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# STRATEGIES TO MITIGATE THE IMPACT OF SHIFTING FROM A MILK AND FORAGE REGIMEN TO A HIGH-CONCENTRATE DIET IN INTENSIVELY REARED BEEF CATTLE


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**UNIVERSIDAD DE ZARAGOZA**
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# Strategies to mitigate the impact of shifting from a milk and forage regimen to a high-concentrate diet in intensively reared beef cattle

SUSANA YUSTE FERNÁNDEZ

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# FACULTAD DE VETERINARIA

*Departamento de Producción Animal y*

*Ciencia y Tecnología de los Alimentos*

# *'Strategies to mitigate the impact of shifting from a milk and forage regimen to a high-concentrate diet in intensively reared beef cattle'*


Memoria presentada por

**SUSANA YUSTE FERNÁNDEZ**

para optar al grado de

Doctora por la Universidad de Zaragoza


Zaragoza, Mayo de 2020

Departamento de
Producción Animal
y Ciencia de los Alimentos
Universidad Zaragoza

ANTONIO DE VEGA GARCÍA Y MANUEL FONDEVILA CAMPS, Profesores del Departamento de Producción Animal y Ciencia de los Alimentos de la Universidad de Zaragoza

CERTIFICAN: Que la presente Memoria con título *''Strategies to mitigate the impact of shifting from a milk and forage regimen to a high-concentrate diet in intensively reared beef cattle''* ha sido realizada bajo nuestra dirección por la licenciada en Veterinaria Doña Susana Yuste Fernández, y reúne los requisitos exigidos para optar al grado de Doctora por la Universidad de Zaragoza, por lo que consideramos pertinente su presentación y defensa.

La Memoria de Tesis que se presenta se corresponde con el Proyecto de Tesis aprobado por la Comisión de Doctorado del programa de Producción Animal el día 16 de febrero de 2016.

Zaragoza, Mayo 2020

Fdo.: Antonio de Vega García

Fdo.: Manuel Fondevila Camps

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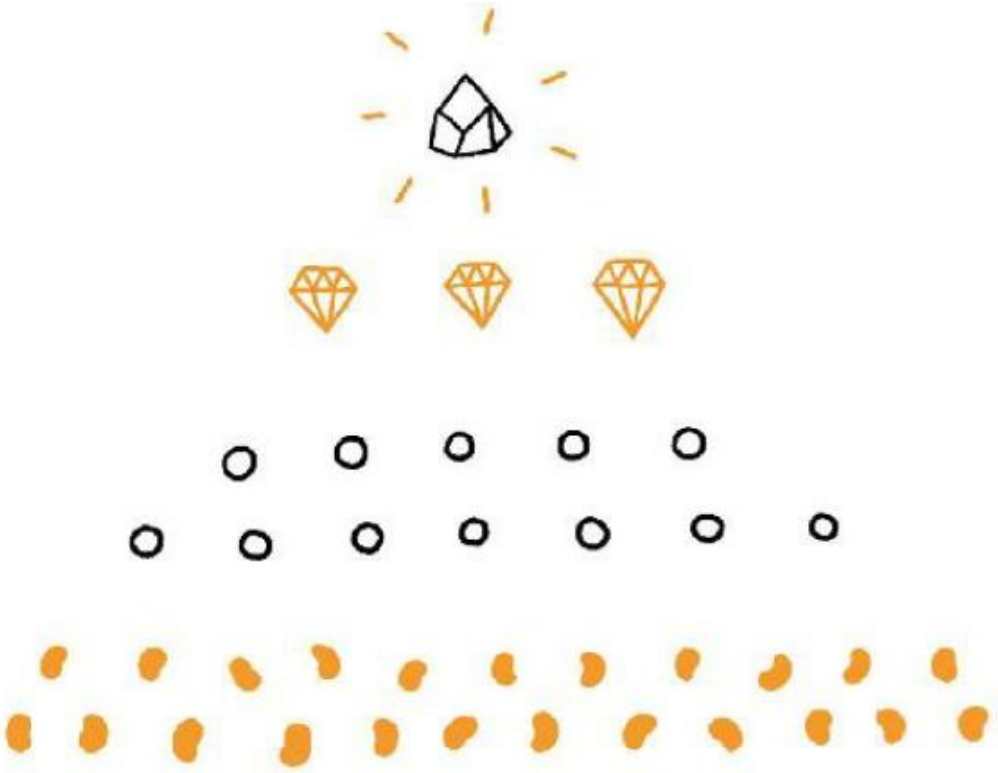
La autora de esta Memoria ha disfrutado una Ayuda Predoctoral para la Formación de Profesorado Universitario (referencia FPU 15/1960) concedida por el Ministerio de Educación, Cultura y Deporte.

*A mis padres*

Estamos rodeados de veintitrés amarillos.
Tenemos doce perlas al año.
Poseemos tres diamantes.
Un trozo de un mismo cristal.
*Albert Espinosa*

***A todos ellos…muchas gracias.***



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# LIST OF ABBREVIATIONS

| | | | |
|---|---|---|---|
| **AC** | Acidogenic capacity | **DFM** | Direct-fed microbials |
| **ADG** | Average daily gain | **DMI** | Dry matter intake |
| **AGV** | Ácidos grasos volatiles | **EO** | Essential oils |
| **AGCM** | Ácidos grasos de cadena media | **FCR** | Feed conversion ratio |
| **ANOSIM** | Analysis of similarity | **GA** | Gradual adaptation |
| **AOAC** | Association of Official Analytical Chemists | **HT** | Hydrolysable tannins |
| **ADF** | Acid detergent fibre | **IGF** | Insulin growth-like factor |
| **ADFom** | ADF fibre expressed exclusive of residual ash | **i.d** | Internal diameter |
| **ARA** | Acute ruminal acidosis | **IGF-1R** | Ruminal receptor for IGF-1 |
| **A:P** | Acetate to propionate ratio | **LPS** | Lipopolysaccharide |
| **AUC** | Area under the curve | **LW** | Live weight |
| **BCFA** | Branched-chain volatile fatty acids | **MCFA** | Medium-chain fatty acids |
| **BOHB** | β-hydroxybutyrate | **ME** | Metabolisable energy |
| **BP** | Sugar beet pulp | **MS** | Materia seca |
| **CCR** | Concentrate conversion ratio | **NDF** | Neutral detergent fibre |
| **CDK** | Cyclin-dependent kinases | **NDFom** | NDF assayed without amylase and expressed exclusive of residual ash |
| **CDMI** | Concentrate dry matter intake | **NE** | Net energy |
| **CO** | Coconut oil | **NEFA** | Non-esterified fatty acids |
| **CP** | Crude protein | **NMDS** | Non-metric Multi-Dimensional Scaling |
| **CT** | Condensed tannins | **OA** | Organic acids |
| **CV** | Coefficient of variation | **PERMANOVA** | Permutational multivariate analysis of variance |

| | | | |
|---|---|---|---|
| **Ph** | Phase | **TJ** | Tight-junctions |
| **PSM** | Plant secondary metabolites | **TMR** | Total mixed ration |
| **PV** | Peso vivo | **TVFA** | Total volatile fatty acids |
| **RA** | Rapid adaptation | **VFA** | Volatile fatty acids |
| **SARA** | Sub-acute ruminal acidosis | | |

# SUMMARY/RESUMEN

# SUMMARY

Current feeding practices for beef cattle rely on high-concentrate diets to achieve fast growth rates and efficiency. The fermentation of these diets increase the production of acids in the rumen, which might cause rumen acidosis. The transition period of beef calves to high-concentrate diets is a critical window of time because calves are often abruptly weaned and immediately switched from a milk and forage to a high-concentrate regime. Within the frame of the present thesis, three experiments were designed to acquire knowledge about the effectiveness of some strategies to reduce the susceptibility to acidosis during the transition period, as well as to assess the real impact of the transition period on animal performance and rumen fermentation.

The Experiment 1 was carried out to evaluate the most adequate pre-weaning feeding management of beef calves that are weaned and subsequently introduced to the intensive feeding system, in terms of rumen development, performance and economic profitability. Thirty female beef calves (15 Parda de Montaña, PM; and 15 Pirenaica, PI) were assigned to three pre-weaning diets (milk only, MO; milk plus hay, MH; and milk plus concentrate, MC). The study consisted of three phases: a) pre-experimental (months 1-2 of age; all calves only consumed milk from their mothers), b) treatment (from three months of age until weaning at five months calves received either meadow hay or starter concentrate *ad libitum*) and c) transition (sixth month, all calves received the same commercial compound feed plus wheat straw *ad libitum*). The intake of milk and solid feeds was recorded. Rumen fluid was sampled to characterise rumen fermentation at weaning and once the calves had been adapted to the feedlot. Blood samples were taken monthly from the second month of the suckling period to analyse rumen blood metabolites. Milk intake did not differ among groups (P=0.46), and solids intake (concentrate or forage) was similar (P=0.46) for MH and MC calves. Pre-weaning supplementation improved live weight (LW) (138, 175 and 196 kg for MO, MH and MC, P<0.001) and daily gains (0.582, 0.988 and 1.184 kg/d for MO, MH and MC, P<0.001) at weaning. During transition, concentrate intake was lower for MO calves (2.77, 3.82 and 3.88 kg dry matter (DM)/d for MO, MH and MC, P<0.001), and MH calves showed the highest feed conversion ratio (2.95, 3.59 and 3.08, for MO, MH and MC, P<0.01). At the end of transition, MC calves showed the highest LW (168, 211 and 236 kg for MO, MH and MC, P<0.001) and daily gain (0.961, 1.067 and 1.275 kg/d for MO, MH and MC, P<0.01), with no differences between MH and MO. Rumen pH was affected by the

interaction diet x sampling day (P<0.01), and the transition to the fattening concentrate resulted in a marked decrease in ruminal pH in those animals that had not received concentrate during the pre-weaning period, whereas MC animals showed similar pH between weaning and at the end of transition. Groups MC and MH showed higher total volatile fatty acids (TVFA) concentration than MO calves at weaning (31, 65 and 66 mmol/L for MO, MH and MC, P<0.001), whereas MC calves showed the lowest values at the end of the transition period (61, 67 and 40 mmol/L for MO, MH and MC, P<0.001). The similar rumen pH between periods together with the lower TVFA concentration in MC calves suggests an increased absorption of the reticulorumen as a result of a higher absorptive capacity of the rumen epithelia, and thus a better adaptation. Ammonia and lactate concentration (105, 36 and 21 mg/L, and 0.13, 0.27 and 0.81 mmol/L for MO, MH and MC, respectively, P<0.001) at weaning were the highest for MO and MC calves, respectively, with no differences between diets at the end of transition. Glucose and non-esterified fatty acids concentrations were only affected by age (P<0.05), and urea and β-hydroxybutyrate (BOHB) concentrations were affected by diet x age the interaction. Concentration of BOHB was similar in MH and MC calves at weaning, which suggests that their rumen was equally efficient in converting butyrate to BOHB. Regardless the type of supplement, offering pre-weaning supplementation resulted in higher economic profitability regardless the type of supplement (389, 438 and 434 € for MO, MH and MC, P<0.01). At the end of the transition, economic profitability (calculated in this case as the difference between calf sale price at the end of the study minus calf purchase price at weaning and the feeding costs associated with the intake of fattening concentrate) ranked MC>MO>MH (25, 20 and 14 €, P<0.01). Therefore, supplementation with concentrates was the best option, whereas supplementation with hay was the least advisable practice if the animals were to be moved to a feedlot after weaning.

Regarding differences between breeds, daily gains during the suckling period were higher for PM than for PI calves (0.966 *vs.* 0.870 kg/d, P<0.01) due to their higher milk intake (7.03 *vs.* 5.87 kg for PM and PI, P<0.01), which provided a better economic margin (437 *vs.* 404 € for PM and PI, P<0.01). During transition, concentrate intake was higher for PM than for PI (3.81 *vs.* 3.16 kg DM/d, P<0.01), but the economic margin was similar (P=0.44).

In conclusion, considering the whole production cycle and in terms of rumen adaptation to high-concentrate diets, concentrate pre-weaning supplementation is the most interesting option for beef calves that are going to be introduced in the feedlot.

The aim of Experiment 2 was to determine the effect of the partial substitution of barley grain with maize and sugar beet pulp (BP) in the diet of beef calves during the transition from milk and pasture to a conventional high-concentrate ration on feed intake, animal performance, and rumen fermentation and microbiota. Further, the effect of different protocols of adaptation to the final growing diet was assessed. Thirty-nine 7-months old beef calves transitioned from a milk and pasture regime to a high-concentrate ration were used. Diets were: a barley-based growing diet (C), and two diets in which barley was partially replaced with maize and 10% BP (BP10) or 22% BP (BP22) on *as fed* basis. Wheat straw was offered *ad libitum*. Three adaptation protocols were followed: in Protocol 1 animals were abruptly shifted to the C diet, whereas calves in Protocols 2 and 3 were gradually adapted and received diets BP10 and BP22, respectively, for 10 d; thereafter a 50:50 mixture of their diet and diet C until day 14, and finally, from day 14, all animals received the final diet (diet C) until slaughter at a target LW of *ca*. 500 kg. Therefore, the experiment had three phases (Ph): Ph1 (0-10d), Ph2 (11-14d) and in Ph3 (15-end), and the transition period comprised the first 14 days. Wheat straw was offered *ad libitum*. Concentrate and straw intake were recorded through the whole experiment and until the end of the Ph2, respectively. The rate of intake of concentrate and straw was also recorded. Rumen fluid was collected over the trial at 0, 3, 6 and 9 h after feeding to characterise fermentation. Samples from 0 h were analysed to assess protozoal (by optical observation through microscope) and bacterial (by DNA sequencing with Ion Torrent Platform) population. Animals in Protocol 3 showed the lowest concentrate intake (4.91, 5.39 and 3.95 kg DM/d for Protocol 1, 2 and 3, $P<0.01$) and the highest straw intake (15, 14 and 24 % of straw on total DM intake for Protocol 1, 2 and 3, $P<0.001$) in Ph2, which could have been due to a self-regulation mechanism in response to the acidogenic capacity of the diet. Daily gains and LW at the end of each phase, total concentrate intake, dressing percentage and concentrate conversion ratio were not affected ($P>0.05$) by the adaptation protocol. Despite the different ingredient composition of the diets, rumen fermentation variables were not affected by the protocol, but varied over time in response to concentrate inclusion. At the end of the trial, a consistent protozoal population persisted in the rumen of all calves (5.89 log cells/mL). Rumen protozoal diversity dropped throughout the study and most protozoa belonged to genera *Entodinium* (97.5%) and *Isotricha* (1.7%). Analysis of the 16S rRNA bacterial gene sequences showed a high individual variability among calves in terms of rumen bacterial population. The multivariate analysis showed that the application of different adaptation protocols had no

effect on rumen bacterial population or diversity (Shannon index and Richness); however, both diversity indexes were affected by day of sampling ($P<0.001$). In fact, most of the analysed bacterial taxa differed over time ($P<0.05$). Although diversity dropped with concentrate inclusion, on day 10 some new genera emerged in the rumen suggesting an adaptation of bacterial community to the new environment

In conclusion, the application of different adaptation protocols had no effect on any of the studied traits, and animal performance and rumen fermentation were not affected by the abrupt transition to the high-concentrate diet. This suggests that calves might cope with different feeding conditions, probably by adapting their feeding behaviour as well as due to the microbial adaptions to the new rumen environment. The use of barley or maize as the prevailing cereal or the inclusion of BP in the adaptation diet have no effect on rumen fermentation or microbial populations of beef calves, and their use will depend on market price or availability.

The Experiment 3 was designed to evaluate the effect of the inclusion of different feed additives, as rumen fermentation modulators, in the adaptation diet for beef calves on feed intake, animal performance, rumen fermentation and rumen microbial composition. Tannins and medium-chain fatty acids (MCFA) were chosen based on the lack of information of their effect on rumen fermentation during the transition period, and according to their modulator effect observed *in vitro* in our laboratory. Eighteen 7-months old beef calves were abruptly weaned and transitioned from a milk and pasture regime to one of these diets: a non-supplemented cereal-based concentrate and wheat straw given *ad libitum* (C), C plus 20 g/kg of a 65:35 chestnut and quebracho tannin extract (T), and C plus 6 g/kg of a mixture of MCFA (M). The experiment lasted 28 d. Rumen fluid was collected over the trial at 0, 3, 6 and 9 h after feeding to characterise fermentation, and samples from 0 h were also analysed for protozoal and bacterial population as in Experiment 2. Intake of concentrate and straw, and their rate of intake were similar ($P>0.05$) among diets and sampling days. Daily gains during the transition period increased over time ($P<0.001$), but were not affected by the inclusion of additives ($P=0.98$). Likewise, no diet effect ($P>0.05$) was found on final LW (248 kg) or on feed conversion ratio (4.48). Rumen fermentation variables were not affected by the addition of additives ($P>0.05$) but differed over time in response to the concentrate inclusion. Rumen protozoal population varied over time ($P<0.05$), and all calves harboured a stable population at the end of the experiment (5.58 log cells/mL). Protozoal diversity decreased

on time, and *Entodinium* (93.1%) and *Isotricha* (4.3%) species were the most abundant at the end of the trial. When occurred, defaunation was transient as protozoa were absent from M calves in three occasions on days 7 and 14 but were refaunated thereafter reaching a consistent population. The addition of tannins or MCFA did not modify ($P=0.92$) the structure of the bacterial community, which was affected by the sampling day ($P<0.001$). The additives did not affect the relative abundances of the main bacterial taxa ($P<0.05$), most of them differing across days ($P<0.001$). Diversity indexes (Shannon and Richness) declined over sampling days ($P<0.05$), although some genera emerged after concentrate inclusion.

At the doses used in the present experiment, tannins and MCFA did not exert any effect on intake, animal performance, rumen fermentation or bacterial population. Addition of tannins did not affect protozoal concentration and diversity, but MCFA might lead to a transient defaunation. An abrupt change from a milk and grass feeding regime to a high-concentrate diet did not impair animal performance or rumen health of beef calves, but concentrate inclusion decreased microbial diversity and strongly altered bacterial composition towards increased amylolytic and acid-tolerant species, suggesting an adaptation of the rumen microbes to the new environmental conditions. Based on our results, there is no necessity to include these additives in the adaptation diet of beef calves.

# RESUMEN

El cebo de terneros en España se lleva a cabo mediante el aporte de dietas con un elevado porcentaje de concentrado en la ración y una mínima parte de forraje, con el fin de lograr un elevado crecimiento diario y acortar el ciclo de producción. Estas dietas son altamente fermentables en el rumen y pueden causar acidosis ruminal. El período de transición de los terneros a las dietas de cebo intensivo es una fase crítica, ya que habitualmente estos animales se destetan de forma abrupta e inmediatamente entran a cebadero. En el contexto de la presente tesis doctoral, se diseñaron tres experimentos con el fin de estudiar la efectividad de algunas estrategias para reducir el riesgo de padecer acidosis ruminal durante el período de transición de terneros de carne.

El Experimento 1 se llevó a cabo para determinar el manejo alimentario previo al destete más adecuado, en términos de desarrollo del rumen, rendimiento animal y rentabilidad económica, para terneros que son destetados de forma abrupta e inmediatamente introducidos al sistema de cebo intensivo. Para ello, se utilizaron treinta terneras (15 Parda de Montaña, PM; y 15 Pirenaica, PI) que se asignaron a tres dietas ofrecidas antes del destete (leche, MO; leche y heno, MH; leche y concentrado, MC). El experimento se dividió en tres fases: a) pre-experimental (meses 1-2 de edad, todas las terneras consumían solo leche de sus madres); b) tratamiento (de los tres meses de edad al destete a los cinco meses, en la que consumieron *ad libitum* heno de prado o concentrado) y c) transición (sexto mes, todas las terneras recibieron el mismo pienso y paja de trigo a voluntad). Se registró la ingesta de concentrado, heno y leche, y se tomaron muestras de líquido ruminal (un día antes del destete y tras el destete) para analizar las variables de fermentación ruminal. Asimismo, desde el segundo mes de lactación, se tomaron muestras de sangre mensuales para analizar metabolitos sanguíneos relacionados con el estado nutricional de las terneras. La ingesta de leche no difirió entre grupos ($P=0,46$), y la de alimentos sólidos (concentrado o forraje) fue similar ($P=0,54$) para las terneras de los grupos MH y MC. La suplementación durante la lactación mejoró el peso vivo (PV) (138, 175, 196 kg en MO, MH y MC, $P<0,001$) y la ganancia media diaria al destete (0,58, 0,98 y 1,18 kg/d en MO, MH y MC, $P<0,001$). Durante la transición, la ingestión de materia seca (MS) de concentrado fue menor para el grupo MO (2,77, 3,82 y 3,88 kg MS/d en MO, MH y MC, $P<0,001$), y las terneras MH mostraron un índice de conversión mayor (2,95, 3,59 y 3,08 en MO, MH y MC, $P<0,01$). Al final de la transición, las terneras MC mostraron el mayor PV (168, 211 y 236 kg en MO, MH y MC, $P<0,001$)

y la mayor ganancia media diaria (0,961, 1,067 y 1,275 kg/d en MO, MH y MC, P<0,01), y no hubo diferencias entre los grupos MH y MO. El pH ruminal se vio afectado por la interacción entre la dieta y el día de muestreo (P<0.01). El cambio al concentrado de engorde resultó en una marcada disminución del pH ruminal en los animales que no habían recibido concentrado, mientras que los animales MC mostraron un pH similar entre períodos. Los grupos MC y MH mostraron una mayor concentración de ácidos grasos volátiles (AGV) que las terneras MO al destete (31, 65 y 66 mmol/L en MO, MH y MC, P<0,001), mientras que las terneras MC mostraron los valores más bajos en al final del período de transición (61, 67 y 40 mmol/L en MO, MH y MC, P<0,001). El similar pH ruminal entre períodos, junto con la menor concentración de AGV en las terneras suplementadas con concentrado, sugiere una mayor absorción del epitelio ruminal y, por lo tanto, una mejor adaptación a la dieta de cebo. Las concentraciones de amoníaco y de ácido láctico (105, 36 y 21 mg/L, y 0,13, 0,27 y 0,81 mmol/L, respectivamente, en MO, MH y MC, P<0,001) en el rumen al destete fueron mayores para las terneras MO y MC, respectivamente, sin diferencias entre grupos al final de la transición. La concentración sanguínea de glucosa y ácidos grasos no esterificados sólo se vio afectada por la edad (P<0,05), mientras que las de urea y β-hidroxibutirato (BOHB) se vieron afectadas por la interacción entre la dieta y la edad. Las terneras MH y MC mostraron concentraciones similares de BOHB al destete, lo que sugiere que su rumen era igualmente eficiente en la conversión de butirato en BOHB. La suplementación previa al destete resultó en una mayor rentabilidad económica al destete, independientemente del tipo de suplemento (389, 438 y 434 € para MO, MH y MC, P<0.01). Al final de la transición, la rentabilidad económica (calculada ésta como la diferencia entre el precio de venta de las terneras al final del estudio menos el precio de compra al destete y los costes de alimentación) siguió el siguiente orden: MC>MO>MH (25, 20 y 14 € para MC, MO y MH, P<0.01). Por lo tanto, la suplementación con concentrado fue la mejor opción, mientras que la suplementación con heno fue la práctica menos recomendable si los animales iban a entrar a cebadero tras el destete.

Con respecto a las diferencias entre razas, la ganancia diaria durante el período previo al destete fue mayor para PM que para PI (0,966 *vs.* 0,870 kg/d, P<0,01) debido a una mayor ingesta de leche en las primeras (7,03 *vs.* 5,87 kg, P<0,01), lo que proporcionó un mejor margen económico. Durante la transición, la ingesta de concentrado fue mayor para PM que para PI (3,81 *vs.* 3,16 kg DM/d, P<0,01), pero el margen económico fue similar entre razas (P=0.44).

En conclusión, teniendo en cuenta todo el ciclo de producción, y en términos de adaptación del rumen a las dietas típicas del sistema intensivo con alto contenido en energía, la suplementación previa al destete con concentrado es la opción más interesante para los terneros de carne que van a entrar a cebadero.

El objetivo del Experimento 2 fue determinar el efecto de la sustitución parcial de la cebada por maíz y pulpa de remolacha (BP) en la dieta de adaptación de los terneros durante la transición a una ración de cebo intensivo sobre la ingestión y el rendimiento de los animales, la fermentación ruminal y la composición microbiana del rumen. Para ese fin, se utilizaron 39 terneros de 7 meses de edad que pasaron de un régimen de leche y pasto a una ración con alto porcentaje de concentrado. Se formularon tres dietas: una dieta de crecimiento a base de cebada (C); y dos dietas en las que la cebada fue parcialmente reemplazada por maíz y 10% (BP10) o 22% de BP (BP22). Se aplicaron tres protocolos de adaptación a la dieta de cebo: en el Protocolo 1, los animales se cambiaron bruscamente a la dieta C, mientras que los terneros en los Protocolos 2 y 3 se adaptaron gradualmente y recibieron las dietas BP10 y BP22, respectivamente, durante 10 días; a partir de entonces, recibieron una mezcla 50:50 de su dieta y la dieta C hasta el día 14, y finalmente, a partir del día 14, los terneros en los Protocolos 2 y 3 recibieron la dieta final hasta el sacrificio con un PV de aproximadamente 500 kg. El experimento tuvo tres fases (Ph): Ph1 (0-10d), Ph2 (11-14d) y en Ph3 (día 15-final), y el período de transición comprendió del día 0 al 14. Los concentrados y la paja de trigo se ofrecieron *ad libitum* en todos los casos. El consumo de concentrado se registró durante todo el experimento, y el de paja solamente durante el período de transición. Asimismo, se registró la velocidad de ingestión del concentrado y la paja en determinados días a lo largo del experimento. También se tomaron muestras de líquido ruminal en diferentes días a las 0, 3, 6 y 9 h tras la oferta del alimento, para caracterizar la fermentación ruminal, y se analizaron las muestras de líquido ruminal de la hora 0 para estudiar la población protozoaria (mediante observación óptica a través del microscopio) y bacteriana (mediante secuenciación de ARNr bacteriano con la plataforma Ion Torrent) del rumen. Los terneros del Protocolo 3 mostraron una menor ingestión de concentrado (4,91, 5,39 y 3,95 kg MS/d en los Protocolos 1, 2 y 3, $P<0,01$) y una mayor ingestión de paja (15, 14 y 24% de paja respecto al total de ingestión de MS para el Protocolo 1, 2 y 3, $P <0,001$) en la Ph2, lo que se podría considerar como un mecanismo de autorregulación en respuesta al poder acidogénico de la dieta. La ganancia media diaria, el PV al final de cada fase, el consumo total de

concentrado a lo largo del experimento, el rendimiento canal y el índice de conversión, así como las variables de fermentación ruminal, no se vieron afectados (P>0,05) por el protocolo de adaptación. En el último muestreo de líquido ruminal, se observó que todos los terneros albergaban una población estable de protozoos en el rumen (5,89 log cels/mL). La diversidad protozoaria del rumen disminuyó a lo largo del estudio, perteneciendo la mayoría a los géneros *Entodinium* (97,5%) e *Isotricha* (1,7%). El análisis de las secuencias del gen 16S del ARNr bacteriano mostró una gran variabilidad individual entre terneros en términos de población bacteriana del rumen. El análisis multivariante reveló que la aplicación de diferentes protocolos de adaptación no tuvo ningún efecto sobre la población o la diversidad bacteriana del rumen (índice de Shannon y Richness); sin embargo, ambos índices se vieron afectados por el día de muestreo (P<0,001). De hecho, la mayoría de los taxones bacterianos analizados se vieron afectados por el día de muestreo (P<0,005). Aunque la diversidad disminuyó con la inclusión de concentrado, en el día 10 aparecieron en el rumen algunos géneros bacterianos, lo que sugirió una adaptación de la comunidad bacteriana al nuevo ambiente.

En conclusión, la aplicación de los diferentes protocolos de adaptación no tuvo efecto en ninguna de las variables estudiadas. Ni el rendimiento animal ni la fermentación ruminal se vieron afectados por la abrupta transición a la dieta de cebo, lo que sugiere que los terneros podrían hacer frente a esas condiciones de alimentación probablemente adaptando su comportamiento alimentario, y mediante la adaptación de la población microbiana al nuevo ambiente ruminal. El uso de cebada o maíz como cereal predominante, o la inclusión de pulpa de remolacha, no tienen ningún efecto sobre la fermentación ruminal o las poblaciones microbianas en los terneros de engorde, y su uso dependerá del precio o la disponibilidad en el mercado de estos ingredientes.

En el Experimento 3 se evaluó el efecto de la inclusión de diferentes aditivos, como moduladores de la fermentación ruminal en la dieta de adaptación de terneros de carne, sobre la ingesta de alimento, el rendimiento animal, la fermentación ruminal y la composición microbiana del rumen. Se valoraron taninos y ácidos grasos de cadena media (AGCM), que fueron elegidos debido a la falta de información sobre su efecto sobre la fermentación ruminal durante el período de transición, y de acuerdo con su efecto modulador observado *in vitro* en nuestro laboratorio. Se utilizaron 18 terneros de 7 meses de edad, que pasaron de una dieta a base de leche y pasto a una de las siguientes: un concentrado a base de cereal y paja de trigo administrada *ad libitum* (C), C con la

inclusión de 20 g/ kg de un extracto de taninos de castaño y quebracho 65:35 (T), o C con la adición de 6 g/kg de una mezcla de AGCM (M). El experimento tuvo una duración de 28 días. Se registró el consumo de concentrado y paja a lo largo del experimento, y sus pautas de ingestión en determinados días. Se tomaron muestras de líquido ruminal a lo largo del experimento a las 0, 3, 6 y 9 h tras la oferta de alimento para caracterizar las variables de fermentación ruminal, y se analizaron las muestras de líquido ruminal obtenidas a las 0 h para evaluar protozoos y bacterias como en el Experimento 2. La ingesta diaria, así como la pauta de ingestión de concentrado y paja fueron similares ($P>0,05$) entre dietas. La ganancia de peso diaria, el PV final y el índice de conversión no se vieron afectados ($P>0,05$) por la inclusión de aditivos. Las variables de fermentación ruminal no difirieron entre grupos ($P>0,05$), pero variaron a lo largo del tiempo en respuesta a la inclusión del concentrado. Aunque la población de protozoos varió a lo largo del experimento, todos los terneros albergaron una población estable al final del ensayo (5,58 log cels/mL). La diversidad protozoaria disminuyó, y los protozoos de las especies *Entodinium* (93.1%) e *Isotricha* (4.3%) fueron los más abundantes al final del ensayo. En tres ocasiones se encontraron terneros del grupo M defaunados, pero ocurrió de forma transitoria. La adición de taninos o AGCM no modificó ($P=0,92$) la estructura de la comunidad bacteriana, la cual se vio afectada por el día de muestreo ($P<0,001$). La inclusión de aditivos no afectó a la abundancia relativa de los principales taxones bacterianos ($P<0,05$), y la mayoría de ellos difirió entre días de muestreo ($P <0.001$). Los índices de diversidad (Shannon y Richness) disminuyeron a lo largo del tiempo ($P<0,05$), aunque algunos géneros surgieron tras la inclusión de concentrados.

A las dosis utilizadas en el presente experimento, los taninos y los AGCM no ejercieron ningún efecto sobre la ingesta, el rendimiento animal, la fermentación ruminal y la población microbiana. La adición de taninos no afectó la concentración y diversidad de protozoos, pero la inclusión de AGCM podría conducir a una defaunación transitoria. El cambio de alimentación de leche y pasto a una dieta alta en concentrado no afectó negativamente el rendimiento animal o la salud del rumen de los terneros. Aunque la inclusión de concentrado disminuyó la diversidad bacteriana, surgieron algunos géneros que indican una posible selección de bacterias más tolerantes a las nuevas condiciones ruminales. De acuerdo con los resultados obtenidos, no parece necesaria la inclusión de estos aditivos en las dietas de transición de los terneros de cebo.

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1: INTRODUCTION

To face the worldwide demand of beef meat, farming is moving to a more specialized activity in which high-energy diets are given to animals aiming to improve efficiency of the production cycle. Calves are often abruptly weaned and immediately switched from a milk and/or forage to a high-concentrate regime. In animals that have consumed forage as the only solid feed the rumen becomes highly specialised in fibre fermentation (Dirksen et al., 1985; Qumar el at., 2016) and thus are particularly susceptible to ruminal acidosis when they are introduced to the feedlot. Ruminal acidosis seems to be a complex and multifactorial nutritional disorder that goes further than a decrease in rumen pH as it may cause important systemic effects. Recovered animals may exhibit poorer performance and impaired feed conversion, what translates into tremendous economic losses for the beef industry. A successful feeding management during the transition period should ensure an encompassed adaptation of the rumen microbial populations, and of the rumen morphology and functional capacity of the epithelium in order to avoid nutritional disorders (Brown et al., 2006; Holtshausen et al., 2013).

The literature review presented in this PhD Thesis is focused on the main aspects that determine the occurrence of ruminal acidosis, especially during the transition period from milk and pasture to a high-concentrate diet, and the principal strategies that would be useful to mitigate the susceptibility to develop this nutritional disorder during such adaptation period. First, as a brief contextualization, a section with data on the current situation of the Spanish beef sector and some generalities on rumen fermentation and rumen microbial population will be presented. Thereafter, the main causes of rumen acidosis will be described. Finally, some of the strategies described in the literature aiming to reduce the incidence of acidosis are compiled.

The experiments performed within the frame of this doctoral thesis aimed to assess different feeding strategies that apparently promote a balanced transition from milk and pasture to a high-concentrate diet, typical of the Spanish fattening system. The strategies assessed in this PhD were chosen based on practical conditions and applicability. The results obtained here demonstrate the capacity of the rumen as a dynamic organ to accommodate to different feeding conditions.

# CHAPTER 2

# LITERATURE REVIEW

# CHAPTER 2: LITERATURE REVIEW

## 2.1 FEEDLOT CATTLE IN THE EUROPEAN UNION AND IN SPAIN

The European Union (EU-28) holds the third largest bovine meat production of the world behind The United States and Brazil, and together the three produce 47% of total world bovine meat produced (EUROSTAT, 2019). In 2019, the EU-28 produced around eight million tons of carcasses representing *ca.* 13% of the global market (EUROSTAT, 2019; Hocquette et al., 2018), and the largest beef meat production was held in France (18.2%), Germany (14.4%), UK (11.7%), Italy (9.9%), and Spain (8.9%) (EUROSTAT, 2019). European beef industry is complex and heterogeneous beef industry with a wide diversity of breeds, animal types (cows, bulls, steers, heifers, veal calves) and farming systems (indoors under intensive system, extensive on permanent pastures, mixed system, breeders, feeders-fatteners, etc.) (Hocquette et al., 2018).

As the 5$^{th}$ largest bovine meat producer in Europe, during 2018 Spain produced 666,632 tons of meat (from *ca.* 2.46 million heads), which accounted for about 17.5% of the final livestock output (S.G. Estadística, MAPA, 2018) and 6.3% of the final agricultural output (MAPAMA, 2019). The Spanish Autonomous Communities with higher beef production were Cataluña (23%), Castilla y León (17%) and Galicia (15%) (MAPA, 2018).

Spanish beef cattle sector is commonly divided into two main segments on the basis of their production system (Figure 2.1). On one hand, there are suckler-beef farms that include breeder farms with nursing cows, who sell calves after weaning, which are mainly located in Castilla y León (26%), Extremadura (23%), Andalucía (11%), Galicia (10%) and Asturias (7%) (SITRAN, 2019). In these farms, calves are allowed to suckle freely while they are grazing and sometimes are supplemented with hay or concentrate when pasture availability is limited. When these calves reach five to seven months, and from 200 to 250 kg, they are weaned and contribute to the supply of calves for fattening.

On the other hand, fattening farms (Figure 2.1) that can be classified into closed-cycle production farms that raise their own calves, or farms that purchase calves for rearing. The latter are farms who buy or import from other EU countries either early weaned young calves of few days or weeks from the dairy sector (<80 kg) or newly weaned beef calves of around 200 kg from beef herds, and fed them indoors with high-energy diets until they reach 10-16 months of age (from 440 to 500 kg market weight). In

general, each type of production system occurs separately and is area-dependent, especially suckler beef farms that are stuck to land, mainly pastures. As worldwide, the beef industry in Spain has followed a decrease in the number of fattening farms, although these farms are becoming more specialized. For instance, since 2010 there has been a simultaneous reduction of 18% of beef farms and an increase of 40.2% in the number of animals per fattening farm (SITRAN, 2019). Although the origin of the animals widely differs from one Spanish region to another, most reared calves are cross-bred with Charolaise or Limousine (SITRAN, 2019), from dairy breeds mainly Holstein-Friesian, from pure breeds such as Limousine, Montbeliard, Belgian-blue, and finally animals from pure autochthonous breeds such as Asturiana de los Valles, Avileña Negra Ibérica, Morucha, Pirenaica, Parda de Montaña, Retinta or Rubia Gallega (SITRAN, 2019).

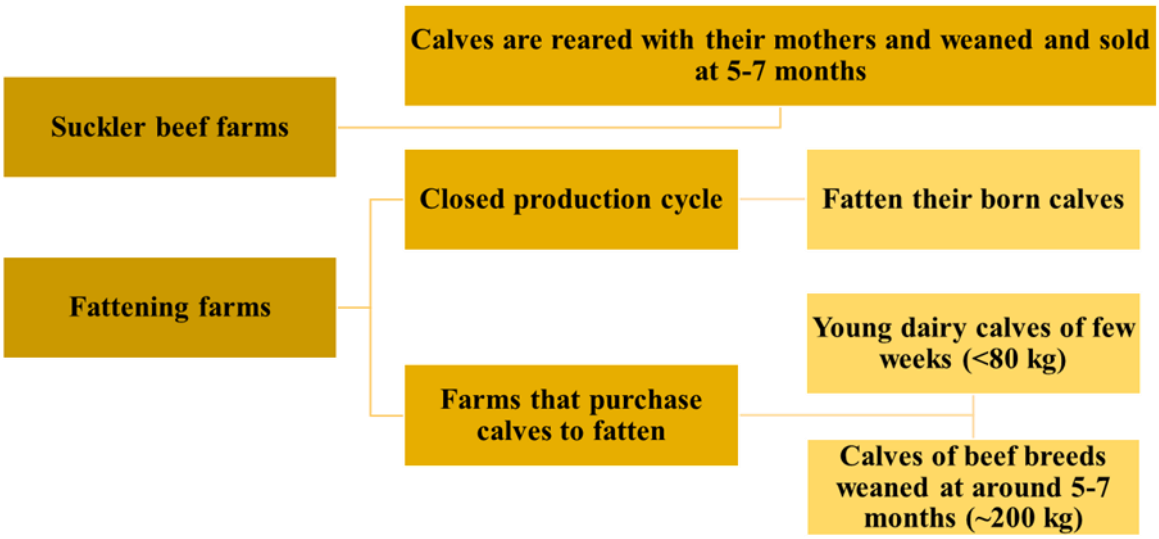


**Figure 2.1** Scheme of the beef production in Spain

## 2.2 CURRENT FEEDING SYSTEMS FOR BEEF CATTLE IN SPAIN

In general, the dry climate conditions and the scarce pluviometry do not allow the growth of large quantities of pastures in Spain and therefore the vast majority of the livestock that supplies the Spanish beef industry is reared intensively on cereals. The pasturelands in the north of Spain or near mountainous regions are used for suckler beef calves herds (Bacha, 2005), although milk cows often have preference over them because of their higher income.

Under the high global demand of meat for an increasing population, the beef meat production industry needs to achieve its maximum productivity and hence beef cattle should gain weight at their maximum potential, shortening the production cycle and making it the more efficient as possible. To attain the actual demand of rapid growth, beef diets are typically energy-dense and are based on the use of high amounts of grain and grain by-products along with protein feeds (Castillo et al., 2004) at the expense of fibre-rich forages. This is a nutritional challenge for ruminants that have evolved to ferment plant materials. Generally, conventional beef-fattening diets contain large quantities (more than 85% of dry matter intake, DMI) of a cereal-based concentrate given *ad libitum* and a small percentage of straw given separately, therefore both offered as free choice. The use of straw adds fibrous structure to the diet, acting as a source of physical effective fibre to stimulate chewing activity and salivary buffer production. Therefore, straw helps to maintain the proper rumen functioning, aiming to avoid nutritional disorders. Concentrates contain mainly up to 60% cereal grains (mostly barley and maize) as a source of energy (de Blas et al., 2008). These diets favour a fast growth (growth rates of 1.1-1.6 kg/d are expected, with a feed conversion of 4-5 kg of total feed per kg of gain), an increase of the productivity and a reduction of the production cycle. Animals are slaughtered at around one year of age (8-12 months for dairy breeds and 12-14 months for beef herds; de Blas et al., 2008), yielding carcass weights of 250-370 kg depending on the breeds.

Under intensive feeding, there are also other feeding managements that are currently used in beef farms as a common practice, such as feeding with wet unifeed (concentrate plus silages offered as a total mixed ration, TMR) or dry unifeed (concentrate and straw as TMR).

## 2.3 RUMEN FERMENTATION AND pH REGULATION

The rumen is an anaerobic fermentative chamber harbouring a microbial consortium that possesses specialized enzymes, who synergically degrade plant materials (celluloses, hemicelluloses, pectins, fructoses, starches and other polysaccharides) to monomeric or dimeric sugars that are fermented resulting in volatile fatty acids (VFA), lactate, methane, carbon dioxide, ammonia, hydrogen, heat and microbial cells (McAllister et al., 1994). Microbial products, VFA (mainly acetate, propionate and butyrate, and in minor proportions valerate, isobutyrate and isovalerate) and bacterial protein, are the main source of energy and protein to the host animal (Bergman, 1990; Van Soest, 1994). Under normal rumen conditions, the VFA readily dissociate, releasing a proton, $H^+$, which reduces the rumen pH. The production of organic acids after feed intake depends on factors such as the nature of the diet (forage to concentrate ratio), meal size and frequency, rate of intake and rate of passage. All these factors affect the extent of feed degradation by rumen microbes (González et al., 2012). Clearance of acids from the rumen and neutralization of protons are some inherent physiological mechanisms aiming to maintain the ruminal acid-base equilibrium to prevent acid accumulation in the rumen (Allen, 1997; Aschenbach et al., 2011); therefore, the amount of acid in the rumen at a given time is the net balance between acid production and acid removal and neutralization.

Removal of VFA from the rumen is carried out either by absorption through the rumen wall or by passage to the omasum (Aschenbach et al., 2009). Absorption of VFA from the rumen is a complex process influenced by factors such as surface area of rumen papillae and availability of transport proteins, epithelial blood flow, rumen motility, and rumen pH (Dirksen et al., 1984; Bannink et al., 2012), and it accounts for the removal of 50 to 85% of the acids produced (Allen, 1997; Aschenbach et al., 2011). Absorption involves two main mechanisms: passive lipophilic diffusion and non-diffusive uptake of VFA by means of specific apical or basolateral transport proteins.

Absorption of undissociated forms of VFA, which are lipophilic, occurs by passive diffusion across the membrane and is followed by a subsequent dissociation of the VFA into the cell. Lipophilic diffusion depends on the proportion of undissociated VFA (which is low at a normal rumen pH of 5.8-6.2; Nagaraja and Titgemeyer, 2007), rumen pH (low rumen pH enhances passive diffusion) and chain length (butyrate>propionate>acetate; Bergman, 1990).

Dissociated VFA require specific protein transporters located in the membrane of the epithelial cells that work as anion exchangers. Absorption of VFA through the rumen epithelium involves complex underlying mechanisms and a plethora of transport proteins (Gäbel and Aschenbach, 2006; Aschenbach et al., 2011). The main pathway of absorption of dissociated VFA is mainly by exchange for intracellular bicarbonate ($VFA^-$ / $HCO_3^-$ exchange), which is imported from the blood via $Na^+$/HCO3$^-$ basolateral cotransport or synthesized *de novo* within the epithelial cell (Dijkstra et al., 2012), and concomitantly secreted into the lumen supplying buffer to the rumen contents (Gäbel and Aschenbach, 2006; Aschenbach et al., 20011; Penner et al., 2009a). The $HCO_3^-$ combines with the $H^+$ pool in the rumen content and by means of the carbonic anhydrase reaction is converted to $H_2CO_3$ with the subsequent decomposition in $CO_2$ and water. Intracellular dissociation of VFA-H and the HCO3$^-$ exported from cells in exchange for $VFA^-$ decrease intracellular pH, and then the Na+/H+ exchangers secrete $H^+$ in apical or basolateral directions to regulate intracellular pH (Aschenbach et al., 2011).

As in the case of undissociated VFA, lactate absorption across the rumen wall mainly occurs via passive diffusion and increases as pH declines (Owens et al., 1998). However, the rate of absorption is much lower because it is a stronger acid since its $pK_a$ is lower than that of VFA (3.9 *vs.* 4.8; Owens et al., 1998). There is not much information on the ability of the rumen epithelium to absorb lactate by active transport, a process that seems to be mediated by monocarboxylate transporters at very slow rates (Aschenbach et al., 2009, 2011). This would explain the rapid accumulation of lactate due to excessive bacterial production during ruminal acidosis.

On the other hand, passage of VFA and lactate with rumen liquid through the lower gastro-intestinal tract as an acid removal mechanism is of minor importance than absorption, since only 29%-39% of total VFA passes into distal parts in dairy cattle and steers (Penner et al., 2009b; Aschenbach et al., 2011; Dijkstra et al., 2012).

Regarding the acid neutralization, the main buffer substances in the rumen are $HCO_3^-$, hydrogen phosphate ($HPO_4^{2-}$) and $NH_4^+$ (Aschenbach et al., 2011). Saliva is an extremely important source of buffer bases because it is rich in $HCO_3^-$ and $HPO_4^{2-}$ (Bailey and Balch, 1961), and cattle can produce up to 150 litres per day. It has been estimated that saliva can neutralize up to 37% of the total acids in the rumen (Allen, 1997). The buffering of the acids produced during rumen fermentation is mainly carried out by the bicarbonate from saliva and that secreted by the rumen epithelium (Gäbel et al., 1991;

Penner et al., 2009a; Schwaiger et al., 2013). As aforementioned, bicarbonate reacts with a proton releasing $CO_2$ and water. Some studies found out that long-term feeding high-concentrate diets increase the net bicarbonate secretion into the rumen and VFA absorption as an adaptive mechanism (Gäbel et al., 1991). Therefore, the increased ability of the rumen epithelium to neutralize acidity is a key point in the adaptation of ruminants to these diets.

## 2.4 THE RUMEN MICROBIAL ECOSYSTEM

The rumen is a highly dynamic ecosystem that harbours a diverse microbial consortium including bacteria, archaea, protozoa, fungi, and bacteriophages (Hungate, 1966). The rumen bacteria are the most numerous ($10^{10}$-$10^{11}$ cells/mL), diverse and metabolically active microorganisms in the rumen. These microorganisms vary in their substrate specificity, and some of them are specialists, which utilize only one substrate or derivate product, whereas others are generalists and can hydrolyse a variety of substrates (Stewart et al., 1997). In general, rumen bacteria fulfil several functional niches including cellulolytic, amylolytic, proteolytic and lipolytic functions (Stewart et al., 1997; Dehority, 2003). Further, based on their location and colonization within the rumen, bacteria have been classified into three groups: those in the liquid phase, those particle-associated bacteria, and epimural bacteria, which are attached to ruminal epithelium.

Methanogenic archaea ($10^{7}$-$10^{9}$ cells/mL) play a vital role in scavenging molecular hydrogen generated during fermentation and reducing hydrogen partial pressure. Archaea are the sole producers of methane in the rumen (Morgavi et al., 2010). They maintain a symbiotic relationship with protozoa, which hold hydrogenosomes that generate $H_2$ that is used by methanogens (Newbold et al., 2015). They also exhibit a syntrophic interaction with anaerobic fungi, which increases the fibre degradation activity of the latter due to interspecies hydrogen transfer and a more efficient regeneration of oxidized nucleotides (such as $NAD^+$, $NADP^+$; Edwards et al., 2017).

The rumen protozoa ($10^{4}$-$10^{6}$ cells/mL) can account for up to 50% of the microbial mass in the rumen (Williams and Coleman, 1992). They engulf starch granules and sugars that are stored intracellularly, avoiding their rapid fermentation by amylolytic bacteria (Mackie et al., 1978; Coleman, 1986; Newbold et al., 2015). The holotrich protozoa are mostly involved in the utilization of non-structural polysaccharides and soluble sugars (Williams and Coleman, 1997) and can store starch as amylopectin, which is intracellularly metabolized to VFA at a slow rate (Coleman, 2016). The entodiniomorphs hardly use sugars but actively ingest starch granules that are slowly metabolized to VFA (Coleman, 1986). Rumen protozoa, especially the holotrichs (Belanche et al., 2015), produce $H_2$ that is used by methanogens through a symbiotic relationship that contributes to methane production; up to 25% of rumen methanogens are associated to rumen protozoa (Newbold et al., 2015). Unlike bacteria, protozoa are apparently not essential for the host (Hungate 1966; Newbold et al., 2015). In fact, defaunation makes the rumen

more efficient in terms of energy spilling and protein metabolism (Mendoza et al., 1993; Hackmann et al., 2013). However, positive effects such as higher fibre digestibility (Newbold et al., 2015), a modulation of starch digestion in the rumen and shifting the site of starch digestion to the small intestine (Nagaraja et al., 1992; Mendoza et al., 1993) are attributed to the presence of rumen protozoa. Further, their predatory role reduces numbers of bacteria, which stabilizes pH (Nagaraja et al., 1992), and lactate clearance is higher in the presence of entodiniomorphs as they consume lactate faster than bacteria (Newbold et al., 1986).

Anaerobic fungi can account for up to 20% of microbial biomass (Edwards et al., 2017). They are the most effective fibre degrading microorganisms in the rumen because of their combined mechanical and extensive enzymatic activity that accelerates lignocellulose degradation (Edwards et al., 2017). On one hand, they degrade fibre by producing fibrolytic enzymes, and on the other hand, they facilitate feed colonization by bacteria because of their ability to pass through the structural barrier of the plant via penetrating rhizoids (Orpin and Joblin, 1997). However, overall contribution of fungi to rumen degradation is relative, because the antifungal activity of bacteria restricts their potential growth and impact.

Bacteriophages ($10^7$-$10^{11}$ cells/mL) are the viruses that infect bacteria, and may help in bacterial mass turnover in the rumen (Nagaraja, 2016).

Rumen microbiota exhibits interesting ecological features such as functional redundancy, plasticity and resilience (Weimer, 2015, Schären et al., 2018; Belanche et al., 2019a). The former refers to the ability of different microbes to produce the same outputs with the same substrates through distinct metabolic pathways (Taxis et al., 2015). In this sense, considerable changes in the ruminal community do not often result in fundamental changes in rumen fermentation products (Weimer, 2015). The rumen plasticity deals with the capacity of microbes to substitute each other in different metabolic niches, to adapt to different substrates and to shift their fermentation products depending on the environment (Schären et al., 2018; Belanche et al., 2019a). The resilience of the rumen microbial community refers to the ability of the rumen microbes to return to its original composition after being disturbed (Allison et al., 2008; Weimer, 2015).

The homeostasis in the rumen ecosystem is essential to the health and productivity of the host animal (Clemmons et al., 2019), and divergences in rumen microbial

communities are associated with different host characteristics including feed efficiency (Myer et al., 2015; Paz et al., 2018; Schären et al., 2018), milk production (Xue et al., 2018), disease states (Khafipour et al., 2009) and methane emission (Lan and Yang, 2019), as well as with differences in host metabolism (Morgavi et al., 2015). Furthermore, there is evidence that epimural bacteria, directly attached to the rumen epithelial lining, exert a significant impact on the development of the rumen epithelium as well as on mucosal immunity and health (Chen et al., 2011; Taschuk and Griebel, 2012; Lin et al., 2019).

### 2.4.1 Factors that modify rumen microbial composition

Ruminal microbiota varies among animals depending on factors such as diurnal changes in rumen environment in response to diet fermentation, age of animals or geographical area (Weimer, 2015; Shaani et al., 2018). Among them, it is well established that the diet is the main factor that alters rumen microbiota (Weimer, 2015; Tapio et al., 2017; Belanche et al., 2019a). Rumen microbiota shows considerable host specificity as animals consuming the same diet reveal different microbial population. This feature was clearly demonstrated in the experiment carried out by Weimer et al. (2010), in which after a complete rumen exchange, rumen community was capable of re-establishing itself. Although the rumen microbial community changes over host lifetime, there is evidence that a 'core microbiome' exists (Jami and Mizrahi, 2012; Henderson et al., 2015; Belanche et al., 2019a), which remains stable irrespective of changes in the diet, animal genetics or geographical area. Additionally, bacterial core seems to remain stable even during acidosis episodes (Petri et al., 2013). Therefore, the rumen core seems a key element in the adaptation capacity of animals to new diets and in the resilience that the rumen microbes exhibit after an environmental perturbation.

There are many reports on the microbial changes after a dietary shift demonstrating that the nature of the diet (forage *vs.* concentrate) and the availability and fermentability of substrates alter the diversity and the composition of microbial population (Tajima et al. 2001; Petri et al., 2013; Belanche et al., 2019a). Ruminants fed on forage-based diets possess a high relative abundance of cellulolytic population (Hungate, 1966; Dehority 2003; Fernando et al., 2010), being higher the numbers of *Fibrobacteres* and *Firmicutes* phyla, and of the genera *Ruminococcus (Ruminococcus flavefaciens* and *Ruminococcus albus*). Additionally, protozoa population is also higher in forage-fed animals. When rumen pH fluctuates as a result of dietary changes, the rumen bacteria community goes

through an ecological succession because the specific niche that each particular species occupies in the rumen environment varies according to their acidic tolerance (Shaani et al., 2018). For example, cellulolytic bacteria have a narrow optimal rumen pH above 6.0 (Russel and Wilson, 1996) and are inhibited when pH is less than 5.5 for an extended period (Calsamiglia et al., 2002). Many genera of rumen protozoa do not tolerate rumen pH lower than 5.5 either (Towne et al., 1990).

