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Strategies to Reduce Nitrogen Excretion from Ruminants: Targeting the Rumen

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Strategies to Reduce Nitrogen Excretion from Ruminants: Targeting the Rumen

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SERGIO CALSAMIGLIA BLANCAFORT, como Catedrático del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona,

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Que al memoria titulada ¨Strategies to Reduce Nitrogen Excretion from Ruminants: Targeting the Rumen¨, presentada por Andreas Foskolos, ha sido realidada bajo mi dirección y, considerada finalizada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que conste a los efectos que correspondan, firmo el presente certificado en Bellaterra, de 22 Octubre de 2012.

Agradecimientos

Esta parte de la jornada esta llegando a su fin. Lo bueno es que este viaje no ha terminado todavía, tiene varios puertos antes de llegar a su destino final, obviamente sólo por motivos naturales, ya que este viaje no tiene como objetivo el dinero, sino el conocimiento y la experiencia. Conocimiento no sólo científico, sino filosófico, cultural e histórico. Cuando llega el momento para irte de un puerto, vuelves la cabeza hacia atrás, cierras los ojos y ves con los ojos de tu mente.

Lo primero que veo es el maestro en la facultad donde estudiaba, en Larisa, sentado en una silla al revés, entre chicos de 19 años, filosofar mientras está hablando de los enlaces químicos del ADN, de cómo se formula una dieta y explicar a su manera cuál es la diferencia entre el buey y el toro. Maestro, Xenoulis Periklis, aunque el viaje para ti ha terminado, gracias por hacerme amar tanto la producción animal como la nutrición animal.

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A continuación, el paisaje esta cambiando. Desde lo alto, se ven las luces de la gran ciudad y a las 5 de la mañana el avión llega a una ciudad que excita la imaginación de cada uno. Aterrizamos a Barcelona, aterrizamos en plural. En este viaje, y como siempre, María estaba a mi lado. Aquí, en la ciudad donde vivimos estos 4 años, en la ciudad que quisimos, quizá no tanto como Atenas o Larisa, pero en la tercera ciudad de nuestro corazón. En la ciudad donde nos casamos, donde obtuvimos Luna. Maraki, te debo mucho a ti, tu gran apoyo en todo, tu esfuerzo por desconectarme del trabajo al estar en casa. Y como una auténtico agrónomo, te debo tu combatividad cuando necesité tu ayuda, tanto en la granja como en el laboratorio.

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List of Abbreviations

Chapter 1:

AA, amino acid; ADF, acid detergent fibre; BEO, blend of essential oils; BNF, biological nitrogen fixation; CAP, capsaicin; CAR, carvacrol; CIN, cinnamldehyde; CNCPS, Cornell net carbohydrate and protein system; CP, crude protein; CT, condensed tannins; DM, dry matter; DMI, dry matter intake; DON, dissolved organic nitrogen; EC, European Commission; EMO, environmental movements; EMPS; efficiency of microbial protein synthesis; ENU-R; efficiency of nitrogen utilization in the rumen; EO, essential oils; EPD, effective protein degradation; EU, European Union; FP7, the seventh framework program; GAR, garlic oil; HAP, hyper ammonia producing; HT, hydrolysable tannins; NIRS, near infrared spectroscopy; MNE, milk nitrogen efficiency; N, nitrogen; N_2, nonreactive nitrogen; NDF, neutral detergent fibre; NH_3, ammonia; NH_4^+, ammonium; Nr, reactive nitrogen; NUE, nitrogen use efficiency; PAbs, polyclonal antibodies; PBV, protein balance in the rumen; PTS, propyl-propylthiosulfinate; PTSO, propyl-propylthiosulfonate; RDP, rumen degradable protein; RUP, rumen undegradable protein; THY, thymol.

Chapter 2-6:

ACl, polyclonal antibodies against *Clostridium aminophilum*; ALL, all samples; APa, polyclonal antibodies against *Peptostreptococcus anaerobius*; APr, polyclonal antibodies against *Prevotella ruminicola*; BCVFA, branch-chained volatile fatty acid; CTR, control; D, detrend; ED, effective degradation; ELISA, enzyme linked immunosorbent assay; FF, forages; LAB, lactic acid bacteria; LPep, large peptides; MSC, multiple scatter correction; NF no forages; NPN, non protein nitrogen; OM, organic matter; PBS, phosphate buffered saline; R^2, coefficient of

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determination for calibration; r^2, coefficient of determination for external validation; r_c^2, coefficient of determination for cross validation; RER: range error ratio; RPD, ratio of performance to deviation; SEC, standard error of calibration; SECV, standard error of cross validation; SEP, standard error of validation; SMT, soybean meal monensin treated; SNT, soybean meal no treated; SNV, standard normal variate; SPep, small peptides; TA tungstic acid; TCA, trichloroacetic acid; TMT, tryptone monensin treated; TN, total nitrogen; TNT, tryptone no treated; TP, true protein; VFA; volatile fatty acids.

RESUMEN

Esta tesis doctoral se llevó a cabo en el marco del proyecto financiado por la Unión Europea Rednex que se centra en la contaminación ambiental con nitrógeno (N) de la ganadería lechera. La agricultura, y en particular la producción animal, es el principal contribuyente al fenómeno llamado la cascada de N que describe la circulación de N reactivo en los ecosistemas causando efectos múltiples en la atmósfera, los ecosistemas terrestres, los sistemas de agua dulce y marina, y la salud humana. El objetivo principal de la tesis fue utilizar tecnologías innovadoras para dar respuestas y proponer soluciones que pueden reducir la excreción de N de los rumiantes al medio ambiente. Así, se llevaron a cabo cuatro estudios para evaluar las diferentes tecnologías e innovaciones: la espectroscopia de infrarrojo cercano como herramienta para mejorar la precisión en la formulación de raciones en la granja, el uso anticuerpos policlonales contra las principal bacterias proteolíticas y desaminadoras en el rumen; y, el uso de compuestos de aceites esenciales como modificadores de la población microbiana responsable de la degradación de proteínas en el rumen y en forraje de raigrás durante el ensilaje.

En el primer estudio, creamos una base de datos con una colección de 809 muestras distintas de alimentos frecuentemente utilizados en la alimentación de rumiantes. Parte de los alimentos se analizaron para la degradación de materia seca (MS) y proteína bruta (PB), y una parte más pequeña (n = 100) para la degradación de fibra neutro detergetnte (FND). Los alimentos se agruparon como forrajes (FF; n = 256) y no forrajes (NF, n = 553). La degradabilidad se describió en términos de la fracción soluble (a), la fracción degradable pero no soluble (b) y su velocidad de degradación (c). La degradabilidad efectiva (DE) de la MS y PB (5% h^{-1} velocidad de transito) y FND (2% h^{-1} velocidad de transito) se calcularon de acuerdo con la ecuación de Ørskov y McDonald (1979). Todas las muestras fueron escaneadas de 1.100 a 2.500 nm,

utilizando un monocromador de exploración NIRSystems 5000 (FOSS, Hoganas, Suecia). La reflectancia se registró cada 2 nm como 1/Reflectancia. Las muestras fueron escaneadas dos veces por duplicado utilizando células en anillo de taza y para cada muestra se calculó el espectro promedio. El software WinISI III (v. 1,6) fue empleado para analizar los espectros y desarrollar los modelos quimiométricos. El método de regresión utilizado para realizar las calibraciones fue la regresión por minimos cuadrados parciales (MPLS) para todas las muestras (ALL), FF y NF. La precisión de las ecuaciones obtenidas fue confirmada por un conjunto de validación externa con el 20% del total de muestras. La DE, las fracciones a y b de MS y PB se predijeron bien, y mejoraron después de separación por grupos (FF y NF). La velocidad de la degradación de la MS y PB no se predijo satisfactoriamente cuando se incluyeron todas las muestras (r^2 <0,7). Sin embargo, cuando las muestras se separaron por grupos mejoró la predicción de la MS (r^2> 0,7) y de la PB para FF (r^2> 0,7) mejoraron. Para la FND, el número de muestras fue menor y la mayoría se agruparon en FF. Las ecuaciones obtenidas predijeron satisfactoriamente la DE y la fracción b de la FND, y la separación por grupos (FF y NF) mejoró las predicciones. Cuando todos los alimentos se incluyeron en el análisis, la velocidad de degradación no se predijo bien (r^2 = 0,4), pero cuando las muestras se agruparon la predicción para FF era aceptable (r^2 = 0,8). En conclusión, la separación en grupos de FF y NF mejoró las predicciones de NIRS, especialmente para la predicción de la velocidad de la degradación. Las ecuaciones son aceptables y permiten la incorporación de NIRS como herramienta de campo para los modelos de la evaluación de alimentosb que requieren la predicción de la velocidad de degradación y degradación efectiva de los nutrientes.

En el segundo estudio, evaluamos el efecto de la adición de compuestos activos de aceites esenciales (AE) en la composición química y la degradación de proteínas en ensilados de raigrás.

Durante el ensilaje de forrajes, la proteína se degrada en forma extensa. Algunos compuestos de AE pueden alterar el metabolismo proteico a través de la inhibición de la peptidolisis y de la desaminación. Por lo tanto, la hipótesis era que la adición de AE en el forraje de raigrás podría afectar la degradación proteica y la desaminación durante el ensilaje. Se prepararon microsilos (n = 74) en bolsas de poliéster con 2,0 kg de forraje fresco de raigrás picado, rociado de acuerdo a los tratamientos y sellado con una máquina de vacío automatica. Los compuestos de los AE probados fueron: timol (THY), eugenol (EUG), cinamaldehído (CIN), capsaicina (CAP) y carvacrol (CAR), en 4 dosis: 0, 50, 500 y 2.000 mg / kg de forraje fresco. Los ensilajes se abrieron 35 días después y se tomaron muestras. Las muestras se analizaron para el pH, las fracciones de nitrógeno (N-amoniacal, péptidos cortos y péptidos largos), materia seca (MS), ácido láctico, t el contaje de bacterias productoras de ácido láctico (LAB) y Clostridium. El pH del ensilaje fue mayor de lo esperado (5,5 a 6,6) y se atribuyó al bajo contenido de MS del forraje y la adición de los AE. La adición de CAP no afectó ninguna de las variables analizadas. La adición de THY, EUG y CAR en dosis altas (2.000 mg / kg de forraje) redujo la concentración de N-amoníacal en los ensilajes de raigrás. Además, CAR redujo la concentración de N-amoniacal en la dosis moderada (500 mg / kg de forraje). La actividad antimicrobiana de estos compuestos redujo la población de LAB, que explica la reducción de la concentración de N amoniacal. La adición de CIN a 2.000 mg / kg de forraje tuvo un efecto general sobre la degradación de la proteína, resultando en silos con 9,7% más de N proteico real, pero no afectó el recuento de LAB o la concentración de ácido láctico de los silos. Estos efectos pueden ser atribuidos a la inhibición de la actividad enzimática de la planta, pero el mecanismo exacto de la acción necesita ser identificado. Los resultados sugieren la contribución de las LAB en el proceso de degradación de la proteína y la desaminación durante el ensilaje. Los compuestos de

AE probados afectaron la degradación de proteínas y la desaminación del forraje de raigrás durante el ensilaje, pero la dosis efectiva fue demasiado alta para ser aplicado en la práctica.

En el tercer estudio, se produjeron y probaron *in vitro* anticuerpos policlonales (APs contra las principales bacterias proteolíticas desaminadoras en el rumen con el objetivo de reducir la concentración de N amoniacal y mejorar la eficiencia del N en el rumen. Recientemente, los APs se han utilizado para el control de bacterias específicas responsables de la acidosis ruminal. Por lo tanto, nuestra hipótesis fue que la adición de APs contra *Prevotella ruminicola*, *Clostridium aminophilum* y *Peptostreptococcus anaerobius* podría neutralizar las bacterias involucradas en la proteólisis y desaminación reduciendo el N-amoniacal en el rumen. Las bacterias se cultivaron de acuerdo a las recomendaciones, se inactivaron con formaldehído, se liofilizaron, y se utilizaron para inmunizar conejos. Se recogieron muestras de sangre después de la cuarta inmunización y la respuesta a los antígenos en suero se analizó mediante ELISA. En el primer experimento, se utilizó la technica de producción de gas e incubaciones *in vitro* durante 24 h para probar los efectos de los APs en la fermentación ruminal a corto plazo. Los tratamientos fueron: control (CTR; suero de animales no vacunados), APs contra *P. ruminicola* (APr), *C. aminophilum* (ACl), *P. anaerobius* (APa), y una mezcla de APs (1:1:1 de APr, ACl y APa, respectivamente; AMix). Los tratamientos se evaluaron a 0,005, 0,05 y 0,5 para la producción de gas y en 0,005 y 0,05 ml de suero / 30 ml de medio para las incubaciones de 24 h. La producción de gas se registró durante 24 h y se tomaron muestras para analizar N-amoniacal y los ácidos grasos volátiles (AGV) de tubos seleccionados a las 3, 12 y 24 h y se tomaron muestras En el segundo experimento, ocho fermentadores de cultivo continuo se inocularon con líquido ruminal de una vaca lechera alimentada con una dieta 50:50 forraje:concentrado, en 2 períodos replicados para probar los efectos de los mismos tratamientos, excepto el AMix, a 3,2 ml de suero /

fermentador / día. Durante los días de muestreo, los fermentadores se muestrearon a 0, 2, 4 y 6 h después de la dosificación de la dieta para analizar las fracciones de N y a las 2 h para analizar los AGV. Las muestras del efluente de las 24 h fueron analizadas para las fracciones de N, los AGV y la digestibilidad de nutrientes. La adición de APs no tuvo efecto sobre la fermentación ruminal a corto plazo. En el estudio de los fermentadores, el N-amoniacal en los efluentes no se afectó por los tratamientos (rango entre 7,31 y 7,91 mg / 100 ml para CTR y APa, respectivamente). La digestibilidad de los nutrientes y la variación horaria de las fracciones del N no fueron diferentes entre los tratamientos. Los APs probados no afectaron al metabolismo proteico ni en la fermentación ruminal a corto ni a largo plazo.

