

As cited above, three experiments were carried out in section II, with different objectives from one to another. Thus, in the first two experiments the objective was to evaluate the potential capacity of certain feed additives at different levels of inclusion in terms of reducing the fermentation of barley, chosen as a model type of concentrate feed, when incubated *in vitro* in closed batch cultures. These additives were tannins (quebracho and grape as condensed tannins, and chestnut and oak as hydrolysable tannins; Experiment 3), and fatty acids and essential oils (a commercial mixture of medium chain fatty acids, palmitic acid and linoleic acid as fatty acids, Experiment 4.1; and cinnamaldehyde, eugenol and a commercial blend of essential oils, Experiment 4.2). In both Experiments, the inoculum used was obtained from calves fed *ad libitum* with a concentrate mixture plus straw, both given *ad libitum*, and the pH of incubation solution was adjusted to about 6.2 to simulate high concentrate feeding conditions. Then, once the different additives were evaluated under conventional *in vitro* batch system conditions, another experiment (Experiment 5) was planned using the semicontinuous *in vitro* incubation system to compare the pattern of response of several additives, that were chosen based on the results of the first two experiments, on their effects on barley fermentation when the *in vitro* conditions were adjusted to the study of the fermentation pattern of high concentrate diets. This experiment was carried out with the same inoculum

type than in the first two experiments of this section, adjusting the buffer solution to a pH of 5.5 during the first six hours and allowing it to rise to around 6.5 from 8 hour onwards.

Effect of type and level of tannins, fatty acids, and essential oils

When unsupplemented barley was compared to the inclusion of different additives included at different levels in Experiments 3 and 4 no marked differences on incubation pH were detected, which means that the adjustment of medium pH around 6.2 was enough to prevent further effects on medium pH. In fact, this pH may be considered as slightly high to study the barley fermentation in conditions of intensive feeding; however, it is important to note that the buffer effect in the incubation solution is exhausted after ≈ 10 h of incubation, as previously proved (Amanzougarene and Fondevila, 2018). On the other hand, the use of inoculum from beef calves fed on a high concentrate diet can reassure the incubation conditions in terms of incubation pH values and also in terms of rumen microbial population adaptation to fermentation of concentrate diets.

In the first Experiment of this section, increasing levels of tannin extracts reduced in a different extent the magnitude of barley fermentation; thus, the extent of reduction was lower with CHT and higher with GCT, that showed a marked effect on microbial fermentation pattern. The effect of tannins on rumen fermentation pattern has been widely demonstrated, and it has been reported that tannins reduced microbial digestion of proteins (Hagerman et al., 1992; McAllister et al., 1994; Frutos et al., 2000) or fiber (Chiquette et al., 1988; Rodríguez et al., 2011). Barros et al. (2012) also reported the presence of interactions between condensed tannins and starch. This reduction in nutrients digestion was reflected in the lower rate and extent of feeds fermentation in the rumen. A lower rate and extent of substrate fermentation by the presence of phenolic compounds has been recently demonstrated by our group (Amanzougarene et al. 2018a; Amanzougarene et al. 2018b). In the present experiment the effect was markedly observed on gas production, and the effect on other parameters especially pH and VFA concentration was of minor magnitude. However, the classification of tannins into condensed and hydrolysable tannins does not imply that their effects are the same within each type, and Mueller-Harvey (2006) reported that the effects of tannins are relatively independent of their chemical composition. Thus, the observed response in this experiment between OHT and CHT, considered as hydrolysable tannins was not the same, neither that between GCT and QCT as condensed tannins extracts.

In Experiment 4.1, when barley was supplemented with fatty acids, its fermentation was reduced, as above. However, the effect of these lipids was of minor magnitude. The effect LIN resulted noteworthy compared to MFA and PAL. Furthermore, the microbial mass was the studied parameter that undergoes remarkable reductions, especially with high level of MFA, and similarly Maczulak et al. (1981) reported that the effect of lipids on rumen environment reduces bacterial growth. These results may be explained by the antimicrobial effect of dietary fats. However, it should be taken into consideration that each type of fatty acids has different characteristics, and Palmquist and Jenkins (1980) and Jenkins (1993) stated that the rumen microbial fermentation activity is more affected by polyunsaturated than by saturated fatty acids. Others (Sinclair et al., 2005; Vazirigohar et al., 2018) have shown reductions in gas production profiles when diets are rich in polyunsaturated fatty acids. In the case of medium-chain fatty acids, although Hristov et al. (2004) reported that these fats may modulate ruminants fermentation and improve nutrients utilisation, Kleverhusen et al. (2011) indicated that these fats exert a strong effect on ruminal bacteria involved in carbohydrates degradation, and it has been observed that nutrients disappearance is reduced after their addition (Soliva et al., 2004; Machmüller, 2006).

The inclusion of certain essential oils in intensively reared ruminant diets may be an important tool to reduce the risk of acidosis. Nevertheless, their effects depend on factors such as the extent of their selective antimicrobial properties against different rumen microorganisms. Thus, in the second experiment of this section (Experiment 4.2), the supplementation of barley with EUG, CIN and CBC showed different responses. Microbial fermentation of barley increased at a higher extent with CBC than with EUG or CIN. Hence, although the reduction in the rate of barley fermentation when it was supplemented with cinnamaldehyde confirms the selective effect of this essential oil over some gram-positive bacteria (Cardozo et al., 2006), the high extent of depression was unexpected. Thus, the medium pH should be lower for reaching positive effects with cinnamaldehyde on rumen bacterial fermentation (Juven et al., 1994; Cardozo et al., 2005), and therefore in our case the adjustment of buffer solution around of 6.2 might be too high, as cited above. In the same way, the observed response with eugenol, especially on total volatile fatty acids and microbial mass can be justified by the strong effect of this essential oil on ruminal bacteria (Dorman and Deans, 2000). In case of the commercial blend of essential oils used herein, its positive effect might be explained by the possible existence of synergistic and/or antagonistic effects among the different compounds of this

mixture (Burt, 2004). In ageement with what was cited regarding cinnamaldehyde, Spanghero et al. (2008) suggested that the effectiveness of the blend of essential oils might be higher under acidic medium conditions.

In any case, based on what was observed in both Experiments 3 and 4, the reduced fermentation with the different additives (except with CBC) cannot be considered as a negative effect, assuming that, *in vivo*, substrate proportions that are not digested in the rumen will reach lower sites of the gastrointestinal tract where can be digested and directly provide nutrients to the animals. On the other hand, none of the additives used in these experiments negatively affected the medium pH.

In the last Experiment from this section, five additives (grape condensed tannins, the mixture of mid-chain fatty acids, linoleic acid, eugenol and cinnamaldehyde) were chosen based on the results observed in both previous Experiments of this section, and they were studied to evaluate and to compare their effects on *in vitro* barley fermentation in intensive feeding ruminant diets, using the semicontinuous *in vitro* incubation system, and thus avoiding some of the methodological problems detected, that might affect the magnitude of the response.

The results of this Experiment were distinguished by the highest pH values recorded with GCT from 6 h onwards. Compared to the results obtained with this additive when included at its medium level in Experiment 3 (when a conventional *in vitro* closed batch system was used), it can be seen that the simple semicontinuous *in vitro* incubation system adjusted to conditions of high concentrate diets fermentation wasd a useful tool to give a clearer vision of the effect of this additive. In the same way, when a conventional system was used, CIN and GCT showed the same trend reducing the barley fermentation without marked effects on medium pH, but lower values of pH were recorded by CIN when the semicontinuous system was used, and both essential oils reduce fermentation respect to barley alone. The minor effects of both fatty acids on barley fermentation pattern observed in this Experiment support those observed with both additives when the *in vitro* closed batch cultures system was used. Furthermore, the results of bacterial biodiversity in this Experiment showed a strong effect of both EUG and CIN on rumen microbiota, whereas supplementation of barley with MFA and LIN resulted in a lower effect on bacterial diversity and the inclusion of GCT did not modify the pattern of bacterial biodiversity.

The unexpected strong negative effect of the essential oils tested in this experiment could be associated to the antimicrobial characteristics of these additives (Busquet et al., 2005; Castillejos et al., 2006). Thus, Castillejos et al. (2006) suggested that eugenol cannot be recommended in diets for intensive beef production. However, contrary to our results, Busquet et al. (2005b) and Cardozo et al. (2005) recommended the use of cinnamaldehyde in intensive beef production diets because of its potential effects to modulate rumen fermentation. This negative effect may be due to the evolution of the buffer capacity of the incubation solution during the whole incubation period, since medium pH may influence the dissociated or undissociated status of essential oils molecules, affecting rumen fermentation in a different sense (Cardozo et al., 2005). The positive effect of GCT and, in a minor extent with LIN and MFA on medium pH may confirm the capacity of these additives to inhibit the activity of certain species involved in carbohydrate fermentation (McAllister, et al., 1994; Kleverhusen et al., 2011; Vazirigohar et al., 2018).

Finally, based on the results from the Experiments of section II, it can be suggested that the *in vitro* semicontinuous system is an adequate tool to evaluate the effects of the different additives on rumen microbial fermentation under intensive feeding conditions compared to the closed batch cultures. Through the use of this system it could be clearly concluded that grape condensed tannins bind to barley components and reduce its *in vitro* fermentation, at the time they may positively affect ruminal environment by maintaining a higher pH than with barley alone. Instead, the fatty acids affected at a minor magnitude both barley fermentation and environmental conditions. Thus, both types of additives may reduce barley acidification potential, reason why their inclusion in diets for fattening of young ruminants could be recommended. In contrast, essential oils, especially cinnamaldehyde, promoted an acute drop on pH and also reduce at a high extent barley fermentation, which cannot be of interest for using in these types of diets.

Chapter X. Conclusions

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Conclusions

Based on the different results of all experiments carried out in this doctoral thesis, the following conclusions can be derived:

1. The inoculum source strongly affects both the microbial fermentation pattern of incubation substrates and the medium pH. The use of rumen liquid from animals fed with a concentrate diet promotes a wider fluctuation of incubation pH and render a higher volume of gas production than when it is obtained from animals given a forage diet that is not well adapted to fermentation of non-fibrous carbohydrates. However, buffering of medium under low pH conditions may overestimate fermentation differences between inocula by increasing indirect gas production.

2. The chemical composition of the substrates determines the rate and extent of microbial fermentation between the different carbohydrate sources. Sources of highly fermentable fibre combined with sugars, such as citrus pulp, or at a lower extent with starch, such as wheat bran may create a more acidic environment than cereal grains. This acidification capacity is also associated with a high fermentation, as indicated by the *in vitro* gas fermentation pattern.

3. The inclusion of feed mixtures including by-products such as sugarbeet pulp at 0.50 of total diet in diets for fattening ruminants may promote positive effects on ruminal fermentation due to synergistic interactions between diet components, promoting a more stable environment without largely affecting the extent of fermentation.

4. The effect of inclusion of the different additives used in this doctoral thesis on *in vitro* microbial fermentation of barley depends on factors related with the experimental conditions (type of incubation system, medium pH), the concentration of the active compound and the dose of inclusion, that may confound their comparison and impede the understanding of their efficiency.

5. Quebracho, grape, chestnut and oak tannins reduced rumen microbial fermentation of barley grain, with maximum and minimum responses being recorded with grape and chestnut tannins, respectively. Except for chestnut, all the other sources already reached their maximum level of response at their first level of inclusion (10 mg/g substrate). Qualitatively, the addition of tannins did not largely affect medium pH or other environmental parameters, except for an increase in butyrate proportion.

