Mediterranean Agronomic Institute of Zaragoza

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Effect of the nature of the carbohydrate source in diets of fattening on ruminal fermentation under *in vitro* conditions

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General index

Index of tables

Index of figures

Abbreviation

ADF: Acid Detergent Fiber

ADL: Acid Detergent Lignin

AOCS: American Oil Chemists' Society

BCFA: Branched-Chain Fatty Acids

CI: Concentrate Inoculum

CP: Crude Protein

DM: Dry Matter

DMd: Dry Matter disappearance

EE: Ether Extract

FI: Forage Inoculum

GP Gas Production

NDF: Neutral Detergent Fiber

OM: Organic Matter

SCFA: Short Chain Fatty Acid

VFA: Volatile Fatty Acid

Summary

In order to determine the capacity of rumen acidification and their level and rate of fermentation in situations that mimic those of high concentrate feeding, the *in vitro* pH and gas production pattern of different sources of carbohydrates, namely three varieties of each of barley (B1, B2, B3), corn (C1, C2, C3) and sorghum (S1, S2, S3) as cereals, plus wheat bran (WB), citrus pulp (CP), sugar beet pulp (BP) and sucrose (SU) as sources of non-starch carbohydrates, were studied according to the nature of the inoculum source (from a concentrate diet, CI, or from forage diet, FI).

A first methodological experiment (Experiment 1.0) was arranged with five levels of pH (6.50; 6.25; 6.00; 5.75 and 5.50), adjusted according to the inclusion of bicarbonate ion in the incubation solution to simulate fermentation conditions under high concentrate feeding. The pH diminished linearly ($P<0.001$) with the buffering of the media, and remained constant throughout the 12 h incubation period except for treatments 5.75 and 5.50, whose pH continued decreasing to 5.51 and 5.31 at 12 h, respectively. Gas production decreased linearly ($P<0.001$) with the medium pH at 2, 4, 6, 8 and 10 h, and quadratically ($P= 0.012$) with the medium pH at 12 h. The total volume of gas produced after 12 h was correlated to pH at 12 h ($R^2= 0.629$, $P<0.01$) and 6 h ($R^2= 0.836$, $P<0.01$). Experiment 1.0 highlighted the close relationship between medium pH and substrate fermentation, and allowed to establish the adequate buffering level for the next studies.

In a second experiment (Experiment 1.1), all the experimental substrates (n= 13) were tested under optimal pH conditions (pH= 6.5) using FI, in order to get a basic comparison of their fermentative characteristics. Throughout all the incubation period (8 h), the medium pH was maintained for all cases in the expected range of 6.5 ± 0.2. The type of substrate did not affect ($P>0.05$) the medium pH at 2 h, but at 4 and 8 h the effect was significant. With these experimental conditions, CP always recorded the highest ($P<0.001$) volume of gas and the three varieties of sorghum recorded the lowest ($P<0.001$). The concentration of total VFA was influenced by the substrate type ($P<0.001$): at 4 h this concentration was highest with CP followed by WB, and the lowest concentration was recorded with the two corn varieties C1 and C2 and the three varieties of sorghum, whereas at 8 h WB was higher than CP, and S2 recorded the lowest concentration. For the VFA profile, similar pattern was observed at both 4 and 8 h: the proportion of acetate was highest with S2 and lowest with WB, whereas WB recorded the high proportion of propionate, together with the barley

varieties and SU, and S2 recorded the lowest proportion; and the lowest butyrate proportion was recorded with CP and BP. This experiment showed marked differences in fermentation characteristics among substrates, as expected, and gives a tool for their relative comparison in other fermentation conditions.

In the third experiment (Experiment 1.2), the nine cereal sources were tested under a medium pH adjusted at 5.5 that is allowing for a maximum response of fermentation on incubation pH. According to the nature of inoculum (FI and CI) two different sets of incubation series were established. The inoculum source and the substrate type influenced significantly the medium pH, which was always lower with CI than FI ($P<0.001$). Among substrates, pH was lower with barley varieties, and higher with sorghum varieties ($P<0.001$), this effect being more apparent with CI. The interaction inoculum x substrate ($P<0.001$) indicates that cereal varieties behaved differently depending on the inoculum source, but no differences in incubation pH were detected among varieties of the same cereal ($P>0.05$). The gas production recorded with the CI as inoculum was always superior to that with FI ($P<0.001$). Despite the inoculum, after 10 h incubation the barley varieties recorded the highest volume of gas ($P<0.001$) followed by corn and sorghum varieties, which recorded the lowest volume mostly with S2 when incubated with FI. A significant interaction between the inoculum type and the substrate was recorded at all incubation times ($P= 0.001$), indicating different behaviour among varieties of each cereal species for each inoculum. The DMd was higher with FI than with CI (0.360 vs. 0.280; $P<0.001$), being highest for barley (on average, 0.511), followed by corn (0.314) and then sorghum varieties (0.136; $P<0.001$). Total VFA concentration was higher with CI than FI ($P<0.001$), with higher ($P<0.001$) proportions of acetate and branched-chain VFA and lower ($P<0.001$) of butyrate and valerate with the latter. Among substrates, total VFA concentration was higher ($P<0.001$) with barley than corn and sorghum varieties. At 4 h there was not difference between corn and sorghum in the total VFA concentration, whereas at 8 h it was higher with corn than sorghum. At 8 h with FI, sorghum varieties were more acetate than barley, whereas no substrate differences were detected with CI. For butyrate, its proportion with CI was lowest with barley but did not differ among substrates with FI inoculum. The lactic acid concentration was affected by the inoculum ($P<0.001$), being higher with CI. Barley was the type of substrate which recorded the highest concentration, and sorghum the lowest. Differences among varieties within species were only recorded with FI at 8 h, and with CI at 4 h. From this experiment it is apparent that barley varieties ferment at a higher extent, and promote a higher drop of

medium pH with both inocula, although the CI inoculum also promotes a lower pH. Differences in gas production among cereal species were maximised with FI. There were only minor differences among cereal varieties, except among those of sorghum, probably because of their proportion of tannins.

In another experiment (Experiment 1.3), four non-starch carbohydrate sources (BP, CP, WB and SU) were tested with inocula from different nature (CI and FI), under medium pH adjusted at 5.5, also including C2 as a control. As in the previous experiment, the drop in pH was more important with CI than FI ($P<0.001$). The interaction inoculum x substrate at 4 ($P<0.05$) and 6, 8 and 10 h ($P<0.001$) indicate that substrates behave differently for both inocula. With CI, at all times of incubation SU recorded the lowest pH ($P<0.001$), whereas the other substrates maintained parallel trends, being lower with CP ($P<0.001$). With FI, SU abruptly dropped from 5.75 at 6 h to 4.87 after 10 h incubation, and CP became lowest ($P<0.001$) at 6 h (5.52). The other substrates maintained medium pH between 6.0 and 5.7 throughout all the incubation period. At all times of incubation, the volume of gas produced with CI was higher ($P<0.001$). The interaction inoculum x substrate ($P<0.05$) indicate differences between CP and BP with CI, at all incubation times, but with FI differences between CP and BP were only manifested from 6 h onwards. After 10 hours of incubation, the *in vitro* gas production of substrates ranked them as: CP, WB > SU, C2 and BP with CI, and CP > WB > BP, SU > C2 with FI. In terms of total VFA concentration, it was higher with CI ($P<0.001$), which showed more butyrate ($P<0.001$) whereas that with FI had more acetate ($P<0.001$). At 4 and 8 h incubation, the VFA concentration was higher ($P<0.001$) with CP. A higher acetate proportion was observed with BP ($P<0.001$), higher propionate with WB, SU, and CP ($P<0.001$), and the highest proportion of butyrate was recorded with WB and SU.

In the Experiments 1.2 and 1.3 strong correlations were finding between medium pH, gas production, total VFA concentration, VFA profile, and lactic acid (for Experiment 1.2).

Resumen

Para determinar la capacidad de acidificación del rumen, el nivel y ritmo de fermentación en situaciones que imitan la alimentación con dietas altamente concentradas de diferentes fuentes de carbohidratos, se emplearon tres variedades de cebada (B1, B2, B3), de maíz (C1, C2, C3) y de sorgo (S1, S2, S3) como cereales, además de salvado de trigo (BM), pulpa de cítricos (CP), pulpa remolacha (BP) y sacarosa (SU) como fuentes de carbohidratos no amiláceos, en función de la naturaleza de la fuente de inóculo (obtenido a partir de una dieta de concentrado, CI, o de dieta de forraje, FI).

Un primer experimento metodológico (Experimento 1.0) se realizó con cinco niveles de pH (6,50; 6,25; 6,00; 5,75 y 5,50) ajustados en función de la concentración de ion bicarbonato en la solución tampón para simular las condiciones de fermentación bajo una alimentación de alto nivel de concentrado. El pH disminuyó de forma lineal ($P<0,001$) con el búfer del medio, y se mantuvo constante durante las 12 h del período de incubación, excepto para los tratamientos 5,75 y 5,50, cuyo pH continuó disminuyendo hasta 5,51 y 5,31, respectivamente. La producción de gas disminuyó con el pH del medio, de forma lineal ($P<0,001$) a las 2, 4, 6, 8 y 10 h, y cuadráticamente ($P= 0,012$) a las 12 h. El volumen total de gas producido después de 12 h se correlacionó con el pH a las 12 h ($R^2= 0,629$, $P<0,01$) y a las 6 h ($R^2= 0,836$, $P<0,01$). El Experimento 1.0 resaltó la relación estrecha entre el pH del medio y la fermentación del sustrato, y permitió establecer el nivel de búfer adecuado para los próximos estudios.

En el segundo experimento (Experimento 1.1), todos los sustratos experimentales (n= 13) fueron probados bajo condiciones de pH óptimas (pH= 6,5) usando FI, para obtener una comparación básica de sus características fermentativas. A lo largo de las 12 h del período de incubación, el pH del medio se mantuvo en todos los casos dentro del rango esperado de 6,5 ± 0,2. El tipo de sustrato no afectó el pH del medio a las 2 h, pero sí ($P<0,05$) a las 4 y 8 h. Con estas condiciones experimentales, CP siempre registró el mayor volumen de gas ($P<0,001$) y las tres variedades de sorgo registraron el más bajo ($P<0,001$). A las 4 h, la concentración de ácidos grasos volátiles (AGV) fue más alta ($P<0,001$) con CP seguido por WB, y la concentración más baja se registró con las variedades de maíz C1 y C2 y las tres variedades de sorgo, mientras que a las 8 h WB fue mayor que CP, y S2 registró la concentración más baja. El mismo patrón se observó sobre el perfil de AGV a las 4 y 8 h: la proporción de acetato fue más alta con S2 y más baja con WB, mientras que WB registró la

mayor proporción de propionato, junto con las variedades de cebada y SU, y S2 registró la proporción más baja, y la menor proporción de butirato se registró con CP y BP. Este experimento mostró diferencias en las características de fermentación entre sustratos, como se esperaba, y aporta una herramienta para su comparación relativa en otras condiciones de fermentación.

En el Experimento 1.2, las nueve fuentes de cereales fueron probadas a un pH del medio ajustado a 5,5, para maximizar la respuesta sobre el pH de incubación, empleando los dos inóculos FI y CI. La fuente de inóculo y el tipo de sustrato afectaron al pH del medio, que siempre fue inferior con CI que con FI ($P<0,001$). Entre sustratos, el pH fue menor con las variedades de cebada, y superior con las variedades de sorgo ($P<0,001$), siendo este efecto más evidente con CI. Las variedades de cereales se comportaron de manera diferente dependiendo de la fuente de inóculo (interacción inóculo x sustrato, $P<0,001$), pero no se detectaron diferencias en el pH de incubación entre variedades del mismo cereal ($P>0,05$). La producción de gas registrada con CI fue siempre superior a la registrada con FI ($P<0,001$). Por el contrario, la dMS fue mayor con FI que con CI (0,360 vs 0,280; $P<0,001$). La concentración total de AGV resultó mayor con CI que con FI ($P<0,001$), con el que se registró una mayor proporción de acetato ($P<0,001$) y de AGV ramificados, y una menor proporción de butirato y valerato ($P<0,001$). La concentración de ácido láctico también fue mayor con CI ($P<0,001$). Independientemente del inóculo, después de 10 h de incubación las variedades de cebada registraron el mayor volumen de gas ($P<0,001$), seguidas de las variedades de maíz y sorgo, que registraron el volumen más bajo con S2 incubado con FI. También sobre la producción de gas la interacción entre el tipo de inóculo y el sustrato en todos los tiempos de incubación ($P= 0,001$) indica un comportamiento diferente entre las variedades de cada especie de cereales por cada inóculo. La dMS fue mayor para las variedades de cebada (en promedio, 0,511), seguido por el maíz (0,314) y el sorgo (0,136; $P<0,001$), así como la concentración total de AGV fue mayor ($P<0,001$) con la cebada que las variedades de maíz y sorgo, que a las 8 h fue mayor con el maíz que con el sorgo. A las 8 h con FI, el acetato se produjo más con las variedades de sorgo que con las variedades de cebada, pero con CI no hubo deferencia entre sustratos. Para el butirato, la cebada registró la menor proporción con CI, pero no hubo deferencias entre sustratos con FI. La cebada fue el tipo de sustrato que registró la mayor concentración de ácido láctico, y el sorgo el más bajo, pero solamente se registraron diferencias entre variedades de cereales con CI a las 4 h y con FI a las 8 h.

A partir de este experimento, es evidente que las variedades de cebada fermentan en mayor medida, y promueven una mayor caída del pH del medio con ambos inóculos, aunque lógicamente el inóculo CI promueve un pH más bajo. Las diferencias en la producción de gas entre especies de cereales se maximizan con FI, pero sólo se detectaron diferencias menores entre las variedades de cereales, excepto entre las de sorgo, probablemente debido a su diferente proporción de taninos.

En el Experimento 1.3, cuatro fuentes de carbohidratos no amiláceos (BP, CP, WB y SU), junto con C2 como control se probados con los inóculos CI y FI, bajo el pH del medio ajustado a 5,5. Igual que en el experimento anterior, la caída del pH fue mayor con CI que con FI ($P<0,001$). La interacción inóculo x sustrato a 4 ($P<0,05$), 6, 8 y 10 h ($P<0,001$) indica que los sustratos se comportan de manera diferente para los dos inóculos. Con CI, en todos los momentos de incubación SU registró el pH más bajo ($P<0,001$), mientras que los otros sustratos mantienen las tendencias paralelas, siendo menor con CP ($P<0,001$). Con FI, SU abruptamente cayó de 5,75 a las 6 h a 4,87 después de 10 h de incubación, y CP se convirtió más bajo ($P<0,001$) en 6 h (5,52). Los otros sustratos mantuvieron el pH del medio entre 6,0 y 5,7 a lo largo de todo el período de incubación. En todos los tiempos de incubación, el volumen de gas producido y la concentración de AGV fueron mayores con CI ($P<0,001$), que presentó mayor proporción de butirato y menor de acetato que FI ($P<0,001$). Con CI, se observaron diferencias entre CP y BP, en todos los tiempos de incubación, mientras con FI las diferencias entre CP y BP solamente se manifestaron a partir de 6 h (interacción inóculo x sustrato, $P<0,05$). Después 10 horas de incubación, la producción de gas *in vitro* de sustratos los clasificó como sigue: CP, WB> SU, C2 y BP con CI, y CP> WB> BP, SU> C2 con FI. A las 4 y 8 h de incubación, la concentración de AGV fue mayor ($P<0,001$) con CP, y se observó una mayor proporción de acetato con BP ($P<0,001$), una alta proporción de propionato con WB, SU, y CP ($P<0,001$), y la mayor proporción de butirato con WB y SU.

En los Experimentos 1.2 y 1.3 se observaron correlaciones importantes entre el pH del medio, la producción de gas, la concentración total y el perfil de AGV y la concentración ácido láctico (en el Experimento 1.2).

Résumé

Pour déterminer la capacité d'acidification du rumen, son niveau et rythme de fermentation dans des conditions qui imitent une alimentation élevée en concentré de différentes sources de glucides, ont été utilisées trois variétés d'orge (B1, B2, B3) de maïs (C1, C2, C3) et de sorgho (S1, S2, S3) comme céréales, plus son de blé (BM), pulpe d'agrumes (CP), pulpe de betterave (BP) et saccharose (SU) comme sources de glucides non-amylacés, en fonction de la nature de la source d'inoculum (obtenue à partir d'une ration concentré, CI, ou d'une ration fourrage, FI).

La première expérience méthodologique (Expérience 1.0) a été réalisée avec cinq niveaux de pH (6,50; 6,25; 6,00; 5,75 et 5,50), ont été ajusté en fonction de la concentration en ion bicarbonate dans la solution tampon pour simuler les conditions de fermentation sous une alimentation riche en concentré. Le pH diminue linéairement ($P<0,001$) avec le pouvoir tampon de milieu, et est resté constant durant toute la période d'incubation (12 h) à l'exception pour les traitements 5,75 et 5,50, dont le pH a continué à décroître jusqu'à 5,51 et 5,31 à 12 h, respectivement. La production de gaz a diminué de manière linéaire ($P<0,001$) à 2, 4, 6, 8 et 10 h, et de manière quadratique ($P= 0,012$) à 12 h avec le pH du milieu. Le volume total de gaz produit après 12 h a été corrélé au pH à 12 h ($R^2= 0,629$, $P<0,01$ h) et à 6 h ($R^2= 0,836$, $P<0,01$). L'Expérience 1.0 a souligné la relation étroite entre le pH du milieu et la fermentation de substrat, et a permis d'établir le niveau de mise en tampon suffisant pour les prochaines études.

Dans une seconde expérience (Expérience 1.1), tous les substrats expérimentaux (n= 13) ont été testés sous des conditions optimales de pH (pH= 6,5) en utilisant FI, afin d'obtenir une comparaison de base de leurs caractéristiques fermentaires. Durant toute la période d'incubation (8 h), le pH du milieu a été maintenu dans tous les cas dans la gamme attendue de 6,5 ± 0,2. Le type de substrat n'a pas affecté ($P>0,05$) le pH du milieu à 2 h, mais à 4 et 8 h l'effet a été significatif. Avec ces conditions expérimentales, CP toujours a enregistré le volume de gaz le plus élevé ($P<0,001$), et les trois variétés de sorgho ont enregistré le plus faible ($P<0,001$). La concentration des acides gras volatil (AGV) a été influencée par le type de substrat ($P<0,001$): à 4 h cette concentration a été la plus élevée avec CP suivie par WB, et la plus faible concentration a été enregistrée avec les deux variétés de maïs C1 et C2 et les trois variétés de sorgho, tandis qu'à 8 h la concentration enregistrée par WB a été plus élevée

que celle enregistrée par CP, et S2 a enregistré la plus faible concentration. Pour le profil des AGV, tendance similaire a été observée à la fois à 4 et à 8 h: la proportion d'acétate a été la plus élevée avec S2 et la plus faible avec WB, alors que WB a enregistré la proportion la plus élevée de propionate, ainsi que les variétés d'orge et SU, et S2 a enregistré la proportion la plus faible; et la plus faible proportion de butyrate a été enregistrée par CP et BP. Cette expérience a montré des différences marquées dans les caractéristiques de fermentation entre substrats, comme prévu, et apporte un outil pour leur comparaison par rapport à d'autres conditions de fermentation.