En el cuarto estudio, se evaluaron los efectos de propil-propylthiosulfonato (PTSO), un compuesto organosulfurado estable del ajo, sobre la fermentación ruminal en un sistema de cultivo de doble flujo continuo. Nuestra hipótesis fue que la adición del PTSO alteraría la fermentación ruminal y el metabolismo del N reduciendo la concentración de N-amoniacal y aumentando la relación acetato a propionato. Se realizaron dos experimentos usando fermentadores de cultivo continuo de doble flujo en dos períodos para cada experimento. Cada período experimental consistió de 5 días de adaptación del fluido ruminal a los tratamientos y 3 días para el muestreo. La temperatura (39 ºC), el pH (6,4), y la velocidad de dilución del líquido ($0,10$ h^{-1}) y sólido ($0,05$ h^{-1}) se mantuvieron constantes. Durante los últimos 3 días, se tomaron muestras a las 2 h después de la dosificación de la dieta por la mañana y efluente de 24 h. Las muestras fueron analizadas para su concentración de AGV, N-amoniacal, péptidos pequeños (PPep), péptidos largos (LPep) y la digestibilidad de la materia orgánica (MO), proteína bruta (PB), fibra neutro detergente (aNDFom) y fibra ácido detergente (ADFom). En el primer experimento, los tratamientos incluyeron un control negativo sin aditivo (CTR), un control

positivo con monensina a 12 mg/l (MON) y dos dosis de PTSO a 30 mg/l (PTSO30) y 300 mg/l (PTSO300). La adición de PTSO30 no afectó a ninguna de las mediciones. El PTSO300 disminuyo drásticamente la concentración de AGV totales en el efluente, redujo la digestibilidad verdadera de MO y la digestibilidad aNDFom y ADFom, lo que indica una fuerte actividad antimicrobiana y la inhibición de la fermentación microbiana. El segundo experimento se desarrollo de forma idéntica al primero y se llevó a cabo para probar dosis crecientes de PTSO (0, 50, 100 y 150 mg / l) sobre la fermentación microbiana ruminal. Los AGV totales y la proporción molar del propionato respondieron cuadráticamente con valores más altos en las dosis intermedias. El butirato aumentó y los AGV ramificados disminuyeron linealmente con las dosis crecientes de PTSO, y las concentraciones de N-amoniacal, PPep y LPep no se afectaron por los tratamientos. En las muestras de los efluentes de 24 h, sólo las concentraciones de AGV totales y AGV ramificados se respondieron de forma cuadrática y lineal con el aumento de la dosis de PTSO, respectivamente. La digestibilidad de la MO, PB, aNDFom y ADFom no se afectaron por los tratamientos. Los resultados sugieren el potencial de PTSO para modificar la fermentación del rumen en una dirección coherente con la mejora de la utilización de energía en dosis eficaces entre 50 y 100 mg/l.

SUMMARY

The current PhD thesis was conducted within the framework of the European Union funded project RedNex, that is focused on the environmental contamination with nitrogen (N) from dairy farming. Agriculture, and particularly livestock production, is the main contributor to the phenomenon called as the N cascade that describes the circulation of reactive N into the ecosystems causing multiple effects in the atmosphere, terrestrial ecosystems, freshwater and marine systems, and human health. The main objective of the thesis was to use innovative and novel technologies to give answers and suggest solutions that may reduce the N excretion from ruminants to the environment. We conducted four studies to evaluate different technologies and innovations: near infrared spectroscopy as a tool to provide better management of nutrient formulation at the farm; polyclonal antibodies against main proteolytic and deaminating bacteria in the rumen; and essential oil compounds as modifiers of microbial populations responsible for protein degradation in the rumen and ryegrass forage during ensiling.

In the first study, we created a large database of a collection of 809 different feedstuffs frequently used in ruminant nutrition. Feedstuffs were analyzed for dry matter (DM) and crude protein (CP) degradation and a smaller part (n = 100) for neutral detergent fibre (NDF) degradation. Feedstuffs were grouped as forages (FF; n = 256) and non-forages (NF; n = 553). Degradability was described in terms of immediately rumen soluble fraction (a), the degradable but not soluble faction (b) and its rate of degradation (c). Overall effective degradability (ED) of DM and CP (5% h^{-1} passage rate), and NDF (2% h^{-1} passage rate) were calculated according to the equation of Ørskov and McDonald (1979). All samples were scanned from 1,100 to 2,500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hoganas, Sweden). Reflectance was recorded in 2 nm steps as log 1/Reflectance. Samples were scanned twice in duplicate using ring

cup cells and mean spectrum was calculated for each sample. A WinISI III (v. 1.6) software was employed for spectra data analysis and development of chemometric models. Calibrations were developed by the modified partial least squares (MPLS) regression technique for all (ALL), FF and NF samples. The precision of the equations obtained was confirmed by an external validation set of 20% of total samples. The ED, a and b fractions of DM and CP were well predicted and improved by group separation. The rate of degradation of DM and CP were not satisfactorily predicted when all samples were included ($r^2 < 0.7$). However, separating samples improved the prediction of DM ($r^2 > 0.7$) and of CP for FF ($r^2 > 0.7$). For NDF, the number of feedstuffs was lower and the majority was grouped in FF. Equations obtained satisfactorily predicted ED and fraction b of NDF and group separation further improved predictions. When all feedstuffs were included the rate of degradation was not well predicted ($r^2 = 0.4$), but when samples were grouped prediction for FF was acceptable ($r^2 = 0.8$). In conclusion, group separation into FF and NF improved NIRS equations especially for prediction of degradation rate. Current equations are acceptable and allow to incorporate NIRS as a field tool for feed evaluation models, that require prediction of the rate of degradation and effective degradation of feedstuffs.

In the second study, we evaluated the effect of the addition of essential oil (EO) compounds on ryegrass silage chemical composition and protein degradation. During ensiling of forages, an extensive degradation of protein has been documented. Some EO compounds may alter protein metabolism through the inhibition of peptidolysis and deamination. Therefore, we hypothesized that the addition of EO to ryegrass forage can affect protein degradation and deamination during ensiling. Microsilos (n=74) were prepared in polyester bags with 2.0 kg of fresh chopped ryegrass forage, sprayed according to treatments and sealed with an automated

vacuum machine. The EO compounds tested were: thymol (THY), eugenol (EUG), cinnamaldehyde (CIN), capsaicin (CAP) and carvacrol (CAR), at 4 doses: 0, 50, 500 and 2,000 mg/kg of fresh forage. Silages were opened after 35 days and sampled. Samples were analyzed for pH, N fractions (large peptide-N, small peptide-N, and ammonia-N), dry matter (DM), lactic acid, lactic acid bacteria (LAB) and clostridia. Silage pH was higher than expected (5.5 to 6.6) and was attributed to the low DM content of the forage and the addition of EO. The addition of CAP did not affect any of the variables tested. The addition of THY, EUG and CAR in high dose (2,000 mg/kg of forage) reduced ammonia-N concentration in ryegrass silages. Moreover, CAR reduced ammonia-N concentration in the moderate dose (500 mg/kg of forage). The antimicrobial activity of these compounds reduced the population of LAB, explaining the reduction of ammonia-N concentration. The addition of CIN at 2,000 mg/kg of forage had an overall effect on protein degradation resulting in silages with 9.7% higher true protein N, but had no effect on LAB counts or lactic acid concentration of silages. These effects might be attributed to the inhibition of plant enzymatic activity, but the exact mechanism of action needs to be identified. Results suggest the contribution of LAB in the process of protein degradation and deamination during ensiling. Tested EO compounds affected protein degradation and deamination of ryegrass forage during ensiling, but the effective dose was too high to be applied in practice.

In the third study, we produced and test *in vitro* polyclonal antibodies (PAbs) against main proteolytic and deaminating bacteria in the rumen with the objective to reduce ammonia-N concentration and improve N efficiency in the rumen. Recently, polyclonal antibodies (PAbs) have been used to control specific bacteria responsible for ruminal acidosis. Thus, we hypothesized that the addition of PAbs against *Prevotella ruminicola*, *Clostridium aminophilum*

and *Peptostreptococcus anaerobius* may neutralize target bacteria reducing ammonia-N in the rumen. Bacteria were grown according to recommendations, inactivated with formaldehyde, freeze dried, and used to immunize rabbits. Blood samples were collected after the 4th immunization and serum responses to the antigens were analyzed by ELISA. In the first experiment, the modified gas production and the 24 h batch culture techniques were used to test the effects of PAbs in short term ruminal fermentation. Treatments were: control (CTR; serum of non-immunized animals), PAbs against *P. ruminicola* (APr), *C. aminophilum* (ACl), *P. anaerobius* (APa), and a mix of PAbs (1:1:1 of APr, ACl and APa, respectively; AMix). Treatments were tested at 0.005, 0.05 and 0.5 for gas production and at 0.005 and 0.05 ml of serum / 30 ml of medium for batch culture. Gas production was recorded for 24 h and selected tubes of the batch culture were withdrawn at 3, 12 and 24 h and sampled for ammonia-N and volatile fatty acids (VFA). In the second experiment, eight continuous culture fermenters were inoculated with ruminal liquid from a dairy cow fed a 50:50 concentrate:forage diet, in 2 replicated periods to test the effects of the same treatments, except the AMix, at 3.2 ml of serum/fermenter/day. During sampling days, fermenters were sampled at 0, 2, 4 and 6 h post feeding for N fractions and at 2 h for VFA. Samples of the 24 h effluent were analyzed for N fractions, VFA and digestibility of nutrients. The addition of PAbs had no effect on ruminal fermentation in short term fermentation. In the fermenters study, ammonia-N in the effluents were not affected by treatments (average of 7.31 to 7.91 mg / 100 ml for CTR and APa, respectively). Nutrient digestibility and the hourly variation of N fractions did not differ among treatments. Tested PAbs did not affect ruminal protein degradation in the short or long term fermentation.

In the forth study, we evaluated the effects of propyl-propylthiosulfonate (PTSO), a stable organosulfurate compound of garlic, on ruminal fermentation in a dual flow continuous culture system. We hypothesized that PTSO addition will alter ruminal fermentation and N metabolism reducing ammonia-N concentration and increasing the acetate to propionate ratio. Two experiments were conducted using dual flow continuous culture fermenters in two replicated periods for each experiment. Each experimental period consisted of 5 d for adaptation of the ruminal fluid to treatments and 3 d for sampling. Temperature (39ºC), pH (6.4), and liquid (0.10 h^{-1}) and solid (0.05 h^{-1}) dilution rates were maintained constant. During the last 3 days, samples were taken at 2 h after the morning feeding and from the 24 h effluent. Samples were analyzed for VFA, ammonia-N, large peptide (LPep), small peptides (SPep) and digestibility of organic matter (OM), crude protein (CP), neutral detergent fibre (aNDFom) and acid detergent fibre (ADFom). In experiment 1 treatments included a negative control without additive (CTR), a positive control with monensin at 12 mg/l (MON) and two doses of PTSO at 30 mg/L (PTSO30) and 300 mg/L (PTSO300). The addition of PTSO30 did not affect any of the measurements. The PTSO300 decreased dramatically the concentration of total VFA in the effluent, reduced true digestibility OM and digestibility of aNDFom and ADFom, indicating a strong antimicrobial activity and the inhibition of microbial fermentation. Experiment 2 was conducted to test increasing doses of PTSO (0, 50, 100 and 150 mg/l) on rumen microbial fermentation. Total VFA and propionate molar proportion responded quadratically with higher values in the intermediate doses. Butyrate increased and BCVFA decreased linearly with increasing doses of PTSO, and concentrations of ammonia-N, LPep and SPep were not affected by treatments. In the samples from the 24-h effluents, only the total VFA and BCVFA concentrations responded quadratically and linearly with increasing doses of PTSO, respectively. Digestibilities of OM,

CP, aNDFom and ADFom were not affected by treatments. Results suggest the potential of PTSO to modify rumen fermentation in a direction consistent with better energy utilization in an effective dose between 50 and 100 mg/l.

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Chapter 1

Literature Review

1. The Environmental Issue

1.1. The Environmental Movement and EU Policy

In recent years the environmental protection has become an element of politics and policy making throughout the European Union (EU) and the United States of America (USA). One important consequence of the institutionalization of environmentalism has been the increasing involvement of environmental movements (EMO) in policy making (Rootes, 1999; Coglianese, 2001). The contemporary rise of EMO started in the '60s in the USA and Western Europe in connection with the development of atomic energy, the chemical revolution in agriculture, the proliferation of synthetic materials, and the increased in power generation and resource extraction technologies (Rome, 2003). The number of organizations involved in EMO grew from several hundred to over three thousand by the '70s in the USA, and the number of citizens joining EMO organizations increased significantly (Coglianese, 2001). In Europe, the peak of environmental organization activities and protests took place in the '80s (Rootes, 2003).

At the same time, the diversity of the aims of the movement grown, including not only nuclear power management but also pollution, forest preservation, biodiversity, animal rights, etc. (Liddick, 2006). Rootes (2003) reported that the five main issues of environmental protest in seven EU countries were: nature conservation, urban and industrial pollution, energy, transport pollution, and animal welfare and hunting.

In the USA, the institutionalization of environmentalism started immediately and the incorporation of major organizations into state structures were almost completed by the mid '70s (Coglianese, 2001; Dryzek, 2003). In contrast, the diversity of cultures and state formations in the EU did not allow a fast incorporation of EMO. Countries like Norway demonstrated an active

inclusion of EMO, but others like United Kingdom exclude environmental groups from state policy (Dryzek et al., 2003). However, within the EU the institutionalization of EMO progressed rapidly and by 1990 environmentalism was institutionalized almost everywhere in the EU territory (Rootes, 2003). The EU itself has provided some of the stimuli, incorporating environmental issues in the Directorate General (Environment). In the EU member states, environmental issues have moved up in policy agenda sometimes as a result of pressure from the European Commission (EC) and usually with the aim of raising and harmonizing standards of environmental protection (Rootes, 2002, 2003).