6. The magnitude and the rate of *in vitro* barley fermentation were reduced by the use of medium-chain fatty acids, palmitic and linoleic. Similarly, a linear depression was observed with cinnamaldehyde, and a negative response although of a lower magnitude was also observed up to dose (d2) of eugenol. In contrast, the commercial blend CRINA Ruminants linearly increased the magnitude and rate of barley fermentation. Both fatty acids and essential oils did not negatively affect medium pH throughout the incubation, suggesting that both types of additives may modulate barley acidification potential. The reduction in the extent of barley fermentation with some of these additives may be nutritionally beneficial to beef cattle, since the increased nutrient proportions that reach the lower sites of the gastrointestinal tract could be digested there.

7. Additives such as grape condensed tannins, linoleic acid and medium-chain fatty acids moderately reduce *in vitro* ruminal fermentation of barley, at the time they positively affect ruminal environment by maintaining a higher pH than barley alone. In contrast, essential oils, especially cinnamaldehyde, negatively affected environmental conditions and notably reduced barley fermentation to an extent that cannot be expected to compensate at the small gut.

8. The choice of the adequate methodology is a key to obtain clear and concrete results in the study of study the *in vitro* microbial fermentation of substrates under conditions simulating high concentrate feeding. Thus, reducing bicarbonate concentration in the incubation solution allows for adjusting medium pH to values of 5.5 to 6.0, as it is highly correlated with the *in vitro* gas production. Besides, to evaluate the *in vitro* microbial fermentation of concentrate substrates, the *in vitro* semicontinuous system used herein allows for the study of fermentation under daily pH pattern and liquid outflow rate that simulate rumen conditions of high concentrate diets.

Conclusiones

Partiendo de los resultados obtenidos en los distintos experimentos realizados en la presente tesis doctoral, se pueden extraer las siguientes conclusiones:

1. La fuente del inóculo afecta notablemnete tanto el patrón de fermentación microbiana de los sustratos de incubación como el pH del medio. El uso de líquido ruminal de animales alimentados con una dieta concentrada promueve una fluctuación más amplia del pH de incubación y genera un mayor volumen de producción de gas comparado con el inóculo procedente de animales alimentados con una dieta a base de forraje, ya que éste no está adaptado a la fermentación de carbohidratos no fibrosos. Sin embargo, el sistema tampón del medio en condiciones de bajo pH puede sobreestimar las diferencias de fermentación entre los inóculos al aumentar la producción indirecta de gas.

2. La composición química de las diferentes fuentes de carbohidratos empleadas como sustratos determina el ritmo y la magnitud de la fermentación microbiana entre. Alimentos ricos en fibra altamente fermentable, asociada con azúcares, como la pulpa de cítricos o en meor medida con almidón, como el salvado de trigo, pueden crear un ambiente más acidótico que los granos de cereales. Esta capacidad de acidificación también está asociada con una alta fermentación, como lo indica el patrón de fermentación de gas *in vitro*.

3. La administración de mezclas incluyendo subproductos tales como la pulpa de remolacha al 0,50 de la dieta total en dietas para rumiantes de engorde puede promover efectos positivos sobre la fermentación ruminal debido a interacciones sinérgicas entre componentes de la dieta, favoreciendo un entorno más estable sin afectar en gran medida el grado de fermentación.

4. El efecto de la inclusión de los diferentes aditivos utilizados en esta tesis doctoral sobre la fermentación microbiana de cebada *in vitro* depende de factores relacionados con las condiciones experimentales (tipo de sistema de incubación, pH del medio), la concentración del compuesto activo y la dosis de inclusión, pudiendo interferir en su comparación e impedir la comprensión de su eficacia.

5. Los taninos de quebracho, uva, castaño y roble redujeron la fermentación microbiana ruminal del grano de cebada, registrando respuestas máximas y mínimas con taninos de uva y castaño, respectivamente. Excepto para el castaño, el resto de los extractos alcanzaron su máximo nivel de respuesta con primer nivel de inclusión valorado (10 mg/g de sustrato). Cualitativamente, la adición de taninos no afectó sustancialmente el pH promedio u otros parámetros ambientales, excepto un aumento en la proporción de butirato.

6. La magnitud y ritmo de fermentación *in vitro* de la cebada se redujeron mediante la adición de ácidos grasos de cadena media, ácido palmítico y ácido linoleico. Del mismo modo, se observó una depresión lineal con el cinamaldehído, y una respuesta negativa, aunque de menor magnitud con el eugenol hasta la dosis d2. En contraste, la mezcla comercial de aceites esenciales mejoró linealmente la magnitud y la tasa de fermentación de la cebada. Los ácidos grasos y los aceites esenciales no afectaron negativamente el pH del medio durante la incubación, lo que sugiere que ambos tipos de aditivos pueden modular el potencial de acidificación de la cebada. La reducción en la extensión de la fermentación de la cebada con algunos de estos aditivos puede tener un efecto nutricionalmente beneficioso para el ganado vacuno, ya que la mayor proporción del alimento que debería llegar a los segmentos más distales del tracto gastrointestinal podría digerirse allí, aportando una mayor proporción de nutrientes a los animales.

7. Aditivos tales como los taninos condensados de uva, el ácido linoleico y los ácidos grasos de cadena media reducen moderadamente la fermentación ruminal *in vitro* de la cebada, en el momento en que afectan positivamente el ambiente ruminal al mantener un pH más alto que la cebada sola. Por el contrario, los aceites esenciales, especialmente el cinamaldehído, afectaron negativamente las condiciones ambientales y redujeron notablemente la fermentación de la cebada hasta alcanzar el punto en que no sea posible compensarse en el intestino delgado.

8. La elección de una metodología adecuada es clave para obtener resultados claros y concretos en el estudio de la fermentación microbiana de sustratos *in vitro* en condiciones que simulen una alta inclusión de concentrados. Para ello, la reducción de la concentración de bicarbonato en la solución de incubación permite ajustar el pH del

medio a valores de 5,5 a 6,0, reduciendo, ya que está altamente correlacionada con la producción de gas *in vitro*. Además, para evaluar la fermentación microbiana de sustratos concentrados, el sistema semicontinuo *in vitro* utilizado en este trabajo permite el estudio de la fermentación bajo un patrón diario de pH y una tasa de salida de líquido que simulan las condiciones del rumen en las dietas consistentes en alto contenido de concentrado.

References

References

Ahmed, M. G., El-Zarkouny, S. Z., EL-Shazli, K. A., and Sallam, S. M. A. (2014). Impact of essential oils blend on methane emission, rumen fermentation characteristics and nutrient digestibility in barki sheep. J Agric Sci. 6-7: 144-156.

Al-Dobaib, S. N. (2009). Effect of different levels of quebracho tannin on nitrogen utilization and growth performance of Najdi sheep fed alfalfa (Medicago sativa) hay as a sole diet. Anim. Sci. J. 80: 532-541.

Allen, M. S. (1997). Relationship between fermentation acid production in the rumen and the requirement for physically effective fibre. J. Dairy Sci. 80: 1447-1462.

Álvarez del Pino, M. C., Hervás, G., Mantecón, A. R., Giráldez, F. J., and Frutos, P. (2005). Comparison of biological and chemical methods, and internal and external standards, for assaying tannins in Spanish shrub species. J. Sci. Food Agric. 85: 583-590.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2017a). *In vitro* fermentation pattern and acidification, potential of different sources of carbohydrates by ruminants giving high concentrate diets. Span. J. Agric. Res. 15: e0602.

Amanzougarene, Z., Yuste, S., Vega, A., and Fondevila, M. (2017b). Fermentación *in vitro* de cebada en función del nivel de inclusión de ácidos grasos en dietas para el cebo de terneros. XVI Jornadas sobre Producción Animal 168-170 May 30-31, Zaragoza, Spain.

Amanzougarene, Z., Yuste, S., Vega, A., and Fondevila, M. (2017c). Efecto de la inclusión de aceites esenciales en dietas para el cebo de terneros sobre la fermentación *in vitro* de cebada. XVI Jornadas sobre Producción Animal 258-260 May 30-31, Zaragoza, Spain.

Amanzougarene, Z., and Fondevila, M. (2018). Fitting of pH conditions for the study of concentrate feeds fermentation by the *in vitro* gas production technique. Anim. Prod. Sci. 58: 1751-1757.

.

Amanzougarene, Z., Yuste, S., Castrillo, C., and Fondevila, M. (2018a). *In vitro* acidification potential and fermentation pattern of different cereal grains incubated with inoculum from animals given forage or concentrate-based diets. Anim. Prod. Sci. 58: 2300-2307.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2018b). Differences in nutritional characteristics of three varieties of sorghum grain determine their *in vitro* rumen fermentation. Span. J. Agric. 16: e0608.

Amanzougarene, Z., Yuste, S., and Fondevila, M. (2019). Addition of several tannin extracts to modulate fermentation of barley meal under intensive ruminant feeding conditions simulated *in vitro* by incubating at pH 6.0–6.2. Anim. Prod. Sci. 59: 1081-1089.

Analytical Software. (2010). Statistix 10 for Windows. Analytical Software, Tallahasee, FL.

Animut, G., Puchala, R., Goetsch, A., Patra, A., Sahlu, T., Varel, V., and Wells, J. (2008). Methane emission by goats consuming different sources of condensed tannins. Anim. Feed Sci. Technol. 144: 228-241.

AOAC (2005). Official Methods of Analysis, 18th ed. (Horwitz W, Latimer GW, eds.), *Association of Official Analytical Chemists*. Gaithersburg. MD. USA.

Araujo-Febres, O., and Vergara-López, J. (2007). Propiedades físicas y químicas del rumen. Arch. Latinoam. Prod. Anim. 15: 133-140.

Ariza, P., Bach, A., Stern, M. D., and Hall. M. B. (2001). Effects of carbohydrates from citrus pulp and hominy feed on microbial fermentation in continuous culture. J. Anim. Sci. 79: 2713-2718.

Bae, H. D., McAllister, T. A., Yanke, J., Cheng, K. J., and Muir, A. D. (1993). Effects of condensed tannins on endoglucanase activity and filter paper digestion by fibrobacter succinogenes S85. Appl. Environ. Microbiol. 59: 2132-2138.

Balandrin, M. F., and Klocke, J. A. (1985). Natural plant chemicals: Sources of industrial and medicinal materials. Science. 228: 1154-1160.

Balch, D. A., and Rowland, S. J. (1957). Volatile fatty acids and lactic acid in the rumen of dairy cows receiving a variety of diets. Br. J. Nutri. 11: 288-298.

Baldwin, R. L., and Allison, M. J. (1983). Rumen Metabolism. J. Anim. Sci. 57: 461-477.

Baldwin, R. L. (1999). The proliferative actions of insulin, insulin-like growth factor-1, epidermal growth factor, butyrate and propionate on ruminal epithelial cells *in vitro*. Small Ruminant Res. 32: 261-268.

Bampidis, V. A., and Robinson, P. H. (2006). Citrus by-products as ruminant feeds: A review. Anim. Feed Sci. Technol. 128: 175-217.

Barker, S. B., Summerson, W. H. (1941). The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138: 535-554.

Barrios-Urdaneta, A., Fondevila, M., and Castrillo, C. (2003). Effect of supplementation with different proportions of barley grain or citrus pulp on the digestive utilization of ammonia-treated straw by sheep. Anim. Sci. 76: 309-317.

Barros, F., Awika, J. M., and Rooney, L. W. (2012). Interaction of tannins and other sorghum phenolic compounds with starch and effects on in vitro starch digestibility. J. Agr. Food Chem. 60: 11609-11617.

Barry, T. N., and Duncan, S. J. (1984). The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 1. Voluntary intake. Brit. J. Nutr. 51: 485-491.