After concentrate inclusion or after increments in concentrate proportion in the diet, the steady-state conditions in the rumen are disrupted. The higher arrival of readily fermentable material leads to increased bacterial growth rate and activity. Then, dysbiosis occurs and there is a succession of changes likely selecting for more acid-tolerant bacteria (Khafipour et al., 2009; Hook et al., 2011; Petri et al., 2013). Numbers of fibrolytic bacteria typically decrease, whereas amylolytic bacteria (such as *Lactobacillus*, *Prevotella*, *Ruminobacter*, *Streptococcus* or *Selenomonas*), which possess a faster growth rate, become dominant in the rumen of grain-fed animals (Mackie and Gilchrist, 1979; Goad et al., 1998; Hook et al., 2011). There is also an increase in lactate-utilizing bacteria (*Selenomonas ruminantium* and *Megasphaera elsdenii*; Mackie and Gilchrist, 1979; Fernando et al., 2010; Hook et al., 2011), which under these conditions are essential to control rumen fermentation to prevent lactate accumulation. Hook et al. (2011) also reported an increased population of succinate-utilizers under a high-grain feeding, which is important to produce propionate. Variations on microbial populations during the transition from high-forage to high-concentrate diets are well described in the literature in steers (Fernando et al., 2010; Lee et al., 2019), goats (Grilli et al., 2016; Lee et al., 2019), and sheep (Seddik et al., 2018). For instance, Fernando et al. (2010) studied the bacterial shifts of steers that were transitioned from a hay-based diet to a high-concentrate diet, and reported a decrease in the *Firmicutes* to *Bacteroidetes* ratio and of fibrolytic bacteria (such as *Fibrobacteres* or *Butyrivibrio*), and increases of species such as *M. elsdenii*, *Streptococcus bovis*, *S. ruminantium*, *Prevotella bryantii* and *Lactobacillus* spp. among others.

On the other hand, bacterial diversity has also been reported to decrease after concentrate inclusion (Tapio et al., 2017; Schären et al., 2018; Belanche et al., 2019a), which may reflect a more functional ecosystem. Lower richness, lower diversity and higher dominance in rumen microbiota have been associated with more efficient animals (Shabat et al., 2016). These authors found that the more diverse use of substrate by inefficient animal's microbiome (higher richness and diversity) resulted in a wide array

of output metabolites, some of which either negatively affected animal energy harvest or could not be utilized by the animal for its energy requirements. On the contrary, for an efficient animal's microbiome, the use of these compounds was dominated by a reduced but more specialized number of pathways that were more relevant for the energy needs of the animal. Additionally, recent studies have found that dietary changes also affect the epimural bacteria (Chen et al., 2011) decreasing their diversity (Seddik et al., 2018).

Regarding rumen protozoa, they may follow the same trend as bacterial population during the transition to a high-concentrate diet in that they increase their numbers as a response to increased arrival of fermentable material in the rumen (Mackie et al., 1968), but diversity decreases because protozoa are susceptible to low pH. Early work by Eadie and Mann (1970) and Slyter (1970) reported that protozoa are more susceptible to low pH and that these microbes were virtually absent in high-concentrate diets. However, there is evidence that high-concentrate diets support a consistent protozoa population (Towne et al., 1990; Hristov et al., 2001), although with a reduced diversity, mainly *Entodinium* species (Franzolin and Dehority, 1996). Both density and diversity of methanogens and anaerobic fungi is also decreased with high-concentrate diets (Orpin and Joblin, 1997; Belanche et al., 2019a).

As stated above, the rumen microbiota exhibits a high degree of resilience (Allison et al., 2008; Weimer, 2015). To achieve an adaptation to new environmental conditions, such as those after concentrate inclusion (higher VFA production and lower pH), and reach stability, the microbes require a period between 14 to 21 days (Dieho et al., 2016). If the animals are abruptly switched to high-concentrate diets without a prior adaptation, the effect of such environmental disturbance can lead to important dysbiosis with detrimental effects for the whole system, as will be discussed later.

## 2.5 THE TRANSITION PERIOD TO HIGH-CONCENTRATE DIETS

A great proportion of calves arriving to feedlots in Spain come from suckler beef herds. These animals graze along with their mothers and are abruptly weaned at 5-7 months and immediately switched from a milk and grass regime to a high-concentrate diet. The transition period typically involves the first 14 to 21 days after the calves are introduced to the intensive feeding. The transition period is often considered as the most dramatic and stressful period in an animal's life since calves often undergo through weaning, marketing, transportation for long distances, reallocation to new facilities and mixing with other animals from different origin. A short-term feed restriction such as that occurring during marketing and transportation may be critical in reaching rumen acidosis after the subsequent re-feeding as this latter may cause a rapid increase in carbohydrate availability and a reduction in rumen pH (Albornoz et al., 2013).

The introduction of concentrates results in a sudden shift of rumen environment that becomes more acidic due to the accumulation of organic acids. Further, in general, suckler calves have usually consumed only forage as a solid feed, and possess an undeveloped and keratinized rumen epithelia (Bacha, 2005) with a limited capacity of absorption (Dirksen et al., 1985; Krause and Oetzel 2006; Qumar et al., 2016). Moreover, when animals have only consumed forage, there is evidence that lactate absorption is null (Qumar et al., 2016). On the other hand, rumen microbiota requires time to adjust to the new ruminal environment. Lactic acid fermenters are slow in adapting to rapid dietary changes, and equilibrium between lactate utilizers and producers should be achieved in order to avoid acid accumulation. Therefore, calves that are introduced to high-grain diets from milk and forage regime are particularly susceptible to develop acidosis.

According to the information in the literature, rumen microbes require a period of around two to three weeks to reach a stable population under the new environmental conditions (Mackie and Gilchrist, 1979; Dieho et al., 2016). Further, the rumen epithelium needs an estimated time of *ca*. four weeks to develop morphological and functional adaptive responses (Dirksen et al., 1985; Bannink, et al., 2012; Dieho et al., 2016). The increase in the absorptive capacity may occur earlier than the morphological adaptation as reported by Etschmann et al. (2009) and Qumar et al. (2016) who found that functional adaptations (changes in ion transport mechanisms) in response to concentrate take place within 1-2 weeks with concentrates. Consequently, the inclusion of concentrates during the transition period should be gradual.

During this period, the feeder's goal is to achieve a maximum feed consumption as soon as possible in order to attain maximum growth and productivity. However, it is even more important to follow a moderate transition and to control the fermentation to prevent the occurrence of digestive disturbances during this period. Otherwise, impaired rumen health during the transition period may have negative outcomes during the fattening period, affecting animal welfare and productivity (Brown et al. 2006). For instance, an abrupt inclusion of high amounts of concentrate may negatively affect rumen epithelium, decreasing absorption, or cause the disruption of barrier function of the rumen epithelium. This latter would lead to systemic inflammation and activation of the immune system, which, if prolonged over long periods of time, might increase host's energy requirements and result in immune suppression and thus in increased susceptibility to other diseases (Zebeli and Metzler-Zebeli, 2012). In terms of performance, cattle that had gone through sub-acute rumen acidosis (SARA) exhibit lower average daily gain (ADG) and higher feed conversion ratio, likely due to variable feed intake and/or damage of the rumen epithelium that affects absorption (Castillo-López et al., 2014).

## 2.6 DIGESTIVE DISSORDERS ASSOCIATED WITH INTENSIVE FEEDING

Beef cattle reared on intensive conditions consume large quantities of high-grain rations and a minimum amount of structural fibre. The commonly used starch-rich diets are rapidly fermented in the rumen yielding high amounts of VFA. Despite this might be desirable from the point of view of cattle nutrition, current feeding practices might overwhelm the physiological mechanisms of the rumen to maintain rumen pH, leading to nutritional disturbances (Aschenbach et al., 2011; Humer et al., 2018a). Nutritionally related pathologies represent the second mortality factor in feedlot cattle after the respiratory diseases (Nagaraja and Titgemeyer, 2007). The most common and well-studied nutritional disorder in cattle fed high-grain diets is rumen acidosis, which results in high economic losses for the producers (Nagaraja and Titgemeyer, 2007; Humer et al., 2018a).

### 2.6.1 Ruminal acidosis

Ruminal acidosis is a non-physiological decrease of rumen pH during a certain period of time that impairs the normal functioning of the rumen. This disorder occurs as a consequence of an overproduction of organic acids (VFA and lactate) that may decrease rumen pH if the acid production surpasses rumen regulation mechanisms. The rumen acidosis involves various degrees of rumen acidity as a result of a complex interaction among diet composition, feed intake, rumen microbial population and individual susceptibility. Some factors predispose to rumen acidosis such as the excessive intake of rapidly fermentable carbohydrates, the insufficient intake of fibre and/or an inadequate adaptation to high-concentrate diets, as it may occur during the transition period (Krause and Oetzel, 2006).

The current beef feeding management, where the concentrate to forage ratio often represents over 85:15, is the ideal scenario to develop rumen acidosis mainly because the oversupply of rapidly fermentable organic matter in the rumen and the lack of fibre to stimulate rumination. Rumen pH in feedlot cattle usually fluctuates between 5.8 and 6.2 (Nagaraja and Titgemeyer, 2007) with a normal variation over the course of the day that can reach up to one pH unit (Nagaraja and Titgemeyer, 2007). On the other hand, the limited rumination promoted by the low inclusion of neutral detergent fibre (NDF) in these diets weakens the buffering capacity of the rumen. Further, NDF stimulates ruminal contractions that move the fibre mat, thus mixing the rumen contents and making VFA

available to absorption through rumen epithelium, decreasing the risk of ruminal acidosis (Allen, 1997).

*2.6.1.1 Pathogenesis and types of ruminal acidosis.*

The decrease of the rumen pH occurs after the excessive consumption, in a short time, of rapidly fermentable carbohydrates, mainly starch, which is followed by an enhanced bacterial activity that increases VFA production (Slyter, 1976). When the rate of acid production exceeds that of absorption, the subsequent accumulation of fermentation products might induce a concomitant depression of rumen pH and an increase of osmolarity (Humer et al., 2018a). As rumen pH declines, acid-tolerant bacteria proliferate and lactate utilizers (*M. elsdenii* and *S. ruminantium*) increase as a mechanism to restore the equilibrium (Goad et al., 1998). However, at rumen pH less than 5.5, a dysfunctional unbalance of rumen microbes occurs (Nocek, 1997) and lactate producers (mainly *S. bovis*) may outnumber lactate fermenters, which results in more VFA and lactate production and accumulation that increases the drop of rumen pH. Acidotic episodes alter bacterial diversity, and are associated with the proliferation of microbes such as *S. bovis*, *Escherichia coli*, *Lactobacillus* spp., *Acetitomaculum*, *Pseudobutyrivibrio*, *Succinivibrio*, *M. elsdenii*, *S. ruminantium*, *Prevotella briantii*, *Anaerovibrio lipolytica*, *Bifidobacterium* spp., and *Atopobium* spp. (Khafipour et al., 2009; Petri et al., 2013; McCann et al., 2016), and with the decrease of others such as *Fibrobacter succinogenes*, *R. albus* and *R. flavefaciens* (Russell and Wilson, 1996). If rumen pH continues falling below 5.0, negative changes in the rumen epithelium occur, cellulolytic bacteria and protozoa are inhibited, *S. bovis* growth is also inhibited (Aschenbach et al., 2019), and there is a bloom of the acid-tolerant lactobacilli. At this point, lactate production exceeds utilization and accumulates, exacerbating the pH decline. Therefore, this scenario contributes to the spiralling process that may cause acute physiological alterations and even lead to animal's death (Nocek, 1997; Figure 2.2).

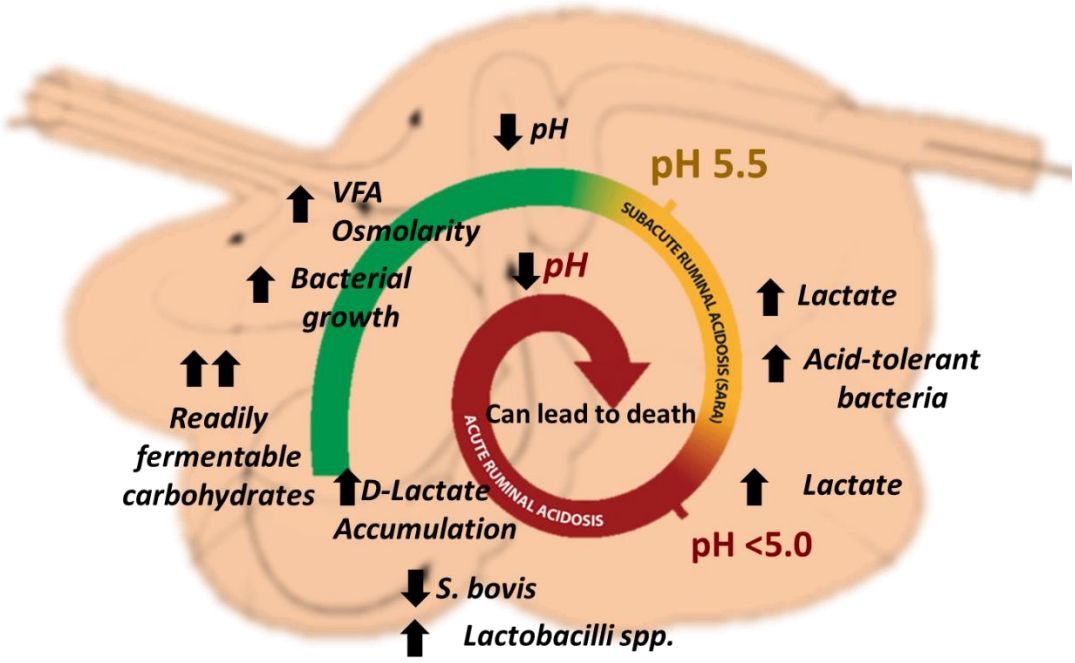


**Figure 2.2** Pathogeny of ruminal acidosis. Adapted from Nocek (1997).

According to the severity of rumen pH depression, the accumulated products (VFA or lactate), and the evidence of clinic manifestations, ruminal acidosis is broadly classified as acute ruminal acidosis (ARA) or subacute rumen acidosis (SARA) as detailed by Nagaraja and Titgemeyer (2007). ARA is a dramatic reduction of rumen pH below 5.0 as a result of lactic acid accumulation (>50 mmol/L, Owens et al., 1998; Nagaraja et al., 2007) for an extended period of time. SARA is the result of a temporary VFA accumulation (>150 mmol/L; Nagaraja and Titgemeyer, 2007) or of a limited rumen buffering capacity that cannot cope with the accumulation of acids during periods of excessive ruminal fermentation. These situations lead to a decrease of rumen pH to values ranging from 5.5-5.6 (Ghozo et al., 2005; Krause and Oetzel, 2006; Nagaraja and Titgemeyer, 2007). There is some discrepancy in the literature about the exact threshold of rumen pH that identifies an episode of SARA. Some authors state that the length of time that rumen pH remains low, and not the mean or minimum pH, is the main factor that affects rumen microbes or epithelium (Gozho et al., 2005; Nagaraja and Titgemeyer, 2007; Humer et al., 2018a), therefore, it is commonly stated that an episode of SARA is characterized by a rumen pH <5.6 for more than 180 min/d (Gozho et al., 2005; Plaizier et al., 2008). More interestingly, Calsamiglia et al. (2012) pointed out that SARA is not only a pH problem but a combination of a reduction in pH and the type of diet fed (*i.e.* feed ingredients) that dictates the shift in microbial populations. During SARA, little or

no lactate is found in the rumen because it is converted to propionate, butyrate and valerate by lactate utilizers (Stone, 2004).

Sub-acute rumen acidosis can be slowly overcome by VFA absorption and usually implies a recovery of the rumen function (Nagaraja and Titgemeyer, 2007; Penner et al., 2010), although mechanisms of barrier restoration are still not clear (Aschenbach et al., 2019). In a limited number of cases, SARA may potentially develop into ARA if the pH fails to recover and keeps lowering (pH <5.0) (Nagaraja and Titgemeyer, 2007). Repeated SARA episodes may trigger adaptation of the rumen epithelium that might decrease the severity of the acidotic episode. For instance, Steele et al. (2009) studied how the rumen adapts during an acidotic episode after a grain challenge and observed increased expression of genes involved in ketogenesis. They concluded that it was an adaptive response of the rumen epithelium due to increased arrival of substrate as an attempt to metabolize and remove the accumulated VFA. In another study assessing four consecutive SARA challenges in bulls, Nagata et al. (2018) reported that rumen pH reached SARA values only in the first two challenges but not during the other two (Figure 2.3), and that bacterial diversity restored on the fourth challenge. Therefore, they concluded that cattle might adapt to repeated SARA challenges due to the dynamic fluctuations of the microbiota and their ability to adapt to rumen environmental changes. In contrast, recovery from ARA is more difficult and an external intervention might be necessary since it can lead to a systemic acidosis (Nagaraja and Titgemeyer, 2007).

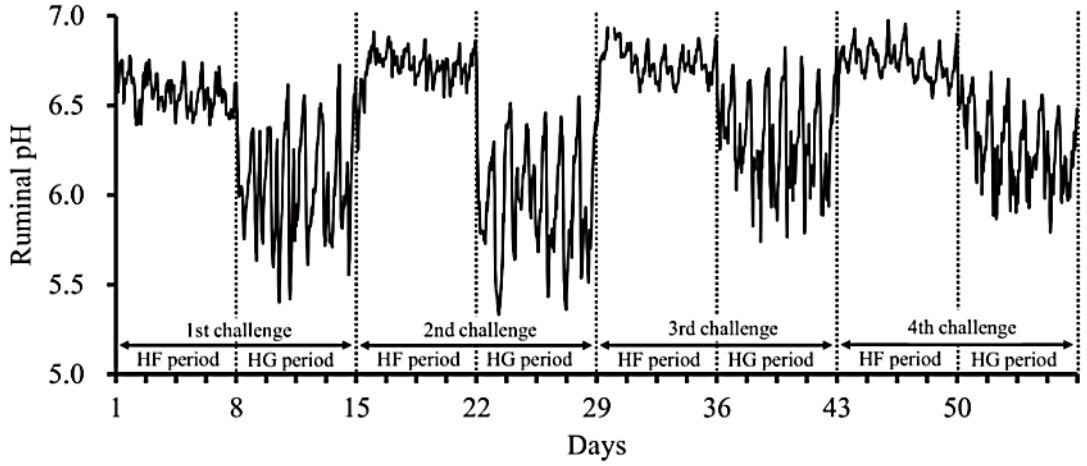


**Figure 2.3** Changes in the rumen pH of bulls during four consecutive SARA challenges. Cattle were fed a high-forage (HF) diet for 7d (HF period) and then a high-grain (HG) diet for 7 d (HG period). A SARA challenge was defined as an HF period followed by a HG period. Source: Nagata et al. (2018).

*2.6.1.2 Individual susceptibility to rumen acidosis*

There is considerable variation in the susceptibility of animals to develop ruminal acidosis even when fed a common high-grain diet and with similar feed intake (Figure 2.4**;** Bevans et al., 2005; Mohammed et al., 2012; Schlau et al., 2012).

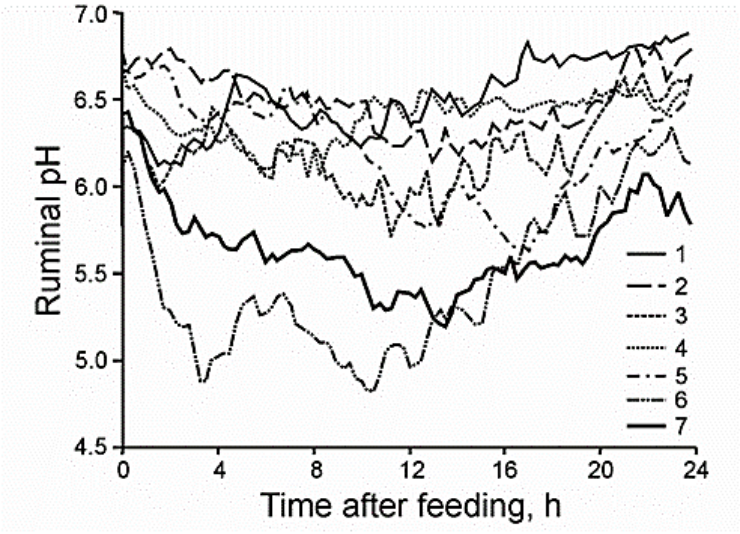


**Figure 2.4** Variation in individual pH in feedlot steers fed the same diet. Source: Krause et al. (1998).

Variations in the susceptibility to rumen acidosis among animals can be caused by physiological (*e.g.* different expression of genes involved in VFA absorption and metabolism by the epithelium, salivation rate and composition of saliva, ruminal fluid outflow, etc.), behavioural (rate of feed intake, feed sorting or chewing behaviour), and microbial (differences in the ruminal microbial diversity) factors (Mohammed et al., 2012; Humer et al., 2018a). However, the extent to which these factors affect the resistance to acidotic episodes is not known. Since absorption accounts for the major proportion of acid removal (Allen, 1997) it would be reasonable that differences in the absorptive capacity of the rumen epithelium are fundamental in affecting the susceptibility to ruminal acidosis. For instance, Penner et al. (2009a) studied whether differences in rumen epithelium uptake capacity were related to lower susceptibility to develop SARA. After SARA induction in sheep with an oral drench of glucose, they reported that animals with greater absorptive capacity (as measured *in vitro* with Ussing chambers) and higher metabolic capacity of the rumen epithelium (higher plasma β-hydroxybutyrate concentration) were less susceptible to SARA. Additionally, the susceptibility of damage in the barrier function may also be affected by the VFA absorption, since there is evidence that a disturbed barrier function is caused by a combination of low pH and high VFA concentration (Meissner et al., 2017). Resistant

animals also have higher butyrate concentrations (Chen et al., 2012; Schlau et al., 2012), which might increase the absorptive surface of rumen epithelium (Shen et al., 2004; see section 2.7). Chen et al. (2012) stated that microbial-host interactions play an essential role in the resistance to acidosis. They reported that a higher population of epimural bacteria was associated with higher pH, and speculated about the stimulating effect of epimural bacteria on VFA absorption. These authors also found a higher expression of genes related to host innate immunity (Toll-like receptors (TLR) 2 and 4) in the rumen papillae of resistant animals. Further, they observed that epimural bacteria might stimulate the rumen epithelial innate immune response of resistant animals with their products. The stimulation of the immune system may lead to better barrier function that prevents damages in the epithelial tissue (for example those exerted by LPS or histamine released during acidotic episodes).

*2.6.1.3 Consequences of acidotic episodes*

Sub-acute rumen acidosis is probably the most prevalent form of ruminal acidosis in beef farms and causes higher economic losses because it lacks pathognomonic manifestations. Nevertheless, animals may manifest unspecific symptoms such as lethargy, transient episodes of diarrhoea and visible grain in the faeces, DMI fluctuation and weight loss (Owens et al., 1998; Plaizier et al., 2008; Humer et al., 2018a). There is evidence that the consequences of the acidotic episodes depend on the severity of the insult and of the duration of the pH decrease (Aschenbach et al., 2019).

Acidotic conditions in the rumen may be associated with pathological changes in the rumen epithelium leading to an impaired function of the rumen epithelia, which can lead to translocation of harmful substances across the rumen epithelial layer (Liu et al., 2013; Meissner et al., 2017; Aschenbach et al., 2019). The rumen epithelial barrier is a vital component of the immune system of ruminants as it is a selective defence barrier that prevents translocation and systemic dissemination of harmful bacteria, toxins, and immunogenic factors. The damage in the rumen epithelia determines whether the acidotic hazard remains in the forestomach or expands causing systemic effects. Ruminal acidosis and/or the continuous stimulus of acidotic diets (lower pH and high concentration of VFA) can lead to structural proliferation and differentiation processes of the rumen epithelia (Steele et al., 2009). This may cause lesions in the rumen wall that start with a sloughing of the *stratum corneum* that may be followed by excessive keratinization of the epithelium leading to parakeratosis (Steele et al., 2009). If the hazard to the rumen

epithelium is prolonged for a long period or the degree of acidity is high, damage of the epithelial barrier function occurs (Steele et al., 2009; Liu et al., 2013; Meissner et al., 2017). There may be a deterioration of the cellular tight-junctions (TJ), which selectively regulate the passage of molecules and ions via the paracellular pathway (Zebeli and Metzler-Zebeli, 2012). In goats fed a high-concentrate, 65% grain diet, Liu et al. (2013) observed that the damage in the barrier function after 7 weeks was mediated by a downregulation of the expression of TJ proteins (claudins and occludin). Meissner et al. (2017) studied *ex vivo*, with Ussing chambers, the effect of different pH conditions and VFA concentration on rumen epithelial barrier function. Two groups of epithelial tissues from the rumen of slaughtered sheep were incubated for 7 h without VFA, at a mucosal pH of 6.1 and 5.1, respectively. Another group was first incubated in a mucosal solution containing 100 mmol of VFA at pH 5.1 for 2 h, and thereafter in a mucosal solution without VFA at pH 6.1 for the remaining 7 h. The authors concluded that rumen epithelia might tolerate a rumen pH of 5.1 for several hours; however, they stated that luminal acidity with a concurrent high VFA concentration induces profound damage to the transmembrane TJ proteins disturbing barrier function and thus increasing permeability. The same research group recently demonstrated that increased permeability was related to a decrease in the abundance of TJ proteins, and that there were no differences between the three major VFA in the impairment of the barrier integrity during acidosis (Greco et al., 2018). Other authors (Penner et al., 2010) reported that a single episode of SARA (pH<5.8 for 100 min) did not affect barrier function, but more episodes are necessary to trigger epithelial damage. The accumulation of high amounts of fermentation products results in hyperosmolarity in the rumen, which increases permeability by widening the paracellular spaces (Aschenbach et al., 2019).

During acidotic episodes (ARA or SARA), some harmful compounds (*e.g.* bacterial endotoxins or biogenic amines such as histamine or ethanolamine) derived from microbial activity, changes in the turnover of epithelial cells and/or lysis of rumen microbiota are released into the rumen (Nocek, 1997; Zebeli and Metzler-Zebeli, 2012; Aschenbach et al., 2019). The accumulation of all these immunogenic compounds, whose exact release mechanism is not known yet, and the concurrent acidity load in the rumen, may increase rumen permeability (Aschenbach et al., 2019).

Impaired integrity of the rumen lining might allow the translocation of rumen bacteria and noxious substances into the blood stream leading to liver abscesses (Nagaraja and Chengappa, 1998), laminitis (Nocek, 1997) or a systemic inflammatory response

(Plaizier et al., 2008; Khafipour et al., 2009). The role of the bacterial endotoxin (or bacterial lipopolysaccharide-LPS-, a bioactive cell wall component of the gram-negative bacteria) has received special attention since high-grain feeding has been linked to increased death and lysis of gram negative bacteria at a low pH (Humer et al., 2018b). The bacterial LPS is regarded as an important immunogenic component of the gastro intestinal tract, and triggers a cascade of events towards an inflammatory response and a release of acute phase proteins into the blood during acidotic episodes (ARA and SARA) (Gozho et al., 2005; Plaizier et al., 2008; Liu et al., 2013). Inflammatory responses may occur if rumen pH is <5.6 for more than 180 min/d (Gozho et al., 2005).

The reduction of VFA absorption occurs in parallel to the damage of the rumen function (Schwaiger et al., 2013; Qumar et al., 2016). Schwaiger et al. (2013) observed a decreased VFA absorption and increased saliva production shortly after a bout of ruminal acidosis. It is interesting the bilateral relationship between absorption and SARA (Aschenbach et al., 2019): a decreased absorption of VFA induces SARA but SARA decreases VFA absorption. Aschenbach et al. (2019) pointed out that the reduction of VFA absorption as well as the increase in saliva production during an acidotic episode may denote a defence mechanism to maintain intraepithelial pH homeostasis in order to avoid the negative consequences of excessive intracellular VFA supply on barrier function (Meissner et al., 2017).

In addition, high amounts of grain in the rumen might lead to increased bypass of starch supply and fermentation into the lower gut increasing the risk of developing lower gut acidosis. This disturbance has similar consequences for epithelial functions and LPS absorption to those described for the rumen acidosis (Steele et al., 2016; Aschenbach et al., 2019). Indeed, disruption of the barrier function in the lower gut may promote more intense systemic inflammation (Steele et al., 2016).

Rumen pH is also important for fibre degradation since extended periods of pH under 6.0 decrease the fibrolytic populations (Russel and Wilson, 1996; Fernando et al., 2010), and this has a detrimental effect on fibre digestibility and feed efficiency (Russell and Wilson, 1996). Calsamiglia et al. (2002) studied the effects of pH fluctuation on microbial fermentation and reported that short and transitory low pH episodes had minor effects on fibrolytic bacteria and did not affect the overall microbial fermentation as long as there was enough time with normal pH for the recovery of the fibrolytic population. Some authors have reported that fibre degradation is compromised at rumen pH<5.8 for

more than 5 h/d (Zebeli and Metzler-Zebeli, 2012). What remains unclear is the exact lapse of time under suboptimal pH that really causes a detrimental effect on rumen microbiota and rumen function.

## 2.7 STRATEGIES TO PREVENT RUMINAL ACIDOSIS

The control of the fermentation process to prevent and minimize rumen acidosis may be achieved by different strategies (Schwartzkopf-Genswein et al., 2003; González et al., 2012; Mazza, 2016), from ingredient variation with different intrinsic properties and different processing methods, through feeding management in the farm, to the addition of some feed additives that have an specific action in rumen microbiota. Manipulation of microbes through immunological methods or by the addition of specific microorganisms has also been used. Manipulation involves a complex process and, in this section, only the most useful methods for control of acidosis will be addressed.

### 2.7.1 Diet composition and feeding management

Strategies classified here aim to enhance rumen development and to modulate rumen fermentation by means of diet composition and feeding management.

#### *2.7.1.1 Strategies to promote rumen epithelia development*

Research has shown that diet type and nutrition level lead to adaptive responses that involve a series of morphological (rumen papillae proliferation), microbiological (proliferation of lactate-utilizer and acid-tolerant bacteria), and molecular changes (increased transcript proteins involved in the transport and metabolism of VFA) (Steele et al., 2016). Promoting an early and adequate rumen development has been a key target of ruminant nutrition (Kertz et al., 2017), and there are well established feeding strategies that may improve rumen development (Diao et al., 2019). In the present section, two main strategies are addressed: concentrate supplementation during pre-weaning period, and butyrate supplementation to enhance rumen development and its absorptive capacity that in turn can prevent rumen acidosis.

Pre-weaning solid feed supplementation is known to improve rumen development as well as to help to establish a stable commensal microbiota to facilitate the weaning process and the post-weaning adaptation to the fattening diets. It is acknowledged that rumen morphological and metabolic maturation relies on solid feed consumption (Baldwin et al., 2004). In this sense, it is well recognized that a forage with an adequate particle size plays a role in the thickening and muscularisation of the rumen, and that concentrate, through the higher production of VFA, stimulates the morphological and metabolic development of the epithelia (Baldwin et al., 2004).

Early workers already observed that high-concentrate diets promoted better rumen development through a higher production of VFA (Tamate et al., 1962; Stobo et al., 1966). Research during the last decades has shown that energy-rich diets induce a series of adaptive mechanisms in the rumen including morphological transformation, changes in gene expression related to transport proteins, cell proliferation and enzymatic activity (Figure 2.5; Gäbel et al., 1991; Penner et al., 2011). Concentrate feeding enhances the rumen epithelial cell proliferation and increases the density of the rumen papillae (Shen et al., 2004) because it might increase the abundance of gene transcripts related to epithelial proliferation (Soomro et al., 2018; Sun et al., 2018), which in turn increases the absorptive capacity of the rumen epithelia (Dirksen et al., 1985). Among the different VFA, it is well recognized that mainly butyrate, and to a lower extent propionate, promote epithelial proliferation (Mentschel et al., 2001; Baldwin et al., 2004). The cell proliferation is associated to changes in the cell cycle mainly regulated by cyclins and cyclin-dependent kinases (CDK) (Norbury and Nurse, 1992). Recent studies (Sun et al., 2018), have shown that concentrate (likely through a major production of butyrate) increases the development of the rumen epithelia by pathways that accelerate cell cycle (*i.e.* increased expression of cyclins) and moderate apoptosis (*i.e.* decrease gene expression of some caspases, which have an important role in apoptosis). It is known that butyrate promotes epithelium proliferation indirectly through the stimulation of release of hormones and growth factors (such as insulin, growth hormone, insulin-like growth factor 1-IGF-1, glucagon) (Gálfi et al., 1991; Baldwin et al., 2004; Penner et al., 2011). Shen et al. (2004) compared two groups of young goats differing in the energy level (high *vs.* low) according to the amount of offered concentrate, and found that high-energy diet produced both greater butyric acid and plasmatic concentrations of IGF-1and its ruminal receptor (IGF-1R). This was in turn related to higher length, width, density and surface of rumen papillae, since IGF-1, via stimulation of the IGF-1R, may induce upregulation of cyclins (cyclin D1) (Lu et al., 2013).

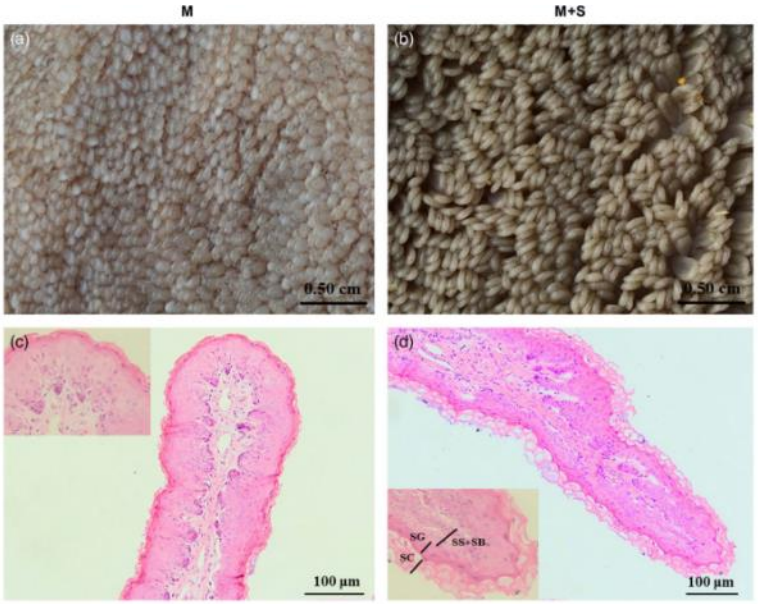


**Figure 2.5** Comparison of the rumen epithelium (a, b) and papillae micrograph (c, d) of lambs fed milk (M) or milk plus starter concentrate (M+S). Note the papillae proliferation enhanced by concentrate supplementation. Source: Sun et al. (2018).

On the other hand, supplementation with exogenous butyrate in different forms and during different stages of rearing (before or after weaning) stimulated forestomach as well as lower gastrointestinal tract development in ruminants (Górka et al., 2018; Figure 2.6). Feed supplementation with butyrate can be achieved as protected or unprotected (whether embedded or not in a lipid matrix) butyrate salts (calcium, sodium, potassium, magnesium) or as butyrins (esters of butyrate and glycerol) (Górka et al., 2018). Sodium butyrate is the most used form of butyrate (Górka et al., 2018). There is a wide range of studies supplementing either solid or liquid feeds (*i.e.* milk replacer) with butyrate, as recently reviewed by Niwińska et al. (2017) and Górka et al. (2018), which report the stimulating effect of butyrate supplementation (*ca*. 0.3% DM) on rumen development as well as on animal performance. After infusion of exogenous butyrate in lambs (0.36 g/kg of BW/d), Liu et al (2019) reported increased concentration of IGF-1 in plasma and higher expression of IGF-1R. Mentschel et al. (2001) found that butyrate infusion stimulated rumen papillae proliferation by reducing cellular apoptosis in calves. In another experiment, Malhi et al. (2013), after infusing sodium butyrate in goats (0.3 g/kg of BW/d), observed higher papillae growth (82% respect to control group), an increase in cyclin D1expression of genes, and an increased VFA absorption in the goats of the experimental group. Additionally, Sun et al. (2018) and Liu et al. (2019) reported that both concentrate feeding and butyrate supplementation causes the hyper-expression of genes regulating the transcription of cyclins (cyclins A, D1, E1, CDK2, CDK4 and

CDK6) and decreases gene expression of caspases (caspases 3 and 8), therefore increasing proliferation of rumen papillae in small ruminants. Apoptosis is also important to maintain the homeostasis in the development of the rumen epithelium, and that is the reason why some authors (*e.g.* Soomro et al., 2018) observed and upregulation of some caspases.

On the other hand, there is also evidence that high-concentrate diets (Yan et al., 2014; Sun et al., 2018) and administration of exogenous butyrate (Liu et al., 2019) enhance functional adaptation by upregulating expression of genes encoding for transport proteins involved in VFA absorption and epithelial metabolism, and of genes responsible for maintaining the intracellular pH of rumen epithelial cells. Furthermore, rumen microbes seem to have also an important role on the proliferation of the rumen epithelium through VFA production. A recent study (Lin et al., 2019) has reported that changes in gene expression related to growth of the epithelium are mediated by the rumen microbiome through their production of acetate and butyrate.

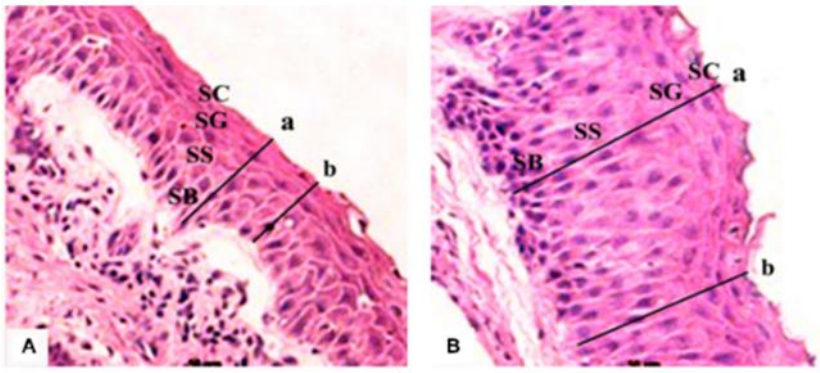


**Figure 2.6** Effect of butyrate infusion on rumen papillae of goats. A: Control group. B: goats infused with 30 g/kg body weight of sodium butyrate. SB, *stratum basale*; SS, *stratum spinosum*; SG, *stratum granulosum*; SC, *stratum corneum*. a: maximum depth of epithelial strata; b: minimum depth of epithelial strata.Source: Soomro et al. (2018).

In view of the adaptive responses to concentrate feeding or exogenous butyrate, pre-weaning supplementation with starter concentrates seems to be a feeding management that may enhance morpho-functional development of the epithelium, which is desirable to adapt the rumen as fast as possible to the fattening diets without detrimental effects. In some beef production systems in Spain (extensive in mountain areas), the common practice is to rear suckler calves only on milk or milk and pastures until weaning since it seems *a priori* to be more economically feasible than providing starter concentrate. In this scenario, it could be interesting to compare the common pre-weaning managements *vs.* providing starter concentrate in order to establish the best strategy to

achieve both adaptation of these calves to the feedlot conditions and economic profitability.

*2.7.1.2 Feed bunk management*

Inadequate feed bunk management might result in erratic feeding behaviour and in drastic changes in rumen fermentation. In addition, there should be a consistent schedule of feed delivering in order to avoid long periods of feed deprivation. Further, the amount of feed provided should be as constant as possible because periods of low feed intake followed by re-feeding can lead to rumen acidosis (Owens et al., 1998).

On a daily basis, it is desired to promote a more stable rumen environment through an even distribution of feed intake that results in a greater meal frequency and small meal size (González at al., 2012). Frequency of feeding may positively affect rumen fermentation in that increasing to more than one daily delivery might lead to a more constant fermentation and reduce post-prandial variation of rumen pH (Schwartzkopf-Genswein et al., 2003). However, some studies found that increasing feeding frequency did not reduce daily rumen pH variations. For instance, Robles et al. (2007) increased the frequency of feeding from one to four times per day in beef heifers and observed that total daily mealtime, meal frequency and meal size were not affected by treatments. In that experiment, neither daily average rumen pH nor total VFA concentration were affected by treatments, but pH after 12 h was higher when animals were fed twice a day at 8:00 and 20:00 h, and the lowest when they were fed once a day at 08:00 h. Schutz et al. (2011) also studied the effect of feeding frequency, from one to three times a day, in steers fed a high-concentrate diet. They only reported performance parameters, which were increased in those animals fed three times a day; however, they reported higher liver condemnation (that decreased as feeding frequency decreased) in those animals (Schutz et al., 2011). The liver condemnation could indicate that those heifers underwent through acidotic episodes (Humer et al. 2018a).

Another key aspect of feeding management is the way to provide the roughage source, which in Spain is commonly cereal straw. It is of particular importance to assure regular intake of straw in order to promote rumination and thus a synchrony between acid production and acid neutralization with the aid of saliva. When cattle are given forages and concentrates *ad libitum*, which is the most common feeding method in Spain, animals sort against the forage component and only consume less than 20% of total DMI as forage

(Forbes and Provenza, 2000). In young cattle, the percentage may be even lower (*ca.* 8% with *ad libitum* diets; Devant et al., 2000; Gimeno et al., 2015a). Feeding diets as TMR might promote more homogeneous intake of forage that may stimulate rumination and a more buffered fermentation. Iraira et al. (2012) compared the intake and feeding behaviour of beef calves offered straw (10%) and concentrate (90%) separately or as TMR. Animals in the TMR group consumed more straw and less concentrate, exhibited lower total rate of intake and spent more time ruminating, which in the end contributed to a more stable rumen environment. On the contrary, Moya et al. (2011) offered a high-grain (85%) ration as TMR or separately, and did not found differences in rumen pH or VFA concentration, stating that cattle can self-regulate their intake. In another experiment, Gimeno et al. (2014) offered concentrate (87%) and straw (13%) together in the form of 30 × 30 × 30 mm briquettes or separated as free choice (straw was then offered in long form, 5-20 cm length), and found that briquettes promoted a lower pH than free choice, which was attributed to the reduced particle size of the straw in the briquettes (30 mm for an homogeneous mixture). In this trial, animals offered a free choice diet auto-regulated their concentrate and straw intake maintaining a stable rumen function as reported in the trial of Moya et al. (2011).

Additionally, in some conditions, beef cattle farmers limit or eliminate straw provision in order to reduce production costs (Faleiro et al., 2011). However, although all-concentrate diets from weaning to slaughter may promote higher daily gains and feed efficiency, they impair rumen health and increase abnormal behaviors reducing welfare (Faleiro et al., 2011). Indeed, Devant et al. (2016) reported that straw deprivation in bulls fed a high-concentrate diet led to a downregulation of genes encoding for signalling molecules involved in the gut-brain axis that determine behaviour, and thus those bulls exhibited more stereotypes. Furthermore, when bulls were deprived of straw had increased rumen lesions and exhibited an upregulation of genes involved in inflammatory response and a downregulation of genes encoding for proteins involved in epithelial barrier function. Therefore, although straw exclusion may reduce production costs, the results of this practice are counterproductive and thus is not advisable since there is an increase of stereotypical occurrence and of rumen morphological lesions, and the loss of the integrity of the epithelial barrier may lead to systemic infections and inflammation.

*2.7.1.3 Diet formulation*

Calsamiglia et al. (2012) pointed out that rumen acidosis is produced by a combination of low rumen pH and the characteristics of the diet (*i.e.* intrinsic characteristics of feed degradation, type of cereal grain and processing methods, etc.). Moreover, as there is a negative relationship between the amounts of rumen degradable starch and rumen pH (Sauvant et al., 1999), it is of great importance to formulate the ration with ingredients with different acidogenic capacity (AC). The AC of the feedstuffs is related to the content of protein, starch, non-fibre carbohydrates and fibre (Rustomo et al., 2006). Energy sources have the highest AC, fibre sources intermediate, and protein sources the lowest AC. The rate of rumen fluid pH depression after incubation is positively correlated with AC ($R^2$=0.74; P<0.001; Rustomo et al., 2006). Further, the AC depends upon the content of starch and its rate of degradation in the rumen (González et al., 2012). Rumen degradation rate and extent are determined by the chemical and physical structure of starch granules such as size, type, or their association level with protein matrices. (Herrera-Saldana et al., 1990; McAllister et al., 2006; González et al., 2012). Grains represent more than 80% of the high-concentrate rations, and according to their starch degradation rate in the rumen can be classified from the fastest to the slowest as follows: oats, wheat, barley, maize, and sorghum (Herrera-Saldana et al., 1990; Offner et al., 2003; González et al., 2012). In Spain, the most used cereals are barley and maize. The starch content of maize is lower than that of barley, but maize promotes a slower fermentation that may prevent and alleviate rumen acidosis. There are some studies assessing the effect of the replacement of barley with maize aiming to reduce acid accumulation; however, there are no consistent results. Some studies with beef cattle fed high-concentrate diets found similar fermentation pattern with either barley or maize as the prevailing cereal (Rotger et al., 2006; Gimeno et al., 2015a,b), although rumen pH decreased to a major extent when barley-based diets were used (Khorasani et al., 2001; Gimeno et al., 2015a,b). However, there are no studies on the effect of cereal substitution during the transition period, in which the substitution of barley with maize could be an interesting option to prevent acid accumulation in the rumen.

The substitution of cereals with high-energy by-products has been proposed as a viable strategy to reduce the incidence of acidosis (González et al., 2012). The use of non-forage fibre sources, such as citrus pulp or sugar beet pulp (BP), may be an interesting option for ruminant diets because they are rich in pectins, which fermentation produces

little or no lactate, and results in an increased acetate/propionate ratio (Hall et al., 1998). Amanzougarene et al. (2017a) compared the *in vitro* fermentation of various sources of carbohydrates commonly used in ruminant diets, and reported that the acidification of the medium was lower with maize and BP. By contrast, citrus pulp is rich in soluble sugars and promoted higher rate of fermentation leading to very low pH of incubation (Amanzougarene et al., 2017a). Further, BP possesses a characteristic chemical structure, which confers a high buffering capacity that can alleviate the over-acidification in the rumen (Marounek et al., 1985; Van Soest et al., 1991; Jasaitis et al., 1997). Studies including BP in the diet for ruminants fed high-concentrate diets report higher rumen pH when BP is included in the diet as a substitute for cereals (Bodas et al., 2007; Mojtahedi and Danesh Mesgaran, 2011), and thus could be an interesting option for adaptation diets.

*2.7.1.4 Feed processing methods*

Feed processing changes the physical features of cereals grains (*i.e.* density, particle size and surface area), and certain methods can alter the starch granules (*e.g.* steam rolling and steam flaking expose grain to moisture and heat, and a proportion of starch is gelatinized). Different processing methods are grinding, pelleting, dry or steam rolling (*i.e.* exposure to steam prior to rolling), steam flaking (*i.e.* longer duration of exposure and higher grain temperature) (McAllister et al., 2006). These processing methods break physical barriers of grains (hull, pericarp and protein matrix) and microbes have easier access to starch granules, which increase considerably the rate and extent of starch digestion in the rumen (Theurer, 1986; McAllister et al., 1994). Within processing method, grain features such as degree of gelatinization after steam flaking, and grain particle size after dry rolling, may differ considerably. Each processing method differs in its nutritional efficacy (Figure 2.7) and varies depending on the grains features**.** For sorghum and maize, processing appears to improve starch utilization by disruption of protein matrix (Theurer, 1986). With barley and wheat less extensive processing (coarse grinding or rolling) is enough to optimize starch digestibility since the protein matrixes are more diffuse and do not impede the access of microbes to starch granules (McAllister et al., 2006). Grinding and pelleting are the most common methods of grain processing for beef cattle (Castillo et al., 2006; Owens and Soderlund, 2006), mainly due to their low cost.

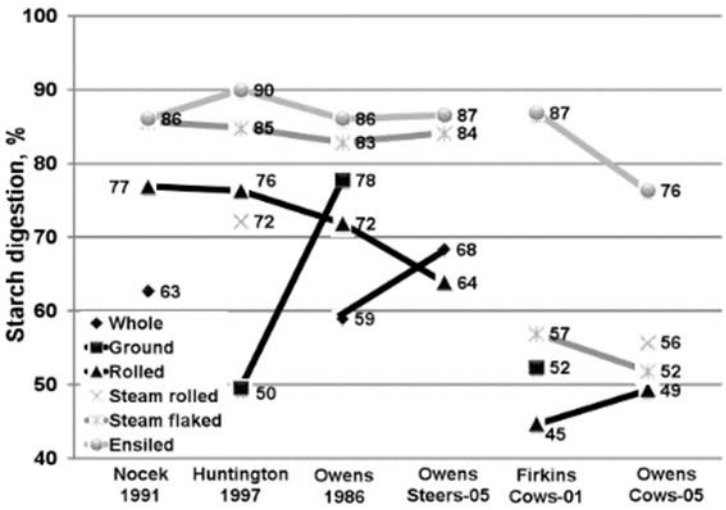


**Figure 2.7** Starch digestion of corn grain processed by various methods in the rumen of cattle. Source: Owens and Soderlund (2006).

As already mentioned, there is a negative relationship between the amounts of ruminally degradable starch and rumen pH (Sauvant et al., 1999). Therefore, increasing the extent of cereals processing may increase the susceptibility to ruminal acidosis (Beauchemin et al., 2001). In addition, chewing time is also reduced when increasing the degree of processing (Beauchemin et al., 2011). When comparing different processing methods (4-mm diameter pellets from grains ground to pass a 3-mm sieve *vs.* grains ground at 5-mm diameter), pelleted ration tended to result in more stable acid-base balance fermentation in feedlot steers (Castillo et al., 2006). In another experiment, Castrillo et al. (2013) compared a pelleted (either at 3.5- or 10- mm diameter) *vs*. finely ground (to pass through a 3-mm diameter sieve) diet for growing calves, and the 3.5-mm pelleted ration increased fermentation rate and resulted in lower pH compared to ground meal. However, pelleting at 10 mm promoted a more homogenous rate of intake, resulting in more stable rumen fermentation. Gimeno et al. (2015b) studied the effect of the prevailing cereal (barley *vs*. maize) in the concentrate and its processing method (ground at 3.5 mm *vs.* ground in the same conditions and thereafter pelleted at 6 mm) in beef calves fed-high concentrate diets, and reported a lack of effect of the processing method maybe due to the low diameter of pellets (6 mm). In another trial of similar characteristics, Gimeno et al. (2015a) studied the effect of the prevailing cereal and of the processing method (grinding *vs.* dry rolling) in beef calves, and dry rolling promoted more buffered environment especially with maize-based diets.

The degree of processing might have an important effect on rumen fermentation during the transition to high-grain diets. Minimizing the degree of processing could maximize chewing and slow down the rate of fermentation, but in a necessary equilibrium with a processing extent that would make the feed more digestible.

*2.7.1.5 Step-up adaptation to high-concentrate diets*

As mentioned previously, the general concept of adaptation encompasses two steps: microbiological and morpho-functional adaptation of the rumen epithelium in order to decrease susceptibility of acidosis. The adaptation has been traditionally accomplished by gradually increasing the proportion of concentrate over a period of 14 to 21 days using a series of step-up diets, at the time forage is proportionally decreased (Bevans et al., 2005). If the change to a high-concentrate diet is carried out abruptly, it may result in acidosis (Goad et al., 1998; Bevans et al., 2005). Several adaptation protocols have been evaluated, as summarized by Brown et al. (2006), and a gradual adaptation is highly recommended to minimize or prevent rumen acidosis development (Bevans et al., 2005; Holtshausen et al., 2013).

Bevans et al. (2005), for instance, compared a rapid *vs*. a gradual adaptation of feedlot cattle to a high-concentrate diet. The rapid adaptation protocol (RA) consisted in changing cattle from 40 to 90% concentrate diets through a single intermediate diet (65% concentrate) fed for 3 days, whereas the gradual adaptation protocol (GA) involved 5 intermediate diets (48, 57, 65, 73, and 82% concentrate) each being fed for 3 days. The adaptation method did not affect significantly rumen pH on the first four days of feeding 65 or 90% of concentrate, although RA exhibited slightly lower ruminal pH (Bevans et al., 2005). Further, the RA group spent more time (higher area under the curve (AUC)) with pH below 6.2, 5.6 and 5.2, and 50% of the heifers in RA group developed SARA on the first day with 65% concentrate. No heifers in the group GA were at risk of acidosis (Bevans et al., 2005). On the first day with 90% concentrate, all heifers developed SARA but the RA group showed an AUC for pH<5.2 higher than that of the GA group, and thus had a higher risk of developing ARA. High lactate concentration was found in two RA and in one GA heifers, which indicated that individual susceptibility to rumen acidosis was different irrespective of the adaptation protocol (Bevans et al., 2005). Therefore, although care must be taken in order to adapt cattle to fattening diets, the individual susceptibility is what mainly determines the risk of acidosis.

### 2.7.2 Manipulation of rumen fermentation

Since the European ban of the use of antibiotics in animal feeds in 2006 (Directive 1831/2003/CEE, European Commission, 2003), a lot of effort has been made to investigate new additives as alternatives to modulate rumen fermentation and to reduce the incidence of rumen acidosis. There are some feed additives that positively modify microbial fermentation reducing methane, improving protein metabolism, enhancing food consumption or preventing the incidence of rumen acidosis. Under the scope of the present review, only feed additives aiming to modulate fermentation and hence minimizing the risk of rumen acidosis are commented.

#### *2.7.1.6 Buffers and neutralizing agents*

Buffers are substances that can resist changes in pH and alkalizers are substances that increase ruminal pH, therefore these compounds are of interest for preventing ruminal acidosis (Calsamiglia et al., 2012). Buffers include sodium bicarbonate, magnesium carbonate, potassium carbonate, and potential alkalizer agents are magnesium oxide, sodium bicarbonate and sodium sesquicarbonate (Erdman, 1988; Calsamiglia et al., 2012). Compounds like sodium bicarbonate and magnesium oxide are used routinely for cattle diets; however, it is known that these compounds only play a supportive role in the overall ruminal acid-base balance, and their role on ruminal pH is relatively small and varies widely depending on diet composition (Askar et al., 2011; Humer et al., 2018a).