The inclusion of EMO in states policy was explained by the "life cycle" theory of social movements. According to this theory, a social movement starts as radical protest against the established order and the movement demands gradually move to become framed in ways acceptable to power holders, and the de-radicalized movement enters the corridors of power (Offe, 1990). However, this approach takes the social structure for granted, as a static reality and does not accept the dynamic nature of it (Dryzek et al., 2003; Cox, 2006). The environmental movements are a good example of social interaction of movements- governments: EMO are still developing their social action by participating in main political scenes, protesting or acting in more radical manner (Brulle, 2000; Rootes, 2003; Liddick, 2006), but at the same time governments are incorporating parts of EMO's principles and aims into their own policy (Rootes, 1999; Coglianese, 2001; Dryzer et al., 2003). The incorporation of social movements leads, on one hand, to the control of social opposition and on the other hand in improvements concerning the particular issue. The moderate version of EMO that have been incorporated into the EU policy making process, provide new challenges for industry and science.

1.2. EU Research on Environmental Issues and the RedNex Project

It is difficult to assess the extent of research on environmental issues supported by the EU due to the diversity of programs and the integration of different scientific areas. The European Commission (EC) is responsible for funding research of different scientific areas mainly through framework programs for research and technological development. The seventh framework program (FP7) covers the period 2007-2013 and has a total budget of over 50 billion €. Within the FP7, environmental science is a main research area. Main topics include: climate change, natural hazards, environmental health, natural resources management, biodiversity, marine environment, land and urban management, environmental technologies, earth observation, sustainable and environmentally friendly Europe, and assessment tools for sustainable development (EC, 2007). The overall budget for environmental sciences was set at 1.9 billion €; 3.8% of the total budget. However, other research areas within FP7 contribute to the final budget for the environmental issue. Main contributing areas are: agriculture, fisheries and forestry, energy research and sustainable development (EC, 2007). Thus, the exact budget dedicated to environmental purposes is difficult to calculate.

The RedNex project (http://www.rednex-fp7.eu/) is one of the EU funded projects that even though is focused on the environmental issue it is included in the agriculture, fisheries and forestry research area. The acronym derives from Reduced (Red) Nitrogen (N) Excretion (ex). Therefore, the objective of the project is to develop innovative and practical management approaches to reduce N excretion from dairy cows into the environment through the optimization of rumen function, an improved understanding and prediction of dietary N utilization for milk production, and excretion in urine and faeces.

The following partners participate to the project: Aberystwyth University (UK), European Association of Animal Production (Italy), Friedrich-Löffler-Institut. Bundesforschungsinstitut für Tiergesundheit (Germany), Institut National de la Recherche Agronomique (France), Slovenske Centrum Polnohospodarskeho Vyskumu (Slovakia), Universitat Autonoma de Barcelona (Spain), Universiteit Gent (Belgium), University of Aarhus (Denmark), University of Reading (UK), and Wageningen Universiteit (Netherlands). The RedNEx programme is organised into 7 interlinked sub-projects (work packages (WP); Figure 1).
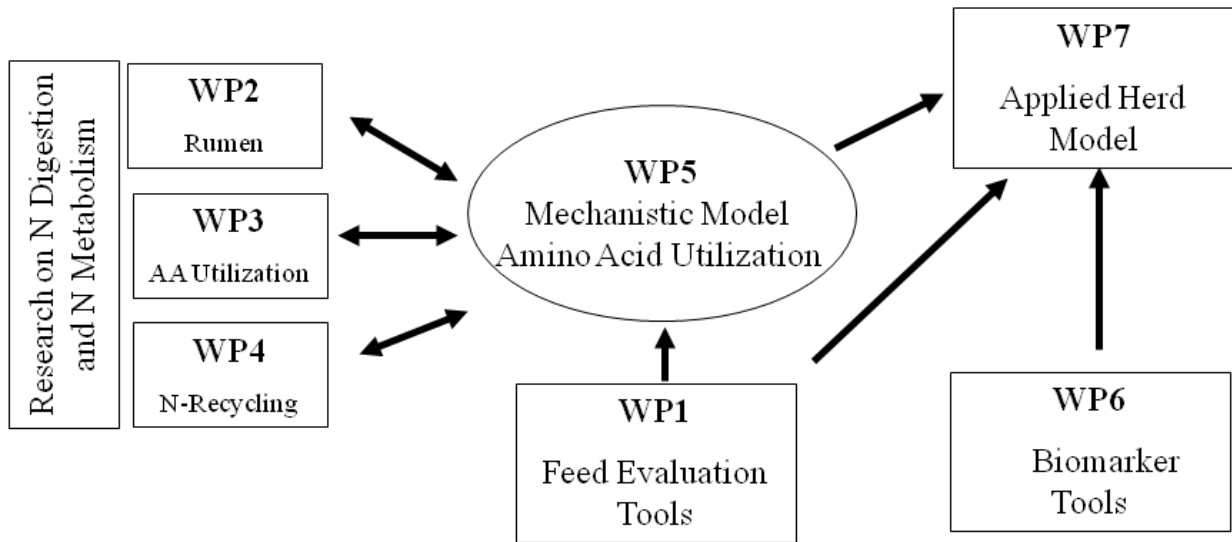
Figure 1. Work Packages (WP) organization of the RedNex project.

In the centre of the framework is WP5 with the aim to develop and apply a mechanistic model of the rumen, gut wall, liver and mammary gland that explains N and amino acid (AA) metabolism, in order to integrate data and concepts and ultimately replace current empirical protein evaluation systems. Data from partners of WP 2, 3 and 4 on rumen N metabolism, N recycling to the rumen and AA absorption and metabolism is used to develop and evaluate the

mechanistic model. This model will dictate feeding strategies to be tested by other work packages, as well as to help identify key elements for the applied farm level model in WP7. The farm level model is meant to facilitate accurate prediction of quantities of N excreted by dairy herds using standardized methodology in the EU countries.

This thesis is developed within the framework of the RedNex project and contributes to WP1 and WP2. The main objective of our contribution to WP1 is to investigate the potential of near infrared spectroscopy (NIRS) to predict the rate and extent of dry matter (DM), crude protein (CP), neutral detergent fibre (NDF) and acid detergent fibre (ADF) degradation in the rumen. The application of a faster and cheaper method to estimate these parameters should improve feed formulation practice with a better balance between energy and protein supply to both rumen micro-organisms and the host animal, therefore reducing N losses during rumen fermentation. Our contribution to WP2 is focused on developing new strategies to alter ruminal microbial protein degradation. The objective is to reduce protein degradation and ammonia-N production that may enhance the flow of proteins leaving the rumen and optimize ruminal microbial protein synthesis.

1.3. Nitrogen Contamination of the Environment

Nitrogen is an essential element of food production determining the productivity of crops and animals (Jensen et al., 2011). However, its extensive use has led to the phenomenon described as the N cascade (Galloway et al., 2003). Agriculture is the main contributor to this phenomenon and the increased efficiency of N use in crops and animal production were proposed as key actions for N management (Sutton et al., 2011a).

In nature there are two forms of N: nonreactive N (N_2), and reactive N (Nr) that includes inorganic N such as ammonia (NH_3) and ammonium (NH_4^+), inorganic oxidized forms like nitrogen oxides (NO_x), and organic compounds like urea and proteins. Gaseous di-nitrogen (N_2) constitutes 78% of the earth's atmosphere and it is a rather inert chemical, being nearly unavailable for the biological cycle (Galloway et al., 2003). Human activity caused a significant accumulation of Nr in the environment (Galloway et al., 1995). Data from USA and EU suggest that inputs of anthropogenic Nr increased dramatically since 1950 (Galloway et al., 2003; Sutton et al., 2011a). In the EU of 27 countries (EU-27) the annual N inputs are estimated between 20-23 Tg (1Tg=1 million tonnes) between 1980-2000 (Figure 2). Similarly, in the USA a production of approximately 25 Tg of Nr per year is estimated since 1997 (Galloway et al., 2003), while in global perspective approximately 187 Tg of Nr per year are produced since 2004 (Galloway et al., 2008).
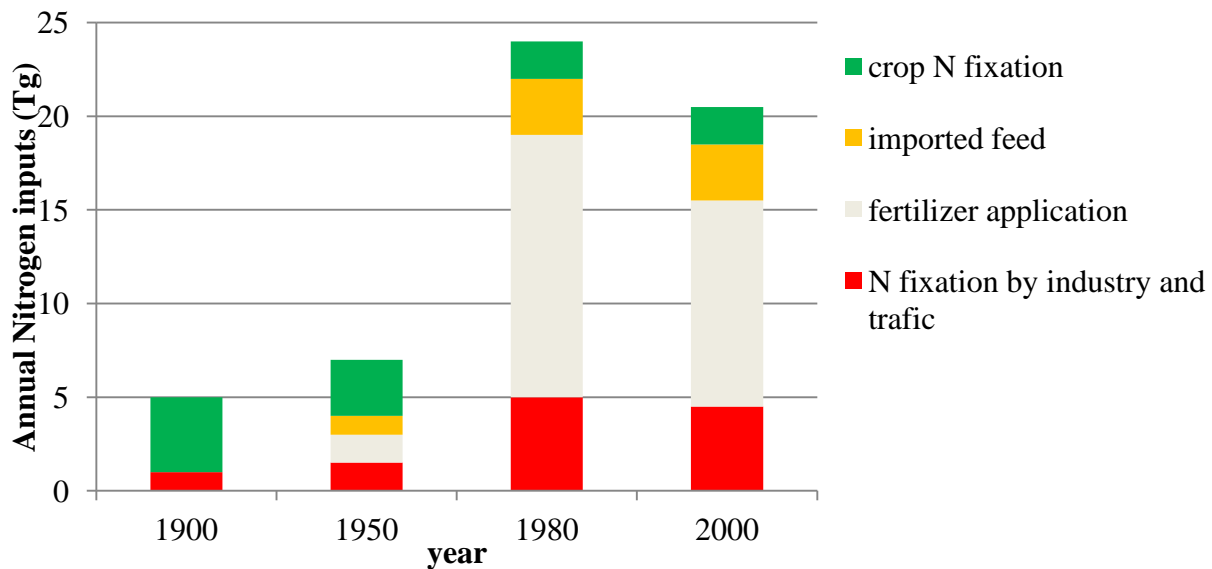
Figure 2. Evolution of reactive nitrogen (Nr) inputs in EU-27 (adapted from Sutton et al., 2011a).

The global increase in Nr production has three main causes: (1) widespread cultivation of legumes, and other crops that promote conversion of N_2 to organic N through biological nitrogen fixation (BNF); (2) combustion of fossil fuels, which converts atmospheric N_2 and fossil N to reactive NOx; and (3) the Haber-Bosch process, which converts nonreactive N_2 to reactive NH_3, which is used for food production and some industrial activities (Galloway et al., 2003; Erisman et al., 2011).

Nature controls the equilibrium between these two forms of N with the process of nitrification and denitrification. Nitrification is defined the conversion of NH_4^+ or NH_3 into nitrate (NO_3^-). Denitrification refers to the reduction by aerobic bacteria of one or both ionic nitrogen oxides (NO_3^- and NO_2^-) to gaseus oxides (NO and N_2O), which may be further reduced to N_2 (Hiscock et al., 1991). However, the rate of anthropogenic Nr production is much higher than that of denitrification (Galloway et al., 2003). Therefore, reactive N accumulates in the environment. This accumulation and its resulting effects on the environment are described by the theory of the N cascade.

1.3.1. The N Cascade

The theory of the N cascade has been proposed by environmental scientists to describe the circulation of anthropogenic Nr in earth's ecosystems (Galloway 1998, Galloway et al., 2003, 2004). According to this theory, one atom of Nr, like the one used in fertilizers, circulates into the ecosystems causing multiple effects in the atmosphere, terrestrial ecosystems, freshwater and marine systems, and human health (Figure 3). The anthropogenic Nr results in intended and unintended consequences. In the intended cascade, Nr contributes to soil fertility and increases yield of crops, providing feeds for livestock and, subsequently, food of animal or plant origin for

human consumption. However, Nr is extremely mobile, with emissions from agriculture, combustion and industry leading to an unintended cascade of Nr into the atmosphere as NH_3, nitric oxide (NO), nitrous oxide (N_2O), or N_2, or is lost into aquatic ecosystems, primarily as nitrate (NO_3). Once transferred to these downstream or downwind systems, the N atom is part of the cascade. Depending on its chemical form, Nr will enter the cascade at different levels (Galloway et al 2003; Erisman et al., 2011; Sutton et al., 2011a).
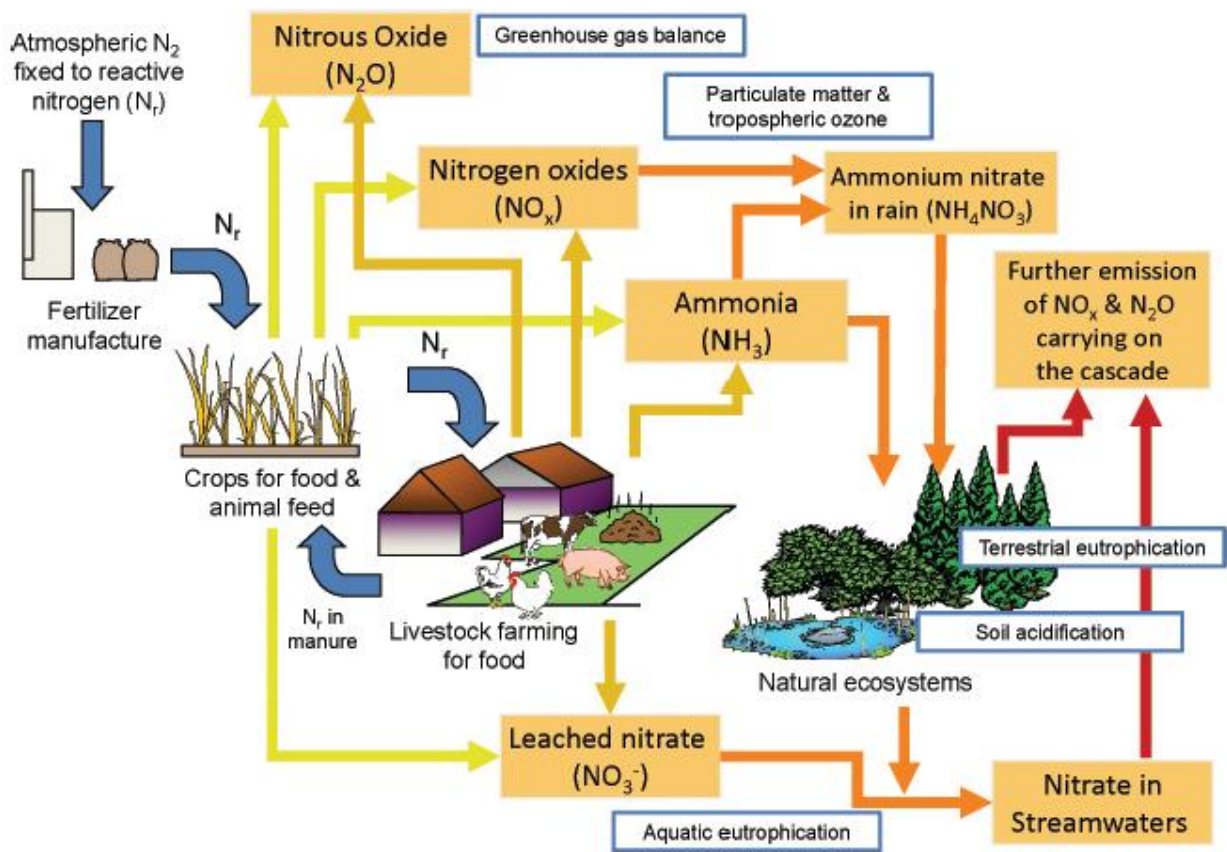
Figure 3. Simplified view of the Nitrogen cascade (Sutton et al., 2011a).