Barry, T. N., and Manley, T. R. (1984). The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep. 2. Quantitative digestion of carbohydrates and proteins. Brit. J. Nutr. 51: 493-504.

Bartley, E. E. (1976). Bovine saliva: production and function. In: M.S. Weinberg and A.L. Sheff- her (Editurs), Buffers in Ruminant Physiology and Metabolism. Church and Dwight, New York, NY, pp. 61-81.

Bate-Smith, E. C., and Swain, T. (1962). Flavonoid compounds. In Comparative Biochemistry. Eds. H S Mason and A M Florkin. Academic Press, New York. pp. 755-809.

Bauman, D. E., Harvatine, K. J., and Lock, A. L. (2011). Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annu. Rev. Nutr. 31: 299-319.

Bayat, A. R., Tapio, I., Vilkki, J., Shingfield, K. J., and Leskinen. H. (2018). Plant oil supplements reduce methane emissions and improve milk fatty acid composition in dairy cows fed grass silage-based diets without affecting milk yield. J. Dairy Sci. 101:1136-1151.

Bayourthe, C., and Ali-Haimoud-Lekhal, D. (2014). Les extraits de plantes chez le ruminant : effets sur les fermentations dans le rumen et la qualité lipidique des produits animaux. INRA. Prod Anim. 27: 317-328.

Beauchemin, K. A., McGinn, S. M., Benchaar, C., and Holtshausen, L. (2009). Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. J. Dairy Sci. 92: 2118-2127.

Beauchemin, K. A., McGinn, S. M., Martinez, T. F., and McAllister, T. A. (2007). Use of condensed tannin extract from quebracho trees to reduce methane emissions from cattle. J Anim Sci. 85:1990-1996.

Belanche, A., Doreau, M., Edwards, J. E., Moorby, J. M, Pinloche, E., and 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. J. Nut. 142: 1684-1692.

Belbis, G. H. (2007). Flore du rumen: origine, composition, évolution, conséquences Physiologiques. PhD thesis, Faculté de Médecine de Créteil, France.

Benchaar, C., Petit, H. V., Berthiaume, R., Whyte, T. D., and Chouinard, P. Y., (2006a). Effects of addition of essential oils and monensin premix on digestion, ruminal fermentation, milk production and milk composition in dairy cows. J. Dairy Sci. 89: 4352-4364.

Benchaar, C., Duynisveld, J. L., and Charmley, E., (2006b). Effects of monensin and increasing dose levels of a mixture of essential oil compounds on intake, digestion and growth performance of beef cattle. Can. J. Anim. Sci. 86: 91-96.

Benchaar, C., Chaves, A. V., Fraser, G. R., Wang, Y., Beauchemin, K. A., and McAllister, T.A. (2007). Effects of essential oils and their components on *in vitro* rumen microbial fermentation. Can. J. Anim. Sci. 87: 413-419.

Benchaar, C., Calsamiglia, S., Chaves, A. V., Fraser, G. R., Colombatto, D., McAllister, T. A., and Beauchemin, K. A. (2008). A review of plant-derived essential oils in ruminant nutrition and production. Anim. Feed Sci. Technol. 145: 209-228.

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

Benchaar, C., Lettat, A., Hassanat, F., Yang, W. Z. Forster, R. J., Petit, H. V., and Chouinard, P.Y. (2012). Eugenol for dairy cows fed low or high concentrate diets: Effects on digestion, ruminal fermentation characteristics, rumen microbial populations and milk fatty acid profile. Anim. Feed Sci. Technol. 178: 139-150.

Bergman, E. N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol. Rev. 70: 567-589.

Bertipaglia, L. M. A., Fondevila, M., Van Laar, H., and Castrillo, C. (2010) 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.

Bevans, D. W., Beauchemin, K. A., Schwartzkopf-Genswein, K. S., McKinnon, J. J., and McAllister, T. A. (2005). Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle. J. Anim. Sci. 83: 1116-1132.

Bhat, T. K., Singh, B., and Sharma, O. P. (1998). Microbial degradation of tannins-A current perspective. Biodegradation. 9: 343-357.

Bhatta, R., Krishnamurty, U., and Mohammed, F. (2000). Effect of feeding tamarind (Tamarindus indica) seed husk as a source of tannin on dry matter intake, digestibility of nutrients and production performance of cross−bred dairy cows in mid lactation. Anim. Feed Sci. Technol. 83: 67-74.

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

Blain, J. C. (2002). Introduction à la nutrition des animaux domestiques. Technique et documentation. 425 p.

Boeckaert, C., Boon, N., Abdulsudi, I. Z., Verstraet, W., and Fievez, V. (2006). Accumulation of biohydrogenation intermediates and changes in rumen protozoal population. Commun. Agric. Appl. Biol. Sci. 71: 83-86.

Bolton, J. R., and Pass, D. A. (1988). The alimentary tract. Clinicopathologic principles for veterinary medicine. Robinson, W. F., and Huxtable, C. R. R. Cambridge, Cambridge University Press: 99-121.

Britton, R. A., and Stock, R. A. (1987). Acidosis, rate of starch digestion and intake. Oklahoma Agricultural Experiment Station.MP. 125-137.

Britton, R., and Stock, R. (1989). Acidosis: A continual problem in cattle fed high grain diets. pp. 8-15 in Proc. Cornell Nutr. Conf. Feed Manuf., Cornell Univ., Ithaca, NY.

Broudiscou, L. P, Offner, A., and Sauvant, D. (2014). Effects of inoculum source, pH, redox potential and headspace di-hidrogen on rumen *in vitro* fermentation yields. Animal. 8: 931-937.

Brown, M. S., Krehbiel, C. R., Galyean, M. L., Remmenga, M. D., Peters, J. P., Hibbard, B., Robinson, J., and Moseley, W. M. (2000). Evaluation of models of acute and subacute acidosis on dry matter intake, ruminal fermentation, blood chemistry, and endocrine profiles of beef steers. J. Anim. Sci. 78: 3155-3168

Brugere-Picoux, J. (2004). Acidose lactique du rumen. Maladies des moutons, 2ème éd. Ed. France Agricole: 133-137.

Bueno, I. C. S., Vitti, D. M. S. S., Louvandini, H., and Abdalla, A. L. (2008). A new approach for *in vitro* bioassay to measure tannin biological effects based on a gas production technique. Anim. Feed Sci. Technol. 141: 153-170.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. Int. J. Food Microbiol. 94: 223-253.

Busquet, M., Calsamiglia, S., Ferret, A., and Kamel. C. (2005a). Screening for effects of plant extracts and active compounds of plants on dairy cattle rumen microbial fermentation in a continuous culture system. Anim. Feed Sci. Technol. 123-124: 597-613.

Busquet, M., Calsamiglia, S., Ferret, A., Cardodo, P. W., and Kamel, C. (2005b). Effect of cinnamaldehyde and garlic oil on rumen microbial fermentation in a dual flow continuous culture. J. Dairy Sci. 88: 2508-2516.

Busquet, M., Calsamiglia, S., Ferret, A., and Kamel, C. (2006). Plant extracts affect *in vitro* rumen microbial fermentation. J. Dairy Sci. 89: 761-771.

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

Calsamiglia, S., Cardozo, P. W., Ferret, A., and Bach, A. (2008). Changes in rumen microbial fermentation are due to a combined effect of type of diet and pH. J. Anim. Sci.86: 702-711.

Calsamiglia, S., Blanch, M., Ferret A., and Moya, D. (2012). Is subacute ruminal acidosis a pH related problem? Causes and tools for its control. Anim. Feed Sci. Technol. 172: 42-50.

Cardozo, P. W., Calsamiglia, S., Ferret, A., and Kamel, C. (2004). Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. J. Anim. Sci. 82: 3230-3236.

Cardozo, P. W., Calsamiglia, S., Ferret, A., and Kamel, C. (2005). Screening for the effects of natural plant extracts at different pH on *in vitro* rumen microbial fermentation of a high-concentrate diet for beef cattle. J Anim. Sci. 83: 2572-2579.

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

Carreño, D., Hervás, G., Toral, P. G., Belnguer, A., and Frutos, P. (2015). Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep. Anim. Feed Sci. Technol. 202: 42-51.

Carro, M. D., Valdés, C., Ranilla M. J., Gonzáles J. S. (2000). Effect of forage to concentrate ration in the diet on ruminal fermentation and digesta flow kinetics in sheep offered food at a fixed and restricted level of intake. Anim. Sci. 70: 127-134.

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

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

Castillejos, L., Calsamiglia, S., Ferret, A., and Losa, R. (2007). Effects of dose and adaptation time of a specific blend of essential oils compounds on rumen fermentation. Anim. Feed Sci. Technol. 132: 186-201.

Cerneau, P., and Michalet-Doreau, B. (1991). *In situ* starch degradation of different feeds in the rumen. Reprod. Nutr. Dev. 31: 65-72.

Chalupa, W., Rickabaugh, B., Kronfeld, D. S., Sklan, D. (1984). Rumen fermentation in vitro as influenced by long chain fatty. acids. J. Dairy Sci. 67: 1439-1444.

Chao, S. C., Young, D. G., Oberg, and C. J. (2000). Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. J. Essent. Oil Res. 12: 639-649.

Chaucheyras-Durand, F., and Durand, H. (2010). Probiotics in animal nutrition and health. Benef. Microbes. 1: 3-9.

Chesson, A. and Forsberg, C. W. (1988). Polysaccharide degradation by rumen microorganisms. In: The Rumen Microbial Ecosystem. Hobson P. N., Ed. Elsev. Sci. Publis. NY. 251-284.

Chevalier, F. H. (2001). La digestion ruminale des amidons: cinétique et applications. PhD Thesis, Université Paul-Sabatier de Toulouse, France.

Chilliard, Y., and Ollier, A. (1994). Alimentation lipidique et métabolisme du tissu adipeux chez les ruminants. Comparaison avec le porc et les rongeurs. INRA Prod. Anim. 7: 293-308.

Chiquette, J., Cheng, K. J., Costerton. J. W., and Milligan, L. P. (1988) Effect of tannins on the digestibility of two isosynthetic strains of birds foot trefoil (Lotus corniculatus L.) using in vitro and in sacco techniques. Can. J. Anim. Sci. 68: 751-760.

Church, D. C. (1988). The Ruminant Animal: digestive physiology and nutrition. Prentice-Hall, Inc. Englewood Cliffs, New Jersey. 564

Cobellis, G., Trabalza-Marinucci, M., and Yu, Z., (2016a). Critical evaluation of essential oils as rumen modifiers in ruminant nutrition: A review. Sci. Total Environ. 545-546: 556-568.

Cobellis, G., Trabalza-Marinucci, M., Marcotullio, M.C., and Yu, Z. (2016b). Evaluation of different essential oils in modulating methane and ammonia production, rumen fermentation, and rumen bacteria in vitro. Anim. Feed Sci. Technol. 215: 25-36.

Colonna, P., Buléon, A., Leloup, V., Thibault, J. F., renard, C., Lahaye, M., and Viroben, G. (1995). In: Nutrition des ruminants domestiques. Jarrige, R., Ruckebusch, Y., Demarquilly, C., Farce, M-H. and Journet, M. INRA. Ed. Chap 3: 83-119.

Costa, D. F. A., Quigley, S. P., Isherwood, P., McLennan, S. R., Sun, X. Q., Gibbs, S. J., and Poppi, D. P. (2017). The inclusion of low quantities of lipids in the diet of ruminants fed low quality forages has little effect on rumen function. Anim. Feed Sci. Technol. 234: 20-28.