#### *2.7.1.7 Organic acids*

Organic acids (OA) are commonly found in biological tissues since they are key intermediates of metabolic cycles and some are produced during fermentation processes (Martin, 1998). Most research on the use of OA in ruminants has been carried out with dicarboxylic acids such as fumaric and malic acids, which are intermediary metabolites in the tricarboxylic acid cycle and in the succinate-propionate pathway in the rumen (Martin, 1998; Carro and Ungerfeld, 2015). In ruminants, malate and fumarate are commonly used as free acids or in salt-form (sodium, calcium, etc.) to prevent lactate accumulation. Their mechanism of action is based on the stimulation of growth and uptake of lactate by *S. ruminantium*, especially malate since it acts as an electron sink for hydrogen that allows the use of lactate by *S. ruminantium* for growth (Nisbet and Martin, 1990; Martin, 1998). Organic acids also increase propionate production by *S. ruminantium* through a double mechanism: stimulation of lactate uptake and its

fermentation to propionate; and fermentation of malate and fumarate by the succinate-propionate pathway to synthesize succinate and propionate. Reported effects of OA addition in ruminants are increased rumen pH, decreased methane production and increased propionate production through lactic acid fermentation (Martin, 1998; Carro and Ungerfeld, 2015). Various *in vitro* studies have shown higher pH, increased VFA, increased propionate and reduced lactate (Carro and Ranilla, 2003; Newbold et al., 2005); however, *in vivo* responses to OA still remain unclear. Carro and Ungerfeld (2015) reviewed some *in vivo* studies in ruminants on the use of OA, and found that there is a wide inconsistency being the apparent contradiction probably due to differences in chemical forms of OA, diets, cereal types, and especially level of supplementation. In general, the inclusion of malate or its salts in doses ranging from 1 to 26.4 g/kg DM in beef steers has been reported to increase rumen pH (Montaño et al., 1999; Khampa et al., 2006), decrease plasma concentrations of lactate (Castillo et al., 2007; Liu et al., 2009), and increase propionate (Khampa et al., 2006). Other authors, however, found no effect of malate supplementation on rumen fermentation at doses of 1.1 g malate/kg concentrate (Carrasco et al., 2012) or 5.9 to 13.4 g of malate/kg DM of concentrate (Vyas et al., 2015). Similarly, the supplementation with fumarate in doses ranging from 12 to 24.4 g/kg DM in beef cattle increased nadir rumen pH and reduced the amount of time with rumen pH<5.8 (de Nardi et al., 2014), increased VFA and propionate concentration (Bayaru et al., 2001; Beauchemin and McGinn, 2006) or did not alter rumen fermentation (McGinn et al., 2004; Vyas et al., 2015). In general, no effect on lactate was reported in *in vivo* studies because lactate levels are not supposed to be high under normal conditions such as those occurring in the aforementioned studies. Even though OA have been proposed in ruminant nutrition to reduce lactate accumulation, and their use would be interesting during the transition period to high-concentrate diets, their inclusion seems to be not economically feasible for their high cost (Carro and Ungerfeld, 2015). Some alternatives to their use might be combining OA or their salts with other additives such as probiotics or plant extracts, or the use of forages with high content of OA, such as alfalfa or bermudagrass (Martin, 1998). No information appears to be available on the use of OA in animals in transition to high-concentrate diets.

*2.7.1.8 Plant secondary metabolites*

Plant secondary metabolites (PSM) are substances produced by plants for defence rather than for growth or reproduction. These compounds include tannins, essential oils (EO), saponins and organosulphur compounds. The PSM are of interest because they can modify rumen fermentation, possess antioxidant and antimicrobial properties, stimulate digestive enzyme secretion, and inhibit methane production (Patra and Saxena, 2009a) and because of their selective effects on rumen microorganisms are considered rumen modulators. The biological effects are variable (Patra and Saxena, 2009a; Patra, 2010.) because they depend on the type of compound, source and concentration in the plant, chemical structure, molecular weight, dose and composition of the diets to which they are added. In general, the antimicrobial effect of PSM may stem from their potential to interact and form complexes with the lipid by-layer of microbial cell membranes, by altering essential enzymatic functions and transport, depriving nutrients for microbial growth, causing damage of membranes and allowing leakage of cell constituents and nutrients, and finally even leading to disruption of membrane structure and cell lysis (Smith et al., 2005; Calsamiglia et al., 2007; Patra and Saxena, 2009b). These compounds are particularly more active against gram-positive rather than gram-negative bacteria because the outer membrane of the latter exerts protection. Much of the emphasis with PSM has been currently focused to reduce methanogenesis, as those compounds may reduce protozoal numbers (Patra, 2010).

Tannins are a very diverse and complex group of polyphenolic PSM that are classified into condensed (CT) and hydrolysable (HT) depending on their chemical structure, and possess the capacity to form complexes with proteins and other molecules such as fibres, starch, nucleic acids and minerals. (Makkar, 2003; Mueller-Harvey, 2006). In general, when included at moderate doses in ruminant diets (<50g/kg DM), the main effect of tannins on rumen fermentation is a reduction in proteolysis and a moderation of rumen fermentation and pH due to their complexation with proteins and structural polysaccharides (Makkar, 2003). However, results are inconsistent because their biological activity is dependent upon their chemical structure, type, source and dosages (Mueller-Harvey, 2006). Most assayed tannins for ruminant diets are from quebracho, acacia, chestnut, oak, mimosa or leucaena. *In vitro* studies have observed a moderate fermentation of cereals with the addition of tannins (50g/kg DM; Martínez et al., 2006). Hassanat and Benchaar (2012) studied *in vitro* the impact of different sources of tannins

from acacia, quebracho, chestnut and oak (doses from 20 to 200 g tannins/kg DM) on fermentation and found that doses higher than 50 g/kg DM increased pH but also decreased VFA concentration, which could denote negative effects for microbes. *In vivo* experiments report inconsistent results. In steers fed high-concentrate diets, the inclusion of 14.9 g/kg DM of chestnut or acacia tannins (Krueger et al., 2010) or 4 g CT/kg DM (Mezzomo et al., 2011) did not exert any effect on rumen fermentation. By contrast, a lower dose (2 g/kg of feed) of a blend of HT and CT tannins led to higher rumen pH compared to control in high-concentrate fed steers (Díaz Carrasco et al., 2017). Most studies carried out to assess the effect of tannin inclusion on rumen fermentation have been performed in adult animals, and there are no studies with animals during their transition to high-concentrate diets. Further, since there is no consistency on the reported results it could be of interest to test the modulator effect of tannins during a critical period for developing acidotic state such as the transition period.

Saponins are high-molecular-weight glycosides in which sugars are linked to a hydrophobic aglycone called sapogenin that may be either of triterpene or steroidal nature (Wallace et al., 2002; Patra and Saxena, 2009a). Different classes of saponins are triterpenes, steroids and the steroid alkaloids. Studies with saponins-containing plants include saponins from yucca (*Yucca schidigera*), bark tree (*Quillaja saponaria*), peanuts, chick-pea, tea or lucerne, among others. Saponins may alter bacterial populations towards decreased number of *S. bovis* and increased *S. ruminantium*. *In vitro* studies showed that saponins from *Yucca schidigera* could reduce growth of *S. bovis* and other amylolytic bacteria, and stimulate growth of *S. ruminantium* (Wang et al., 2000; Narvaez et al., 2013). *In vivo*, including intrarruminally 20 or 60 gr/d of yucca in a high-grain diet for beef heifers neither affected amylolytic activity nor rumen pH, but increased propionate concentration compared to control, and the authors suggested that it could be due to a stimulation of *S. ruminantium* (Hristov et al., 1999). These studies suggest that saponins exert a selective manipulation of the rumen microbes that might be helpful to reduce the incidence of rumen acidosis (Patra and Saxena, 2009b), although the different structures of saponins and their different sources may elicit different responses on rumen metabolism. In addition, Cardozo et al. (2005) reported a stronger antimicrobial activity at low pH (pH 7 *vs.* pH 5.5) *in vitro*, which may suggest that the effect of saponins may be stronger with high-concentre diets.

Essential oils are extracts derived from plants and spices obtained by steam distillation or organic-solvent extraction that consist mainly of cyclic hydrocarbons and their alcohol, aldehyde or ester derivatives. (Wallace et al., 2002). These compounds possess a strong antimicrobial activity because are lipophilic and interact with cell walls of bacteria. The more extensively studied EO in ruminants have been thymol (thyme), carvacrol (oregano), eugenol (clove, cinnamon), cinnamaldehyde (cinnamon), anethol (anise, fennel) and peppermint oils (Calsamiglia et al., 2007; Benchaar et al., 2008). The effects of EO vary depending on the individual oils or mixtures, doses and source of essential oil, and effects appear to be pH-dependent (Cardozo et al., 2005; Calsamiglia et al., 2007). Addition of carvacrol (400 mg/L), cinnamon (400 mg/L), thymol (200 mg/L) and eugenol (800 mg/L) resulted in a pH increase *in vitro* that was not accompanied with a decrease in total VFA. However, ruminal fermentation pattern changed differently depending on the source of EO, indicating different effects on rumen bacteria (Benchaar et al., 2007). *In vivo*, the addition of 5 g/d of either thyme or cinnamon in calves fed a high-concentrate diet did not affect rumen pH or total VFA compared to control group, although it changed the individual concentrations of VFA (Vakili et al., 2013). Eugenol or cinnamaldehyde supplementation at different doses (400 mg/d, 800 mg/d or 1600 mg/d) did not affect rumen fermentation (pH, VFA or proportions of individual VFA) in growing beef cattle given high-grain diets (Yang et al., 2010 a, b). No effect on rumen pH, total VFA or VFA concentrations were observed even with higher doses (*ca.* 1.49 or 2.9 g/d) of cinnamaldehyde in a TMR for dairy cows (Chapman et al., 2019). Regarding blends of EO, no differences in rumen fermentation were found in hay-fed steers supplemented (1 or 2 g/d) with a blend of EO containing thymol, eugenol, vanillin, limonene and guaiacol (*i.e.* commercial product CRINA®) (Tomkins et al., 2015). Inclusion of CRINA ® during the pre-weaning period did not affect rumen pH but increased VFA concentration in young dairy calves (Kazemi-Bonchenari et al., 2018). Santos et al. (2015) supplemented a blend of EO (carvacrol, cineole, cinnamaldehyde and pepper oil; ACTIVO ®) either between milk replacer (400 mg/kg) or distributed in milk replacer (200 mg/kg) and starter feed (200 mg/kg), and did not found differences in rumen pH, VFA, amylolytic and cellulolytic bacteria of dairy calves.

In most cases, *in vitro* effects are evident but *in vivo* significant effects are hardly observed, possibly because doses that elicit favourable effects on fermentation *in vitro* are too high to be used *in vivo* due to the toxic effects, the negative effect on palatability

and the high cost (Beauchemin et al., 2009). In addition, the microbial adaptation to EO (Castillejos et al., 2007) and their degradation in the rumen (Newbold et al., 1997; Makkar and Becker, 1997) are also causes of the lack of long-term effects *in vivo*. Therefore, their inclusion seems not advisable during the transition period.

*2.7.1.9 Medium-chain fatty acids*

Medium-chain fatty acids (MCFA) refer to a family of saturated fatty acids that includes caproic ($C_6$), caprylic ($C_8$), capric ($C_{10}$) and lauric ($C_{12}$) acids. These compounds possess antimethanogenic and antimicrobial activity (Machmüller, 2006). The MCFA modify the microbial cell wall permeability leading to a membrane destabilization, incorporation of the MCFA into the cell, and alteration of essential processes, and may lead to cell death (Desbois and Smith, 2010). The effects on rumen fermentation have been reported to be influenced by the type of fatty acid (Dohme et al., 2001), dose, experimental approach, and by the type of diet (forage- *vs*. concentrate- based diets; Machmüller et al., 2001). *In vitro*, Amanzougarene et al. (2017b) studied the effect of a blend of MCFA (doses: 2, 4 and 6 g/kg DM), and reported a tendency to increased rumen pH, without adverse effects on VFA, after 24 h of incubation of barley. Other authors, however, studied *in vitro* the effect of $C_8$, $C_{10}$ and $C_{12}$ (10-40 mg per 60 mL of incubation liquid) and reported decreased pH with all MCFA compared to the control (Ajisaka et al., 2002), which was explained by the significant reduction of rumen protozoa. The same was reported by Hristov et al. (2004a) who studied *in vitro* the effect of $C_6$, $C_8$, $C_{10}$ (0.625, 1.25 and 2.5 g/L of incubation liquid) and $C_{12}$ (2.5, 5.0, 10 g/L) and observed that effects on fermentation were dependent upon the individual fatty acids and dose. Also *in vitro*, Klevenhusen et al. (2011) assessed the effect of monolaurin addition (50 g/kg of DM) on target rumen bacteria, and interestingly found an increase of *M. elsdenii* when this monoglyceride was included. As *M. elsdenii* can ferment lactate, the use of monolaurin in adaptation diets could be a useful strategy to prevent lactate accumulation. In ruminant feeding, the vast majority of studies have been carried out with the coconut oil (CO) as a common and practical source of MCFA (Dohme et al., 2000), and apart of CO, there is no much information in the literature about the *in vivo* effects of MCFA on rumen fermentation. In addition, *in vivo* results are even more inconsistent due to the different nature of diets, and most of the studies have been carried out with the addition of CO or $C_{12}$ (Liu et al., 2011; Hristov et al., 2012). For instance, Jordan et al. (2006) supplemented beef heifers with 250 g CO/d and reported decreased VFA concentration, which suggests

it was toxic for rumen microbes. Although it seems there is no literature about this topic, MCFA inclusion in high-concentrate diets during the transition period could help to attenuate rumen fermentative activity, reducing the risk of ruminal acidosis.

#### *2.7.1.10 Direct-fed microbials*

Direct-fed microbials (DFM) are feed products that contain a source of live or naturally occurring microorganisms such as bacteria, yeasts or moulds (McAllister et al., 2011). Some researchers refer to this group as 'probiotics', but DFM may be more focused as the term 'probiotics' also includes microbial culture extracts and enzymes in addition to live microbes (McAllister et al., 2011). In ruminants, these additives improve microbial balance enhancing gastrointestinal tract health and thus animal productivity (Beauchemin et al., 2006). The vast majority of studies using bacterial DFM to prevent rumen acidosis have focused on enhancing lactate metabolism through inoculation of lactate-producing or lactate-fermenting bacteria (McAllister et al., 2011). Lactate-producing bacteria (*e.g. Lactobacillus* spp., *Streptococuus* spp. or *Enterococcus* spp.) have been largely used because they seem to stimulate the growth of lactate utilizing bacteria as well as the adaptation of other bacteria to the presence of lactate (Beauchemin et al., 2006). Therefore, these additives might prevent rumen acidosis, but in general there is no consistency on the effects of bacterial DFM supplementation in cattle because of the huge variability between studies was due to strains diet, grain types, the addition of buffers, doses and types of DFM (Beauchemin et al., 2006; Lettat et al., 2012). Beauchemin et al. (2003) and Kenney et al. (2015) pointed out that bacterial DFM would be a useful tool when the rumen is not adapted to lactate such as during the transition period, when they might prevent lactate accumulation. On the other hand, inoculation with lactic-fermenting bacteria (*i.e. M. elsdenii* and *S. ruminantium*) may also be protective against rumen acidosis, especially during periods of dietary transition (Henning et al., 2010; McAllister et al., 2011). In a recent study with beef heifers fed high-grain diets, *M. elsdenii* ATCC 17,753 inoculation into the rumen increased protozoal counts, decreased *S. bovis* numbers and VFA concentration, and minimized drop in pH during a SARA event (Arik et al., 2019). Unfortunately, although this practice has some advantages, cultured rumen bacteria frequently fail to establish in the rumen and their effects are not long lasting, which forces to a daily administration (McAllister et al., 2011) what makes supplementation with bacterial DFM a not very feasible practice under farm conditions.

As there is recent evidence that rumen ecosystem can be manipulated during the early life, and changes may persist in the adulthood (Abecia et al., 2014), microbiota engineering would be an interesting practice to attenuate ruminal acidosis problems if permanent lactic fermenters could be established since the early-life. However, the window of time of intervention is still unknown (Yáñez-Ruiz et al., 2015), and persistence of the effects is not clear (Belanche et al., 2019b).

Regarding yeasts, the common used strain of *Saccharomyces cerevisiae* has been widely used in cattle nutrition because it seems to have a potential to prevent or alleviate the negative consequences of ruminal acidosis (Desnoyers et al., 2009; Humer et al., 2018b). The stabilizing effect of live yeasts has been ascribed to reduced lactate accumulation due to either nutritional competition between yeasts and *S. bovis*, or to stimulation of growth and lactate fermentation by *M. elsdenii* and *S. ruminantium* (Chaucheyras et al., 1996; Pinloche et al., 2013) by providing essential nutrients and growth factors such as B vitamins, amino acids and organic acids, which seem essential for lactate-fermenting bacteria, even acting as a prebiotic when added as dead cells (Chaucheyras-Durand et al., 2008; Oeztuerk, 2009; Vyas et al.,2014). In dairy cows, most reported outcomes of live yeast supplementation on rumen fermentation seem to be increased rumen pH and VFA, decreased lactate concentration (Desnoyers et al., 2009), and decreased toxic compounds (LPS, biogenic amines, etc.) during low ruminal pH episodes (Humer et al., 2018b). However, studies with beef steers are inconsistent probably due to the variability of diets and types of animals used in the studies (Ding et al., 2014; Vyas et al., 2014; Jiao et al., 2017). Interestingly, Jiao et al. (2017) found higher numbers of protozoa with either yeast treatments, which may be an indirect stabilizing effect on pH, as also found in another study with sheep (Silberberg et al., 2013). Additionally, live-yeasts-supplemented sheep responded ‘better’ to reiterative acidosis challenges (Silberberg et al., 2013). Although the effects of yeast supplementation seem promising, the outcomes on rumen fermentation seem to depend on many biotic and abiotic factors such as the strain of yeast and its viability, the dose rate, yeast storage and delivery, and diet (Desnoyers et al., 2009). Further, yeast must be supplied continuously in feeds because once in the rumen they do not survive due to the anaerobic conditions (Jouany and Morgavi, 2007).

## 2.8 RECAPITULATION

Most of the calves that enter in the feedlot in Spain had been fed on milk and/ or grass and are abruptly weaned and directly changed to a high-concentrate diet. These animals are predisposed to develop ruminal acidosis because the inclusion of concentrates deeply disrupts the microbial ecosystem. Further, these animals do not have a fully developed rumen in terms of absorptive capacity, and thus the introduction of concentrates might lead to an acid accumulation in the rumen. Somehow, the transition period could be compared to the rumen conditions during a SARA episode in that dysbiosis occurs, there is an accumulation of VFA and the epithelium cannot absorb and neutralize the acids. Further, the increased acidity and osmolarity might damage the integrity of the rumen epithelium disrupting barrier function and leading to a cascade of consequences that can depress immunity system. The consequences of acidotic episodes during the transition period may be costly not only from a point of view of a daily basis during this period (reduced intake, lethargy, etc.), but also because epithelial damage may lead to harmful effects that might promote numerous secondary diseases and impair performance and productivity throughout the fattening period. Therefore, it seems that the transition period is a critical window of time in which is essential to promote good rumen conditions and development to keep pace with the fattening diets.

A lot of research has evidenced that there are possible helpful strategies that may modulate rumen microbial populations and alleviate the risk of rumen acidosis. Most strategies (pre-weaning management, the use of different ingredients and/or different processing methods, supplementation with feed additives, etc.) have been tested under different conditions and mostly in adult animals with a fully developed rumen. Straightforward strategies, such as pre-weaning supplementation, are known to stimulate rumen development, which may favour a smooth transition to high-concentrate diets. However, this practice is not fully carried out in many areas due to that it seems less economically feasible. On the other hand, when feed additives have been assessed under dietary changes from high-forage to high-concentrate diets, this has been only carried out with adult animals, mainly dairy cows, and thus there are no studies during the transition period of calves to high-concentrate diets. In addition, the effects of these additives on rumen fermentation are mostly inconsistent because there are some biotic (*e.g.* DFM viability) and abiotic (*i.e.* doses, chemical composition, experimental approach, rearing stage, type of diet, etc.) factors that determine the effect of the feed additives to prevent

acidosis. Since the interactions that may occur among all these factors are complex, there would be essential to investigate some strategies under the specific conditions of each particular production system.

# CHAPTER 3

# HYPOTHESIS, OBJECTIVES AND EXPERIMENTAL APPROACH

# CHAPTER 3: HYPOTHESIS, OBJECTIVES AND EXPERIMENTAL APPROACH

The present doctoral thesis was designed to acquire knowledge about the effectiveness of some strategies to reduce the susceptibility of ruminal acidosis during the transition period of beef calves to a high-concentrate diet.

## HYPOTHESES

The main hypotheses of the present thesis are:

1. Pre-weaning supplementation with solid feeds may promote a better rumen development and thus animals would be better adapted to the high-concentrate diets during the transition period, which in turn would result in higher profitability for the beef producer.

2. Formulating the adaptation diets with ingredients with slower starch degradation rate or higher fibre contents, may temper rumen fermentation and stabilize the rumen environment, favouring rumen health and animal performance.

3. The inclusion of feed additives in the adaptation compound feed might modulate microbial activity and thus slow down fermentation, avoiding acid accumulation.

## OBJECTIVES

To test and verify these hypotheses, the objectives of this research were the following:

1. To assess the most adequate pre-weaning strategy (only milk or milk plus either forage or concentrate supplementation) for beef calves that are weaned and subsequently introduced to the intensive feeding system, in terms of rumen development, feed intake, animal performance, and economic profitability.

2. To determine the effect of the partial substitution of barley grain by maize as a slowly fermentable starch source and sugar beet pulp as a source of fibre, in the adaptation diet of beef calves during the transition from milk and pasture to a conventional high-concentrate ration on feed intake, animal performance, rumen fermentation, and rumen microbial composition.

3. To evaluate the effect of the inclusion of different feed additives as rumen fermentation modulators for the adaptation diet of beef calves on feed intake, animal performance, rumen fermentation and rumen microbial composition. Tannins and medium-chain

fatty acids were chosen based on the lack of information of their effect on rumen fermentation during the transition period, and according to their modulation effects previously observed *in vitro* in our laboratory. These *in vitro* results are part of another doctoral thesis included in the same research project (Amanzougarene, 2020).

## EXPERIMENTAL APPROACH

To address these objectives, three experiments were designed, intending to resemble the feeding practices that are currently carried out for beef production in Spain.

### Experiment 1

This experiment was designed to achieve the first objective. Three pre-weaning managements (milk only, milk plus hay and milk plus concentrates) were evaluated with local breeds, following the typical beef production management in the Pyrenees. As such, calves were group-loose-housed with their mothers and allowed to milk during the entire nursing period. From three months of age, two groups of animals received the solid supplements during the suckling period until weaning at five months. Thereafter, calves were abruptly weaned and immediately moved to another facility where all calves received the same compound feed for one month. After that, animals were kept in the same facility for breeding or commercial purposes. Feed consumption, productive performance, rumen fermentation variables and blood metabolites were evaluated throughout the experiment. Further, economic performance was evaluated with partial budgeting, which assessed the effects of a change from the current to a new scenario, including only the resources that change between scenarios, in order to assess the most profitable practice.

### Experiment 2

The second experiment met the second objective. Thirty-nine Limousine crossbred male calves, reared with their dams on pasture and allowed to suckle freely while were grazing were used. These animals were abruptly weaned at an average age of seven months and transported to the experimental facilities. Three adaptation protocols were followed: in Protocol 1 animals were abruptly shifted to a barley-based fattening diet, whereas calves in Protocols 2 and 3 were gradually adapted and received diets in which barley was partially replaced by different proportions of maize and sugar beet pulp for 10 days, thereafter a mixture of their diet and the fattening diet, and finally after 14 days all animals received the fattening diet. Production and rumen environment variables

were evaluated to determine the short- and long-term effects of the different feeding management during the transition period on animal performance, and a histopathologic exam was also carried out to determine the occurrence of ruminal lesions.

**Experiment 3**

Lastly, the third experiment accomplished the third objective for which eighteen calves of the same characteristics as in the second experiment were used. Tannins and MCFA were chosen for its apparent capacity of modulating rumen fermentation and its feasible applicability in practical conditions and were compared with a non-supplemented control diet. The experiment lasted 28 days as the adaptation period, and thereafter animals were kept in the experimental facilities until they reached on average *ca.* 500kg. The variables studied in this experiment were the same as in Experiment 2, except that for the current trial ruminal tissue samples were not taken.

# CHAPTER 4

# EXPERIMENT 1

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Effect of pre-weaning diet on performance, blood metabolites, and ruminal fermentation around weaning in suckling calves from two beef breeds. Animal Production Science, 2020, 60: 1018-1027. doi: 10.1071/AN19152.

# CHAPTER 4: EXPERIMENT 1

## Effect of pre-weaning diet on performance, blood metabolites, and ruminal fermentation around weaning in suckling calves from two beef breeds


### Abstract

The aim of the present study was to assess the optimal feeding strategies during the pre-weaning period of beef calves for an adequate adaptation to high-concentrate diets. Thirty female beef calves (15 Parda de Montaña, PM; and 15 Pirenaica, PI) were assigned to three pre-weaning diets (Milk only, MO; Milk plus hay, MH; Milk plus concentrate, MC), fed from the third month until weaning at five months. During the subsequent transition period (one month), all animals received a fattening concentrate plus wheat straw *ad libitum*. Milk intake did not differ among diets, and solids intake was similar for MH and MC calves. Pre-weaning supplementation improved performance at the end of the suckling period (MC>MH>MO). During transition, concentrate intake was lower for MO calves, and MH calves showed the lowest feed efficiency. At the end of transition, MC calves showed the highest live weight and average daily gain, with no differences between MH and MO. Rumen pH at weaning did not differ from that at the end of transition in MC calves, whereas MH and MO animals showed lower values at the latter. Supplemented calves showed higher VFA concentration than MO calves at weaning, whereas MC calves showed the lowest values at the end of the transition period. Ammonia and lactate concentration at weaning were the highest for MO and MC calves, respectively, with no differences between diets at the end of transition. Glucose and non-esterified fatty acids concentrations were only affected by age. Urea and β-hydroxybutyrate concentrations were affected by the interaction between diet and age. Economic profitability at weaning was higher for supplemented (MH=MC) than for MO calves, but it ranked MC>MO>MH at the end of the transition period. Daily gains during the suckling period were higher for PM than for PI calves due to higher milk intake, which provided a better economic margin. During transition, concentrate intake was higher for PM than for PI, but the economic margin was similar. Considering the whole production cycle, pre-weaning supplementation with concentrates is the most interesting option in terms of performance and profitability.

## Introduction

Gross income and profitability in the calf-producing industry is highly dependent on the production of calves with high weaning weights (Martin et al., 1981). In this sense, a number of studies (Myers et al., 1999; Blanco et al., 2008) have addressed the effect of supplementing suckling beef calves on performance before and after weaning. Solid feed supplementation prior to weaning is considered fundamental for rumen development (Baldwin et al., 2004; Khan et al., 2016), being crucial for the later adaptation to the fattening diets.

Previous research about the influence of supplementation during the suckling period has been conducted indoors using a starter concentrate (Casasús et al., 2001; Blanco et al., 2008) or a mixture of concentrates and forages (Tamate et al., 1962; Stobo et al., 1966; Coverdale et al., 2004). However, to the best of our knowledge no studies have been conducted to assess the short-term influence of concentrate or forage supplementation during the suckling period on the early stages of the subsequent feedlot fattening of beef cattle. Besides, the effects might be breed-dependent, since milk consumption by lactating calves during the suckling period is highly determined by the yield potential of their dams. On the other hand, the profitability of pre-weaning supplementation will depend on the price of feedstuffs in relation to the value of weaned calves, and on the duration of the fattening period (Lardy and Maddock, 2007).

The hypothesis tested in the present study was that supplementation with solid feeds during the suckling period would improve rumen fermentation and animal performance at weaning, and increase the economic profitability of the farm. Therefore, this work aimed to assess the effects of different feeding strategies in the pre-weaning period on animal performance, blood metabolites, ruminal fermentation, and economic performance of autumn-born beef calves at the end of both suckling and transition periods.

## Materials and methods

The experimental procedures were in compliance with the guidelines of the European Union (Directive 2010/63/EU) on the protection of animals used for scientific purposes.

**Animals and management**

The study was conducted at La Garcipollera Research Station, in the central Pyrenees of Spain, using Parda de Montaña (PM, dual-purpose breed derived from Brown Swiss) and Pirenaica (PI, local beef cattle breed) cow-calf pairs. Fifteen autumn-born (2015) purebred PM (37± SD 7.8 kg) and fifteen PI (32±3.8 kg) female calves were used in a 2 (breeds) x 3 (diets) factorial experiment. Animals were randomly assigned at calving, within each breed, to one of three pre-weaning supplementation strategies: milk only (MO); milk plus hay (MH); and milk plus starter concentrate (MC). Concentrate, ground through a 6-mm sieve, and hay, offered in long form (Table 4.1), were both given *ad libitum* in a single daily meal (08:00 h). The study consisted of three phases (Figure 4.1): a) pre-experimental (months 1-2 of age), in which all calves consumed only milk from their mothers; b) treatment, in which solid supplements (either meadow hay or starter concentrate) were offered *ad libitum* on a group basis from three months of age until weaning at five months; and c) transition (sixth month) in which all calves received the same commercial compound feed.

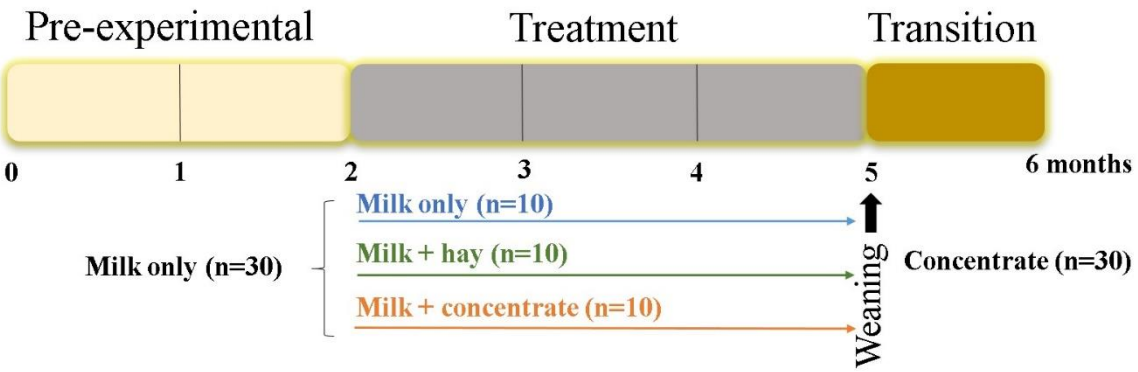


**Figure 4.1**. Diagram of the experimental design.

Calves and their dams were group-, loose-housed during the entire nursing period, in separate pens for each breed and calf diet. Dams were group-fed daily 12 kg (as fed) of meadow hay plus 2 kg of ground barley per head to meet their nutritive requirements for maintenance and an estimated milk production of 7 kg/d (ARC, 1980). All calves were allowed to suckle their mothers twice daily for 30 minutes at 08:00 and 16:00 h. Restricted suckling is a common practice during the indoor suckling period in mountain suckler beef cattle farms, in order to alleviate the negative effects of *ad libitum* suckling on the resumption of ovarian activity of the cows (Álvarez-Rodríguez et al., 2009). During the rest of the day, calves were kept in wood shavings-bedded cubicles adjacent to their dams, with two pens per breed and treatment (one pen with two and the other with three calves,

3 $m^2$ per calf). Cross-suckling was not observed. All animals had *ad libitum* access to water and vitamin-mineral blocks throughout the experiment.

**Table 4.1** Ingredients and composition of the different feedstuffs received by the calves throughout the experiment.

| Item | Meadow hay[1] | Concentrate | |
|---|---|---|---|
| | | Starter[2] | Fattening[3] |
| Ingredient (as-fed basis), g/kg | | | |
| Corn | | 439 | 164 |
| Barley | | 183 | 127 |
| Corn gluten feed | | 150 | 171 |
| Corn middling | | | 269 |
| Palm kernel flour | | 70 | 100 |
| Soybean flour | | 70 | |
| Sugar beet pulp | | 30 | |
| Palm oil | | 25 | |
| Wheat | | | 97 |
| Wheat bran | | | 30 |
| Calcium carbonate | | 12 | 18 |
| Urea | | 6 | |
| Sodium chloride | | 5 | 5 |
| Vitamin-mineral premix | | 6 | 10 |
| Nutrient composition | | | |
| DM, g/kg | 884 | 874 | 900 |
| CP, g/kg DM | 81 | 163 | 155 |
| NDFom, g/kg DM | 620 | 312 | 342 |
| ADFom, g/kg DM | 347 | 94 | 94 |
| Lignin (sa) g/kg DM | 54 | 15 | 30 |
| Ash, g/kg DM | 72 | 62 | 64 |
| ME, MJ/kg DM | 9.4 | 13.7 | 13.7 |

DM= dry matter; CP=, crude protein; NDFom = neutral detergent fibre assayed without amylase and expressed exclusive of residual ash; ADFom = acid detergent fibre expressed exclusive of residual ash; Lignin (sa): Acid detergent lignin; ME: metabolisable energy. [1]Fed to MH calves (see M&M) from 3 to weaning at 5 months. [2]Fed to MC calves (see M&M) from 3 to weaning at 5 months. [3]Fed to all calves from 5 months until the end of the study.

At the end of the fifth month (day 150), all calves were abruptly weaned and moved to an adjacent fattening barn, whereas their dams were turned out to outdoor pastures. All calves from the same breed were located in the same pen during the transition phase, provided with an automatic feeding station (ALPRO®, Alfa Laval Agri, Tumba, Sweden) that was therefore shared by 15 calves. Each calf had a transponder and, therefore, the individual intake was recorded by the automatic feeding system. During the first week after weaning, calves received the fattening concentrate in gradually increasing amounts until *ad libitum* intake was reached. Barley straw was also provided *ad libitum* from

weaning on a pen (breed) basis.

**Measurements**

All calves were weighed fortnightly at 08:00 h without prior deprivation of feed and water. Average daily gain was obtained by linear regression of LW on date during the different phases (pre-experimental, treatment and transition), and also on a monthly basis along the treatment phase. Milk intake was determined during the treatment phase using the weigh-suckle-weigh technique (Le Neindre, 1973) on days 60, 90, 120 and 150 of the milk-feeding period. Solid feed intake was recorded daily during the treatment phase on a per-group basis. During the transition phase, concentrate intake was individually recorded daily and barley straw intake was recorded on a pen basis. Metabolisable energy (ME) of hay was calculated from acid detergent fibre (ADF) content according to the equation proposed by Mertens (1983) (Net energy, NE = 2.469-0.0351x %ADF; $R^2$=0.849; ME = NE/0.61), whereas ME of concentrates was calculated taking into account ingredient composition and ME values from FEDNA (2010). Daily energy and protein intake from solid feeds were estimated from the energy and protein content of the different feedstuffs.

**Rumen fermentation**

Samples of approximately 200 mL were collected via oesophageal tube connected to an electric vacuum pump 02:30 h after suckling (day 150) and approximately 02:30 h after the first meal (day 170). The pH was immediately measured using a portable pH-meter (model Seven2GO, Mettler-Toledo AG, Schwerzenbach, Switzerland). Rumen fluid was strained through a 1-mm metal mesh and aliquots were taken, in duplicate, for ammonia, lactate and VFA analysis. For the determination of ammonia, 4 mL of 0.1 N HCl were added to 4 mL of filtered rumen fluid. Samples for VFA analysis were prepared by adding 1 mL deproteinising solution (2% (v/v) of orthophosphoric acid and 0.2% (w/v) of 4-methyl valeric acid) to 4 mL of strained rumen contents. The same amount of rumen liquor (4 mL) was taken for the determination of lactic acid. Samples obtained each day were frozen (-20 ºC) for further analysis.

Concentration of ammonia in rumen fluid samples was determined colorimetrically following the method by Chaney and Marbach (1962), and that of VFA by gas chromatography in an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid, Spain) fitted with a capillary column (Model HP-FFAP polyethylene glycol

TPA-treated, 30m x530µm i.d.x1µm film thickness). Total lactate concentration was measured using the colorimetric method proposed by Barker and Summerson (1941).

**Blood sampling**

Calves were bled at 08:00 h, before feeding, on days 60, 90, 120 and 150 of the milk-feeding period (treatment phase), and 30 days after weaning (transition phase), by puncture of the jugular vein. The samples to determine plasma glucose, non-esterified fatty acids (NEFA), and urea concentrations were collected into 4 mL tubes containing EDTA (Vacuette, Spain), and those used to determine BOHB were collected into 4 mL heparinized tubes (Vacuette, Spain). Blood samples were centrifuged at 1,500 g for 15 min at 4°C immediately after collection, and the plasma was harvested and frozen at −20°C until analysis. Plasma concentrations of glucose (glucose oxidase/peroxidase method), BOHB (enzymatic-colorimetric method) and urea (kinetic UV test) were determined with an automatic analyser (GernonStar, RAL/TRANSASIA, Dabhel, India). The mean intra- and inter-assay coefficients of variation (CV) for these metabolites were < 5.4% and < 5.8%, respectively. The plasma NEFA were analysed with an enzymatic method using a commercial kit (Randox Laboratories Ltd., Crumlin Co., Antrim, UK). The mean intra- and inter assay CV were 5.1% and 7.4%, respectively.

**Economic analysis**

According to Blanco et al. (2008), the different treatments were economically compared considering only the technical and economic aspects that varied among strategies. Costs associated to cow management, calf veterinary costs, yardage or other expenses were not considered as they did not vary between treatments. Feeding costs during the treatment and transition phases were determined considering solid feed intake, and the price of hay and that of starter and fattening concentrates (hay: 0.149 €/kg; starter concentrate: 0.409 €/kg; fattening concentrate: 0.384 €/kg; prices correspond to spring of 2016). Calf sale prices (493 € per a 200-kg weaned female ± 1.65 € per kg above or below 200 kg; prices correspond to spring of 2016) at the end of the treatment and transition phases were calculated on the basis of calf weights at weaning and at the end of the experiment, respectively. The economic margin at the end of the treatment phase was calculated as the difference between calf sale price at weaning and feeding costs associated with hay and starter concentrate intake. The economic margin at the end of the transition phase was calculated as the difference between calf sale price at the end of the

study minus calf purchase price at weaning and the feeding costs associated with the intake of fattening concentrate.

### Statistical analysis

Calf ADG during the pre-experimental phase, feed to gain ratio during the transition phase, and economic performance in treatment and transition phases were analysed with the GLM procedure of SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA) with breed, calf diet during the treatment phase and their interaction as fixed effects, and animal as random. Rumen fermentation variables were analysed with the GLM procedure considering diet, breed and sampling day, and their interactions as fixed effects. For analysis involving measurements collected over time (calf weights, ADG, milk intake and blood metabolites determined at monthly intervals) repeated measures analyses were conducted using the MIXED procedure of SAS with breed, calf diet, time and their interactions as fixed effects, and animal as the random effect. A similar analysis was performed to analyse solid feed intake during the treatment phase (weekly averages of DM, energy and protein intake from solid feed per replicate pen), and concentrate intake during the transition phase (weekly averages of individual DM, energy and protein intake). Breed, calf diet during the treatment phase, week and their interactions were considered as fixed effects. The random effect was either the pen during the treatment phase or the animal during the transition phase. The variance-covariance structure was selected on the basis of the lowest Akaike information criterion. Means were separated using the LSMEANS procedure, and pairwise comparisons were conducted using the PDIFF command. Differences were considered significant at $P<0.05$, whereas differences were considered to indicate a trend to significance when $0.05 \leq P \leq 0.10$.

## Results

The interaction between calf diet and breed was not significant for any of the studied traits; therefore, these main effects were examined separately.

### Performance

No differences were found among calf diets in LW from birth to the end of the pre-experimental phase (Table 4.2). During the pre-weaning phase, differences in LW among diets were observed from the fourth month (123, 146 and 153 kg for MO, MH and MC, respectively; $P<0.01$), and persisted until the end of the study. At weaning, solid feed supplementation improved performance ($P<0.001$), and MC calves were heavier than MH

calves. At the end of the transition phase, LW was affected (P<0.001) by the previous pre-weaning dietary strategy and ranked MO<MH<MC.

Calf LW was affected by the interaction between breed and age (P<0.01). Parda de Montaña calves were significantly heavier than PI calves from the third month (P<0.05) until the end of the transition phase (Table 4.2). Calf ADG was influenced by the interaction between diet and age (P<0.001). No differences were observed among diets during the pre-experimental phase but they were present during the treatment phase (MO<MH<MC; P<0.001).

**Table 4.2** Performance from birth to the sixth month of age of calves according to the pre-weaning diet (Milk only: MO; Milk plus hay: MH; Milk plus concentrate: MC) and the beef cattle breeds (Parda de Montaña: PM; Pirenaica: PI).

| | Diet (D) | | | Breed (B) | | | P-value[1] | |
|---|---|---|---|---|---|---|---|---|
| Phase | MO | MH | MC | PM | PI | SEM | D | B |
| Live weight (kg)[2] | | | | | | | | |
| Pre-experimental (months 1-2)[3] | 87 | 91 | 92 | 96 | 83 | 1.1 | 0.87 | 0.09 |
| Treatment (months 3-5) | $138^{b}$ | $178^{a}$ | $196^{a}$ | $181^{s}$ | $159^{t}$ | 1.2 | <0.001 | <0.01 |
| Transition (month 6)[4] | $168^{c}$ | $211^{b}$ | $236^{a}$ | $218^{s}$ | $192^{t}$ | 1.5 | <0.001 | <0.01 |
| ADG (kg/day) | | | | | | | | |
| Pre-experimental (months 1-2) | $0.907^{x}$ | 0.923 | $0.908^{y}$ | $0.916^{y}$ | $0.910^{y}$ | 0.0167 | 0.96 | 0.91 |
| Treatment (months 3-5) | $0.582^{c,y}$ | $0.988^{b}$ | $1.184^{a,x}$ | $0.966^{s,y}$ | $0.870^{t,y}$ | 0.0276 | <0.001 | <0.01 |
| Transition (month 6) | $0.961^{b,x}$ | $1.067^{b}$ | $1.275^{a,x}$ | $1.156^{x}$ | $1.045^{x}$ | 0.0305 | <0.01 | 0.09 |

[1] The interaction between calf diet and breed was not significant in any case.[2] Live weight at the end of each phase. [3]During the pre-experimental phase, all calves consumed only milk from their dams. [4]During the transition phase, all calves received a commercial compound feed plus barley straw *ad libitum.*

[a, b, c] Means with different letters within a phase differ significantly among calf diets (P<0.05). [s, t] Means with different letters within a phase differ significantly between breeds (P<0.05). [x, y] Means with different letters within breed or calf diet differ significantly between phases (P<0.05).

On a monthly basis, differences among diets were already observed at the third month, with lower ADG for the calves in the MO group (0.672 kg/d) than for those receiving supplements (P<0.001), which had similar gains (0.954 and 1.025 kg/d for MH and MC, respectively; P=0.20). In months four and five, gains differed significantly among the three groups (P<0.001). During the transition phase, MC calves showed higher ADG than the other two groups, which did not differ (P<0.001). Parda de Montaña calves

showed higher ADG than PI calves across the study (1.013 *vs.* 0.941 kg/d, respectively; P=0.05). Differences between breeds were only significant during the treatment phase, but also tended to be in the transition period (Table 4.2).

**Feed Intake**

During the treatment phase, milk consumption was affected by calf age (P<0.001) and breed (P<0.01) with PM calves showing higher intake than PI calves (Table 4.3). No interactions were observed between calf age and diet (P=0.23) or breed (P=0.40). Daily solid feed DMI was only affected by week (P<0.001), increasing steadily from 367 g DM/d on the first to 2456 g DM/d on the last week. Daily energy and protein intake from solid feed were lower for MH than for MC calves (11.6 *vs.* 18.9 MJ ME/d, respectively; P<0.01; and 108 *vs.* 235 g CP/d; P<0.01). When expressed in terms of metabolic weight, solid feed DMI increased with calf age (P=0.001) from 12.2 in the first to 48.5 g DM/kg $LW^{0.75}$/d in the last week. Dry matter (P=0.07) and energy intake from solid feeds (P=0.08) tended to be higher for PM than for PI calves, with no differences in protein intake (P=0.11). When expressed in terms of metabolic weight, solid feed intake did not differ between breeds (P=0.12).

During the transition phase, straw DMI averaged 345 g/d, with no differences between breeds. Calves that had been supplemented during the milk-feeding period showed a higher concentrate DMI than MO calves during the whole four-week period, with no differences between MC and MH. In terms of metabolic weight, concentrate DMI during transition was also affected by the interaction between pre-weaning diet and week (P<0.01), being lower for MO calves than for the other groups only in the first two weeks, with no differences thereafter. Calves on MO showed a lower daily energy and protein intake from concentrates with no differences between MH and MC animals (P<0.001). Feed to gain ratio was affected by the pre-weaning diet (P<0.01), with higher values for the MH calves. Concentrate DMI during transition (both as kg DM/d –P<0.001- or in terms of metabolic weight –P<0.01) was influenced by the interaction between breed and week. Daily intake did not differ between breeds on the first week, but intake by PM calves was higher thereafter. Similarly, energy and protein intake from concentrate were affected by the interaction between breed and week (P<0.001), PM calves showing higher values than PI calves from the second week to the end of the study. In terms of metabolic weight, no differences between breeds were found in the first two weeks, but higher values for PM calves appeared thereafter.

**Table 4.3** Milk and solid feed intake (kg/d) from the third to the sixth month of age of calves according to the pre-weaning diet (Milk only: MO; Milk plus hay: MH; Milk plus concentrate: MC) and the beef cattle breeds (Parda de Montaña: PM; Pirenaica: PI).

| | Diet (D) | | | | Breed (B) | | | P-value[1] | |
|---|---|---|---|---|---|---|---|---|---|
| | MO | MH | MC | SEM | PM | PI | SEM | D | B |
| Treatment phase (months 3-5) | | | | | | | | | |
| Milk intake | 6.07 | 6.68 | 6.61 | 0.370 | 7.03 | 5.87 | 0.302 | 0.46 | <0.01 |
| Solid feed intake[2] | | | | | | | | | |
| kg DM/d | - | 1.33 | 1.44 | 0.122 | 1.58 | 1.19 | 0.122 | 0.54 | 0.07 |
| g DM/kg $LW^{0.75}$/d | - | 33.9 | 32.1 | 2.78 | 36.6 | 29.3 | 0.122 | 0.67 | 0.12 |
| Transition phase (month 6)[3] | | | | | | | | | |
| Concentrate intake | | | | | | | | | |
| kg DM/d | 2.77$^{b}$ | 3.82$^{a}$ | 3.88$^{a}$ | 0.194 | 3.81$^{s}$ | 3.16$^{t}$ | 0.158 | <0.001 | <0.01 |
| g DM/kg $LW^{0.75}$/d | 64.3 | 73.4 | 68.7 | 3.02 | 71.9 | 65.6 | 2.47 | 0.12 | 0.08 |
| Feed to gain ratio[4] | 2.95$^{b}$ | 3.59$^{a}$ | 3.02$^{b}$ | 0.235 | 3.29 | 3.08 | 0.157 | <0.01 | 0.20 |

[1] The interaction between calf diet and breed was not significant in any case. [2]During the treatment phase, supplemented calves were offered either meadow hay (MH) or a commercial starter concentrate (MO) *ad libitum*. [3] During the transition, phase all calves received a commercial compound feed plus barley straw *ad libitum*. [4]Kg of feed dry matter intake per kg live weight gain.

[a, b, c] Means with different letters within a phase differ significantly among calf diets (P<0.05).

[s, t] Means with different letters within a phase differ significantly between breeds (P<0.05).

**Rumen fermentation**

Rumen fermentation variables were affected by the interaction between pre-weaning diet and sampling day (P<0.05; Table 4.4) but not by breed.

Rumen pH at the end of suckling period did not differ among diets, whereas in the transition phase higher values were observed for MC, intermediate for MH and lower for MO (P=0.01). Within diets, rumen pH of MO and MH calves but not those of MC calves decreased from suckling period to transition (Table 4.4). Ammonia concentration at weaning was higher in MO calves (P<0.001) with no differences between diets during the transition phase. Lactate concentration at weaning was higher for MC calves (P<0.001) with no differences between diets thereafter. Within groups, lactate concentration was lower at the end of the suckling period than during the transition phase for MO and MH (P<0.001) whereas the opposite was found with diet MC (P<0.01). Total VFA (TVFA) concentration was lower in MO calves at weaning, and in MC calves at the end of the transition (Table 4.5). Molar proportions of all individual VFA were affected by the interaction between pre-weaning diet and sampling day (P<0.05). The ratio acetate-to-

propionate (A:P) was also affected by the mentioned interaction with a pre-weaning diet effect at weaning (MO>MH>MC; $P<0.001$) but no differences during transition. Within diets, the ratio A:P was lower during transition than at weaning for all groups.

**Table 4.4** Rumen pH, ammonia (mg/L) and lactate (mmol/L) in rumen fluid of beef cattle at weaning (at 5 months of age) and at the end of the transition phase[1] (at 6 months of age) according to the pre-weaning diet (Milk only: MO; Milk plus hay: MH; Milk plus concentrate: MC).

| | | Diet (D) | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sampling day (S) | MO | MH | MC | SEM[2] | SEM[3] | D | S | D x S |
| Rumen pH | Weaning | 7.62 $^{x}$ | 7.58 $^{x}$ | 7.16 | 0.136 | 0.114 | 0.52 | <0.001 | <0.01 |
| | Transition | 6.43 $^{b,y}$ | 6.83 $^{ab,y}$ | 7.30 $^{a}$ | | | | | |
| Ammonia | Weaning | 105 $^{a,x}$ | 35.7 $^{b}$ | 21.1 $^{b}$ | 5.14 | 4.19 | <0.001 | <0.001 | <0.001 |
| | Transition | 32.2 $^{y}$ | 31.0 | 24.1 | | | | | |
| Lactate | Weaning | 0.13 $^{b,y}$ | 0.27 $^{b,y}$ | 0.81 $^{a,x}$ | 0.043 | 0.035 | <0.001 | <0.001 | <0.001 |
| | Transition | 0.58 $^{x}$ | 0.65 $^{x}$ | 0.56 $^{y}$ | | | | | |

[1]During the transition phase, all calves received a commercial compound feed plus straw *ad libitum.* [2] Standard error of the mean for comparison among diets. [3] Standard error of the mean for comparison between days.
$^{a, b, c}$ Means with different letters within a sampling day differ significantly among calf diets (P<0.05). $^{x, y}$ Means with different letters within a diet differ significantly between sampling days (P<0.05).

**Table 4.5** Total concentration of volatile fatty acids (VFA, mmol/L) and molar proportions of individual VFA (mol/100 mol) in rumen fluid of beef calves at weaning (at 5 months of age) and at the end of the transition phase[1] (at 6 months of age) according to the pre-weaning diet (Milk only: MO; Milk plus hay: MH; Milk plus concentrate: MC).

| | | Diet (D) | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|
| | Sampling day (S) | MO | MH | MC | SEM[2] | SEM[3] | D | S | D x S |
| VFA | Weaning | 30.6 $^{b,x}$ | 65.0 $^{a}$ | 65.5 $^{a,x}$ | 5.14 | 3.89 | 0.012 | 0.70 | <0.001 |
| | Transition | 60.5 $^{a,y}$ | 66.8 $^{a}$ | 40.4 $^{b,y}$ | | | | | |
| Acetate | Weaning | 77.4 $^{a,x}$ | 65.5 $^{b,x}$ | 62.3 $^{b,x}$ | 1.87 | 1.53 | 0.003 | <0.001 | 0.036 |
| | Transition | 47.6 $^{y}$ | 48.8 $^{y}$ | 43.7 $^{y}$ | | | | | |
| Propionate | Weaning | 13.0 $^{b,x}$ | 17.7 $^{b,x}$ | 29.5 $^{a,x}$ | 1.49 | 1.22 | <0.001 | <0.001 | 0.003 |
| | Transition | 40.3 $^{y}$ | 40.7 $^{y}$ | 41.7 $^{y}$ | | | | | |
| Butyrate | Weaning | 5.10 $^{b}$ | 13.82 $^{a,x}$ | 5.28 $^{b,x}$ | 0.532 | 0.435 | <0.001 | 0.14 | <0.001 |
| | Transition | 6.61 $^{b}$ | 5.84 $^{b,y}$ | 8.99 $^{a,y}$ | | | | | |
| Isobutyrate | Weaning | 1.52 $^{a,x}$ | 1.03 $^{b,x}$ | 0.38 $^{c,y}$ | 0.070 | 0.056 | 0.002 | <0.001 | <0.001 |
| | Transition | 0.59 $^{b,y}$ | 0.50 $^{b,y}$ | 0.86 $^{a,x}$ | | | | | |
| Valerate | Weaning | 0.85 $^{b,y}$ | 0.87 $^{b,y}$ | 1.88 $^{a,y}$ | 0.203 | 0.149 | 0.03 | <0.001 | 0.030 |
| | Transition | 3.91 $^{a,x}$ | 3.07 $^{b,x}$ | 3.47 $^{ab,x}$ | | | | | |
| Isovalerate | Weaning | 2.05 $^{a,x}$ | 1.10 $^{a}$ | 0.72 $^{b,y}$ | 0.136 | 0.102 | 0.07 | 0.05 | <0.001 |
| | Transition | 0.62 $^{b,y}$ | 0.83 $^{ab}$ | 1.27 $^{a,x}$ | | | | | |
| Ratio A:P | Weaning | 5.35 $^{a,x}$ | 3.97 $^{b,x}$ | 2.70 $^{c,x}$ | 0.227 | 0.185 | <0.001 | <0.001 | 0.003 |
| | Transition | 1.25 $^{y}$ | 1.34 $^{y}$ | 1.10 $^{y}$ | | | | | |

[1]During the transition phase all calves received a commercial compound feed plus barley straw *ad libitum.* [2] Standard error of the mean for comparison among diets. [3] Standard error of the mean for comparison between day $^{a, b, c}$ Means with different letters within a sampling day differ significantly among calf diets (P<0.05). $^{x, y}$ Means with different letters within a diet differ significantly between sampling days (P<0.05).