An important characteristic of the phenomenon is that once Nr enters into the ecosystem loses its connection with the original source. This provides an additional opportunity to control the phenomenon not only on its production but also at different sites of the ecosystem.

1.3.2. Agriculture: TheMmain Contributor is Livestock Production

The main contributors to the N cascade are industry and agriculture. In the EU-27, industry accounts for 21%, fertilizer manufacture for 70% and crop BNF for 8% of total Nr production (Sutton et al., 2011a). Therefore, agriculture is the main contributor accounting for approximately 78% of total Nr production. World Nr fertilizer consumption in 2000 was 81.7 Tg; Europe, India and the US consumed 11-12 Tg each, and China consumed more than twice that amount (Fixen and West, 2002). Moreover, the European Nr flow in crop production is mainly supplied by Nr in fertilizers that account for 48% of total Nr inputs into agricultural soils; followed by crop residues (15%), manure application (15%), manure in grazing (12%), atmospheric deposition (8%) and BNF (4%; Leip et al., 2011). However, more than 80% of the crop production is used as feed for livestock: calculations in EU-27 demonstrated that 8.7 Tg Nr per year from domestic feed production plus 3.1 Tg Nr per year from imported feeds, provide a total of 11.8 Tg Nr per year for livestock production (Jensen et al., 2011). Similarly, in the USA, 70% of crops that were not exported are fed to livestock (Howarth et al., 2002). Therefore, livestock production is the dominant human driver altering the nitrogen cycle.

1.3.3. A European Model to Assess N Cycle and Generated Emissions of Livestock

Recently, efforts to control the N cascade included an assessment of the current situation in the EU-27, where a farm model was created and used to calculate N inputs and emissions in different farming systems (Jarvis et al., 2011).

A schematic representation of N flows at the farm level is presented in Figure 4. According to this analysis Nr inputs on a farm level derived from imported animal feed (1), bedding (2), fertilizers and BNF (3). Outputs include exported products such as crops (4), milk and meat (5), and manure (6). Other outputs include losses as gases to the atmosphere from the

components of livestock production (7, 8) and cropped or grazed fields as NH_3, N_2, N_2O or NO (9), or in run-off liquids or leaching as NO_3^-, NH_4^+ or dissolved organic N (DON; 10). The farm N cycle also involves many internal transfers and transformations. In grazing systems, N from urine and feces is deposited on the fields during grazing (11) or in animal housing, animal holding areas and feedlots (12). From there, it is either applied directly to land or enters the manure management system (13, 14, and 15). Moreover, internal N transfers are the uptake into the crop either to be consumed directly by livestock (16, 17) or into tillage-crop production (18). There are also many internal transfers and transformations in the soil (19; Jarvis et al., 2011).
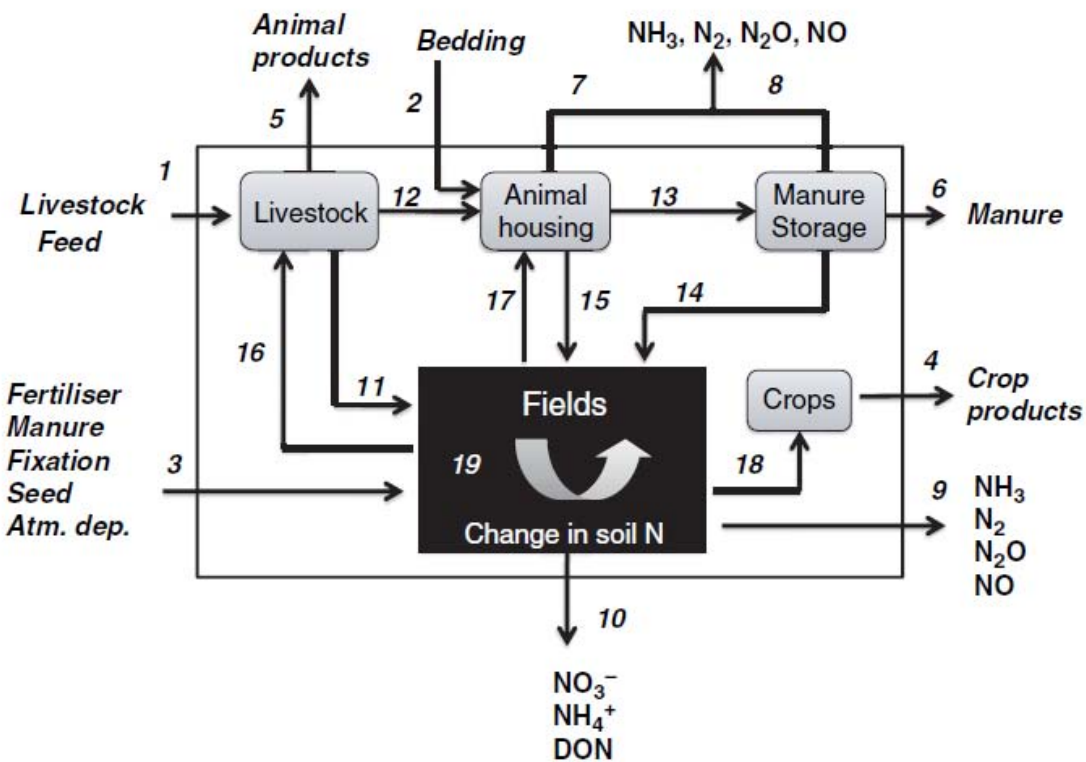
Figure 4. Shematic diagram of annual nitrogen flows on a farm (Jarvis et al., 2011)

There are five different dairy systems identified in the EU (i. High input/output; ii. Low input/output; iii. Mountain; iv. Mediterranean; and v. Organic), of which the high input/output represents more than 85% of European milk production (CEAS, 2000). Therefore, Jarvis et al.

(2011) utilized the high input/output system to assess the impact of dairy production into the N cascade (Figure 5).
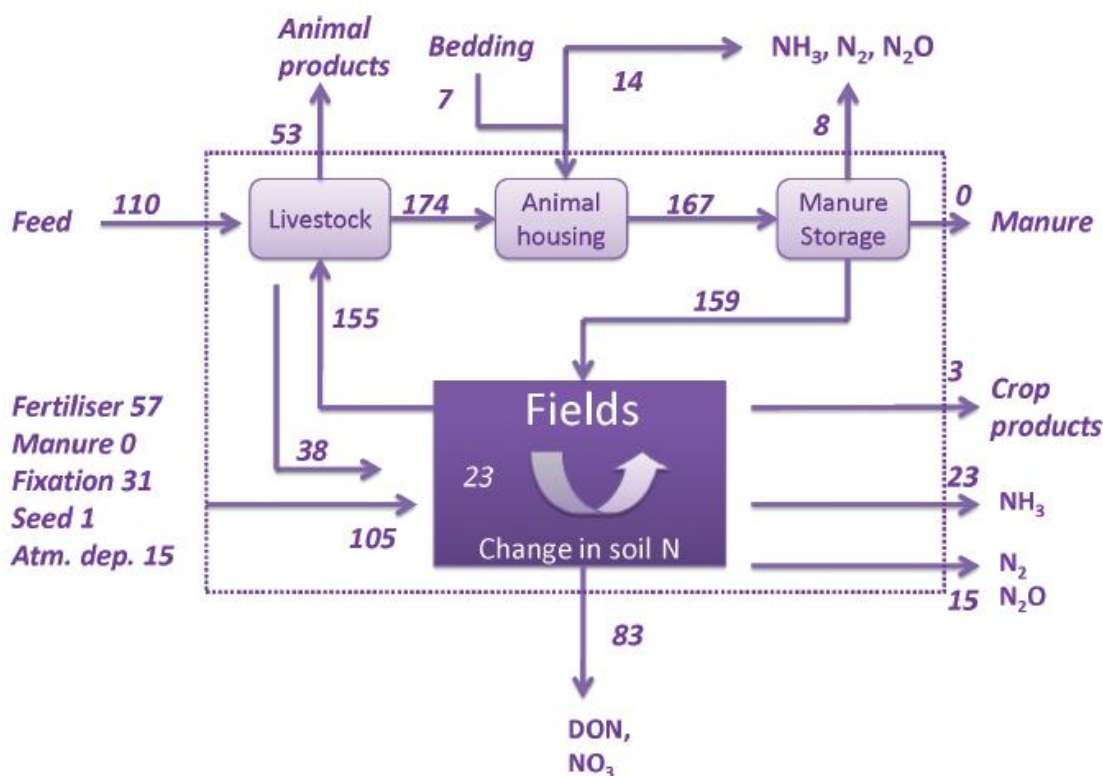
Figure 5. Annual nitrogen flows (kg/ha) in a dairy farming system (Jarvis et al., 2011).

In a high input/output dairy system, annual inputs of Nr derived from imported feeds (110 kg/ha) and fertilizers (57 kg/ha). However, the main Nr input to farm's fields comes from its own manure (159 kg/ha). Dairy cows receive annually 265 kg/ha of Nr, derived from imported and domestic feedstuffs (110 and 155 kg/ha, respectively), while they produce 53 kg/ha in animal products, indicating an animal efficiency of 20%. Utilizing the same approach, an efficiency of 35.5% was calculated for pig farming. Overall annual losses of Nr to the environment from dairy farming calculated at 143 kg/ha. From total losses, 58% is in the form of DON and NO_3, and 42% in the form of NH_3, N_2O and N_2. It should be taken under consideration

that the current study was conducted utilizing data from 27 EU countries, where diversity of practices is extremely high. The application of fertilizers, for example, used in this study is very low compared with other studies. In a high input/output system where restrictions of manure utilization are implemented, like in The Netherlands, application of 250-300 kg/ha of N fertilizer is more common (CEAS, 2000; Kuipers and Mandersloot, 1999). Despite the limitations of this approach, it can be concluded that dairy farming has a low N efficiency compared with pig farming, and that the main N losses are derived from manure Nr concentration in the form of NH_3.

1.3.4. N Emissions from Dairy Farming

Manure is the main N outflow from cows (75-80% of total N output; Tamminga, 1992), contributes significantly to the total amount of N applied to farm's fields (26.5% of total N applied to fields; Jarvis et al., 2011) and is the major pool of N losses in the form of NH_3 and NO_3 during storage, grazing and application to fields as fertilizers.

Oenema et al. (2007) calculated for EU-27 that 70-80% of animal excreta were collected in housing systems and the remaining 20-30% was deposited during grazing, and that 48% of the N excreted in manure is lost during storage and field application. The main gaseous loss is through NH_3 volatilization (Tamminga 1992, 1996; Butterbach-Bahl et al., 2011; Jensen et al., 2011). For the EU-27, calculated losses in the form of NH_3 accounted for 19% of total Nr during storage and 17% of total Nr during field application; another 11% was lost via nitrification and denitrification and 4% via Nr leaching and runoff during storage (Oenema et al., 2007). Nitrification and denitrification are major biological process resulting in Nr losses in terrestrial ecosystems, but more attention is given at the soil level rather than manure storage before application (Butterbach-Bahl et al., 2011). Thus, the main Nr emission from dairy farming is

NH_3 in manure that derives from urea in urine, which is hydrolyzed to ammonium (NH_4^+), and then is converted to ammonia in an alkaline environment. The main N source for urea synthesis in the liver of ruminants derives from NH_3 absorbed in the rumen (Reynolds, 2006).

1.4. Conclusions

The environmental movements of the '70s and '80s stimulated and expressed public concern on the environmental impact of the modern production systems. The institutionalization of moderate parts of the movements stimulated state policies and scientific research. The N cascade is among the main environmental issues due to its impact on different ecosystems that are independent from the origin source. The main contributor to the phenomenon is agriculture, and, particularly, livestock production. The dairy farming sector is one of the most intensive sectors of the EU. Direct emissions of reactive N from dairy that contribute directly to N cascade are derived from manure, where urea is transformed into NH_3, being the main gaseous loss is through volatilization, although nitrification and denitrification also contribute to the process.

2. Strategies to Reduce N Excretion from Dairy Cows

Different strategies to reduce N excretion from ruminants have been proposed. Some are focused on the production system (system-oriented) and others on the animal (cow-oriented), but both approaches are indented to improve the efficiency of N utilization. However, the concept of N efficiency can be confusing because efficiency may refer to the animal, the farm or the entire system. Therefore, before analyzing strategies to reduce N excretion the theoretical concept of N efficiency is discussed.