Dans la troisième expérience (Expérience 1.2), les neuf sources de céréales ont été testées dans un milieu ajusté à pH 5,5 pour maximiser la réponse sur le pH d'incubation en utilisant les deux inoculums FI et CI. Deux séries d'incubation différentes ont été établies. La source d'inoculum et le type de substrat ont influencé de façon significative le pH du milieu, qui a été toujours inférieur avec CI qu'avec FI ($P<0,001$). Entre substrats, le pH a été inferieur avec les variétés d'orge, et supérieur avec les variétés de sorgho ($P<0,001$), cet effet a été plus apparent avec CI. L'interaction inoculum x substrat ($P= 0,001$) indique que les variétés des céréales se sont comportées différemment en fonction de la source d'inoculum, mais entre les variétés de la même céréale, des différences de pH d'incubation n'ont pas été détectées ($P>0,05$). La production de gaz enregistrée avec CI a été toujours supérieure à celle enregistrée par FI ($P<0,001$). Malgré l'inoculum, après 10 h d'incubation les variétés d'orge ont enregistré le volume de gaz le plus élevé ($P<0,001$), suivies par les variétés de maïs et de sorgho, qui ont enregistré le volume le plus faible surtout avec S2 quand a été incubé avec FI. Une interaction significative entre le type d'inoculum et le substrat a été enregistrée à tous les temps d'incubation ($P<0,001$), indiquant un comportement différent entre variétés de chaque espèce de céréale pour chaque inoculum. La dMS a été plus élevée avec FI qu'avec CI (0,360 vs 0,280; $P<0,001$), étant la plus élevée pour l'orge (en moyenne, 0,511), suivie par le maïs (0,314) et puis les variétés de sorgho (0,136; $P <0,001$). La concentration totale des AGV est plus élevée avec CI qu'avec FI ($P<0,001$), avec ce dernier les proportions d'acétate et des acides gras á chaîne ramifiée ont été plus élevées ($P<0,001$), et celles de butyrate et de valerate ont été les plus faible ($P<0,001$). Parmi les substrats, la concentration totale des AGV est plus élevée ($P<0,001$) avec l'orge qu'avec les variétés de maïs et de sorgho. À 4 h il n'y avait pas de différence entre le maïs et le sorgho dans la concentration totale des AGV, tandis qu'à 8 h, la concentration des AGV est plus élevée avec le maïs que le sorgho. À 8 h avec FI, les proportions d'acétate enregistrées par les variétés de sorgho ont

été plus élevée que celles enregistrées par les variétés de l'orge, mais avec CI il n'y avait pas de différences entre les substrats. Pour le butyrate, l'orge a enregistré les proportions les plus faibles avec CI, mais avec FI il n'y avait pas de différences entre les substrats. La concentration en acide lactique a été affectée par le type de l'inoculum (P<0,001), étant supérieure avec CI. L'orge a été le substrat qui a enregistré la concentration la plus élevée, et le sorgho a enregistré la plus faible. Les différences entre les variétés au sein des espèces ont été enregistrées uniquement avec FI à 8 h, et avec CI à 4 h.

A partir de cette expérience, il est évident que les variétés d'orge fermentent à une mesure plus élevée, et promeuvent une baisse importante de pH du milieu avec les deux inocula, même si l'inoculum CI promeut aussi la diminution de pH. Les différences dans la production de gaz entre les espèces de céréales ont été maximisées avec FI. Il y avait seulement petites différences entre les variétés de déférentes céréales, à l'exception de celles du sorgho, probablement en raison de leur proportion en tanins.

Dans l'Expérience 1.3, quatre sources de glucides non amylacés (BP, CP, WB et SU) ont été testées avec inocula de nature différente (CI et FI), sous un pH ajusté à 5,5, comprenant également C2 comme contrôle. Comme dans l'expérience précédente, la chute du pH a été plus élevée avec CI qu'avec FI (P<0,001). L'interaction inoculum x substrat à 4 (P<0,05) et 6, 8 et 10 h (P<0,001) a indiqué que les substrats comportent différemment pour les deux inocula. Avec CI, à tout temps d'incubation SU a enregistré le pH le plus bas (P<0,001), tandis que les autres substrats ont maintenu une tendance parallèles, étant inférieure avec le CP (P<0,001). Avec FI, SU a brusquement chuté de 5,75 à 6 h à 4,87 après 10 h d'incubation, et CP est devenu plus faible (P<0,001) à 6 h (5,52). Les autres substrats ont maintenu le pH du milieu entre 6,0 et 5,7 durant toute la période d'incubation. Durant toute la période d'incubation, le volume de gaz produit avec CI est plus élevé (P<0,001). L'interaction inoculum x substrat (P<0,05) a indiqué des différences entre CP et BP avec CI à tous les temps d'incubation, mais avec FI ces différences entre CP et BP se sont manifestées à partir de 6 h. Après 10 heures d'incubation, le classement des substrats selon le volume de gaz produit est le suivant: CP, WB> SU, C2 et BP avec CI et CP> WB> BP, SU> C2 avec FI. En terme de la concentration totale des AGV, elle est plus élevée avec CI (P<0,001), qui a montré une concentration plus élevée en butyrate (P<0,001), alors que FI a montré une concentration plus élevée en acétate (P<0,001). A 4 et 8 h d'incubation, la concentration en AGV est plus élevée (P<0,001) avec CP. Une proportion d'acétate élevée a été observée avec

BP (P<0,001), le propionate a été plus élevé avec WB, SU, et CP (P<0,001), et la proportion la plus élevée en butyrate a été enregistrée avec WB et SU.

Dans les Expériences 1.2 et 1.3 de fortes corrélations ont été trouvées entre le pH du milieu, la production de gaz, la concentration totale des AGV, profil des AGV, et l'acide lactique (pour l'Expérience 1.2).

INTRODUCTION

Introduction

The specific characteristics of cattle farming, associated with the climatological constraints of Spain, leads to the development of a specific production model, different from the rest of Europe, where the farms in closed cycle are frequent (birth, farming and fattening on the same site). In Spain, birth and fattening take place in different, separated farms (Robles, 2006). The largest part of beef production comes from young animals slaughtered at 8-10 months of age with an average weight of 350-400 kg in early and dairy breeds, and 12 months of age and 450-500 kg in beef breeds, with animals raised with their mothers at pasture (García-Rebollar et al., 2008). Up to 54% of the calves fattened in Spain in 2010 were imported alive, either sucklers of less than 80 kg (60%) or held at pasture of 80-160 kg (21%), assuming that the fattening of the sucklers reaches about one third of the total (MARM, 2012). In Spain, calves in intensive fattening are mostly fed with high-energy compound feeds and cereal straw distributed ad libitum, being the concentrates about 90% of total consumption (Calsamiglia and Ferret, 2002). There are farms with mother cows that produce weanlings, where diets are based on the consumption of forages or pastures from the farm that are complemented occasionally. In this system, the production level is low, in contrast to the intensive system in which the diet is based on cereal-rich concentrates, where the yield is very high. The production system which is particularly interesting in the context of this work is that of feedlots, that use early-weaned animals from dairy herds, who are fattened to slaughter with diets based on cereal-rich concentrates. In this type of production, the digestive problems would be responsible for 30% of total mortality. Among them, the most frequent problem is that of rumen lactic acidosis (Bermúdez, 2002). The development of the rumen starts at two to three weeks of age, but it reaches its full capacity a few months later. This development implies anatomical, physiological, and metabolic changes resulting the transition of monogastric digestion to a polygastric (Church, 1974). From this period onwards, the microorganisms are essential for the digestion of feeds. For a stable activity of these microorganisms, Fournier (1999) recommends that the first transition phase will start from the second to the third week of age and will continue as long as the milk is offered. During this phase animals will gradually increase concentrates consumption and decrease milk consumption, which would favour the development of the rumen papillae and facilitate the weaning of the young animal. According to BAMN (1997) and Hutjens (2001), for a good adaptation to solid feed the concentrate is more effective for transition, and forage should be included from the fifth or sixth week of life. The volatile fatty

acids produced from the consumption of concentrate, mainly propionic and butyric acids, are responsible for the establishment of a functional rumen (Quigley, 1997). The second phase starts with the weaning of the animal and ends with the preparation of the young ruminant for fattening. Such change can be done abruptly or progressively to avoid the multiple stressing situations coinciding. To ensure the correct development of this step, animals must be healthy (Fournier, 1999). The fattening of young ruminants is commonly associated to high growth rates that are obtained by intensive feeding systems based on cereal-rich rations, often associated with low forage supply. In the intensive fattening, the most critical moment for animals who are adapted to digest and metabolise predominantly forage diets, is during the abrupt change to a high energy diet (rich in concentrate). A frequent consequence of this practice is the onset of latent or subclinical acidosis processes associated with a very high production of volatile fatty acids from microbial fermentation that cannot be balanced by regulatory factors like the buffering capacity of saliva produced during rumination (Martin et al., 2006). According to Mialon et al. (2008), the rumen pH is normally situated above 6.25 to ensure the optimal conditions for the majority of microbial species. This pH, which is typically achieved by forage rich diets, may attain values below 6.0 or 5.5 for abnormally long duration during the day when high concentrate diets are offered. In such cases the ruminal flora is perturbed, leading to digestive troubles such as decreased ingestion, decreased growth, or even infectious and locomotor complications, with considerable economic importance (Stone, 1999). The type and composition of diet strongly condition the microbial populations (Jouany and Ushida, 1998). Thus, their populations are modulated by the proportion of concentrate feed in the ration, the increase of cereals in diets for intensive fattening decreases in the salivation and the arrival of bicarbonates in the rumen, which leads to reduce the rumen pH, causing a reduction of the cellulolytic flora, together with the increase of lactic flora. From this point, it is necessary to know in an accurate way the process of microbial fermentation of feeds commonly used in concentrate diets, in order to better understand nutritive processes of intensively reared ruminants and to establish strategies to minimise the negative impact of this practice.

Having into account the importance of rumen fermentation, several analytical procedures have been developed for facilitate their study, either *in situ* (Mehrez and Orskov, 1977) or *in vitro* (Tilley and Terry, 1963; Menke et al., 1979). Among them, the gas production method (Menke and Steingass, 1988; Theodorou et al., 1994) indirectly estimates the rate and extent of microbial fermentation from the gas produced under *in vitro* conditions. The application

conditions of such technique are suitable to mimic high forage diets, by maintaining an incubation pH over 6.5 using a highly buffered medium. These conditions, however, are not adapted to the study of fermentative processes in intensive feeding, and therefore estimation of concentrate fermentation is biased. However, there are no difference between media in initial pH, but there are large differences in the final pH when adding from 2 to 12 mmol propionic acid to the medium, with the media of Steingass (1983) and Goering and Van Soest (1970) maintaining a pH above 5.5, while others had a pH of 5.42 (Theodorou, 1993) and 5.29 (Huntington et al., 1998). In a study reported by Fakhri et al. (1997), pH at 72 h of incubation of wheat and corn grains was much lower with the Steingass (1983) medium, but there was little difference between the media of Goering and Van Soest (1970) and Theodorou (1993). The concentration of bicarbonate ion is lower in the Theodorou, (1993) and Huntington et al. (1998) media compared with those of Goering and van Soest (1970) and Steingass (1983).

LITERATURE REVIEW

I. Ruminal acidosis in feedlot

I.1. Physiology of ruminal pH

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

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

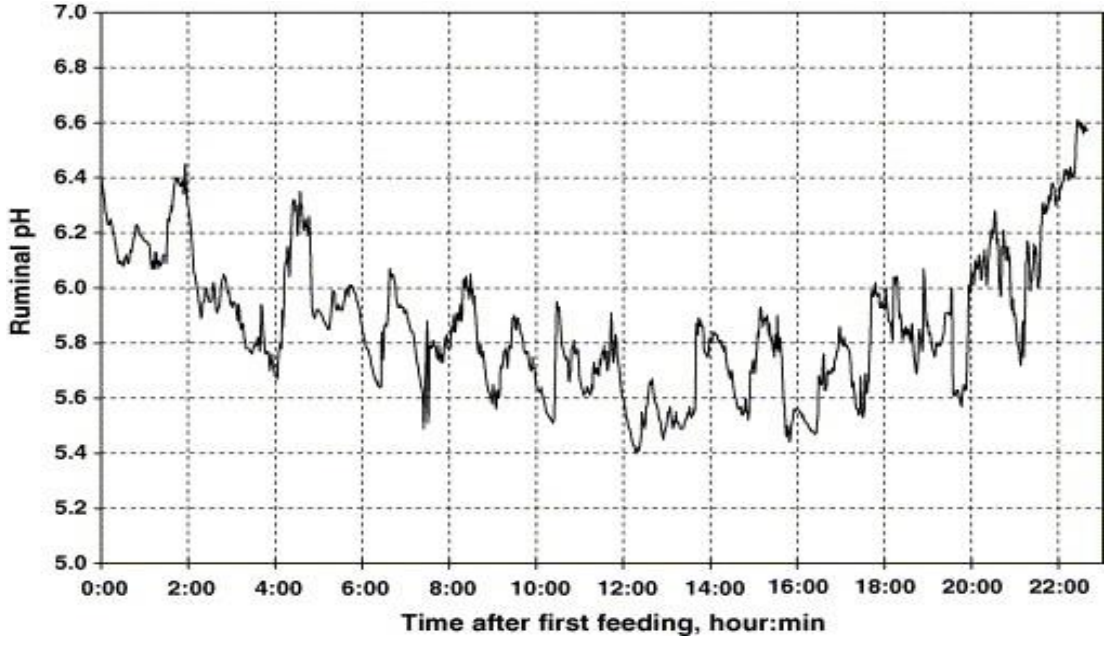
The variations in the amount of CO_2 dissolved in the medium automatically vary the amount of H^+ ions (Marden et al., 2005). Then, the pH of the rumen is defined by:

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

According to Counotte et al. (1979) and Erdman (1988), the most prevalent ruminal buffer is HCO_3^-. Araujo-Febres and Vergara-López (2007) reported that the rumen buffer system is very complex, based on an abundant production of saliva (mainly from secretions of bicarbonate and phosphate which buffer rumen fermentation), the elimination of VFA by their absorption through the rumen wall, and the mineral salts which react with the organic acids from plants producing CO_2. The saliva secreted by ruminants has a pH of 8.2, and high concentration of sodium, bicarbonate and phosphate ions and behaves as lubricant of the feed consumed, characteristics which allows their buffer action on the rumen fluid (Krause and Oetzel, 2006). The measurement of the buffer capacity of rumen contents is useful to express the ability of this organ to remain more or less stable at certain pH range. The pH of the rumen undergoes diurnal fluctuations and reflects the balance of acid production and absorption, as well as the buffering function provided by the bicarbonate ion from the saliva. Ruminal pH varies considerably during the course of a day, and is particularly driven by the amount of fermentable carbohydrate in each meal (Krause and Oetzel, 2006). Shifts of 0.5 to 1.0 pH units within a 24h period are common (Dado and Allen, 1993; Nocek et al., 2002). This represents a five to ten-fold change in the hydrogen ion concentration in the rumen (Krause and Oetzel, 2006). A typical pattern of

ruminal pH variation during the day is presented in Figure 1. After feeding, VFA production from fermentation increases resulting in a drop of rumen pH. As the rate of VFA production decreases while absorption continues in the subsequent hours, the rumen pH will progressively rise again (Crater et al., 2007) until the next feeding. Among the factors which contribute to change the rumen pH, the nature of the diet is a key factor in ruminal pH fluctuations, although ruminants have a highly developed system to maintain the pH within the physiological limits of 5.5 to 7.0 based, on the above mentioned processes of salivation and absorption through the rumen wall, as well as by inversing rate of passage, (Krause and Oetzel, 2006). The high consumption of forage stimulates saliva secretions, and the carbohydrates of these forages are slowly digestible, while the consumption of cereal grains or feeds rich in starch or soluble sugars that are rapidly fermentable generates a high concentration of organic acids (Fischer et al., 1994). Thus, rumen pH drops below the physiological levels when ruminants consume excessive amounts of rapidly fermentable (non-fiber) carbohydrates. Besides, in intensive production systems where the use of concentrates is high, the rate and extent of degradation of the fibrous fraction of feed, which proportion is already reduced, is diminished by the low activity of cellulases of rumen microorganisms at low pH (Araujo-Febres and Vergara-López, 2007). Under these conditions, the rumination decreases as well as the secretion of saliva, and thus the buffering capacity of the rumen fluid decreases.

Figure 1. Post-feeding variations in ruminal pH over a period of 24 h. The cow was fed dry, cracked corn grain and finely chopped alfalfa silage twice daily, at 12 h interval (Krause and Combs, 2003).



I.2. Rumen acidosis

Microbes in the rumen degrade and ferment carbohydrates into volatile fatty acids which are absorbed through the rumen wall into the blood stream, in addition to gases such as carbon dioxide and methane that are expelled by eructation. Volatile fatty acids are the main energy source for ruminants, providing approximately 70% of the total energy requirements (Bergman, 1990). The three main volatile fatty acids produced in ruminants are acetic acid, propionic acid, and butyric acid (VFAs; C2, C3 y C4 respectively). The fattening of young ruminants with ad libitum feeding of diets high in grains and other highly fermentable carbohydrates and proteins, and often low in fiber promotes a high rate and extent of rumen microbial fermentation, thus producing high amounts of VFAs. A frequent consequence of this feeding practice is acidosis. The acidosis has become an important problem in ruminant feeding. In practice, there are two levels of acidosis: acute ruminal acidosis (ARA) and sub-acute ruminal acidosis (SARA).

Acute rumen acidosis occurs with rapid grain overload offer in non-adapted animals, and may result in severe illness, liver abscesses, and even in death of the animal. Ruminal pH of 5.0 or below, approaching 4.5 or lower is considered the benchmark for acute ruminal acidosis (Britton and Stock, 1989; Owens et al., 1998; Krause and Oetzel, 2006). The main reason for pH to reach 4.5 or below is lactic acid accumulation (Nagaraja and Titgemeyer, 2006), which is the result of increased lactic acid production and decreased lactic acid fermentation. Lactic acid is a compound derived from the intermediary metabolism of carbohydrates that is found in very small amounts in normal ruminal conditions. This acid is stronger than the VFAs, and is the first responsible for the alterations observed in cases of acute ruminal acidosis. Subsequent absorption of organic acids into the bloodstream might overwhelm the bicarbonate buffering system, the excretion rate of acids and the capacity of tissues and organs to metabolize acids, resulting in systemic acidosis (Brown et al., 2000). When the ruminal pH is below 5.0, the action of Lactobacillus spp, and Streptococcus bovis enhances, thus increasing lactic acid production. The maintaining of the pH drop on time determines the death of a considerable number of rumen bacterial species among them some lactate utilizing bacteria, such as Megasphaera elsdenii and Selenomonas ruminantium, which are not adapted to survive under these conditions. This aggravates the ruminal dysfunction at the time it releases endotoxins to the medium that can be absorbed into the body (Sienra, 2009). The direct action of acids on rumen epithelium determines its inflammation and the destruction of large areas, and finally the accumulation of lactic acid determines a significant increase in the osmotic pressure, so the

water can pass from the organism into the rumen (Radostits et al., 1994; Owens et al., 1998; Sienra, 2009). The affected animals show symptoms in two to four hours after grain intake. The symptoms include complete anorexia, abdominal pain, tachycardia, tachypnea, diarrhoea, lethargy, spasms, and death.