**Blood metabolites**

The concentrations of the different metabolites are presented in Figure 4.2. Breed and the interaction between breed and age were not significant, except in the case of urea. Glucose (P<0.001) and NEFA (P=0.02) concentrations were only affected by age, generally decreasing and increasing across the study, respectively. Urea concentrations were affected by the interactions between pre-weaning diet and age (P<0.001; Figure 4.2), and between breed and age (P<0.01).

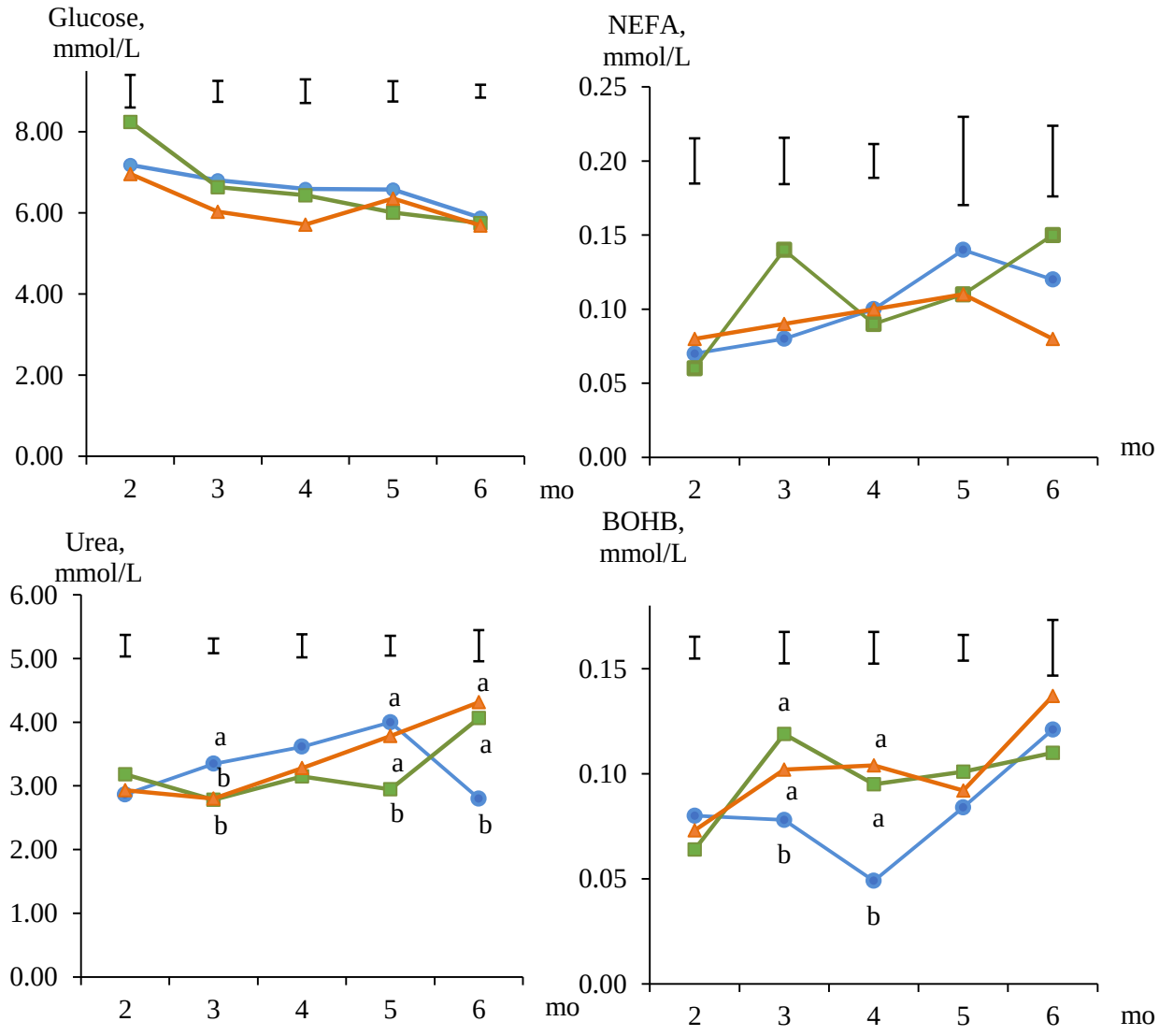


**Figure 4.2** Blood concentrations of glucose, non-esterified fatty acids (NEFA), urea and β – hydroxybutyrate (BHOB; mmol/L) in beef calves according to the pre-weaning diet (●: milk only; ■: milk plus hay; ▲: milk plus concentrate) from month 2 until the end of the study at 6 months (mo). [a,b] Means at a given month with different superscripts differ significantly (P<0.05). Upper bars show the standard error of means.

In this sense, PM calves showed a higher urea concentration than PI calves at the end of transition (4.24 *vs.* 3.21 mmol/L; P<0.01). β-hydroxybutyrate concentration was also affected by the interaction between pre-weaning diet and calf age (P<0.001; Figure 4.2).

**Economic performance**

Feeding costs at the end of weaning differed among diets (P<0.001), with higher values for MC, intermediate for MH, and nil for MO calves (Table 4.6). The sale price, dependent on weaning LW, differed among diets and ranked MC>MH>MO (P<0.01). The economic margin was higher for MH and MC calves than for MO calves (P<0.01). In this phase, feeding costs did not differ between breeds but calf sale price (P<0.001) and economic margin (P<0.01) were higher for PM than for PI calves due to differences in LW at weaning.

**Table 4.6** Economic performance (euros, €) at weaning (at 5 months of age) and at the end of the transition phase[1] (at 6 months of age) according to the pre-weaning diet (Milk only: MO; Milk plus hay: MH; Milk plus concentrate: MC) and the breed (Parda de Montaña: PM; Pirenaica: PI).

| | Diet (D) | | | | Breed (B) | | | P-value[2] | |
|---|---|---|---|---|---|---|---|---|---|
| | MO | MH | MC | SEM | PM | PI | SEM | D | B |
| Weaning | | | | | | | | | |
| Feeding costs | 0$^{c}$ | 18$^{b}$ | 54$^{a}$ | 3.4 | 27 | 21 | 2.3 | <0.001 | 0.16 |
| Calf sale price | 389$^{c}$ | 456$^{b}$ | 488$^{a}$ | 7.9 | 464$^{s}$ | 425$^{t}$ | 5.3 | <0.01 | <0.001 |
| Economic margin | 389$^{b}$ | 438 $^{a}$ | 434$^{a}$ | 8.4 | 437$^{s}$ | 404$^{t}$ | 5.6 | <0.01 | <0.01 |
| Transition | | | | | | | | | |
| Feeding costs | 29$^{a}$ | 41$^{b}$ | 42$^{b}$ | 2.0 | 41$^{s}$ | 34$^{t}$ | 1.3 | <0.01 | 0.012 |
| Calf sale price | 439$^{c}$ | 512$^{b}$ | 554$^{a}$ | 10.0 | 524$^{s}$ | 479$^{t}$ | 6.6 | <0.01 | <0.001 |
| Economic margin[3] | 20$^{b}$ | 14$^{c}$ | 25$^{a}$ | 1.4 | 19 | 20 | 1.0 | <0.01 | 0.44 |

[1] During the transition phase, all calves received a commercial compound feed plus barley straw *ad libitum*. [2] The interaction between calf diet and breed was not significant in any case.
[3] Difference between calf sale price at the end of the study minus calf purchase price at weaning and feeding costs during transition.
[a, b, c:] Means with different letters within a phase differ significantly among calf diets (P<0.05).
[s, t:] Means with different letters within a phase differ significantly between breeds (P<0.05).

At the end of the transition phase, feeding costs were lower for MO than for MC and MH calves, with no differences between these latter. Calf sale prices differed according to LW at the end of this phase, and the economic margin was lower for MH, intermediate for MO and higher for MC calves (P<0.01). Parda de Montaña calves had higher feeding costs but also higher sale price than PI calves (P<0.001). Therefore, the

economic margin did not differ between breeds.

## Discussion

### Calf performance

Calves in the present study were managed under a restricted suckling since it is the common practice in the mountain areas. One may think that performance of unsupplemented calves would be enhanced if they were allowed *ad libitum* access to their dams. However, when cows are fed according to their nutritional requirements, as is the case of the present study, the gains of unsupplemented, milk-fed calves are independent of nursing frequency (Álvarez-Rodríguez et al., 2009). On the contrary, restricted suckling leads to lower gains than *ad libitum* access to dams when cows are underfed (Álvarez-Rodríguez et al., 2009), which was not the case in the current experiment.

Providing solid supplements from the third month of the suckling period improved performance at weaning. Despite the same DMI of solid feeds in MH and MC supplemented calves, differences in ADG were probably due to the different energy density of the diets. In addition, the similar LW of these two groups at weaning was probably due to the higher gut fill in MH animals (Jahn et al., 1970). Improvement of performance at weaning by pre-weaning supplementation has been reported previously (Myers et al., 1999; Blanco et al., 2008). However, the effect when animals are subsequently fed a fattening ration in a feedlot is not clear. In studies with PM calves in similar conditions to those in the present study (Casasús et al., 2001; Blanco et al., 2008), no effect of pre-weaning supplementation on feedlot gains was observed. However, differences in LW at the end of suckling period affected the length of the fattening period, which is in contrast with the results of Myers et al. (1999). Our data show that supplemented animals were 26 % (MH) to 40% (MC) heavier than non-supplemented animals at the end of the trial.

The lack of an effect of supplementation on milk intake, and of the nature of the solid feed on DMI, which increased as calves aged, could be attributed to the increased capacity of the gastro intestinal tract to process and ferment higher amounts of solid feed with supplementation (Baldwin et al., 2004). Khan et al. (2011a) also stated that voluntary feed intake in calves after weaning is influenced by their digestive capacity. In the current study, supplemented calves reached a higher intake of concentrate in the transition, although in terms of intake per kg of metabolic weight there were no differences.

Concerning feed efficiency, our results agree with those of Faulkner et al. (1994) who reported that supplemented calves converted energy less efficiently that those fed only on milk. In the current study, hay-supplemented calves showed the lowest feed efficiency. Blaxter and Wainman (1964) reported that utilization of ME for tissue gain was lower for all-roughage diets due to their higher energy cost of digestion and metabolism.

During the pre-experimental phase, the milk yield was not measured, but Casasús (1998) observed that PI cows had lower milk yield than PM cows (7.57 *vs.* 9.87 kg/d; $P<0.001$) that was compensated with higher ($P<0.01$) fat (4.06% *vs.* 3.31%), lactose (4.86% *vs.* 4.27%), and non-fat solids (9.49% *vs.* 8.90%) hence allowing similar growth in both breeds. During the treatment phase, the tendency for lower solid feed intake in PI calves together with the lower milk intake led to lower LW and ADG. During transition, there were no differences in ADG between breeds but the higher intake per kg of metabolic weight of PM during the last two weeks resulted in higher LW of those animals.

**Rumen fermentation**

The transition from the pre-weaning diets to the fattening concentrate resulted in a marked decrease in ruminal pH in those animals that had not received concentrate during the pre-weaning period, whereas MC animals showed similar rumen pH between weaning and the end of transition. It is well known that concentrate feeding enhances the rumen epithelial proliferation as well as the density and the absorption capacity of the rumen papillae (Shen et al., 2004; Sun et al., 2018) because it increases the abundance of gene transcripts related to epithelial proliferation and absorption (Sun et al., 2018). Therefore, the similar rumen pH between periods together with the lower TVFA concentration in MC calves might indicate an increased absorption of the reticulorumen as a result of a higher absorptive capacity of the rumen epithelia. Increased absorption of VFA has been shown to decrease the susceptibility to ruminal acidosis (Schwaiger et al., 2013); hence, MC calves might have developed a protective response to high-grain feeding. Concerning the effect of pre-weaning diet on rumen TVFA concentration at weaning, MO calves showed lower values than supplemented animals. Although in milk-fed calves the closure of the oesophageal groove enhances the passage of liquid into the abomasum, Suárez et al. (2007) observed in veal calves that 21 to 35% of the ingested milk replacer can enter the rumen and, consequently, milk could constitute the first substrate for fermentation. In the present study, the VFA concentration, the high molar proportions of branched-chain

VFA and the presence of a high concentration of ammonia in the rumen liquor of MO calves suggest fermentation of milk in rumen.

At weaning, MC calves had the lowest ammonia concentration in spite of the higher arrival of dietary protein in the rumen. This could be the consequence of an increased absorption of ammonia enhanced by the development of papillae, a reduction of deamination and enhanced microbial capture of released amino acids (Agle et al., 2010), and (or) an increased capture of released ammonia by microorganisms for protein synthesis (Rey et al., 2012). Ammonia concentration decreased at the end of the transition, which may indicate an improved utilization by rumen microorganisms (Anderson et al., 1987) and (or) an increased absorption across a more mature rumen epithelium.

Molar proportions of valerate usually increase as a result of increased carbohydrate and protein in the rumen (Anderson et al., 1987) and, in this sense, MC calves showed the highest concentrations before weaning. In addition, an increase of valerate was observed in all groups in the transition compared to pre-weaning period. Molar proportion of propionate, which was higher in MC calves before weaning, increased in the transition in all groups, resulting in a decline of the ratio A:P. Low A:P ratio (less than 2) generally reflects intake of high-grain diets by young calves (Anderson et al., 1987).

**Blood metabolites**

Glucose concentration decreased as calves grew older indicating a shift in the source of nutrients (Baldwin et al., 2004). Calves on MC showed numerically lower glucose concentration on third and fourth months despite the intake of starter concentrate. By contrast, Rodríguez-Sánchez et al. (2015) found higher glucose concentrations, before the morning feeding, in suckling calves fed a high-concentrate *vs.* a milk-only diet from the beginning of the suckling period. They attributed those differences to the starter intake, which increased the propionate available in the rumen from ruminal fermentation and hence gluconeogenesis. Although similar animals were used in our experiment (PM), and concentrate intake was similar (1.44 kg/d in the present work *vs.* 1.23 kg/d from three to six months of age in the assay by Rodríguez-Sánchez et al. (2015)), it must be taken into account that, in our experiment, access to concentrate was allowed only from the third month of lactation, and that its composition was different.

Calves on MO showed the highest urea concentrations from the third month to the end of the suckling period in spite of the higher intake of protein in the supplemented

groups. A likely explanation could be the large quantity of ammonia in rumen liquor, as a consequence of milk passing into the rumen, which would lead to a high uptake of ammonia through the rumen wall and its conversion into urea in the liver. In the transition phase, differences among groups could be mostly because of differences in concentrate intake. Concerning the breed effect, differences were mainly due to the different intake of protein.

Concentrations of NEFA were unaffected by pre-weaning diet. It could have been expected that MO calves showed higher NEFA concentrations at weaning, which would suggest a negative energy balance and fat mobilization attributable to a decline in dam's yield of milk. However, the positive ADG values indicated that all animals remained in an anabolic state throughout the experiment.

ß-hydroxybutyrate increased with age regardless of pre-weaning diet as shown by Coverdale et al. (2004). Non-supplemented calves had the lowest concentrations of BOHB in months three and four of the suckling period, which may be due a lower rumen development. Similar concentrations of BOHB in hay- or concentrate-supplemented animals may indicate that the rumen epithelium was equally efficient in converting butyrate to BOHB (Khan et al., 2011b). By contrast, at weaning at five months, all groups showed similar concentrations regardless of diet. In supplemented animals, solid intake may have led to an increased metabolic activity of ruminal epithelium, increasing BOHB levels in plasma, especially in concentrate-supplemented animals (Quigley et al., 1992).

**Economic performance**

Pre-weaning supplementation improved economic performance at weaning, as in other studies (Blanco et al., 2008; Mollenhorst et al., 2016). In the current scenario, with a restricted suckling regimen, offering supplements resulted in higher economic profitability regardless the type of supplement, as it led to higher weaning weights and better economic return. Therefore, pre-weaning supplementation would be advisable for producers who sell calves at weaning. At the end of the transition, the lower feed efficiency of MH calves resulted in an improved economic margin for MO and MC calves. Consequently, supplementation with concentrates was the best option whereas supplementation with hay was the least advisable practice if the animals were to be moved to a feedlot after weaning. Nevertheless, pre-weaning supplementation with hay could be interesting depending on its market price or if producers have their own forage crops. Our

results are in accordance with Bernués et al. (2001) who found a higher economic profitability when concentrates were offered in closed-cycle or finishing farms.

## Conclusions

Offering solid feeds during the suckling period improves performance and economic income, especially if calves are sold at weaning. Solid feed supplementation not only increases calf growth but also provides better rumen development, which is essential for a smooth transition to the fattening diets. Concentrate supplementation could be an interesting option for weaner producers and for closed-cycle farms, whereas hay supplementation could be feasible depending on its cost.

# CHAPTER 5

# EXPERIMENT 2

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Effects of partial substitution of barley with maize and sugar beet pulp on growth performance, rumen fermentation and microbial diversity shift of beef calves during transition from a milk and pasture regimen to a high-concentrate diet. Livestock Science. 2020, 238: 10471. doi: 10.1016/j.livsci.2020.10407.

# CHAPTER 5: EXPERIMENT 2

**Effects of partial substitution of barley with maize and sugar beet pulp on growth performance, rumen fermentation and microbial diversity shift of beef calves during transition from a milk and pasture regimen to a high-concentrate diet.**

## Abstract

The effects of partial substitution of barley with maize and sugar beet pulp (BP) on animal performance, rumen fermentation and rumen microbial populations were evaluated in thirty-nine newly weaned calves transitioned from a milk and pasture regime to a high-concentrate ration. Diets were: a barley-based growing diet (C); and two diets in which barley was partially replaced with maize and 10% BP (BP10) or 22% BP (BP22) on as fed basis. Wheat straw was offered *ad libitum* in all cases. Three adaptation protocols were followed: in Protocol 1 animals were abruptly shifted to the C diet, whereas calves in Protocols 2 and 3 were gradually adapted and received diets BP10 and BP22, respectively, for 10 days; thereafter a 50:50 mixture of their diet and diet C until day 14, and finally, from day 15 until the end of the experiment they received diet C. All animals were slaughtered at a target LW of *ca*. 500 kg. The experiment had then three phases (Ph): Ph1 (0-10d), Ph2 (11-14d) and Ph3 (15 d-slaughter). Concentrate intake and proportion of straw in the diet were affected ($P<0.01$) by the interaction dietary protocol by phase, showing animals in Protocol 3 the lowest concentrate intake and the highest straw proportion in Ph2. Daily gains, LW at the end of each phase, dressing percentage (59 ± 0.4%) and total concentrate intake throughout the experiment (995 ± 21.3 kg DM) were similar ($P>0.05$) among adaptation protocols. Daily rumen pH, and concentrations of lactate and VFA were not affected by the feeding protocol. At the end of the trial, protozoa persisted in the rumen of all calves with an average concentration of 5.89 log cells/mL. Diversity dropped throughout the study; most protozoa belonged to genera *Entodinium* (97.5%) and *Isotricha* (1.7%), with minor presence of *Dasytricha* (0.05%), *Epidinium* (0.43%) and Subfamily *Diplodiniinae* (0.23%). The adaptation protocol had no effect on rumen bacterial population or diversity; however, both were affected by day of sampling indicating a shift in bacterial community in response to the concentrate inclusion. Although diversity dropped with concentrate inclusion, there was an increase after day 10. In conclusion, the application of different protocols to adapt beef cattle to high-concentrate diets had no effect on animal performance and rumen fermentation,

which were not negatively affected by the abrupt transition to the high-concentrate diet. This suggests that calves might cope with those feeding conditions probably due to the microbial adaptions to a new rumen environment.

## Introduction

Conventional beef production in Spain is commonly carried out under intensive feeding conditions. Beef calves are reared with their mothers on pasture and allowed to suckle freely for 5-7 months of age. After weaning, these calves face a feeding challenge since they are immediately shifted to a cereal-based high-concentrate diet. Under farm conditions, the transition period involves the first 14-21 days after the calves arrive in the feedlot where they receive a high-grain adaptation compound feed to stimulate the rumen papillae development and to acclimatise to the fattening concentrate (de Blas et al., 2008). Adaptation diets containing high proportions of starch are rapidly fermented in the rumen resulting in high VFA and/or lactate concentrations, which contribute to an increased risk of ARA or SARA (Nagaraja and Titgemeyer, 2007). The transition is a challenging period for the rumen microbiota since it undergoes through an important dietary shift, which is known to be one of the major driving factors modifying rumen bacterial composition (Weimer, 2015). A gradual transition (Bevans et al., 2005), the use of feedstuffs with different rate of starch fermentation, and the substitution of cereals with high-energy by-products have been proposed as viable strategies to reduce the incidence of acidosis (González et al., 2012), and to improve the rumen health during this period, which affects animal health and production during the whole feeding cycle (Brown et al., 2006). The choice of the main cereal determines the acidogenic power of the diet (González et al., 2012). In this sense, maize grain has a lower rate and extent of ruminal degradation than barley grain (Herrera-Saldana et al., 1990). Previous studies have addressed the effect of replacing barley with maize as the prevailing cereal on rumen fermentation characteristics (Khorasani et al., 2001; Gimeno et al., 2015) but results are inconsistent. On the other hand, sugar beet pulp is a non-forage fibre source rich in NDF and pectins (*ca.* 25% of DM; FEDNA, 2010) which chemical structure confers a high buffering capacity that can alleviate the over-acidification in the rumen. Pectin fermentation, although extensive and rapid, produces little or no lactate and gives an acetate/propionate ratio higher than starch (Van Soest et al., 1991). *In vitro* studies have reported that BP possesses a stabilizing effect of rumen pH and an acetogenic capacity (Marounek et al., 1985; Amanzougarene et al., 2017a), which has been supported by *in vivo* studies with dairy cows (Mahjoubi et

al., 2009) and beef steers (Mojtahedi and Danesh Mesgaran, 2011). Little is known about the effect of partial substitution of barley with maize and BP in adaptation diets to a high-concentrate regimen in beef cattle. This practice may be beneficial in raising and stabilizing the rumen pH during the transition period, which comprises a substantial risk of reaching low reticulo-ruminal pH for newly weaned calves that are not adapted to high-grain diets. On the other hand, although previous research explored the rumen microbiome dynamics during transition from forage to concentrate rations (Petri et al., 2013; Fernando et al., 2010), or during an acidotic challenge (Petri et al., 2013; Nagata et al., 2018), there is no much information on weaned calves transitioned from a milk/grass regime to a high-concentrate diet. We hypothesized that partially replacing barley with maize and BP in the adaptation diet of beef calves during transition from milk and pasture to a conventional high-concentrate ration would attenuate ruminal fermentation and reduce the risk of acidotic conditions during this critical period. Therefore, the objectives of the current experiment were to study the effect of the partial substitution of barley grain with maize and BP in the adaptation diet of beef calves during transition from milk and pasture to a conventional high-concentrate ration on feed intake and daily gains, rumen fermentation, and rumen microbial composition of beef calves.

## Materials and Methods

### Animals

Thirty-nine autumn-born Limousine crossbred male calves reared with their dams on pasture, and allowed to suckle freely while they were grazing, were used. Calves (250 ± SD 24.3 kg) were abruptly weaned and transported to the Servicio de Experimentación Animal (University of Zaragoza) where they were individually housed in pens (1.7 x 3.4 m) provided with slatted concrete floor, automatic water dispenser and two separate troughs for concentrate and wheat straw. From the arrival at the facilities, calves were offered fescue (*Festuca pratensis*) hay (g/kg: organic matter, OM 904; crude protein, CP 128; neutral detergent fibre, NDF 654; acid detergent fibre, ADF 322; lignin 58) *ad libitum* for their adaptation to the farm environmental conditions. One week after their arrival, twelve calves were fitted in the dorsal sac of the rumen with a 150-mm long, 15-mm internal diameter (i.d.) permanent cannula, and allowed two more weeks for recovery from surgery. Then, all animals were blocked by LW and randomly assigned to one of the three experimental diets described below (n=thirteen animals, including four cannulated, per experimental diet, ensuring homogeneous average LW and SD per

group). Animals began the experiment with a LW of 258 kg (± 24.1 kg), and an age of 215 d (± 35.4 d). Animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. The care and management of animals were performed according to the Spanish Policy for Animal Protection RD 53/2013, which meets the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes.

**Diets and experimental procedures**

Three isoenergetic and isoproteic diets were used: a control barley-based growing compound feed (C; including 9.3 g palm oil/kg and 57 g corn gluten feed/kg), and two concentrates formulated with an inclusion of either 10% (BP10; including 54 g corn gluten feed/kg, but no palm oil) or 22% (BP22; including 29.5 g palm oil/kg and 92 g corn gluten feed/kg) of dried sugar beet pulp on an as fed basis (Table 5.1). Diets were formulated taking into account the different starch degradation rates of the ingredients, and starch/NDF ratios. In this sense, 66% of barley was replaced in the BP10 diet for 49.5% maize and 16.5% BP. In the BP22 diet, there was a 75% barley replacement (30% with maize, 37% BP, and the rest with other ingredients: corn gluten feed, palm oil, etc.). Three adaptation protocols were followed (Figure 5.1): in Protocol 1 animals were abruptly shifted from milk and pasture to the C diet. Animals in Protocol 2 received diet BP10 and those in Protocol 3 diet BP22 for 10 days, then a mixture of 50:50 of their diet and diet C until day 14, and finally, from day 15 until the end of the study all calves received diet C. All animals were slaughtered at a target LW of *ca*. 500 kg. The experiment was therefore divided into three phases (Ph): Ph1 from days 0 to 10, Ph2 from day 11 to day 14, and Ph3 from day 15 until slaughter on day 154. Hence, the transition period involved the first 14 d (Ph1 and Ph2).

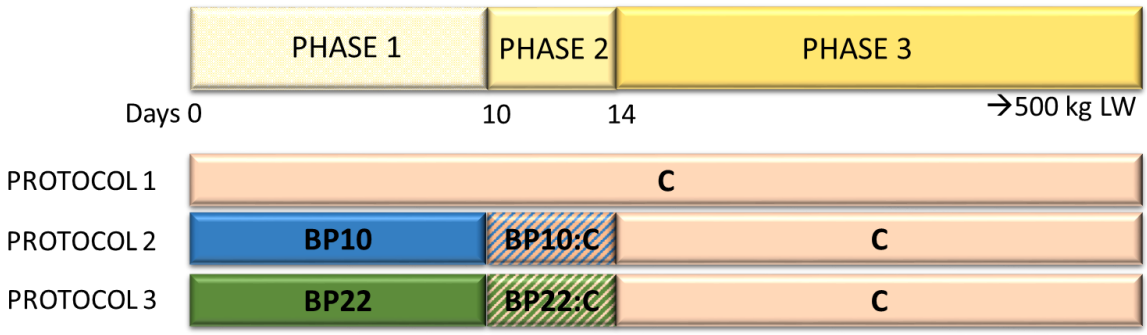



**Figure 5.1** Scheme of the experimental design.

Animals were weighed weekly prior to feed distribution. The ADG was calculated as the regression coefficient of individual LW on time. Concentrates (ground to 3.5 mm) were offered *ad libitum* once a day at 09:00 h, and the amount was daily adjusted to ensure at least 10% of refusals. Wheat straw, chopped coarsely to approximately 10 cm in length, was offered three times daily (9:00 h, 12:00 h and 18:00 h) to ensure *ad libitum* access. The initial amount of concentrate offered per animal was 8 kg fresh matter. The amounts of concentrate and straw offered to each animal were recorded daily throughout the experiment. Straw intake was only recorded throughout the transition period (until the end of Ph2). Representative samples of each compound feed and straw were taken weekly, pooled at the end of the experiment and analysed for chemical composition (Table 5.1). Concentrate and straw refusals (these latter only during transition) were collected daily and weekly, respectively, pooled on an animal basis and weighed weekly. Representative samples of offered feeds and refusals were dried at 104ºC for 24 h, to determine individual dry matter intake (DMI). Concentrate conversion ratio (CCR) was calculated from total amount of concentrate DMI and weight gained by the animals throughout the study. The dressing percentage was calculated as the relationship between cold carcass weight and LW at day 154 of the trial.

**Table 5.1** Ingredient and nutrient composition of the experimental diets.

| | Diets | | | |
|---|---|---|---|---|
| Ingredients (as fed basis), g/kg | C | BP10 | BP22 | Wheat straw |
| Barley | 590.0 | 200.0 | 150.0 | |
| Maize | 150.5 | 466.1 | 333.4 | |
| Sugar beet pulp | - | 102.3 | 220.0 | |
| Corn gluten feed (200gCP/ kg fresh matter) | 57.0 | 53.9 | 91.9 | |
| Soy meal (470 g CP/ kg fresh matter) | 172.1 | 163.7 | 161.8 | |
| Palm oil | 9.3 | - | 29.5 | |
| Urea | 0.6 | - | - | |
| Calcium carbonate | 8.5 | 8.2 | 2.0 | |
| Dicalcium phosphate | 5.0 | - | 6.4 | |
| NaCl | 5.0 | 3.8 | 3.0 | |
| Vitamin-mineral premix[1] | 2.0 | 2.0 | 2.0 | |
| Nutrient composition (g/kg DM) | | | | |
| OM | 947.6 | 951.8 | 948.4 | 956.1 |
| CP | 171.2 | 174.0 | 170.9 | 18.2 |
| EE | 29.7 | 25.3 | 53.5 | 11.0 |
| Starch | 449.0 | 457.0 | 298.0 | 5.0 |
| NDF | 150.6 | 148.8 | 192.9 | 749.0 |
| ADF | 48.7 | 61.0 | 89.0 | 437.0 |
| ME (MJ/kg DM) | 11.41 | 11.45 | 11.75 | 6.40 |
| Lignin (sa) | 5.2 | 6.8 | 8.0 | 50.3 |

[1] Vitamin-mineral premix declared composition (per kg): 4x106 IU vitamin A, 0.8x106 IU vitamin D3, 5x103 IU alpha-tocopherol, 100 g sepiolite, 150 mg etoxiquin, 10 g Mn oxide, 7.5 g Fe carbonate, 20 g Zn oxide, 250 mg K-iodate, 300 mg Co, 2.5 g Cu, and 500 mg Na selenite, 10 mg antioxidants. OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ME, metabolisable energy; Lignin (sa), acid detergent lignin.

The rate of intake of both concentrate and straw was measured on days 9 (Ph1), 14 (Ph2) and 20 (Ph3) of the experiment. The rate of intake of concentrate was recorded at two-hour intervals from 9:00 to 21:00 h, and from 21:00 to 9:00 h of the next day. Rate of intake of straw was recorded every four hours from 9:00 to 21:00 h and from 21:00 to 09:00 h of the next day. Trying to reduce the effect of a likely contamination of the refusals with saliva, a correction of the DM actually ingested in each measuring interval was carried out on the following basis: the difference between the feedstuff DM content and that of the refusals (both measured in the week corresponding to each pattern of intake measurement) was divided by 24, and the result multiplied by the number of hours in each measuring interval. The value obtained for each interval was assumed to represent the decrement to be applied to the DM content of the concentrate and straw. When the daily sum of the DM theoretically consumed in each interval applying this correction differed more than ± 5% compared to the recorded daily DMI, all the values obtained for the

different intervals of that day were considered as missing data. When the DMI recorded at a determined interval was negative, the value was considered a missing datum.

**Rumen sampling**

Rumen fluid samples were obtained at 0 h (before feeding) on day 0, and at 0, 3, 6 and 9 h after feeding at the end of Ph1 and Ph2 (days 10 and 14), and on days 21, 30 and 42 in Ph3. Since on day 0 we only collected rumen fluid at 0 h, two different statistical analyses were conducted: one with results at 0 h over all sampling days to study the effect of the concentrate inclusion, and another with all days and sampling intervals except d 0 to study the fermentation pattern of the different diets. About 200 mL were removed from the rumen of each animal on each sampling time, using a customized vacuum device connected to a 0.6 cm i.d. semi-rigid tube with 2-mm pores. Representative samples were taken moving the tube in all directions inside the rumen while sampling. A 5-mL subsample was taken and immediately frozen in liquid nitrogen at -80ºC until molecular analyses of bacterial DNA was carried out. Another subsample of 2 mL (only taken at 0 and 6 h after feeding) was pipetted with a wide mouth tip (3 mm) into a tube containing 2 mL of 18.5% formaldehyde for quantification of the rumen protozoa. Then, rumen fluid was strained through a 1-mm pore size metal mesh sieve, and pH was immediately measured using a portable pH-meter (model Seven2GO, Mettler-Toledo AG, Schwerzenbach, Switzerland). Rumen fluid aliquots were taken, in duplicate, for ammonia, lactate and VFA analysis. For the determination of ammonia, two samples of 4 mL of 0.2 N HCl were added to 4 mL of filtered rumen fluid. Samples for VFA analysis were prepared by adding 1 mL deproteising solution (2% (v/v) of orthophosphoric acid and 0.2% (w/v) of 4-methyl valeric acid) to 4 mL of strained rumen contents. The same amount of rumen liquor (4 mL) was taken for the determination of lactate. Samples obtained each day were frozen (-20 ºC) until analysis.

**Histological examination of the digestive tract**

A subset of four non-cannulated animals of each treatment was chosen randomly and euthanized on day 20 of the experiment following the European Guidelines for Animal Welfare (Directive 86/609 EEC) with an intravenous injection of pentobarbital (Dolethal, Vetoquinol, Lure, France). A total of twelve tissue samples of 1 x 3 cm of rumen (two from dorsal sac, two from pillars and three from the ventral sac), abomasum (cranial and caudal), duodenum, ileum and jejunum of each animal was harvested,

emptied, and rinsed with cold water. Similarly, the same number of samples were taken at the slaughterhouse of the rest of the animals during the sacrifice day. For histological examination, tissue samples were excised and preserved by immersion in individual containers with 10% phosphate-buffered formalin, routinely processed and embedded in paraffin. Sections 5 μm thick were stained with haematoxylin and eosin and were observed through a microscope. The most relevant histopathological findings were indicated.

**Chemical and microbiological analyses**

Samples of the different feedstuffs were ground in a hammer mill fitted with a 1-mm sieve size, and analysed following the procedures of AOAC (2005) for DM (ref 934.01), OM (ref. 942.05), CP (ref. 976.05) and ether extract (ref. 2003.05). Concentration of NDF was analysed using an Ankom 200 Fiber Analyzer (Ankom Technology) as described by Mertens (2002), using α-amilase and sodium sulphite, and results were expressed exclusive of residual ashes. Acid detergent fibre and acid detergent lignin were analysed as described by AOAC (2005; ref. 973.18), and 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). Metabolisable energy of wheat straw was calculated from ADF content according to the equation proposed by Mertens (1983) (NE=2.469-0.0351*%ADF; $R^2$=0.849; ME = NE/0.61), whereas ME of concentrates was calculated taking into account the ingredient composition and ME values from FEDNA (2010).

Concentration of ammonia in rumen samples was determined colorimetrically following the method described by Chaney and Marbach (1962), and that of VFA by gas chromatography in an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid, Spain) fitted with a capillary column (Model HP-FFAP polyethylene glycol TPA-treated, 30m x530μm i.d.x1μm film thickness). Total lactate concentration was measured using the colorimetric method proposed by Barker and Summerson (1941).

Rumen samples taken at 6 h after feeding on days 10, 14, 30 and 42 of the experiment were chosen for protozoal quantification by optical observation using a 10x eyepiece with x10 and x40 objectives through a microscope (Axiolab, Carl Zeiss Jena, Germany) in a Sedgewick-Rafter counting chamber following the procedure of Dehority (1993), except for those cases with extremely low protozoal numbers, when the entire

chamber was counted. During each counting, the numbers of different genera in the protozoal population were recorded, identified and grouped into the genera *Isotricha* and *Dasytricha* from the Family *Isotrichidae*; genus *Epidinium* from the Subfamily *Ophryoscolecinae*; genus *Entodinium* from the Subfamily *Entodiniinae*; and genera *Diplodinium*, *Metadinium*, *Eudiplodinium*, *Enoploplastron*, *Ostracodinium* and *Polyplastron* from the Subfamily *Diplodiniinae*. Total protozoal and groups concentration for each calf were calculated and transformed into logarithmic basis ($log_{10}$/mL) to assume normality before being subjected to statistical analysis; however, for a better interpretation, relative abundances of different protozoal groups are presented as percentage together with the standard error. Absence of total protozoa or any protozoal group was considered as zero in the statistical analyses.

**DNA extraction and Ion Torrent sequencing**

Samples taken before feeding (0h) on days 0, 10, 14, 21 and 30 were chosen for sequencing analyses using Ion Torrent Next Generation Sequencing. Samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The microbial 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 maximize bacterial cell lysis. Concentration and purity of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). Sequencing of the 16S rRNA gene was conducted following the procedure described by de la Fuente et al. (2014) and Schauf et al. (2018). Briefly, the bacterial V1-V3 hypervariable region was amplified by PCR using barcoded fusion primer pairs 27F and 338R (Wang et al., 2014). Sequencing of the PCR products was performed in the Ion Torrent Personal Genome Machine system (Life Technologies, Carlsbad, CA, US) using the Ion Personal Genome Machine Sequencing 200 kit v2 (Life Technologies, Carlsbad, CA). Following sequencing, data were combined and sample identification numbers assigned to multiplexed reads using Ion Reporter™ 5.10. Software (Thermo Fisher Scientific). Sequencing amplicon reads were subjected to trimming, denoising, and chimera removal and clustered into Operational Taxonomic Units at 97% identity using UPARSE pipeline (Edgar, 2013). Reads were subjected to quality filtering (quality score of 20 in a 1 to 40 scale) and trimmed at a maximum length of 250 bp. Taxonomic assignation of 16S rRNA sequences was established by comparison against the Ribosomal Data Project II database

(Cole et al., 2003), considering a bootstrap value of 0.80 for annotation, leaving successive taxon levels as unclassified.

To maximize the comparability across samples, the number of reads was manually normalised to the sample with the lowest number of reads (85,442). Number of reads of each microbial taxon were $\log_{10}$-transformed (nº of reads + 1) prior to statistical analysis to assume normality; however, for a better interpretation, relative abundances of different bacterial taxa are presented as percentage together with the standard error. Two measures of diversity were calculated: Shannon index and Richness according to the number of observed genera using R software (v.3.5.3; R Core Team, 2014).

**Statistical analyses**

Data of final LW of each phase, concentrate DMI (CDMI), total CDMI, proportion of wheat straw in the ration, ADG, CCR and dressing percentage were corrected by covariance using the initial LW and age as covariates. Concentrate intake, proportion of straw in the ration, ME intake, ADG and LW at the end of each phase were analysed as repeated measures using the PROC MIXED of SAS (v. 9.4; SAS Inst. Inc., Cary NC, US) with protocol, phase and their interactions as fixed effects, and animal as random. For total CDMI, CCR and dressing percentage, the effect of protocol was the fixed effect, and animal the random effect. Pattern of intake of concentrate and straw, rumen fermentation variables, rumen protozoa concentration, concentration of the different bacterial taxa (excluding those with relative abundances lower than 0.1% of total sequences), different ratios between phyla, and diversity indexes were analysed as repeated measures with the MIXED procedure with protocol, sampling time interval nested within day (when applicable), sampling day and all possible interactions as fixed effects, and animal as random. Sampling time nested within day or sampling day was used as repeated measure, as appropriate. For both bacterial and protozoal concentrations, treatment means were compared under a conservative Bonferroni adjustment. The variance-covariance structure was selected based on the lowest Akaike information criterion. For sequencing data, multivariate statistical analyses were performed using package “vegan” from the R statistical program. To determine the impact of the diet and time on overall microbial community structure, a non-parametric permutational multivariate analysis of variance (PERMANOVA) was conducted based on the Bray-Curtis dissimilarity. Non-metric multi-dimensional scaling (NMDS) plot was generated representing the Bray-Curtis distance. Spearman´s correlation coefficients were calculated to assess the relationships

between the ruminal fermentation characteristics at hour 0 and the bacterial diversity indexes, the log-transformed data of the main bacterial taxa and protozoal concentrations using R software. Only Spearman correlations with $r \geq 0.30$ or $r \leq -0.3$ and $P<0.05$ are shown (Belanche et al., 2019a). For all data, differences were considered significant if $P<0.05$, whereas differences were considered to indicate a trend to significance when $0.05 \leq P \leq 0.10$.

## Results

### Feed intake and animal performance

Concentrate intake was affected by the interaction between dietary protocol and phase ($P<0.01$; Table 5.2), and in this sense, no differences between treatments were found in Ph1 and Ph3, but in Ph2 animals calves in Protocol 3 showed the lowest values. Proportion of straw on total DMI was also affected by the interaction between adaptation protocol and phase ($P<0.01$; Table 5.2), showing calves in Protocol 3 the highest values in Ph2. Hence, the amount of ME ingested (from concentrate and straw) during transition (0-14d) was lower for animals in Protocol 3 during Ph2 (62, 67 and 52 MJ of ME for Protocols 1, 2 and 3, respectively), with no differences between protocols in Ph1 and Ph3 (significant interaction protocol x phase; $P<0.01$).

**Table 5.2** Effect of the adaptation protocol of beef calves to a high-concentrate diet[1] on concentrate and wheat straw dry matter (DM) intake (DMI), average daily gains (ADG) and live weight (LW) during transition and the fattening period.

| | | Phase 1 | Phase 2 | Phase 3 | | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | | (0-10 d) | (11-14 d) | (15 d-End) | SEM | Protocol | Phase | ProtocolxPhase |
| Concentrate intake | Protocol 1 | 4.72 | 4.91[a] | 6.75 | 0.262 | 0.53 | <0.001 | <0.01 |
| (kg DM/d) | Protocol 2 | 4.92 | 5.39[a] | 6.57 | 0.386 | | | |
| | Protocol 3 | 4.72 | 3.95[b] | 7.01 | 0.232 | | | |
| Straw intake[2] | Protocol 1 | 24 | 15[b] | - | 0.3 | 0.15 | <0.001 | <0.001 |
| (% of total DMI/d) | Protocol 2 | 24 | 14[b] | - | 0.30 | | | |
| | Protocol 3 | 22 | 24[a] | - | - | | | |
| ADG | Protocol 1 | 0.63 | 0.75 | 1.78 | 0.100 | 0.64 | <0.001 | 0.36 |
| (kg/d) | Protocol 2 | 0.66 | 0.74 | 1.43 | 0.100 | | | |
| | Protocol 3 | 0.66 | 0.69 | 1.49 | 0.101 | | | |
| LW at the end of each Phase | Protocol 1 | 268 | 279 | 507 | 5.8 | 0.29 | <0.001 | 0.70 |
| (kg) | Protocol 2 | 270 | 278 | 499 | 5.9 | | | |
| | Protocol 3 | 268 | 274 | 488 | 5.8 | | | |

[1] In Phase 1, calves received C (Protocol 1), BP10 (Protocol 2) or BP22 (Protocol 3) diets (see Table 1) from days 0 to 10; in Phase 2, animals on BP10 or BP22 groups received a mixture of 50:50 (w/w) of C and either BP10 or BP22 from day 11 to 14; Phase 3 comprised from day 15 to the end of the experiment (when animals reached *ca.* 500 kg), and all calves received diet C. [2]Straw intake was recorded only during Phases 1 and 2. [a, b] Different letters within a column indicate differences between protocols at P<0.05.

The rate of intake of concentrate, pooled on 4-h intervals for an easier comparison with results of rate of intake of straw is given in Figure 5.2, and was not affected by the adaptation protocol (P=0.64) or sampling day (P=0.55), but it was affected by time interval (P<0.001) and animals consumed 31% of the concentrate during the first 4 h. The rate of straw intake (Figure 5.2) was affected neither by dietary protocol (P=0.74) nor by sampling day (P=0.61), but it was affected by time interval (P<0.001). In this regard, the highest intake of straw was observed from 12-24 h after feeding, when calves consumed, on average, 34% of the daily straw intake. The highest total DMI was observed during the first 4 h after the morning feeding (P<0.001), when animals consumed, on average, 30% of the ration, decreasing to 19% in the following 4 h. Total CDMI at the end of the study did not differ among groups (1002, 978 and 1010 kg of DM for Protocols 1, 2 and 3, P=0.69).

Regarding daily gains, no differences were found among protocols (P=0.64; Table 5.2), and ADG increased over time (0.65, 0.73 and 1.58 kg/d in Ph1, Ph2 and Ph3, respectively, P<0.001). All groups exhibited similar (P> 0.10) LW at the end of each phase (Table 5.2), CCR (4.21 ± 0.100) as well as dressing percentage (average 59 ± 0.4%).

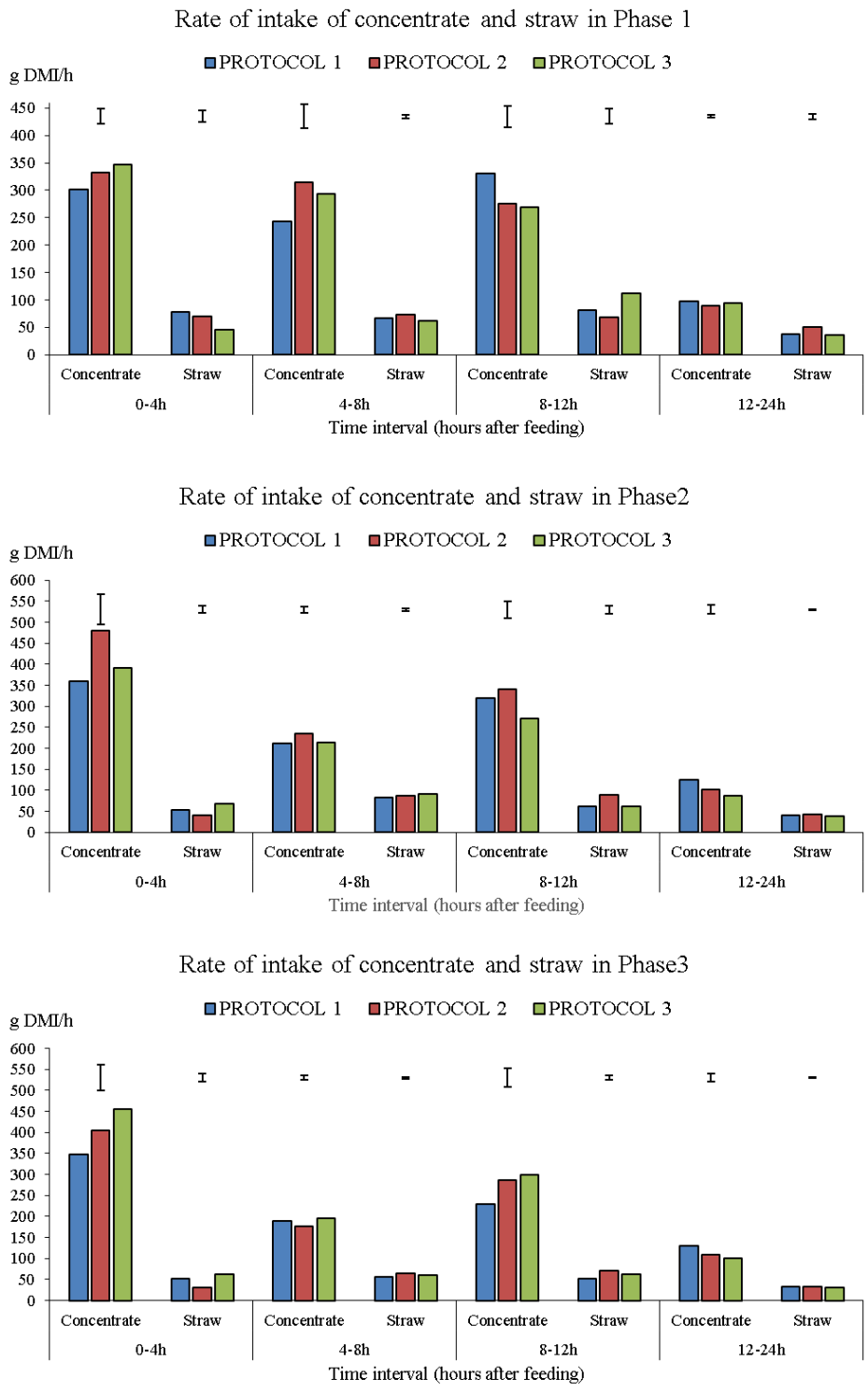



**Figure 5.2** Effect of different dietary adaptation protocols (See Table 5.2) fed during the transition period of beef calves from milk/grass to a high-concentrate diet on the rate of intake of concentrates and straw recorded on day 6 (Phase1), 14 (Phase 2) and 20 (Phase 3). Rates of intake of concentrates or straw were not affected by diet or day of sampling ($P>0.05$).

**Rumen fermentation**

Analyses of hour 0 of all sampling days showed that concentrate inclusion, regardless the diet, modified the rumen environment (Table 5.3). Compared to day 0, rumen pH decreased ($P<0.001$), lactate ($P<0.001$) and total VFA ($P<0.01$) increased, and acetate ($P<0.001$) and the acetate/propionate ratio ($P<0.05$) decreased on the following days. Shifts towards increased propionate ($P<0.05$), butyrate ($P<0.001$), valerate ($P<0.001$), and branched-chain volatile fatty acids (BCFA: sum of isovalerate and isobutyrate; $P<0.01$) were also found. Ammonia concentrations at hour 0 remained stable throughout the study ($P=0.65$).

Analysis of the hourly evolution of rumen fermentation variables (0, 3, 6 and 9 h after feeding) throughout the experiment showed that all rumen fermentation variables (except for ammonia ($P=0.06$) and lactate ($P=0.74$) concentration, and molar proportions of propionate ($P=0.08$) and valerate ($P=0.30$)) were affected ($P<0.05$) by sampling time after feeding. However, adaptation protocol or its interaction with sampling time was not significant in any case ($P>0.10$). Rumen pH decreased ($P<0.001$) to a major extent during the first 3 h after feeding, on average -0.25 units. The lowest rumen pH was always recorded at 9 h after feeding. Minimum and maximum rumen pH, and the pH variation within a day (standard deviation) were not affected by the sampling day ($P>0.10$). Sampling day affected ($P<0.05$) lactate concentration (highest at the end of transition), VFA concentration (which decreased over time) and proportions of propionate, valerate and BCFA, and rumen pH tended to differ across days ($P=0.08$). Given that the lowest pH was always recorded at 9 h after feeding, coinciding with the highest VFA concentration, rumen fermentation variables at hour 9 after feeding were analysed separately for an easier comparison of the fermentability of the diets. Rumen pH at 9 h did not differ among protocols or sampling days (Table 5.4), whereas lactate concentration was not affected by protocol but it was by sampling day, the lowest value appearing on day 35. Ammonia concentration at 9 h was highly variable among animals and tended to be affected by the interaction between protocol and sampling day ($P=0.05$; Table 5.4). Total VFA and proportions of the main VFA were not affected by the adaptation protocol but differed across sampling days (except for butyrate; Table 5.5).

**Table 5.3** Effect of concentrate inclusion on rumen pH, lactate (mmol/L), ammonia (mg/L), concentration of volatile fatty acids (VFA; mmol/L) and of the main VFA (mol/100 mol) before feeding in the rumen of beef calves subjected to different adaptation protocols[1] to a high-concentrate diet during transition (0-14 d) and the first weeks of the fattening period.

| | Days | | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 14 | 21 | 30 | 42 | SEM | Protocol (P) | Day (d) | Pxd |
| Rumen pH | 7.48$^{a}$ | 6.47$^{b}$ | 6.39$^{b}$ | 6.69$^{b}$ | 6.44$^{b}$ | 6.65$^{b}$ | 0.111 | 0.92 | <0.001 | 0.99 |
| Lactate | 0.26$^{c}$ | 0.94$^{bc}$ | 2.22$^{ab}$ | 3.77$^{a}$ | 0.50$^{c}$ | 1.06$^{bc}$ | 0.533 | 0.13 | <0.001 | 0.12 |
| Ammonia | 101 | 122 | 114 | 115 | 120 | 109 | 14.1 | 0.95 | 0.65 | 0.45 |
| Total VFA | 52$^{c}$ | 85$^{ab}$ | 127$^{a}$ | 73$^{b}$ | 99$^{ab}$ | 76$^{b}$ | 9.4 | 0.85 | <0.001 | 0.07 |
| Acetate | 72.4$^{a}$ | 52.2$^{b}$ | 66.0$^{a}$ | 51.4$^{b}$ | 54.9$^{b}$ | 50.5$^{b}$ | 2.58 | 0.34 | <0.001 | 0.78 |
| Propionate | 16.4$^{b}$ | 20.3$^{ab}$ | 16.0$^{b}$ | 19.5$^{b}$ | 21.5$^{a}$ | 23.9$^{a}$ | 1.60 | 0.43 | <0.01 | 0.65 |
| Butyrate | 6.9$^{c}$ | 18.8$^{a}$ | 12.4$^{ab}$ | 20.4$^{a}$ | 16.5$^{ab}$ | 18.7$^{a}$ | 1.72 | 0.09 | <0.001 | 0.95 |
| Valerate | 0.6$^{c}$ | 1.8$^{b}$ | 1.3$^{bc}$ | 2.3$^{ab}$ | 2.3$^{ab}$ | 3.0$^{a}$ | 0.33 | 0.29 | <0.001 | 0.85 |
| BCFA | 3.5$^{b}$ | 6.7$^{a}$ | 4.1$^{b}$ | 6.1$^{a}$ | 4.4$^{b}$ | 3.6$^{b}$ | 0.58 | 0.58 | <0.01 | 0.11 |
| Acetate/propionate ratio | 4.50$^{a}$ | 2.77$^{b}$ | 4.46$^{a}$ | 2.95$^{b}$ | 3.39$^{ab}$ | 2.29$^{b}$ | 0.504 | 0.88 | <0.001 | 0.83 |

[1] See Table 5.2.

[a, b,c] Different letters within a row indicate differences between treatments at P<0.05.