2.1. N Efficiency

Efficiency of N utilization is defined as the amount of N retained in animal products per amount of N offered, and can be calculated either at farm or at animal level. In the first case, we refer to N use efficiency (NUE) and takes into account total outputs of the farm (milk, meat, live animals, crops and manure), and total inputs (fertilizers, imported feedstuffs and domestic crop production; Groot et al., 2006; Powell et al., 2010). When N efficiency is calculated at the animal level we should define productive goals. For dairy cows the N efficiency is calculated as milk N efficiency (MNE) and is defined as the amount of N produced in milk per amount of N intake (Huhtanen and Hristov, 2009).

2.1.1. N Efficiency at Farm Level

Literature reflects a wide variation of NUE in dairy farms, ranging from 8 to 64% (Rotz et al., 2005; Hristov et al., 2006; Ovens et al., 2008; Powell et al., 2010). This high range should be attributed mainly to methodological problems in the calculation of NUE. To estimate NUE, all imported and farm produced N should be considered. However, it is difficult to calculate the contribution of BNF from home made legumes (Powell et al., 2010) and estimations for BNF are often used (Hristov et al., 2006). Moreover, a quantitative manure calculation is essential to

estimate N flows. In grazing based dairy systems a significant amount of manure is directly applied to fields complicating further the calculation of total manure produced (Del Prado et al., 2006; Ryan et al., 2011) and the form that manure Nr enters the ecosystem (NH_3 or NO_3). Despite the methodological considerations, whole farm NUE is frequently used when the dairy farm is the target of assessing a strategy to reduce N cycle in a farm level.

2.1.2. N Efficiency at Animal Level

In high producing Holstein cows, Lund et al. (2008) reported an efficiency of N utilization of 24-32% with different feeding strategies. Castillo et al. (2000) also reported an average efficiency of 28% in high producing dairy cows and Borsting et al. (2003) reported an average efficiency of 25% in Denmark. Recently, Huhtanen and Hristov (2009) constructed a large database from 739 different diets from North American (NA) studies and 998 diets from North European (NE) studies and reported an average MNE of 24.7 and 27.7 % and a range of 14.0 - 45.3% and 16.4 – 40.2% for NA and NE studies, respectively. This efficiency is very low compared with monogastric animals, where a N retention efficiency as high as 59- 66% has been reported for piglets (van den Borne et al., 2006; Li et al., 2007).

It has been assumed that MNE increases when milk yield increases due to the reduced maintenance cost (Tamminga, 1992; Rotz, 2004); however, when annual milk yield is already high only small progress can be expected (Tamminga, 1996; Van Bruchem et al., 1999). Recent studies utilizing large data sets indicated a poor relationship between milk yield and MNE (Huhtanen et al., 2008; Hunhtanen and Hristov, 2009). In the first study, a poor but significant relationship between milk yield and MNE ($R^2 = 0.14$) was detected using a simple regression model, but when a mixed model analysis was used no relationship between these two variables was detected (Figure 6; Huhtanen et al., 2008). When CP concentration of the offered diet was

added into the model, the R^2 value increased to 0.73. Authors concluded that diet composition rather than milk yield is the main determinant of MNE. When data from NA (Hristov et al., 2004, 2005) was incorporated results verified the strong relationship between CP concentration of diets and MNE, and that milk yield was a poor but significant predictor of MNE (Huhtanen and Hristov, 2009).

Diet CP concentration had a negative relation with MNE (MNE = -1.2 x CP + 475), therefore MNE decreased as dietary CP concentration increased (Huhtanen et al., 2008). Moreover, CP concentration was more important for MNE than CP intake in both data sets. Castillo et al. (2000) reported reduced MNE with increasing levels of N intake especially when it exceeded 400 g/d. Huhtanen et al. (2008) included DM intake (DMI) into the model of CP intake alone and predictions were improved. The coefficient of CP intake + DMI was positive, while the coefficient of CP concentration negative. Therefore, the effect of increased CP intake on MNE was strongly dependent on how CP intake was increased (increased dietary CP concentration vs. increased DMI). Similarly, Huhtanen and Hristov (2009) demonstrated a combined effect of CP intake and DMI on MNE.

In the study of Huhtanen et al. (2008), the variable that predicted best MNE was protein balance in the rumen (PBV; MNE = -1.58 x PBV + 289), indicating the importance of rumen function on MNE. The term PBV is used in the NorFor feed evaluation system and it is an estimator of rumen N losses (Volden, 2011). It is defined as the balance between rumen degradable protein supply (RDP) and microbial requirements of RDP. The supply of RDP (g/d) was calculated as effective protein degradability (EPD) $\times$ CP intake (g/d) and microbial CP requirement (g/kg) as $0.179 \times$ DMI (kg/d) $\times$ [digestible carbohydrates (g/kg of DM) + EPD $\times$ CP (g/kg of DM)]. Børsting et al. (2003) suggested that maximum milk yield can be reached when

PBV values are close to zero. The negative regression coefficient of PBV indicates that when the supply of RPD is higher than microbial requirements, MNE is reduced (Huhtanen et al., 2008).
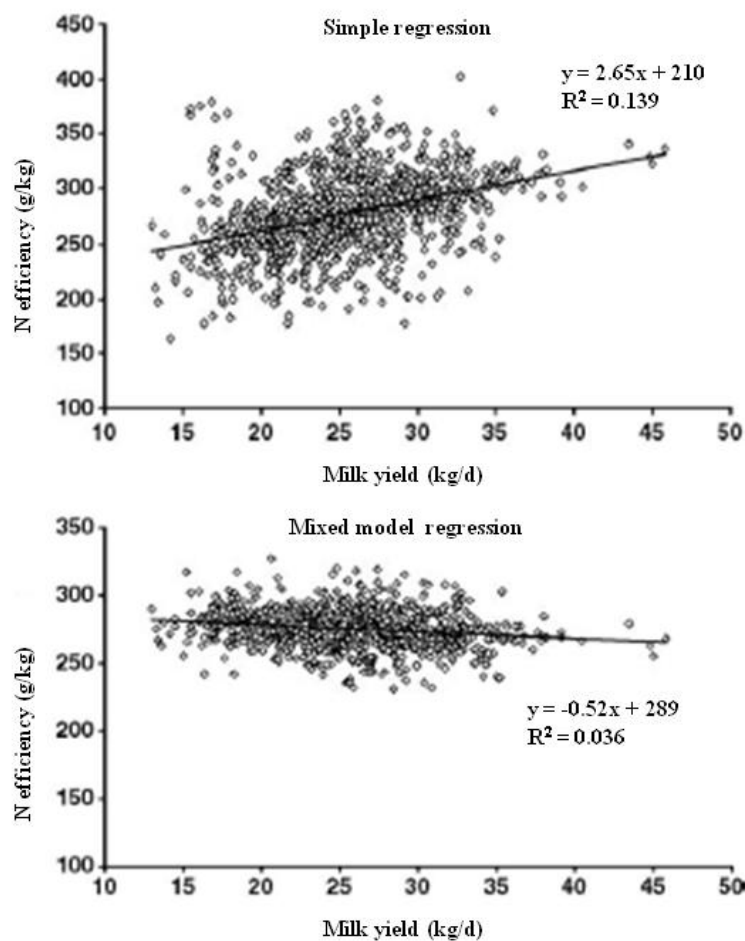
Figure 6. Relationship between milk yield and milk nitrogen efficiency (MNE) analyzed by a simple or mixed model regression (Huhtanen et al., 2008).

Despite the importance of rumen PBV, RDP alone, or in a combination with rumen undegradable protein (RUP) did not improve prediction of the CP intake model (Huhtanen et al., 2008; Huhtanen and Hristov, 2009). However, a strong correlation between CP and RDP ($R^2 =$

0.78) and RUP ($R^2 = 0.53$) has been reported (NRC, 2001). Therefore, the lack of improvement should be attributed to the difficulty to separate CP from its components (RDP and RUP), and that RDP does not take into account the microbial requirements, as does PBV.

2.2. Systemic Strategies

The systemic approach to control N emissions from dairy farming include the organic dairy farming as well as its opposite (the high input/output system), and legislative restrictions on the N use and manure management.

2.2.1. Organic vs High Input / Output

Organic agriculture is the direct result of environmental movements and actions on both governmental policy and the agribusiness sector. Different ideologies and ideas within Europe have contributed to a common basis for organic farming as it is known today (von Borell and Sorensen, 2003). In the EU, organic farming has experienced a dynamic development since the end of last century, mainly due to the support of the European Common Agricultural Policy (CAP) on environmentally friendly systems and their consideration in policy measures (Harring, 2003). However, EU-27 organic dairy sector represents only 2.7% of EU dairy sector and member States with the largest share of certified organic cows in total number of cows are Austria (15.6%), Denmark (9.6%) and Italy (3.2%; EC, 2010).

Dalgaard et al. (1998) reported farm NUE of 28 vs 20% in organic vs conventional dairy, respectively. Similarly, comparative studies between organic and conventional dairy in Denmark indicated higher farm NUE in organic farms (Halberg et al., 1995). This difference is expected if we consider that in conventionally dairy farming the main Nr input comes from fertilizers, while there is no use of chemical fertilizers in organic dairying. Khalili et al. (2002) investigated the

effect of different supplements on milk production and MNE of organic dairy production. Supplements contained different CP concentrations, ranging from 12.9 – 18.1% of DM. They reported an average MNE of 26.3 – 29.5 %, with the highest MNE from the lowest CP diet, but with a significant reduction of milk production. The reported average MNE is similar to those observed in conventional dairy farming (Huhtanen and Hristov, 2009). However, organic dairy farms have lower milk production compared with conventional (Cederberg and Mattsson, 2000; Sato et al., 2005) mainly due to the limited use of concentrates in the feed ration (Rosati and Aumaitre, 2004).

Jarvis et al. (2011), utilized the same farm model used to assess N cycle for conventional dairy (see section 1.3.3.; Figure 4) to assess N cycle in organic dairy farming (Figure 7). For organic dairy farming, annual inputs of Nr derived from imported feeds (25 kg/ha), and BNF was the main within farm producer of Nr (75 kg/ha). Organic dairy cows require annually 162 kg/ha of Nr, that derive from imported and domestic feedstuffs (25 and 137 kg/ha, respectively), while they produce 32 kg/ha in animal products, indicating an animal efficiency of 19.7%. This efficiency is similar to that of high input/output dairy system (20%; see section 1.3.3). Should be noticed that the calculated efficiency is lower than MNE, because authors include also Nr in meat produced by the dairy cows in the farm (model variable: animal products, see Figure 6 and 7 for intensive and organic dairy farming, respectively). Organic system manages to reduce N losses at the farm and improve farm NUE mainly through the restriction of fertilizers. However, when losses are expressed per unit N in products (animal and crops) the losses are about 30% greater in the organic system than the high input/output dairy system (Jarvis et al., 2011).
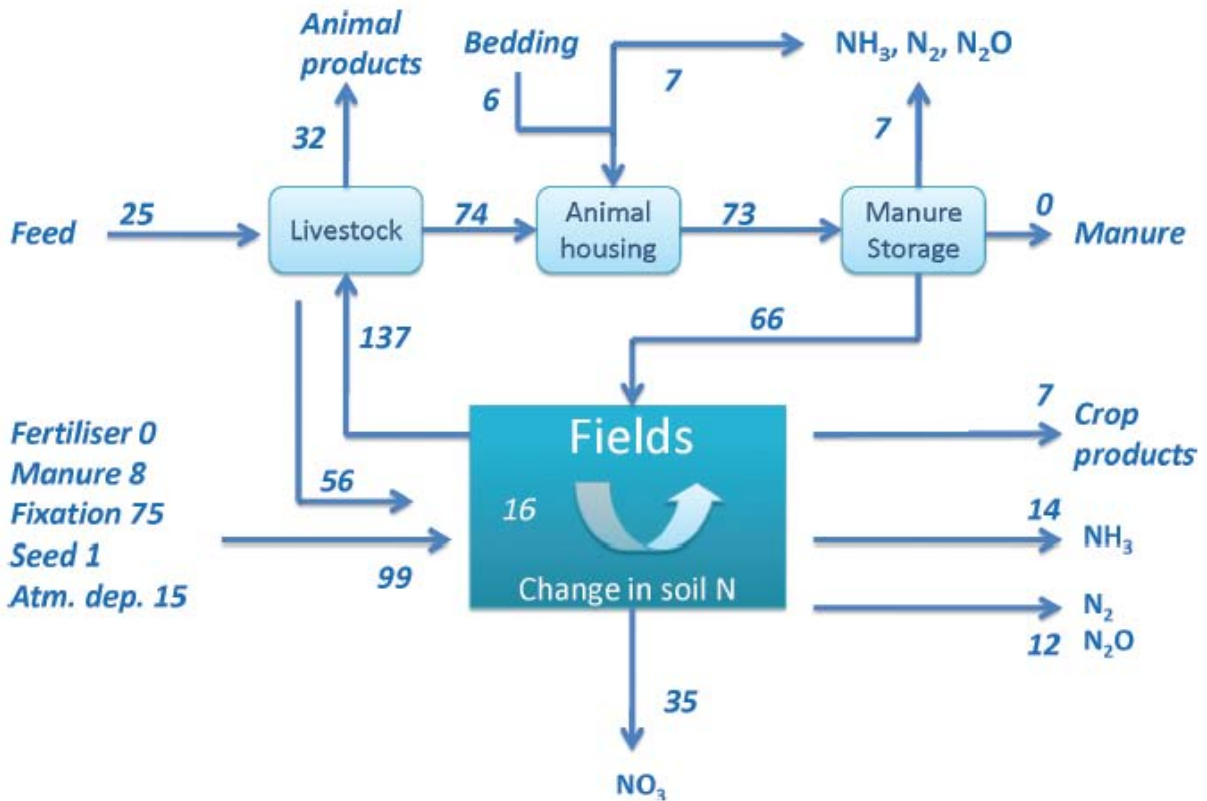
Figure 7. Annual nitrogen flows (kg/ha) in an organic dairy system (Jarvis et al., 2011).