Sub-acute ruminal acidosis, also known as chronic or sub-clinical acidosis, is a well-recognized digestive disorder that is an increasing health problem in most herds (fattening or dairy). SARA is defined by periods of moderately depressed ruminal pH from about 5.5 to 5.0 (Krause and Oetzel, 2006). This drop in ruminal pH is a result of the breakdown of dietary carbohydrates (e.g. starch), particularly from cereal grains such as corn and barley. Grains are high in readily fermentable carbohydrates that are rapidly broken down by ruminal bacteria, leading to the production of VFAs and lactic acid. Under normal feeding conditions, VFAs are readily absorbed by papillae (small finger-like projections) from the rumen wall. According to Nagaraja and Titgemeyer (2006), SARA results from excessive VFAs production that exceeds the ability of the ruminal papillae to absorb them; thus, volatile fatty acids accumulate in the rumen and, as a result, reduce ruminal pH. Lactic acid does not consistently accumulate in the ruminal fluid of ruminants affected with SARA (Oetzel et al., 1999) as it supported by beef feedlot data (Britton and Stock, 1986). However, transient spikes of ruminal lactate up to 20mM can be discovered if ruminal lactate concentrations are frequently recorded during the day (Kennelly et al., 1999). Some clinical signs often observed during SARA are: reduced rumination, mild diarrhea, foamy feces containing gas bubbles, appearance of undigested grain (particles larger than 6 mm) in feces, episodes of laminitis, weight loss and poor body condition, and unexplained abscesses.

A common cause of SARA occurs at fattening, when cattle often undergo acidic challenges in the feedlot when they are transitioned from forage-based to concentrate-based diets as part of normal feedlot management. Nagaraja and Titgemeyer (2006) explain that an abrupt dietary change does not allow ruminal bacteria and ruminal papillae for the adequate time to adapt, thereby leading to a rapid production and accumulation of VFA. Another common cause of SARA is an inadequate balance of mixed rations, such that the effective fiber content falls below recommended levels or particle size is too small. This suppresses rumination and reduces stimulation of the production of saliva, reducing the possibility buffer changes in ruminal pH. In order to limit the development of acidosis in transition, the young ruminant must adapt their feeding behaviour to very high concentrate rations offered ad libitum (Mialon et al., 2008). According to Brugere-Picoux (2004), changes in ruminal bacterial populations when

exposed to high concentrate rations require from 2 to 3 weeks, and it is recommended that concentrate levels be increased at 5 to 7 day intervals during this period to avoid SARA. The adaptation of ruminal papillae when higher concentrate diets are fed takes longer, approximately 4 to 6 weeks (Mutsvangwa, 2003). To prevent acute acidosis, Brugere-Picoux (2004) recommended that the starch dietary concentration must be less than 30%, and the rate of the concentrate must be below 60% of the total dry matter. So, there is a direct relationship between diet, ruminal ecosystem and the appearance of acidosis (Sauvant and Peyraud, 2010). Fermentation acids production in the rumen needs to be balanced with fermentation acids removal and neutralisation in order to achieve optimal ruminal conditions and optimal production (Krause and Oetzel, 2006).

To minimize the risk of acidosis in ruminants, it is often recommended the supplementation of rations with chemical buffers, with the purpose of maintaining the optimum pH. The most used are the sodium bicarbonate and sodium sesquicarbonate; $NaHCO_3$, Na_2CO_3 (Meschy et al., 2004). In addition to chemical buffers, biological additives such as enzymes, yeasts and fungi are used to prevent the latent acidosis (Chaucheyras-Durand and Durand, 2010; Desnoyers et al., 2009).

II. Digestion of dietary carbohydrates

Carbohydrates as nutrients can be classified into two categories according to their distribution in the plant cell. The first category corresponds to the cytoplasmic carbohydrates; they are either intermediates of cellular metabolism of carbohydrates or reserve carbohydrates. The former are water-soluble carbohydrates that include mono and disaccharides that directly contribute to the metabolism of the plant cell. The principal reserve carbohydrates of plant is starch that is stored in grain, tubers, and roots. Starch is a complex carbohydrate, made of many glucose units joined by chemical bonds. The two main components of starch are amylose, who is a helical polymer made of α-D-glucose units, bound to each other through α (1→4) glycosidic bonds, and amylopectin where the glucose units are linked in a linear way with α (1→4) glycosidic bonds. Branching takes place with α (1→6) bonds occurring every 24 to 30 glucose units, resulting in a soluble molecule that can be quickly degraded as it has many end points onto which enzymes can attach.

The second category of carbohydrates corresponds to structural carbohydrates; they are constituents of the cell wall, and include cellulose, hemicelluloses, lignin and pectins. The cellulose is a polymer of glucose linked with β 1-4 bonds, and is the main component of the plant wall. These linear chains are associated among them by hydrogen-type bonds to form cellulose micro-fibrils. The hemicelluloses differ from the cellulose by the simultaneous presence of β 1-4 linkages, β 1-3 and α 1-6. (Grenet and Besle, 1991). Hemicelluloses and cellulose are systematically related to constitute the base of the plant cell wall. Lignin is not a carbohydrate, but a polyphenolic structure. However, due to its importance in the plant cell wall, it is often classified as a vegetable fiber components. It offers greater resistance to plant cell walls. Pectins are uronic acid polymers and sugars, and are considered as soluble fiber. They are principally acids polymers α-galacturonic and rhamnose linked by α-1-4 linkages.

II.1. Carbohydrate degradation

According to Hungate (1966), carbohydrates are the main source of energy for ruminal microorganisms, and are quantitatively the most important component in the diet of ruminants. In addition to provide energy to the microorganisms and the ruminant, when present in form of fiber they have a physical role in maintaining the optimal functioning of the rumen. The non-fibrous carbohydrates are efficiently used as energy source, as they are fermented quickly and to a high extent, but increase the risk of ruminal acidosis, as previously stated. In contrast, the fibrous carbohydrates are more resistant to degradation, but modulate transit of digesta and stimulate rumination and saliva production which acts as a buffer of rumen contents. Pectins and soluble fiber are also rapidly used by rumen microorganisms.

Some bacterial and protozoal species are implicated in the hydrolysis of starch. The amylolytic bacteria are favoured by a ruminal pH between 5.0 and 6.0 (Belbis, 2007). The microorganisms produce amylases that are extracellularly active, randomly attacking the α-glucosidic bonds, releasing glucose as the final product. The degradation of starch begins with the adhesion of the microorganism to the substrate, and then continues with the action of several enzymes that degrade the structure of starch to oligosaccharides, which can be absorbed and fermented by micro-organisms. The differences in starch structure from one plant species to another, or even among varieties, is what influences its fermentation (Samantha and Van Barneveld, 1999). Rooney and Pflugfelder (1986); Colonna et al. (1995) reported that the enzymatic digestibility of starch is in general inversely related to its amylose content, or to their

ratio amylose/amylopectin (Offner et al., 2003). This degradability is also influenced by the structure of the endosperm of the cereal grain. Michalet-Doreau and Doreau (1986); Huntington (1997); Evers et al. (1999) reported that the endosperm is composed of two parts:

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

Since the endosperm of barley is floury, it is most fermentable than the endosperm of corn and sorghum, which are hard (Chevalier, 2001). Besides, the presence of phenolic compounds in the grains, such in the case of sorghum which have starch and proteins highly related among them by the effect of tannins (Kristen et al., 2015), influence their fermentation (Kim et al., 2006). Often, the amount of starch among cereal species is higher with sorghum and corn than barley varieties. This results have been observed by several authors, (72%, 63%, and 59% respectively; Laurent (1988); 74% for corn and sorghum, and 59.5% for barley; Chevalier (2001); 64%, 63%, and 53%, respectively; FEDNA (2010)). However, the rate of starch fermentation is higher with barley (40% to 60%/h depending on the variety) than corn and sorghum (15% to 30%/h) (Sauvant and Michalet-Doreau, 1988). As it is shown in Table 1. The fermentation of soluble sugar occurs immediately after the food ingestion, carried out by a very large number of bacteria (Hungate, 1966).

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

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

The fibrous carbohydrates are also degraded by the rumen microorganisms, though at a lower and variable extent (Chesson and Forsberg, 1988; Orskov and Ryle, 1998), giving a lower proportion of energy, and may limit intake because of their bulk effect. The fibrolytic bacteria, fungi and certain protozoa are implicated in the degradation of fiber, and their action is facilitated by a pH above 6.5 (Dusart, 2014). The microorganisms secrete many hydrolases to the rumen medium except for the fibrolytic activity of protozoa, which occurs intracellularly after intake of fibrous particles. Three types of cellulases act in synergy: endo β 1-4 glucanases, cellobiosidases and β –glucosidases. The first type attacks to the cellulose to form cello-oligosaccharides, the second attacks the non-reducing end of the cellulose to give the cello-biose units, and the third type hydrolyses the cellobiose and cello-oligosaccharides of low degree of polymerization to give glucose (Jouany, 1994). Concerning the hemicelluloses, the xylanases are secreted by a large number of bacterial species, and protozoa and fungi also produce cellulases and hemicellulases. For the hydrolysis of the pectin, pectinolytic enzymes have been identified in bacteria and protozoa but not in fungi, microorganism that quickly hydrolyse this polysaccharide (Grenet and Besle, 1991).

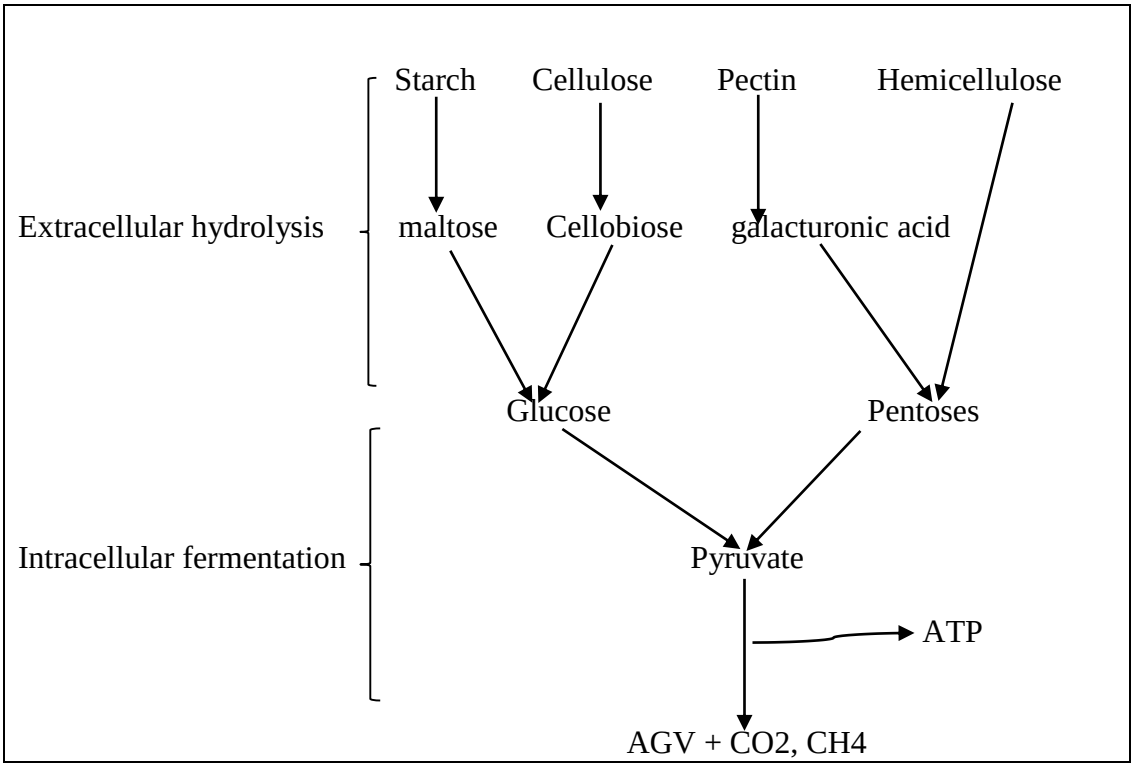
The fermentation of the fibrous carbohydrate depends on the lignin content in the feeds, being negatively related to its proportion and the magnitude of linkages with structural carbohydrates (Chesson and Forsberg, 1988). Despite having a considerable fibrous fraction, several agro-industrial by-products used in the nutrition of ruminants, such as sugar beet pulp, citrus pulp, and wheat bran, present the advantage of low proportion of lignin (1.69%, 078%, and 3.4% respectively; FEDNA, 2010). Besides, these feeds have a considerable proportion of easily-fermentable hemicelluloses and pectin (in sugar beet pulp and mainly in citrus pulp), which renders high amount of energy when fermented in the rumen (Nocek and Tamminga, 1991).

II.2. Metabolic pathways of carbohydrate fermentation by rumen bacteria

The sugars released by hydrolysis will be absorbed and then fermented into pyruvate by the Emden-Meyerhof and pentose phosphate pathways. The pyruvate is metabolised to VFAs like acetic acid (C2), propionic acid (C3), butyric acid (C4) and a minor proportion of valeric acid and branched-chain VFA, and in some cases to lactic acid. Gases (carbon dioxide, hydrogen gas and methane) and energy are released, in the latter form of adenosine tri-phosphate (ATP). The two steps of the microbial fermentation of carbohydrates are shown in

Figure 2. A high proportion of fiber fraction, causes an increase in the proportion of C2, because the fibrolytic rumen bacteria generally use this metabolic pathway, whereas a high starch content will promote the production of C3. The presence of soluble carbohydrates (sucrose, lactose, inulin) contained in foods such as beets or whey will increase the proportion of C4 (Grenet and Besle, 1991). Dusart (2014) reports that in balanced diets, the C2 represents between 60 and 65% of ruminal VFA, the C3 from 18 to 20%, the C4 from 10 to 15%, and the other VFAs from 2 to 5%. Table 2 shows the differences in proportion of VFA in function of the nature of the diets.

Figure 2. The fermentation and utilization of carbohydrate by ruminal bacteria (Dusart, 2014)



The rumen pH varies mainly according to the level of accumulation of VFAs (Tamminga and Van Vuuren, 1988), and the proportions of VFAs depend on the nature of the diet. With a forage-based diet, the pH remains relatively constant throughout the day, this due to the buffer capacity of the hay and that of saliva that is produced in large quantities during chewing (Michalet-Doreau and Sauvant, 1989). But the rich grain rations bring into the rumen a significant amount of rapidly fermentable starch, causing an increase of the VFA concentration

and an important drop in pH (Mackie et al., 1978). The variations in pH promote changes in the microbial population of the rumen: knowing that the fibrolytic bacteria develop at a pH above 6.5 (Dusart, 2014) and that the amylolytic bacteria preferably grows at a pH between 5.0 and 6.0 (Belbis, 2007), a low pH will cause higher proportions of C3 whereas a high pH will cause higher proportions of C2 (Blain, 2002). The decrease in the percentage of C2 with starch-rich diets is directly related to the drop in rumen pH, and presumably to the increase of concentration of amylolytic bacteria. In such situations, significant disruptions in the rumen fermentations and the onset of increasing amounts of lactic acid are common (Michalet-Doreau and Sauvant, 1989). Blain (2002) reports than the quantity of VFA in the rumen is also dependent on the rate of substrate degradation: the faster this substrate is degraded, the higher the quantity of VFA is produced. Thus, high proportions of starch in the diet will cause high ruminal VFA production, promoting a rapid drop in ruminal pH that can cause ruminal acidosis. The fibrous fraction is digested at a lower rate than starch.

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

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

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

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

III. In vitro gas measuring techniques

Various methods have been developed to evaluate the nutritive value of feeds for ruminants, classified as *in vivo*, *in situ*, and *in vitro* methods according to their analytical procedures. The estimation by *in vivo* methods has the inconvenient of being expensive, laborious, and not applicable to a large scale. Hence, alternative methods are put in place. Since *in situ* methods require preparation and maintenance of a significant number of rumen cannulated animals (Mehrez and Orskov, 1977), the *in vitro* methods seem a more practical alternative. *In vitro* methods not only have the advantage of being less expensive and less time consuming, but also give the chance to maintain experimental conditions under control more precisely than *in vivo* trials permit (Raab et al., 1983). Three major types of biological digestion techniques are currently available to determine the nutritive value of ruminant feeds *in vitro*: 1) measuring the extent of microbial digestion in terms of substrate disappearance, as in the Tilley and Terry (1963) method; 2) degradation with cell-free fungal cellulases (De Boever et al., 1986); and 3) indirect estimation of fermentation by measuring gas production (Menke et al., 1979; Theodorou et al., 1994). These biological methods are more meaningful than simple chemical methods, since microorganisms and enzymes are more sensitive to factors influencing the rate and extent of digestion (Van Soest, 1994). The *in vitro* gas measurement technique is actually used not only for evaluating the nutritive value of feeds, but also is being widely used for obtaining a better insight into rumen fermentation processes. Considering the characteristic of the work conducted for this study, the gas production techniques were chosen as experimental procedure, and thus a major effort will be based on their explanation.

III.1. The pressure measuring systems

This method is based on the measurement of the accumulated gas pressure in batch systems (glass bottles of 125 ml), containing the substrate, the artificial saliva and the ruminal fluid. The incubation is carried out in a heater or in a water bath, at 39 ° C. The pressure recording can be done manually, according to the Theodoreau et al. (1994) technique, or automatically according to several systems (Pell and Shofield, 1993; Cone et al., 1996; Davis et al., 2000).