**Table 5.4** Rumen pH, and lactate (mmol/L) and ammonia (mg/L) concentration 9 h after feeding in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet during transition (0-14 d) and the first weeks of the fattening period.

| | | Protocol[1] | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | Mean | SEM[2] | SEM[3] | Protocol (P) | Day (d) | Pxd |
| Rumen pH | d 10 | 6.12 | 5.91 | 6.30 | 6.11 | 0.186 | 0.107 | 0.50 | 0.82 | 0.84 |
| | d 14 | 6.11 | 5.86 | 6.20 | 6.05 | 0.178 | 0.102 | | | |
| | d 21 | 6.42 | 6.22 | 6.07 | 6.24 | 0.251 | 0.145 | | | |
| | d 35 | 6.20 | 5.96 | 6.09 | 6.08 | 0.192 | 0.111 | | | |
| | d 42 | 5.98 | 6.21 | 6.30 | 6.16 | 0.214 | 0.123 | | | |
| Lactate (mmol/L) | d 10 | 1.06 | 1.28 | 1.31 | 1.22[a] | 0.305 | 0.176 | 0.69 | <0.001 | 0.19 |
| | d 14 | 1.31 | 0.99 | 1.31 | 1.20[a] | 0.292 | 0.152 | | | |
| | d 21 | 0.84 | 1.26 | 0.67 | 0.92[ab] | 0.387 | 0.202 | | | |
| | d 35 | 0.52 | 0.30 | 0.61 | 0.48[b] | 0.145 | 0.084 | | | |
| | d 42 | 1.21 | 1.67 | 0.95 | 1.28[a] | 0.176 | 0.101 | | | |
| Ammonia (mg/L) | d 10 | 117 | 49 | 101 | 89 | 17.2 | 9.9 | 0.21 | 0.59 | 0.052 |
| | d 14 | 63 | 105 | 100 | 89 | 9.7 | 5.6 | | | |
| | d 21 | 60 | 102 | 56 | 72 | 15.9 | 9.2 | | | |
| | d 35 | 59 | 103 | 113 | 92 | 9.5 | 5.5 | | | |
| | d 42 | 86 | 80 | 89 | 85 | 18.9 | 10.9 | | | |

[1] See Table 5.2.
[2] Standard error of the mean for comparisons between protocols within days.
[3] Standard error of the mean for comparisons between days within protocols.
[a, b] Different letters within a column indicate differences between sampling days at P<0.05.

**Table 5.5** Total volatile fatty acids (mmol/L;VFA) and main VFA concentration (mol/100 mol) at 9 h after feeding in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet during transition (0-14 d) and the first weeks of the fattening period.

| | | Protocol[1] | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Day | 1 | 2 | 3 | Mean | SEM[2] | SEM[3] | Protocol (P) | Day (d) | Pxd |
| VFA | d 10 | 116 | 113 | 99 | 109[a] | 5.8 | 10.2 | 0.54 | <0.01 | 0.42 |
| | d 14 | 124 | 105 | 135 | 121[a] | 14.9 | 25.9 | | | |
| | d 21 | 91 | 92 | 145 | 109[a] | 10.9 | 19.0 | | | |
| | d 30 | 85 | 82 | 79 | 82[b] | 8.2 | 14.4 | | | |
| | d 42 | 92 | 67 | 64 | 74[b] | 7.9 | 13.7 | | | |
| Acetate | d 10 | 61.4 | 59.3 | 58.4 | 59.7[a] | 2.06 | 3.57 | 0.13 | <0.001 | 0.27 |
| | d 14 | 59.6 | 56.3 | 68.5 | 61.5[a] | 3.42 | 5.94 | | | |
| | d 21 | 57.5 | 57.4 | 73.2 | 62.7[a] | 3.01 | 5.22 | | | |
| | d 30 | 51.1 | 55.9 | 53.4 | 53.5[b] | 1.50 | 2.61 | | | |
| | d 42 | 51.8 | 44.9 | 46.6 | 47.8[c] | 1.64 | 2.85 | | | |
| Propionate | d 10 | 16.4 | 17.2 | 13.1 | 18.9[b] | 1.14 | 0.66 | 0.47 | 0.001 | 0.30 |
| | d 14 | 18.9 | 16.8 | 16 | 17.2[bc] | 2.97 | 1.71 | | | |
| | d 21 | 15.5 | 16.8 | 11.3 | 14.5[c] | 1.57 | 0.91 | | | |
| | d 30 | 29.3 | 22.1 | 17.6 | 23.1[a] | 2.95 | 1.7 | | | |
| | d 42 | 24.6 | 22.7 | 26.2 | 24.5[a] | 3.10 | 1.79 | | | |
| Butyrate | d 10 | 14.6 | 18.0 | 14.7 | 15.7 | 2.94 | 1.70 | 0.36 | 0.45 | 0.25 |
| | d 14 | 15.1 | 19.4 | 12.0 | 15.5 | 4.83 | 2.79 | | | |
| | d 21 | 21.5 | 18.8 | 11.4 | 17.2 | 3.53 | 2.04 | | | |
| | d 30 | 14.1 | 16.4 | 22.7 | 17.7 | 2.75 | 1.59 | | | |
| | d 42 | 18.1 | 24.2 | 19.2 | 20.5 | 3.09 | 1.79 | | | |
| Valerate | d 10 | 1.71 | 1.21 | 1.32 | 1.41[c] | 0.204 | 0.124 | 0.53 | <0.01 | 0.77 |
| | d 14 | 2.04 | 2.16 | 1.15 | 1.78[bc] | 0.589 | 0.349 | | | |
| | d 21 | 2.37 | 2.05 | 0.94 | 1.79[bc] | 0.510 | 0.295 | | | |
| | d 30 | 3.09 | 2.42 | 2.45 | 2.65[a] | 0.712 | 0.412 | | | |
| | d 42 | 3.32 | 3.56 | 4.03 | 3.64[a] | 0.833 | 0.483 | | | |

[1] See Table 5.2.
[2] Standard error of the mean for comparisons between diets within days.
[3] Standard error of the mean for comparisons between days.
[a, b, c] Different letters within a column indicate differences between sampling days at P<0.05.

**Rumen histology examination**

Histological examination of tissues revealed minimal rumen mucosal damage attributable to dietary differences at the end of transition. Visual observation revealed no obvious differences in morphology of rumen papillae. Microscopic observation indicated that there was a great individual variability irrespectively of the applied protocol (Figure 5.3), since some calves had numerous and short papillae whereas others showed less density with longer and thicker papillae. Only one animal in Protocol 1 and another in Protocol 3, presented rumen epithelium damage, showing parakeratosis and focal rumenitis, respectively. Samples taken from the abomasum and intestinal tract revealed common lesions corresponding to chronic enteritis caused by coccidiosis, which was considered normal taking into account the origin of the calves. In general, at the end of transition the histological examination revealed minor rumen mucosa alterations with no evidence of inflammation or parakeratosis, except in the abovementioned animals.

**Figure 5.3** Section of the rumen epithelium of two calves at the end of the transition period.

Histological examination of the samples taken at the end of the study revealed the occurrence of inflammatory lesions of the rumen with focal intraepithelial pustules in the rumen papillae in all animals. Besides, all calves presented evidence of parakeratosis of rumen mucosa and sloughing of the epithelium. Findings in the samples from the abomasum and intestines were of minor importance with no significant abnormalities.

**Rumen microbial population**

Rumen protozoa concentration was not affected by protocol (P=0.76) or sampling day (P=0.19), and averaged 6.22, 6.00, 5.21 and 5.89 log cells/mL on days 10, 14, 35 and 42 of the experiment. Protozoal population was highly variable among animals and days of sampling, and ranged from 2.73 to 6.83 log cells/mL. Only one calf from Protocol 3

was defaunated on day 35, but recovered a stable population thereafter. It is worth mentioning that at the end of transition (day 14) all animals harboured a consistent protozoal population and diversity despite the abrupt dietary change. Figure 5.4 shows the evolution of the concentration of the different protozoa groups over time. *Entodinium* population remained stable thought the experiment (P=0.20). *Dasytricha* and protozoa from genus *Epidinium* and those from Subfamily *Diplodiniinae* decreased over time (P<0.01) and *Isotricha* followed a decreasing trend (P=0.08). On the last sampling day, the observed protozoa belonged mostly to the genera *Entodinium* (97.5 ± 0.72%); although *Isotricha* was also present (1.74 ± 0.47%). Protozoa of the genera *Dasytricha* (0.05 ± 0.057%), *Epidinium* (0.43 ± 0.372%) and those of the Subfamily *Diplodiniinae* (0.23 ± 0.135%) were virtually absent on day 42. No significant correlations (P>0.05) were found with protozoal concentrations and rumen fermentation variables.

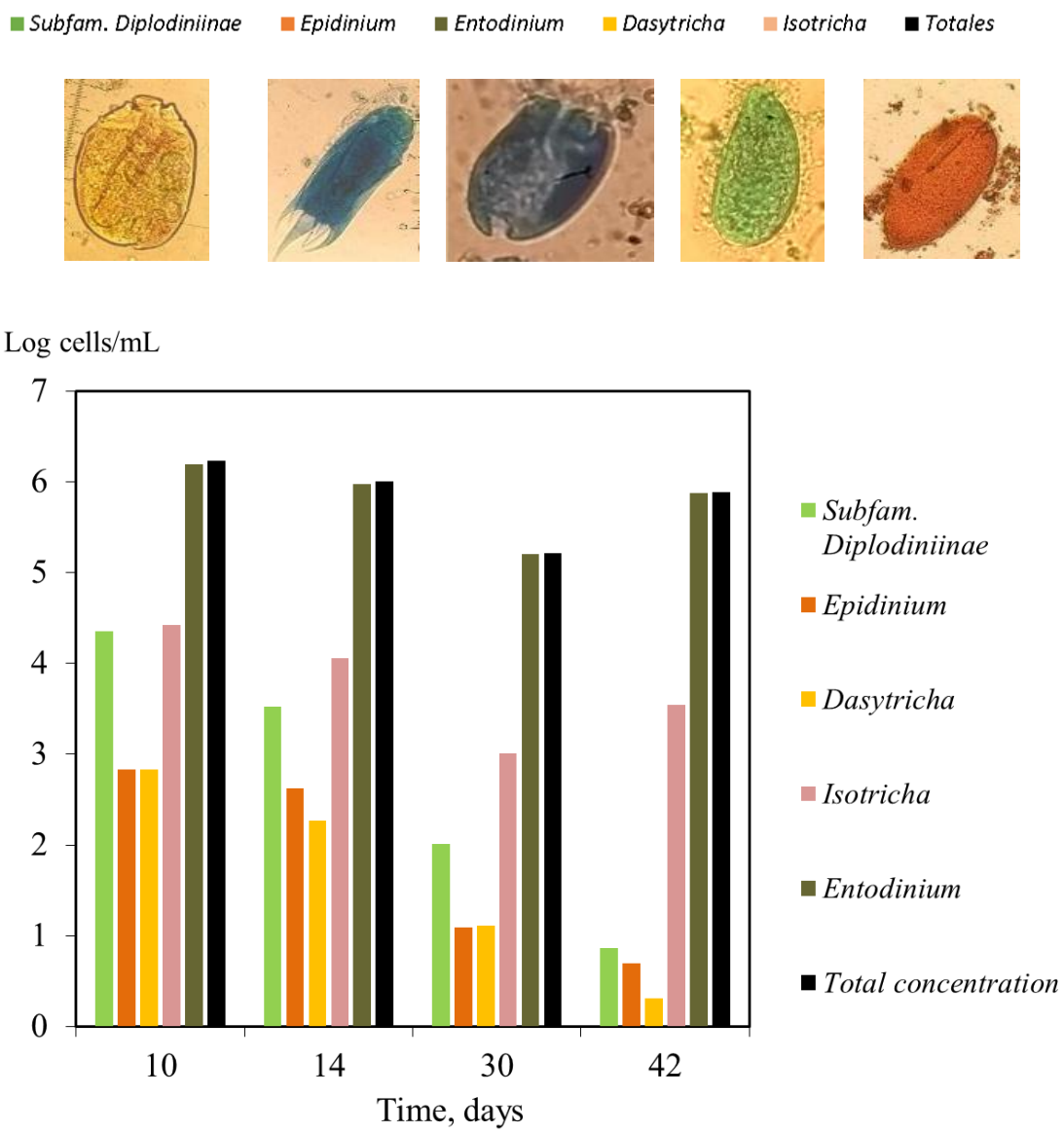



**Figure 5.4** Evolution of total concentration and rumen protozoa groups (Log cells/mL) in beef calves that followed different adaptation protocols to a high-concentrate diet. Days 10, 14, 30 and 42 after concentrate inclusion. The effect of protocol was not significant in any case (P>0.05).

Regarding rumen bacterial population, the sequences obtained from 16S rRNA gene were assigned to 96 genera belonging to 224 families from 24 different phyla. High individual variability was found among calves (CV from 29 to 270%). Collectively, four phyla represented more than 95% of sequences (Figure 5.5): *Bacteroidetes* (45.8 ± 3.88%), *Firmicutes* (43.0 ± 3.11%), *Actinobacteria* (5.9 ± 3.10%), and *Proteobacteria* (3.5 ± 1.66%). Of the two major phyla, *Bacteroidetes* were dominated by a single genus (*Prevotella*), while *Firmicutes* comprised several families and genera.

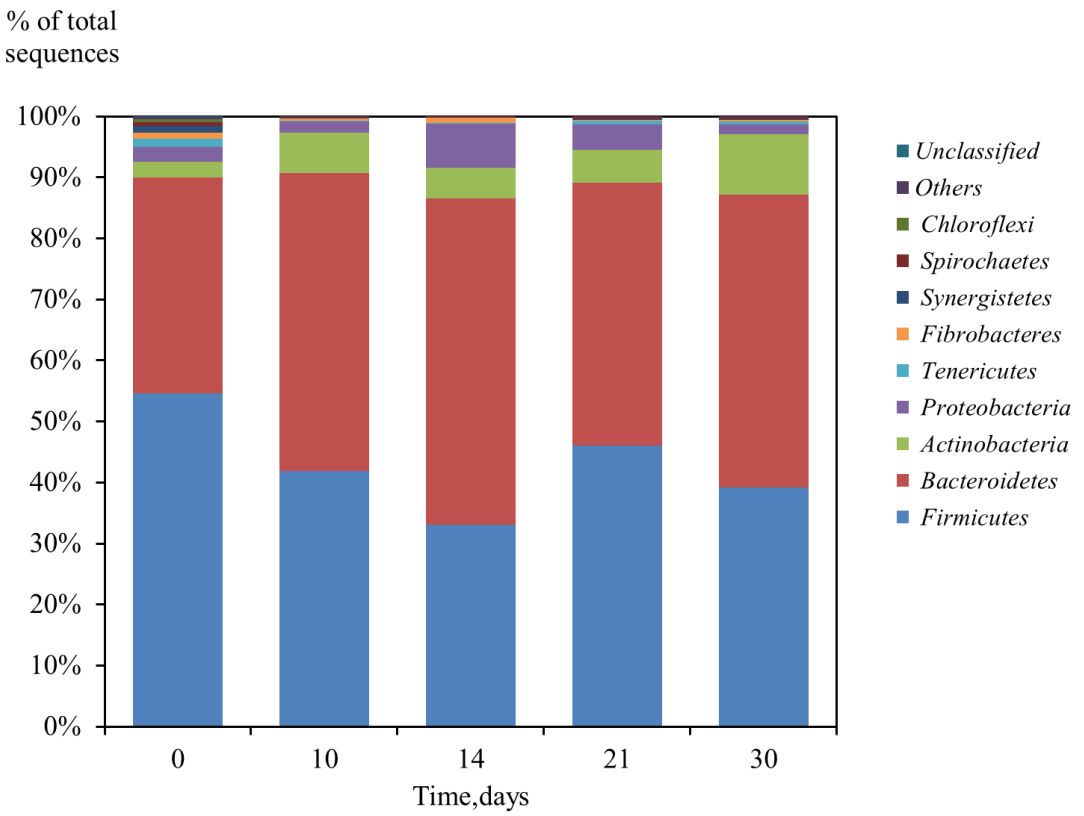
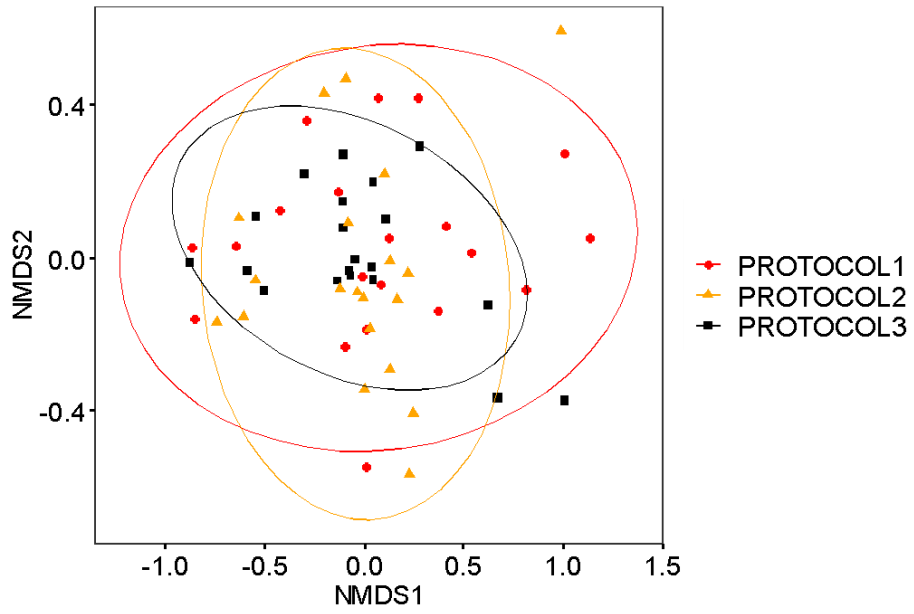



**Figure 5.5** Structure of the bacterial community at phyla level of beef calves that followed different adaptation protocols to a high-concentrate diet. Days 0, 10, 14, 21 and 30 after concentrate inclusion. The effect of protocol was not significant in any case (P>0.05)

The PERMANOVA revealed no effect of the adaptation protocol (P=0.46, $R^2$=0.03) but a significant effect of time on overall microbial composition (P<0.001; $R^2$=0.09). In addition, ANOSIM also showed no differences among treatments (P=0.61; R=-0.01) but a significant effect of time (P<0.001; R=0.34). The NMDS plot exhibited that bacterial communities did not cluster by protocol (Figure 5.6A), and there was a slight clustering by sampling days (Figure 5.6B) in which samples from days 0 and 10 seemed to be more homogeneous and thereafter more dispersed.

**5.6A**




**5.6B**

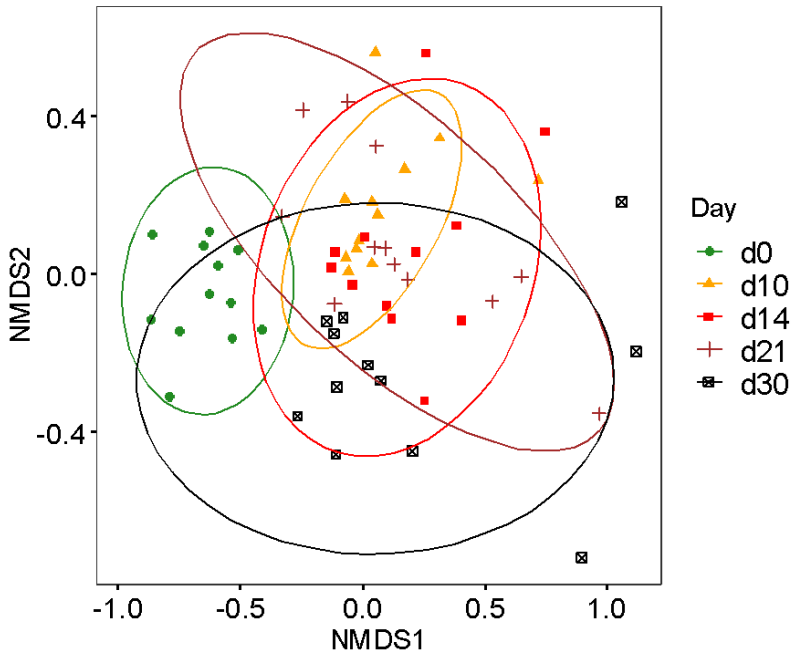



**Figure 5.6** Non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis dissimilarity showing the relationship among bacterial populations in the rumen of beef calves regarding the different adaptation protocols (see Table 5.2) to a high-concentrate diet (A) or in the different sampling days (B) during the transition from milk and forage to a high-concentrate diet.

Like the rumen fermentation variables, the effect of protocol was not significant for any bacterial taxon (Table 5.6). The abundance (data expressed as $\log_{10}$ of the number of reads +1) of most taxonomic levels differed across sampling days (Table 5.6). Of the four major phyla, only *Firmicutes* varied over time (P<0.001), and showed lower abundance on day 30 compared to day 0, and the similar trend was observed on other minor phyla such as *Chloroflexi* (P<0.001), *Synergistetes (*P<0.001), and *Tenericutes* (P<0.001). *Proteobacteria* and the ratio *Proteobacteria* (calculated as the abundance of *Proteobacteria* sequences divided by the sum of the abundance of *Firmicutes* and *Bacteroidetes* sequences) remained stable over time (P>0.05), and on day 14 two calves in Protocol 1 showed ratios higher than 0.19. The ratio *Firmicutes*/*Bacteroidetes* tended to differ over time (P=0.08), and on day 30 it was lower compared to day 0 (1.63 *vs.* 0.89; Table 5.6). After concentrate inclusion, on day 10 some genera appeared in the rumen: *Bifidobacterium*, *Lactobacillus*, *Roseburia*, *Sharpea* and *Succinomonas*. During the transition period (day 0 to day 14), regardless the protocol, there was a decline (P<0.05) in the members of the phyla *Chloroflexi*, *Synergistetes* and *Tenericutes*, and of the genera *Atopobium*, *Clostridium*, *Butyrivibrio*, *Coprococcus*, *Pseubutyrivibrio* and *Streptococcus*. In addition, there was an increase of *Bifidobacterium*, *Prevotella*, *Roseburia*, *Lactobacillus*, *Selenomonas*, *Anaerovibrio* and *Sharpea* on day 14 compared to day 0 (P<0.05). Overall, on day 30 compared to day 0, at the genus level, *Bifidobacterium*, *Roseburia*, *Lactobacillus* and *Succiniclasticum* increased (P<0.001), and *Eubacterium*, *Butyrivibrio*, *Pseudobutyrivibrio*, *Coprococcus*, *Selenomonas* and *Anaerovibrio* decreased (P<0.001; Table 5.6). Diversity indexes (Shannon and genera Richness) were not affected by the adaptation protocol (P>0.05), but were altered across sampling days (P<0.05; Table 5.6).

**Table 5.6** Effect of the diet and day of sampling on the structure of the bacterial community and on bacterial diversity indexes in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet[1] during transition (0-14 d) and the first weeks of the fattening period.

| | | | Protocols[1] (P) | | | | Day (d) | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Familiy | Genus | 1 | 2 | 3 | SEM | 0 | 10 | 14 | 21 | 30 | SEM | P | d | Pxd |
| *Actinobacteria* | | | 3.52 | 3.41 | 3.28 | 0.13 | 3.33 | 3.48 | 3.41 | 3.4 | 3.38 | 0.147 | 0.48 | 0.96 | 0.71 |
| | *Coriobacteriaceae* | | 3.4 | 3.19 | 3.09 | 0.105 | 3.24 | 3.23 | 3.31 | 3.22 | 3.14 | 0.135 | 0.16 | 0.92 | 0.50 |
| | | *Olsenella* | 3.11 | 2.9 | 2.84 | 0.113 | 2.90 | 3.04 | 3.00 | 2.9 | 2.92 | 0.146 | 0.20 | 0.93 | 0.32 |
| | | *Atopobium* | 1.8 | 1.43 | 1.56 | 0.259 | $2.11^{a}$ | $1.25^{bc}$ | $1.14^{c}$ | $1.53^{abc}$ | $1.95^{ab}$ | 0.223 | 0.60 | <0.001 | 0.33 |
| | *Bifidobacteriaceae* | | 1.89 | 2.09 | 1.65 | 0.29 | $0.27^{b}$ | $2.58^{a}$ | $2.29^{a}$ | $2.00^{a}$ | $2.25^{a}$ | 0.317 | 0.57 | <0.001 | 0.44 |
| | | *Bifidobacterium* | 1.71 | 1.85 | 1.52 | 0.243 | $0.00^{b}$ | $2.56^{a}$ | $2.26^{a}$ | $1.86^{a}$ | $1.78^{a}$ | 0.302 | 0.64 | <0.001 | 0.38 |
| *Bacteroidetes* (B) | | | 4.51 | 4.58 | 4.61 | 0.035 | 4.47 | 4.6 | 4.62 | 4.54 | 4.59 | 0.044 | 0.18 | 0.12 | 0.42 |
| | *Prevotellaceae* | | 4.32 | 4.45 | 4.44 | 0.038 | 4.17 | 4.53 | 4.55 | 4.37 | 4.4 | 0.047 | 0.07 | <0.001 | 0.15 |
| | | *Prevotella* | 3.22 | 3.33 | 3.35 | 0.08 | $2.85^{c}$ | $3.93^{a}$ | $3.37^{b}$ | $3.23^{bc}$ | $3.11^{bc}$ | 0.104 | 0.47 | <0.001 | 0.28 |
| *Chloroflexi* | | | 1.19 | 0.93 | 1.06 | 0.159 | $2.41^{a}$ | $0.44^{c}$ | $0.37^{c}$ | $0.80^{bc}$ | $1.28^{b}$ | 0.189 | 0.54 | <0.001 | 0.50 |
| *Fibrobacteres* | | | 1.94 | 2.17 | 2.45 | 0.19 | 2.85 | 1.99 | 2.32 | 1.84 | 1.93 | 0.246 | 0.17 | 0.03 | 0.55 |
| | *Fibrobacteraceae* | | 1.94 | 2.17 | 2.45 | 0.19 | 2.85 | 1.99 | 2.32 | 1.84 | 1.93 | 0.246 | 0.17 | 0.03 | 0.55 |
| | | *Fibrobacter* | 1.82 | 2.02 | 2.3 | 0.186 | $2.74^{a}$ | $1.85^{ab}$ | $2.12^{ab}$ | $1.69^{b}$ | $1.84^{ab}$ | 0.24 | 0.18 | 0.02 | 0.66 |
| *Firmicutes* (F) | | | 4.58 | 4.55 | 4.5 | 0.027 | $4.67^{a}$ | $4.54^{abc}$ | 4.42c | $4.58^{ab}$ | $4.51^{bc}$ | 0.035 | 0.09 | <0.001 | 0.59 |
| | *Clostridiaceae* | | 3.85 | 3.82 | 3.83 | 0.053 | $4.11^{a}$ | $3.86^{b}$ | $3.74^{b}$ | $3.74^{b}$ | $3.70^{b}$ | 0.057 | 0.90 | <0.001 | 0.89 |
| | | *Clostridium* | 2.56 | 2.74 | 2.71 | 0.094 | $2.90^{ab}$ | $3.01^{a}$ | $2.38^{c}$ | $2.59^{abc}$ | $2.47^{bc}$ | 0.118 | 0.41 | 0.0015 | 0.50 |
| | *Eubacteriaceae* | | 3.15 | 3.07 | 3.12 | 0.064 | $3.60^{a}$ | $3.06^{b}$ | $2.95^{b}$ | $2.95^{b}$ | $3.01^{b}$ | 0.083 | 0.67 | <0.001 | 0.99 |
| | | *Eubacterium* | 2.12 | 2.1 | 2.27 | 0.119 | $2.59^{a}$ | $2.31^{ab}$ | $2.02^{ab}$ | $2.04^{ab}$ | $1.86^{b}$ | 0.154 | 0.57 | 0.01 | 0.66 |

**Table 5.6** Effect of the diet and day of sampling on the structure of the bacterial community and on bacterial diversity indexes in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet[1] during transition (0-14d) and the first weeks of the fattening period. (Continued).

| | | | Protocols[1] (P) | | | | Day (d) | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Family | Genus | 1 | 2 | 3 | SEM | 0 | 10 | 14 | 21 | 30 | SEM | P | d | Pxd |
| | | *Lachnospiraceae* | 3.86 | 3.86 | 3.84 | 0.052 | 3.97 | 3.91 | 3.73 | 3.79 | 3.86 | 0.066 | 0.96 | 0.10 | 0.98 |
| | | *Butyrivibrio* | 2 | 2.38 | 2.38 | 0.209 | $3.25^{a}$ | $2.47^{ab}$ | $2.10^{b}$ | $1.75^{b}$ | $1.68^{b}$ | 0.21 | 0.38 | <0.001 | 0.38 |
| | | *Blautia* | 1.89 | 1.7 | 1.83 | 0.132 | 1.97 | 1.76 | 1.92 | 1.75 | 1.64 | 0.144 | 0.60 | 0.42 | 0.11 |
| | | *Catonella* | 1.67 | 1.68 | 1.88 | 0.109 | 1.68 | 2.14 | 1.66 | 1.65 | 1.6 | 0.141 | 0.32 | 0.05 | 0.17 |
| | | *Coprococcus* | 1.17 | 1.31 | 1.38 | 0.153 | $2.71^{a}$ | $1.58^{b}$ | $1.01^{bc}$ | $0.86^{bc}$ | $0.28^{c}$ | 0.198 | 0.59 | <0.001 | 0.72 |
| | | *Lactonifactor* | 1.85 | 1.86 | 1.69 | 0.171 | 1.41 | 2.13 | 2.12 | 1.91 | 1.43 | 0.205 | 0.74 | 0.02 | 0.57 |
| | | *Moryella* | 1.47 | 1.66 | 1.84 | 0.137 | 1.46 | 1.55 | 1.74 | 1.74 | 1.78 | 0.177 | 0.17 | 0.63 | 0.10 |
| | | *Pseudobutyrivibrio* | 1.15 | 1.4 | 1.74 | 0.154 | $2.49^{a}$ | $1.55^{b}$ | $1.37^{bc}$ | $1.05^{bc}$ | $0.69^{c}$ | 0.186 | 0.16 | <0.001 | 0.23 |
| | | *Roseburia* | 1.01 | 1.29 | 1.16 | 0.241 | $0.00^{b}$ | $1.75^{a}$ | $1.51^{a}$ | $1.28^{a}$ | $1.18^{a}$ | 0.27 | 0.7 | <0.001 | 0.20 |
| | | *Lactobacillaceae* | 2.77 | 2.55 | 2.3 | 0.171 | 2.43 | 2.68 | 2.3 | 2.77 | 2.52 | 0.221 | 0.16 | 0.57 | 0.90 |
| | | *Lactobacillus* | 1.75 | 1.36 | 0.96 | 0.366 | $0.00^{b}$ | $1.81^{a}$ | $1.18^{a}$ | $1.89^{a}$ | $1.86^{a}$ | 0.305 | 0.35 | <0.001 | 0.37 |
| | | *Veillonellaceae* | 2.68 | 2.87 | 2.73 | 0.1 | $2.20^{b}$ | $3.25^{a}$ | $3.00^{a}$ | $2.98^{a}$ | $2.37^{b}$ | 0.129 | 0.38 | <0.001 | 0.95 |
| | | *Selenomonas* | 2.02 | 2.37 | 2.16 | 0.117 | $1.72^{b}$ | $2.85^{a}$ | $2.73^{a}$ | $2.57^{a}$ | $1.04^{c}$ | 0.125 | 0.16 | <0.001 | 0.14 |
| | | *Anaerovibrio* | 1.55 | 1.59 | 1.6 | 0.134 | $0.43^{b}$ | $2.38^{a}$ | $1.97^{a}$ | $2.08^{a}$ | $1.03^{b}$ | 0.172 | 0.96 | <0.001 | 0.93 |
| | | *Ruminococcaceae* | 3.91 | 3.89 | 3.68 | 0.073 | $3.65^{b}$ | $3.83^{ab}$ | $3.77^{ab}$ | $4.03^{a}$ | $3.85^{ab}$ | 0.083 | 0.09 | 0.03 | 0.15 |
| | | *Ruminococcus* | 2.84 | 2.8 | 2.74 | 0.129 | $3.02^{a}$ | $3.33^{a}$ | $2.84^{a}$ | $2.94^{a}$ | $1.86^{b}$ | 0.162 | 0.87 | <0.001 | 0.07 |
| | | *Streptococcaceae* | 1.54 | 1.89 | 1.85 | 0.17 | $2.52^{a}$ | $1.85^{ab}$ | $1.36^{b}$ | $1.29^{b}$ | $1.79^{a}$ | 0.212 | 0.11 | 0.01 | 0.72 |
| | | *Streptococcus* | 1.21 | 1.75 | 1.47 | 0.179 | $2.21^{a}$ | $1.50^{ab}$ | $1.24^{b}$ | $1.09^{b}$ | $1.35^{ab}$ | 0.231 | 0.51 | 0.002 | 0.94 |
| | | *Acidaminococcaceae* | 2.82 | 2.74 | 2.84 | 0.069 | 2.5 | 2.78 | 2.87 | 2.81 | 3.03 | 0.089 | 0.99 | 0.70 | 0.97 |

**Table 5.6** Effect of the diet and day of sampling on the structure of the bacterial community and on bacterial diversity indexes in the rumen fluid of beef calves subjected to different adaptation protocols to a high-concentrate diet[1] during transition (0-14d) and the first weeks of the fattening period. (Continued).

| | | | Protocols[1] (P) | | | | Day (d) | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Family | Genus | 1 | 2 | 3 | SEM | 0 | 10 | 14 | 21 | 30 | SEM | P | d | Pxd |
| | | *Succiniclasticum* | 2.45 | 2.44 | 2.45 | 0.101 | 2.31 | 2.43 | 2.57 | 2.45 | 2.48 | 0.13 | 0.68 | <0.001 | 0.83 |
| | *Erysipelotrichaceae* | | 2.95 | 2.92 | 3.04 | 0.1 | $3.46^{a}$ | $2.67^{bc}$ | $2.50^{c}$ | $3.16^{a}$ | $3.07^{ab}$ | 0.112 | 0.23 | 0.03 | 0.21 |
| | | *Sharpea* | 1.09 | 0.7 | 0.55 | 0.236 | $0.00^{b}$ | $0.57^{ab}$ | $1.33^{a}$ | $0.97^{ab}$ | $1.03^{ab}$ | 0.304 | 0.35 | 0.02 | 0.79 |
| *Proteobacteria* (Pr) | | | 3.22 | 2.95 | 3.16 | 0.128 | 3.3 | 2.89 | 3.26 | 3.36 | 2.74 | 0.154 | 0.87 | 0.02 | 0.24 |
| | *Succinivibrionaceae* | | 2.18 | 2.06 | 2.19 | 0.204 | 1.63 | 2.39 | 2.55 | 2.46 | 1.7 | 0.252 | 0.99 | 0.18 | 0.64 |
| | | *Ruminobacter* | 1 | 1.02 | 0.99 | 0.307 | 0.62 | 1.13 | 1.54 | 1.16 | 0.57 | 0.335 | 0.68 | 0.24 | 0.83 |
| | | *Succinimonas* | 0.49 | 0.25 | 0.48 | 0.212 | 0.00 | 0.35 | 0.20 | 0.78 | 0.69 | 0.274 | 0.83 | 0.004 | 0.21 |
| | | *Succinivibrio* | 1.55 | 1.63 | 1.78 | 0.265 | $1.44^{ab}$ | $1.92^{a}$ | $1.98^{a}$ | $1.86^{a}$ | $1.06^{b}$ | 0.225 | 0.96 | 0.02 | 0.64 |
| *Spirochaetes* | | | 1.89 | 1.88 | 1.97 | 0.24 | $2.69^{a}$ | $1.61^{b}$ | $1.82^{ab}$ | $1.71^{ab}$ | $1.75^{ab}$ | 0.257 | 0.91 | <0.001 | 0.96 |
| *Synergistetes* | | | 1.58 | 1.49 | 1.59 | 0.178 | $2.90^{a}$ | $1.33^{b}$ | $1.16^{b}$ | $1.32^{b}$ | $1.05^{b}$ | 0.217 | 0.24 | <0.001 | 0.30 |
| *Tenericutes* | | | 2.03 | 2.21 | 2.35 | 0.133 | $3.00^{a}$ | $1.78^{bc}$ | $1.59^{c}$ | $2.36^{ab}$ | $2.26^{bc}$ | 0.171 | 0.78 | <0.001 | 0.06 |
| Shannon Index | | | 2.22 | 2.13 | 2.11 | 0.122 | 2.51a | $1.85^{c}$ | $2.02^{bc}$ | $2.20^{b}$ | $2.17^{b}$ | 0.11 | 0.80 | <0.001 | 0.04 |
| Richness | | | 31.3 | 31.2 | 30.8 | 0.83 | $35.7^{a}$ | $32.5^{b}$ | $30.2^{b}$ | $30.5^{b}$ | $26.3^{c}$ | 1.04 | 0.92 | <.0001 | 0.63 |
| Ratio F/B | | | 1.09 | 0.95 | 1.41 | 0.172 | 1.63 | 0.99 | 0.89 | 1.35 | 0.89 | 0.222 | 0.17 | 0.08 | 0.69 |
| Ratio Pr | | | 0.02 | 0.04 | 0.08 | 0.023 | 0.03 | 0.02 | 0.11 | 0.05 | 0.02 | 0.029 | 0.17 | 0.16 | 0.18 |

[1] See Table 5.2.

The number of reads was normalised to 85,442 reads and $\log_{10}$-transformed (nº reads+1). Only bacteria taxa that represented on average more than 0.1% of total sequences are shown. *Proteobacteria* ratio was calculated as the abundance of *Proteobacteria* sequences divided by the sum of the abundance of *Firmicutes* and *Bacteroidetes* sequences. [a,b,c] Different superscript letters within a row indicate differences between treatments or between days at P<0.05, as obtained using Bonferroni's test.

Correlation analysis between relative abundances of the bacterial taxa and rumen fermentation variables is shown in Table 5.7. Most of the correlations were consistent under the three Protocols; however, others such as the phylum *Actinobacteria* or the genus *Prevotella* correlated differently for each group (Table 5.7). Diversity indexes did not show significant correlations with fermentation variables.

Certain bacterial genera were detected ubiquitously in the rumen of all calves across the experiment, and therefore, were assumed as the 'core microbiome'. The rumen bacterial core included members of the major phyla *Bacteroidetes* (including families: *Porphyromonadaceae, Flavobacteriaceae, Sphingobacteriaceae, Cytophagaceae,* and the genus *Prevotella*), *Firmicutes* (Families: *Erysipelotrichaceae*, *Paenibacillaceae, Acidaminococcaceae, Peptostreptococcaceae*, *Syntrophomonadaceae*, *Lactobacillaceae, Bacillaceae*, *Peptococcaceae*, *Marinilabiliaceae*; and the genera *Blautia, Butyrivibrio, Catonella, Clostridium, Eubacterium, Lactonifactor, Moryella, Ruminococcus, Schwartzia, Selenomonas, Streptococcus, Succiniclasticum,* and *Syntrophococcus*), *Actinobacteria* (genera *Atopobium* and *Olsenella),* and *Proteobacteria* (Family *Desulfovibrionaceae,* and genus *Succinivibrio*). From minor phyla: *Fibrobacteres* (genus *Fibrobacter*), *Tenericutes* (*Families Spiroplasmataceae* and *Anaeroplasmataceae), Spirochaetes* (genus *Treponema*) and *Synergstetes* (Family *Synergistaceae*) were also part of the rumen core. The abundance of the shared taxa in the overall bacterial community was highly diverse ranging from 0.01% to 55% of total bacteria. Protocols did not show differences regarding core taxa.

**Table 5.7** Spearman´s correlations (r) between bacterial taxa (P: phylum; F: family; G: genus) and rumen fermentation variables of beef calves subjected to different adaptation protocols (P1, P2, P3; See Table 2) to a high-concentrate diet during transition (0-14d) and the first weeks of the fattening period. Only Spearman correlations with r≥0.3 or r ≤-0.3 and P<0.05 are shown (n=60).

| | Rumen pH | | | Lactate | | | Ammonia | | | Total VFA | | | Acetate | | | Propionate | | | Butyrate | | | Valerate | | | BCFA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial taxa | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 | P1 | P2 | P3 |
| *P. Actinobacteria* | -0.35 | -0.42 | 0.44 | | | -0.31 | -0.66 | | -0.59 | 0.44 | | -0.44 | | 0.32 | | | 0.38 | -0.34 | | 0.46 | -0.48 | 0.51 | 0.52 | | | | -0.49 |
| *F. Coriobacteriaceae* | -0.33 | -0.42 | 0.45 | | | | -0.66 | | -0.50 | 0.40 | | | | | | | | | 0.43 | 0.35 | -0.52 | | | | | | |
| *G.Olsenella* | -0.38 | -0.51 | 0.44 | | | -0.44 | -0.55 | | | 0.43 | 0.45 | | 0.33 | | | | 0.38 | -0.36 | 0.44 | 0.59 | -0.60 | 0.51 | 0.63 | | | | -0.52 |
| *G.Atopobium* | 0.35 | 0.39 | | -0.35 | -0.67 | | | | | | -0.38 | | | | | | | -0.50 | | -0.46 | | | | | | -0.57 | |
| *F.Bifidobacteriaceae* | -0.43 | -0.51 | -0.43 | | | 0.50 | | | | 0.55 | 0.64 | | | 0.52 | | 0.55 | 0.58 | 0.43 | 0.53 | 0.50 | 0.51 | 0.57 | 0.50 | 0.60 | | | |
| *G.Bifidobacterium* | -0.43 | -0.59 | -0.41 | 0.40 | 0.34 | 0.51 | -0.45 | | | 0.55 | 0.63 | 0.34 | | 0.48 | | 0.52 | 0.56 | 0.43 | 0.54 | 0.54 | 0.49 | 0.52 | 0.56 | 0.54 | | | |
| *P.Bacteroidetes* | | | | 0.70 | | | | 0.58 | | | | 0.36 | | | | | | 0.34 | | | 0.48 | | | | | | 0.56 |
| *F. Prevotellaceae* | | -0.39 | -0.41 | | 0.86 | 0.31 | | | 0.34 | | | 0.51 | | | | 0.52 | | 0.51 | | | | | | | 0.50 | | |
| *G.Prevotella* | | | -0.57 | 0.45 | 0.67 | 0.54 | | | | 0.34 | | 0.57 | -0.35 | | 0.43 | 0.44 | | 0.66 | 0.44 | 0.33 | 0.54 | 0.34 | | | 0.44 | 0.56 | |
| *P.Chloroflexi* | 0.52 | 0.51 | 0.74 | -0.68 | -0.47 | -0.72 | | | | -0.34 | -0.66 | -0.73 | | -0.50 | -0.53 | -0.39 | -0.47 | -0.80 | -0.63 | -0.59 | -0.68 | -0.62 | -0.54 | -0.60 | -0.50 | | |
| *P.Fibrobacteres* | 0.60 | 0.63 | 0.44 | -0.51 | -0.33 | -0.35 | | 0.48 | | | -0.79 | | | -0.58 | | | -0.67 | -0.35 | -0.58 | -0.81 | -0.36 | -0.65 | -0.76 | -0.54 | | -0.42 | |
| *F.Fibrobacteraceae* | 0.60 | 0.63 | 0.44 | -0.51 | -0.33 | -0.35 | 0.48 | | | | -0.79 | | | -0.58 | | | -0.67 | | -0.58 | -0.81 | | -0.65 | -0.76 | -0.54 | | | |
| *G.Fibrobacter* | 0.65 | 0.63 | 0.53 | -0.52 | -0.32 | -0.38 | 0.41 | | | | -0.78 | -0.38 | | -0.59 | | | -0.64 | -0.42 | -0.60 | -0.77 | -0.40 | -0.67 | -0.75 | -0.57 | | -0.42 | |
| *P.Firmicutes* | 0.40 | 0.48 | 0.34 | | -0.44 | -0.31 | | | | | -0.54 | | | -0.52 | | -0.73 | | -0.49 | | | -0.36 | | | | | | |
| *F.Clostridiaceae* | 0.64 | 0.55 | | | | -0.54 | | | | | -0.51 | | | | | -0.64 | -0.61 | | | -0.57 | -0.54 | -0.60 | -0.70 | -0.68 | | | -0.50 |
| *F.Eubacteriaceae* | 0.59 | 0.50 | 0.48 | -0.43 | -0.63 | -0.49 | | | | -0.35 | | | | | | -0.49 | | | -0.45 | | -0.52 | -0.52 | | -0.66 | -0.53 | | -0.51 |
| *G.Eubacterium* | | 0.49 | | | -0.57 | -0.31 | -0.47 | -0.38 | | | | -0.54 | | | -0.41 | | | | | | -0.58 | | | -0.58 | -0.44 | | -0.66 |
| *G.Butyrivibrio* | 0.82 | 0.68 | 0.58 | -0.53 | | -0.31 | 0.46 | | | -0.49 | -0.58 | -0.57 | | -0.32 | -0.46 | -0.47 | -0.64 | -0.49 | -0.55 | -0.63 | -0.45 | -0.81 | -0.70 | -0.66 | | | |
| *G.Blautia* | | 0.55 | | | | | -0.34 | 0.38 | | | -0.45 | | 0.30 | | | | -0.40 | | | -0.52 | | | | | 0.35 | | |
| *G.Coprococcus* | 0.60 | 0.62 | 0.46 | -0.52 | -0.43 | -0.44 | 0.32 | | | -0.38 | -0.56 | -0.52 | | -0.36 | -0.32 | | -0.58 | -0.48 | | -0.53 | -0.47 | -0.54 | -0.65 | -0.63 | | | |
| *G.Lactonifactor* | -0.31 | | -0.50 | | 0.81 | 0.41 | | 0.43 | | | | 0.36 | | | | | | 0.36 | 0.39 | | 0.49 | | | | 0.50 | 0.69 | 0.60 |
| *G.Pseudobutyrivibrio* | 0.69 | 0.50 | 0.44 | | -0.45 | -0.39 | | | | -0.56 | | | | | | -0.51 | -0.63 | -0.35 | -0.57 | -0.56 | -0.62 | -0.75 | -0.72 | -0.70 | | | |
| *G.Roseburia* | | -0.63 | -0.43 | 0.71 | | 0.48 | | | | | 0.53 | 0.34 | | | | | | 0.43 | | 0.65 | 0.53 | | 0.59 | 0.60 | | | |
| *F.Lactobacillaceae* | | -0.34 | | | | | | | | | 0.59 | | | 0.54 | | | 0.54 | | | 0.51 | | | 0.49 | | | |
| *G.Lactobacillus* | | -0.76 | -0.43 | 0.43 | | | | | | | 0.61 | | -0.45 | 0.34 | | | 0.63 | 0.38 | 0.44 | 0.74 | 0.38 | 0.53 | 0.76 | | | | |
| *F.Veillonellaceae* | | -0.44 | | 0.41 | 0.58 | | | | | | | | -0.52 | | | -0.45 | 0.40 | | | 0.52 | | | | | 0.61 | 0.60 | |
| *G.Selenomonas* | | | | | 0.68 | | | | | | | | -0.58 | | | | | 0.52 | | | | | | | 0.52 | 0.50 | |
| *G.Anaerovibrio* | | | | 0.51 | 0.68 | 0.52 | | | | | | | -0.54 | | | | | 0.54 | 0.37 | 0.44 | | | | | 0.49 | 0.71 | |
| *F.Ruminococcaceae* | | | | 0.67 | 0.47 | | | | | | | | -0.44 | -0.35 | | | | | 0.38 | | | | | | 0.53 | 0.54 | |
| *G.Ruminococcus* | | 0.36 | | | 0.62 | -0.35 | | 0.30 | -0.63 | -0.34 | | -0.34 | | | | -0.36 | | -0.37 | -0.32 | | | | | | | | |
| *F.Streptococcaceae* | 0.73 | | | -0.81 | -0.54 | | | | | | -0.54 | | | | | | -0.65 | | -0.59 | -0.57 | | -0.59 | -0.68 | -0.58 | -0.55 | | |
| *G.Streptococcus* | 0.73 | | | -0.59 | | | 0.46 | | | -0.45 | -0.51 | | | -0.35 | | -0.34 | -0.61 | | -0.54 | -0.49 | | -0.67 | -0.61 | | | | |
| *F.Acidaminococcaceae* | | | | | | | | 0.43 | 0.62 | | | | | | | 0.50 | | | | | | | | | | 0.50 | |
| *G.Succiniclasticum* | | | | | | | | 0.31 | 0.63 | | | | | -0.50 | | 0.35 | | | | | | | | | | 0.59 | |
| *F.Erysipelotrichaceae* | 0.61 | 0.41 | 0.61 | | -0.57 | -0.47 | | | | -0.65 | | -0.62 | | | -0.51 | -0.61 | -0.52 | -0.55 | -0.66 | -0.40 | -0.54 | | | | | -0.57 | |
| *G.Sharpea* | -0.46 | -0.59 | | 0.35 | | | -0.31 | | | 0.44 | 0.47 | | | 0.46 | | 0.43 | | | | | | 0.68 | | | | | |
| *P.Spirochaetes* | 0.59 | 0.60 | 0.44 | -0.51 | | -0.37 | 0.37 | | | -0.53 | -0.50 | -0.32 | | -0.32 | | | -0.45 | -0.41 | -0.57 | -0.51 | | -0.56 | -0.60 | -0.53 | | -0.52 | |
| *P.Synergistetes* | 0.61 | 0.73 | 0.41 | -0.55 | -0.37 | -0.43 | | | | -0.60 | -0.54 | -0.53 | | -0.32 | -0.40 | | -0.62 | -0.60 | -0.48 | -0.54 | | -0.70 | -0.65 | -0.64 | | | |
| *P.Tenericutes* | 0.76 | 0.56 | 0.57 | -0.55 | | -0.53 | | | | -0.57 | | -0.61 | | | -0.49 | -0.52 | -0.48 | -0.67 | -0.77 | -0.51 | | -0.76 | -0.60 | | | -0.50 | |

## Discussion

Three protocols to adapt beef calves from a milk and pasture diet to a high-concentrate ration have been evaluated in this study. For this purpose, barley was partially replaced by maize and BP in the adaptation diet during the transition period. Protocol 1 was assumed as an abrupt shift to the fattening diet, whereas animals in Protocols 2 and 3 were supposed to be gradually transitioned. Adaptation diets with lower acidogenic capacity due to a slower degradation rate of starch (González et al., 2012), and to a starch to NDF ratio lower than 2:1 (Bacha, 2002), are desirable to prevent ruminal acidosis. Diet C had the greatest acidogenic power since barley possesses a higher rate of fermentation than maize (Herrera-Saldana et al., 1990), and this diet had a starch:NDF ratio of 3:1. Although diet BP10 showed the same starch/NDF ratio, its acidogenic capacity was lower because of the lower proportion of barley and the inclusion of BP itself. On the other hand, BP is unique in its composition and possesses a high buffer capacity, which is partly due to the galacturonic acid in its structure and its high fibre content (Van Soest et al., 1991; FEDNA, 2010). Williams et al. (1987) reported that the replacement of barley with BP considerably increased the capacity of the diet to neutralize hydrogen ions, since buffer capacity of BP is greater than that of cereals (Jasaitis et al., 1987). Attention must be drawn to the fact that diets C and BP22 also included palm oil (9.3 and 29.5 g/kg, respectively) and this might have helped to alleviate the risk of ruminal acidosis in animals consuming those diets. In addition, corn gluten feed was included in the formulae (57, 54 and 92 g/kg for diets C, BP10 and BP22, respectively), and the higher level in diet BP22 could have also had an effect on rumen fermentation.

### Feed intake and animal performance

Concentrate intake during Ph1 and Ph3 was similar among protocols. However, in Ph2 (when calves in Protocols 2 and 3 received a 50:50 mixture of their BP diets with the C diet) calves in Protocol 3 showed the lowest intake of concentrate and consumed more straw, suggesting an self-regulation mechanism to increase the buffering capacity of the rumen (González et al., 2012) in response to the acidogenic capacity of the new diet. In Ph3, animals in Protocol 2 seemed to be more adapted to ration C, which might be due to the higher acidogenic power of the previous BP10 diet. Throughout Ph3, DMI and ADG were similar among protocols. Similarly, Bevans et al. (2005) reported that a gradual or rapid adaptation to high-concentrate diets had no effect on DMI thereafter in the feedlot period.

With respect to the use of barley or maize in the diet, previous studies have also found no differences on intake among groups in steers (Surber and Bownman, 1998; Gimeno et al., 2015) or lambs (Yahaghi et al., 2012) fed corn- or barley-based diets. Regarding barley replacement with BP, Bodas et al. (2007) reported lower intake in fattening lambs when replacing 12% of barley with BP. In contrast, Maktabi et al. (2016) replaced BP for grains (barley and corn) in the starter diet of dairy calves and reported higher DMI.

Regarding the rate of intake of concentrates and wheat straw, no differences were found among protocols or phases in agreement with the results reported by Gimeno et al. (2015), who studied the effect of the main cereal (proportions of barley to maize 75:25 or 25:75) on the rate of intake of concentrate and straw in beef steers. These authors attributed the lack of differences to the higher straw intake observed in animals receiving the barley-based concentrate. In the present experiment, animals consumed substantially more straw (*ca*. 20%) than that reported in other experiments with cattle fed high-concentrate diets (*ca*. 5% in Devant et al., 2000; *ca*.7% in Gimeno et al., 2015) and this could explain the lack of differences between adaptation protocols.

**Rumen fermentation variables**

Results obtained from the analyses of rumen fermentation variables at hour 0 throughout the experiment mirrored the inclusion of concentrates, and were consistent with the general features reported after switching hay-adapted animals to a high-grain diet (Goad et al., 1998). Despite the different characteristics of the dietary ingredients, no differences among adaptation protocols were observed for any of the daily average rumen fermentation characteristics or for their post-prandial evolution.