Organic dairy production it is considered by definition an environmentally friendly way of production and it actually manages to decrease N excretion at the farm level, but with a considerable reduction of milk production. Environmentalism includes more issues than pollution, such as animal welfare, maintenance of biodiversity as well as the control of human consumption of dairy products. Therefore, a potential decrease of milk production in region, state or global level should be the prize that to be paid for the protection of the environment.

On the other hand, the high input/output system due to the increased milk production manages to excrete less Nr per unit of product. Therefore, if maintaining the same level of milk production is also requested, the high input / output is the most adequate one. Moreover, efforts to reduce further N losses by intensive dairy production are ongoing.

2.2.2. Manure Management and Fertilizers Restriction

An alternative system-oriented approach could be to make more precise use of Nr inputs from fertilizers and to better manage Nr outputs in manure. The last decades EU environmental policy has guided changes in the dairy production system through legislation. The EU has imposed two major directives to mitigate the effects of N emissions to the environment: (i) The Nitrates Directive (1991/696/EC) aiming to reduce nitrate water pollution in the nitrate vulnerable zones of Europe, and (ii) The Water Framework Directive (2000/60/EC) aiming to protect groundwater resources.

The Netherlands and Denmark are the leading countries on the development and implementation of these legislations (Sonneveld and Bouma, 2003; Bouma, 2011). These countries together with Belgium, Luxemburg and Germany have the highest N excess per ha; Southern European countries, such as Spain, Italy and Greece have the lowest N excess per ha (Maas et al., 2012).

In The Netherlands the implementation of new manure management rules and the development of housing systems with solid floor design, lead to a reduction of NH_3 emissions from dairy farming (Figure 8). Moreover, the lower manure and fertilizer use and the less grazing time of dairy cows reduced NO_3 concentration in the upper water from 150 to 70 mg/l for 1990 and 2007, respectively (Vellinga et al., 2011). Groot et al. (2006) calculated that farm NUE increased from 20% to 30% in a 5 years plan. In Denmark the state policies on N pollution focused on mandatory fertilizer and crop rotation plans, and limit the manure N applied to fields (Kronvang et al., 2008). As a result, consumption of commercial fertilizer decreased from 395,000 tonnes N in 1990 to 196,000 tonnes N in 2003, while the amount of N applied as manure slightly decreased from 244,000 tonnes N to 237,000 tonnes N.
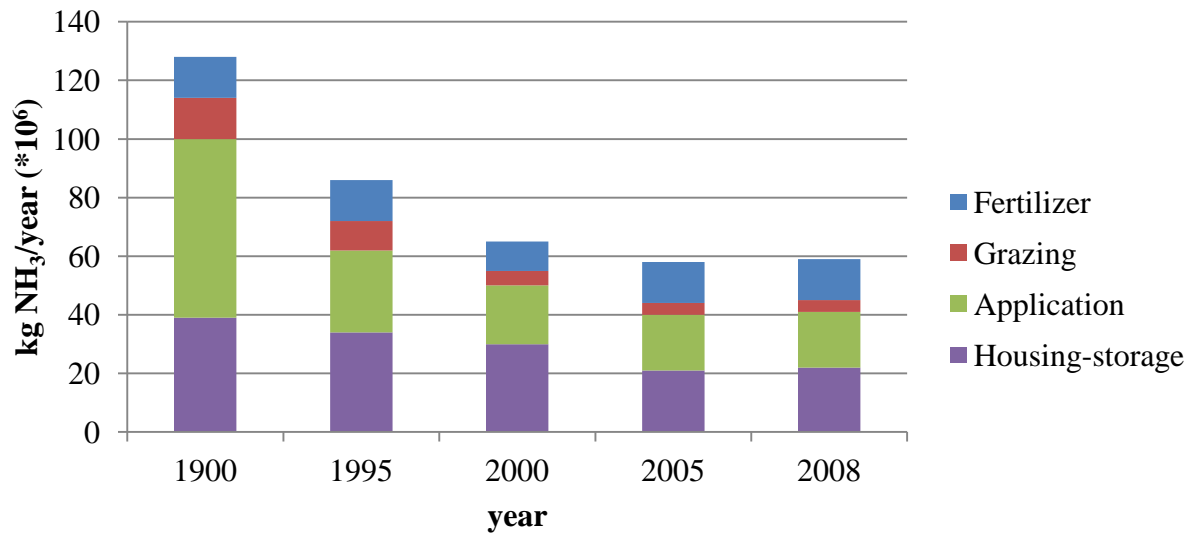
Figure 8. Evolution of ammonia (NH_3) emissions in The Netherlands (Vellinga et al., 2011).

Therefore, non-cow strategies, such as rational use of fertilizer and concentrate N imports to the farm and improved efficiency of N uptake from the soil, provide an effective strategy to reduce Nr emissions to the environment (Van Bruchem et al., 1999; Virtanen and Nousiainen, 2005).

2.3. Cow-oriented strategies

Cow-oriented strategies include improvements in N utilization through better genetic cattle, improved reproduction management and more precise nutrition. Due to the purpose of the current work, nutritional strategies that are using nutrition are reviewed. Among them, those that are targeting the rumen are analyzed in a separate section (section 3.0).

2.3.1. Controlling CP Overfeeding

According to the Cornell net carbohydrate and protein system (CNCPS; Fox et al., 1992), a dairy cow of 650 kg BW with daily milk production of 35 kg needs a feed ration with CP concentration of approximately 16.4% of DM. However, CP is usually overfed in dairy herds.

Average CP concentration of 19.1% with a maximum of 21.5% of DM was reported for Wisconsin (Gunderson et al., 1998). However, CP concentrations have been steadily decreasing in the last 15 years, ranging from 16.7 to 18.0% of DM in the same region (Powell et al., 2006). Similarly, Chase (2003) reviewed 62 published papers indicated an averaged CP concentration of 17.5% of DM. Huhtanen and Hristov (2009) utilizing large data sets from NA and NE studies, reported that NE diets had a CP concentration of 16.5% and NA diets had a 17.8% of DM; a 8% difference.

A more precise feeding that will adjust CP diet level to animal requirements would have substantial effects on MNE (Leonardi et al., 2003; Groof and Wu, 2005; Schwab et al., 2005). Colmenero and Broderick (2006) reported MNE of 25.4 % of N intake when dairy cows were overfed CP (19.4% of DM) and MNE of 30.8 % of N intake when cows were fed according to CP requirements (16.5% of DM); an improvement of 21.2% (Table 1).

Overfeeding has been intentionally practiced to provide a safety margin against uncertain CP concentration of forages and feed (Satter et al., 2002; Firkins and Reynolds, 2005). To overcome this variation of forage CP concentration, a better control of feedstuffs composition, feed ratio balance, TMR preparation and feeding management are required at farm level. Jonker et al. (2002) reported that utilizing monthly milk yield and feed component analysis to reformulate diets increased MNE by 4.2% without adjusting CP concentration. A future incorporation of NIRS in feed evaluation and diet formulation would provide a fast, cheap and pragmatic method to balance diets more precisely without overfeeding dairy cows.

2.3.2. Reducing Further CP Concentration

Several studies suggested that lower levels of CP than requirements could be fed maintaining the same milk yield, reducing N excretion and improving MNE (Colmenero and Broderick, 2006; Agle et al., 2010; Lee et al., 2012). Lee et al. (2012) demonstrated that reducing CP level from 16.7 to 14.8 % of DM reduced NH_3 emissions from fresh dairy cow manure incubated in a controlled environment and from manure-amended soil. In addition, Colmenero and Broderick (2006) utilizing a wide range of CP (13.5, 15.0, 16.5 and 17.9 % of DM) reported that MNE and fecal N excretion (% of N intake) decreased linearly and urinary N excretion (% of N intake) increased linearly by increasing CP concentration (Table 1). Moreover, MNE for the low CP diet (13.5 % of DM) was improved by 18.5% compared with feeding CP according requirements (16.5%), and 43.7% compared with overfeeding CP diet (17.9% of CP). However, other studies reported a reduced milk yield and DMI when CP concentration was reduced below requirements (Alstrup and Weisbjerg, 2012; Weisberg et al., 2012).

Table 4. Effect of CP content on milk production and composition and on N metabolism of dairy cows (Colomeno and Broderick, 2006).

Item[1]	Dietary CP (% of DM)					SE	P-value[2]		
	13.5	15.0	16.5	17.9	19.4		diet	L	Q
DMI (kg/d)	22.3	22.2	23	22.3	22.9	0.50	0.25	0.22	0.93
Milk Yield (kg/d)	36.3	37.2	38.3	36.6	37.0	1.01	0.17	0.65	0.10
N metabolism									
N intake (g/d)	483^a	531^b	605^c	641^d	711^e	13	0.01	0.01	0.75
MNE (% N intake)	36.5^a	34.0^b	30.8^c	27.5^d	25.4^e	0.80	0.01	0.01	0.8
Urinary N excretion (% N intake)	23.8^a	26.6^b	29.8^c	33.2d	36.2^e	0.9	0.01	0.01	0.19
Fecal N excretion (% N intake)	40.3^a	32.9^b	32.0^{bc}	30.5^{cd}	29.6^d	1.00	0.01	0.01	0.01

[1] DMI: dry matter intake; MNE: milk nitrogen efficiency
[2] L: linear; Q: quadratic

Table 5. Effects of feeding dairy cows ratios with crude protein (CP) concentration below requirements on dry matter intake (DMI) and milk yield (MY) with or without altering only rumen degradable protein (RDP).

Lactation stage[1]	CP level (%DM)	RDP	DMI	MY	Reference
Early	15.2 vs 17.4	Yes	−	↓4-13%	*Kalscheur et al., 1998*
Mid	13.3 vs 15.3	Yes	−	−	*Kalscheur et al., 1998*
Late	14.2 vs 12.6	Yes	↓2-6%	−	*Kalscheur et al., 1998*
Early	15.0 vs 18.0	No	−	−	*Bach et al., 2000*
Mid	15.1 vs 16.7	No	↓3%	↓7%	*Broderick, 2003*
Early - Mid	14.8 vs 16.8	No	↓6%	↓6%	*Ipharraguerre and Clark, 2005*
Mid	12.3 vs13.9 vs 15.5 vs 17.1	Yes	−	↓5-6%	*Kalscheur et al., 2006*
Mid	13.5 vs 15.0 vs 16.5 vs 17.9	No	−	−	*Colmenero and Broderick, 2006*
Early and Mid mixed	13.5 vs 16.1	Yes	−	−	*Gresslay and Armentano, 2007*
Mid – Late	12.9 vs 13.4 vs 15.4	No	−	−	*Agle et al., 2010*
Not specify	14.0 vs 16.0	No	↓3%	↓3%	*Alstrup and Weisbjerg, 2012*
Not specify	12.1 vs13.4 vs15.0 vs 16.7	No	↓2-6%	↓2-13%	*Weisbjerg et al., 2012*

[1] Early lactation: ≤ 90 days in milk; Mid lactation: 91 ≤ days in milk ≤ 200; Late lactation: ≥ 201 days in milk.

In Table 2 are summarized several studies where diets with CP concentration below requirements were fed to dairy cows. In six out of twelve experiments a reduced by 6.6% on average milk yield was observed due to CP reduction, while in the remaining 6 studies no difference among CP levels was observed. Moreover, in 5 out of 6 experiments the reduced milk yield was accompanied with reduced by 4% on average DMI, and in 1 experiment the reduced

CP decreased DMI without altering milk yield. However, results are confounded from other parameters studied in these experiments, like AA profile adjustment (Bach et al., 2000) and that the reduction of CP was realized by reducing only RDP fraction (Kalscheur et al., 1998, 2006; Gresslay and Armentano, 2007). Hristov and Huhtanen (2008) reported the lack of relationship between DMI and CP concentration (Figure 9) when a wide range of CP diets were studied. However, diets with CP below requirements (<16% of DM) represented a small portion of the data.

DMI (kg/d) = 20.22 + 0.140 * CP (%)
R^2 = 0.02; P = 0.003
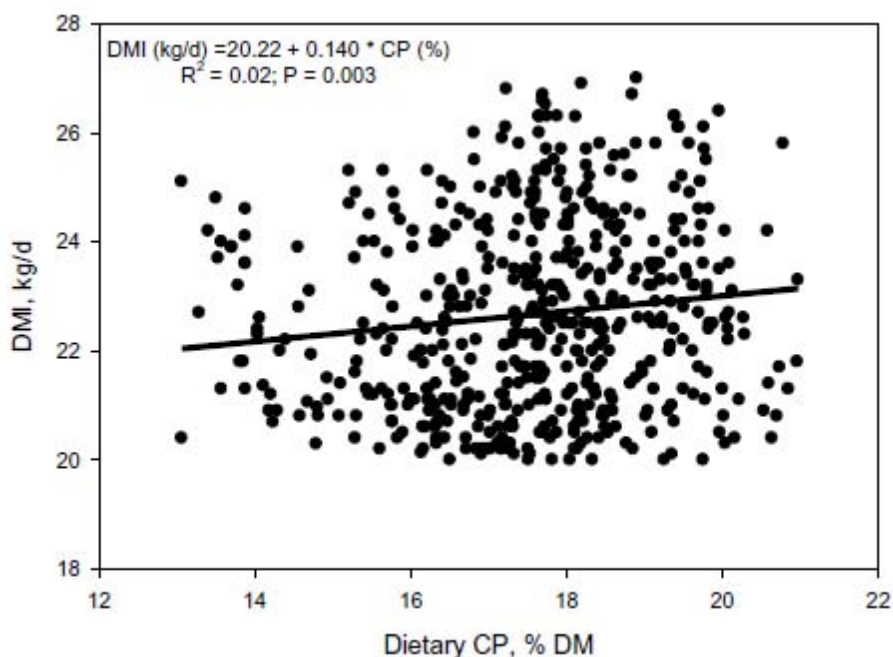
Figure 9. Relationship between dietary CP concentration and dry matter intake (DMI) in dairy cows (Hristov and Huhtanen, 2008).