III.2. Origins of gas

Blümmel and Orskov (1993) reported that when a substrate is incubated *in vitro* with buffered rumen fluid, the carbohydrates are fermented to VFA, producing gases, mainly CO_2 and CH_4, and microbial cells. Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate (Wolin, 1960; Beuvink and Spoelstra, 1992; Blummel and Ørskov, 1993). The amount of gas released from carbohydrates will depend on their chemical structure which determines the metabolic pathway (as stated above) and the relationship with other feed components (lignin, protein, and tannins) that, partially masking the carbohydrate structures, will modulate the rate and extent of fermentation. Gas production from protein fermentation is relatively small as compared to carbohydrate fermentation (Wolin, 1960). In fact, Raab et al. (1983) stated a negative linear regression between ammoniac concentration released from fermentation of crude protein of plants and gas production, the same conclusion was concluded from results found by Gonzalez et al. (1998), whose observed a significant inverse relationship between the gas production and nitrogen (N) concentration, this was explicated by these authors from the lower productions of gas recorded which coincided with the higher protein content of forage samples. In this way, Menke and Steingass (1988) observed that protein fermented *in vitro* produces ammonium bicarbonate from the carbon dioxide and ammonia resulting from its fermentation. Furthermore, ammonia produced in protein breakdown may be directly used by bacteria for protein synthesis. Hence, if carbon dioxide and ammonia are given off in a lower extent in the gas phase, it will result in a lower amount of gas recorded, which will underestimate the extent of fermentation. Hess (1991) added that a high correlation exists between the level of NH_3-N and CP contents in the plants, as for each unit of the increase in the protein content in the forage, the level of NH3-N increased approximately 21 units.

The contribution of fat to gas production is negligible: when coconut oil, palm kernel oil and/or soybean oil were incubated, only 2.0 to 2.8 ml/g OM of gas were produced, mostly from the glycerol released from hydrolysis of triglycerides, while a similar amount of casein and cellulose produced about 23.4 ml and 80 ml gas (Menke and Steingass, 1988; Getachew et al., 1998). Besides, Moore et al. (1986); Palmquist (1988) stated that the fat supplementation may alter rumen fermentation by reducing digestibility of other dietary ingredients, especially fiber. It is generally assumed that adding fats, especially unsaturated, at or over a proportion of 0.05 in forage or mixed diets reduces rumen microbial fermentation and DM disappearance (Devendra and Lewis, 1974; Kowalczyc et al., 1977; Moore et al., 1986; Tesfa, 1992). Wu and

Palmquist (1991) observed that degradation of long-chain fatty acids to CO_2 and VFA was less than 0.01 when incubated with rumen microorganisms *in vitro*.

The molar proportions of different VFAs (acetate, propionate and butyrate) produced is dependent on the type of substrate (Beuvink and Spoelstra, 1992; Blummel and Ørskov, 1993). Therefore, the molar ratio of acetate to propionate was used to evaluate substrate related differences (Getachew et al., 1998). The same authors added that rapidly fermentable carbohydrates yield relatively higher propionate as compared to acetate, and the contrary takes place when slowly fermentable carbohydrates are incubated. The following reactions show the estimation of the stoichiometry of volatile fatty acid production in the rumen:

Hexose + 2 NAD + 2 ADP → 2 pyruvate + 2 NADH + 2 ATP
2 pyruvate + 2 ADP + 2 H_2O → 2 acetate + 2 CO_2 + 2 H_2 + 2 ATP
2 pyruvate + 4 NADH + 2 ADP → 2 propionate + 2 H_2O + 4 NAD + 2 ATP
2 pyruvate + 2 NADH + 1 ADP → 1 butyrate + 2 H_2 + 2 CO_2 + 2 NAD + 1 ATP
CO_2 + 4 H_2 → CH_4 + 2 H_2O + 1 ATP

The gas generated comes either directly from substrate fermentation, or indirectly from the liberation of carbon dioxide following neutralisation of the VFAs by the carbonate/bicarbonate ion in the buffer (Beuvink and Spoelstra, 1992). Following Blümmel and Orskov (1993); Makkar et al. (1995), a highly significant correlation has been observed between SCFA and gas production. Wolin, (1960); Hungate, (1966); Van Soest, (1994), reported that the gas is produced mainly when substrate is fermented to acetate and butyrate. Substrate fermentation to propionate yields gas only from buffering of the acid and, therefore, relatively lower gas production is associated with propionate production (Table 3). Mauricio et al. (1998), reported that when the substrate is fermented mainly to propionic acid, their fermentation is underestimated.

Table 3. Molar proportions (per mol of hexose) of direct (from microbial fermentation) and indirect (from buffering) gas produced in the rumen fermentation *in vitro* (Beuvink and Spoelstra, 1992).

VFA, mmol	Direct gas, mmol	Indirect gas, mmol	Total gas, mmol,
2 acetic acid	2 CO_2	2 CO_2	4 CO_2
2 propionic acid	-	2 CO_2	2 CO_2
1 butyric acid	2 CO_2	1 CO_2	3 CO_2
2 lactic acid	-	2 CO_2	2 CO_2

III.3. Effect of pH on *in vitro* fermentation

Another aspect to be taken into consideration is the incubation pH, which determines the equilibrium of microbial population (Hungate, 1966), and is therefore a key factor in the development of fermentative activity. Conventional *in vitro* evaluation systems (Tilley and Terry, 1963; Menke et al., 1979; Theodorou et al., 1994) maintain the medium pH between 6.5 and 7.0 by HCO_3^-/CO_2 equilibrium from the bicarbonate buffer from (Goering and van Soest, 1970), although a small proportion of phosphate buffer is also included and participates in buffering although it does not contribute to gas production. This buffer is adequate for routinary evaluating fibrous feedstuffs given that, at the current HCO_3^- concentration (0.11 M), it adjusts the pH to around 6.7–6.8 and keeps it stable along the incubation, which is the initial and main aim of the technique. However, it is not so suitable for approaching pH to around or below 6.0 like when rapidly fermentable carbohydrates are supplemented by feeding mixed and concentrate diets (Grant and Mertens, 1992; Fondevila et al., 2002). For simulating *in vivo* conditions of high concentrate feeding, the conventional buffer from Goering and van Soest (1970) does not allow for maintaining pH conditions between 6.5 and 5.5 (Kohn and Dunlap, 1998). Continuous culture systems (Hoover et al., 1976; Czerkawski and Breckenridge, 1977) control pH by both the inclusing of bicarbonate ion in the artificial saliva and the infusion of concentrated solutions of NaOH and HCl, although strong acids and bases have a lower buffering capacity than the weak ones (Kohn and Dunlap, 1998) and their inclusion may alter microbial activity. Reducing the concentration of bicarbonate buffer may be a simple way of maintaining a low pH (Kohn and Dunlap, 1998) but with this practice its buffering capacity is also considerably reduced and may be exceeded when VFA production increases during incubation (Beuvink and Spoelstra, 1992) however, it has also be considered that fermentation

of highly concentrated feeds occurs rapidly, making unnecessary monitoring for times longer than 12-16 hours. A way to overcoming this problem would be to periodically inoculate fresh low-concentrate buffer into the incubation medium as it has been applied in a semi-continues incubation system by Fondevila and Perez Espés (2008).

IV. Problem and objectives

The transition from weaning to fattening with high concentrate feeds for young ruminants needs some time for the microbial population to be adapted in order to allow for minimizing the risk of acidosis, which is at present one of the most common problems during intensive fattening of ruminants. In such situation, a sound knowledge about the fermentative behaviour of different sources of carbohydrates is needed, including the study of their potential for rate and extent of environmental pH reduction, in order to choose the better alternative for an adequate transition to a high concentrate feeding. For achieving that in a practical way, routine *in vitro* nutritive evaluation techniques must be adapted for the study of this type of feeds, with emphasis on the incubation pH, which has a major impact on rumen microbial activity.

This thesis aims to develop a strategy to mitigate the impact of the transition from a forage diet to a diet high in concentrate for the early fattening of ruminant by assessing the effect of the source of carbohydrate supplementation on pH and overall microbial fermentation, under *in vitro* conditions.

Other specific objectives are the following:

1. To study under *in vitro* conditiones, how the dietary components given during the transition from a forage to a concentrate diet can modulate the characteristics of ruminal fermentation.
2. To determine how inocula from different feeding conditions modulate the fermentation response, in order to estemate the magnitude of the importance of adaptation of rumen microbial conditiones to diet characteristics.
3. To evaluate under *in vitro* conditions, the adequate carbohydrate type for a transition from a ruminal environment based on forage to another rich on concentrate.

EXPERIMENTATION

Experimentation

I. Material and methods

I.1. Substrates

Thirteen feeds were studied as substrates of incubation, which were kindly provided by E. Dorado (**CADEBRO),** M.D. Carro and V. Jimeno (Universidad Politécnica de Madrid), and G. Fondevila (**NUTRECO)**. Feeds were grouped as cereals (three varieties of corn, three of sorghum and three of barley), or other carbohydrate sources (sugar beet pulp, citrus pulp, wheat bran and sucrose). All of the substrates except sucrose were milled with a sieve of 1 mm using a hammer mill (Retsch Gmbh/SK1/417449), and then packaged until use in airtight plastic bottles. Chemical composition of substrates is given in Table 4, and the total phenolics and tannins concentration (g/kg DM) in the three sorghum varieties (S1, S2 and S3) is given in Table 5.

Table 4. Chemical composition (g/kg DM) of feeds used as incubation substrates

Substrates	Code	OM	CP	EE	Starch	NDF	ADF	ADL
CORN DEKALB 6815	**C1**	987	85	38.3	693.4	88	28	5.2
CORN DEKALB 666746	**C2**	986	75	34.3	705.7	91	25	2.0
CORN PIONEEL 75 (transgenic)	**C3**	986	73	41.4	706.5	93	27	5.2
WHITE SORGHUM	**S1**	984	109	39.6	689.4	98	47	14.4
BROWN SORGHUM	**S2**	985	106	40.8	637.6	93	60	16.7
BROWN SORGHUM	**S3**	979	113	10.5	647.4	97	60	5.2
BARLEY GUSTAV	**B1**	978	105	24.4	672.1	173	56	17.5
BARLEY SIGNORA	**B2**	978	112	18.0	634.7	152	44	9.3
BARLEY GRAPHIL	**B3**	977	91	17.7	650.5	189	55	9.1
BEET PULP	**BP**	953	107	4.8		437	272	74.6
CITRUS PULP	**CP**	940	59	13.8		192	207	20.9
WHEAT BRAN SARINA NOGAL	**WB**	944	161	31.4	244.9	499	145	37.0
SUCROSE	**SU**	1000						

Table 5. Total phenolics and tannins concentration (g/kg DM) in the three sorghum varieties (S1, S2 and S3).

Substrate	total phenolics	total tannins
S1	1,54	0,200
S2	19,30	8,080
S3	2,56	1,320

I.2. Microbial inoculum source

The rumen fluid was obtained from four adult Rasa Aragonesa ewes (average weight 70 ± 3 kg) fitted with a rumen cannula, housed in the facilities of the Servicio de Apoyo a la Experimentación Animal of the Universidad de Zaragoza. In order to obtain the adequate inoculum characteristics for the planned experimental objectives, these animals were fed either 600 g of alfalfa hay plus 300 g of straw for the first part of the experimental design (forage inoculum, FI), or 500 g of concentrate (0.60 barley, 0.20 corn, and 0.20 soybean meal) plus 300 g of alfalfa hay for the second part (concentrate inoculum, CI). The rumen contents (approximately 300 ml) were extracted before the morning feeding, pooled, filtered through a cheesecloth and transferred to the laboratory in thermos bottles preheated to 39° C.

I.3. Incubation procedures

The fermentation kinetics of experimental feeds were determined by their *in vitro* incubation following the procedure of Theodorou et al. (1994), in 116 ml glass bottles containing 500 mg of substrates (except for sucrose, 400 mg). The bottles were filled with 80 ml of incubation solution, which consisted of 0.10 rumen inoculum and 0.90 of an incubation mixture containing (ml/l): 474 ml distilled water; 238 ml of buffering solution made up with sodium bicarbonate ($NaHCO_3$) and ammonium bicarbonate ($(NH_4)HCO_3$); 238 ml of macro-minerals solution made up with 5.7 g disodium hydrogen phosphate (Na_2HPO_4), 6.2 g potassium di-hydrogen orthophosphate (KH_2PO_4), and 0.6 g magnesium sulphate ($MgSO_4.7H_2O$); and 50 ml of reducing solution made up with 47.5 ml distilled water, 2 ml 1N NaOH and 313 mg HCl-cysteine. This incubation medium did not contain micro-minerals nor resazurin (Mould et al., 2005). All these ingredients were mixed and allowed to be reduced

under a CO_2 atmosphere, using heat to accelerate the reaction. Bottles were filled with the incubation solution under a CO_2 stream, sealed with rubber caps and aluminium caps, and incubated at 39 ºC. On each incubation run, three additional bottles without substrate were also included as blanks.

Pressure was recorded every two hours, throughout the incubation, by means of an HD 2124.02 manometer fitted with a TP804 pressure gauge (Delta Ohm, Caselle di Selvazzano, Italy). The gas produced was allowed to evacuate after each measurement, in order to prevent any pressure effect on microbial activity (Rymer et al., 2005). Readings were converted into volume by a pre-established linear regression equation between the pressure recorded in the same bottles under the same conditions and known air volumes (n= 103; R^2= 0.996). The gas volume recorded for each incubation time was expressed per unit of incubated organic matter (OM).

On every experimental series, some bottles were randomly chosen at certain incubation times, open after the pressure reading and the pH of their contents was immediately measured (CRISON micropH 2001, Barcelona, España). Some of these bottles were also sampled for volatile fatty acid analysis (2 ml sample, collected over 0.5 ml of a deproteinizing mixture of 0.5M PO_4H_3 with 2 mg/ml 4-methyl valeric acid) and lactate (2 ml). At the end of each experimental series, the contents of each remaining bottle were filtered through nylon bags (45 µm pore size), and dried at 60ºC for 48 hours to determine dry matter disappearance (DMd).

I.4. Experimental design

Attending to the main objective of this work, four experiments were conducted according to the concentration of the buffer solution, the type and variety of substrate, and the type of inoculum.

1.4.1. Experiment 1.0

Control of the *in vitro* incubation pH for the simulation of fermentation under concentrate feeding conditions.

This preliminary experiment was carried out to adjust the experimental *in vitro* conditions to fit the incubation pH according to the level of inclusion of bicarbonate ion in the incubation

solution, and to study its effect on the kinetics of fermentation of the substrate. For this experiment, five levels of incubation pH (6.50; 6.25; 6.00; 5.75 and 5.50) were fixed by adjusting the concentration of bicarbonate ion in the incubation solution (Table 6), based on the calculation from Kohn and Dunlap (1998) in order to simulate the fermentation conditions under high concentrate feeding. An amount of 500 mg of B3 was included as a model substrate. Two *in vitro* incubation series were carried out for 12 h with seven bottles for each treatment, using FI as inoculum. The gas production was recorded every two hours (at 2, 4, 6, 8, 10 and 12 h of incubation), and immediately after one bottle of each treatment was opened to determine the incubation pH. The evolution of gas production was estimated from the two bottles maintained for 12 h.

Table 6. Bicarbonate salt concentration in the buffer solution for adjusting the incubation pH, and final concentration of bicarbonate ion in the incubation medium.

pH	**$NaHCO_3$, g/l**	**$(NH_4)\ HCO_3$, g/l**	**HCO_3^-, mol/l**
6.5	18.3	1.9	0.058
6.25	10.3	1.07	0.032
6.0	5.7	0.6	0.018
5.75	3.17	0.25	0.01
5.5	1.91	0.12	0.006

1.4.2. Experiment 1.1

Fermentation of different sources of carbohydrates under optimal pH conditions (pH=6.5)

The purpose was to compare the fermentation pattern of all the substrates (13 feeds) tested in the others experiments, when incubation pH was maintained at an optimum level for carbohydrate fermentation. Two incubation series of 8 h were carried out, with triplicated bottles for each of the 13 substrates tested, plus 3 bottles without substrate as blanks. The FI was used and the incubation pH was adjusted to 6.5 by using the adequate amount of bicarbonate salts (Table 6). The volume of gas production was measured every two hours, and samples of the incubation medium were taken at 4 and 8 h of the experiment for volatile fatty acids and lactate.

1.4.3. Experiment 1.2

In vitro fermentation of cereal sources with inocula from different nature.

The objective of this experiment was to compare the *in vitro* fermentation pattern of cereal substrates according to the nature of the inoculum, in poorly buffered medium to mimic the natural fluctuations in rumen pH. At the same time, variations among varieties of the same cereal species according to composition of different varieties were studied.

Two different sets of incubation series were established, according to the nature of the inoculum (FI and CI), to compare *in vitro* fermentation of 3 varieties of corn, 3 varieties of sorghum and 3 varieties of barley, as substrates. Animals were accustomed to the corresponding diets for 14 days before being used as donors of rumen inoculum. The incubation solution was buffered at pH 5.5, according to the results from the experiment 1.0. For each inoculum, 3 *in vitro* incubation series of 10 h were carried out, with five bottles per substrate plus three additional bottles without substrate as blanks. The gas production was recorded every two hours, and then one bottle for each treatment was opened to determine the incubation pH. At 4 and 8 h of the experiment, samples were taken from the opened bottles for further determination of volatile fatty acids and lactate. At the end of incubation (10 h), the contents of bottles were filtered and dried at 60ºC for 48 hours to determine DMd.

1.4.4. Experiment 1.3

In vitro fermentation of non-starch carbohydrate sources with inocula from different nature.

The objective of this experiment was to compare the *in vitro* fermentation pattern of other non-starch carbohydrate sources according to the nature of the inoculum, in poorly buffered medium to mimic the natural fluctuations in rumen pH. Two different sets of incubation series were established, according to the nature of the inoculum (FI and CI), to compare *in vitro* fermentation of BP, CP, WB and SU. In addition, C2 (as a control). Animals were accustomed to the corresponding diets for 14 days before being used as donors of rumen inoculum. Having into account the results from the experiment 1.0, the incubation pH was fixed at 5.5. Three 10 h *in vitro* incubation series were carried out for each inoculum, with five bottles per substrate

plus three additional bottles without substrate as blanks. The gas production was recorded every two hours, and after pressure reading one bottle for each treatment was opened to determine the incubation pH. Besides, at 4 and 8 h of the experiment, samples from the opened bottles were also taken for determination of volatile fatty acids and lactate. At the end of incubation (10 h), the contents of bottles were filtered and dried at 60ºC for 48 hours to determine DMd.

I.5. Chemical analysis

Substrates were analysed following the procedures of AOAC (2005). The dry matter (DM) of substrates was determined in a forced air oven stove at 105 ºC during 24 h (ref. 934.01). The OM was determined by incineration at 550 ºC for 8 h (ref. 942.05). The crude protein (CP) was analysed by the determination of total nitrogen by Kjeldahl method (ref. 976.05), using a Kjeltec apparatus (Foss Tecator, 2300 Kjeltec Analyzer unit). Ether extract (EE) was determined by soxhlet analysis (ref. 2003.05) following AOCS official procedure AM 5-04 using ANKOM^{XT15} extraction system. The neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were determined in ANKOM^{220} Fiber Analyzer (ANKOM; New York) equipment according to the method proposed by Van Soest et al. (1991). The neutral detergent fiber 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 from samples ground to 0.5 mm using a commercial kit (Total Starch Assay Kit K-TSTA 07/11, Megazyme, Bray, Ireland). The total phenolic and tannin content was analysed following the colorimetric method of Makkar et al. (1993) using the Folin-Ciocalteau reagent and with tannic acid (MERCK Chemicals, Madrid, Spain) as the reference standard. The frozen samples of incubation media were thawed and centrifuged at 20,000 g for 15 minutes for their analysis of lactic acid and VFAs. The VFAs were determined by gas chromatography on an Agilent 6890, apparatus equipped with a capillary column (HP-FFAP Polyethylene glycol TPA, 30 m x 530 µm Id). The lactic acid concentration was determined by the colorimetric method proposed by Barker and Summerson (1941).