Our data agree with most studies that did not detect differences on daily average rumen pH comparing barley- or maize- based diets in dairy cows (Casper et al., 1999; Khorasani et al., 2001) or beef cattle (Rotger et al., 2006; Gimeno et al., 2015). However, the extent of post-prandial decrease of rumen pH was higher when barley was the prevailing cereal (Khorasani et al., 2001; Gimeno et al., 2015). As mentioned above, in the present study calves consumed a considerable amount of straw that could explain the lack of differences in the decrease of rumen pH after feeding, given the different nature of diet ingredients. Regarding the BP inclusion, previous experiments reported higher ruminal pH when BP increased at the expense of barley in dairy cows (Mahjoubi et al.,

2009), steers fed low-forage diets (Mojtahedi and Danesh Mesgaran, 2011), and fattening lambs (Bodas et al., 2007). In these research, barley was the only grain source accounting for 23% and 33% of DM, respectively, and thus replacing grain for BP in these diets (from 33% to 100% replacement) is likely to result in an increase in pH due to the buffer capacity of BP. The lack of effect of BP in our study is masked by the partial substitution of barley with maize as well. In the study of Bodas et al. (2007), the higher rumen pH could have probably been due to the lower intake (P=0.03) promoted by the BP replacing 12% of barley and the numerically higher straw intake in the BP group (14 *vs.* 19 g DM/d).

Lactate concentration in our study was similar among diets in agreement with the results of Gimeno et al. (2015). In general, an overall increase of lactate concentration, which was not accompanied by a low rumen pH (6.20 on average), was observed at the end of transition. Lactate concentrations in our study (except for one animal that reached 7.05 mmol/l in one sampling) were below the risk values considered as benchmark of SARA (>5 mmol/L; Nagaraja and Titgemeyer, 2007).

Ammonia concentration was highly variable among animals but the different ingredient compositions of the diets did not exert any effect. Casper et al. (1999) reported lower ammonia concentrations with barley-based diets that was attributed to the higher degradability of barley and thus high available energy for microbes to capture N for protein synthesis. Other authors, however, did not find any difference between barley and maize (Gimeno et al., 2015). In other studies, the substitution of barley with BP resulted in lower ammonia, which was attributed to the extensive utilization of ammonia by fibrolytic bacteria when diet contained BP (Mojthaedi and Danesh Mesgaran, 2011). In contrast, others authors found similar ammonia concentrations in lambs fed concentrates including or not BP (Bodas et al., 2007).

Overall, VFA concentration decreased over time despite the higher concentrate intake, suggesting an increased absorption due to the adaptation of the rumen epithelium. Further, no effect of adaptation protocol was found on VFA concentration or on proportions of individual VFA despite differences in ingredient composition of the three diets. In agreement with our results, Rotger et al. (2006) and Gimeno et al. (2015) did not report differences between barley and corn diets in beef cattle; however, Surber and Bownman (1998) found higher VFA production (and higher butyrate and lower propionate) with barley than with maize, even with identical DMI between diets. In dairy cows, Casper et al. (1999) reported greater VFA concentration in corn-fed cows, while

Khorasani et al. (2001) reported higher concentrations with barley, which was attributed to its higher degradability. The inconsistency among experiments might be explained by differences in varieties of these cereals, the processing method, and the forage to concentrate ratio. In previous studies, partially replacing barley with BP resulted in lower VFA concentrations (Bodas et al., 2007; Mojtahedi and Danesh Mesgaran, 2011). Sugar beet pulp is known by its acetogenic capacity (Marounek et al., 1985) and thus most studies reported increased acetate/propionate ratio (Mahjoubi et al., 2009; Maktabi et al., 2016). Others have also reported higher butyrate, and authors speculated about the capacity of BP to stimulate butyrate-producing microbiota (Mahjoubi et al., 2009; Mojtahedi and Danesh Mesgaran, 2011).

**Rumen microbial populations**

Regardless the applied dietary protocol, at the end of transition, a well-established protozoa population persisted in the rumen of all animals despite the abrupt dietary change from milk and grass to concentrate. These findings support those of Towne et al. (1990) and Hristov et al. (2001) who observed that, although diversity decreased, total protozoal concentration was not affected, and a consistent protozoa population was present in steers fed high-concentrate diets.

In agreement with other reports of rumen bacterial composition in beef cattle, we observed *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* to be the dominant phyla (Fernando et al., 2010; Petri et al., 2013). In our study, the *Firmicutes*/*Bacteroidetes* ratio fluctuated over time, and declined on day 30 compared to day 0, which is consistent with results reported by Fernando et al. (2010) since *Bacteroidetes* are usually more abundant with high-concentrate diets. A *Proteobacteria* ratio above 0.19 suggests an unstable microbial community (Auffret et al., 2017), and was detected on day 14 in two calves in Protocol 1 in which *Proteobacteria* increased up to 42% and 19% of the total population, respectively. Unbalanced microbial populations can be associated with acidotic events. Probably, that might be the reason of those transient *Proteobacteria* abundances, since one of these calves showed a relatively high VFA concentration (155 mmol/L), and the other showed a high lactate concentration (7.05 mmol/L) before the morning feeding. In addition, another animal in Protocol 2 had a 64% abundance of *Actinobacteria*, a rumen pH of 5.79 and a high VFA concentration (257 mmol/L) at 0 h on day 30, likely indicating a transient acidotic episode. Other authors have found *Proteobacteria* and *Actinobacteria* to increase after an acidotic

challenge (Petri et al., 2013). It is acknowledged that the rumen as an ecosystem responds to dietary changes with an ecological succession of compositional changes that develop more tolerant bacteria under the new environment (Weimer, 2015). In the present study, changes in bacterial composition as well as in the rumen fermentation variables paralleled the higher supply of fermentable substrates (mainly starch) favouring the growth of amylolytic and acid-tolerant bacteria (Goad et al., 1998). For instance, the introduction of concentrates promoted the appearance of some bacteria, such as *Bifidobacterium*, *Lactobacillus, Roseburia* and *Sharpea* on day 10. Interestingly, these genera (and others that also increased during transition, such as *Prevotella*, *Selenomonas*, and *Anaerovibrio*) were positively correlated with butyrate, propionate, VFA, and lactate, and negatively with rumen pH. Conversely, the genera *Butyrivibrio, Coprococcus,* and *Pseubutyrivibrio,* and the phyla *Cloroflexi, Synergistetes,* and *Tenericutes* decreased during the transition, and were positively correlated with rumen pH, and negatively with VFA and lactate.

Genera richness and Shannon index deeply decreased after 10 days of concentrate inclusion as recorded by other authors during the transition from a forage to a high-concentrate diet (Petri et al., 2013; Nagata et al., 2018); however, the Shannon index slightly increased from day 14 until the end of the study. Along with the recovery of the abundances of some genera to the initial values, the increase of the Shannon index may suggest an adaptation and development of some genera that are tolerant to the new rumen environment. This is in agreement with the results reported by Nagata et al. (2018), who studied the effect of four consecutive SARA challenges on rumen microbiota and diversity in Holstein bulls, and reported that in the forth challenge diversity indexes were greater than in the first one. This might refer to the resilient capacity of the rumen microbiota (Weimer, 2015). Together with the lack of effect of diet on bacterial population or on rumen fermentation, the inconsistency of some correlations between rumen fermentation and bacterial taxa across diets might suggest that bacteria can shift their metabolic pathways depending on the substrates available without altering the final products (Taxis et al., 2015). This feature refers to the principle of the functional redundancy of the rumen bacteria (Taxis et al., 2015; Weimer, 2015).

Most taxa reported in our 'core microbiome' agreed with those previously reported by other authors (Petri et al., 2013). Although the bacterial taxa that composed the rumen core were not equally abundant and some genera in the core represented as low as 0.01% of total bacterial community, the fact that were found in every animal across sampling

days suggests that are important for rumen function as already pointed out by Jami and Mizrahi (2012).

Overall, the most interesting finding obtained in the current experiment was the lack of effect of the different adaptation protocols. All groups responded similarly to the transition without impaired effects in terms of animal performance during the whole trial, ruminal fermentation, and rumen microbiota. Further, given the lack of differences in intake and ADG during the transition, and the price of the concentrates (268, 271 and 292 €/ton for C, BP10 and BP22, respectively), under practical farm management it does not seem necessary to provide an adaptation concentrate and follow an adaptation protocol since no subsequent improvements or economic profit are achieved.

## Conclusions

Collectively, our observations demonstrate that an abrupt transition from milk and pasture regimen to a high-concentrate diet did not greatly impair animal performance or rumen health of beef calves. Animals can compensate for the higher acidogenic capacity of the diet increasing the straw intake, and it seems that the resilience of the rumen microbiota and the stability of the core microbes are key points to prevent the detrimental effects on rumen fermentation, and thus on animal performance. The use of barley or maize as the prevailing cereal or the inclusion of BP have no effect on rumen fermentation or microbial populations of beef calves, and their use will depend on market price or availability.

# CHAPTER 6

# EXPERIMENT 3

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This chapter has been splitted for publication as follows:

-Rumen protozoal dynamics during the transition from milk/grass to high-concentrate based diet in beef calves as affected by the addition of tannins or medium-chain fatty acids. Animal Feed Science and Technology. 2019, 257: 114273. doi: 10.1016/j.anifeedsci.2019.114273.

-Growth, rumen fermentation and bacterial dynamics of beef calves during transition from milk and pasture to a high-concentrate diet added with tannins or medium-chain fatty acids (Under Review in Animal Production Science).

# CHAPTER 6: EXPERIMENT 3

## Growth, rumen fermentation and microbial dynamics of beef calves during transition from milk and pasture to a high-concentrate diet added with tannins or medium-chain fatty acids

**Abstract**

The effects of the addition of tannins and medium-chain fatty acids (MCFA) to the adaptation diet from milk and pasture to a high-concentrate ration on feed intake, animal performance, rumen fermentation and rumen microbial composition were studied in eighteen Limousine crossbred beef calves. Calves of 7 months (212 ± SD 27.0 kg) were abruptly weaned and transitioned from a milk/grass regime to one of these diets: a non-supplemented high-energy diet consisting of a cereal-based concentrate plus wheat straw, both given *ad libitum* (C), C plus 20 g/kg of a 65:35 chestnut and quebracho tannin extract (T), and C plus 6 g/kg of a commercial mixture of MCFA (M). The experiment lasted 28 days. Concentrate and straw intake, and their rate of intake were recorded. Rumen fluid was collected on days 0, 1, 7, 14, 21 and 28 at 0, 3, 6 and 9 h after feeding to characterize fermentation. Samples from 0 h were analysed to assess rumen protozoa and bacterial population. Feed intake of concentrates (P=0.95), straw (P=0.48), and their rate of intake throughout the day were similar (P>0.05) among diets, and animals showed similar (P>0.05) ADG and final LW. Rumen fermentation variables were not affected by diet (P>0.05) but differed over time in response to the concentrate inclusion. Rumen protozoal population was not affected by the addition of additives (P>0.05) and changed over time. Diversity decreased towards a population with mainly and *Entodinium* and *Isotricha* species. The addition of tannins or MCFA did not modify (P=0.98) the general structure of the bacterial community, which was affected by sampling day (P<0.001). Similarly, the inclusion of additives did not affect relative abundances of the main bacterial taxa (P<0.05), most of them differing across days (P<0.001). There was a decrease (P<0.01) of phyla *Firmicutes*, *Bacteroidetes*, *Fibrobacteres* and *Tenericutes*, and an increase of *Proteobacteria* and *Actinobacteria* on day 28 compared to day 0. Diversity indexes (Shannon and Richness) declined over sampling days (P<0.05), although some genera such as *Bifidobacterium* and *Lactobacillus* appeared after concentrate inclusion. Tannins and MCFA, at the doses used in the present experiment, did not exert any effect on intake, animal performance, rumen fermentation and microbial population. However, transition from milk and pasture to a high-concentrate diet modified the rumen environment and the

general structure of the bacterial community. Although diversity decreased, some genera emerged indicating a possible selection of more tolerant bacteria to the new environmental conditions.

## Introduction

Under the conventional intensive beef production in some Mediterranean countries, calves are abruptly weaned at an age of 6 months and immediately switched from a milk and/or high-forage regime to a high-concentrate diet. Initially, a 14-21 day transition period is applied, in which calves receive a compound feed to stimulate the rumen papillae development and to allow microbial adaptation to the fattening concentrate. Ruminal fermentation of these diets, rich in readily fermentable carbohydrates, yields a large amount of VFA and lactate, which may lead to an accumulation of fermentation products if the rate of acid production exceeds acid removal from the rumen. This ruminal imbalance increases the risk of a marked drop in rumen pH below 5.0 or 5.6, level generally considered as threshold for acute or subacute ruminal acidosis, respectively (Owens et al. 1998; Krause and Oetzel, 2006). In such situation, it is of great interest to slow down the rate of fermentation and maintain the rumen pH above the mentioned threshold.

The use of feed additives to modulate the rate and extent of microbial fermentation is common to mitigate the consequences of dietary shift during transition period (González et al., 2012). Tannins (Baah et al., 2007; Amanzougarene et al., 2019) and certain MCFA (Matsumoto et al., 1991; Hristov et al., 2004a) have been assessed for their potential to interact with rumen microbiota modifying the rumen environment (Henderson, 1973; Makkar, 2003) and slowing down rumen fermentation, thus preventing the onset of acidotic episodes. The information on the effect of tannins is not consistent since their biological activity widely depends not only on their polyphenolic nature or plant sources (Amanzougarene et al., 2019), but also on the basal diet fed to the animals (Mueller-Harvey, 2006). Reports on the effects of tannins in high-concentrate diets fed to beef cattle are limited and results on rumen metabolism and animal performance are inconclusive (Krueger et al., 2010; Mezzomo et al., 2011). Additionally, studies on the effects of tannin inclusion on rumen microbial composition under this type of diets are scarce (Vasta et al., 2019), especially with beef calves during the transition period.

With regard to MCFA, these fatty acids have also been evaluated for their potential to interact with bacteria (Henderson, 1973) and modulate rumen fermentation (Amanzougarene et al., 2017b). These feed additives have been extensively assessed for their inhibition of rumen methanogenesis (Machmüller, 2006). Most studies have been carried out with lauric acid ($C_{12}$) or with coconut oil (CO) as a source of MCFA, both *in vitro* (Dong et al., 1997; Patra and Yu, 2013) and *in vivo* (Yabuuchi et al., 2007; Hristov et al., 2012) with adult cattle (steers or dairy cows). However, there are no studies during the adaptation of newly weaned beef calves. Moreover, a great deal of studies has been carried out *in vitro* focusing on the effect of other MCFA (*e.g.* caprylic -$C_8$ or capric-$C_{10}$) on rumen metabolism (Dohme et al., 2001). The *in vitro* experimental approach is not representative of *in vivo* dosing and rumen conditions (Soto et al., 2012; Martínez-Fernández et al., 2013), and the possible adaptation of microbiota might not be assessed. In any case, findings on the effects of MCFA on rumen fermentation and microbes diverge among studies due to the high variety of fatty acids and sources of oils, doses and animal diets, as well as chemical forms of MCFA (Liu et al., 2011; Hristov et al., 2012).

On the other hand, rumen microbial shifts after concentrate inclusion may affect the ability of the host to better adapt to the new diet. The rumen protozoa contribute to slow down rumen starch digestion by ingesting starch granules and store them as amylopectin, which reduces starch availability for bacteria and thus prevents its rapid fermentation (Williams and Coleman, 1992; Jouany and Ushida, 1999). In addition, entodiniomorphid protozoa actively contribute to the ruminal metabolism of lactic acid (Newbold et al., 1987), reducing the risk of rumen pH to decline. Some studies in cattle fed high-concentrate diets have reported a reduction or even absence of rumen protozoa (Eadie et al., 1970; Lyle et al., 1981); however, others have found a considerable protozoa population in feedlot cattle (Towne et al., 1990; Franzolin and Dehority, 1996). The role of the rumen protozoa during the transition period may be of importance and there are no studies assessing protozoa populations in the rumen of calves during the transition to a high-concentrate diet.

In view of the paucity of *in vivo* studies carried out with beef calves during the transition period from milk and pasture to a high-concentrate feeding, and the inconclusive results obtained with the use of tannins and MCFA, the first objective of this study was to assess the effect of the inclusion of tannins and MCFA in the adaptation diet of beef calves on feed intake, growth performance, rumen fermentation and rumen

microbial composition. Further, we aimed to study the rumen protozoal dynamics after the transition from milk and pasture diet to a high-concentrate ration. We hypothesized that tannins and MCFA would interact with rumen microbiota and might attenuate rumen fermentation decreasing the risk of acidosis without greatly affect availability of energy by the host animal when added to a high-concentrate diet. In addition, since high-concentrate diets seem to reduce protozoal population we hypothesized that calves that had been abruptly weaned and introduced to a high-concentrate diet have reduced protozoal population in a sense that can affect rumen fermentation towards an acidification of the environment and the risk of acidosis.

## Material and methods

### Animals, dietary treatments and experimental design

Animal care, handling and surgical procedures were approved by the Ethics Committee of the University of Zaragoza. The care and management of animals were performed according to the Spanish Policy for Animal Protection RD 53/2013, which meets the EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Eighteen 7-month old (212 ± SD 27.0 kg and 224 ± 54.3 d) Limousine crossbreed male calves from different Spanish locations (seven from Extremadura, six from Castilla y León, four from Cantabria and one from Asturias) were used. Animals were reared with their dams on pasture and allowed to suckle freely while they were grazing. Then, calves were abruptly weaned and transported to the Servicio de Experimentación Animal (University of Zaragoza) where they received grass hay (g/kg: organic matter, OM 901; crude protein, CP 112; neutral detergent fibre, NDF 571; acid detergent fibre, ADF 343; lignin 45) *ad libitum* for their adaptation to the farm environmental conditions. Animals were individually housed in a 3.4 x 3.4 m pens provided with slatted concrete floor, automatic water dispenser and separate troughs for concentrate and roughage. One week after their arrival, calves were fistulated in the dorsal sac of the rumen with a 150-mm long, 15-mm i.d. permanent cannula. Animals were allowed to recover from surgery for two weeks, and therefore the experiment began three weeks after the arrival of the animals.

The experiment was carried out for 28 days, and thereafter animals were kept in the experimental facilities and fed on a common fattening compound feed until they reached a target LW of *ca*. 500 kg. On the first day of the experiment, calves were abruptly

switched to a ration consisting of a cereal and soybean meal-based concentrate (Table 6.1) in meal form (ground through a 3.5-mm sieve) plus wheat straw (offered in long form), both given *ad libitum*. The three dietary treatments were: a non-supplemented diet, considered as a control (C); C plus 20 g/kg (as fed) of a commercial 65:35 chestnut (*Castanea* spp.) and quebracho (*Schinopsis* spp.) tannin extract containing over 65% of tannins (Vinitanon, Agrovin, Spain; T); and C plus 6 g/kg (as fed) of a commercial mixture of MCFA (OptimaPLUS, Nutrika, Belgium; M). Analysed proportions of fatty acids in M were: caproic acid, 10%; caprylic acid, 20%; capric acid, 20%; and lauric acid, 50%. Dose and source of tannins extract were chosen based on *in vitro* results (Amanzougarene et al., 2019) as well as on market availability and price. Dose of the MCFA additive, commonly recommended for dairy cows or adult beef cattle but with no indications for calves during the transition period, was selected according to the statement of the manufacturer for the former animals. These stated doses had also been previously studied *in vitro* (Amanzougarene et al., 2017b).

Six calves were randomly allocated to each experimental diet, ensuring homogeneous average LW and SD per group. Concentrate was offered *ad libitum* once a day at 08:00 h, with the ration daily adjusted to ensure at minimum 10% refusals. Straw was offered three times daily to ensure *ad libitum* access, intending to keep at least 0.5 kg straw at any time in the feeder. The amounts of concentrate and straw offered and refused were daily recorded throughout the experiment. Weekly samples of both concentrate and straw were taken for chemical analysis. Animals were weighed at the beginning of the experiment and then weekly prior to feed distribution. The ADG was calculated as the regression coefficient of individual LW on time. The feed conversion ratio (FCR) was calculated as the ratio between the total amount of DMI (concentrate plus straw) and weight gained throughout the study.

The rate of intake of concentrate and straw was measured on days 6, 13, 20 and 27, recording concentrate intake at two-hour intervals from 8:00 to 20:00 h, and from 20:00 to 8:00 h of the next day. Rate of intake of straw was recorded every four hours from 8:00 to 20:00 h and from 20:00 to 08:00 h of the next day. Trying to reduce the effect of a likely contamination of the refusals with saliva, a correction of the DM actually ingested in each measuring interval was carried out on the following basis: the difference between the feedstuff DM content and that of the refusals (both measured in the week corresponding to each pattern of intake measurement) was divided by 24, and the result multiplied by the number of hours in each measuring interval. The value obtained for

each interval was assumed to represent the decrement to be applied to the DM content of the concentrate and straw. When the daily sum of the DM theoretically consumed in each interval applying this correction differed more than ± 5% compared to the recorded daily DMI, all the values obtained for the different intervals of that day were considered as missing data. When the DMI recorded at a determined interval was negative, the value was considered a missing datum.

**Table 6.1** Ingredient and chemical composition of diets. Composition of wheat straw is also included.

| | Concentrates | | | Wheat |
|---|---|---|---|---|
| | C | T | M | straw |
| Ingredients (as fed basis), g/kg | | | | |
| Barley | 590 | 572 | 587 | |
| Maize | 151 | 148 | 150 | |
| Corn gluten feed (200g CP/kg) | 57 | 56 | 57 | |
| Soybean meal (470 g CP/kg) | 172 | 169 | 171 | |
| Palm oil | 9.3 | 9.1 | 9.3 | |
| Urea | 0.6 | 0.6 | 0.6 | |
| Calcium carbonate | 8.5 | 8.3 | 8.4 | |
| Dicalcium phosphate | 5.0 | 4.9 | 5.0 | |
| Sodium chloride | 5.0 | 4.9 | 5.0 | |
| Vitamin-mineral premix[1] | 2.0 | 2.0 | 2.0 | |
| OptimaPLUS (Nutrika, Belgium) | - | - | 6 | |
| Vinitanon (Agrovin, Spain) | - | 20 | - | |
| Nutrient composition (g/kg DM) | | | | |
| OM | 945 | 947 | 947 | 937 |
| CP | 164 | 168 | 169 | 22 |
| EE | 33 | 29 | 32 | 12 |
| Starch | 471 | 484 | 500 | 5 |
| NDF | 155 | 149 | 157 | 814 |
| ADF | 49 | 46 | 47 | 473 |
| Lignin | 4 | 3 | 3 | 42 |

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre. [1] Vitamin-mineral premix declared composition (per kg): 4x106 IU vitamin A, 0.8x106 IU vitamin D3, 5x103 IU alpha-tocopherol, 100 g sepiolite, 150 mg etoxiquin, 10 g Mn oxide, 7.5 g Fe carbonate, 20 g Zn oxide, 250 mg K-iodate, 300 mg Co, 2.5 g Cu, and 500 mg Na selenite, 10 mg antioxidants.

Rumen fluid samples were obtained at 0 h (before feeding) on day 0, and at 0, 3, 6 and 9 h after feeding on days 1, 7, 14, 21 and 28. Since on day 0 we only collected rumen fluid at 0 h, two different statistical analyses were conducted: one with all 0 h over all sampling days to study the effect of the concentrate inclusion, and another with all days and sampling intervals except day 0 to study the fermentation pattern of the different

diets. About 200 mL were removed from the rumen of each animal on each sampling time, using a customized vacuum device connected to a 0.6-cm i.d. semi-rigid tube with 2-mm pores. Representative samples were taken moving the tube in all directions inside the rumen while sampling. For the analysis of the rumen bacterial population, a 5-mL subsample was taken and immediately frozen in liquid nitrogen at -80ºC molecular analyses of bacterial DNA was carried out. For protozoa quantification, 2-mL subsamples were taken with a wide-mouth (3 mm) pipette, diluted in an equal volume of 18.5% formaldehyde and kept at room temperature until the protozoal counts were performed. Then, rumen fluid was strained through a 1-mm pore size metal mesh sieve, and rumen pH was immediately measured using a portable pH-meter (model Seven2GO, Mettler-Toledo AG, Schwerzenbach, Switzerland). Three 4-mL aliquots of rumen fluid were taken in duplicate for ammonia (in 4 mL 0.1 N HCl), lactate and volatile fatty acids (VFA; in 1-mL solution made up with 2% orthophosphoric acid and 0.2% 4-methyl valeric acid) analysis. Samples were frozen (-20 ºC) until further analyses.

**Chemical analyses**

Concentrate and straw refusals were collected daily and fortnightly, respectively, pooled on an animal basis and weighed weekly and fortnightly. Dry matter of feeds and refusals was determined by oven drying at 65ºC to a constant weight. Feeds were analysed following the procedures of AOAC (2005) for their OM (ref. 942.05), CP (ref. 976.05) and ether extract (ref. 2003.05) content. Concentration of NDF was analysed using an Ankom 200 Fiber Analyzer (Ankom Technology, New York) as described by Mertens (2002); both ADF and lignin in sulphuric acid were determined as described by Robertson and Van Soest (1981). The NDF is expressed exclusive of residual ashes, and α–amylase was used in the analysis; sodium sulphite was not used. Total starch content was determined enzymatically in samples ground to 0.5 mm by using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland).

The concentration of VFA in rumen samples, and the concentration of individual MCFA in the additive, were determined by gas chromatography in an Agilent 6890 apparatus (Agilent Technologies España S.L., Madrid, Spain) fitted with a capillary column (Model HP-FFAP polyethylene glycol TPA-treated, 30m x530µm i.d. x1µm film thickness). Ammonia and total lactate concentrations were measured colorimetrically following the methods proposed by Chaney and Marbach (1962), and Barker and Summerson (1941), respectively.

**Microbial analyses**

Rumen protozoal quantification was performed on rumen liquid samples from 0 h of days 0, 7, 14, 21 and 28 by optical observation using a 10x eyepiece with x10 and x40 objectives through a microscope (Axiolab, Carl Zeiss Jena, Germany) in a Sedgewick-Rafter counting chamber following the procedure of Dehority (1993), except for those cases with extremely low protozoal numbers, when the entire chamber was counted. Genera and some species were identified as outlined by Ogimoto and Imai (1981), and Dehority (1993). During each counting, the numbers of different genera in the protozoal population were recorded, identified and grouped into the genera *Isotricha* and *Dasytricha* from the Family *Isotrichidae*; genus *Epidinium* from the Subfamily *Ophryoscolecinae*; genus *Entodinium* from the Subfamily *Entodiniinae*; and genera *Diplodinium, Metadinium, Eudiplodinium, Enoploplastron, Ostracodinium* and *Polyplastron* from the Subfamily *Diplodiniinae*. Total protozoal and groups concentration for each calf were calculated and transformed into logarithmic basis ($log_{10}$/mL) before being subjected to statistical analysis.

For bacterial analysis, rumen liquid samples taken before feeding (0 h) on days 0, 14, 21 and 28 were chosen for sequencing analyses using Ion Torrent Next Generation Sequencing. Samples were freeze-dried, thoroughly mixed and disrupted (Mini-Bead Beater, Biospec Products, Bartlesville, OK, USA). The microbial 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 maximize bacterial cell lysis. Concentration and purity of extracted DNA was tested in Nanodrop ND-1000 (Nano-Drop Technologies, Inc., Wilmington, DE, USA). Sequencing of the 16S rRNA gene was conducted following the procedure described by de la Fuente et al. (2014) and Schauf et al. (2018). Briefly, the bacterial V1-V3 hypervariable region was amplified by PCR using barcoded fusion primer pairs 27F and 338R (Wang et al., 2014). Sequencing of the PCR products was performed in the Ion Torrent Personal Genome Machine system (Life Technologies, Carlsbad, CA, US) using the Ion Personal Genome Machine Sequencing 200 kit v2 (Life Technologies, Carlsbad, CA). Following sequencing, data were combined and sample identification numbers assigned to multiplexed reads using Ion Reporter™ 5.10. Software (Thermo Fisher Scientific). Sequencing amplicon reads were subjected to trimming, denoising, and chimera removal and clustered into Operational Taxonomic Units at 97% identity using

UPARSE pipeline (Edgar, 2013). Reads were subjected to quality filtering (quality score of 20 in a 1 to 40 scale) and trimmed at a maximum length of 250 bp. Taxonomic assignation of 16S rRNA sequences was established by comparison against the Ribosomal Data Project II database (Cole et al., 2003), considering a bootstrap value of 0.80 for annotation, leaving successive taxon levels as unclassified. To maximize the comparability across samples, the number of reads was manually normalised to the sample with the lowest number of reads (15,935). Number of reads of each microbial taxon were $log_{10}$-transformed (nº of reads + 1) prior to statistical analysis to assume normality; however, for a better interpretation, relative abundances of different bacterial taxa are presented as percentage (mean± SEM) in the results section. Three measures of diversity were calculated: β-diversity, Shannon index and genera richness using R software (v.3.5.3 R Core Team, 2014).

**Statistical analyses**

Analyses of concentrate and straw DMI, the rate of intake of concentrate and straw, ADG, rumen fermentation variables, protozoa concentration, bacterial taxa abundance (excluding those with relative abundances lower than 0.1% of total sequences), different ratios between phyla, and diversity indexes were analysed as repeated measures with the MIXED procedure of SAS (v.9.4 SAS Inst. Inc., Cary NC, US) considering the diet, sampling day and their interaction as fixed effects, and animal within diet as random effect. Sampling time after feeding within day or sampling day were used as repeated measure, as appropriate. Analysis of final LW, total concentrate DMI and FCR were carried out with the PROC MIXED with diet as the fixed effect and animal as random effect. Initial age and LW were included in the model as covariates for analysis of DMI, final LW and ADG.

Polynomial (linear, quadratic and cubic) contrasts were established using the CONTRAST statement of SAS in order to assess the evolution of protozoa population over the experimental period. Absence of total protozoa or any protozoal group was considered as zero in the statistical analyses. Results for the relative abundance of protozoal groups is presented as arithmetic means with their standard deviation. For both bacterial and protozoal abundance, treatment means were compared under a conservative Bonferroni adjustment. In all statistical analyses, the variance-covariance structure was selected based on the lowest Akaike Information Criterion.

For sequencing data, multivariate statistical analyses were performed using the package "vegan" from the R statistical program. To determine the impact of the diet and time on overall microbial community structure, a non-parametric permutational multivariate analysis of variance (PERMANOVA) was conducted based on the Bray-Curtis dissimilarity. Differences in bacterial composition among diets as well as among sampling days were assessed using analysis of similarity (ANOSIM). Non-metric multi-dimensional scaling (NMDS) plot was generated representing the Bray-Curtis distance. To evaluate differences in community structure (β-diversity), an ANOVA was conducted on the distances to the centroids for each day or in each diet.

Spearman correlation coefficients were calculated using the CORR procedure of SAS to assess the relationships between the ruminal fermentation characteristics at hour 0 and either the protozoal concentration, the log-transformed data of the main bacterial taxa concentrations or different ratios between phyla and diversity indexes. For rumen protozoa and bacterial population, only Spearman correlations with $r>0.30$ or $r<-0.3$ and $P<0.05$ are shown (Belanche et al., 2019a). For all data, differences were considered significant if $P<0.05$, whereas differences were considered to indicate a trend to significance when $0.05 \leq P \leq 0.10$.

## Results

### Feed intake and animal performance

No treatment differences were found in concentrate or straw intakes (Table 6.2) throughout the experiment ($P>0.05$); although they respectively increased and decreased over time ($P=0.01$). Consequently, proportion of concentrate increased ($P<0.001$) from day 14 to day 28, although again no differences among treatments were found ($P=0.67$). Total DMI during the whole experiment did not differ among groups ($P=0.55$) and averaged 153 ± 7.98 (SEM) kg.

**Table 6.2** Average dry matter (DM) intake (kg/d) of concentrate (from 0 to 28 days) and straw (from 0 to 28 days), and concentrate proportion of total dry matter intake (%) of beef calves given a non-supplemented diet (C); C plus 20 g/kg of a chestnut and quebracho tannin extract (T); and C plus 6 g/kg of a mixture of medium-chain fatty acids (M) during the adaptation period to a high-concentrate feeding.

| | | Diet | | | | | P- value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | C | T | M | Average | SEM | D | S | DxS |
| Concentrate | 0-14 d | 4.37 | 3.89 | 3.90 | 4.05 | 0.221 | 0.56 | 0.002 | 0.32 |
| (kg DM/d) | 15-28 d | 4.72 | 4.7 | 4.97 | 4.79 | 0.305 | | | |
| Straw | 0-14 d | 0.90 | 0.78 | 0.69 | 0.79 | 0.069 | 0.88 | 0.006 | 0.83 |
| (kg DM/d) | 15-28 d | 0.52 | 0.51 | 0.44 | 0.49 | 0.053 | | | |
| Concentrate | 0-14 d | 82.7 | 83.2 | 84.2 | 83.3 | 1.33 | 0.67 | <0.001 | 0.98 |
| in the ration (%) | 15-28 d | 89.4 | 90.1 | 91.8 | 90.4 | 1.04 | | | |

[1] Probability effects for Diet (D), Sampling day (S) and their interaction (DxS)

The rate of intake of concentrate, pooled on 4-h intervals for an easier comparison with results of rate of intake of straw is given in Figure 6.1. There were no differences among treatments (P=0.80) or sampling days (P=0.56). However, it was affected by time interval after feeding (P<0.001), and calves showed the highest intake of concentrate during the first 4 h after feeding consuming on average 30% of the concentrate. Similarly, the rate of intake of straw was neither affected by diet (P=0.83) nor by day (P=0.25). However, it was affected by sampling time interval (P<0.001) and calves exhibited the highest straw intake 8 to 12 h after feeding (Figure 6.1), averaging 32% of total straw intake.

The ADG during the transition period was not affected by the inclusion of additives (P=0.98) and averaged 0.85 ± 0.073 kg/d throughout the trial, increasing over time (P<0.001) from 0.78 ± 0.038 kg/d on the first week to 0.95 ± 0.053 kg/d on the last week of the experiment. Likewise, no diet effect was found on final LW (248 ± 4.24 kg, P=0.83) or on FCR (4.48 ± 0.038, P=0.87).

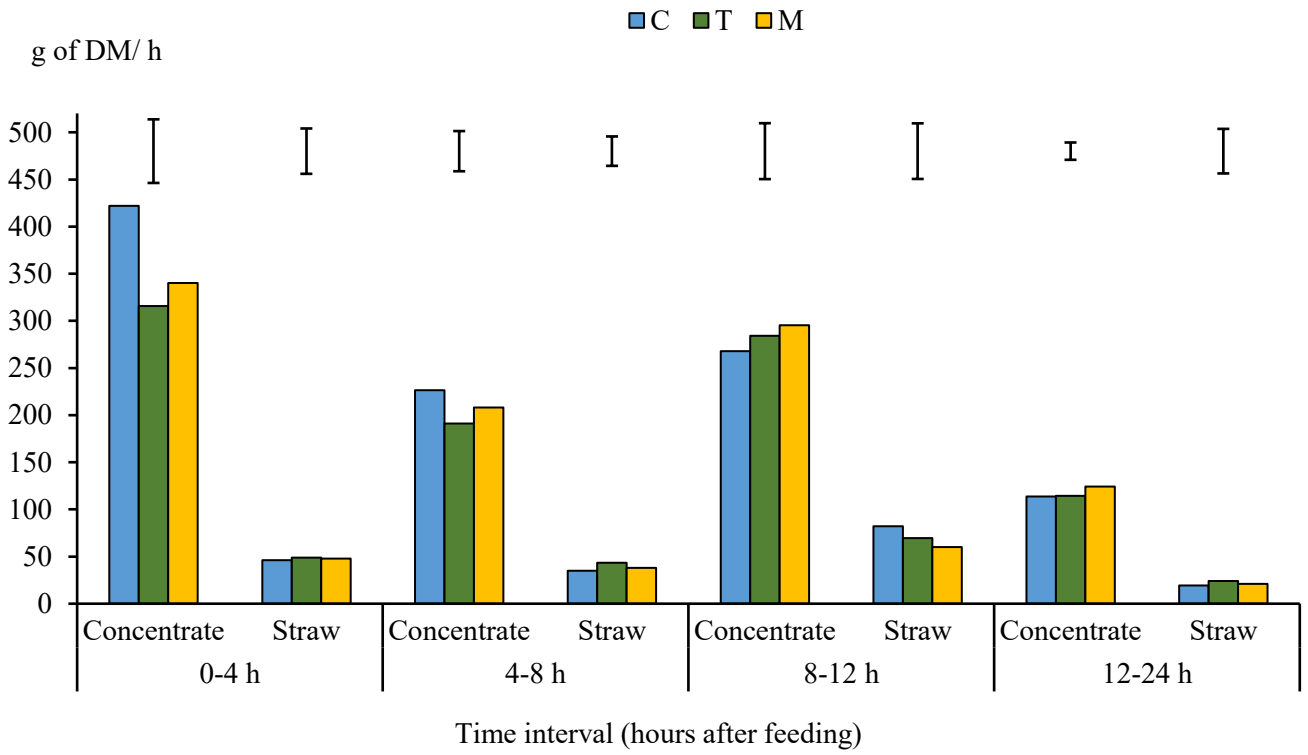


**Figure 6.1** Average intake of concentrate and straw of beef calves fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) when the rate of intake was recorded. Upper bars represent the standard error to the means.

**Rumen fermentation variables**

Analyses of hour 0 of all sampling days showed that concentrate inclusion, regardless the diet, modified the rumen environment (Table 6.3). Rumen pH was unaffected by diet ($P=0.16$) but decreased over time ($P<0.001$). Even though there were no differences among diets on rumen lactate concentration on days 0 and 7, values in C calves became the highest on day14 with no differences between T and M, and those differences disappeared thereafter (interaction diet x time, $P=0.03$). Ruminal ammonia concentration was unaffected by diet or by sampling day ($P>0.10$). Total VFA concentration was higher ($P=0.03$) in calves given diet C than in those given diet T, with intermediate values for animals on M diet. Total VFA increased on day 28 ($P<0.001$) with respect to the previous dates. Molar VFA proportions were not affected by diet ($P>0.10$), but all of them except propionate ($P=0.13$) varied among sampling dates ($P<0.05$). Except for day 28, butyrate, valerate and branched-chain fatty acids (BCFA) proportions increased at the expense of acetate.

**Table 6.3** Rumen pH, lactate (mmol/L), ammonia (mg/L), total volatile fatty acids (VFA) concentration (mmol/L) and molar VFA proportions (mol/100 mol) before feeding (0 h) of beef calves given different diets (a non-supplemented diet, C; C plus 20 g/kg of a chestnut and quebracho tannin extract, T; and C plus 6 g/kg of a mixture of medium-chain fatty acids, M) during the adaptation period to a high-concentrate feeding.

| | Diet (D) | | | | Sampling day (S) | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C | T | M | SEM | 0 | 7 | 14 | 21 | 28 | SEM | D | S | DxS |
| pH | 6.51 | 6.66 | 6.54 | 0.056 | 6.92$^{a}$ | 6.63$^{b}$ | 6.57$^{b}$ | 6.44$^{cb}$ | 6.30$^{c}$ | 0.072 | 0.16 | <0.001 | 0.17 |
| Lactate | 0.65 | 0.54 | 0.55 | 0.066 | 0.15 | 0.54 | 0.75 | 0.61 | 0.84 | 0.071 | 0.43 | <0.001 | 0.03 |
| Ammonia | 82.5 | 81.6 | 80.2 | 9.08 | 88.6 | 85.8 | 95.8 | 81.9 | 56.0 | 10.95 | 0.98 | 0.11 | 0.66 |
| VFA | 87.9$^{a}$ | 73.8$^{b}$ | 77.1$^{ab}$ | 3.93 | 80.7$^{b}$ | 70.0$^{b}$ | 73.8$^{b}$ | 72.5$^{b}$ | 100.8$^{a}$ | 5.08 | 0.035 | <0.001 | 0.32 |
| Acetate | 57.3 | 58.2 | 53.6 | 1.57 | 68.3$^{a}$ | 52.9$^{bc}$ | 54.1$^{bc}$ | 49.4$^{c}$ | 57.2$^{b}$ | 1.84 | 0.12 | <0.001 | 0.79 |
| Propionate | 20.7 | 19.1 | 23.9 | 1.62 | 18.7 | 21.2 | 20.3 | 24.7 | 21.4 | 1.71 | 0.14 | 0.13 | 0.87 |
| Butyrate | 16.0 | 16.7 | 15.9 | 1.16 | 9.3$^{b}$ | 18.7$^{a}$ | 18.5$^{a}$ | 18.7$^{a}$ | 15.8$^{a}$ | 1.42 | 0.89 | <0.001 | 0.5 |
| Valerate | 1.6 | 1.8 | 2.3 | 0.03 | 0.8$^{c}$ | 1.5$^{b}$ | 2.4$^{a}$ | 2.5$^{a}$ | 2.4$^{a}$ | 0.21 | 0.12 | <0.001 | 0.06 |
| BCFA | 4.4 | 4.2 | 4.2 | 0.53 | 2.9$^{c}$ | 5.7$^{a}$ | 4.8$^{ab}$ | 4.7$^{ab}$ | 3.2$^{bc}$ | 0.64 | 0.93 | 0.013 | 0.93 |

$^{a, b,c}$ Different letters within a row indicate differences between diets or days at P<0.05.

Analyses of the evolution of rumen fermentation variables (0, 3, 6 and 9 h after feeding) throughout the experiment showed that all variables (except rumen pH and lactate concentration) were affected ($P<0.05$) by sampling time interval. The interaction diet by sampling day or diet by time interval was not significant ($P>0.10$) in any case. Average daily rumen pH was unaffected by the addition of feed additives ($P=0.59$) and varied over time ($P<0.001$), and both minimum and maximum rumen pH did not differ among groups ($P>0.10$). Rumen pH daily variation (standard deviation) did not differ among treatments ($P=0.73$) or sampling days ($P=0.51$). Lactate concentration was unaffected by diet or day ($P>0.05$). Ammonia concentration was similar between diets ($P=0.34$) and decreased over time ($P<0.01$). The inclusion of feed additives did not affect ($P>0.05$) TVFA concentration or molar proportions of the main VFA, which all varied across sampling days ($P<0.05$). The hourly evolution of rumen fermentation variables for the days 1, 14 and 28 is shown in Table 6.4 in order to provide information about the fermentability of the diets throughout the experiment, although all groups responded similarly during the transition period. Of the sampling hours, hour 6 was chosen as representing the maximum fermentation since the lowest rumen pH and the highest VFA concentration were found at that time. Again, no effect of diet was found in any of the rumen characteristics at hour 6 (Table 6.5) and a significant effect of the sampling day was found in all variables except for lactate and propionate.

**Table 6.4** Daily evolution (0, 3, 6 and 9 h after feeding) of rumen fermentation variables in the rumen fluid of beef calves fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 65% of tannins, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) during the transition to a high-concentrate diet.

| | | Day 1 | | | | | Day 14 | | | | | Day 28 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet | 0 h | 3 h | 6 h | 9 h | SEM | 0 h | 3 h | 6 h | 9 h | SEM | 0 h | 3 h | 6 h | 9 h | SEM | P-value[1] |
| Rumen pH | C | 5.72 | 6.06 | 5.92 | 5.90 | 0.110 | 6.30 | 6.45 | 6.65 | 6.71 | 0.098 | 6.28 | 6.14 | 6.04 | 5.96 | 0.090 | 0.28 |
| | T | 6.21 | 5.97 | 5.85 | 5.88 | | 6.66 | 6.47 | 6.47 | 6.53 | | 6.52 | 6.18 | 6.16 | 6.30 | | |
| | M | 6.30 | 6.17 | 6.14 | 6.04 | | 6.75 | 6.54 | 6.48 | 6.51 | | 6.11 | 6.09 | 6.25 | 5.84 | | |
| Lactate (mmol/L) | C | 0.39 | 0.41 | 0.52 | 0.37 | 0.354 | 1.07 | 1.20 | 1.08 | 1.00 | 0.740 | 0.79 | 0.86 | 1.53 | 1.37 | 0.218 | 0.53 |
| | T | 1.86 | 1.58 | 0.99 | 1.70 | | 0.64 | 1.12 | 0.86 | 0.84 | | 0.75 | 0.98 | 0.84 | 0.95 | | |
| | M | 0.25 | 0.45 | 1.23 | 2.38 | | 0.55 | 0.65 | 0.68 | 0.52 | | 0.97 | 1.00 | 1.52 | 1.91 | | |
| Ammonia (mg/L) | C | 54 | 157 | 153 | 174 | 17.9 | 95 | 114 | 93 | 79 | 13.4 | 60 | 74 | 73 | 49 | 10.7 | 0.83 |
| | T | 80 | 88 | 93 | 88 | | 97 | 86 | 84 | 72 | | 52 | 53 | 44 | 41 | | |
| | M | 61 | 130 | 144 | 160 | | 95 | 87 | 72 | 47 | | 56 | 64 | 55 | 46 | | |
| VFA (mmol/L) | C | 111 | 106 | 113 | 87 | 7.0 | 89 | 90 | 83 | 74 | 5.9 | 123 | 113 | 103 | 132 | 8.1 | 0.95 |
| | T | 83 | 105 | 117 | 109 | | 70 | 72 | 79 | 74 | | 86 | 109 | 99 | 119 | | |
| | M | 94 | 112 | 108 | 111 | | 63 | 82 | 89 | 87 | | 94 | 111 | 113 | 124 | | |

[1] Probability for the interaction diet by hour within day.

**Table 6.4** Daily evolution (0, 3, 6 and 9 h after feeding) of rumen fermentation variables in the rumen fluid of beef calves fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 65% of tannins, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) during the transition to a high-concentrate diet. (Continued)

| | | Day 1 | | | | | Day 14 | | | | | Day 28 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Diet | 0 h | 3 h | 6 h | 9 h | SEM | 0 h | 3 h | 6 h | 9 h | SEM | 0 h | 3 h | 6 h | 9 h | SEM | P-value[1] |
| Acetate | C | 55.4 | 53.9 | 57.7 | 50.9 | 1.71 | 53.7 | 53.4 | 50.7 | 48.8 | 1.85 | 60.5 | 51.2 | 51.2 | 60.2 | 2.29 | 0.96 |
| (mol/100 mol) | T | 52.1 | 55.1 | 57.6 | 49.6 | | 48.7 | 51.9 | 48.3 | 55.7 | | 59.8 | 57.7 | 57.8 | 64.3 | | |
| | M | 54.0 | 60.7 | 59.1 | 53.3 | | 52.8 | 53.8 | 53.5 | 51.4 | | 51.4 | 52.6 | 56.7 | 61.1 | | |
| Propionate | C | 27.7 | 27.4 | 25.3 | 28.0 | 1.48 | 20.7 | 21.0 | 21.9 | 22.8 | 0.84 | 19.1 | 24.3 | 24.8 | 19.5 | 2.09 | 0.99 |
| (mol/100 mol) | T | 31.3 | 29.0 | 27.2 | 31.0 | | 16.9 | 21.0 | 20.4 | 21.6 | | 21.6 | 22.4 | 22.1 | 18.9 | | |
| | M | 29.5 | 25.0 | 26.4 | 30.3 | | 23.3 | 23.3 | 23.3 | 24.5 | | 23.4 | 24.5 | 22.6 | 18.9 | | |
| Butyrate | C | 13.8 | 15.2 | 14.1 | 17.5 | 1.09 | 18.4 | 17.9 | 19.1 | 19.7 | 1.81 | 15.7 | 18.8 | 18.3 | 16.3 | 1.31 | 0.73 |
| (mol/100 mol) | T | 13.2 | 13.1 | 13.0 | 16.7 | | 20.8 | 23.3 | 21.7 | 23.5 | | 13.4 | 15.1 | 15.0 | 13.0 | | |
| | M | 12.5 | 11.3 | 11.5 | 13.3 | | 16.3 | 15.9 | 16.7 | 17.5 | | 18.1 | 17.0 | 15.3 | 15.0 | | |

[1] Probability for the interaction diet by hour within day.

**Table 6.5** Rumen pH, lactate (mmol/L), ammonia (mg/L), total volatile fatty acids (VFA) concentration (mmol/L) and molar VFA proportions (mol/100 mol) 6 h after feeding of beef cattle fed different diets (a non-supplemented diet, C; C plus 20 g/kg of a commercial 65:35 chestnut and quebracho tannin extract containing over 65% of tannins, T; and C plus 6 g/kg of a commercial mixture of medium-chain fatty acids, M) during the transition to a high-concentrate diet.

| | | Sampling day (S) | | | | | | P-value | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Diet (D) | 1 | 7 | 14 | 21 | 28 | SEM | D | S | DxS |
| Rumen pH | C | 5.92$^{c}$ | 6.64$^{a}$ | 6.65$^{a}$ | 6.48a$^{b}$ | 6.04$^{bc}$ | 0.176 | 0.95 | <0.001 | 0.42 |
| | T | 5.85$^{c}$ | 6.87$^{a}$ | 6.47$^{ab}$ | 6.27$^{bc}$ | 6.16$^{bc}$ | | | | |
| | M | 6.14$^{ab}$ | 6.56$^{a}$ | 6.48$^{ab}$ | 6.08$^{b}$ | 6.25$^{ab}$ | | | | |
| Lactate | C | 0.52 | 1.57 | 1.08 | 0.89 | 1.53 | 1.297 | 0.27 | 0.56 | 0.64 |
| (mmol/L) | T | 0.99 | 4.76 | 0.86 | 3.79 | 0.84 | | | | |
| | M | 1.23 | 0.77 | 0.68 | 1.13 | 1.52 | | | | |
| Ammonia | C | 153$^{a}$ | 74$^{b}$ | 93$^{b}$ | 60$^{b}$ | 73$^{b}$ | 21.2 | 0.34 | <0.01 | 0.48 |
| (mg/L) | T | 93 | 67 | 84 | 43 | 44 | | | | |
| | M | 144$^{a}$ | 75$^{b}$ | 72$^{b}$ | 78$^{b}$ | 55$^{b}$ | | | | |
| VFA | C | 113$^{a}$ | 92$^{ab}$ | 83$^{ab}$ | 76$^{b}$ | 103$^{ab}$ | 11.8 | 0.6 | <0.001 | 0.92 |
| (mmol/L) | T | 117$^{a}$ | 70$^{b}$ | 80$^{b}$ | 69$^{b}$ | 99$^{ab}$ | | | | |
| | M | 108$^{ab}$ | 79$^{b}$ | 89$^{ab}$ | 86$^{ab}$ | 113$^{a}$ | | | | |
| Acetate | C | 57.6$^{ab}$ | 59.9$^{a}$ | 50.7$^{bc}$ | 48.1$^{c}$ | 51.2$^{bc}$ | 3.14 | 0.77 | <0.01 | 0.2 |
| | T | 57.5$^{a}$ | 59.1$^{a}$ | 51.9$^{ab}$ | 47.4$^{b}$ | 57.8$^{a}$ | | | | |
| | M | 59.1$^{a}$ | 48.3$^{b}$ | 53.5$^{ab}$ | 49.0$^{b}$ | 56.6$^{ab}$ | | | | |
| Propionate | C | 25.3 | 17.3 | 21.8 | 27.5 | 24.7 | 3.04 | 0.24 | 0.07 | 0.69 |
| | T | 27.2 | 17.0 | 20.3 | 22.2 | 22.1 | | | | |
| | M | 26.4 | 25.8 | 23.2 | 27.2 | 22.6 | | | | |
| Butyrate | C | 14.1 | 15.7 | 19.1 | 18.0 | 18.2 | 2.47 | 0.52 | <0.01 | 0.33 |
| | T | 12.9$^{b}$ | 17.8$^{ab}$ | 21.6$^{a}$ | 23.6$^{a}$ | 14.9$^{b}$ | | | | |
| | M | 11.5$^{b}$ | 20.1$^{ab}$ | 16.7$^{ab}$ | 16.1$^{ab}$ | 15.2$^{ab}$ | | | | |

$^{a, b,c}$ Different letters within a row indicate differences between sampling days at $P$<0.05.

**Rumen protozoal counts**

Average rumen protozoal concentration was 5.82 log cells/mL although fluctuations were observed throughout the experimental period. Individually, eight calves (four from diet C, three from T and one from M) maintained a stable protozoal population on time with a concentration over 5 log cells/mL, whereas another two animals (both from diet M) were defaunated (absent of protozoa) on days 7 and 14, but were recolonized after one or two weeks reaching consistent concentrations thereafter. The interaction between diet and sampling day showed a trend for an effect on protozoal concentration across the trial (P=0.07; Figure 6.2); thus, on days 7 and 14, animals on diet M had a lower protozoal concentration whereas no differences were found between the other diets (6.11, 6.34 and 4.12 log cells/mL on day 7, and 6.63, 6.52 and 4.82 log cells/mL on day 14, for diets C, T and M, respectively).

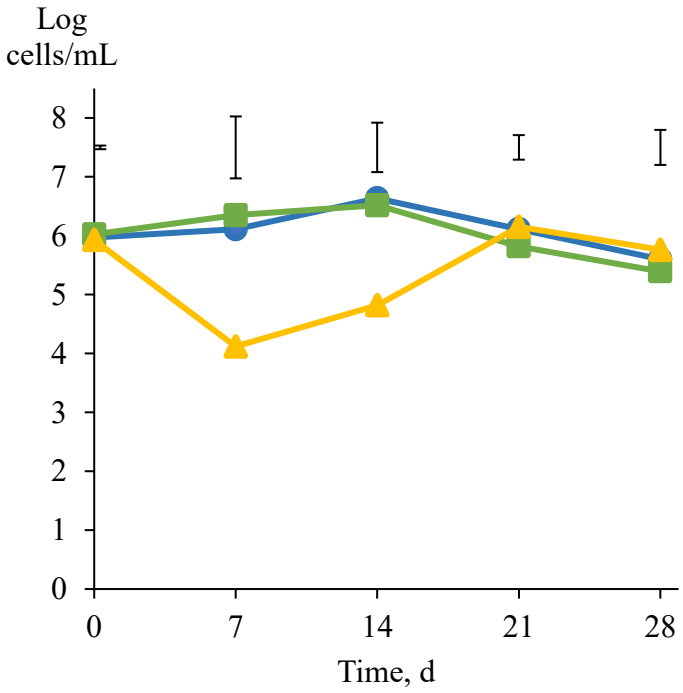


**Figure 6.2** Rumen protozoa concentration (log cells/mL) in beef calves during the adaptation period to a high-concentrate ration without supplement (C: ●), with 2 g/kg of tannin extract (T: ■), or with 6 g/kg of medium-chain fatty acids mixture (M: ▲). Days 0, 7, 14 and 28 after concentrate inclusion. Upper bars show the standard error of the means.