From the reported studies, it can be concluded that reducing CP concentration below requirements will improve MNE and reduce N excretion from dairy cows. However, effects on milk yield and DMI are contradictory between studies. Therefore, the question if and how much below CP requirements can we go remains to be investigated.

2.4. Conclusions

System and cow-oriented strategies to reduce N excretion from ruminants have been proposed. Organic dairy production manages to reduce net values of N excretion per farm, but due to the lower milk production, N losses per unit of milk are 30% higher than intensive dairy systems. The restriction of fertilizers and manure application through legislation is an effective strategy in because it manages to reduce N inputs in a farm level. However, cow-oriented strategies are also needed to benefit from the potentials of improving the low MNE observed in dairy cows. The first action would be to control CP overfeeding, developing fast and applied tools that would improve nutritional management at the farm level, and adjusting CP concentration to animal requirements. Reduce CP concentration below animal requirements has been suggested, but it should be further investigated due to the contradicted results on DMI and milk yield observed in several studies.

3. Targeting the Rumen

Tamminga (1992) calculated that 75-85% of the ingested N is excreted in faeces and urine, and identify the most important pathways for N losses: (i) urinary excretion of urea synthesized from ammonia lost in the rumen, (ii) fecal and urinary excretion resulting from indigestible or endogenous excretion, and (iii) urinary excretion because of an inefficient utilization of absorbed protein for maintenance and for the synthesis of milk and body protein. Because of the larger losses and easier intervention, the rumen, and particularly the N losses in the form of NH_3, was proposed to be the most appropriate step for modification (Tamminga, 1992, 1996).

In the rumen, NH_3 is produced via deamination of amino acids or non protein nitrogen compounds, like urea and amides, which are converted to ammonia in the rumen (van Soest, 1994; Bach et al., 2005). Ammonia then may be used for microbial growth if energy is available, escape at the lower gastrointestinal tract, or be absorbed through the rumen wall and transferred to the blood and liver. In the liver, ammonia is transformed to urea, which is either transfered back to the rumen through saliva and the rumen wall, or it is excreted in the urine (van Soest 1994; Dijkstra et al., 1996). An important factor in this process is the availability of energy in the rumen. When energy is available in the rumen, amino acids and ammonia are used for microbial synthesis, but if energy is limiting, amino acids will be deaminated.

Huhtanen et al. (2008) demonstrated the importance of rumen N balance on MNE using *the* Scandinavian term PBV, which is the balance between RDP supply and microbial requirements of RDP. Research to improve N utilization in the rumen is focused on two approaches: (i) to optimize microbial protein synthesis, and (ii) to limit protein degradation (Hristov and Jounary, 2005).

3.1. Protein Degradation in the Rumen

Ruminal protein degradation was recently characterized as *the catabolic cascade of proteolysis* since many different microorganisms (bacteria protozoa and anaerobic fungi) or even plant enzymes are participating. However, ruminal bacteria are playing the most important role (Walker et al., 2005).

The process of proteolysis follows these steps (Figure 10): (i) attachment/adsorption of bacteria to feed particles, (ii) hydrolysis of protein that leads to the formation of oligopeptides, which are further hydrolyzed to smaller peptides; (iii) peptides can either be transported into bacteria or further hydrolyzed into amino acids (iv) amino acids transported into bacteria are either transaminated or deaminated to form ammonia. In the first step of protein breakdown, different proteolytic microorganism are involved, such as bacteria, protozoa and anaerobic fungi; in the hydrolysis of oligopeptides to dipeptides the principal bacteria involved are: *Prevotella* spp., *Streptococcus bovis* and *Ruminobacter amylophilus*; in the breakdown of dipeptide to animo acids are involved protozoa and bacteria, such as *Prevotella* spp.*, Fibrobacter succinogenes, Megashaera elsedenii* and *Lachnospira multipara*; in the deamination of amino acids are mainly involved the hyper ammonia producing bacteria (HAP) and *Prevotella* spp. (Wallace, 1996; Rychlik and Russell, 2000; Walker et al., 2005).

Two groups of bacteria involved in the process of protein degradation are of particular interest, those of the genus *Prevotella* and the HAP bacteria. The genus *Prevotella* is the most common proteolytic bacterium (Wallace and Cotta, 1988), where four main species have been identified: *Prevotella brevis*, *Prevotella bryantii*, *Prevotella albensis*, and *Prevotella ruminicola* (Avgustin et al., 1997). They are involved in most steps of protein degradation and are among the most abundant bacteria in the rumen (Walker et al., 2005). They can ferment amino acids

producing NH$_3$ in relatively slow rate, but due to their abundance in the rumen are consider major deaminating bacteria (Rychlik and Russell, 2000).
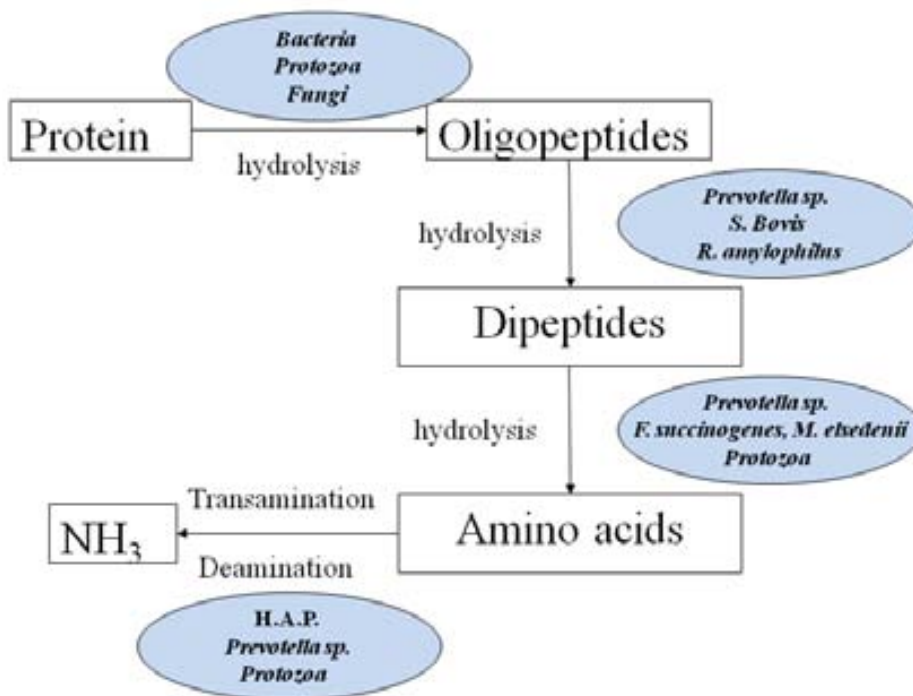
Figure 10. Proteolysis in the rumen.

Another important group of bacteria that are involved in protein degradation are the HAP, which are non-saccharolytic amino acid fermenters and rapid producers of ammonia from amino acids. The first HAP species isolated were *Clostridium aminophilum*, *Clostridium sticklandii* and *Peptostreptococcus anaerobius* (Chen and Russell, 1988, 1989; Paster et al., 1993; Russell et al., 1988, 1991). Subsequently, other HAP bacteria have been isolated from cattle or sheep in New Zealand (Attwood et al., 1998), Australia (McSweeney et al., 1999) and UK (Eschenlauer et al., 2002; Wallace et al., 2003; 2004). They are present in the rumen in low concentrations, but their high deamination rate makes them the principal deaminating ruminal bacteria (Rychlik and Russell, 2000).

The accumulation of NH_3 in the rumen at rates higher that microbes can utilize for their growth leads to substantial losses of N from the rumen (Walker et al., 2005). Bach et al. (2005) reported a strong relationship between efficiency of N utilization in the rumen (ENU-R) and NH_3 concentration in continuous culture ($Y = 43.6 - 0.469ENU$; $R^2 = 0.78$; RMSE = 4.53). Therefore, the reduction of ruminal NH_3 without affecting microbial protein synthesis, and the increase in dipeptide and amino acid outflow from the rumen might be an effective strategy to improve ENU-R and MNE (Calsamiglia et al., 2010).

3.2. Efficiency of N Utilization in the Rumen

The efficiency of rumen function is generally assessed with the efficiency of microbial protein synthesis (EMPS), which is calculated as g of bacterial N per kg of fermentable energy (Bach et al., 2005; Calsamiglia et al., 2010). However, EMPS is calculated according to available energy in the rumen and not available N. Bach et al. (2005) proposed the use of the efficiency of N utilization in the rumen, which is measured as the ratio between g of bacterial N per g of rumen available N, where available N represents RDP and endogenous N (including recycled N). Using data from in vitro continuous culture studies they reported a quadratic relationship between EMPS and ENU-R (Figure 11). The concept of ENU-R may be valid when *in vitro* data is assessed, but estimates *in vivo* are more difficult to obtain because of the need to estimate endogenous N (Calsamiglia et al., 2010).
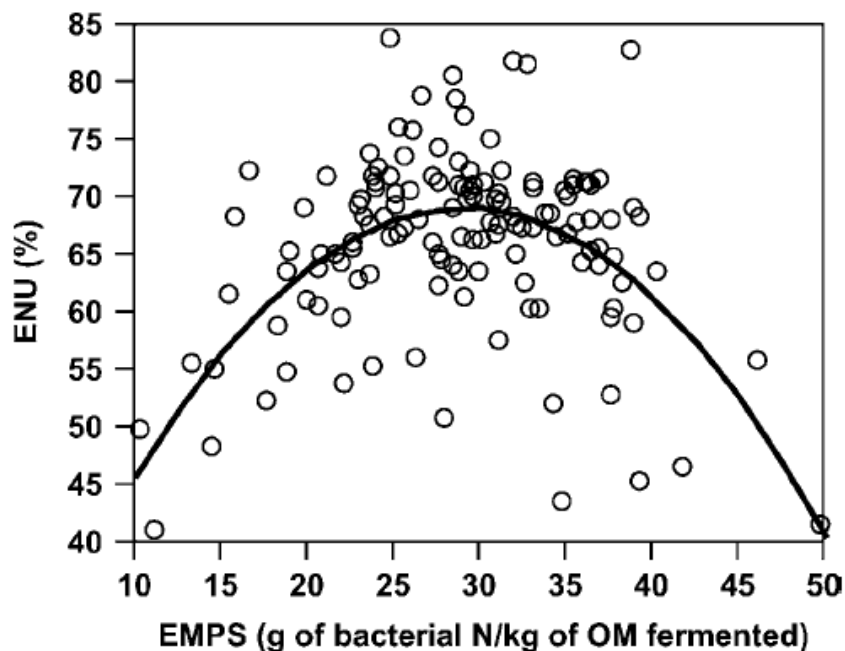
Figure 11. Relationship between efficiency of microbial protein synthesis (EMPS) and

efficiency of N utilization in the rumen (ENU, %; Bach et al., 2005).

3.3. Strategies that Target Ruminal Protein Degradation

Many strategies to reduce ruminal protein degradation have been tested and can be

categorized in two groups: those that affect feed protein and those that target rumen microbes.

The first include methods that intend to change ruminal availability of CP by decreasing RDP

and increasing RUP content of feeds. Those that target rumen microbes include different feed

additives that act as modulators of ruminal microbial population.

3.3.1. Feedstuff Processing and Manipulation

Heat processing is the most common method used to decrease RDP by denaturation of

proteins and the formation of protein-carbohydrate (Maillard reactions) and protein-protein cross

links (Satter, 1986). Different processing technologies have been developed, such roasting,

flaking, extruding, and expanding. The effectiveness of the techniques depends on the processed

feed and processing conditions (Van der Poel et al., 2005). Several *in situ* studies indicated a reduction of CP soluble fraction, degradable fraction and reduced rate of degradation of the degradable fraction (Goelema et al., 1999; Prestlokken, 1999). However, heat treatment may also reduce the digestibility of RUP. Stern et al. (2006) reported the variability of RUP and intestinal digestibility of heat processed feedstuffs, including animal by products. . Intestinal protein digestion of soybean meal treated with various techniques ranged from 57.7% to 83.8%, suggesting a considerable variation caused by processing.

Chemical treatment of feed proteins includes three categories: chemicals that induce cross links with proteins, chemicals that alter protein structure by denaturation, and chemicals that bind proteins but with little or no interaction of protein structure (Broderick et al., 1991). Protein feedstuffs, and especially soybean meal, have been treated with sodium hydroxide or formaldehyde (Santos et al., 1999), but the most common chemical treatment is formaldehyde. Formaldehyde forms revisable cross linkages with amino acids and amide groups which reduce protein degradability in the rumen (Waltz and Stern, 1989).

Recently, the use of essential oils as modifiers of protein degradation during ensiling was tested (Kung et al., 2008). However, the low dose of a commercially available mixture used (40 and 80 mg of EO / kg of fresh forage) and the selection of maize as the ensiling crop, limited the possibility of EO to affect protein degradation during ensiling.

3.3.2. Targeting Microbial Population in the Rumen

Ionophores have successfully reduced N losses and improved animal performance, but due to the increased public concern for potential transfer of into meat and milk, the EU prohibited its use in 2006 (Official Journal of the European Union, 2003). This stimulated

research in evaluating other alternatives to modulate rumen fermentation, including the use of yeasts, enzymes, organic acids, probiotics, plant extracts and recently, polyclonal antibodies.

(i) Ionophores

There are several ionophores registered as feed additives, such as laidlomycin, lasalocid, monensin and narasin. Monensin is the main ionophore used in beef and dairy production (Tedeschi et al., 2003). Even though their use is not allowed in the EU, its effects and mechanism of action will be discussed because of their effectiveness in manipulating of ruminal microbial population and their use in experiments as positive control when alternatives are tested (Busquet et al., 2005a; Castillejos et al., 2006).