I.6. Statistical analysis

Results were analysed by ANOVA using the Statistix 10 software package (Analytical Software, 2010), considering the average of the two bottles of the same treatment on each incubation time as the experimental unit and the series as a block for all the experiments. For

the Experiment 1.0, when the results were significant ($P<0.05$), the evolution of treatment values was studied by orthogonal polynomials. For the Experiment 1.1, the effect of the source of carbohydrates on pH, *in vitro* gas production, total VFA concentration, VFA profile and lactic acid was studied for each time of incubation as a one-way ANOVA. In the Experiments 1.2 and 1.3, the effect of the type of inoculum, the type of substrate and their interaction on the gas production, pH, total VFA concentration, VFA profile and lactic acid was studied for each time of sampling.

The differences were considered significant when $P<0.05$, and it was considered that a trend for significance was considered when $0.05<P<0.10$. The Tukey method ($P<0.05$) was used for the multiple comparison between means. The relationship between results from the different parameters was studied by linear or multiple regression.

RESULTS

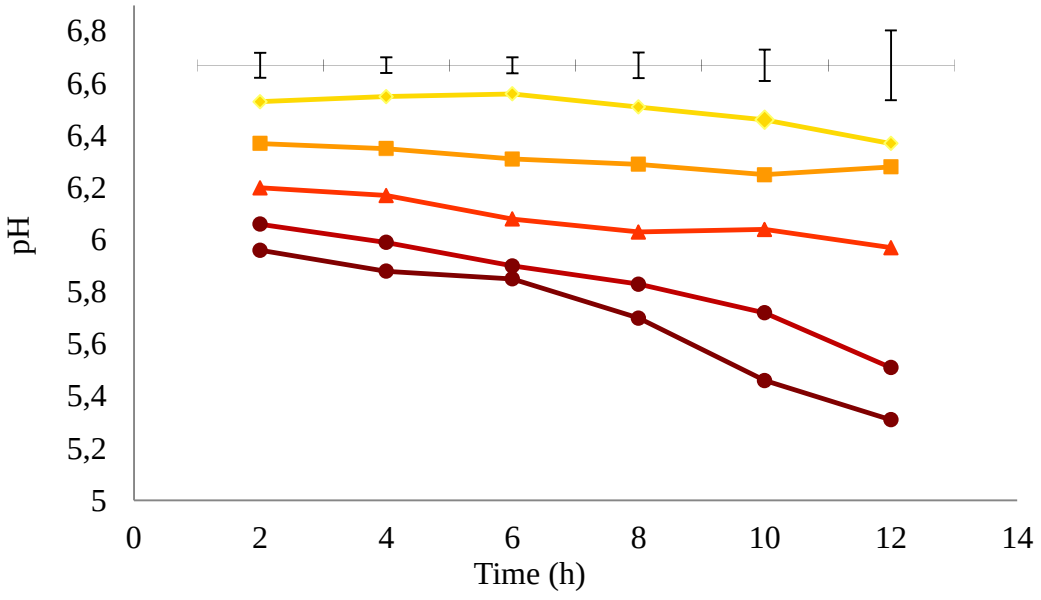
II. Results

II. 1. Experiment 1.0

II. 1. 1. Pattern of incubation pH

The pH of the rumen inoculum was 6.62 and 6.50 in the incubation series 1 and 2, respectively. A linear drop (P<0.001) in the medium pH according to the decrease of the buffering capacity was observed at all times of sampling (Figure 3). Besides, pH at 4 h tended (P= 0.081) to drop quadratically with the level of bicarbonate, this pattern being significant at 6 h (P= 0.006) because of the lack of differences between media 5.75 and 5.50 (pH of 5.90 and 5.85, respectively). The different concentration of bicarbonate ion in the buffer allowed to reach (± 0.1 units) the pH expected at 4 (6.35), 6 (6.08), 8 (5.83) and 10 (5.46) h of incubation for the treatments 6.25, 6.00, 5.75 and 5.50, respectively. From these times of incubation onwards, the medium pH was maintained within the range provided with the treatments 6.25 and 6.00, while it dropped slightly respect to the expected values with treatments 5.75 and 5.50 (final pH after 12 h of 5.51 and 5.31, respectively). Besides, at 4 h of incubation only treatments 5.75 and 5.5 achieve pH values of less than 6.0. For its part, from the beginning of incubation the pH of the medium 6.50 was maintained within the range fitted (pH 6.53 at 2 h), with minor modifications of 0.1 pH units until 12 h of incubation, when it decreased slightly (to pH 6.37).

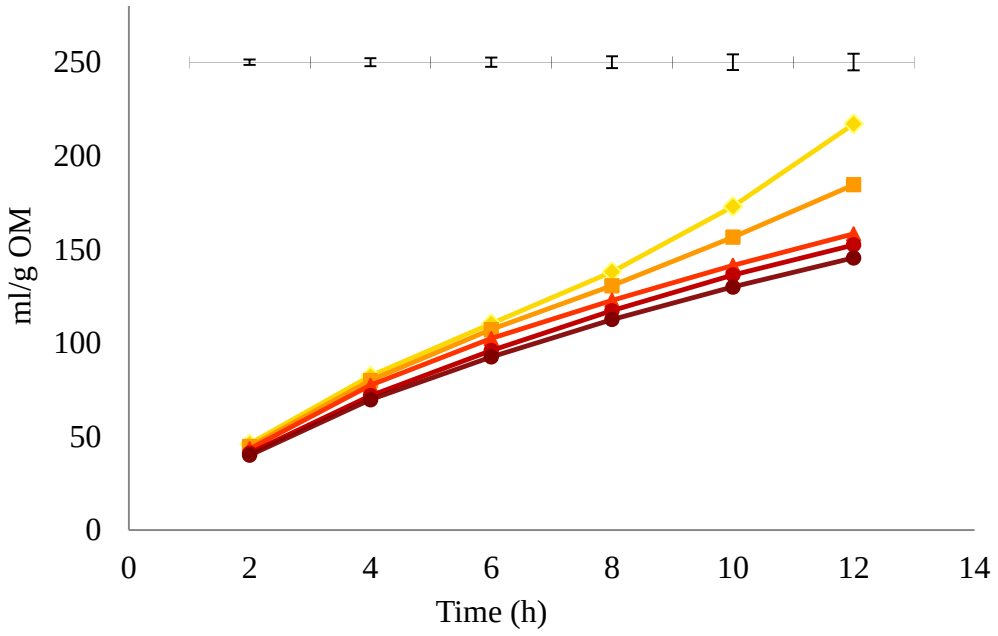
Figure 3. Pattern of incubation pH according to the fitted pH based on the concentration of bicarbonate ion in the incubation solution (pH 6.5 ♦; pH 6.25 ■; pH 6.00 ▲; pH 5.75 ●; pH 5.50 ●).



II. 1. 2. *In vitro* gas production

The volume of gas produced *in vitro* decreased linearly (P<0.05) with the pH of the incubation medium promoted at all incubation times (Figure 4), although this effect also showed a quadratic pattern (P= 0.012) at 12 h, because of the increased differences between the volume of gas produced with the medium at pH 6.50 (217 ml/g OM), and to a lesser extent at pH 6.25 (184 ml/g OM), compared to the increases in gas production with the other treatments at this time of incubation (158, 152, and 145 ml/g OM for pH 6.00, 5.75, and 5.5 respectively). In this sense, from 8 h afterwards, treatments which stabilized the pH of incubation at 0.2 units or more above 6.0 (pH 6.50 and 6.25) maintained a positive trend to increase the volume of gas produced, whereas the volume of gas produced in this period tended to decrease in treatments which medium was maintained at pH 6.0 or below. A significant correlation between the production of gas at 12 h and the pH of the medium was detected, not only at the end of the incubation period (R^2 = 0.629; P= 0.004), but also between the final volume at 12 h and pH recorded at previous incubation times, especially 6 h (R^2 = 0.836; P<0.001).

Figure 4. Pattern of the volume of gas produced *in vitro* (ml/g OM) according to the planned pH based on concentration of bicarbonate ion in the incubation solution (pH 6.5 ♦; pH 6.25 ■; pH 6.00 ▲; pH 5.75 ●; pH 5.50 ●).



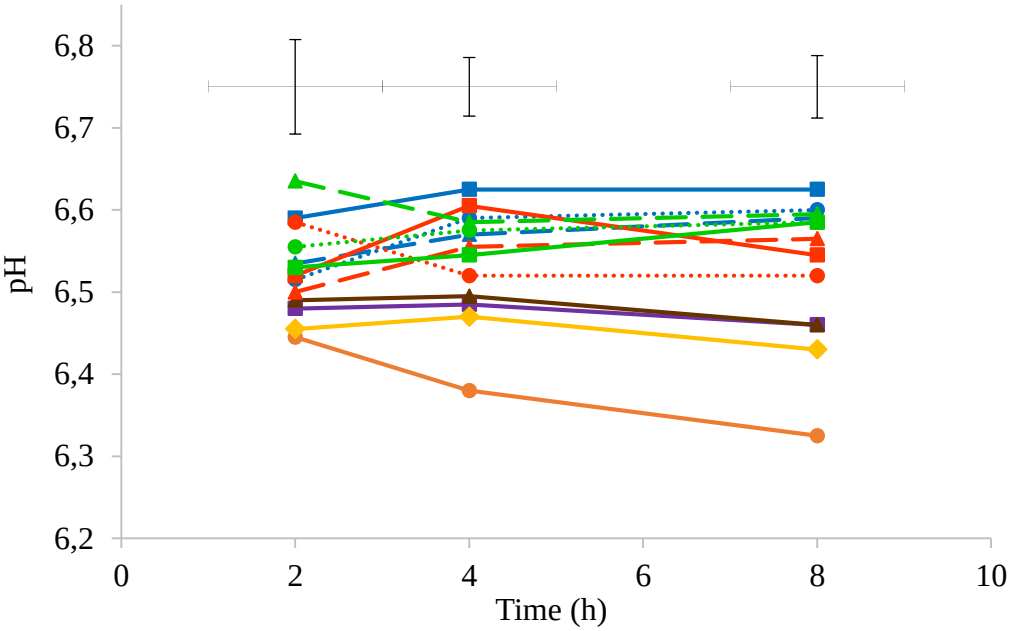
II. 2. Experiment 1.1

- ***In vitro* fermentation of the substrates at pH 6.5**

II. 2. 1. Pattern of incubation pH

The average pH of the rumen inoculum was 7.16. At 2 h of incubation, the type of substrate did not affect (P>0.05) the pH of the incubation medium, but at 4 and 8 h an effect was appreciated (P<0.001). In any case, throughout all the incubation period (8 h) the incubation pH was maintained for all cases within the expected range of 6.5 ± 0.2 (Figure 5).

Figure 5. Pattern of incubation pH according to the source of carbohydrates (C1 ■; C2 ▲; C3 ●; S1 ■; S2 ▲; S3 ●; B1 ■; B2 ▲; B3 ●; BP ■; CP ●; WB ▲; SU ♦)



II. 2. 2. *In vitro* gas production

The *in vitro* gas production when pH was fixed to 6.5 was affected by the substrate type (P<0.001). At every time of measurement, the volume of gas recorded with CP was always highest (P<0.001; Table 7). At 2 and 4 h, the volume of gas recorded with the three varieties of sorghum (S1, S2 and S3) and C3 were the lowest, and at 6 and 8 h the other corn varieties (C1

and C2) did not differ from this group. Barley varieties (B1, B2 and B3), as well as BP, WB and SU were grouped at a higher level, but always below the volume of gas produced by CP.

Table 7. Pattern of the volume of gas produced *in vitro* (ml/g OM) at different incubation times, from the different sources of carbohydrates when incubated at pH 6.5.

	Time of incubation (h)			
Substrates	2	4	6	8
C1	11.8^{bc}	25.2^{bcdef}	36.3^{cde}	48.6^{cd}
C2	11.4^{bc}	25.7^{bcdef}	37.5^{cde}	50.4^{cd}
C3	08.7^{c}	21.4^{cdef}	32.0^{cde}	43.9^{cd}
S1	08.4^{c}	16.3^{def}	22.4^{de}	29.9^{d}
S2	07.3^{c}	12.9^{f}	16.6^{e}	20.8^{d}
S3	07.5^{c}	15.3^{ef}	21.1^{de}	27.7^{d}
B1	13.7^{bc}	30.7^{bcde}	46.8^{bc}	67.4^{bc}
B2	13.7^{bc}	30.8^{bcde}	48.5^{bc}	71.9^{bc}
B3	13.8^{bc}	32.0^{bcd}	49.8^{bc}	72.7^{bc}
BP	18.8^{ab}	39.1^{b}	63.1^{b}	84.6^{b}
CP	27.8^{a}	55.4^{a}	92.8^{a}	137.6^{a}
WB	11.9^{bc}	36.0^{bc}	63.0^{b}	92.8^{b}
SU	12.7^{bc}	27.0^{bcdef}	41.9^{bcd}	68.6^{bc}
SEM	1,67	2,81	4,20	5,45

^{a,b,c,d,e,f,} Means within a column with different superscripts differ (P<0.001). SEM: standard error of the means.

II. 2. 3. Volatile fatty acids production

In terms of total VFA concentration, their production was affected by the substrate type (P<0.001; Tables 8 and 9). At 4 h incubation (Table 8), the concentration of VFA was higher with CP followed by WB. In a different level, BP and the two varieties of barley (B2 and B3), and SU, B1 and C3 were grouped successively after, and before the lowest group which included two varieties of corn (C1 and C2) and the three varieties of sorghum (S1, S2 and S3). At 8 h (Table 9), the highest concentration of VFA was recorded by WB followed by BP and CP, and the lowest concentration was recorded by S2.

Regarding the VFA profile the heterogeneous chemical nature of substrates made the comparison confusing. At 4h (Table 8), the proportion of acetate was higher (P<0.05) with S2 than with WB, whereas propionate proportion was higher with WB than with C1 and the sorghum varieties S1 and S2, which recorded the lowest proportion (P<0.05). The three corn

and the three sorghum varieties, together with B1, recorded higher proportions of butyrate than CP. The proportion of branched-chain VFA, considered as the sum of both isobutyrate and isovalerate, was higher with S3 than B1 and SU, and then B2, B3, WB and BP, recording the lowest value with CP. For valerate, BP, CP, SU and B3 recorded the lowest values. Differences in the VFA molar pattern were minimised at 8 h (Table 9). In terms of acetate, differences were recorded between S2 and WB and B2 ($P<0.05$), whereas the proportion of propionate was higher with WB, barley varieties, BP and SU, and was lower with S2. The proportion of butyrate recorded with CP and BP was the lower than the other substrates ($P<0.05$). Besides, the highest proportion of branched-chain VFA was observed with the sorghum varieties S2 and S3, and the lowest was observed with SU, BP and CP. The higher proportion of valerate, was recorded by WB and the lower was recorded by BP, CP, B3 and SU.

Table 8. Total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 4 h of incubation with different sources of carbohydrates.

Substrate	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
C1	09.16^{c}	0.551^{ab}	0.169^{bc}	0.055^{a}	0.018^{abc}	0.005^{a}
C2	10.82^{c}	0.546^{ab}	0.179^{abc}	0.052^{a}	0.018^{abc}	0.004^{abc}
C3	11.28^{bc}	0.543^{ab}	0.188^{abc}	0.053^{a}	0.018^{abc}	0.004^{abc}
S1	09.79^{c}	0.554^{ab}	0.166^{bc}	0.055^{a}	0.020^{ab}	0.005^{ab}
S2	09.35^{c}	0.579^{a}	0.144^{c}	0.052^{a}	0.020^{ab}	0.004^{abc}
S3	09.66^{c}	0.543^{ab}	0.177^{abc}	0.053^{a}	0.021^{a}	0.004^{abc}
B1	12.68^{bc}	0.525^{ab}	0.202^{ab}	0.052^{a}	0.015^{bcd}	0.004^{abc}
B2	13.98^{abc}	0.529^{ab}	0.202^{ab}	0.050^{ab}	0.0146^{cd}	0.004^{abc}
B3	13.24^{abc}	0.534^{ab}	0.198^{ab}	0.050^{ab}	0.0143^{cd}	0.003^{bc}
BP	13.80^{abc}	0.560^{ab}	0.182^{abc}	0.043^{ab}	0.011^{de}	0.002^{c}
CP	17.58^{a}	0.569^{ab}	0.183^{abc}	0.036^{b}	0.008^{e}	0.003^{bc}
WB	15.82^{ab}	0.505^{b}	0.227^{a}	0.049^{ab}	0.013^{cd}	0.004^{abc}
SU	11.68^{bc}	0.529^{ab}	0.202^{ab}	0.049^{ab}	0.015^{bcd}	0.003^{bc}
SEM	1.2054	0.0168	0.0129	0.0037	0.0012	0,0003

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

Table 9. Total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 8 h of incubation with different sources of carbohydrates

Substrate	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
C1	13.58^{bcd}	0.543^{ab}	0.189^{ab}	0.052^{a}	0.013^{bc}	0.004^{bcd}
C2	13.70^{bcd}	0.539^{ab}	0.188^{ab}	0.053^{a}	0.014^{ab}	0.004^{bcd}
C3	13.76^{bcd}	0.540^{ab}	0.187^{ab}	0.053^{a}	0.014^{ab}	0.004^{bcd}
S1	11.38^{cd}	0.537^{ab}	0.182^{ab}	0.058^{a}	0.018^{ab}	0.005^{abcd}
S2	09.53^{d}	0.566^{a}	0.153^{b}	0.055^{a}	0.020^{a}	0.005^{abcd}
S3	10.55^{cd}	0.535^{ab}	0.183^{ab}	0.056^{a}	0.021^{a}	0.005^{abcd}
B1	18.31^{abcd}	0.512^{ab}	0.218^{a}	0.052^{a}	0.013^{bc}	0.004^{bcd}
B2	17.85^{abcd}	0.496^{b}	0.231^{a}	0.055^{a}	0.013^{bc}	0.004^{bcd}
B3	18.61^{abc}	0.512^{ab}	0.219^{a}	0.053^{a}	0.011^{bcd}	0.003^{cd}
BP	22.32^{ab}	0.545^{ab}	0.211^{a}	0.036^{b}	0.005^{d}	0.003^{cd}
CP	21.70^{ab}	0.551^{ab}	0.203^{ab}	0.037^{b}	0.005^{d}	0.003^{cd}
WB	22.91^{a}	0.497^{b}	0.232^{a}	0.052^{a}	0.013^{bc}	0.006^{a}
SU	16.87^{abcd}	0.524^{ab}	0.216^{a}	0.049^{a}	0.007^{cd}	0.003^{cd}
SEM	2.1850	0.0153	0.0136	0.0029	0.0018	0.0004

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

II. 3. Experiment 1.2

- ***In vitro* fermentation of the substrates at pH 5.5**

II. 3. 1. Pattern of incubation pH

At the start of the incubations, the inoculum pH averaged 6.34 ± 0.2 and 6.72 ± 0.2 for the concentrate (CI) and forage (FI) inocula, respectively, and dropped in ± 0.5 and ± 0.7 pH units respectively in the first two hours of incubation (Table 10). During the whole incubation period, average pH with CI was lower than that of FI (P<0.001), differences increasing with time from 0.17 to 0.51 pH units at two and 10 h.