Protozoal concentration in group M followed a cubic response (P=0.018) across the trial; the marked drop in concentration found in this group on days 7 and 14 was mainly due to the aforementioned defaunation of two animals, and to low concentrations in another two calves (2.82 log cells/mL in one animal on day 7, and 3.06 log cells/mL in

the other calf on day 14). A low rumen protozoal concentration (below 4 log cells/mL) was also observed in one animal from diet T on day 21, and in one animal per treatment on day 28.

The concentration pattern of the different protozoal groups throughout the study is shown in Figure 6.3. Differences in concentrations of *Isotricha* among diets were observed over time (interaction diet x sampling day, P=0.04); thus, in C calves tended to follow a quadratic evolution (P=0.09), in T calves it showed a linear decrease (P<0.001) and in M a sharp quadratic decrease (P=0.03). In contrast, concentration of *Dasytricha*, which on day 0 showed a relative abundance over 20% of total protozoa in six out of 18 calves, was quadratically (P=0.001) reduced, and subsequently disappeared from the rumen of all calves from days 14 (C), 21 (M) and 28 (T). No dietary differences were observed for *Dasytricha* concentration. Despite *Entodinium* spp. concentration across diets behaved similarly than that for total protozoa on day 7 and day 14, *Entodinium* spp. were not affected by diet (P=0.16) and maintained average concentrations between 5.35 and 6.63 log cells/mL. Despite its constant concentration throughout the experiment (P=0.70), relative abundance of *Entodinium* widely differed among calves, ranging from below 40% in three calves to over 70% in another four animals on day 0, and increasing to over 90% of total protozoa from day 7 onwards (Table 6.6).

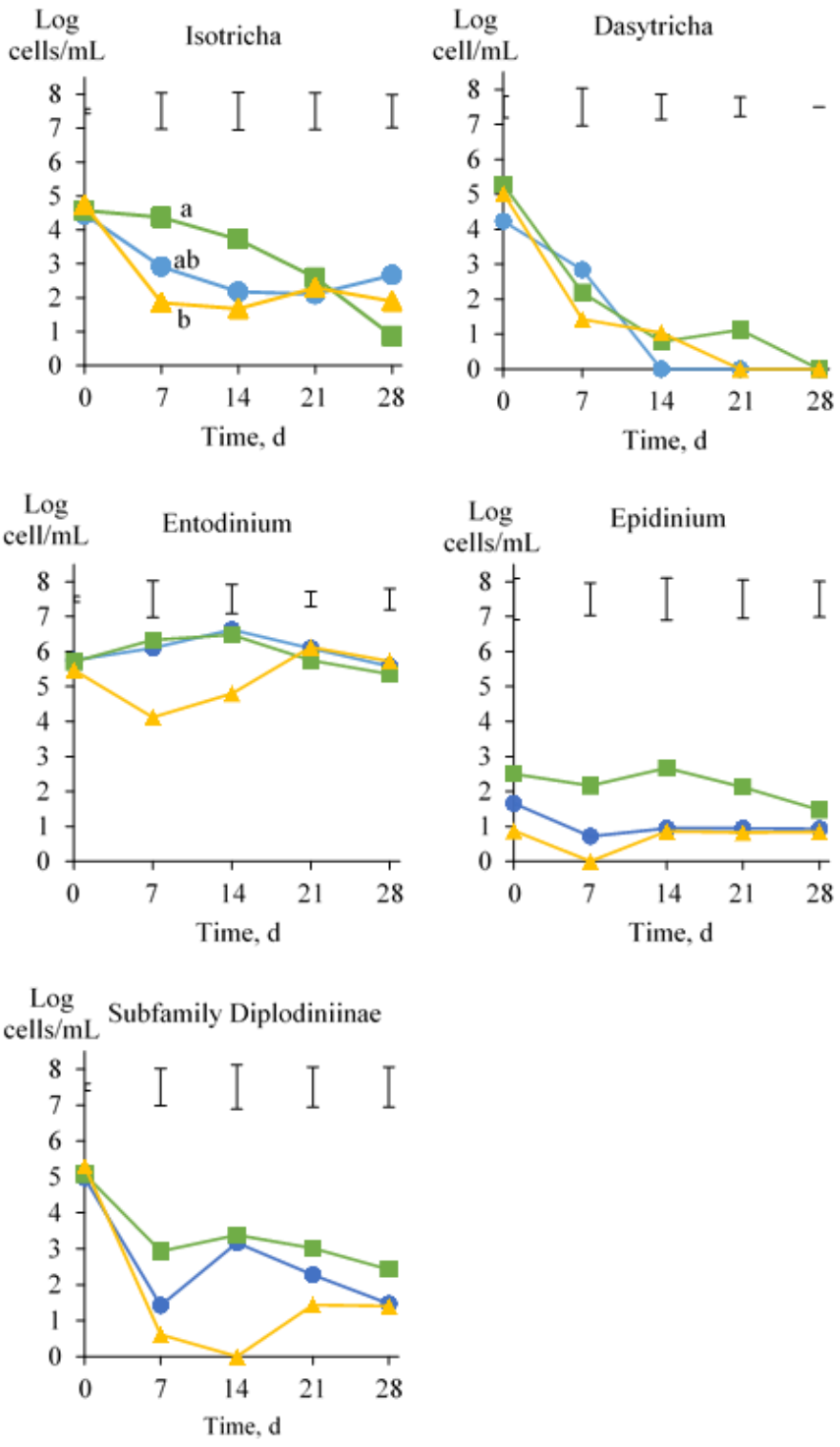


**Figure 6.3** Ruminal concentration of protozoal groups (*Isotricha*, *Dasytricha*, *Entodinium*, *Epidinium* and *Subfamily Diplodiniinae*) in beef calves during the adaptation period to a high concentrate ration without supplement (C: ●) with 2 g/kg of tannin extract (T: ■), or with 6 g/kg of medium-chain fatty acids mixture (M: ▲). Days 0, 7, 14 and 28 after concentrate inclusion. a, b: Different letters indicate statistical differences at P<0.05. Upper bars show the standard error of the means.

Initially (day 0), the genus *Epidinium* spp. was detected in only six animals in low (from 0.83 to 2.67 log cells/mL) but relatively constant concentrations (Figure 6.3) and remained present in four out of 18 calves on day 28 (Table 6.6). No effects of diet (P=0.45) or sampling day (P=0.20) were found. Protozoa from six genera of the Subfamily *Diplodiniinae* (*Diplodinium, Metadinium, Eudiplodinium, Enoploplastron, Ostracodinium* and *Polyplastron)* were detected in all animals at the beginning of the experiment, with an average concentration of 5.11 log cells/mL. This group of protozoa was not affected by diet (P=0.14) but changed over time (average values of 1.65, 2.18, 2.24 and 1.77 log cells/mL on day 7, 14, 21 and 28, respectively; P< 0.001), remaining present in only seven out of the 18 calves at the end of the trial (Table 6.6). It is worth mentioning that, in most cases, the presence of protozoa of this group was supported by the genera *Polyplastron* and *Eudiplodinium*, whereas *Metadinium* and *Enoploplastron* completely disappeared from day 7. At the end of the study (day 28), only *Polyplastron* (in five calves), *Eudiplodinium* (in three calves) and *Diplodinium* (in one calf) were detected.

**Table 6.6** Relative abundance (%)of the main groups of protozoa (±SD) and number of harbouring beef calves (out of 18) given different diets (a non-supplemented diet ,C; C plus 20 g/kg of a chestnut and quebracho tannin extract, T; and C plus 6 g/kg of a mixture of medium-chain fatty acids, M) during the adaptation period to a high-concentrate feeding.

| **Day** | ***Isotricha*** | ***Dasytricha*** | ***Entodinium*** | ***Epidinium*** | ***Subfamily Diplodiniinae*** |
|---|---|---|---|---|---|
| 0 | 5.0 (±3.01) | 19.9 (±13.81) | 53.7 (±20.34) | 13.1 (±10.77) | 18.2 (±10.61) |
| n | 18 | 17 | 18 | 6 | 18 |
| 7 | 1.4 (±1.42) | 0.5 (±0.36) | 98.1 (±2.01) | 1.5 (±0.97) | 0.4 (±0.32) |
| n | 12 | 9 | 16 | 4 | 7 |
| 14 | 0.9 (±0.88) | 4.3 (±5.50) | 95.5 (±4.81) | 7.2 (±4.76) | 2.3 (±1.98) |
| n | 10 | 3 | 17 | 5 | 8 |
| 21 | 3.2 (±5.01) | 6.1 (±7.54) | 92.8 (±10.43) | 11.3 (±3.64) | 3.2 (±4.86) |
| n | 10 | 2 | 18 | 5 | 9 |
| 28 | 8.6 (±7.23) | - | 93.2 (±7.59) | 6.6 (±5.62) | 2.7(±4.65) |
| n | 9 | 0 | 18 | 4 | 7 |

A wide and diverse protozoal community was observed in weaned calves at the start of the experiment, with protozoal diversity (Types A and B; Eadie, 1962) partly depending on the origin of calves. The four calves from Cantabria had a Type B population including *Entodinium*, holotrichs (*Isotricha* and *Dasytricha*), *Diplodinium* and *Ostracodinium* species, and *Eudiplodinium* and/or *Epidinium* together or separately, whereas that from Asturias (which is a neighbour region) had Type A protozoa, containing *Entodinium* and holotrichs species plus *Polyplastron multivesiculatum* as the predominant large entodiniomorph. Moreover, calves from Extremadura and from Castilla y León harboured either Type A or Type B protozoa. Although most of the initially observed protozoal species were present in all animals on day 0, some degree of specificity occurred, and *Metadinium affine* and *Epidinium caudatum* were only present in the seven calves from Extremadura, whereas *Entodinium rostratum* was present in all animals except in those from this region; similarly, *Ostracodinium mammosum* and *Eudiplodinium dilobum* were found in the six animals from Castilla y León, and *Epidinium parvicaudatum* was only present in the four animals from Cantabria.

Even though species description was not the main objective of this work, up to 26 protozoal species were identified at the start of the experiment (day 0). *Isotricha* spp. (mostly *I. prostoma*) was present in all animals, and *Dasytricha ruminantium* was detected in 17 calves. Six *Entodinium* species were identified (the number of calves harboring those species is showed in brackets): *E. nanellum* (18), *E. longinucleatum* (11), *E. exiguum* (10), *E. dubardi* (10), *E. caudatum* (9) and *E. rostratum* (7), as well as three *Epidinium species*: *E. eucaudatum* (6), *E. caudatum* (3) and *E. parvicaudatum* (3). Among the Subfamily Diplodiniinae, three *Diplodinium* (*D. dentatum*, 5; *D. monolobosum*, 4; and *D. lobatum*, 3); three *Eudiplodinium* (*E. bovis*, 7; *E. maggi*, 7; and *E. dilobum*, 5); six *Ostracodinium* (*O. gracile*, 7; *O. obtusum*, 2; *O. rugoloricatum*, 2; *O. mammosum*, 2; and *O. trivesiculatum*, 1); two *Metadinium* (*M. medium*, 4 and *M. affine*, 3); *Enoploplastron triloricatum* (4) and *Polyplastron multivesiculatum* (12) were observed.

Significant correlations were observed between concentrations of some protozoal groups and some rumen fermentation variables (Table 6.7). No relationships were established for *Dasytricha* and *Epidinium* because of the low number of data pairs (31 and 24, respectively, out of 90 possible counts). Spearman's correlation coefficients were relatively low, because of the major importance of the bacterial role in fermentation. Total protozoa and *Entodinium* were positively correlated with lactate (not for *Entodinium*),

ammonia concentration and BCFA (P<0.01). The Subfamily *Diplodiniinae* was positively correlated with pH (P<0.001), and negatively with butyrate, valerate and lactate (P<0.01). Similarly, a relationship between the proportion of straw in the ration and total protozoa concentration was found (r= 0.43, P<0.01).

**Table 6.7** Spearman's correlations (r) between concentrations of different protozoal groups and of rumen fermentation variables. Only Spearman correlations with r>0.30 r<-0.3 and P<0.05 are shown

| | n | Rumen pH | Lactate | Ammonia | Butyrate | Valerate | BCFA |
|---|---|---|---|---|---|---|---|
| Total protozoa | 87 | | 0.38 | 0.35 | | | 0.48 |
| *Entodinium* | 87 | | | 0.32 | 0.30 | | 0.48 |
| Subf. Diplodiniinae | 50 | 0.54 | -0.39 | | -0.35 | -0.62 | |

No relationships were established between *Dasytricha* or *Epidinium* and any fermentation parameters because of the low number of data pairs (31 and 24, respectively).

**Rumen bacterial populations**

The sequences obtained from 16S rRNA gene were assigned to 67 genera belonging to 217 families from 21 different phyla. High individual variability was found among calves (CV from 21% to 302%). Collectively, four phyla represented more than 97% of the total sequences (Figure 6.4): *Bacteroidetes* (43.5 ± 1.21%), *Firmicutes* (38.3 ± 1.49%), *Proteobacteria* (11.3 ± 1.98%), and *Actinobacteria* (4.35 ± 0.80%), which, with other seven phyla, represented on average 99.9% of total sequences. Of the two major phyla, *Bacteroidetes* was dominated by a single genus (*Prevotella*), while *Firmicutes* comprised several families and genera.

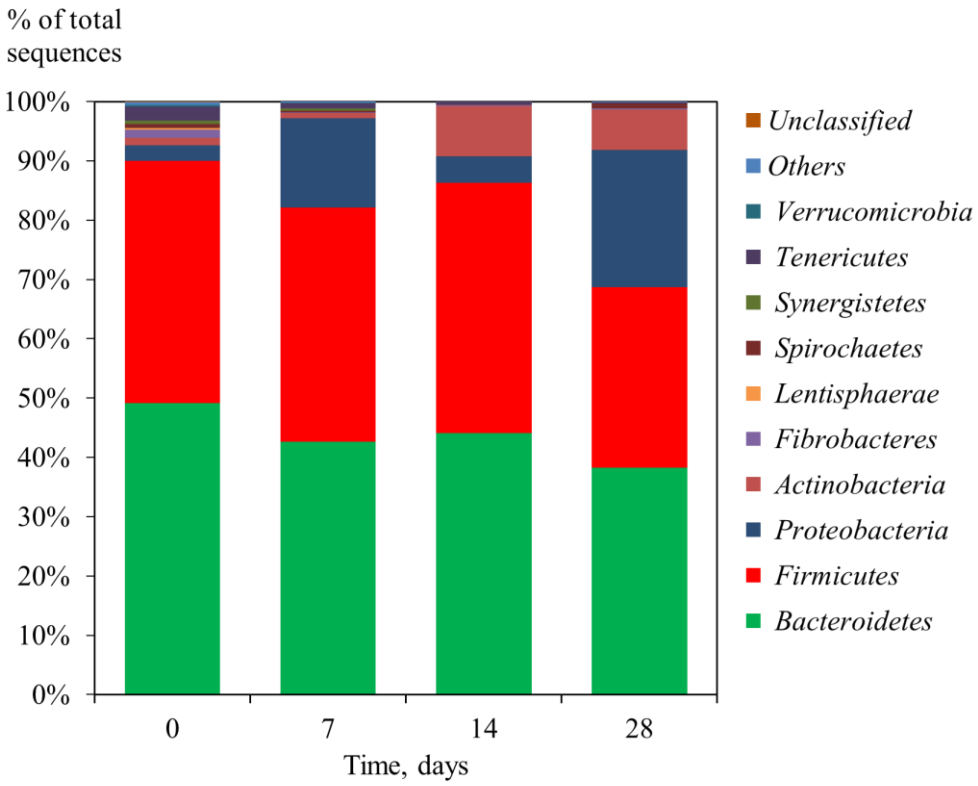


**Figure 6.4** Structure of the bacterial community (% of total sequences) at phyla level of beef calves fed different diets during the transition from milk and forage to a high-concentrate diet. Days 0, 7, 14 and 28 after concentrate inclusion. Diet effect was not significant in any case (P>0.05)

The PERMANOVA showed no effect of feed additives (P=0.92, $R^2$=0.007) whereas day of sampling exerted a significant effect on overall microbial composition (P<0.001; $R^2$=0.32). Likewise, ANOSIM revealed no differences among treatments (P=0.97; R=-0.03) and a significant effect of time (P<0.001; R=0.40). The NMDS plot exhibited that bacterial communities did not cluster by diet (Figure 6.5A). However, it revealed a slight clustering by sampling days separating days 0 and 7, while samples from days 14 and 28 exhibited higher heterogeneity (Figure 6.5B). Analysis of β-diversity, which is the variation in composition among samples within each factor (*i.e.* diet and day) as measured by the distance to the centroid, showed that dispersion within each dietary group was homogeneous (P=0.12). However, dispersion to the centroid differed among

days (P=0.011), and the pairwise comparison showed that dispersion of day 0 *vs.* day 28 differed significantly.

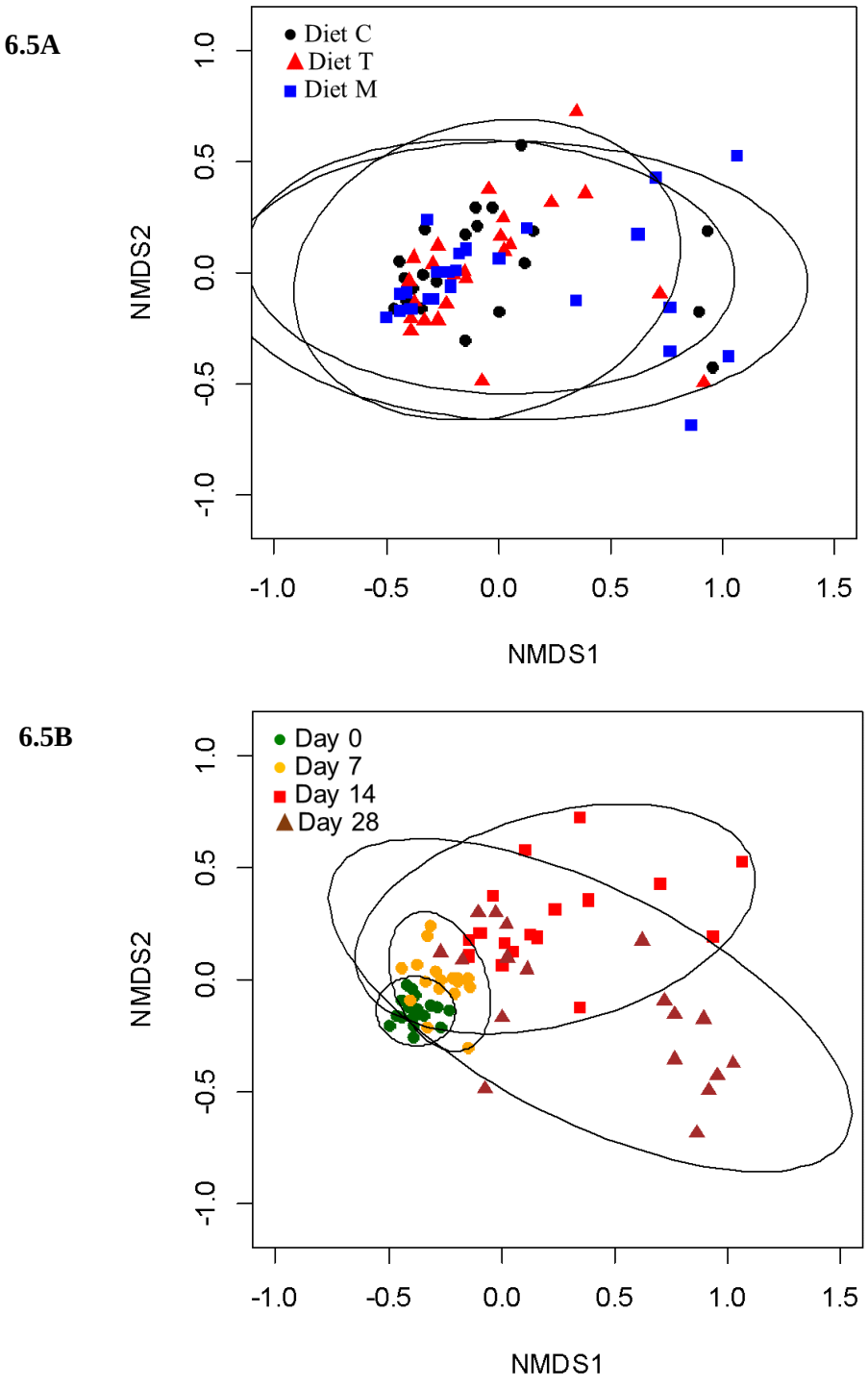


**Figure 6.5** Non-metric multidimensional scaling (NMDS) ordination plot based on Bray-Curtis dissimilarity showing relationship among bacterial populations in rumen of beef calves fed different diets (A; for diet composition see Table 6.1) and in different sampling days (B) during the transition from milk and forage to a high-concentrate diet.

Table 6.8 shows the data of the bacterial community of calves expressed as $\log_{10}$ of the number of reads +1. The addition of tannins or MCFA did not show any effect on the abundance of the most analysed taxa or diversity indexes (P>0.10), therefore only differences among days are commented. Comparing days 0 *vs*. 28, the most noticeable change was the decrease of relative abundance of the two major phyla (*Bacteroidetes* and *Firmicutes*) with an increase of *Proteobacteria* and, to a lower extent, of *Actinobacteria*. In addition, the minor phyla *Chloroflexi*, *Spirochetes*, *Synergistetes* and *Tenericutes* also decreased (P<0.001) at the end of the study. Relative abundance of *Proteobacteria* differed across sampling days showing the highest numerical values on day 28 (23 ± 5.0%) (P<0.001). Individual relative abundance of this phylum ranged from 0.58% to as high as 64% of the total sequences. The ratio *Proteobacteria* (calculated as the abundance of *Proteobacteria* sequences divided by the sum of the abundance of *Firmicutes* and *Bacteroidetes* sequences) was constant until day 14, increasing on the last sampling day (day 28; P<0.001; average value of 0.48; Table 6.8). The ratio *Firmicutes*/*Bacteroidetes* remained stable over time (P=0.18), and was not affected by diet (P=0.54). One week after concentrate inclusion, the most noticeable change compared to day 0 was an increase of the abundance of *Proteobacteria* from 2.57% to 15.1%, with a significant increase of the genus *Ruminobacter* within this phylum (from <0.1% to 6.32%); also, there was a manifest increase of *Streptococcus* on day 7 (from 0.28% to 5.28%) that recovered initial abundance thereafter. Genera *Bifidobacterium*, *Lactobacillus* and *Sharpea* emerged 14 d after concentrate inclusion irrespectively of diet. Compared to day 0 when animals had been fed only on milk and pasture, at the end of the study there was a different genera composition irrespectively of the tannins or MCFA inclusion; for instance, there was a substantial (P<0.001) increase of *Succinivibrio, Bifidobacterium, Lactobacillus, Roseburia, Anaerovibrio, Sharpea,* and a decrease (P<0.001) in *Butyrivibrio, Pseudobutyrivirio, Clostridium, Coprococcus*, and *Fibrobacter*. Shannon index and genera richness were only affected by sampling day and decreased over time (P<0.001).

**Table 6.8** Effect of the diet and day of sampling on the structure of the bacterial community, and on diversity indexes of beef calves fed different diets (C, T and M; for diet composition see Table 6.1) during the transition period (28 d) from milk and pasture diet to a high concentrate ration.

| | | | Diets | | | | Day | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Familiy | Genus | C | T | M | SEM | 0 | 7 | 14 | 28 | SEM | Diet | Day | DietxDay |
| *Actinobacteria* | | | 2.52 | 2.59 | 2.38 | 0.094 | 2.30$^{bc}$ | 2.08$^{c}$ | 2.93$^{a}$ | 2.69$^{ab}$ | 0.108 | 0.30 | <0.001 | 0.98 |
| | *Coriobacteriaceae* | | 2.14 | 2.20 | 2.09 | 0.092 | 2.14 | 2.03 | 2.25 | 2.17 | 0.107 | 0.70 | 0.520 | 0.84 |
| | | *Olsenella* | 1.66 | 1.92 | 1.78 | 0.119 | 1.61 | 1.62 | 1.95 | 1.98 | 0.137 | 0.31 | 0.102 | 0.96 |
| | | *Atopobium* | 1.09 | 1.06 | 1.06 | 0.132 | 1.55$^{a}$ | 1.32$^{ab}$ | 0.40$^{c}$ | 1.01$^{b}$ | 0.130 | 0.98 | <0.001 | 0.51 |
| | *Bifidobacteriaceae* | | 1.31 | 1.30 | 1.15 | 0.137 | 0.03$^{b}$ | 0.06$^{b}$ | 2.73$^{a}$ | 2.19$^{a}$ | 0.158 | 0.64 | <0.001 | 0.79 |
| | | *Bifidobacterium* | 1.21 | 1.18 | 1.03 | 0.110 | 0.03$^{c}$ | 0$^{c}$ | 2.68$^{a}$ | 1.86$^{b}$ | 0.128 | 0.48 | <0.001 | 0.70 |
| *Bacteroidetes* (B) | | | 3.81 | 3.83 | 3.85 | 0.022 | 3.89$^{a}$ | 3.82$^{ab}$ | 3.83$^{ab}$ | 3.77$^{b}$ | 0.025 | 0.55 | 0.012 | 0.97 |
| | *Prevotellaceae* | | 3.66 | 3.66 | 3.70 | 0.028 | 3.62$^{b}$ | 3.66$^{ab}$ | 3.75$^{a}$ | 3.67$^{ab}$ | 0.030 | 0.57 | 0.017 | 0.98 |
| | | *Prevotella* | 2.73 | 2.78 | 2.80 | 0.079 | 2.57$^{b}$ | 2.76$^{ab}$ | 2.95$^{a}$ | 2.79$^{ab}$ | 0.091 | 0.83 | 0.045 | 0.73 |
| *Chloroflexi* | | | 0.77 | 0.70 | 0.86 | 0.091 | 1.64$^{a}$ | 1.10$^{b}$ | 0.22$^{c}$ | 0.15$^{c}$ | 0.105 | 0.45 | <0.001 | 0.60 |
| *Fibrobacteres* | | | 0.98 | 1.27 | 1.35 | 0.135 | 2.26$^{a}$ | 0.85$^{b}$ | 1.03$^{b}$ | 0.64$^{b}$ | 0.156 | 0.14 | <0.001 | 0.20 |
| | *Fibrobacteraceae* | | 0.98 | 1.27 | 1.35 | 0.135 | 2.27$^{a}$ | 0.85$^{b}$ | 1.03$^{b}$ | 0.64$^{b}$ | 0.156 | 0.14 | <0.001 | 0.20 |
| | | *Fibrobacter* | 0.85 | 1.14 | 1.20 | 0.130 | 2.15$^{a}$ | 0.67$^{b}$ | 0.91$^{b}$ | 0.54$^{b}$ | 0.150 | 0.13 | <0.001 | 0.29 |
| *Firmicutes* (F) | | | 3.76 | 3.76 | 3.77 | 0.030 | 3.81$^{a}$ | 3.7$^{a}$ | 3.81$^{a}$ | 3.63$^{b}$ | 0.033 | 0.99 | <0.001 | 0.70 |
| | *Clostridiaceae* | | 2.98 | 3.05 | 2.95 | 0.051 | 3.19$^{a}$ | 3.03$^{a}$ | 2.98$^{ab}$ | 2.77$^{b}$ | 0.056 | 0.38 | <0.001 | 0.96 |
| | | *Clostridium* | 1.94 | 1.87 | 1.86 | 0.079 | 2.05$^{a}$ | 2.01$^{a}$ | 2.13$^{a}$ | 1.38$^{b}$ | 0.085 | 0.75 | <0.001 | 0.26 |
| | *Eubacteriaceae* | | 2.44 | 2.50 | 2.41 | 0.067 | 2.78$^{a}$ | 2.63$^{a}$ | 2.08$^{b}$ | 2.32$^{b}$ | 0.077 | 0.62 | <0.001 | 0.98 |
| | | *Eubacterium* | 1.57 | 1.56 | 1.63 | 0.156 | 2.00$^{a}$ | 1.60$^{a}$ | 1.16$^{b}$ | 1.59$^{a}$ | 0.128 | 0.95 | <0.001 | 0.78 |
| | *Lachnospiraceae* | | 3.16 | 3.11 | 3.19 | 0.045 | 3.14$^{ab}$ | 3.15$^{a}$ | 3.24$^{a}$ | 3.09$^{b}$ | 0.041 | 0.44 | 0.045 | 0.33 |
| | | *Butyrivibrio* | 2.04 | 2.11 | 1.72 | 0.114 | 2.56$^{a}$ | 2.52$^{a}$ | 1.82$^{b}$ | 0.94$^{c}$ | 0.132 | 0.05 | <0.001 | 0.15 |
| | | *Blautia* | 1.13 | 0.99 | 0.99 | 0.138 | 0.90$^{bc}$ | 1.20$^{ab}$ | 1.41$^{a}$ | 0.63$^{c}$ | 0.120 | 0.72 | <0.001 | 0.25 |

**Table 6.8** Effect of the diet and day of sampling on the relative abundance of the main bacterial taxa, and on diversity indexes of beef calves fed different diets (C, T and M; for diet composition see Table 6.1) during the transition period (28 d) from milk and pasture diet to a high concentrate ration. (Continued).

| | | | Diets | | | | Day | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Familiy | Genus | C | T | M | SEM | 0 | 7 | 14 | 28 | SEM | Diet | Day | DietxDay |
| | | *Catonella* | 1.08 | 1.11 | 1.32 | 0.097 | $1.12^{ab}$ | $1.15^{ab}$ | $1.45^{a}$ | $0.97^{b}$ | 0.103 | 0.19 | 0.012 | 0.16 |
| | | *Coprococcus* | 0.97 | 1.15 | 1.08 | 0.129 | $1.67^{a}$ | $1.54^{a}$ | $0.60^{b}$ | $0.46^{b}$ | 0.129 | 0.62 | <0.001 | 0.81 |
| | | *Lactonifactor* | 0.54 | 0.93 | 0.48 | 0.117 | $0.28^{b}$ | $0.65^{ab}$ | $1.06^{a}$ | $0.61^{b}$ | 0.117 | 0.03 | <0.001 | 0.27 |
| | | *Pseudobutyrivibrio* | 1.05 | 1.14 | 0.97 | 0.090 | $1.64^{a}$ | $1.34^{a}$ | $0.66^{b}$ | $0.59^{b}$ | 0.103 | 0.44 | <0.001 | 0.28 |
| | | *Roseburia* | 0.68 | 0.55 | 0.82 | 0.145 | $0.03^{b}$ | $0.18^{b}$ | $1.26^{a}$ | $1.26^{a}$ | 0.168 | 0.43 | <0.001 | 0.22 |
| | *Lactobacillaceae* | | 1.30 | 1.52 | 1.63 | 0.137 | $1.71^{a}$ | $1.08^{b}$ | $1.5^{ab}$ | $1.64^{ab}$ | 0.150 | 0.26 | 0.019 | 0.51 |
| | | *Lactobacillus* | 0.29 | 0.36 | 0.49 | 0.131 | $0^{b}$ | $0.02^{b}$ | $0.69^{a}$ | $0.81^{a}$ | 0.135 | 0.58 | <0.001 | 0.46 |
| | *Veillonellaceae* | | 2.02 | 2.00 | 2.11 | 0.106 | $1.81^{b}$ | $1.78^{b}$ | $2.33^{a}$ | $2.26^{a}$ | 0.113 | 0.26 | 0.019 | 0.51 |
| | | *Selenomonas* | 1.46 | 1.60 | 1.46 | 0.152 | $1.07^{c}$ | $1.28^{bc}$ | $2.04^{a}$ | $1.63^{ab}$ | 0.141 | 0.77 | <0.001 | 0.77 |
| | | *Schwartzia* | 0.31 | 0.56 | 0.79 | 0.113 | $0.54^{ab}$ | $0.09^{b}$ | $0.80^{a}$ | $0.78^{a}$ | 0.130 | 0.02 | <0.001 | 0.70 |
| | | *Anaerovibrio* | 0.88 | 1.11 | 1.11 | 0.117 | $0.36^{b}$ | $1.03^{a}$ | $1.42^{a}$ | $1.32^{a}$ | 0.135 | 0.29 | <0.001 | 0.90 |
| | *Ruminococcaceae* | | 2.83 | 2.74 | 2.83 | 0.096 | 2.78 | 2.78 | 2.98 | 2.65 | 0.111 | 0.76 | 0.216 | 0.79 |
| | | *Ruminococcus* | 2.11 | 1.97 | 2.13 | 0.127 | $2.26^{a}$ | $1.78^{b}$ | $2.55^{a}$ | $1.69^{b}$ | 0.146 | 0.63 | <0.001 | 0.80 |
| | *Streptococcaceae* | | 1.48 | 1.33 | 1.50 | 0.123 | 1.78 | 2.45 | 0.96 | 0.56 | 0.142 | 0.57 | <0.001 | 0.70 |
| | | *Streptococcus* | 1.42 | 1.40 | 1.20 | 0.123 | $1.54^{b}$ | $2.42^{a}$ | $0.94^{cd}$ | $0.47^{d}$ | 0.142 | 0.38 | <0.001 | 0.66 |
| | *Acidaminococcaceae* | | 2.22 | 2.24 | 2.21 | 0.051 | $2.18^{b}$ | $2.43^{a}$ | $2.12^{b}$ | $2.16^{b}$ | 0.059 | 0.86 | <0.01 | 0.73 |
| | | *Succiniclasticum* | 1.70 | 1.91 | 1.75 | 0.102 | $1.73^{ab}$ | $2.13^{a}$ | $1.70^{ab}$ | $1.57^{b}$ | 0.118 | 0.32 | <0.01 | 0.80 |
| | *Erysipelotrichaceae* | | 2.10 | 2.35 | 2.16 | 0.098 | $2.63^{a}$ | $2.23^{b}$ | $1.71^{c}$ | $2.23^{b}$ | 0.096 | 0.21 | <0.001 | 0.32 |
| | | *Sharpea* | 0.39 | 0.38 | 0.53 | 0.160 | $0^{b}$ | 0b | $0.85^{a}$ | $0.88^{a}$ | 0.185 | 0.77 | <0.001 | 1.00 |

**Table 6.8** Effect of the diet and day of sampling on the relative abundance of the main bacterial taxa, and on diversity indexes of beef calves fed different diets (C, T and M; for diet composition see Table 6.1) during the transition period (28 d) from milk and pasture diet to a high concentrate ration. (Continued).

| | | | Diets | | | | Day | | | | | *P*-values | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Phylum | Familiy | Genus | C | T | M | SEM | 0 | 7 | 14 | 28 | SEM | Diet | Day | DietxDay |
| *Proteobacteria* (Pr) | | | 2.65 | 2.62 | 2.70 | 0.172 | 2.44$^{b}$ | 3.23$^{a}$ | 1.70$^{c}$ | 3.27$^{a}$ | 0.178 | 0.95 | <0.001 | 0.95 |
| | *Succinivibrionaceae* | | 1.60 | 1.92 | 1.77 | 0.190 | 1.04$^{b}$ | 2.62$^{a}$ | 0.87$^{b}$ | 2.53$^{a}$ | 0.215 | 0.51 | <0.001 | 0.68 |
| | | *Ruminobacter* | 0.65 | 0.91 | 0.75 | 0.168 | 0.11$^{b}$ | 2.31$^{a}$ | 0.13$^{b}$ | 0.54$^{b}$ | 0.181 | 0.56 | <0.001 | 0.59 |
| | | *Succinivibrio* | 0.81 | 1.09 | 0.94 | 0.156 | 1.00$^{b}$ | 0.45$^{b}$ | 0.37$^{b}$ | 1.97$^{a}$ | 0.180 | 0.47 | <0.001 | 0.76 |
| *Spirochaetes* | | | 0.98 | 0.90 | 1.19 | 0.167 | 1.82$^{a}$ | 1.17$^{b}$ | 0.38$^{c}$ | 0.73$^{bc}$ | 0.167 | 0.48 | <0.001 | 0.91 |
| *Synergistetes* | | | 1.20 | 1.18 | 1.13 | 0.099 | 1.84$^{a}$ | 1.76$^{a}$ | 0.53$^{b}$ | 0.55$^{b}$ | 0.114 | 0.90 | <0.001 | 0.58 |
| *Tenericutes* | | | 1.78 | 1.71 | 1.79 | 0.124 | 2.42$^{a}$ | 2.06$^{a}$ | 1.40$^{b}$ | 1.15$^{b}$ | 0.143 | 0.87 | <0.001 | 0.45 |
| Shannon Index | | | 1.96 | 2.06 | 1.99 | 0.078 | 2.43$^{a}$ | 1.93$^{b}$ | 1.81$^{b}$ | 1.84$^{b}$ | 0.092 | 0.58 | <0.001 | 0.23 |
| Richness | | | 25 | 27 | 27 | 0.91 | 28$^{a}$ | 26$^{b}$ | 25$^{b}$ | 25b | 0.88 | 0.49 | 0.01 | 0.38 |
| Ratio F/B | | | 1.02 | 0.86 | 0.94 | 0.097 | 0.83 | 1.04 | 1.09 | 0.80 | 0.116 | 0.54 | 0.48 | 0.45 |
| Ratio Pr | | | 0.23 | 0.17 | 0.2 | 0.066 | 0.03$^{b}$ | 0.20$^{b}$ | 0.07$^{b}$ | 0.48$^{a}$ | 0.078 | 0.83 | <0.001 | 0.8 |

The number of reads was normalised to 15,935 reads and $\log_{10}$-transformed (nº reads+1). Only bacteria taxa that represented on average more than 0.1% of total sequences are shown. *Proteobacteria* ratio was calculated as the abundance of *Proteobacteria* sequences divided by the sum of the abundance of *Firmicutes* and *Bacteroidetes* sequences. $^{a,b,c}$ Different superscript letters within a row indicate differences between treatments or between days at ($P<0.05$), as obtained using Bonferroni's test .

Certain bacterial genera were found to be ubiquitous across all samples throughout the experiment, and therefore, were defined as the 'bacterial core'. Comparisons were also done among diets but there were no differences regarding core taxa. From the phylum *Bacteroidetes*, the following taxa were part of the core: the families *Prevotellaceae* (and the genus *Prevotella* withins this family), *Porphyromonadaceae, Flavobacteriaceae, Sphingobacteriaceae,* and *Cytophagaceae.* From *Firmicutes* the families *Erysipelotrichaceae, Eubacteriaceae* (and genus *Eubacterium*), *Costridiaceae* (and genus *Clostridium*), *Paenibacillaceae, Acidaminococcaceae* (and genus *Succiniclasticum*), *Peptostreptococcaceae*, f. *Ruminococcaceae* (and genus *Ruminococcus*), *Lactobacillaceae, Marinilabiliaceae, Oscillospiraceae, Veillonellaceae* (and genera *Anaerovibrio* and *Selenomonas)* and from the family *Lachnopiraceae* the genera *Butyrivibrio* and *Catonella*. From the phylum *Actinobacteria,* the family *Coriobacteriaceae* with the genus *Olsenella,* and from *Proteobacteria* the families *Desulfovibrionaceae* and *Succinivibriococcaceae* with the genus *Succinivibrio.* From minor phyla, only *Tenericutes* was also part of the rumen core. The abundance of the shared taxa in the overall bacterial community was highly diverse ranging from 0.08% to 64% of total bacteria.

Correlation analysis between relative abundances of the bacterial taxa, diversity indexes and rumen fermentation variables were not always consistent across diets (Table 6.9), except from the Shannon index that was negatively correlated with lactate concentration under the three diets. Interestingly, diets T and M showed similar correlations. Although it did not meet our criteria of considering a valid correlation when $r>0.30$ or $r<-0.3$, the *Proteobacteria* ratio showed significant ($P<0.05$) correlations under the three diets with TVFA ($r=0.28$, $P=0.02$), propionate concentration ($r=0.28$, $P=0.02$) and rumen pH ($r=-0.32$, $P=0.01$).

**Table 6.9** Spearman correlations between bacterial taxa (P: Phylum, F: Family; G:Genus) and Shannon Index, and rumen fermentation variables of beef calves fed different diets (C, T and M; see Table 6.1) during the transition period from milk and pasture diet to a high concentrate ration. Only Spearman correlations with r>0.30 r<-0.3 and P<0.05 are shown (n=72).

| | Rumen pH | | | Lactate | | | $NH_3$ | | | Total VFA | | | Acetate | | | Propionate | | | Butyrate | | | Valerate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial Taxa | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M |
| *P. Actinobacteria* | | -0.36 | | | | | | -0.36 | | | | | | | | | | | | 0.31 | | 0.35 | 0.34 | 0.45 |
| *F. Coriobacteriaceae* | | -0.40 | | | | | | -0.40 | | | | | | | | | | | | | | | | 0.37 |
| *G.Olsenella* | | -0.57 | | | | | | -0.57 | | | | | | | | | | | | | | | 0.41 | 0.40 |
| *G.Atopobium* | 0.36 | | | -0.50 | | | 0.36 | | | | | | | | | | | | -0.41 | | | -0.48 | -0.35 | |
| *F.Bifidobacteriaceae* | -0.44 | -0.37 | -0.31 | 0.55 | 0.30 | 0.44 | -0.44 | -0.37 | -0.31 | | | | | | | | | | 0.50 | 0.35 | | 0.64 | 0.53 | 0.62 |
| *G.Bifidobacterium* | -0.44 | -0.33 | -0.34 | 0.59 | | 0.43 | -0.44 | -0.33 | -0.34 | | | | | | | | | | 0.47 | | 0.37 | 0.56 | 0.46 | 0.65 |
| *P.Bacteroidetes* | 0.49 | | 0.55 | | -0.35 | | 0.49 | | 0.55 | | | -0.36 | | | | -0.36 | -0.30 | | | | -0.35 | | -0.38 | -0.47 |
| *F. Prevotellaceae* | | | | | | 0.39 | | | | | -0.54 | | -0.40 | -0.48 | -0.31 | | -0.47 | | | | | | | |
| *G.Prevotella* | | | | | | | | | | | | | | | | | | | | | 0.31 | | 0.31 | |
| *P.Chloroflexi* | 0.35 | 0.51 | 0.42 | -0.47 | -0.52 | -0.61 | 0.35 | 0.51 | 0.42 | | | | | | | | | | -0.46 | -0.39 | -0.41 | -0.45 | -0.77 | -0.77 |
| *P.Fibrobacteres* | 0.41 | 0.33 | 0.49 | -0.37 | -0.62 | -0.49 | 0.41 | 0.33 | 0.49 | | | | 0.31 | | | | | | | -0.47 | -0.56 | -0.40 | -0.50 | -0.61 |
| *F.Fibrobacteraceae* | 0.41 | 0.33 | 0.49 | -0.37 | -0.62 | -0.49 | 0.41 | 0.33 | 0.49 | | | | 0.31 | | | | | | | -0.47 | -0.56 | -0.40 | -0.50 | -0.61 |
| *G.Fibrobacter* | 0.43 | 0.38 | 0.50 | | -0.63 | -0.51 | 0.43 | 0.38 | 0.50 | | | | 0.32 | | | | | | -0.31 | -0.49 | -0.59 | | -0.52 | -0.60 |
| *P.Firmicutes* | 0.33 | | | | | | 0.33 | | | -0.40 | | -0.39 | -0.31 | | -0.38 | | | | | | | -0.39 | | |
| *F.Clostridiaceae* | 0.49 | 0.33 | 0.40 | -0.46 | | -0.40 | 0.49 | 0.33 | 0.40 | | | | | | | -0.32 | | | | | -0.58 | -0.32 | -0.50 | -0.49 |
| *G. Clostridum* | | 0.60 | 0.55 | | | -0.45 | | 0.60 | 0.55 | | | -0.38 | | | | | -0.45 | | | | -0.64 | | -0.51 | -0.40 |
| *G.Eubacterium* | | 0.34 | | | | -0.41 | | 0.34 | | | | | | | | | -0.32 | | | | -0.36 | | | -0.52 |
| *G.Butyrivibrio* | | | | | 0.31 | | | | | | | | | -0.50 | | | -0.47 | -0.31 | | 0.37 | | | | |
| *G.Blautia* | 0.41 | 0.68 | 0.73 | | | -0.60 | 0.41 | 0.68 | 0.73 | -0.48 | | | | | | -0.34 | -0.32 | | -0.44 | | -0.49 | | -0.61 | -0.79 |
| *G.Coprococcus* | | | 0.56 | | | | | | 0.56 | | -0.33 | -0.50 | | -0.35 | -0.44 | | -0.49 | | | | -0.34 | | | |
| *G.Lactonifactor* | | 0.66 | 0.60 | -0.45 | -0.55 | -0.74 | | 0.66 | 0.60 | | | -0.35 | | | | | | | -0.37 | -0.34 | -0.63 | | -0.74 | -0.81 |
| *G.Pseudobutyrivibrio* | | | | | 0.34 | | | | | | -0.42 | | | -0.44 | | | -0.51 | | 0.47 | | | 0.35 | | |
| *G.Roseburia* | | 0.60 | | 0.37 | | -0.38 | | 0.60 | | | | | | | | | | | -0.50 | | -0.47 | -0.47 | -0.55 | |

**Table 6.9** Spearman correlations between bacterial taxa (P: Phylum, F: Family; G: Genus) and Shannon Index, and rumen fermentation variables of beef calves fed different diets (C, T and M; see Table 6.1) during the transition period from milk and pasture diet to a high concentrate ration. Only Spearman correlations with r>0.30 r<-0.3 and P<0.05 are shown (n=72) (Continued).

| | Rumen pH | | | Lactate | | | $NH_3$ | | | Total VFA | | | Acetate | | | Propionate | | | Butyrate | | | Valerate | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial Taxa | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M | C | T | M |
| *F.Lactobacillaceae* | -0.56 | -0.63 | | | 0.37 | 0.32 | -0.56 | -0.63 | | 0.38 | | | | | | 0.32 | | -0.39 | 0.51 | | | 0.67 | 0.58 | 0.34 |
| *G.Lactobacillus* | -0.33 | -0.49 | | | 0.39 | 0.35 | -0.33 | -0.49 | | | | | | | | 0.35 | | -0.36 | | | | 0.51 | 0.41 | 0.60 |
| *F.Veillonellaceae* | -0.36 | -0.39 | | | | | -0.36 | -0.39 | | | | | | | | 0.31 | | | | | | 0.36 | 0.35 | 0.43 |
| *G.Selenomonas* | -0.59 | -0.36 | | 0.64 | | 0.35 | -0.59 | -0.36 | | 0.30 | | | | | -0.33 | | | | 0.54 | | | 0.47 | | 0.33 |
| *G.Anaerovibrio* | -0.49 | -0.47 | | | | 0.37 | -0.49 | -0.47 | | | | | | -0.32 | -0.31 | 0.34 | | | 0.31 | 0.38 | 0.32 | 0.51 | 0.44 | 0.47 |
| *F.Ruminococcaceae* | | | | | | 0.40 | | | | | | | | | | -0.31 | | | 0.37 | | | | | |
| *G.Ruminococcus* | | | | | | | | | | | | -0.31 | | | -0.33 | | | | | | | | | |
| *F.Streptococcaceae* | | | 0.52 | | | -0.54 | | | 0.52 | -0.33 | | | | | | | | | -0.34 | | | | -0.39 | -0.65 |
| *G.Streptococcus* | | | 0.45 | | | -0.46 | | | 0.45 | -0.36 | | | | | | | | | | | | | -0.35 | -0.53 |
| *F.Acidaminococcaceae* | | | | | | | | | | | | | | | | | | | | | | | | |
| *G.Succiniclasticum* | | | | | | | | | | | | | | | | | | | | | | | | |
| *F.Erysipelotrichaceae* | 0.51 | | | -0.68 | -0.39 | -0.31 | 0.51 | | | | | | | 0.40 | 0.45 | -0.30 | | | | | | -0.36 | -0.42 | -0.32 |
| *Proteobacteria (P)* | -0.34 | -0.53 | | | | 0.33 | -0.34 | -0.53 | | | 0.36 | | | | | | 0.53 | | 0.54 | | | 0.40 | 0.56 | 0.54 |
| *Succinivibrionaceae* | | -0.34 | -0.36 | | | | | -0.34 | -0.36 | | 0.34 | 0.33 | 0.33 | | | | 0.44 | 0.37 | | | | | | |
| *Ruminobacter* | | | -0.40 | | | 0.35 | | | -0.40 | | | | | | | | | | | | 0.39 | | | |
| *Succinimonas* | | | | | | | | | | | | | -0.30 | | -0.39 | | | | | | | | | |
| *Succinivibrio* | | | | | | | | | | | | | | | | | | | | | | | | |
| *G.Sharpea* | | | | -0.33 | -0.32 | 0.31 | | | | | | 0.34 | 0.31 | 0.31 | 0.37 | | 0.37 | | | | | | | |
| *P.Spirochaetes* | | | 0.45 | | -0.60 | -0.49 | | | 0.45 | | | | | | | | -0.49 | | | | -0.33 | | -0.57 | -0.69 |
| *P.Synergistetes* | 0.41 | | 0.51 | -0.51 | -0.45 | -0.67 | 0.41 | | 0.51 | | | | | | | | | | -0.41 | | -0.45 | -0.35 | -0.51 | -0.78 |
| *P.Tenericutes* | 0.60 | 0.34 | 0.69 | -0.64 | -0.42 | -0.83 | 0.60 | 0.34 | 0.69 | | | | | | | | | | -0.70 | | -0.51 | -0.43 | -0.50 | -0.69 |
| *Shannon Index* | | | | -0.50 | -0.57 | -0.55 | | | | | | | | | | | | | | | | | | |

## Discussion

This study was conducted to investigate the effect of two additives (tannins and MCFA) on intake and animal performance, rumen environment and microbial (protozoal and bacterial) population in beef calves that were transitioned from a milk and pasture regime to a high-concentrate diet under conventional conditions.

Overall, no effect of the tested additives was found on any of the studied traits. Despite the high fermentability of the diet, none of the animals showed rumen pH values lower than 5.5 or lactate concentrations higher than 5 mmol/L, which could be indicative of sub-acute acidosis (Nagaraja and Titgemeyer, 2007). In contrast, irrespective of diet, abrupt concentrate inclusion strongly altered the rumen microbial composition and rumen fermentation. At the end of the study, rumen ecosystem significantly differed from that at the beginning. In this section, rumen microbial populations will be dealt with first, and thereafter the effect of tannins and medium-chain fatty acids will be discussed.

### Rumen protozoal population

When young ruminants are abruptly switched from a milk/forage to a high-grain diet, the protozoal population markedly changes. If the dietary proportion of concentrate exceeds 60% of the total diet, a subsequent depression in ruminal pH leads to a decrease in the protozoal concentrations and diversity (Mackie et al., 1978) that eventually might result in the rumen defaunation (Eadie et al., 1970; Lyle et al., 1981). However, some studies have demonstrated that feedlot cattle fed high- or all-concentrate diets *ad libitum* even possess consistent protozoal concentrations (Franzolin and Dehority, 1996; Hristov et al., 2001), suggesting that a protozoal adaptation to ruminal conditions occurs progressively, depending this response on individual variability. When the rumen is under development, the microbial population might be more affected by dietary changes (Yáñez-Ruiz et al., 2015) but animals with a fully functional rumen because of age and forage intake, such as those in the present study, might be more prepared to withstand metabolic challenges after an abrupt weaning.

Rumen pH was assumed to be a key factor in the establishment and maintenance of the ruminal protozoa population, and defaunation in cattle consuming grain-rich diets has been ascribed to the low pH (5.5 and below) caused by these diets. However, Lyle et al. (1981) found that protozoa were eliminated in steers despite rumen pH values between 5.7 and 6.0, whereas Franzolin and Dehority (1996) found consistent protozoa population in steers that had rumen pH values lower than 5.7 for 12h/d. Therefore, the rumen pH and

the time that pH values remain low are not the only factors responsible for a marked decrease in protozoal population or even defaunation as pointed out by Franzolin and Dehority (1996). In the present study, despite the high fermentability of the diet and the sudden dietary shift, rumen pH values were below 6.05 only in 19 out of 72 measurements. Throughout the experiment, in three cases, M calves were defaunated or harboured a low protozoal concentration (below 3 log cells/mL) on day 7 and day 14. Ruminal pH on that sampling date in those animals was 6.22, 7.26 and 7.08, respectively, values well over the rumen pH considered as threshold for growth of ruminal protozoa. Therefore, protozoal changes cannot be explained because of rumen pH, which remained quite stable. Instead, it might be due to the toxic effect of MCFA on rumen protozoa, as discussed below. However, those aforementioned animals recovered their population in subsequent dates, as reported by Towne et al. (1990). Refaunation could have been endogenous, with protozoa surviving in the rumen at undetectable levels or migrating from the omasum (Towne and Nagaraja, 1990), or exogenous, by contact from a faunated neighbour calf (Eadie and Mann, 1970). Defaunation or large reductions in ruminal fauna are generally associated with physical and chemical modifications of the rumen environment (Williams and Coleman, 1992), being a decrease in ruminal ammonia the most consistent effect of defaunation, because of a decreased proteolytic activity and bacterial lysis after elimination of protozoa (Jouany et al., 1988). In our work, positive correlations between total protozoal concentration and rumen ammonia and BCFA, as an index of proteolysis, were observed (Table 6.6). The positive correlation between total protozoa and lactate can be related to the normal lactate production derived from starch utilization, since no high lactate concentration or low pH were found. On the contrary, protozoa in the Subfamily *Diplodiniinae*, which are mainly fibrolytic, showed a negative correlation with lactate and a positive relationship with pH suggesting that this group is sensitive to high concentrations of lactate and the subsequent low pH. Similarly, the absence of protozoa is associated in the meta-analysis conducted by Newbold et al. (2015) with a clear reduction of ammonia concentration and butyrate, but no major effect on lactate concentration and a lack of response on pH or VFA concentration.