Counotte, G. H. M., Van't Klooster, A. T., Van der Kuilen, J. and Prins, R. A. (1979). An analysis of the buffer system in the rumen of dairy cattle. J. Anim. Sci. 49: 1536-1544.

Crater, A. R., Barboza, P. S. and Forster, R. J. (2007). Regulation of rumen fermentation during seasonal fluctuations in food intake of muskoxen. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 146: 233-241.

Czerkawski, J. W., and Breckenridge, G. (1977). Design and development of a long term rumen simulation technique (RUSITEC). Brit. J. Nutri. 38: 371-384.

Czerkawski, J. W. (1986). An Introduction to Rumen Studies. Oxford, Pergamon Press.

Dado, R. G. and Allen, M. S. (1993). Continuous computer acquisition of feed and water intakes, chewing, reticular motility, and ruminal pH of cattle. J. Dairy Sci. 76: 1589-1600.

Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A., and Phillipson, A. T. (1945). The mechanism of absorption from the rumen as exemplified by the behavior of acetic, propionic and butyric acids. J. Exp. Biol. 22: 75-84.

Davidson, P. M., and Naidu, A. S. (2000). Phyto-phenols. In: Natural Food Antimicrobial Systems. Naidu, A. S. ed. CRC Press. Boca Raton. FL. pp. 265-293.

de la Fuente, G., Belanche, A., Girwood, S. E., Pinloche, E., Wilkinson, T., and Newbold, C. J. (2014). Pros and cons of ion-torrent next generation sequencing versus terminal restriction fragment length polymorphism T-RFLP for studying the rumen bacterial community. PLoS One 9: e101435.

Dean, S. G., and Ritchie, G. (1987). Antibacterial properties of plant essential oils. Int. J. Food Microbiol. 5: 165-180.

Dehority, B. A. (2003). Rumen Microbiology. Nottingham University Press, Nottingham, UK. 372.

Demarquilly, C., and Andrieu, J. (1988). Les fourrages. In : Alimentation des bovins ovins et caprins. INRA Editions. 3: pp 57-74.

DePeeters, E. J., Fadel, J. G., and Arosemena, A. (1997). Digestion kinetics of neutral detergent fiber and chemical composition within some selected by-product feedstuffs. Anim. Feed Sci. Technol. 67: 127-140.

Desbois, A. P., and Smith, V. J. (2010). Antibacterial free fatty acids: Activities, mechanisms of action and biotechnological potential. Appl. Microbiol. Biotechnol. 85: 1629-1642.

Desnoyers, M., Giger-Reverdin, S., Bertin, G., Duvaux-Ponter, C., and Sauvant, D. (2009). Meta-analysis of the influence of Saccharomyces cerevisiae supplementation on ruminal parameters and milk production of ruminants. J. Dairy Sci. 92: 1620-1632.

Devant, M., Ferret, A., Calsamiglia, S., Casals, R., and Gasa, J. (2001). Effect of nitrogen source in high-concentrate, low-protein beef cattle diets on microbial fermentation studied *in vivo* and *in vitro*. J. Anim. Sci. 79: 1944-1953.

Dijkstra, J., Kebreab, E., Bannink, A., France, J., and López, S. (2005). Application of the gas production technique to feed evaluation systems for ruminants. Anim. Feed Sci. Technol. 123-124: 561-578.

Doreau, M., Legay, F., and Bauchart, D. (1991). Effect of source and level of supplemental fat on total and ruminal organic matter and nitrogen digestion in dairy cows. J. Dairy Sci. 92. 74: 2233-2242.

Doreau, M., and Ferlay, A. (1994). Digestion and utilisation of fatty acids by ruminants. Anim. Feed Sci. Technol. 45: 379-396.

Doreau, M., and Ferlay, A. (1995). Effect of dietary lipids on nitrogen metabolism in the rumen: a review. Livest. Prod. Sci. 43: 97-110.

Doreau, M., and Chilliard, Y. (1997). Digestion and metabolism of dietary fat in farm animals. Br. J. Nutr. 78: S15-S35.

Doreau, M., Aurousseau, E., and Martin, C. (2009). Effects of linseed lipids fed as rolled seeds, extruded seeds or oil on organic matter and crude protein digestion in cows. Anim. Feed Sci. Technol. 150: 187-196.

Dorman, H. J. D., and S. G. Deans. (2000). Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88: 308-316.

Drackley, J. K. (2008). Calf nutrition from birth to breeding. Vet. Clin. Food Anim. Pract. 24: 5-86.

Dunbar, J., Ticknor, L. O., and Kuske, C.R. (2000). Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66: 2943-2950.

Dusart, C. (2014). La digestion ruminale: mise en place d'un modèle d'études *in vitro* à long terme en culture batch. PhD thesis, Université Paul-Sabatier de Toulouse, France.

Erdman, R. A. (1988). Dietary buffering requirements of the lactating dairy cow: A review. J. Dairy Sci. 71: 3246-3266.

Evers, A. D., Blakeney, A. B., and O'Brien, L. (1999). Cereal structure and composition. Aust. J. Agric. Res. 50: 629-650

Faleiro, A. G., Gonzalez, L. A., Blanch, M., Cavini, S., Castells, L., Ruíz de la Torre, J. L., Manteca, X., Calsamiglia, S., and Ferret, A. (2011). Performance, ruminal changes, behaviour and welfare of growing heifers fed a concentrate diet with or without barley straw. Animal, 5: 294-303.

FEDNA, (2010). In: De Blas, C., Mateos, G.G. and García-Rebollar, P. (Eds.), Tablas de composición y valor nutritivo de alimentos para la fabricación de piensos compuestos, 3rd ed., Fundación Española para el Desarrollo de la Nutrición Animal, Madrid, Spain.

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

Fernando, S. C., Purvis, H. T., Najar, F. Z., Sukharnikov, L. O., Krehbiel, C. R., Nagaraja, T. G., Roe, B. A., and de Silva, U. (2010). Rumen microbial population dynamics during adaptation to a high-grain diet. Appl. Environ. Microbiol. 76: 7482-7490.

Firkins, J. L., Eastridge, M. L. St-Pierre, N. R., and Noftsger, S. M. (2001). Effects of grain variability and processing on starch utilization by lactating dairy cattle. J. Anim. Sci. 79: E218-E238.

Fischer, J. M., Buchanan-Smith, J. G., Campbell, C., Grieve, D. G., and Allen, O. B. (1994). Effects of forage particle size and long hay for cows fed total mixed rations based on alfalfa and corn. J. Dairy Sci. 77: 217-229.

Flores, A. J., Garciarena, A. D., Vieyra, J. M. H., Beauchemin, K. A., and Colombatto, D. (2013). Effects of specific essential oil compounds on the ruminal environment, milk production and milk composition of lactating dairy cows at pasture. Anim. Feed Sci. Technol. 186: 20-26.

Fondevila, M., Castrillo, C., Guada, J. A., and Barcells, J. (1994). Effect of ammonia treatment and carbohydrate supplementation of barley straw on rumen liquid characteristics and substrate degradation by sheep. Anim. Feed Sci. Technol. 50: 137-155.

Fondevila, M., Barrios-Urdaneta, A., Balcells, J., and Castrillo, C., (2002). Gas production from straw incubated in vitro with different levels of purified carbohydrates. Anim Feed Sci Technol, 101: 1-15.

Fondevila, M., and Pérez-Espés, B. (2008). A new *in vitro* system to study the effect of liquid phase turnover and pH on microbial fermentation of concentrate diets for ruminants. Anim. Feed Sci. Technol. 144: 196-211.

Fonty, G., Gouet, Ph., Jouany, J. P., and Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. J. Gen. Microbiol. 133: 1835-1843.

Frutos, P., Hervás, G., Giráldez, F. J., Fernández, M., and Mantecón, A. R. (2000). Digestive utilization of quebracho-treated soya-bean meal in sheep. J. Agric. Sci. 134: 101-108.

García-Rebollar, P., Bacha, F., and Jimeno-Vinatea, V. (2008). Alimentación del ternero en sistemas intensivos de cebo. En "Producción de ganado vacuno de carne y tipos

comerciales en España. Sañudo, C., Jimeno, V. and Cervino M. (ed)". Schering-Plough. Alcobendas (Madrid). 75-88.

Gershenzon, J., and Croteau. R. (1991). Terpenoids. In Herbivores: Their interactions with secondary plant metabolites. Vol. 1. Rosenthal, G. A., and Berenbaum, M. R., ed. Academic Press, San Diego, CA. pp. 165-219.

Getachew, G., DePeters, E. J., Robinson, P. H. and Taylor, S. J. (2001). *In vitro* rumen fermentation and gas production: influence of yellow grease, tallow, corn oil and their potassium soaps. Anim. Feed Sci. Technol. 93: 1-15.

Getachew, G., Pitroff, W., Putnam, D. H., Dandekar, A., Goyal, S., DePeeters, E. J. (2008). The influence of addition of gallic acid, tannic acid, or quebracho tannins to alfalfa hay on *in vitro* rumen fermentation and microbial protein synthesis. Anim. Feed Sci. Technol**.** 140: 444-461.

Giesecke, D., and Stangassinger, M. (1980). Lactic acid metabolism. In Digestive Physiology and Metabolism in Ruminants. Ruckebusch, Y., and Thivend, P. ed. AVI Publ. Co., Westport, CT. pp. 523-539.

Giger-Reverdin, S., Duvaux-Ponter, C., Sauvant, D., Martin, O., do Prado, I. N., and Muller, R. (2002). Intrinsic buffer capacity of feedstuffs. Anim. Feed Sci. Technol. 96: 83-102.

Giner-Chávez, B. I. (1996). Condensed tannins in tropical forages. Doctoral Thesis. Cornell University. Ithaca, NY, USA.

Goad, D. W., Goad, C. L., and Nagaraja, T. G., (1998). Ruminal microbial and fermentative changes associated with experimentally induced subacute acidosis in steers. J. Anim. Sci. 76: 234-241.

Goering, H. K., and van Soest, P. J. (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). USDA Agriculture Handbook No. 379. USDA-ARS. Washington. DC. USA.

Govil, K., Yadav, D. S., Patil, A. K., Nayak, S., Baghel, R. P. S., Yadav, P. K., Malapure, C. D., and Thakur, D. (2017). Feeding management for early rumen development in calves. J. Entomol. Zool. Stud. 5: 1132-1139.

Grainger, C., Clarke, T., Auldist, M., Beauchemin, K., McGinn, S., Waghorn, G., and Eckard, R. (2009). Potential use of Acacia mearnsii condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. Can. J. Anim. Sci. 89: 241-251.

Grant, R. H., and Mertens, D. R. (1992). Influence of buffer pH and raw corn starch addition on *in vitro* fiber digestion kinetics. J. Dairy Sci.75: 2762-2768.

Greathead, H. (2003). Plant and plant extract for improving animal productivity. Proc. Nutr. Soc. 62: 279-290.

Grenet, E., and Besle, J. M. (1991). Microbes and fiber degradation. In rumen microbial metabolism and ruminant digestion. INRA. ISBN 2-7380-0345-1: 107-129.

Guerrero, M., Cerrillo-Soto, M. A, Ramírez, R. G., Salem, A. Z. M., and González, H. (2012). Influence of polyethylene glycol on *in vitro* gas production profiles and microbial protein synthesis of some shrub species. Anim. Feed Sci. Technol. 176, 32–39.

Guimarães-Beelen, P. M., Berchielli, T. T., Beelen, R., and Medeiros, A. N. (2006). Influence of condensed tannins from Brazilian semi-arid legumes on ruminal degradability, microbial colonization and ruminal enzymatic activity in Saanen goats. Small Ruminant Res. 61: 35-44.