Besides, the cereal species used as substrate also influenced (P<0.001) the drop in pH (Figures 6 and 7), but the interaction inoculum x substrate (P<0.001) indicates that cereal varieties behaved differently depending on the inoculum source. When the CI was used (Figure 6), pH from 8 h onwards was highest with sorghum varieties, recording intermediate and minimum values with corn and barley (average pH of 5.63, 5.44 and 5.13, respectively, at 8 h).

With this inoculum, differences within cereal species at 10 h incubation were apparent (P<0.05) for sorghum varieties S1 and S2 (5.42 vs. 5.61) and corn varieties C3 and C1 (5.16 and 5.32, respectively). The pH recorded with these corn varieties (C1 and C3) at 8 h onwards was lower than with sorghum varieties, whereas C1 and S1 did not differ (P>0.05). When cereals were compared using FI (Figure 7), the pH from 2 to 10 h incubation was reduced at a higher extent with barley (on average, 5.93 and 5.51) than with corn (6.00 and 5.84) and sorghum (6.01 and 5.91) varieties. No differences in incubation pH were detected among varieties of the same cereal, and differences neither existed between sorghum and corn varieties at 2 and 4 h incubation, between sorghum varieties, C2 and C3 at 6 and 8 h incubation, and between sorghum varieties and C1 at 10 h incubation (P>0.05).

Table 10. Average of pH between inocula from concentrate (CI) or forage (FI) diets.

	Time of incubation (h)				
inoculum	2	4	6	8	10
CI	5.81[b]	5.74[b]	5.61[b]	5.40[b]	5.24[b]
FI	5.98[a]	5.94[a]	5.90[a]	5.85[a]	5.75[a]
SEM	6.990	0.014	0.016	0.016	0.017

[a,b] Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means.

Figure 6. Pattern of incubation pH according to the different varieties of corn, sorghum, and barley (C1 ■; C2 ▲; C3 ●; S1 ■; S2 ▲; S3 ●; B1 ■; B2 ▲; B3 ●) with inoculum from a concentrate diet (CI).

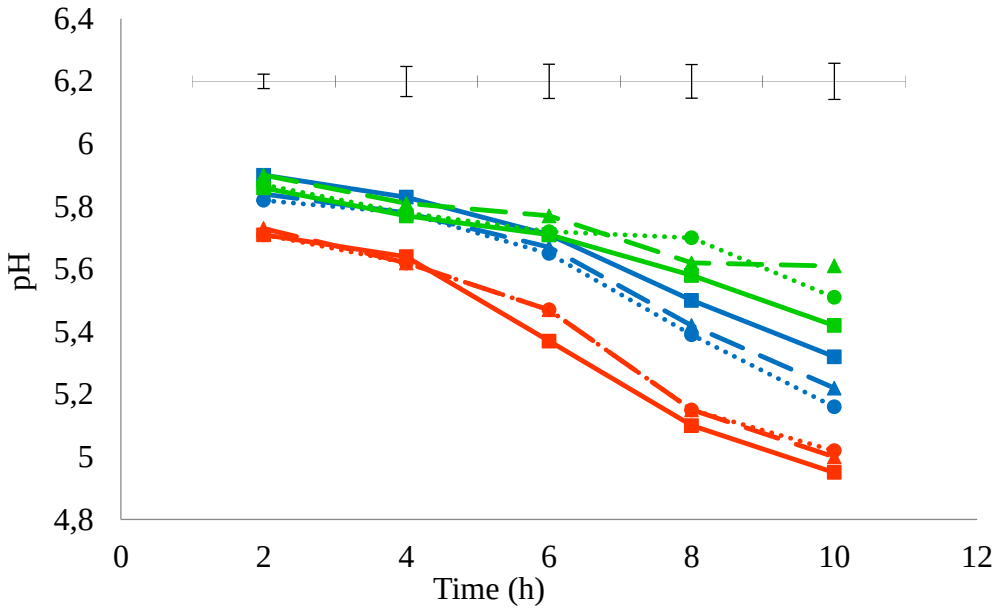
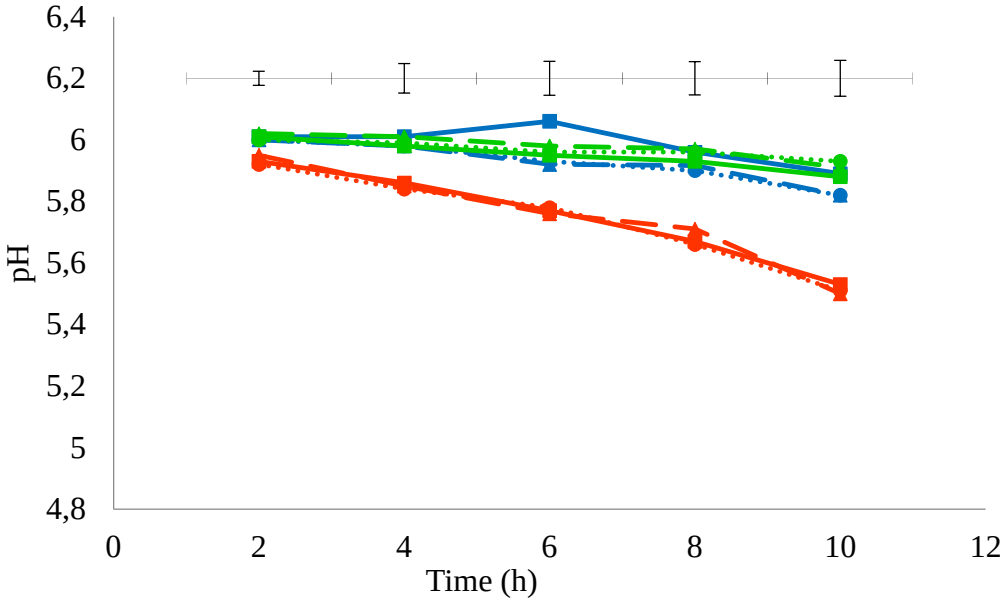


Figure 7. Pattern of incubation pH according to the different varieties of corn, sorghum, and barley (C1 ■; C2 ▲; C3 ●; S1 ■; S2 ▲; S3 ●; B1 ■; B2 ▲; B3 ●) with inoculum from a forage diet (FI).



II. 3. 2. *In vitro* gas production

The comparison of the gas production between inocula at each time of measurement shows that the gas production recorded with the CI was always superior to the gas production recorded with the FI ($P<0.001$; Table 11). Average differences of gas produced between inocula increased with incubation time, from 8 to 31 ml/g OM. After 10 h of incubation, the average volume of gas produced (ml/g OM) with both inocula ranked the substrates as follows: the barley varieties recorded the highest gas volume (on average, 71.8 ml/g OM), followed by corn (54.1 ml/g OM) and sorghum varieties recording the lowest (30.1 ml/g OM).

A significant interaction between the inoculum type and the substrate was recorded at all incubation times ($P= 0.001$), indicating different behaviour among cereal varieties of each cereal species for each inoculum, and therefore the evolution of the gas production throughout the incubation period for the different cereal substrates by inocula are reported separately in Figures 8 and 9. These figures show marked differences in the *in vitro* gas production between the different substrates, mainly between sorghum varieties and those from barley and corn ($P<0.001$). Thus, with the CI (Figure 8), the volume of gas recorded was highest with the three

varieties of barley (82.5 ml/g OM), which did not differ among them. Corn varieties did not differ among them (73.0 ml/g OM), and S1 (68.4 ml/g OM) was also grouped with them, whereas gas production with the other sorghum varieties (S2 and S3) was lowest (31.1 and 39.7 ml/g OM, respectively). However, with the FI (Figure 9) differences at 2 and 4 h between barley varieties (6.1 and 17.4 ml/g OM) and both C1 (7.5 and 16.9 ml/g OM) and C2 (8.8 and 17.1 ml/g OM) were not detected. Again, after 10 h of incubation the sorghum varieties S3 (17.5 ml/g OM) and S2 (0.7 ml/g OM) were lowest with this inoculum, the fermentation of the latter being negligible.

Table 11. Average volume of gas produced (ml/g OM) between inocula from concentrate (CI) or forage (FI) diets.

	Time of incubation (h)				
inoculum	2	4	6	8	10
CI	13.6[a]	26.5[a]	38.6[a]	52.1[a]	67.2[a]
FI	05.3[b]	12.8[b]	20.5[b]	28.5[b]	36.7[b]
SEM	0.45	0.64	0.76	1.00	1.37

[a,b] Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means.

Figure 8. Pattern of the volume of gas produced *in vitro* (ml/g OM) at different incubation times, with different varieties of corn, sorghum, and barley (C1 ■; C2 ▲; C3 ●; S1 ■; S2 ▲; S3 ●; B1 ■; B2 ▲; B3 ●) when incubated at pH 5.5 with inoculum from a concentrate diet (CI).

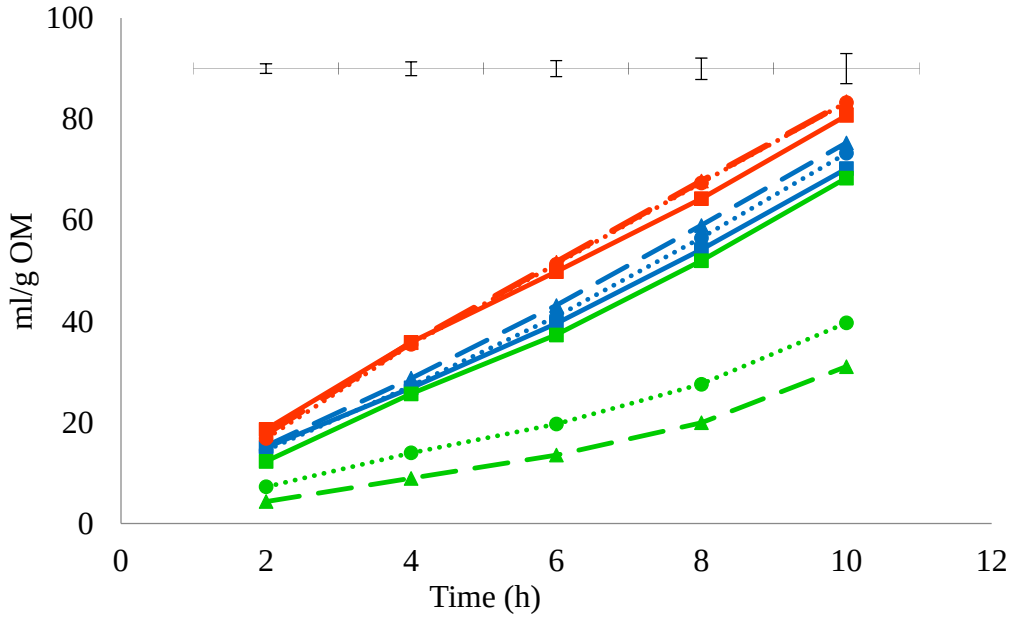
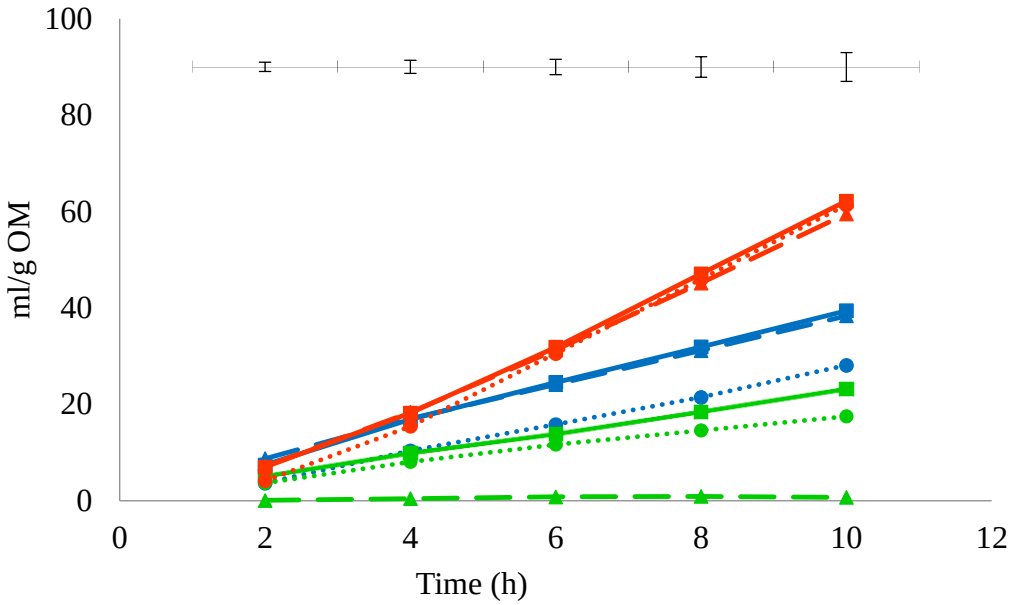


Figure 9. Pattern of the volume of gas produced *in vitro* (ml/g OM) at different incubation times, with different varieties of corn, sorghum, and barley (C1 ■; C2 ▲; C3 ●; S1 ■; S2 ▲; S3 ●; B1 ■; B2 ▲; B3 ●) when incubated at pH 5.5 with inoculum from a forage diet (FI).



II. 3. 3. Dry matter disappearance

The proportion of DM disappeared after 10 h incubation (DMd) was higher with FI than with CI (0.360 vs. 0.280; P<0.001). The interaction inoculum x substrate did not reach significance (P= 0.12), indicating that substrates behaved similarly despite the source of inoculum. As it was observed with the gas production results, DMd was highest for barley (on average, 0.511), followed by corn (0.314) and then sorghum varieties (0.136; P<0.001), not existing differences among varieties within the cereal species.

II. 3. 4. Volatile fatty acids production

Tables 12 and 13 show the average values of total volatile fatty acid concentration and molar VFA proportions at 4 and 8 h of incubation, respectively. Among inocula, the production of VFA was higher (P<0.001) with CI than FI at both 4 and 8 h incubation, and maintained a similar VFA profile throughout the incubation period, CI showing lower (P<0.001) proportions of acetate and branched-chain VFA, and higher (P<0.001) proportions of butyrate and valerate than FI, whereas propionate proportion did not differ among inocula.

Table 12 shows the average values for total VFA concentration and molar proportions of VFA for the different substrate incubated for 4 h incubation for both types of inocula, since the interaction inoculum x substrate resulted non-significant ($P>0.05$) for all studied parameters. On average, the VFA concentration at 4 h was higher ($P= 0.001$) with the barley variety B3 than all corn and sorghum varieties, and acetate proportion was higher ($P<0.001$) in S2 and S1 than B1 and B3, whereas propionate proportion was higher ($P<0.001$) for barley than sorghum varieties. No substrate differences were recorded in butyrate proportion at 4 h, although the interaction inoculum x substrate tended ($P= 0.067$) for differences between S2 and B1 only with concentrate inoculum. The BCFA proportion was higher ($P= 0.01$) in S3 than in B2, B3 and the corn varieties, whereas no differences were recorded in butyrate and valerate.

After 8 h, a higher ($P>0.001$) VFA concentration was detected with barley varieties B1 and B2 compared with sorghum varieties, those of corn recording intermediate values (Table 13). No differences were detected among varieties of the same cereal species. The interaction inoculum x substrate ($P<0.001$) for acetate proportion indicates that, with FI as inoculum, barley varieties fermented with a lower proportion that the three sorghums, recording corn intermediate values, whereas no substrate differences were detected with CI as inoculum. For butyrate, the interaction inoculum x substrate ($P>0.001$) indicates that its proportion for barley was lowest with CI, but did not differ among substrates with FI inoculum. In terms of propionate, barley varieties and C1 and C3 promoted a higher ($P>0.001$) proportion than sorghum S1 and S2, but C2 and S3 did not differ. Despite the ANOVA showed a substrate effect ($P= 0.026$), the means comparisons by the tukey test did not reveal any treatment difference.

Table 12. Average total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 4 h of incubation for both concentrate (CI) and a forage (FI) inocula and the different cereal sources incubated as substrates.

	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
Inocula						
CI	22.8^{a}	0.479^{b}	0.177	0.107^{a}	0.013^{b}	0.009^{a}
FI	14.8^{b}	0.542^{a}	0.177	0.052^{b}	0.020^{a}	0.006^{b}
SEM	0.565	0.0022	0.0016	0.0006	0.0005	0.0004
Substrates						
C1	16.52^{b}	0.517^{abcd}	0.176^{bc}	0.078	0.016^{b}	0.007
C2	17.14^{b}	0.517^{abcd}	0.176^{bcd}	0.078	0.016^{bd}	0.007
C3	17.63^{b}	0.511^{abcd}	0.180^{bc}	0.080	0.016^{b}	0.007
S1	16.39^{b}	0.524^{ab}	0.165^{cde}	0.080	0.011^{ab}	0.007
S2	15.96^{b}	0.527^{a}	0.159^{de}	0.082	0.018^{ab}	0.007
S3	17.11^{b}	0.522^{abc}	0.159^{e}	0.082	0.021^{a}	0.010
B1	20.56^{ab}	0.500^{cd}	0.191^{ab}	0.079	0.017^{ab}	0.008
B2	21.01^{ab}	0.502^{bcd}	0.191^{ab}	0.080	0.016^{b}	0.007
B3	23.48^{a}	0.495^{d}	0.197^{a}	0.080	0.014^{b}	0.007
SEM	1.2230	0.0047	0.0036	0.0014	0.0010	0.0011
Probability of effects						
Inoculum	<0.001	<0.001	0.75	<0.001	<0.001	<0.001
Substrate	0.001	<0.001	<0.001	0.37	0.010	0.78
Inoc x Subs	0.32	0.60	0.11	0.067	0.17	0.84

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

Table 13. Average total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 8 h of incubation for both concentrate (CI) and a forage (FI) inocula and the different cereal sources incubated as substrates.