In the present experiment, maintenance of a stable concentration of total protozoa might be associated with a balanced rumen fermentation, by preventing a low ruminal pH. The symbiotic relationship between protozoa and host might be particularly important during the transition from high-forage diets to rations high in readily fermentable carbohydrates (Brown et al., 2006), through the protozoal ability to uptake

readily fermentable starch granules, reducing the rate and extent of digestion in the rumen (Mendoza et al., 1993; Fondevila and Dehority, 2001). For instance, holotrichs rapidly assimilate sugars, fermenting and storing them as amylopectin, and entodiniomorphs actively ingest starch granules and have an important role in the lactic acid clearance in the rumen (Newbold et al., 1987). This provides the rumen with buffering capacity and prevents the detrimental effect that might result from an overload of fermentable material with the subsequent over-acidification of the environment.

Even though diversity decreased throughout the experiment, *Entodinium* was not the only genus present, as holotrich protozoa (*Isotricha*) and *Polyplastron* were also present in some calves on day 28. Granja-Salcedo et al. (2016) observed a stable rumen protozoal concentration over 6.0 log cells/mL in steers given concentrate proportions increasing from 30% to 80%, with *Entodinium* spp. as 99% of total population and only minor presence (around 3.0 log cells/mL) of *Eudiplodinium* and *Eremoplastron* with the highest level of concentrate, averaging a rumen pH of 6.0. Our results support those studies, as we observed a consistent and relatively stable protozoal population after 7 days with a high (83% to 90% of total intake) concentrate ration.

**Rumen bacterial composition**

At the end of the study, the rumen ecosystem significantly differed from that at the beginning. These findings agree with other reports on dynamics of rumen bacterial population in cattle transitioned from high-forage to high-concentrate diets (Fernando et al., 2010; Petri et al., 2013). Although there are many factors responsible for the high variability of bacterial composition, the predominant phyla observed here are consistent with other reports in beef cattle (Petri et al., 2013). It is noteworthy that we observed slightly high *Proteobacteria* abundances with respect to the abovementioned studies, especially on the last day of sampling. The ratio *Proteobacteria* is an indicator of bacterial disturbance or dysbiosis when is higher than 0.19, and is generally observed after changes in ruminal VFA potentially associated with low rumen pH (Auffret et al., 2017). In agreement with that, we found that the *Proteobacteria* ratio was correlated with TVFA concentration ($r$=0.28), and rumen pH($r$=-0.32). In addition, irrespectively of diet, 39% and 56% of the animals showed a *Proteobacteria* ratio higher than 0.19 on days 7 and 28, respectively. Therefore, although animals did not reach acidotic conditions based on rumen pH or lactate values and the benchmark of acidosis (Nagaraja and Titgemeyer, 2007), there was an unbalanced rumen condition, which could indicate that there was a

selection of new bacteria involved in starch metabolism in order to adapt to the environment. In fact, regardless of the inclusion of additives, and even though diversity decreased, some amylolytic genera emerged after concentrate inclusion. Overall, the change in the bacterial composition and thus on rumen fermentation mirrored the higher arrival of fermentable carbohydrates in the rumen enhancing the growth of amylolytic and acid-tolerant bacteria (Goad et al., 1998; Fernando et al., 2010). We identified some bacterial taxa that were ubiquitous in all calves representing the rumen core, which agreed with the bacterial core reported by other authors (Petri et al., 2013; Mannelli et al., 2019).

**Effects of tannin inclusion**

Tannins are a very diverse and complex group of polyphenolic plant secondary metabolites that are classified into condensed (CT) and hydrolysable (HT) depending on their chemical structure, and possess the capacity to form complexes with proteins, and to a lesser extent with other macromolecules such as fibre, starch, nucleic acids, minerals, etc. (Makkar, 2003; Mueller-Harvey, 2006).

In the current study, we did not observe differences in feed intake or daily gains with the inclusion of tannin extract compared to the control diet. Likewise, in steers fed high-concentrate diets, similar feed intake was observed with mimosa or chestnut tannins extracts included at 14.9 g/kg DM (Krueger et al., 2010) or when quebracho was added at 4 g CT/kg DM on the concentrate (Mezzomo et al., 2011). In finishing lambs supplemented with chestnut tannins (20.8 g/kg DM), Frutos et al. (2004) also observed similar intakes and ADG than the control group. By contrast, Barajas et al. (2010) reported an enhanced feed efficiency in growing bulls supplemented with 2 g CT/kg DM, and Rivera-Méndez et al. (2016), regardless the type of tannins (CT, HT or mixture), observed increased intake and ADG in finishing steers supplemented with 6 g tannins/kg DM.

The effects of tannins addition on rumen fermentation and rumen microbiota are unclear. In general, tannins are regarded as bacteriostatic and bactericide compounds. Their mode of action on rumen microbiota has been linked to their ability of binding with microbial extracellular polymers, inducing morphological changes and even leading to disruption of bacterial cell wall (Jones et al., 1994), inhibition of extracellular microbial enzymes, and deprivation of nutrients for microbial growth (Smith et al., 2005). Among other factors, tannin-bacterial interactions are strongly dependent on the type and chemical structure of tannins. In this sense, CT possess stronger inhibition activity than

HT, and the inhibitory power is directly proportional to their molecular weight (Saminathan et al., 2016). Nevertheless, both types of tannins can be hydrolysed by some bacteria producing acetate and butyrate, and used by bacteria as energy source (Bhat et al., 1998), being HT more susceptible to bacterial hydrolysis (Smith et al., 2005). Further, there are some tannin-resistant bacteria able to develop mechanisms such as production of extracellular glycocalyx or an overproduction of tannin-degrading enzymes (McSweeney et al., 2001; Smith et al., 2005), therefore diminishing tannin-protein complexation. Some species of the genera *Prevotella*, *Selenomonas*, *Streptococcus*, *Treponema* and *Ruminobacter* have shown to be tannin-resistant rumen bacteria (Jones et al., 1994), and, among them, *Selenomonas* and *Streptococcus* can degrade tannins (Bhat et al., 1998). In general, the main effect of tannins on rumen fermentation is a reduction in proteolysis and plant cell wall digestion mainly due to their complexation with proteins and structural polysaccharides (Makkar, 2003).

In the literature, there is no consistency in the effect of tannins on rumen fermentation. For instance, Hassanat and Benchaar (2012) studied the impact of different sources of tannins from acacia, quebracho, chestnut and valonea (in doses ranging from 20 to 200 g tannins/kg DM) on fermentation *in vitro*, and indicated that responses of tannin inclusion widely differed among plant sources, types (CT or HT) and doses. In general, in that study, a dose of less than 50 g/kg DM of tannins did not affect total VFA concentration or pH, whereas ammonia concentration was reduced with 20 g/kg in all cases except for quebracho tannins where a minimum dose of 50 g/kg DM was necessary to reduce ammonia concentration. Further, Martínez et al. (2006) found different responses to tannin inclusion (50 g/kg DM) in diets with corn or wheat, suggesting that tannin effect was grain-type dependent (according to the different endosperm architecture). *In vivo*, in forage-fed animals, the inclusion of *ca.* 20 g of CT/kg DM resulted in lower ammonia and BCFA concentration in the rumen fluid suggesting a reduced ruminal proteolysis in sheep (acacia CT, Carulla et al., 2005) and beef cattle (quebracho CT, Beauchemin et al., 2007). These studies also reported lower acetate/propionate ratio with tannins, which can be expected due to the depressive effect of tannins on fibrolytic bacteria. While Carulla et al. (2005) found no effect on TVFA or rumen pH, Beauchemin et al. (2007), using similar doses, reported lower VFA concentration, evidencing that the effect of tannins on fermentation is variable and clearly dependent on source of tannins. In the present study, we did not find changes in the ruminal fermentation in response to tannin addition. This might be due to the lower dose

compared to the mentioned studies, although Díaz Carrasco et al. (2017) used an even lower dose (2 g/kg of feed) and found higher rumen pH in steers supplemented with a blend of HT and CT tannins. In agreement with our results, in concentrate-fed lambs, Salami et al. (2018) did not find any effect of different CT (mimosa and gambier) or HT (chestnut and tara) tannins extracts, added at 40 g/kg of concentrate, on rumen fermentation variables compared to the control, although comparing among tannins sources, they observed different responses. Likewise, in steers fed high-concentrate diets, the inclusion of 14.9 g/kg DM chestnut or acacia tannins (Krueguer et al., 2010) or 4 g CT/kg DM (Mezzomo et al., 2011) did not exert any effect on rumen fermentation. Interestingly, Salami et al. (2018) pointed out that the difference between concentrate and forage on digestion kinetics is one factor to take into consideration when comparing *in vivo* studies, since concentrate diets are rapidly digested compared to forage diets, and therefore formation of tannin complexes can be limited by time. This could explain why in forage-based diets there is a reduction in proteolysis or an effect on ruminal fermentation characteristics whereas in concentrate- based diets usually there are not. However, this assumption should be taken with caution since, for example, Hervás et al. (2003) did not observe an effect on rumen fermentation in sheep fed a forage-based diet with quebracho tannins orally dosed up to 83 g/kg DM, whilst Díaz Carrasco et al. (2017) found higher rumen pH in high-concentrate fed steers supplemented with a blend of HT and CT tannins (2 g/kg of feed). One possible reason for the differences could be that Hervás et al. (2003) administered quebracho tannins orally (dissolved in 400 mL of distilled water) before feeding, and the quick passage of the tannin solution through the rumen might have limited tannin complexation. By contrast, Díaz Carrasco et al. (2017) incorporated the tannins blend in the concentrate so the slower rate of passage could have allowed the formation of tannin complexes.

The effects of tannins on rumen protozoa are controversial. Positive (Vasta et al., 2010), negative (Makkar et al. 1995; Hristov et al., 2003) or absence (Śliwiński et al., 2002; Benchaar et al., 2008) of effects on the protozoal population have been reported. Moreover, Jayanegara et al. (2012) revealed in their meta-analysis that there is no clear relationship between dietary tannins and rumen protozoa. In fact, comparison among studies should be made with caution since the effects depend on supplementation level, origin, molecular weights and type (CT *vs.* HT) of tannins (Patra and Saxena, 2009; Saminathan et al., 2017). Besides, the effect of tannins on protozoal dynamics under a high-concentrate feeding regime is not well documented, since most studies have been

conducted in animals given all-forage or mixed forage-grain diets, or under *in vitro* conditions, where protozoal survival is reduced and the applicability to *in vivo* conditions is limited. Most of the information in the literature is about the effect of CT (*i.e.* from quebracho or *Leucaena leucocephala*), while the effect of HT (*i.e.* from chestnut or tannic acid) on rumen protozoa *in vivo* is scarce. We used a 65:35 commercial mixture of chestnut and quebracho tannins included at 20 g/kg of concentrate, and we found no effect of tannins on any of the studied protozoal groups. Carulla et al. (2005) reported a reduction in holotrich protozoa in a forage-base diet with the addition of 40 g/kg DM condensed tannins extract from *Acacia* in sheep, whereas total protozoal population was unaffected. On the contrary, an increase in total protozoa was observed in lambs supplemented with 100 g/kg of quebracho tannins (Vasta et al., 2010). No effects on holotrichid or entodiniomorphid protozoa were observed by Piñeiro-Vazquez et al. (2018) after two weeks of adaptation to a diet including *L. leucocephala* (containing 20 g/kg of CT), and similar results were observed by Śliwiński et al. (2002) in growing lambs fed a 1:1 hay and concentrate diet and supplemented with the same proportion of chestnut tannins extract. However, Saminathan et al. (2017) reported an overall decrease of protozoa population (increase of *Diplodinium* and a decrease of *Entodinium*, *Eudiplodinium*, *Polyplastron* and *Metadinium* proportions) measured by molecular techniques, but results were obtained after only 24 h of *in vitro* culture of bovine rumen fluid with a forage added with 30 g/kg of condensed tannins from *L. leucocephala*. Also *in vitro*, Makkar et al. (1995) observed a general decrease in protozoa, but of higher magnitude on holotrichs, with quebracho tannins *in vitro* (0.1-0.4 g/L). In terms of protozoal types, in the present study no major effect of tannins addition compared to the control diet was observed on concentration of *Entodinium* or *Dasytricha* (disappearance of the latter was retarded in two weeks), and a slight attenuation of changes in *Epidinium* and Subfamily *Diplodiniinae* groups was apparent. However, the decrease in *Isotricha* was linear and its defaunation was observed in four calves given diet T. Based on these results, there is no apparent effect of tannins on protozoal community or on rumen fermentation variables.

Regarding the tannins effect on bacterial population, in the current study it was hypothesized that tannin inclusion would elicit some inhibition on bacterial groups, as tannin-containing diets have the potential to induce shifts in the rumen bacterial population. However, the addition of tannins did not exert any effect on bacterial composition, which is directly linked to the lack of effects on rumen fermentation.

Condensed tannins usually are stronger microbial inhibitors than HT (Costa et al., 2018). Salami et al. (2018) reported stronger inhibitory effect with chestnut HT compared to control when studying the effect of different sources of CT or HT. Tannins generally inhibit either directly or indirectly proteolytic and fibrolytic bacteria due to their binging capability (Jones et al., 1994; McSweeney et al., 2001). Jones et al. (1994) studied *in vitro* bacterial growth with different levels of sainfoin CT (100-600µg CT/mL), and observed an inhibition of some proteolytic bacteria (*Butyrivibrio fibrisolvens* and *Streptoccocus bovis*); however, *Prevotella ruminicola* was found to be tolerant because it developed some resistance mechanisms. In another experiment, Díaz Carrasco et al. (2017), in high-concentrate fed steers with a mixture of HT and CT added at 2 g tannins/kg of feed reported decreases in ureolytic bacteria (*Butyrivibrio*, *Treponema* and *Succinivibrio*), and reduced *Prevotella*. On the effects of tannins on fibre-degrading bacteria, some studies have reported a decrease of *Fibrobacter* and *Ruminococcus* (Díaz Carrasco et al., 2017; Costa et al., 2018). For instance, Costa et al. (2018) studied the effects of CT, HT or their mixture added at 100 g/kg DM in oil-supplemented sheep on some target bacterial species, and found lower fibrolytic bacteria and higher *Selenomonas* and *Prevotella* with CT *vs.* with HT. In addition, Salami et al. (2018) observed a decrease in the phylum *Fibrobacter* with chestnut HT at 40 g/kg as fed. In that study, authors reported disparity within tannins of the same type, such as lower *Prevotella* and higher *Ruminobacter* in animals fed CT from gambier than in those fed CT from mimosa. Other authors have reported increases of *Fibrobacter* with CT (Saminathan et al., 2016). On the effects of tannins on the overall structure of bacterial community, some studies with HT from chestnut reported a lack of profound changes or disturbance of bacterial composition. For example, Díaz Carrasco et al. (2017) with a mixture of 66:33 chestnut and quebracho tannin extract (very similar to that used in the present experiment) reported a decreased richness but intact Shannon index, which indicates that the structure of the community was not affected by tannins. In addition, these authors reported increases in some genera: *Succiniclasticum*, *Selenomonas* and *Anaerovibrio.* Further, Mannelli et al. (2019) included 16 g/kg chestnut tannins in a forage-based diet for ewes and reported only slight changes in the composition of microbial composition due to the tannin inclusion (e.g. higher *Anerovibrio*, *Streotoccocus*, *Pseubutyrivibrio*). In the present study, the lack of effect of tannins might indicate that bacteria hydrolysed the tannins (since HT are more susceptible and our extract contained 65% HT), or that the dose used here was not severe to interact with rumen bacteria. Nevertheless, the dose used in the present experiment was

within the range of doses used in the experiments mentioned above, so a plausible explanation may be the degradation of the tannin source. Another explanation of the lack of effect could be the adaptation of the microbial population to tannins. However, it could be discarded because, although we did not perform a sequencing of bacterial DNA samples prior to day 7 (a time during which adaptation could have been occurred), there were no differences in rumen fermentation compared to the control. Therefore, it can be assumed that no differences in microbial populations occurred. From the information in the literature, it can be concluded that there is no general rule on the biological effect of tannins, since it is dependent on many factors. First, tannin complexation with nutrients will depend on chemical nature of tannin (CT *vs.* HT), dose, molecular weight, basal diet (forage *vs*. concentrate), etc. Moreover, their effects on rumen bacteria will depend on ability of bacteria to be resistant or to degrade tannins, bacterial strain, plant source of tannin, etc. All these factors, will determine, in turn, the overall effect of tannins on ruminal fermentation and animal performance.

**Effects of MCFA inclusion**

Medium-chain fatty acids refer to a family of saturated fatty acids ($C_6$-$C_{12}$) that have extensively been assessed as feed additives in ruminant diets for their role in reducing ruminal methanogenesis through their suppressive effect on rumen protozoa and methanogens (Machmüller, 2006). These additives have also been used as antimicrobial agents preventing gastrointestinal disorders and increasing animal performance in monogastrics (Khosravinia, 2015; Hanczakowska, 2017). The inhibitory effect of certain rumen bacteria by MCFA is well known (Henderson, 1973), especially by $C_{10}$ and $C_{12}$ (Dohme et al., 2001). The mechanisms for their antimicrobial activity have been suggested to be due to changes in permeability leading to a membrane destabilization that enables the incorporation of the MCFA into the cell (Desbois and Smith, 2010). Dissociation of MCFA inside bacterial cell alters intracellular pH homeostasis, lead to a suppression of enzymatic activities, impairing nutrient intake, and in the end leading to cell death (Desbois and Smith, 2010). Further, MCFA can induce autolysis of the bacterial cell, which may be result of a decrease in membrane fluidity (Desbois and Smith, 2010). In ruminant feeding, most studies have used CO as the most common and practical source of MCFA containing 7.4% (of total fatty acids) of $C_8$, 6.2% of $C_{10}$ and 47.1% of $C_{12}$ (Dohme et al., 2000). Apart from the use of CO, there is no much information in the literature about the *in vivo* effect of MCFA on rumen metabolism and microbes.

In the present study, no differences on intake were observed on group M compared to control. When CO as a source of MCFA was included in the ration of sheep (25 g/kg or 37.58 g CO/d), Liu et al. (2011) observed similar intake than control, but decreased intake was noted in beef heifers supplemented with 125, 250 or 375 g CO/d (Jordan et al., 2006). Machmüller and Kreuzer (1999) reported also decreased intake in sheep when including 70 g/d of CO in the diet. Intake depression can be due to reduced palatability and/ or lower OM and NDF digestibility of the diet. In our study, the dose used was far below those used in the mentioned studies, which can explain the lack of effect.

The effects on rumen fermentation have been reported to be influenced by type of fatty acid (Dohme et al., 2001), doses, experimental approach, and by type of diet (forage-*vs*. concentrate-based diets; Machmüller et al., 2001). Some studies have reported a decrease in ammonia concentration as a result of MCFA addition to the diet (Hristov et al., 2004b), which might be in part linked to the suppressive effect of MCFA on rumen protozoa. Amanzougarene et al. (2017b) studied *in vitro* the effect of the same additive as used in the present experiment (doses: 2, 4 and 6 g/kg DM), and reported a tendency to increased rumen pH, without adverse effects on VFA, after 24 h of incubation of barley. Other authors, however, studied *in vitro* the effect of $C_8$, $C_{10}$ and $C_{12}$ (10-40 mg per 60 mL of incubation liquid) and reported decreased rumen pH with all MCFA compared to the control (Ajisaka et al., 2002), which was explained by the significant reduction of rumen protozoa. The effects of MCFA on total VFA concentration and molar proportions of the main VFA are not consistent. For instance, Dohme et al. (2001) investigated *in vitro* the effect of pure individual MCFA ($C_8$- $C_{12}$) at 50 g/kg DM, and the responses on fermentation characteristics were different depending on the type of fatty acid indicating that each fatty acid interacts differently with microbes. The same was reported by Hristov et al. (2004a) who studied *in vitro* the effect of $C_6$, $C_8$, $C_{10}$ (0.625, 1.25 and 2.5 g/L of incubation liquid) and $C_{12}$ (2.5, 5.0, 10 g/L) and observed that effects on fermentation were dependent upon the individual fatty acids and dose. In another experiment, Goel et al. (2009) incubated increasing doses of $C_{10}$ (from 0 to 30 mg per 50 mL of incubation liquid) and reported linear decreases of VFA and shifts in fermentation pattern towards high butyrate (with 20 mg of $C_{10}$) or towards high propionate (with 30 mg of $C_{10}$). It is noteworthy to mention that in *in vivo* conditions there is not only one individual MCFA present in the rumen at a determined time. *In vivo* results in cattle are even more inconsistent due to the different nature of diets, and most of the studies have been carried out with the addition of CO or $C_{12}$ (Liu et al., 2011; Hristov et al., 2012).

The MCFA are commonly used as additives to modulate rumen microbiota, as they have a selective effect on certain microorganisms (Henderson, 1973). In their meta-analysis, Guyader et al. (2014) reported a decrease of protozoa with MCFA addition. *In vivo*, Faciola and Broderick (2014) reported a 40% decrease of rumen protozoa with 13 g/kg DM of $C_{12}$ in lactating dairy cows, and Matsumoto et al. (1991) observed defaunation after 2 days with either 50 g/kg (as fed) of $C_{10}$ or $C_{12}$, and reductions to around 10% with the same proportion of $C_8$ in goats. Similarly, Machmüller and Kreuzer (1999) reported a reduction in the protozoal population in sheep after 21 days with a dietary inclusion of 35 g/kg of CO, which resulted in average intakes of 5.0, 2.8 and 16.7 g/d of $C_8$, $C_{10}$ and $C_{12}$, respectively. In another experiment, Jordan et al. (2006) found reduced protozoal numbers in beef heifers supplemented with 250 g/d of CO. In an *in vitro* experiment, Hristov et al. (2004a) reported a consistent inhibition with $C_8$ at 1.25 g/L, and elimination of protozoa with $C_{10}$ and $C_{12}$ at doses ranging from 0.6 to 2.5 g/L, whereas no effect of $C_6$ acid on total protozoal numbers was found. Despite the high variability among studies regarding feeding conditions and experimental approach, it is clear that MCFA possess a strong antiprotozoal activity. It is worth considering that doses used in the mentioned studies were much higher than that used in the present work (6 g/kg of concentrate). In any case, the two animals that were defaunated on day 7, as well as those in which low protozoal counts were found, were given diet M, so such effect could be attributed to the inclusion of MCFA even at such a low dose. Regarding the effect of MCFA on the dynamics of protozoal types, there were no major differences (except for *Isotricha*) between M and the other diets (Figure 6.3). Besides, defaunation was transient and protozoal concentration was recovered, suggesting an adaptation of the ruminal microbiota to the additive. Some studies have reported that microbial populations exhibit a remarkable resilience and ability to adapt rapidly to a wide variety of antimicrobial agents (Newbold et al., 1977; Baah et al., 2007). Newbold et al. (1977) suggested that it is not the protozoal population *per se* that is resistant to the antiprotozoal agent, but certain bacterial population is capable of degrading the antiprotozoal component.

In the present study, the addition of a MCFA mixture did not affect the structure of the bacterial population or any bacterial taxon, which is consistent with the lack of effect on rumen fermentation pattern. The additive used here presented 50% of $C_{12}$ (*i.e.* 15 g $C_{12}$/d), which is lower than other doses with which other authors have observed a depressive effect on rumen bacteria. Several studies have reported that MCFA are

inhibitory for cellulolytic bacteria (Henderson, 1973; Patra and Yu, 2013), although this effect can be diet-dependent as found by Dong et al. (1997), who observed that in concentrate diets cellulolytic activity was not inhibited even with 10% CO. In any case, there is no consensus for the general effects of MCFA on bacterial composition. For instance, Hristov et al. (2012) intraruminally dosed 240 g/d of lauric acid and reported decreased populations of *Prevotella*, *Bacteroides*, whereas proportions of *Eubacterium*, *Butyrivibrio*, *Olsenella* and *Lactobacillus*, among others, increased. These authors observed that the most important shifts in bacterial composition in response to these MCFA were within the phylum *Firmicutes*. Others, however, did not observe differences on analysed bacteria with the inclusion (25 or 50 g/d) of $C_{12}$ in a high-concentrate diet for steers (Yabuuchi et al., 2007). The lack of effect in our study could have been due to microbial adaptation, since some microbes can adapt to MCFA, for instance, by upregulating the expression of genes encoding proteins involved in cell wall synthesis (Desbois and Smith, 210). This way, bacteria possess a thicker cell wall preventing the entrance of MCFA. However, as commented in the case of tannins, the lack of effect on rumen fermentation since day 1 might denote that the additive was innocuous for bacteria. Therefore, the most plausible explanation for the lack of effect is the low dose.

## Conclusions

An abrupt change from a milk and pasture feeding regime to a high-concentrate diet did not impair animal performance or rumen health of beef calves. Concentrate inclusion decreased protozoal and bacterial diversity and shifted bacterial composition towards increased amylolytic and acid-tolerant species. The presence of protozoa was probably important to maintain stable rumen conditions avoiding the risk of acidosis, although it increased proteolysis and ammonia concentration. This fact, together with the microbial adaptation to the new rumen environmental conditions, was likely of vital importance for the animals to cope with the abrupt dietary shift without going through acidotic episodes. At the doses used in the present experiment, the addition of tannins and medium-chain fatty acids in the adaptation diet of newly weaned beef calves did not negatively affect productivity and did not result in major effects on rumen fermentation or rumen bacterial populations, probably due to a microbial degradation of tannins and, for the MCFA, to the low dose used.

# CHAPTER 7

# GENERAL DISCUSSION

# CHAPTER 7: GENERAL DISCUSSION

The present thesis was carried out within the frame of a broader research project which aim was to assess the effectiveness of different pre-fattening feeding strategies for reducing the risk of ruminal acidosis in newly weaned beef calves that are switched from milk and forage to high-concentrate diets.

Within the production system of beef cattle, the transition period is considered the phase in which beef calves may experience higher risk and incidence of low rumen pH (Bevans et al., 2005; Brown et al., 2006). There are many strategies involving feeding management and manipulation of the rumen fermentation that seek to prevent and minimize rumen acidosis (Schwartzkopf-Genswein et al., 2003; González et al., 2012). However, there is no agreement in the literature on the results of the application of the different strategies; moreover, comparison among studies is difficult mainly because the differences in research methods and approaches, adaptation protocols, feeding management, animals and breeds or production systems. From the repertoire of strategies to control rumen fermentation (González et al., 2012) we chose those whose applicability under the practical conditions of the conventional beef production in Spain would be more feasible in terms of profitability and labour, and we apply them within the frame of this research project.

The aim of Experiment 1 was to assess the short-term influence of concentrate or forage supplementation during the suckling period on animal performance, rumen fermentation and economic profitability throughout the subsequent feedlot fattening of beef calves. Early workers already pointed out that concentrate supplementation during the suckling period leads to a better rumen epithelial development mainly because of the stimulating effect of the fermentation products (mostly butyrate) on rumen epithelia (Brownlee, 1956; Tamate et al., 1962). This assumption has been confirmed in recent studies where either concentrate supplementation or butyrate infusion increased the abundance of gene transcripts related to epithelial proliferation (Soomro et al., 2018; Sun et al., 2018). Additionally, it is widely known that pre-weaning concentrate feeding would affect animal growth towards a better animal performance at weaning (Faulkner et al., 1994; Myers et al., 1999; Blanco et al., 2008). However, this practice is not fully carried out in many areas due to its lower economic feasibility (Myers et al., 1999; Casasús et al., 2001; Blanco et al., 2008).

Complex interactions may occur among pre-weaning management, the use of different ingredients and/or different processing methods, supplementation with feed additives, etc., and hence it is essential to investigate the different strategies under the specific conditions of each particular production system. The vast majority of livestock that supplies the Spanish beef industry is reared intensively on cereals but comes from beef herds kept in the green pasturelands of Northern Spain or near mountain regions (Bacha, 2005). As a result, it is common to wean calves that have consumed only milk and grass. Hence, we designed Experiment 1 considering autochthonous breeds and their common management in the mountain areas of Spain.

The results showed that providing solid supplements (either concentrate or forage) from the third month of the suckling period improves performance at weaning, and favours concentrate intake during the transition period. Calves fed on only milk exhibited lower concentrate intake on the first two weeks of the transition period, and therefore this is not advisable under a productive point of view. Additionally, supplemented (either with concentrate or forage) animals were heavier at weaning than animals fed on milk only, which could have led to a shortening of the feedlot period, in agreement with the results of Myers et al. (1999).

In terms of rumen fermentation, animals that received concentrate during the suckling period exhibited better adaptation (similar rumen pH and lower total VFA concentration comparing pre-weaning and transition phases) in the transition period. Although we did not characterise rumen papillae samples (density, thickness, width and length), other authors have stated that the better adaptation is due to the stimulating effect of concentrate on rumen epithelia (Shen et al., 2004; Sun et al., 2018), increasing the absorptive capacity of the reticulorumen. Besides, increased absorption of VFA has been shown to decrease the susceptibility to ruminal acidosis (Schwaiger et al., 2013). Thus, calves supplemented with concentrate during the suckling period might have better epithelial adaptation and hence a protective response to rumen acidotic episodes during the fattening period.

Regarding the economic profitability (according to data from 2016, when the experiment was carried out), supplementation with either concentrates or forage during the suckling period was the best option when selling animals at weaning. Concentrate supplementation yielded the best economic return when selling calves at the end of the transition (emulating a closed production farm). Our results were in agreement with those of Bernués et al. (2001) who found a higher economic profitability when concentrates

were offered in closed production cycle farms or finishing farms. In contrast, within their price scenario they found that when selling the animals at weaning, the most profitable option was to not supplement suckling calves (Bernués et al., 2001). Therefore, in view of the different results among scenarios, it is of paramount importance to assess the most feasible practice for each production system and for each market juncture.

In the conditions where the experiment was carried out, concentrate supplementation of calves to be fattened during the suckling period would be the most advisable feeding strategy as it decreased the susceptibility of rumen acidosis during the transition period, and resulted in better animal performance, rumen adaptation and economic yield.

One of the limitations of Experiment 1 was that the trial only lasted for 6 months due to that calves (all female) were destined to replacement. It could have been interesting to study the effect of the pre-weaning management until the end of the whole fattening period. Additionally, although early studies already covered the effect of the solid supplementation on development of rumen epithelia (Brownlee, 1956; Tamate et al., 1962), it could have been interesting to take rumen wall samples in order to assess the effect of the solid supplementation on rumen papillae development. In addition, it would have been advisable to study the evolution of rumen microbial populations under the different feeding managements. All those aspects will be considered for future studies.

The strategies implemented in Experiment 2 (the use of different ingredients in the adaptation diet to a high-concentrate regime) and Experiment 3 (inclusion of different additives in the adaptation diet) were first studied *in vitro*, and were part of another PhD thesis from our research group (Amanzougarene, 2020). Those *in vitro* strategies that provided better results in terms of modulation of fermentation as well as applicability to *in vivo* practices were those assessed in the present work.

In order to evaluate the potential of the experimental diets in Experiments 2 and 3 to promote a more tempered rumen fermentation, the control diet in both experiments was formulated with 59% of barley. Since the acidogenic capacity of diets increases with starch degradation rate (González et al., 2012), and when the ratio starch-NDF is higher than 2:1 (Bacha, 2002), the control diet was considered to have a high acidogenic power. This was so because rate of starch degradation of barley is high (Herrera-Saldana et al., 1990; Offner et al., 2003), and the control diet had a starch:NDF ratio of 3:1. Increased fermentation of starch may overload the absorptive capacity of the rumen, intensifying

reductions in rumen pH (Owens et al., 1998). Our hypothesis was that comparing the assessed strategies with an acidogenic (control) diet would give us an idea of the modulating capacity of the studied experimental treatments.

In Experiment 2, the starting hypothesis was that formulating diets with ingredients with a slow degradation rate of starch would attenuate postprandial drop in rumen pH, and would reduce the incidence of rumen acidosis. It is acknowledged that ingredients possess different rates and extent of degradation in the rumen (Offner et al., 2003) as well as different intrinsic buffer capacity (Jasaitis et al., 1987; Van Soest et al., 1991). In this experiment, three different adaptation protocols to a high-concentrate diet were applied during transition, and for that purpose two experimental diets were formulated in which barley was replaced with different levels of maize and sugarbeet pulp, intending to promote a slower degradation rate of starch (see Chapter 5).

It was expected that the partial replacement of barley with maize and sugarbeet pulp would modulate ruminal pH due to the slower rate of degradation of maize starch (Herrera-Saldana et al., 1990) and the buffering capacity of sugar beet pulp (Jasaitis et al., 1987; Amanzougarene et al., 2017a). However, despite the different ingredient composition of the diets applied during the transition period, no significant effect was observed on feed intake or animal performance. Similarly, no effect was found neither on the postprandial evolution of rumen fermentation variables nor in the evolution of rumen microbial populations (bacteria and protozoa).

Published results on the effect of substitution of barley with maize are highly variable since they are dependent on many factors such as the basal diet, cereal varieties, type of production system and the initial state of the animal. Thus, *in vivo* research reported different fermentation pattern of diets with barley or maize towards either a greater pH reduction with barley (Surber and Bownan, 1998; Khorasani et al., 2001) or a null effect (Rotger et al., 2006; Gimeno et al., 2015).

Sugar beet pulp, rich in soluble fibre and unique in its composition, is reported to possess a high buffer capacity, which is partly due to the presence of galacturonic acid in its structure and its high fibre content (Van Soest et al., 1991). Williams et al. (1987) reported that the replacement of barley with BP considerably increased the capacity of the diet to neutralize hydrogen ions, given the greater buffer capacity of BP compared to that of cereals (Jasaitis et al., 1987). Our research group (Amanzougarene et al., 2017a) carried out *in vitro* incubations, simulating fermentation of fattening diets in the rumen, with inoculum obtained from calves fed *ad libitum* on concentrate and straw. In these

studies, sugar beet pulp promoted a stable *in vitro* fermentation and helped to slow down rumen fermentation, which supported the inclusion of BP in the adaptation diets of our experiment.

Our results showed no differences among the studied feeding protocols on rumen fermentation, which could be explained, at least in part, by the *ad libitum* straw provision. This resulted in a straw intake of, on average, 18% of total feed intake that probably helped to stabilize rumen pH. Additionally, a higher intake of straw was exhibited in animals in Protocol 3 compared to their counterparts when they were switched to the mixture with the final fattening diet (Ph 2; see Chapter 5). This could be due to a self-regulation mechanism intending to compensate for the higher acidogenic power of the diet as it was already observed by other authors (Moya et al., 2011; Gimeno et al., 2014).

It is assumed that a period of 14-21 days is required for adaptation of the rumen microbiota and rumen epithelium to high-concentrate diets (Mackie and Gilchrist, 1979; Dieho et al., 2016). Our results indicated that there were no harmful effects on animal performance, rumen epithelium or rumen fermentation and microbial population in all the animals, regardless of the adaptation protocol, and thus they responded in the same sense to the transition to high-concentrate feeding. Further, the presence of a consistent rumen protozoa population, even in control animals, indicated that animals were well adapted to their dietary conditions (Doré and Gouet, 1991). Based on the results of Experiment 2, we can suggest that animals of characteristics such as those in the present trial (weaned at around 5-7 months) would not need an adaptation period to the fattening diet since they may express self-regulation behaviour mechanisms (*e.g.* increased straw intake in *ad libitum* systems) to facilitate their acclimatisation to the high-concentrate diet. In addition, taking into account the price of the concentrate mixtures (C<BP10<BP22; see Chapter 5) and the lack of differences on rumen fermentation pattern and feed intake, there is apparently no need to provide an adaptation diet since no subsequent improvements or economic profit are achieved. Further, the results obtained in the present thesis are in agreement with previous results obtained by our research group in that the choice of barley or maize as the prevailing cereal to formulate beef cattle diets should be exclusively a matter of availability and market price, since no differences have been found on animal performance (Al Alami, 2012) or rumen fermentation variables (Gimeno, 2015).

Another strategy that was intended for reducing the risk of acidosis during the transition period was the inclusion of feed additives in the adaptation diet. In ruminant nutrition, there is a great variety of feed additives commercially available with different active compounds and mechanisms of action that aim to manipulate rumen fermentation for reducing the risk of rumen acidosis (González et al., 2012). The additives used in Experiment 3 (tannins and MCFA) were previously assessed *in vitro* (Amanzougarene, 2020), showing modulating effects in conditions simulating those occurring in the rumen of beef cattle fed high-concentrate diets. Our original hypothesis was that using those additives *in vivo* would reproduce the results previously obtained *in vitro* in the sense of rumen fermentation modulation. Although there are some studies assessing the effect of inclusion of tannins and MCFA in cattle (Krueger et al., 2010; Mezzomo et al., 2011; Liu et al., 2011; Hristov et al., 2012), differences in the production system, provided diets, type of tannins and fatty acids, and doses, clearly affect the outcomes (Dohme et al., 2001; Hristov et al., 2004a,b; Mueller-Harvey, 2006).

Contrary to our hypothesis, the feed additives assessed *in vivo* in Experiment 3 did not result in a noticeable effect on performance, rumen fermentation or rumen microbiota. The disagreement between results observed *in vitro* and *in vivo*, when testing the same additives and doses, has previously been reported (Mohammed et al., 2004; Martínez-Fernández et al., 2013). These authors detailed greater effect *in vitro* than *in vivo*, differences that were attributed to different degradation rates of compounds (Martínez-Fernández et al., 2013), greater dilution rate *in vivo* (Martínez-Fernández et al., 2013) or decreased bacterial and protozoal population *in vitro* due to the processing and incubation methodology of the rumen fluid (Soto et al., 2012). All these variables, together with the short-term period of the *in vitro* incubations, might explain why *in vitro* experiments usually report significant effects of additives on fermentation variables whereas *in vivo* experiments usually do not.

Tannins have the capacity to form complexes with other molecules (*e.g.* proteins, carbohydrates, fibre), reducing the rate of substrate fermentation in the rumen (Makkar, 2003). The effect of tannins on feed intake and animal performance is highly variable in the literature, but in agreement with our results no effect has been reported in beef steers fed high-concentrate diets when similar doses of tannin extracts than those used in our work were included (Krueger et al., 2010; Mezzomo et al., 2011). The effects of tannin inclusion in the diet on rumen fermentation *in vivo* are also highly variable. When included at moderate doses (<50g/kg DM) in ruminant diets, tannins generally exhibit

some potential to improve rumen fermentation and utilization of dietary protein (Makkar, 2003). This fact leads to an increased flux of amino acids to the lower gut, and thus increases the absorption of amino acids, which may promote animal performance in beef steers (Rivera-Méndez et al., 2016). On the other hand, some authors have reported a modulating effect (Díaz Carrasco et al., 2017) or no effect (Krueger et al., 2010; Mezzomo et al., 2011) when tannin extracts were included in high-concentrate diets for steers. Differences among studies might be related to dietary composition, since Martínez et al. (2006) found different responses to tannin inclusion (50 g/kg DM) in diets based on different grains.

Regarding the effect on rumen microbes, no major effect of tannin inclusion was found in the present work, probably due to a partial degradation of tannins by rumen bacteria. Hydrolysable tannins are more susceptible to bacterial hydrolysis (Smith et al., 2005) and the tannin extract used here contained 65% HT from chestnut. However, other studies assessing the effect of HT on microbes have reported microbial changes in response to tannin inclusion (Díaz Carrasco et al., 2017; Mannelli et al., 2019). In any case, classification of tannins in terms of their response to hydrolysis for explaining their effect in microbial activity is largely discussed (Mueller-Harvey, 2006). Once again, differences might be attributed to the different nature of diets, tannins and doses.

As in the case of tannins, the addition of MCFA did not show any effect on the analysed variables (intake, animal performance, and rumen fermentation and rumen microbes). The MCFA are supposed to exert some effect on rumen fermentation since they interact with rumen microbes (Desbois and Smith, 2010) and reduce their fermentative activity (Henderson, 1973; Dohme et al., 2001; Amanzougarene et al., 2017b). Most studies assessing the effect of MCFA inclusion in ruminant diets have been carried out with individual fatty acids or with CO as a practical source of fatty acids either *in vitro* (Dohme et al., 2000, 2001; Hristov et al., 2004a,b; Amanzougarene et al., 2017b) or *in vivo* (Liu et al., 2011; Hristov et al., 2012). Therefore, comparison among studies is difficult due to the variety of approaches and types of fatty acids, which differently affect rumen fermentation (Dohme et al., 2001).

In our study, the dose recommended by the manufacturer of the commercial MCFA mixture (6g/kg DM, which resulted in *ca.* 31g of MCFA/d) was far below those used in *in vivo* experiments reported in the literature, which might explain the lack of effect on the analysed variables. The effects of MCFA on rumen fermentation might vary depending on the type of fatty acid (Dohme et al., 2001; Hristov et al., 2004a), doses,

experimental approach and type of diet (forage- *vs*. concentrate-based diets; Machmüller et al., 2001). In any case, Amanzougarene et al. (2017b) studied *in vitro* the same MCFA blend used in Experiment 3, and reported increased pH, without adverse effects on VFA, after 24 h of incubation of barley. This was the reason for choosing this additive to be tested *in vivo*. It should be pointed out that *in vitro* experiments are carried out under controlled conditions (*i.e.* buffered rumen fluid or established liquid turnover rates) in which only a few variables are handled and varied, whereas *in vivo* trials involve much more complex scenarios where many variables, mostly associated with feeding behaviour and digestive physiology, might be unknown and then out of control. We can speculate about the fact that animals have a homeostatic system that makes them able to cope reasonably with factors that would induce acidosis in an *in vitro* environment. Contrarily, and for the same reason, treatments able to reduce the risk of acidosis *in vitro* could not necessarily induce the same response *in vivo*.

As already pointed out, there was also a lack of effect of the additives on rumen microbes and rumen fermentation, probably due to the degradation of the tannin source and the low dose of MCFA used. In view of the results obtained in Experiment 3, it does not seem necessary to include such additive to moderate rumen fermentation during transition, as animals in the control group did not exhibit impaired rumen fermentation or abnormal feeding behaviour, which could denote acidotic episodes in any case.

Despite we intended to promote acidotic conditions in Experiments 2 and 3 with the abrupt change to the fattening diet, and the high acidogenic capacity of the adaptation ration, most registered values of rumen pH and lactate concentration over the two trials were above the benchmark of acidosis. In Experiment 2, of a total of 240 pH measurements taken at different times over the experimental period, only 8 rumen pH measures (3.3%) were below 5.6, with the lowest rumen pH being 5.07. Regarding the Experiment 3, of 72 pH measurements only 4 (5.6%) were below 5.6, with the lowest being 4.49. In fact, both lowest values in Experiments 2 and 3 may be considered as an error, since the concentration of VFA and lactate were 101 mmol/L and 0.63 mmol/L, respectively, in the first case, and 66 mmol/L and 0.32 mmol/L, respectively, in the second. Therefore, despite rumen pH reached values around or below 6.0 (pH at 9 h after feeding ranged from 6.05 to 6.16 in Experiment 2 and from 5.90 to 6.30 in Experiment 3) rumen fermentation variables were within the range of physiological values, so we can consider that animals adapted well to the high-concentrate diet. However, it is obvious

that the lack of challenge in rumen physiology of experimental animals could reduce the margin of response to the strategies used as potential solutions.

Regarding rumen bacterial and protozoal populations of Experiments 2 and 3, a large variation was observed in microbial profiles of individual calves. In general, the different strategies assessed in the present thesis showed no effect in the rumen microbial composition, which was strongly affected by concentrate inclusion (regardless the type of ingredient or the inclusion of additives). As reported in other studies (Fernando et al., 2010; Petri et al., 2013), the addition of concentrates affected the structure of bacterial community by altering the relative abundance of the main phyla and genera, and decreasing diversity. The fact that some amylolytic genera emerged after concentrate inclusion, together with the variations in the bacterial composition, denotes the potential of the rumen as an ecosystem to rapidly adapt to new conditions. Interestingly, the new genera that emerged after concentrate inclusion positively correlated with propionate, lactate and total VFA concentrations, being these characteristic products of the ruminal fermentation of intensive diets.

A major limitation of Experiments 2 and 3 was that animals were individually housed and fed, conditions that are not representative of the real commercial settings. Lack of social competition certainly could have resulted in a feeding behaviour that differed from that of commercial settings, with animals probably exhibiting a more homogenous pattern of intake over the day than it should be in practical conditions. However, it is important to highlight that our aim was to control as much as possible the factors that might enhance the risk of acidosis, and to study the specific effect of the diet in the development of this disorder, which justifies their individual housing. Another limitation of our experiments was the low number of animals within each experimental treatment, especially for assessing the effect of the treatments on rumen microbes and on rumen fermentation that has shown high inter-animal variability. This was due to the specific conditions of our experimental facilities, which did not allow for raising a larger number of calves, but also because monitoring rumen fermentation in a large number of rumen cannulated calves is not an easy task. Anyway, for this kind of studies, a higher number of animals should be considered in order to improve the statistical power.

As a general consideration after completing this thesis, and taking into account previous research from our group with beef cattle under intensive feeding conditions (Al

Alami, 2012; Gimeno, 2015), in general, cattle are able to deal with challenging diets with a high proportion of concentrates, provided that are not affected by health or stress processes. It seems that the microbiota plays an important role in the adaptation of the animals to the high-concentrate rations. As an ecosystem, the rumen has a high resilient capacity (Weimer, 2015) in that once it is disrupted it is able to restore the initial conditions. In terms of rumen acidosis, it seems that animals fed on concentrate and straw *ad libitum* might self-regulate their intake to prevent rumen pH drop. Therefore, in beef production systems with animals in similar conditions to those in the experiments described above, the risk of acidosis during the transition period might be overestimated, or at least related more with parameters associated to feeding behaviour than to ingredient and nutrient composition of diets. Further, it does not seem necessary to follow an adaptation protocol to a high-concentrate diet since animals in the control group responded similarly than those in the experimental diets, with no sign of impaired rumen health.

# CHAPTER 8

# CONCLUSIONS/CONCLUSIONES

# CHAPTER 8: CONCLUSIONS

The results obtained under the experimental conditions of this thesis allow to conclude that:

1. Pre-weaning supplementation with concentrates is the most advisable practice for improving calf performance, rumen adaptation to the high-concentrate diets and economic profitability in fattening calves, compared to other feeding strategies such as non-supplementation or forage supplementation.
2. An abrupt transition from a milk and forage regime to a high-concentrate diet based mainly on barley grain as the cereal source is not necessarily manifested into impaired rumen health or acidotic conditions. Therefore, it is not necessary to provide an adaptation concentrate during the transition period, provided that animals are not in health risk.
3. High-concentrate diets (>80% concentrate) during the post-weaning transition period do not lead to defaunation since a consistent and stable rumen protozoa population is maintained in the rumen of beef calves, although rumen protozoal diversity decreases, and ciliate rumen population becomes mainly composed by species of the genera *Entodinium* and, to a minor extent, of *Isotricha*.
4. Dietary inclusion of high proportions of concentrate in the transition period decreases the diversity of rumen bacterial population of beef calves. However, some genera emerge thereafter indicating an adaptation of the rumen as an ecosystem to the new environmental conditions resulting from carbohydrates being rapidly fermented.
5. The use of barley or maize as the prevailing cereal or the inclusion of sugar beet pulp in the diet for beef calves during transition has no major effect on animal performance, rumen fermentation variables, their postprandial evolution or microbial populations. Therefore, their choice will depend on market price and availability.
6. The inclusion of 20 g/kg of a 65:35 chestnut and quebracho tannin extracts or 6 g/kg of a mixture of medium-chain fatty acids in the diet for beef calves during the adaptation period to a high-concentrate diet does not affect feed intake or animal performance and does not show any major effect on rumen fermentation variables or in their postprandial evolution. Similarly, no effect of the additive inclusion is observed in the rumen bacterial population.

7. The inclusion of 6 g/kg of a mixture of medium-chain fatty acids in the diet for beef calves during the adaptation period reduces protozoal concentration, and may even lead to temporary ruminal defaunation, although protozoa adapt to this additive thereafter.

## CONCLUSIONES

Con los resultados obtenidos en las condiciones experimentales de la presente tesis se puede concluir que:

1. En comparación con otras estrategias de alimentación, como la no suplementación o el aporte de forraje, la suplementación con concentrado durante la lactación mejora el rendimiento animal, la adaptación del rumen a las dietas de cebo intensivo y la rentabilidad económica, por lo que puede considerarse la práctica más recomendable para terneros de engorde que van a entrar a cebadero.
2. Una transición abrupta de una dieta a base de leche y forraje a otra alta en concentrados, formulada con una proporción mayoritaria de cebada como fuente de cereal, no se traduce necesariamente en problemas de salud ruminal o condiciones acidóticas en el rumen. Por lo tanto, no es necesario ofrecer una dieta de adaptación al cebo intensivo, de no ser que los animales estén en riesgo sanitario.
3. Las dietas con alto contenido de concentrado (>80%) en el periodo de adaptación al cebo no conducen a la defaunación, ya que los terneros mantienen una abundante población de protozoos ruminales. Sin embargo, estas dietas disminuyen la diversidad de protozoos ruminales, resultando una población dominante de *Entodinium* y una mínima proporción de *Isotricha*.
4. La inclusión de concentrado en dietas de transición al cebo de terneros disminuye la diversidad de la población bacteriana ruminal en terneros de engorde. Sin embargo, algunos géneros bacterianos emergen posteriormente indicando una adaptación del ecosistema ruminal a las nuevas condiciones ambientales derivadas de la fermentación de dietas ricas en carbohidratos rápidamente fermentables.
5. El uso de cebada o maíz como cereal predominante en la dieta para terneros de carne, o la inclusión de pulpa de remolacha, no tiene un efecto manifiesto sobre el rendimiento animal, las variables de fermentación ruminal, sus evoluciones postprandiales o las poblaciones microbianas. Por lo tanto, su uso dependerá del precio y la disponibilidad del mercado.

6. La inclusión de 20 g/kg de un extracto de taninos (65% castaño y 35% quebracho) o de 6 g/kg de una mezcla de ácidos grasos de cadena media en la dieta para terneros de engorde durante el período de adaptación a las dietas de cebo intensivo, no afecta el consumo de alimento o el rendimiento animal, y no tiene un efecto manifiesto sobre las variables de fermentación ruminal o sobre su evolución postprandial. Del mismo modo, no se observa un efecto de la inclusión de estos aditivos en la población bacteriana del rumen.
7. La inclusión de 6 g/kg de una mezcla de ácidos grasos de cadena media en la dieta para terneros durante el período de adaptación reduce la concentración de protozoos e incluso puede conducir a una defaunación ruminal temporal, aunque los protozoos posteriormente se adaptan al aditivo.

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# ANNEX

# ANNEX

## Scientific publications

Yuste, S., Amanzougarene, Z., de la Fuente, G., Fondevila, M., de Vega., A., 2020. Effects of partial substitution of barley with maize and sugar beet pulp on growth performance, rumen fermentation and microbial diversity shift of beef calves during transition from a milk and pasture regimen to a high-concentrate diet. Livestock Science. 2020. 238: 10471. doi: 10.1016/j.livsci.2020.10407.

Yuste, S., Amanzougarene, Z., de Vega, A., Fondevila, M., Blanco, M., Casasús, I., 2020. Effect of pre-weaning diet on performance, blood metabolites, and ruminal fermentation around weaning in suckling calves from two beef breeds. Animal Production Science 2020, 60: 1018-1027. doi: 10.1071/AN19152.

Yuste, S., Amanzougarene, Z., de la Fuente, G., de Vega., A., Fondevila, M., 2019. Rumen protozoal dynamics during the transition from milk/grass to high-concentrate based diet in beef calves as affected by the addition of tannins or medium-chain fatty acids. Animal Feed Science and Technology, 253, 114273. doi:10.1016/j.anifeedsci.2019.114273

## Scientific communications

Yuste S, Amanzougarene Z, de la Fuente G, Fondevila M, de Vega A. Effects of tannins and medium-chain fatty acids on rumen bacterial community of beef calves during the transition period to a high-concentrate diet. 70th EAAP Annual Meeting, 2019, Ghent, Belgium. Book of Abstracts of the 70th EAAP Annual Meeting. Vol 25 P: 166

Yuste S, Amanzougarene Z, Fondevila M, de Vega A. Effect of beet pulp inclusion in the diet during the transition of beef calves to a high-concentrate ration. XVIII Jornadas sobre Producción Animal. Asociación Interprofesional para el desarrollo agrario (AIDA). Zaragoza, Spain. 2019. Book of Abstracts: Pp: 80-83.

Yuste S, Amanzougarene Z, de la Fuente G, Fondevila M, de Vega A. Temporal dynamics in the bacterial community of newly weaned beef calves during the transition period to a high-concentrate diet. XVIII Jornadas sobre Producción Animal. Asociación Interprofesional para el desarrollo agrario (AIDA). Zaragoza, Spain. 2019. Book of Abstracts: Pp: 83-86.

Yuste S, Amanzougarene Z, Fondevila M, de Vega A. Feed additives as rumen fermentation modulators during transition of beef calves. 69th EAAP Annual Meeting, 2018, Dubrovnik, Croatia. Book of Abstracts of the 69th EAAP Annual Meeting. Vol 24 P: 381

Yuste S, de la Fuente G, Amanzougarene Z, de Vega A, Fondevila M. Rumen protozoal dynamics of beef calves along transition from a milk/grass diet to a high-concentrate based diet: effect of tannins or medium-chain fatty acids as additives. ASAS Annual Meeting, 2018, Vancouver, Canada. J. Anim. Sci. Vol. 96, Suppl. S3. P416

Yuste S, Blanco M, de Vega A, Fondevila M, Casasús I. Effect of pre-weaning supplementation of beef calves on technical and economic performance during lactation and transition to intensive fattening. XVII Jornadas sobre Producción Animal. Asociación Interprofesional para el desarrollo agrario (AIDA). Zaragoza, Spain. 2017. Book of Abstracts: Pp: 303-306.

Yuste S, Amanzougarene Z, Blanco M, de Vega A, Fondevila M, Casasús I. Effect of pre-weaning diet on rumen fermentation and blood metabolites in beef calves. XVII Jornadas sobre Producción Animal. Asociación interprofesional para el desarrollo agrario (AIDA). Zaragoza, Spain. 2017. Book of Abstracts Pp: 306-309.

**Research stays**

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