Hagerman, A. E., and Butler, L.G. (1991). Tannins and lignins. In: Herbivores: their interactions with secondary plant metabolites, Vol I: The chemical participants, (Rosenthal G.A. and Berenbaum M.R., eds.), Academic Press, NY (USA), pp. 355-388.

Hagerman, A. E, Robbins, C. T, Weerasuriya, Y., Wilson, T. C, and McArthur, C. (1992) Tannin chemistry in relation to digestion. J. Range Manag. 45: 57-62.

Hall, M. B., Lewis, B. A., Van Soest, P. J., and Chase, L. E. (1997). A simple method for estimation of neutral detergent-soluble fibre. J. Sci .Food Agr. 74: 441-449.

Hall, M. B., Pell, A. N., and Chase, L. E. (1998) Characteristics of neutral detergent-soluble fiber fermentation by mixed ruminal microbes. Anim. Feed Sci. Technol. 70: 23-39.

Hall, M. B., and Herejk, C. (2001). Differences in yields of microbial crude protein from *in vitro* fermentation of carbohydrates. J. Dairy Sci. 84: 2486-2493.

Harborne, J. B. (1999). An overview of antinutritional factors in higher plants. In: Secondary plants products. Antinutritional and beneficial actions in animal feeding (Caygill J.C. and Mueller-Harvey I., eds.). Nottingham Univ Press, UK, pp. 7-16.

Harfoot, C. G. and Hazlewood, G. P. (1988). Lipid metabolism in the rumen. The rumen microbial ecosystem. Elsevier, UK. p. 285-322.

Hariadi, B. T., and Santoso, B. (2010). Evaluation of tropical plants containing tannin on *in vitro* methanogenesis and fermentation parameters using rumen fluid. J. Sci. Food Agric. 90: 456-461.

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

Hatfield, R.D., and Weimer, P.J. (1995). Degradation characteristics of isolated and in situ cell wall lucerne pectic polysaccharides by mixed ruminal microbes. J. Sci. Food Agric. 69: 185-196.

Heinrichs, J. (2005). Rumen development in the diary calf. Adv. Dairy Technol. 17: 179-187

Helander, I. M., Alakomi, H. L., Latva-Kala, K., Mattila-Sandholm, T., Pol, L., Smid, E. J., Gorris, L. G. M., and von Wright, A. (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. J. Agric. Food Chem. 46: 3590-3595.

Henderson, C. (1973). The effects of fatty acids on pure cultures of rumen bacteria, J. Agric. Sci. (Camb.) 81: 107-112.

Herrera-Saldana, R. E., Huber, J. T., and Poore, M. H. (1990). Dry matter, crude protein and starch degradability of five cereal grains. J. Dairy Sci. 73: 2386-2393.

Hervás, G., Frutos, P., Serrano, E., Mantecón, A. R., and Giráldez, F. J. (2000). Effect of tannic acid on rumen degradation and intestinal digestion of treated soya bean meals in sheep. Agric. Sci. 135: 305-310.

Hervás, G., Pérez, V., Giraldez, F.J., Mantecon, A.R., Almar, M.M., and Frutos, P. (2003a). Intoxication of sheep with quebracho tannin extract. J. Comp. Pathol. 129: 44-54.

Hervás, G., Frutos, P., Giráldez, F. J., Mantecón, A. R., Álvarez del Pino, M. C. (2003b). Effect of different doses of quebracho tannins extract on rumen fermentation in ewes. Anim. Feed Sci. Technol**.** 109: 65-78.

Hiltner, P., and Dehority, B. A. (1983). Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. Appl. Environ. Microbiol. 46: 642-648.

Hongoh, Y., Yuzawa, H., Ohkuma, M., and Kudo, T. (2003). Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. FEMS Microbiol. Lett. 221: 299-304.

Hoover, W. H., Crooker, B. A., and Sniffen, C. J. (1976). Effects of differential solid liquid removal rates on protozoa numbers in continuous cultures of rumen contents. J. Anim. Sci. 43: 528-534.

Hristov, A. N., McAllister, T. A., Van Herk, F. H., Cheng, K. J., Newbold, C. J., and Cheeke, P. R., (1999). Effect of Yucca schidigera on ruminal fermentation and nutrient digestion in heifers. J. Anim. Sci. 77: 2554-2563.

Hristov, A. N., Ivan, M., and McAllister, T. A. (2004). *In vitro* effects of individual fatty acids on protozoal numbers and on fermentation products in ruminal fluid from cattle fed a high-concentrate, barley-based diet. J. Anim. Sci. 82: 2693-2704.

Hsu, J. T, and Fahey, G. C. (1990). Effect of centrifugation speed and freezing on composition of ruminal bacterial samples collected from defaunated sheep. J. Dairy Sci**.** 73: 149-152.

Hungate, R. E. (1966). 'The rumen and its microbes.' (Academic Press: New York)

Huntington, G. B. (1997). Starch utilization by ruminants: from basics to the bunk. J. Anim. Sci. 75: 852-867.

Huws, S. A., Lee, M. R. F., Muetzel, S., Scott, M., Wallace, R. J., and Scollan, N. D., (2010). Forage type and fish oil cause shifts in rumen bacterial diversity. FEMS Microbiol. Ecol. 73: 396-407.

Jasaitis, D. K., Wohlt, J. E., and Evans, J. L. (1987). Influence of feed ion content on buffering capacity of ruminant feedstuffs in vitro. J. Dairy Sci. 70: 1391-1403

Jayanegara, A., Leiber, F., and Kreuzer, M. (2012). Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. J. Anim. Physiol. An. N. 96: 365-375.

Jenkins, T. C. (1993). Lipid metabolism in the rumen. J. Dairy Sci. 76: 3851-3863.

Jenkins, T. C. (1994). Regulation of lipid metabolism in the rumen. J. Nutr. 124: 1372-1376.

Jiao, J., Li, X., Beauchemin, K. A., Tan, Z., Tang, S., and Zhou, C. (2015). Rumen development process in goats as affected by supplemental feeding v. grazing: age-related anatomic development, functional achievement and microbial colonisation. Br. J. Nutr. 113: 888-900.

Jones, G. A., McAllister, T. A., Muir, A. D., and Cheng, K. J. (1994). Effects of sainfoin (Onobrychis vicifolia Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Environ. Microbiol. 60: 1374-1378.

Jones, W. T., and Mangan, J. L. (1977). Complexes of the condensed tannins of sainfoin (Onobrychis viciifolia scop.) with fraction 1 leaf protein and with submaxillary mucoprotein and their reversal by polyetheleneglycol and pH. J. Sci. Food Agric. 28: 126-136.

Jouany, J. P. (1994). Les fermentations dans le rumen et leur optimization. INRA Prod. Anim. 7: 207-225.

Jouany, J. P., Broudiscou, L., Prins, R. A., and Komisarczuk-Bony, S. (1995). Métabolisme et nutrition de la population microbienne du rumen. In: Nutrition des ruminants domestiques. Jarrige, R., Ruckebusch, Y., Demarquilly, C., Farce, M-H. and Journet M. INRA. Ed. Chap 9: 350-381.

Jouany, J. P., and Ushida, K. (1998). The role of the protozoa in feed digestion. Asian-Australian. J. Anim. Sci. 12:113-128.

Juven, B. J., Kanner, J., Schved. F., and Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J. Appl. Microbiol. 76: 626-631.

Kennelly, J. J., Robinson, B., and Khorasani, G. R. (1999). Influence of carbohydrate source and buffer on rumen fermentation characteristics, milk yield, and milk composition in early-lactation Holstein cows. J. Dairy Sci. 82: 2486-2496.

Khan, M. A., Bach, A., Weary, D. M., and Von Keyserlingk, M. A. G. (2016). Invited review: transitioning from milk to solid feed in dairy heifers. J. Dairy Sci. 99: 1-18.

Khorasani, G. R., Okine, E. K., and Kennelly, J. J. (2001). Effects of Substituting Barley Grain with Corn on Ruminal Fermentation Characteristics, Milk Yield, and Milk Composition of Holstein Cows. J. Dairy Sci. 84: 2760-2769.

Kim, H. O., Park, S. W., and Park, H. D. (2004). Inactivation of *Escherichia coli* O157:H7 by cinnamic aldehyde purified from *Cinnamomum cassia* shoot. Food Microbiol. 21: 105-110.

Kim, K. H., Tsao, R., Yang, R., and Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem. 95: 466-473.

Kim, S. H., Mamuad, L. L., Kim, E. J., Sung, H. G., Bae, G. S., Cho, K. K., Lee, C., and Lee, S. S. (2018). Effect of different concentrate diet levels on rumen fluid inoculum used for determination of *in vitro* rumen fermentation, methane concentration, and methanogen abundance and diversity. Ital. J. Anim. Sci. 17: 359-367.

Klevenhusen, F., Bernasconi, S. M., Hofstetter, T. B., Bolotin, J., Kunz, C., and Soliva, C. R. (2009). Efficiency of monolaurin in mitigating ruminal methanogenesis and modifying C-isotope fractionation when incubating diets composed of either C_3 or C_4 plants in a rumen simulation technique (Rusitec) system. Brit. J. Nutr. 102: 1308-1317.

Klevenhusen, F., Meile, L., Kreuzer, M., and Soliva, C. R. (2011). Effects of monolaurin on ruminal methanogens and selected bacterial species from cattle, as determined with the rumen simulation technique. Anaerobe. 17: 232-238.

Klieve, A. V., Hennessy, D., Ouwerkerk, D., Forster, R. J., Mackie. R.I., Attwood, G. T. (2003). Establishing populations of Megasphaera elsdenii YE34 and Butyrivibrio fibrisolvens YE44 in the rumen of cattle fed high grain diets. J. Appl. Microbiol. 95: 621-630.

Kohn, R. A., and Dunlap, T. F. (1998). Calculation of the buffering capacity of bicarbonate in the rumen and *in vitro*. J. Anim. Sci. 76: 1702-1709.

Krause, K. M., Combs, D. K., and Beauchemin, K. A. (2002). Effects of forage particle size and grain fermentability in midlactation Cows. II. Ruminal pH and Chewing Activity. J. Dairy Sci. 85: 1947-1957.

Krause, K. M., and Combs, D. K. (2003). Effects of forage particle size, forage source and grain fermentability on performance and ruminal pH in midlactation cows. J. Dairy Sci. 86: 1382-1397.

Krause, K. M. and Oetzel, G. R. (2006). Understanding and preventing subacute ruminal acidosis in dairy herds: A review. Anim. Feed Sci. Technol. 126: 215-236.

Kraus, T. E. C., Yu, Z., Preston, C. M., Dahlgren, R. A., and Zasoski, R. J. (2003). Linking chemical reactivity and protein precipitation to structural characteristics of foliar tannins. J. Chem. Ecol. 29: 703-730.

Krause, D. O., Smith, W. J. M., Brooker, J. D., and McSweeney, C. S. (2005). Tolerance mechanisms of streptococci to hydrolysable and condensed tannins. Anim. Feed Sci. Technol**.** 121: 59-75.

Kristen, L. D., Liyi, Y., Audrey, G., Scott, B., and Joseph, M. A. (2015). Interaction of sorghum tannins with wheat proteins and effect on in vitro starch and protein digestibility in a baked product matrix. J. Agri. Food Chem. 63: 1234-1241.

Krueger, W. K., Gutierrez-Bañuelos, H., Carstens, G. E., Min, B. R, Pinchak, W. E., Gomez, R. R., Anderson, R. C., Krueger, N. A., and Forbes, T. D. A. (2010). Effects of dietary tannin source on performance, feed efficiency, ruminal fermentation, and non-carcass traits in steers fed a high-grain diet. Anim. Feed Sci. Technol**.** 159: 1-9.