	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
Inocula						
CI	30.96^{a}	0.469^{b}	0.192	0.106^{a}	0.012^{b}	0.009^{a}
FI	19.86^{b}	0.531^{a}	0.189	0.055^{b}	0.019^{a}	0.006^{b}
SEM	0.5990	0.0015	0.0017	0.0006	0.0004	0.0004
Substrate						
C1	24.22^{bc}	0.495^{cd}	0.196^{ab}	0.082^{abc}	0.014	0.007
C2	26.01^{abc}	0.504^{bc}	0.188^{bcd}	0.080^{abc}	0.014	0.007
C3	25.22^{abc}	0.499^{bcd}	0.193^{abc}	0.081^{abc}	0.014	0.007
S1	23.84^{bc}	0.510^{ab}	0.177^{de}	0.084^{a}	0.015	0.007
S2	21.64^{c}	0.522^{a}	0.164^{e}	0.081^{abc}	0.018	0.009
S3	21.53^{c}	0.506^{bc}	0.178^{cde}	0.083^{ab}	0.018	0.008
B1	28.34^{ab}	0.487^{d}	0.207^{a}	0.077^{bc}	0.015	0.008
B2	30.49^{a}	0.486^{d}	0.207^{a}	0.078^{abc}	0.015	0.008
B3	27.37^{abc}	0.492^{cd}	0.204^{ab}	0.077^{c}	0.014	0.007
SEM	1.271	0.0031	0.0035	0.0013	0.0009	0.0009
Probability of effects						
Inoculum	<0.001	<0.001	0.15	<0.001	<0.001	<0.001
Substrate	<0.001	<0.001	<0.001	0.002	0.026	0.88
Inoc x Subs	0.84	<0.001	0.68	<0.001	0.69	0.99

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

II. 3. 5. Lactic acid

The concentration of lactic acid at 8 h of incubation (Table 14) was higher (P<0.001) with CI than FI. On average, at 4 h incubation, the lactic acid concentration was higher (P<0.001) with barley varieties B1 and B2 than with C2, C3 and the three sorghum varieties, B3 and C1 recording intermediate values. However, at 8 h these differences maximised, and the three barley varieties showed the highest concentration of lactic acid (P<0.001). The interaction inoculum x substrate did not reach significance (P>0.05) for the lactic acid concentration at any studied time.

Table 14. Lactic acid concentration, recorded for each incubated substrate at 4 h and 8 h of incubation with the inocula from a concentrate (CI) or forage (FI) diets.

	4 h	8 h
Inocula		
CI	0.481	2.312^{a}
FI	0.459	1.330^{b}
SEM	0.0476	0.2253
Substrate		
C1	0.405^{bcd}	0.748^{b}
C2	0.333^{d}	0.964^{b}
C3	0.344^{cd}	1.165^{b}
S1	0.223^{d}	0.350^{b}
S2	0.166^{d}	0.262^{b}
S3	0.214^{d}	0.256^{b}
B1	0.912^{a}	3.923^{a}
B2	0.818^{ab}	3.939^{a}
B3	0.815^{abc}	3.971^{a}
SEM	0.1009	0.4780
Probability of effects		
Inoculum	0.74	0.017
Substrate	<0.001	<0.001
Inoc x Subs	0.88	0.87

^{a,b,c,d,} Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means.

II. 4. Experiment 1.3

- ***In vitro* fermentation of the substrates at pH 5.5**

II. 4 1. Pattern of incubation pH

At the start of incubations, the average pH for the CI was 6.44 ± 0.04, and for the FI was 6.90 ± 0.2. Such values already dropped in 0.6 and 0.9 pH units, respectively, in the first two hours of incubation, further dropping in 0.8 and 0.4 pH units until the end of incubation (Table 15). The type of inoculum influenced the reduction of medium pH ($P<0.001$), the magnitude of this drop in pH being more important in CI than in FI inoculum. The interaction inoculum x substrate at 4 ($P<0.05$) and at 6, 8 and 10 h ($P<0.001$) indicate that substrates behave differently for both inocula. When CI was used as inoculum (Figure 10), SU recorded the lowest pH ($P<0.001$) from 2 to 10 h of incubation, the decrease in pH being from 5.5 at 4 h to 4.8 at 6 h, and further decreasing to values of 4.1 and 4.0 at 8 and 10 h incubation. The other substrates maintained parallel trends, being lower with CP than WB, BP and C2 ($P<0.001$). With FI (Figure 11), pH at 2 h incubation did not differ among substrates, but afterwards pH with CP became lowest ($P<0.001$) at 6 h (5.52), being reduced thereafter in less than 0.2 pH units while that of SU abruptly dropped from 5.75 at 6 h to 4.87 after 10 h incubation. With this inoculum, the other substrates maintained medium pH between 6.0 and 5.7 throughout all the incubation period, being highest for BP ($P<0.001$).

Table 15. Average of incubation pH between inocula from concentrate (CI) or forage (FI) diets.

	Time of incubation (h)				
inoculum	2	4	6	8	10
CI	5.79^{b}	5.62^{b}	5.39^{b}	5.16^{b}	5.04^{b}
FI	5.94^{a}	5.90^{a}	5.81^{a}	5.69^{a}	5.55^{a}
SEM	0.014	0.015	0.012	0.025	0.021

^{a,b} Means within a column with different superscripts differ ($P<0.05$). SEM: standard error of the means

Figure 10. Pattern of incubation pH according to the source of carbohydrates (BP ■; CP ●; WB ▲; SU ♦; C2 ▲) with inoculum from a concentrate diet (CI).

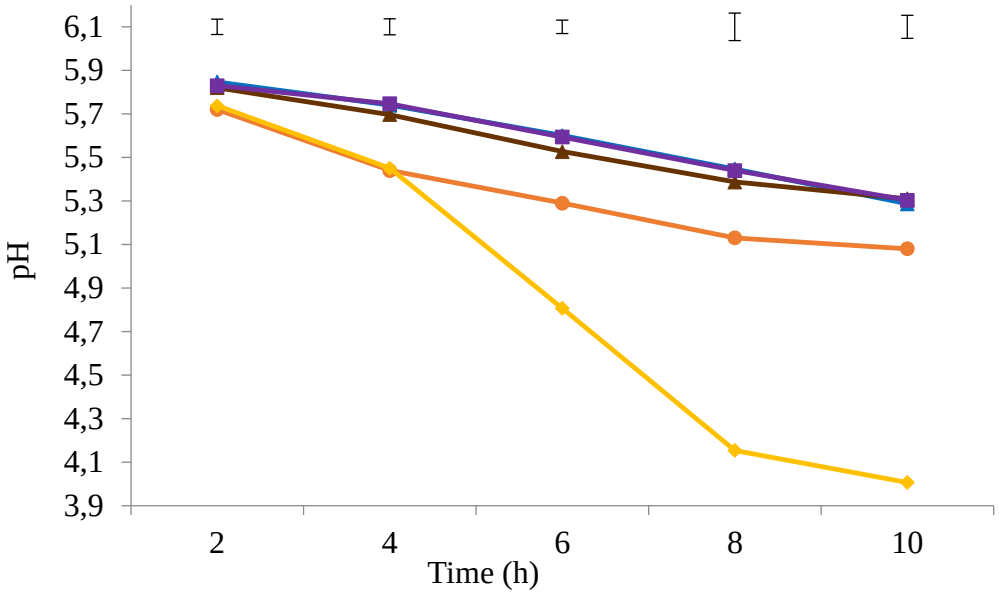


Figure 11. Pattern of incubation pH according to the source of carbohydrates (BP ■; CP ●; WB ▲; SU ♦; C2 ▲) with inoculum from a forage diet (FI).

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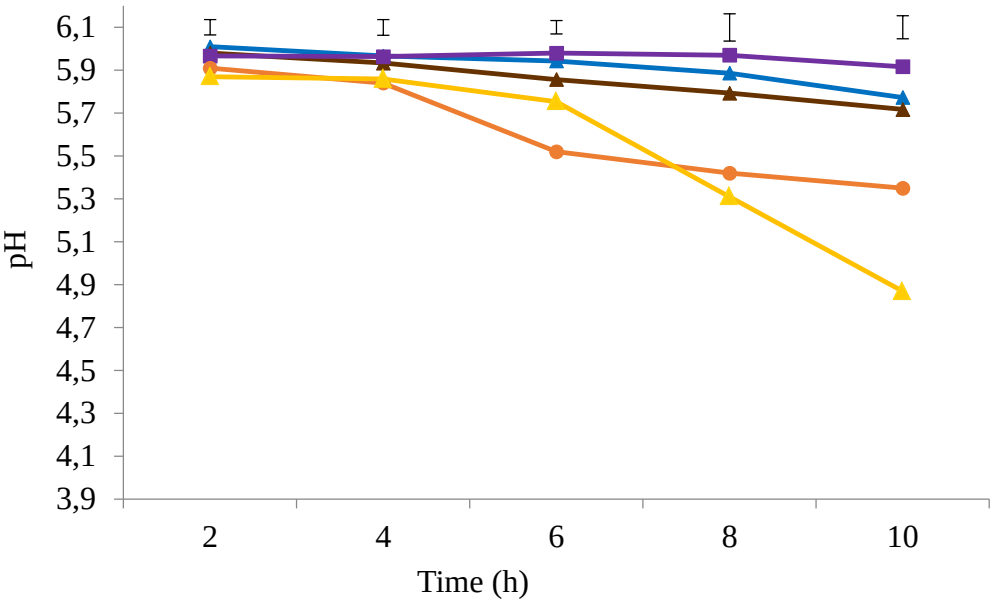


II. 4 2. *In vitro* gas production

For this experiment, the carbohydrate sources tested were: sugar beet pulp (BP), citrus pulp (CP), wheat bran (WB), and sucrose (SU), also including the corn variety C2 as a reference control. At all times of incubation, the volume of gas produced when the inoculum was obtained from animals given a concentrate diet was higher than results obtained with forage inoculum ($P<0.001$; Table 16).

However, since the interaction inoculum x substrate was also significant ($P<0.001$), for an easier understanding the gas production results with CI or FI are showed separately (Figures 12 and 13, respectively). With CI as inoculum (Figure 12), a higher volume of gas was produced from CP ($P<0.05$) at all incubation times than SU, C2 and BP. differences between CP and WB did not existed only at the end of the incubation period (8 and 10 h). Besides, with CI, at 2, 4, 6, and 8 h incubation, SU recorded a higher gas volume than BP and C2 ($P<0.005$). With FI (Figure 13) CP resulted higher than SU and C2 throughout all the incubation period, but differences between CP and the rest of substrates increased from 6 h onwards, thus resulting higher than BP and WB, No differences were detected at 2, 4, and 6 h between the substrates C2 and SU. In summary, after 10 h of incubation, the *in vitro* gas production of substrates ranked them as: CP, WB > SU, C2 and BP with CI (Figure 12), and CP > WB > BP, SU > C2 with FI (Figure 13).

Table 16. Average of volume of gas produced (ml/g OM) between inocula from concentrate (CI) or forage (FI) diets.

	Time of incubation (h)				
inoculum	2	4	6	8	10
CI	21.6[a]	43.1[a]	60.6[a]	74.5[a]	86.4[a]
FI	10.7[b]	22.3[b]	35.2[b]	49.1[b]	62.5[b]
SEM	0.572	0.967	1.117	1.399	2.008

[a,b] Means within a column with different superscripts differ ($P<0.05$). SEM: standard error of the means.

Figure 12. Pattern of the volume of gas produced *in vitro* (ml/g OM) at different incubation times, according to the source of carbohydrates (BP ■; CP ●; WB ▲; SU ♦; C2 ▲) when incubated at pH 5.5, with inoculum from a concentrate diet (CI).

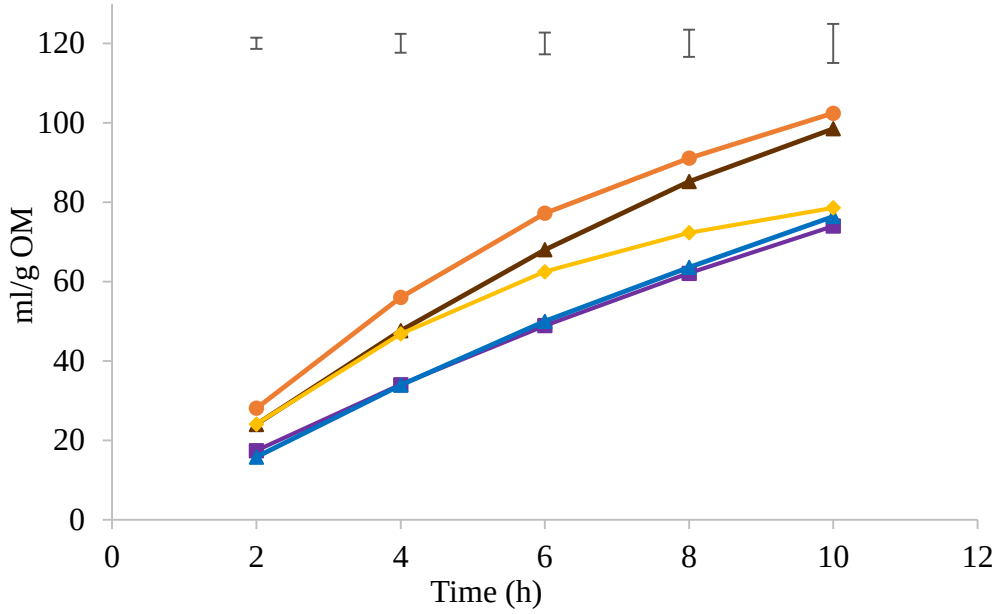
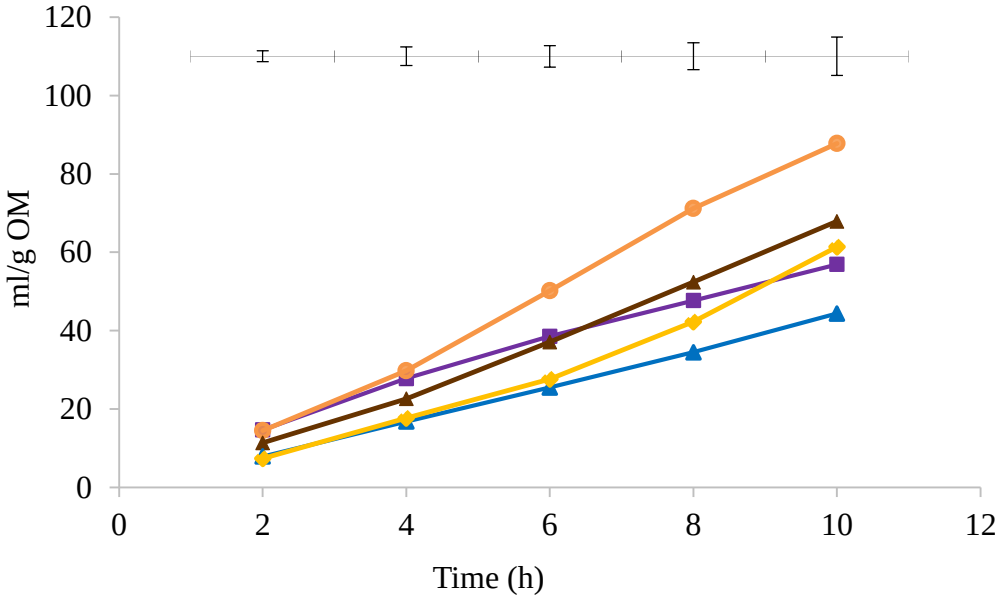


Figure 13. Pattern of the volume of gas produced *in vitro* (ml/g OM) at different incubation times, according to the source of carbohydrates (BP ■; CP ●; WB ▲; SU ♦; C2 ▲) when incubated at pH 5.5, with inoculum from a forage diet (FI).



II. 4. 3. Dry matter disappearance

Among inocula, the proportion of DM disappeared after 10 h of incubation (DMd) was higher with FI than with CI as inoculum (0.518 vs. 0.430; P<0.001). The interaction inoculum x substrate (P<0.05) indicates that each substrate behaved differently depending on the inoculum source. Among substrates, the ranking according to the proportion of DMd was as following: SU (0.985) > CP (0.456) > WB (0.402) > C2 (0.333), BP (0.282).

II. 4. 4. Volatile fatty acids production

The average values of total volatile fatty acid concentration and molar VFA proportions at 4 and 8 h of incubation are shown in Tables 17 and 18. The production of VFA was significantly affected by the inoculum. The production was higher with CI than FI at 4 h as well as at 8 h incubation (P<0.001). In term of VFA profile, the *in vitro* fermentation pattern with CI showed a higher proportion of butyrate, branched-chain VFA, and valerate (P<0.001), whereas that with FI had more acetate (P<0.001). For propionate proportion did not differ among inocula.

At 4 h incubation, the average values for total VFA concentration and molar proportions of VFA for the different substrate incubated for both types of inocula, since the interaction inoculum x substrate resulted non-significant (P>0.05) for all studied parameters (Table 17). At 4 h incubation, the effect of substrate on the total VFA concentration, acetate, butyrate and branched-chain VFA did not significant (P>0.005). Whereas, propionate proportion was higher (P<0.05) for WB and lower for C2.

Table 18 shows the average values for total VFA concentration and molar proportions of VFA for the different substrate incubated for 8 h incubation for both types of inocula, the interaction inoculum x substrate was a trend (P= 0.061) for the total VFA, and it was significant for branched-chain VFA (P<0.05), but it was non-significant (P>0.05) for the others proportion of VFA. For substrate, CP recorded the higher (P<0.001) concentration of total VFA, and C2 recorded the lower (P<0.001). For the VFA profile, a higher proportion for acetate was recorded with BP and CP, and the lower was recorded with WB, but the higher proportion of propionate was recorded with this latter, and the lower was recorded with BP and C2. Besides, butyrate proportion was higher with WB, C2 and SU. Finally, for branched-chain VFA proportion was

higher with C2 followed by BP and SU, and was lower with CP. The effect of substrate on valerate did not significant (P>0.05).

Table 17. Average total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 4 h of incubation for both concentrate (CI) and a forage (FI) inocula and the different carbohydrate sources incubated as substrates.

	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
Inocula						
CI	23.4[a]	0.461[b]	0.187	0.120[a]	0.024[a]	0.007[a]
FI	14.3[b]	0.538[a]	0.195	0.050[b]	0.012[b]	0.004[b]
SEM	1.150	0.0131	0.0075	0.0045	0.0012	0.0004
Substrates						
BP	17.47	0.527	0.170[ab]	0.079	0.017	0.005
CP	20.79	0.504	0.199[ab]	0.076	0.014	0.005
WB	20.53	0.477	0.210[a]	0.087	0.018	0.006
C2	17.94	0.523	0.168[b]	0.082	0.019	0.005
SU	18.03	0.483	0.200[ab]	0.092	0.018	0.006
SEM	1.9935	0.0228	0.0130	0.0079	0.0021	0.0007
Probability of effects						
Inoculum	<0.001	<0.001	0.268	<0.001	<0.001	<0.001
Substrate	0.426	0.148	<0.05	0.277	0.098	0.932
Inoc x Subs	0.919	0.964	0.982	0.679	0.826	0.842

[a,b,] Means within a column with different superscripts differ (P<0.05). SEM: standard error of the means. * BCFA: branched-chain fatty acids (sum of isobutyrate + isovalerate)

Table 18. Average total volatile fatty acids (VFA) concentration and molar VFA proportions recorded at 8 h of incubation for both concentrate (CI) and a forage (FI) inocula and the different carbohydrate sources incubated as substrates.