Kubelková, P., Jalč, D., Jančík, F., and Homolka, P. (2018). *In vitro* ruminal fermentation and fatty acid production by various oil seeds. S. Afr. J. Anim. Sci. 48.

Kumar, R., and Vaithiyanathan, S. (1990). Occurrence, nutritional significance and effect on animal productivity of tannins in tree leaves. Anim. Feed Sci. Technol. 30: 21-38.

Lanzas, C., Fox, D. G., and Pell, A. N. (2007). Digestion kinetics of dried cereal grains. Anim. Feed Sci. Technol. 136: 265-280.

Laurent, F. (1988). Utilisation du blé et des céréales dans la ration des vaches laitières. Ann. Zootech. 37: 117-132.

Le Ruyet, P., Tucker, W. B., Hogue, J. F., Aslam, M., Lema, M., Shin, I. S., Miller, T. P., and Adams, G. D. (1992). Influence of dietary fiber and buffer value index on the ruminal milieu of lactating dairy cows. J. Dairy Sci. 75: 2394-2408.

Losa, R. (2001). The use of essential oils in animal nutrition. In: Feed Manufacturing in the Mediterranean Region. Improving Safety: From Feed to Food. Proceedings of the III Conference of Feed Manufacturers of the Mediterranean, Reus, Spain, March 2000, Cahiers Options Méditerranéennes. 54: 39-44.

Macheboeuf, D., Morgavi, D. P., Papon, Y., Mousset, J. L., and Arturo-Schaan, M. (2008). Dose response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Anim. Feed Sci. Technol. 145: 335-350.

Machmüller, A. (2006). Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agric. Ecosyst. Environ. 112: 107-114.

Maczulak, A. E., Dehority, B. A, and Palmquist, D. L. (1981). Effects of long-chain fatty acids on growth of rumen bacteria. Appl. Environ. Microbiol. 42: 856-862.

Maes, C., and Delcour, J. A. (2001) Alkaline hydrogen peroxide extraction of wheat bran non-starch polysaccharides. J. Cereal Sci. 34: 29-35.

Makkar, H. P. S., Blummel, M., Borowy, N. K., and Becker, K. (1993). Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61: 161-165.

Makkar, H. P. S., Blummel, M., and Becker, K. (1995). Formation of complexes between polyvinyl pyrrolidone or polyethylene glycol and tannins, and their implications in gas production and true digestibility in *in vitro* techniques. Br. J. Nutr. 73: 897–913.

Makkar, H. P. S. (2003). Effects and fate of tannins in ruminant animals, adaptation to tannins and strategies to overcome detrimental effects of feeding tannin rich feeds. Small Rumin. Res. 49: 241-256.

Maktabi, H., Ghasemi, E., and Khorvash, M. (2016). Effects of substituting grain with forage or non-forage fiber source on growth performance, rumen fermentation, and chewing activity of dairy calves. Anim. Feed Sci. Technol. 221: 70-78.

Mangan, J. L. (1988). Nutritional effects of tannins in animal feeds. Nutr. Res. Rev. 1: 209-231.

Mao, S. Y., Huo, W. J., and Zhu, W. Y. (2016). Microbiome–metabolome analysis reveals unhealthy alterations in the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model. Environ. Microbiol. 18: 525-541.

Marden, J. P., Bayourthe, C., Enjalbert, F., and Moncoulon, R. (2005). A new device for measuring kinetics of ruminal pH and redox potential in dairy cattle. J. Dairy Sci. 88: 277-281.

Marounek, M., Bartos, S., and Brezina, P. (1985). Factors influencing the production of volatile fatty acids from hemicellulose, pectin and starch by mixed culture of rumen microorganisms. J. Anim. Physiol. Anim. Nutr. 53: 50-58.

Martin, C., Brossard, L., and Doreau, M. (2006). Mécanismes d'apparition de l'acidose ruminale latente et conséquences physiopathologiques et zootechniques. INRA Prod. Anim. 19: 93-108.

Martínez, T. F., McAllister, T. A., Wang, Y., and Reuter, T. (2006). Effects of tannic acid and quebracho tannins on *in vitro* ruminal fermentation of wheat and corn grain. J. Sci. Food Agric. 86: 1244-1256.

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

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

McAllister, T. A., Cheng, K.J., Rode, L. M., and Forsberg, C. W. (1990). Digestion of barley, maize, and wheat by selected species of ruminal bacteria. Appl. Environ. Microbiol. 56: 3146-3153.

McAllister, T. A., Philippe, R. C., Rode, L. M. and Cheng, K. J. (1993). Effect of the protein matrix on the digestion of cereal grains by ruminal microorganisms. J. Anim Sci. 71: 205-212.

McAllister, T. A, Bae, H. D., Jones, G. A, and Cheng, K. J. (1994). Microbial attachment and feed digestion in the rumen. J. Anim. Sci. 72: 3004-3018.

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

McIntosh, F. M., Newbold, C. J., Losa, R., Williams, P., and Wallace. R. J. (2000). Effects of essential oils on rumen fermentation. Reprod. Nutr. Dev. 40: 221-222.

McIntosh, F. M., Williams, P., Losa, R., Wallace, R. J., Beever, D. A., and Newbold, C. J. (2003). Effects of essential oils on ruminal microorganisms and their protein metabolism. Appl. Environ. Microbiol. 69: 5011-5014.

McLeod, M. N. (1974). Plant tannins - Their role in forage quality. Nutr. Abst. Rev. 44: 803-812.

McMahon, L. R., McAllister, T. A., Berg, B. P., Majak, W., Acharya, S. N., Popp, J. D., Coulman, B. E., Wang, Y., and Cheng, K. J. (2000). A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. Can. J. Plant Sci. 80: 469-485.

McSweeney, C. S., Palmer, B., McNeill, D. M., and Krause, D. (2001). Microbial interactions with tannins: nutritional consequences for ruminants. Anim. Feed Sci. Technol. 91: 83-93.

Menke, K. H., and Steingass, H. (1988). Estimation of the energy feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Ani. Res. Develop. 28, 7-55.

Mertens, D. R. (2002). Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85: 1217-1240.

Meschy, F., Bravo, D., and Sauvant, D. (2004). Analyse quantitative des réponses des vaches laitières à l'apport de substances tampon. INRA Prod. Anim. 17: 11-18.

Meyer, N. F., Erickson, G. E., Klopfenstein, T. J., Greenquist, M. A., Willams, P., and Losa, R. (2007). Effect of CRINA RUMINANTS AF, a mixture of essential oil compounds, on finishing beef steer performance. Nebraska Beef Cattle Reports. 71-80.

Mezzomo, R., Paulino, P. V. R., Detmann, E., Valadres, Filho, S. C., Paulino, M. F., Monnerat, J. P. I. S., Duarte, M. S., Silva, L. H. P., and Moura, L. S. (2011) Influence of condensed tannin on intake, digestibility, and efficiency of protein utilization in beef steers fed high concentrate diet. Livest. Sci. 141: 1-11.

Mialon, M. M., Commun, L., Martin, C., Menassol, J. B., Dubroeucq, H., Veissier, I., Garcia-Launay, F., and Micol, D. (2008). Adaptation du comportement alimentaire des ruminants à des rations acidogènes. Renc. Rech. Ruminants. 15: 335-338.

Michalet-Doreau, B., and Doreau, R. T. (1986). Maize genotype and ruminant nutrition. Sci. Aliments. 19: 349-365.

Michalet-Doreau, B., and Sauvant, D. (1989). Influence de la nature du concentré, céréales ou pulpe de betterave, sur la digestion chez les ruminants. INRA. Prod. Anim. 2: 235-24.

Mould, F. L., and Ørskov, E. R. (1983). Manipulation of rumen fluid pH and its influence on cellulolysis *in sacco*, dry matter degradation and the ruminal microflora of sheep offered either hay or concentrate. Anim. Feed Sci. Technol. 10:1-14.

Mould, F. L., Orskov, E. R., and Mann, S.O. (1983). Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen fluid pH on cellulolysis *in vivo* and dry matter digestion of various roughages. Anim. Feed Sci. Technol. 16: 15-30.

Mould, F. L., Morgan, R., Kliem, K. E., and Krystallidou, E. (2005a). A review and simplification of the *in vitro* incubation medium. Anim. Feed Sci. Technol. 123-124: 155-172.

Mould, F. L., Kliem, K. E., Morgan, R., and Mauricio, R. M. (2005b). *In vitro* microbial inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123–124: 31–50.

Mueller-Harvey, I., and McAllan, A. B. (1992). Tannins. Their biochemistry and nutritional properties. In: Advances in plant cell biochemistry and biotechnology, Vol. 1 (Morrison I.M., ed.). JAI Press Ltd., London (UK), pp. 151-217.

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86: 2010-2037.

Münnich, M., Khiaosa-ard, R., Klevenhusen, F., Hilpold, A., Khol-Parisini, A., and Zebeli, Q. (2017). A meta-analysis of feeding sugar beet pulp in dairy cows: Effects on feed intake, ruminal fermentation, performance, and net food production. Anim. Feed Sci. Technol. 224: 78-89.

Nagadi, S., Herrero, M., and Jessop, N. S. (2000). 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.

Nagaraja, T. G., and Titgemeyer, E. C. (2007). Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. J. Dairy Sci. 90: E17-E38.

Nagaraja, T. G., and Lechtenberg K. F. (2007). Acidosis in feedlot cattle. Vet. Clin. Food Anim. Pract. 23: 333-350

Nagaraja, T. G. (2016). Microbiology of the rumen. In: Millen DD, Arrigoni MDB, Pacheco RDL (eds) Rumenology. Springer International Publishing, Cham, pp 39-61.

Nagata, R., Kim, Y. H., Ohkubo, A., Kushibiki, S., Ichijo, T., and Sato, S. (2018). Effects of repeated subacute ruminal acidosis challenges on the adaptation of the rumen bacterial community in Holstein bulls. J. Dairy Sci. 101: 4424-4436.

Nocek, J. E., and Tamminga, S. (1991). Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy Sci. 74: 3598-3629.

Nocek, J. E. (1997). Bovine acidosis: Implications on laminitis. J. Dairy Sci. 80: 1005-1028.

Nocek, J. E., Kautz, W. P., Leedle, J. A. Z., and Allman, J. G. (2002). Ruminal supplementation of direct-fed microbials on diurnal pH variation and *in situ* digestion in dairy cattle. J. Dairy Sci. 85: 429-433.

O´Brien, L. (1999). Genotype and environment effects on feed grain quality. Austr. J. Agric. Res. 50: 703-719.

Oetzel, G. R., Nordlund, K. V., and Garrett, E. F. (1999). Effect of ruminal pH and stage of lactation on ruminal lactate concentrations in dairy cows. J. Dairy Sci. 82: 38.

Offner, A., Bach, A., and Sauvant, D. (2003). Quantitative review of *in situ* starch degradation in the rumen. Anim. Feed Sci. Technol. 106: 81-93.

Opatpatanakit, Y., Kellaway, R. C., Lean, I. J., Annison, G., and Kirby, A. (1994). Microbial fermentation of cereal grains *in vitro*. Aust. J. Agric. Res. 45: 1247-1263.

Orlandi, T., Kozloski, G., Alves, T., Mesquita, F., and Ávila, S. (2015). Digestibility, ruminal fermentation and duodenal flux of amino acids in steers fed grass forage plus concentrate containing increasing levels of Acacia mearnsii tannin extract. Anim. Feed Sci. Technol. 210: 37-45.

Orskov, E. R., and Ryle, M. (1998). Energy nutrition in ruminants. Chalcomb Publications Painshalll, Church Lane. Welton. Lincoln. LN2 3 LT, UK.

Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J. P., and Begin, A. (1997). Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms. Int. J. Food Microbiol. 37: 155-162.

Overton, T. R., Cameron, M. R., Elliott, J. P., Clark, J. H., and Nelson, D. R. (1995). Ruminal fermentation and passage of nutrients to the duodenum of lactating cows fed mixtures of corn and barley. J. Dairy Sci. 78: 1981-1998.

Owens, F. N., Zinn, R. A., and Kim, Y. K. (1986). Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63: 1634-1648.

Owens, F. N., Secrist, D. S., Hill, W. J., and Gill, D. R. (1998). Acidosis in cattle: A review. J. Anim. Sci. 76: 275-286.

Palmquist, D. L., and Jenkins, T.C. (1980). Fat in lactation rations: Review. J. Dairy Sci. 63: 1-14.

Pan, X. H., Yang, L., Xue, F. G., Xin, H. R., Jiang, L. S., Xiong, B. H., and Beckers, Y. (2016). Relationship between thiamine and subacute ruminal acidosis induced by a high-grain diet in dairy cows. J. Dairy Sci. 99: 8790-8801.

Pantoja, J., Firkins, J. L., and Eastridge, M. L, (1995). Site of digestion and milk production by cows fed diets differing in saturation, esterification and chain length. J. Dairy Sci. 78: 2247-2258.

Patra, A., Kamra, D. and Agarwal, N. (2006). Effect of plant extracts on in vitro methanogenesis, enzyme activities and fermentation of feed in rumen liquor of buffalo. Anim. Feed Sci. Technol. 128: 276- 291.

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

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

Patra, A. K., and Saxena, J. (2011). Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. J. Sci. Food Agric. 91: 24-37.

Penner, G. B., Beauchemin, K. A., and Mutsvangwa, T. (2007). The severity of ruminal acidosis in primiparous Holstein cows during the periparturient period. J. Dairy Sci.90: 365-375.

Penner, G. B., Aschenbach, J. R., Gäbel, G., Rackwitz, R., and Oba. M. (2009). Epithelial capacity for apical uptake of short chain fatty acids is a key determinant for intraruminal pH and the susceptibility to subacute ruminal acidosis in sheep. J. Nutr. 139: 1714-1720.

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

Priolo, A., Waghorn, G. C., Lanza, M., Biondi, L., and Pennisi, P. (2000). Polyethylene glycol as a means for reducing the impact of condensed tannins in carob pulp: effects on lamb growth, performance and meat quality. J Anim. Sci. 78: 810-816.

Quigley, III, J. D., Caldwell, L. A., Sinks, G. D., and Heitmann, R. N. (1991). Changes in blood glucose, nonesterified fatty acids, and ketones in response to weaning and feed intake in young calves. J. Dairy Sci. 74: 250-257.

Quigley, III. J. D. (1997). Raising heifers from birth to weaning. Advances in Dairy Technology. Western Canadian Dairy Seminar.

Raab, L., Cafantaris, B., Jilg, T., and Menke, K. H. (1983). Rumen protein degradation and biosynthesis. A new method for determination of protein degradation in rumen fluid *in vitro*. Brit. J. Nutri. 50: 569-582.

Radostits, O., Blood, D. C., and Gay, C. C. (1994). Veterinary Medicine. 8th (Eds), Baillière Tindall. pp.1354.

Ramírez-Restrepo, C. A., Barry, T. N., Pomroy, W. E., López−Villalobos, N., McNabb, W. C., and Kemp, P. D. (2005). Use of Lotus corniculatus containing condensed tannins to increase summer lamb growth under commercial dryland farming conditions with minimal anthelmintic drench input. Anim. Feed Sci. Technol. 122: 197-217.

Rey, M., Enjalbert, F., and Monteils, V. (2012). Establishment of ruminal enzyme activities and fermentation capacity in dairy calves from birth through weaning. J. Dairy Sci. 95: 1500-1512.

Reynolds, C. K., Dürst, B., Lupoli, B., Humphries, D. J., and Beever, D. E. (2004). Visceral tissue mass and rumen volume in dairy cows during the transition from late gestation to early lactation. J. Dairy Sci. 87: 961-971.

Robertson, J. B, and Van Soest, P. J. (1981). The detergent system of analysis and its application to human foods. In: The analysis of dietary fiber in foods, James W.P.T., Theander O. (Eds.), Marcel Dekker, New York, USA, pp. 123-158

Rodríguez, R., Britos, A., Rodriguez-Romero, N., and Fondevila, M (2011). Effect of plant extracts from several tanniferous browse legumes on *in vitro* microbial fermentation of the tropical grass *Pennisetum purpureum.* Anim. Feed Sci. Technol. 168: 188–195.

Rodríguez, R., de la Fuente, G., Gómez, S., and Fondevila, M. (2014). Biological effect of tannins from different vegetal origin on microbial and fermentation traits *in vitro*. Animal Prod. Sci. 54: 1039-1046.

Rooney, L. W., and Pflugfelder, R. L. (1986). Factors affecting starch digestibility with special emphasis on sorghum and corn. J Anim. Sci. 63: 1607-1623.

Russell, J. B., and Dombrowski, D. B. (1980). Effect of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol. 39: 604-610.

Russell, J. B., and Hino, T. (1985). Regulation of lactate production in streptococcus bovis: A spiraling effect that contributes to rumen acidosis. J. Dairy Sci. 68: 1712-1721.

Salem, A. Z. M., Robinson, P. H., El-Adawy, M. M., and Hassan, A, A. (2007) *In vitro* fermentation and microbial protein synthesis of some browse tree leaves with or without addition of polyethylene glycol. Anim. Feed Sci. Technol. 138: 318-330.

Sañudo, C., Sanchez, A., and Alfonso, M. (1998). Small Ruminant Production systems and factors affecting lamb meat quality. Meat Sci. 49: S29-S64.

Sari, M., Ferret, A., and Calsamiglia, S. (2015). Effect of pH on in vitro microbial fermentation and nutrient flow in diets containing barley straw or non-forage fiber sources. Anim. Feed Sci. Technol. 200: 17-24.

Saro, C., Ranilla, M. J., Tejido, M. L., and Carro, M. D. (2014). Influence of forage type in the diet of sheep on rumen microbiota and fermentation characteristics. Livest Sci. 160: 52-59.

Sauvant, D., Dorleans, M., Delacour, C., Bertrand, D., and Giger, S. (1986). La modélisation des cinétiques de dégradation des constituants pariétaux et cellulaires des aliments dans le rumen. Reprod. Nutr. Develop. 26 : 303-304.

Sauvant, D., and Michelet-Doreau, B. (1988). Les aliments concentrés. In: Alimentation des bovins, ovins, caprins. Chap 17. INRA. 337-349.

Sauvant, D., Meschy, F., and Mertens D. (1999). Les composants de l'acidose ruminale et les effets acidogenes de l'acidose. INRA Prod. Anim. 12: 49-60.

Sauvant, D., Giger-Reverdin, S., and Meschy, F. (2006). Le contrôle de l'acidose ruminale latente. INRA Prod. Anim. 19 : 69-78**.**

Sauvant, D., and Peyraud, J. L. (2010). Calculs de la ration et évaluation du risque acidogène. INRA. Prod. Anim. 23: 333-342.

Scalbert, A. (1991). Antimicrobial properties of tannins. Phytochemistry 30: 3875-3883

Schofield, P., Mbugua, D. M., and Pell, A. N. (2001). Analysis of condensed tannins: a review. Anim. Feed Sci. Technol. 91: 21-40.

Schwartzkopf-Genswein, K. S., Beauchemin, K. A., Gibb, D. J., Crews, D. H. Jr., Hickman, D. D., Streeter, M., and McAllister, T. A. (2003). Effect of bunk management on feeding behavior, ruminal acidosis and performance of feedlot cattle: A review. J. Anim. Sci. 81: E149-E158.

Segel, I. H. (1976). Biochemical Calculations. (2nd Ed.). John Wiley and Sons, NY.

Serment, A. (2012). Dynamique et intensité de biotransformation dans le rumen. PhD thesis Agro Paris Tech UMR INRA-Agro Paris Tech 791 Modélisation Systémique Appliquée aux Ruminants.

Sharpe, M. E., Latham, M. J. Garvie, E. I. Zirngibi, J., and Kandler. O. (1973). Two new species of Lactobacillus isolated from the rumen, Lactobacillus ruminis sp. nov. and Lactobacillus vitulinus sp. nov. J. Gen. Microbiol. 77: 37-49.

Sheu, C.W., and Freese, E. (1972). Effect of fatty acids on growth and envelope proteins of Bacillus subtilis. J. Bacteriol. 111: 516-524.

Sienra, R. (2009). Acidosis en bovinos. Depto. de Rumiantes y Suinos, Facultad de Veterinaria. UR. Uruguay. www.produccion-animal.com.ar.

Sinclair, L. A., Cooper, S. L., Huntington, J. A., Wilkinson, R. G., Hallett, K. G., Enser, M., and Wood, J. D. (2005). *In vitro* biohydrogenation of *n*−3 polyunsaturated fatty acids protected against ruminal microbial metabolism. Anim. Feed Sci. Technol. 123-124: 579-596.

Sivropoulou, A., Kokkini, S., Lanaras, T., and Arsenakis, M. (1995). Antimicrobial activity of mint essential oils. J. Agric. Food Chem. 43: 2384-2388.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., and Arsenakis, M. (1996). Antimicrobial and cytotoxic activities of Origanum essential oils. J. Agric. Food Chem. 44: 1202-1205.

Slyter, L. L. (1976). Influence of acidosis on rumen function. J. Anim. Sci. 43: 910-929.

Smith-Palmer, A., Stewart, J., and Fyfe, L. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Let. Appl. Microbiol. 26: 118-122.

Soder, K. J., Brito, A. F., and Rubano, M. D. (2013). Short communication: Effect of oilseed supplementation of an herbage diet on ruminal fermentation in continuous culture. J. Dairy, Sci. 96: 2551-2556.

Soliva, C. R., Meile, L., Cieslak, A., Kreuzer, M., and Machmüller, A. (2004). Rumen simulation technique study on the interactions of dietary lauric and myristic acid

supplementation in suppressing ruminal methanogenesis. Brit. J. Nutr. 92: 689-700.

Söllinger, A., Tveit, A. T., Poulsen, M., Noel, S. J., Bengtsson, M., Bernhardt, J., Frydendahl Hellwing, A. L., Lund, P., Riedel, K., Schleper, C., Højberg, O., and Urich, T. (2018). Holistic assessment of rumen microbiome dynamics through quantitative metatranscriptomics reveals multifunctional redundancy during key steps of anaerobic feed degradation. mSystems, 3: e00038-18.

Spanghero, M., Zanfi, C., Fabbro, E., Scicutella, N., and Camellini, C. (2008). Effects of a blend of essential oils on some end products of in vitro rumen fermentation. Anim. Feed Sci. Technol. 145: 362-372.

Staples, C. R., and Lough, D. S. (1989). Efficacy of Supplemental Dietary Neutralising Agents for Lactating Dairy Cows. A Review. Anim. Feed Sci. Technol. 23: 277-303

Strobel, H. J., and Russel, J. B. (1986). Effects of pH and energy spilling on bacterial protein synthesis by carbohydrate-limited cultures of mixed rumen bacteria. J. Dairy Sci. 69: 2941-2947.