	VFA	Acetate	Propionate	Butyrate	BCFA*	Valerate
Inocula						
CI	32.0^{a}	0.474^{b}	0.195	0.103^{a}	0.020^{a}	0.006^{a}
FI	15.0^{b}	0.529^{a}	0.204	0.051^{b}	0.011^{b}	0.004^{b}
SEM	0.5910	0.7176	0.0050	0.0018	0.0004	0.0002
Substrates						
BP	22.21^{bc}	0.530^{a}	0.180^{b}	0.069^{b}	0.014^{b}	0.004
CP	27.85^{a}	0.514^{a}	0.203^{ab}	0.066^{b}	0.011^{c}	0.004
WB	25.19^{ab}	0.471^{b}	0.222^{a}	0.083^{a}	0.016^{ab}	0.005
C2	19.97^{c}	0.506^{ab}	0.186^{b}	0.083^{a}	0.019^{a}	0.005
SU	22.36^{bc}	0.495^{ab}	0.205^{ab}	0.079^{a}	0.014^{b}	0.005
SEM	1.0236	1.2430	0.0087	0.0031	0.0008	0.0004
Probability of effects						
Inoculum	<0.001	<0.001	0.069	<0.001	<0.001	<0.001
Substrate	<0.001	0.002	<0.05	<0.001	<0.001	0.402
Inoc x Subs	0.061	0.747	0.768	0.056	<0.05	0.935

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

DISCUSSION

Discussion

In this experiment, our major interest was not only to compare the fermentation pattern of different carbohydrate sources, not even considering potential differences according to the adaptation of the microbial inoculum to one or other environmental conditions. Rather, we aimed to consider the interactions of both factors, considering the potential application of results to situations of feeding transition from a fibrous to a high concentrate diet, when young ruminants are adapted to intensive rearing conditions. In such cases, it is worth knowing the potential of acidification of the different substrates, since this may determine either an easier or a more difficult adaptation of the rumen, thus minimising at possible the risk of acidosis. In this scenario, we previously had to adapt the conventional gas production technique to a low pH incubation medium.

1. Adjustment of pH of the incubation medium and their effect on the GP

Based on these premises, a conventional *in vitro* system (Theodorou et al., 1994) was adapted for allowing the control of both incubation pH and the feed fermentation, bearing in mind the concept of simplicity and low cost. The ion bicarbonate (HCO_3^-) is considered as the most prevalent ruminal buffer (Counotte et al., 1979; Erdman, 1988), and is the basis of the most *in vitro* media used for fermentation studies (Goering and Van Soest, 1970). Anyway, in addition to HCO_3^-, that will exert its buffering activity in combination with the environmental CO_2 thus rendering H_2CO_3, these authors also include a minor proportion of phosphate buffer. The HPO_4^- concentration in this case is approximately 0.02 mol/L, if the mixed buffer is equilibrated under 1 atm of pCO_2. Based on the proportion of the regular buffer used by (Goering and Van Soest, 1970), HCO_3^- is included at a concentration of 0.111 mol/l and it is, by far, the predominant buffer (Kohn and Dunlap, 1998). Under these conditions, incubation pH is generally fixed to 6.7-5.9, and hardly drops below 6.5 during the fermentation process. Thus, the incubation conditions are far from those generally occurring in practical conditions when animals are fed with high proportions of starch-rich concentrate feeds, and therefore the results from the *in vitro* system cannot be directly extrapolated to such feeding practice. The pH that results mainly from the concentration of VFA in the rumen (Sauvant et al., 2006) is the major determinant of the changes in gas production, and the decrease in the gas production in response to a low medium pH could be partly due to the reduced microbial fermentative activity caused by the less favourable conditions, or to the gas produced chemically by the buffering

activity of the acids produced in the fermentation (direct or indirect gas, respectively; Beuvink y Spoelstra, 1992).

Rymer et al. (1998) examined the composition of a variety of buffering media commonly used in gas production techniques, such as those from Goering and van Soest (1970), Theodorou (1993), Steingass (1983) and Huntington et al. (1998), with the objective of determining the effect of media compositions on the volume of indirect gas (proportion of gas released by buffering), when adding from 2 to 12 mmol propionic acid to the medium. There was no difference between the media in initial pH, but the media of Steingass (1983) and Goering and van Soest (1970) maintaining a final pH above 5.5, while the others had a pH of 5.42 (Theodorou, 1993) and 5.29 (Huntington et al., 1998).

In our case, changes in the incubation medium pH were approached by modifying the concentration of bicarbonate ion in the incubation solution, according to the calculations made by Kohn and Dunlap (1998). Previous approaches from our group in this sense, but using a semi-continuous *in vitro* incubation system, had been already published (Fondevila and Pérez-Espés, 2008; Bertipaglia et al., 2010). In the practice, we succeed in manipulating the incubation pH, and the range of pH was established during the 12 h incubation period of Experiment 1.0 according to the concentration of the bicarbonate ion, especially with the medium calculated for pH 6.50, 6.25 and 6.00, that included concentrations of 0.52, 0.29 and 0.16 of that used in the Goering and van Soest (1970) buffer. In any case, the final incubation pH with the media 5.75 and 5.50, corresponding to 0.09 and 0.05 of the mentioned standard buffering capacity, dropped slightly from 10 to 12 h, down to pH 5.51 and 5.31. The continuous drop of pH, which undoubtedly depends on the low proportion of bicarbonate, would allow for a clearer study of the acidifier capacity of a substrate under the conditions induced by high concentrate diets, and therefore were chosen for the later studies.

2. Effect of pH, inoculum source and different sources of carbohydrates on the in vitro fermentation kinetics

In order to minimise potential carry-over effects of the source of inoculum (or even of the incubation run) in Experiments 1.2 and 1.3, as well as for an easier comparison of results from these with those from Experiment 1.1, blanks bottles containing the inoculum without addition of substrate were used. These bottles allow for correcting data from the residual fermentable OM included in the inoculum (Rymer et al., 2005).

- **Effect of the inoculum source**

In both Experiments 1.2 and 1.3, the medium pH differences among inoculum sources (CI and FI) ranged from 0.15-0.17 at 2 h to 0.51 at 10 h. However, the drop of medium pH dropped at a higher extent in Experiment 1.3, since pH of rumen inoculum was 0.1 to 0.2 units higher, but it was 0.2 pH units lower after 10 h of incubation. This suggests that the extent of fermentation was higher in Experiment 1.3, but differences among inocula remained. The effect of the source of rumen fluid on the *in vitro* fermentation has been recently demonstrated (Broudiscou et al., 2014; Matthew et al., 2014). As with the results recorded in these Experiments (1.2 and 1.3), it is widely accepted that forage based diets maintain higher incubation medium pH than diets including a major proportion of concentrate. So, the inclusion of concentrate in ruminant diets influences the pH of the incubation medium. Further, considering the scarce buffering capacity of the experimental incubation medium, such limitation became more important with the concentrate inoculum, and therefore the pH of the incubation medium decreased (Sauvant et al., 2006).

For both Experiments 1.2 and 1.3, the CI rendered more gas than FI, even after subtracting the value of blanks for correcting the contribution of solubles. In the case of Experiment 1.2, the higher production from cereals when incubated with CI instead of FI can be explained by the similarity between the chemical composition of cereal substrates used for incubation and those of the diet given to the animals, and therefore the microorganism of inoculum should be better adapted than those from a fibrous diet for the fermentation of this kind of substrates (Dusart, 2014). For the other carbohydrate sources (Experiment 1.3), the high utilisation of substrates which are rich in rapidly fermentable fiber, as well as the soluble sugars and pectin fractions included in some of them may easily counterbalance the lack of adaptation of the microbial population (Chesson and Monro, 1982; Hatfield and Weimer, 1995; Barrios et al., 2003). Total VFA concentration agreed with results of gas production, being greater in CI than in FI, in coincidence with those found by Calsamiglia et al. (2008). However, in our experiment acetate and BCFA proportions were lower (in Experiment 1.2), and that of butyrate higher, with CI (in the both Experiments 1.2 and 1.3), not showing differences in propionate proportion, whereas Calsamiglia et al. (2008) did not observe any inoculum effect on acetate and butyrate concentrations. From the results of the lactic acid concentration in Experiment 1.2, it is evident that the presence of a concentrate in a ruminant diet influences the lactic acid concentration.

In contrast, the proportion of dry matter disappearance (DMd) was on average higher with forage than with concentrate inoculum for the two Experiments 1.2 and 1.3. This is difficult to justify, but it might be partly explained by the fact that, when the rumen contents were sampled (08:30) the forage inoculum may still contain an important proportion of solubles from the previous day feeding, thus maintaining a high microbial activity, whereas in the concentrate diet solubles may had been utilised by microbial population at a faster rate, thus a minor proportion would remain when sampling (Dusart, 2014). In other way, the experimental incubation conditions should be worse with CI, since the incubation pH in both Experiments 1.2 and 1.3 was lower (pH= 5.5) with this inoculum, and this could also reduce microbial activity and affect the substrates fermentation, which contributes to explain the higher DMd with the FI, which promoted more favourable incubation conditions. A lower DM digestibility because of a low rumen pH was also reported by Sari et al. (2015) in *in vivo* conditions.

- **Effect of the substrate type**

In Experiment 1.1, the pH was maintained throughout all the incubation period within the expected range of 6.45 to 6.65, due to the buffering capacity of the incubation solution (pH 6.5), except for the case of CP, which slightly dropped to 6.3 after 8 h incubation. These results confirmed the ability for controlling the incubation pH observed in Experiment 1.0. The volume of gas produced from all substrates in Experiment 1.1 was higher than that recorded by the same substrates in the Experiments 1.2 and 1.3. This is caused by both a higher activity of the microbial population in better conditions for their activity, thus releasing more direct gas and because of the buffering effect of the incubation solution, releasing indirect CO_2 by maintaining equilibrium with a higher concentration of bicarbonate ion (Beuvink and Spoelstra, 1992). This can also be appreciated from the results found in the Experiment 1.0, and thus it can be said that the fermenter fluid pH is the major determinant of in *in vitro* gas production. Comparing substrates within their nature in this range of incubation pH, the highest volume of gas production was achieved with CP, followed by WB and BP, and then the three barley varieties and SU. These results in gas production are supported by the VFA concentration that after 8 h incubation followed a similar trend, although differences did not reached significance on either total VFA concentration or their molar profile. However, incubation pH resulted slightly lower with the high-fermentable by-products (CP, BP or WB) than the barley varieties, which supports the differences in gas production. Therefore, citrus pulp and wheat bran had an acidic capacity of even higher magnitude than cereal sources. It is clear that concentrates of different

nature have different effects on pH and other fermentation parameters. In Experiment 1.3, SU and CP cause larger declines in pH than the others substrates, despite the inoculum. The BP has an important proportion of the readily fermentable pectin (Chesson and Monro, 1982; Hatfield and Weimer, 1995; Hall et al., 1998), and therefore an important drop of pH should be expectable, but the pH of incubation medium was maintained probably because of its own buffering capacity (FEDNA, 2010). It is expected that the inclusion of high levels of cereal-based concentrates decreases rumen pH (Fondevila et al., 1994; Carro et al., 2000). The different structure of starch among cereal species, or even among varieties (Samantha and Van Barneveld, 1999), and particularly the structure of the endosperm of each species (Michalet-Doreau et al., 1986; Huntington, 1997; Evers et al., 1999), together with the proportion of amylose (Offner et al., 2003; Montiel and Elizalde, 2004) may affect the rate and extent of starch fermentation. Compared with corn and sorghum, it is expected that the starch of barley degrades more rapidly (Thivend and Vermorel, 1971). In our work, when cereals were compared (Experiment 1.2), it is apparent that barley was fermented at a faster rate, and consequently the drop of medium pH occurred earlier and was of greater magnitude. However, whereas the volume of gas produced was clearly higher than that of the other cereal species with the forage inoculum, the magnitude of its effect on pH was not so great, partly because of the high inherent buffering capacity of FI, discussed above. This would imply that the acidifying capacity of barley is not so important when the rumen is changing from a forage to a concentrate diet, as occurs in the transition from weaning to intensive feeding. Differences in gas production between barley and corn were lower when the inoculum is adapted to a highly concentrate diet, existing clear differences in the latter depending on the source of inoculum. In this sense, it is worth noting that fermentation of both corn and sorghum varieties is clearly low when FI was used, suggesting that the microbial population must be adapted to the more recalcitrant type of starch in these cereals.

Further, whereas S1, with a low tannin content bound to the cell wall (Kim et al., 2006) behaved similarly to corn varieties, the certain proportion of tannins in S2 varieties (Table 5) reduced rate and extent of fermentation. Tannins in the brown sorghums interact with proteins but also starch reducing their availability for fermentation (Kristen et al., 2015). Among the brown varieties of sorghum, reactivity may be important in determining their effect on fermentation, thus explaining the lower extent of fermentation in S2 which has a high concentration of tannin. This aspect deserves further study, but might be useful for applying to a slow adaptation of rumen environment to concentrate diets, also might be that the bacteria were sensitive to the phenolic effect. In any way, despite the case of sorghum, probably because

of the above mentioned tannin content is S2. Differences among varieties of both barley and corn were of minor extent, and validates to a certain extent the extrapolation of fermentation results with these cereals. Differences in the volume of gas produced and in VFA between cereal grains and the other carbohydrate sources tested (BP, CP, WB and SU) in the Experiment 1.1 as well as in the Experiments 1.2 and 1.3 respond to their different chemical composition. This fact may be due, at least in part, to the important proportion of pectin and high fermentable fiber fraction in BP (Table 4), and pectin and sugar in CP as well as composition tables from FEDNA (2010). The citrus pulp recorded a higher volume of gas production than sugar beet pulp and wheat bran either in Experiment 1.1 or in Experiment 1.3 due to its content of both soluble sugars and pectin (23 and 25%, respectively; FEDNA, 2010). Besides, the lower protein content of CP may also contribute to increase differences: several results found by different authors (Raab et al., 1983; González et al., 1998; Getachew et al., 2004) showed that ammonia released from protein degradation reacts with CO_2 from fermentation, and this artefact, together with the lower fermentability of proteins compared to carbohydrates, makes that the *in vitro* gas production is negatively related with ammonia or protein content of substrates. In the Experiment 1.3, when incubated with the CI, the low volume of gas was recorded by BP, which is rich in fermentable NDF, compared to CP could be explicated by the effect of the interaction inoculum x substrate on the fermentation kinetics, and the same should explain the low volume of gas recorded with C2 when incubated with the FI (Broudiscou et al., 2014). The proportion of the VFA profile produced showed that this substrate, which has an important content in highly fermentable starch and soluble sugars like WB, barley and SU produced more propionic proportion than other substrates like CP and BP which were more acetic because of their higher content of fermentable fiber and pectin. Concerning corn and sorghum varieties, despite their high level of starch, their proportion of propionate was always less than that barley and the proportion of the other substrates because of a slower rate of starch fermentation. From the interaction between inoculum source and substrate type, even in the presence of a different substrate than the diet of the donor animals, the microbial populations orientated the fermentations to the same VFA profile as the VFA profile in the rumen of the donor animals (Serment et al., 2015), suggesting that the profile of VFA was much more influenced by the inoculum than by the substrate added. This confirms a certain inertia with respect to changing the VFA profile. For the lactic acid concentration it is clear that different kinds of concentrates have different effects on this factor, barley produced higher concentration of lactic acid than corn and sorghum probably due to the structure of starch between them.

- **Relationship between fermentation parameters**

In the Experiments 1.1, 1.2 and 1.3, different correlations were found among the several measured parameters: volume of gas produced, medium pH, total VFA concentration, VFA profile and lactic acid (in the Experiment 1.2).

As expected, it was verified that the incubation pH depends on the total VFA concentration, decreasing with the increase in fermentation (R^2= 0.94 and R^2= 0.81, for Experiments 1.2 and 1.3 respectively). Correlations between the decrease in medium pH and the gas production were also observed in Experiments 1.2 (R^2= 0.61) and 1.3 (R^2= 0.70), highlighting the major relationship between pH and production of either direct or indirect gas. Logically, the concentration of total VFA at 8 h incubation was significantly correlated in the three experiments with the volume of gas produced (R^2= 0.89, R^2= 0.80, and R^2= 0.98, for Experiments 1.1, 1.2, and 1.3, respectively).

The effect of buffer, inoculum and substrate source, volume of gas produced, and their interactions determined not only on the drop in pH of the incubation but also the total VFA. From our results, it was also noted that when the incubation pH was low, and when the inoculum source and the substrates were based on concentrates, the propionate proportion increased respect to that of acetate, and in this case the ratio of C2/C3 was lower. These different interactions and correlations have been found by other authors (Getachew et al., 2004; Calsamiglia et al., 2008; Broudiscou et al., 2014; Matthew et al., 2014).

As the barley varieties showed larger pH declines than corn and sorghum especially with CI, this explained the positive and strong correlation between medium pH and lactic acid concentration (R^2 = 0.98; at 8 h incubation). The positive effect of low pH on lactate production also founded in results from (Russell and Hino, 1985) and most recently by (Broudiscou et al., 2014). Lactate accumulation has also been suggested as a common mechanism in anaerobic bacteria to control intracellular pH through hydrogen removal (Thauer et al., 1977).

CONCLUSION

Conclusion

1. The *in vitro* study of microbial fermentation of concentrate substrates needs to be carried out under low pH conditions that can be achieved by adjusting the medium pH through a reduction of the concentration of bicarbonate ion in the incubation solution. However, the stability on time of the adjustment decreases with bicarbonate concentrations lower than 0.010 M, corresponding to a pH of about 5.75.

2. The *in vitro* gas production is highly correlated with the medium pH, either in terms of direct gas, coming from microbial fermentation of substrates, or indirect gas the indirect gas resulting from the buffering capacity of the medium. When the medium is poorly buffered, highly fermentable substrates exhaust the buffering capacity of the medium, thus increasing the extent and rate of the pH drop.

3. The nature of the inoculum source affects both the medium pH and the ability of microbial community for fermenting the substrates. In this way, there is a strong interaction between the type of inoculum and the substrate, and cereals with slowly fermentable starch such as corn or sorghum produce a low volume of gas with an inoculum from a forage diet whereas differences among inocula are reduced on cereal sources with highly fermentable starch, such as barley.

4. There are some non-starch carbohydrate sources that acidified in a higher extent the medium and promoted a higher gas production than cereals, especially citrus pulp, which is rich in sugars and pectins, whereas fermentation of others such as wheat bran and sugar beet pulp was of similar magnitude to that of barley. In such cases, the effect was of a higher magnitude with an inoculum from a concentrate rather than a forge diet. Only minor differences were detected in fermentation traits among the studied cereal varieties of barley and corn; however, differences existed among sorghum varieties, based on the amount and reactivity of tannins.

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