Svihus, S. B., Uhlen, A. K., and Harstad, O. M. (2005). Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: a review. Anim. Feed Sci. Technol. 122: 303-320.

Tager, L. R., and Krause, K. M. (2010). Effects of cinnamaldehyde, eugenol, and capsicumon fermentation of a corn-based dairy ration in continuous culture. Can. J. Anim. Sci. 90: 413-420.

Tamate, H., McGilliard, A. D., Jacobson, N. L., and Getty R. (1962). Effect of various dietaries on the anatomical development of the stomach in the calf. J. Dairy Sci. 45: 408-420.

Tapio, I., Fischer, D., Blasco, L., Tapio, M., Wallace, R. J., Bayat, A. R., Ventto, L., Kahala, M., Negussie, E., Shingfield, K. J., and Vilkki, J. (2017). Taxon abundance, diversity, co-occurrence and network analysis of the ruminal microbiota in response to dietary changes in dairy cows. PLoS ONE. 12: e0180260.

Taxis, T. M., Wolff, S., Gregg, S. J., Minton, N. O., Zhang, C., Dai, J., Schnabel, R. D., Taylor, J. F., Kerley, M. S., Pires, J. C., Lamberson, W. R., and Conant, G. C. (2015). The players may change but the game remains: network analyses of ruminal microbiomes suggest taxonomic differences mask functional similarity. Nucleic Acids Res. 43. 9600-9612.

Theodoridou, K., Aufrére, J., Andueza, D., Le Morvan, A., Picard, F., Pourrat, J., and Baumont, R. (2012). Effects of condensed tannins in wrapped silage bales of sainfoin (Onobrychis viciifolia) on *in vivo* and in situ digestion in sheep. Animal, 6: 245-253.

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

Tomkins, N. W., Denman, S. E., Pilajun, R., Wanapat, M., McSweeney, C.S., and Elliott, R. (2015). Manipulating rumen fermentation and methanogenesis using an essential oil and monensin in beef cattle fed a tropical grass hay. Anim. Feed Sci. Technol. 200: 25-34.

Valente, T. N. P., Sampaio, C. B., Lima, E. D. S., Deminicis, B. B., Cezário, A. S., and Santos, W. B. R. D. (2017). Aspects of acidosis in ruminants with a focus on nutrition: A Review. J. Agric. Sci. 9: 90-97.

Van Barneveld, S. L. (1999). Chemical and physical characteristics of grains related to variability in energy and amino acid availability in ruminants: A review. Aust. J. Agric. Res. 50: 651-666.

Van Houtert, M. F. J. (1993). The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. Anim. Feed Sci. Technol. 43: 189-225.

Van Soest, P. J. (1994). Nutritional ecology of the ruminant, 2nd ed. Cornell Univ Press. Ithaca, NY, USA. 476. p.

Vargas, J. E., Andrés, S., Snelling, T. J., López-Ferreras, L., Yáñez-Ruiz, D. R, García-Estrada, C., and López, S. (2017). Effect of sunflower and marine oils on ruminal microbiota, *in vitro* fermentation and digesta fatty acid profile. Front. Microbiol. 8: 1124.

Vazirigohar, M., Dehghan-Banadaky, M., Rezayazdi, K., Nejati-Javaremi, A., Mirzaei-Alamouti, H., and Patra, A. K. (2018). *Short communication:* Effects of diets containing supplemental fats on ruminal fermentation and milk odd- and branched-chain fatty acids in dairy cows. J. Dairy Sci. 101: 1-9.

Waldo, D. R. (1973). Extent and partition of cereal grain starch digestion in ruminants. J. Anim. Sci. 37: 1062-1074.

Wales, W. J., Kolver, E. S., Thorne, P. L., and Egan, A. R. (2004). Diurnal variation in ruminal pH on the digestibility of highly digestible perennial ryegrass during continuous culture fermentation. J. Dairy Sci. 87: 1864-1871

Wallace, R. J. (2004). Antimicrobial properties of plant secondary metabolites. Proc. Nutr. Soc. 63, 621-629.

Walsh, S. E., Maillard, J. Y., Russell, A. D., Catrenich, C. E., Charbonneau, D. L., and Bartolo, R. G. (2003). Activity and mechanisms of action of selected biocidal agents on gram-positive and -negative bacteria. J. Appl. Microbiol. 94: 240-427.

Wang, Y., Douglas, G. B., Waghorn, G. C., Barry, T. N., Foote, A. G., and Purchas, R. W., (1996). Effect of condensed tannins upon the performanceof lambs grazing Lotus corniculatus and lucern (Medicago sativa). J. Agric. Sci. 126: 87-98.

Wang, Y. H., Xu, M., Wang, F. N., Yu, Z. P., Yao, J.H., Zan, L. S., and Yang, F. X. (2009). Effect of dietary starch on rumen and small intestine morphology and digesta pH in goats. Livest Sci. 122: 48-52.

Wischer, G., Boguhn, J., Steingass, H., Schollenberg, M., and Rodehutscord, M. (2013) Effects of different tannin-rich extracts and rapeseed tannin monomers on methane formation and microbial protein synthesis *in vitro*. Animal. 7: 1796-1805.

Wohlt, J. E., Jasaitis, D. K., and Evans, J. L. (1987). Use of acid and base titrations to evaluate the buffering capacity of ruminant feedstuffs *in vitro*. J. Dairy Sci. 70: 1465-1470.

Yang, W. Z., Beauchemin, K. A., and Rode L. M. (2001). Effects of grain processing, forage to concentrate ratio, and forage particle size on rumen pH and digestion by dairy cows. J. Dairy Sci.84: 2203-2216.

Yang, W. Z., Ametaj, B. N., Benchaar, C., and Beauchemin, K. A. (2010). Dose response to cinnamaldehyde supplementation in growing beef heifers: ruminal and intestinal digestion. J. Anim. Sci. 88: 680-688.

Zebeli, Q., Dijkstra, J., Tafaj, M., Steingass, H., Ametaj, B. N., and Drochner, W. (2008). Modeling the adequacy of dietary fiber in dairy cows based on theresponses of ruminal pH and milk fat production to composition of the diet. J. Dairy Sci. 91: 2046-2066.

Annex

Annex

Scientific Publications

Amanzougarene, Z., Yuste, S., and Fondevila, M. (2020). Fermentation pattern of several sarbohydrate sources incubated in an *in vitro* semicontinuous system with inocula from ruminants given either forage or concentrate-based diets. Animals. 10: 261. doi: 10.3390/ani10020261.

Amanzougarene, Z., Tejeda, M. P., Calvo, H., de la Fuente, G., and Fondevila, M. (2020). Microbial fermentation of starch- or fibre-rich feeds added with dry or pre-activated Saccharomyces cerevisiae studied in vitro under conditions simulating high-concentrate feeding for ruminants. J. Sci. Food Agric. doi: 10.1002/jsfa.10249.

Amanzougarene, Z., Yuste, S., and Fondevila, M. (2019). Addition of several tannin extracts to modulate fermentation of barley meal under intensive ruminant feeding conditions simulated *in vitro* by incubating at pH 6.0–6.2. Anim. Prod. Sci. 59: 1081-1089.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2019). *In vitro* study of the effect of combinations of cereals and sugar beet pulp on pH and gas production pattern in concentrate or forage-based diets for ruminants. Options Méditerranéennes, A, no. 123, Innovations for Sustainability in Sheep and Goats.

Amanzougarene, Z., and Fondevila, M. (2018). Fitting of pH conditions for the study of concentrate feeds fermentation by the *in vitro* gas production technique. Anim. Prod. Sci. 58: 1751-1757.

Amanzougarene, Z., Yuste, S., Castrillo, C., and Fondevila, M. (2018). *In vitro* acidification potential and fermentation pattern of different cereal grains incubated with inoculum from animals given forage or concentrate-based diets. Anim. Prod. Sci. 58: 2300-2307.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2018). Differences in nutritional characteristics of three varieties of sorghum grain determine their *in vitro* rumen fermentation. Span. J. Agric. 16: e0608.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2017). *In vitro* fermentation pattern and acidification, potential of different sources of carbohydrates by ruminants giving high concentrate diets. Span. J. Agric. Res. 15: e0602.

.

Communication to Scientific Meeting

Amanzougarene, Z., Yuste, S., de la Fuente, G., de Vega, A., and Fondevila, M. (2019) "Efecto de distintos tipos de aditivos sobre la fermentación *in vitro* de cebada, en cultivos semicontínuos". XVIII Jornadas de Producción Animal (AIDA). Zaragoza, España. Page 227-229.

Amanzougarene, Z., Yuste, S., and Fondevila, M. (2019). Fermentation of diets for milking cows including clay minerals (zeolite, bentonite and sepiolite) measured in a semicontinuous in vitro system. Proceedings of the XIIIth International Symposium on Ruminant Physiology (ISRP 2019), Leipzig, Germany. Page 511.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2019). *In vitro* fermentation and acidification potential of several carbohydrates sources used in concentrate-based diets for growing ruminants. Joint Seminar of FAO-CIHEAM Network on Sheep and Goats, Meknes-Morocco.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2018). Effect of additives from different nature on barley fermentation in diets for intensive beef production in an *in vitro* semicontinuous system. 10Th International Symposium on the Nutrition of Herbivores. Clermont-Ferrand, France. Page 392.

Amanzougarene, Z., Tejeda, M.P., Calvo, H., and Fondevila, M. (2018). *In vitro* fermentation of barley or sugarbeet pulp in response to dry or pre-activated *Saccharomyces cerevisiae* yeasts additive under an incubation pH dropping to 6.0. 10Th International Symposium on the Nutrition of Herbivores. Clermont-Ferrand, France. Page 362.

Amanzougarene, Z., Yuste, S., Vega, A., and Fondevila, M. (2017). Fermentación *in vitro* de cebada en función del nivel de inclusión de ácidos grasos en dietas para el cebo de terneros. XVI Jornadas sobre Producción Animal 168-170 May 30-31, Zaragoza, Spain.

Amanzougarene, Z., Yuste, S., Vega, A., and Fondevila, M. (2017). Efecto de la inclusión de aceites esenciales en dietas para el cebo de terneros sobre la fermentación *in vitro* de cebada. XVI Jornadas sobre Producción Animal 258-260 May 30-31, Zaragoza, Spain

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2017) "*In vitro* study of the effect of combinations of cereals and sugarbeet pulp on pH and gas production pattern in concentrate or forage-based diets for ruminants". Seminario conjunto de la red FAO- CIHEAM de ovinos y caprinos. Vitoria, España. 49.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2017) "Effect of activated yeast on *in vitro* fermentation of barley in diets for intensive beef production". *68th Meeting of the* European Federation of Animal Science (EAAP). Tallinn, Estonia. Page281.

Amanzougarene, Z., Yuste, S., de Vega, A., and Fondevila, M. (2017) "*In vitro* fermentation and acidification potential of several carbohydrates sources with two inocula". 68th Meeting of the European Federation of Animal Science (EAAP). Tallinn, Estonia. Page281.

Amanzougarene, Z., Gimeno, A., Fondevila, M. (2015). *In vitro* rumen fermentation of three varieties of sorghum grain, with or without polyethylene glycol. 66th Meeting of the European Federation of Animal Science (EAAP). Warsaw, Poland. pp 428.

Amanzougarene, Z., Schauf, S. Fondevila, M. (2015). Control del pH *in vitro* para la simulación de la fermentación en condiciones de alimentación concentrada. XVI Jornadas sobre Producción Animal (ITEA). Zaragoza, España. Revista de la Asociación Interprofesional para el Desarrollo Agrario I. pp 212- 214.