The mechanism of action of ionophores consists on their attachment to the lipid bilayer of the external membrane of gram positive bacteria and protozoa forming complexes with sodium channels (Na^+). This facilitates the net exchange of intracellular K^+ for extracellular protons (H^+) and Na^+ across the membrane. The accumulation of H^+ causes a drop of intracellular pH and therefore the cell responds by expelling intracellular H^+ and Na^+ at the expense of ATP. Finally the loss of energy reduces the growth and replication of the microbe, resulting in its reduction or elimination from the rumen (Russell, 1987; Russell and Strobel, 1989).

The addition of monensin in continuous culture affected ruminal fermentation reducing the acetate to propionate ratio, without affecting total VFA production, and reduced NH_3 concentration (Busquet et al., 2005a; Castillejos et al., 2006). Similarly, in *in vitro* pure and mixed cultures of ruminal bacteria reduced NH_3 concentration, suggesting the inhibition of deamination (Russell and Martin, 1984; Chen and Russell, 1989, 1990). Further studies demonstrated that these effects were due to the sensitivity of HAP bacteria to monensin addition

(Paster et al., 1993; Rychlik et al., 2002). As referred previously, HAP bacteria are the main producers of ruminal NH_3 and therefore the main bacterial target for strategies that are seeking to improve N utilization in the rumen. *In vivo* studies verified the effects of monensin on ruminal N metabolism (Yang and Russell, 1993). Thus, the use of monensin was proposed as a strategy to reduce N excretion from ruminants (Tedeschi et al., 2003).

(ii) Essential Oils

In the last decade, extensive research has been conducted on plant extracts as an alternative to ionophores (Calsamiglia et al., 2007; Benchaar et al., 2008). Essential oils (EO) are blends of plant secondary metabolites obtained by steam or hydro distillation from the plant volatile fraction. They are complex natural mixtures which can contain about 20–60 components at quite different concentrations, although pure components have also been purified and used (Calsamiglia et al., 2007).

Due to the large number of compounds, a universal mechanism of action for all EO compounds does not exist (Benchaar et al., 2008). The antibacterial activity of EO is associated with their lipophilic properties, which allow them to interact with lipids the lipidic bilayer of bacterial membrane (Sikkema et al., 1994; Ultee et al., 1999), disrupting the cytoplasmic membrane causing an increase in membrane permeability and leakage of cytoplasmic constituents (Sikkema et al., 1994; Helander et al., 1998). Bacteria use ionic pumps to counterbalance these effects and consequently large amounts of energy are wasted slowing down bacterial growth (Griffin et al., 1999; Ultee et al., 1999; Cox et al., 2001). It has been suggested that gram-positive bacteria are more susceptible to EO than gram-negative, as gram-negative have an outer layer that limits the access of hydrophobic compounds (Burt, 2004; Chao and Young, 2000). However, the low molecular weight of some EO, such as thymol and carvacrol,

allows them to cross the cell wall by diffusion and interact with the lipid bilayer of gram-negative bacteria (Helandder et al., 1998; Griffin et al., 1999; Dorman and Deans, 2000).

Several *in vitro* studies suggested that some EO compounds may alter protein metabolism mainly through the inhibition of peptidolysis or deamination (Table 3). Early research of Borchers (1965) and Broderick and Balthrop (1979) indicated that thymol (THY) reduced NH_3 concentration in rumen fluid. Further research in *in vitro* bath culture (Cardozo et al., 2005) and in continuous culture (Castillejos et al., 2006) also reported the inhibition of deamination. In a continuous culture study, low doses (2.2 mg/l) of clove bud oil, which contains eugenol (EUG) by 85% affected N metabolism, increasing peptide N and numerically decreasing AA N concentrations, suggesting that EUG decreased the peptidolytic activity in the rumen (Busquet et al., 2005). In an *in vitro* batch culture (Busquet et al., 2006) and in continuous culture (Castillejos et al., 2006) studies, the addition of EUG reduced NH_3 concentration, suggesting the inhibition of deamination.

The effects of cinnamldehyde (CIN) on N metabolism have been inconsistent. Cardozo et al. (2004), in a continuous culture experiment, reported an accumulation of peptide N and a numerical increase of AA N by the addition of cinnamon oil (0.22 mg/l of rumen fluid), suggesting an inhibition of proteolysis and / or peptidolysis. Ferme et al. (2004), utilizing samples from the study of Cardozo et al. (2004), reported that the addition of CIN resulted in a reduction in *Prevotella* spp., major peptidolytic and deaminating bacteria, providing evidence of a mechanism of action. Busquet et al. (2006) reported the reduction of NH_3 concentration in a 24 h batch culture by the addition of CIN and cinnamon oil (3,000 mg/l), although CIN had stronger effects compared with cinnamon oil. In contrast, in a continuous culture study the addition of

CIN (31.2 and 312 mg/l) had no effect on N metabolism in the rumen (Busquet et al., 2005c), although doses tested were lower than that of Busquet et al. (2006).

Table 6. The effect of some essential oils compounds on nitrogen metabolism in the rumen as indicated by *in vitro* studies.

Essential oil	**N- metabolism**	**References**
Thymol	deamination ↓	*Borchers, 1965; Cardozo et al., 2005; Castillejos et al., 2006*
Eugenol	peptidolysis ↓	*Busquet et al., 2005c*
	deamination ↓	*Busquet et al., 2006; Castillejos et al., 2006*
Cinnamldehyde	proteolysis ↓	*Cardozo et al., 2004; Ferme et al., 2004*
	deamination ↓	*Busquet et al., 2005a; Ferme et al., 2004*
Anethol	peptidolysis ↓	*Cardozo et al., 2004*
	deamination ↓	*Cardozo et al., 2005*
Garlic oil	deamination ↓	*Cardozo et al., 2004, 2005; Ferme et al., 2004*
Capsaicin	deamination ↓	*Cardozo et al., 2005;*
Carvacrol	peptidolysis ↓	*Busquet et al., 2005c*

The effects of capsaicin (CAP) on N metabolism in the rumen in short and long term fermentations have been negligible when rumen fluid from dairy cattle fed a 60% alfalfa hay and 40% concentrate diet was used (Cardozo et al., 2004). However, Cardozo et al. (2005)

demonstrated that the effects were different in an *in vitro* system with rumen fluid from beef cattle fed a 10:90 straw:concentrate diet, and reported that at low (5.5) pH decreased NH_3 concentration, suggesting an inhibition of deamination. Busquet et al. (2005c) reported that in vitro, carvacrol (CAR; 2.2 mg/l) decreased large peptide concentrations and increased ammonia N concentrations 2 h after feeding, suggesting that CAR either inhibited proteolysis or, most likely, stimulated peptidolysis.

Garlic oil (GAR) is a mix of a large number of different molecules that are found in the plant or as the result of changes occurring during oil extraction and processing, including sulfur compounds (thiosulfinates, allyl sulfides, glutamylcysteines, allicin), enzymes, free AA, sterols, steroids, triterpenoid glycosides, flavonoids, phenols, and organoselenium compounds (Lawson, 1996). The effects of GAR and its main active components on N metabolism have been variable. Cardozo et al. (2004) reported that GAR in continuous culture reduced NH_3 and increased peptide and AA N concentrations, suggesting that deamination was inhibited. Moreover, Ferme et al. (2004), utilizing samples from Cardozo et al. (2004), reported that GAR modified the microbial population profile reducing the population of *Prevotella* spp. (mainly *P. ruminicola* and *P. bryantii*) that are involved in AA deamination. In a following study, utilizing a beef diet (10:90 forage to concentrate) at 2 pH levels (5.5 and 7.0) the addition of GAR reduced NH_3 concentration in both pH levels (Cardozo et al., 2005). However, Busquet et al. (2005a, b) reported only small and variable effects of GAR on N metabolism in the rumen. Busquet et al. (2005b) examined the effect of GAR and four of its main compounds, i.e. diallyl sulphide, diallyl disulphide, allyl mercaptan and allicin, on rumen fermentation using an *in vitro* batch and a continuous culture system. Main effects were on VFA profile, where GAR and its components

reduced acetate and increased propionate molar proportion, while effects on N metabolism were negligible.

Recently, two stable organosulfurate compounds of garlic were obtained by decomposition of alliin and allicin: propyl-propylthiosulfinate (PTS) and propyl-propylthiosulfonate (PTSO; Figure 12). Both compounds are structurally similar and only differ in the presence of one more oxygen in PTSO, which makes it more polar and less volatile. The invention is under patent consideration (patent number: US2010/0035984 A1). Their antimicrobial effects were tested on the gastrointestinal microbiota of pigs and PTSO demonstrated a strong antimicrobial activity against main microbial groups, and against *Eschericichia coli* and *Salmonella typhimurium* (Ruiz et al., 2010). However, their effects on ruminal microbial environment remain to be demonstrated.
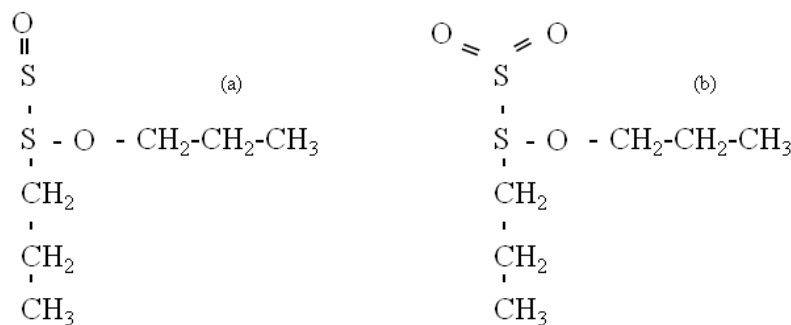
Figure 12. Chemical structure of propyl-propylthiosulfinate (PTS; a) and propyl-propylthiosulfonate (PTSO; b), two garlic derived compounds.

Several commercial mixtures of EO compounds are available in the market as feed additives. A blend of EO (BEO; Crina® ruminants; Akzo Surface Chemistry Ltd., Herfordshire, UK), has been produced and tested *in vitro* with ruminal fluid from dairy and beef cattle. The Crina® supplement contains 100–300 g/kg of phenolic compounds including cresol, resorcinol,

thymol, guaiacol and eugenol (Rossi, 1999). Several *in vitro* studies reported inhibition of deamination (McIntosh et al., 2003; Newbold et al., 2004) due to the inhibition of growth of some HAP bacteria (*i.e.*, *Clostridium sticklandii* and *Peptostreptococcus anaerobius*). However, other HAP bacteria (e.g., *Clostridium aminophilum*) were less sensitive (McIntosh et al., 2003).

Moreover, when BEO (0.72 and 2 g/day) was supplied to dairy cows no change was reported in ruminal NH_3 concentration, N retention, and N digestibility (Benchaar et al., 2006, 2007). Assuming a rumen volume of 100 litres and an outflow rate of 0.1/h for an adult dairy cow, ruminal concentration of BEO would have been 3.1 and 8.3 mg/l for each of the doses, respectively, which are much lower than the ones used in vitro (35–360 mg/l; McIntosh et al., 2003). Kung et al. (2008) reported an increase of daily DMI (1.9 kg) and milk production (2.7 kg) of cows supplemented with 1.2 g/day/cow of BEO, but with no effects on protein metabolism or protein milk content. The higher DMI of this study is in contrast with the results of Tassoul and Shaver (2009), where the same BEO was supplemented to transition cows and DMI was 1.8 kg/day lower compared with control cows. Recently, Giannenas et al. (2011) tested the same BEO in dairy ewes at 3 doses (50, 100 and 150 mg/kg of TMR) for the first five months of lactation. The addition of BEO increased overall milk production (318 l vs 235 l for BEO 150 mg/kg of TMR and control ewes, respectively) without affecting milk composition. The two highest doses reduced counts of HAP bacteria causing a tendency to decrease ruminal NH_3 concentration. Gerasi et al. (2012) compared a mixture of EO compounds (1:1:1: for EUG, CIN and CAP, respectively; 400 mg/day/animal; Pancosma SA, Geneva, Switzerland) with monensin (46.7 mg/kg of DM), supplemented to feedlot cattle fed a high concentrate diet. They reported no effect on feed intake and performance, but steers fed EO had lower ruminal NH_3 (10.78 mg/dl vs 20.05 mg/dl, for EO and monensin, respectively).

Another microencapsulated mixture of EO (RumaXol Feed, Soda Feed Ingredients, MC 98000 Monaco) made from oregano, cinnamon, thyme and orange peel oil was tested *in vitro* and *in vivo*, but N metabolism was not affected (Spanghero et al., 2008, 2009). In the *in vivo* experiment, the maximum dose of 0.96 g/d of the EO mixture was supplemented to dairy cows, with minor changes in milk composition (Spanghero et al., 2009). Santos et al. (2010) administrated 1 g/d in dairy cows of a commercial mixture of EO containing eugenol, geranyl acetate and coriander oil as major components (Agolin Ruminant, AGOLIN SA, Bière, Switzerland) and reported no differences on whole tract digestion of CP. Although *in vivo* studies were unsuccessful to detect changes from the administration of EO, the doses tested were lower than the ones suggested by the *in vitro* studies.

Among EO, eugenol, cinnamaldehyde, thymol, capsaicin, carvacrol and garlic oil seem to be more promising on altering the microbial N metabolism in the rumen towards the desired direction. The decrease of ruminal NH_3 and the increase in AA concentration in the rumen suggested that the main mechanism of action is through the inhibition of deamination.

(iii) Other Additives

Microbial enzymes are used in order to improve feed quality mainly due to their effects to remove anti-nutritional factors and toxins and increase digestibility of existing nutrients. However, they do not have a direct effect on rumen protein degradation of dairy cows (Bonneau and Laarveld, 1999). Some organic acids (e.g. aspartic, malic and fumaric acids) have been reported to induce changes in ruminal pH, methane production and/or VFA profile in a way similar to monensin. However, organic acids do not affect N metabolism in the rumen (Nisbet and Martin, 1993). Probiotics are defined as microorganisms which, when administrated to animals, may provide beneficial effects to the host by improving the environment of the

indigenous microflora. Desnoyers et al. (2009) conducted a meta-analysis of 110 published papers, with 157 experiments and 376 treatments where yeast was supplemented in ruminants. They reported that the addition of *Saccharomyces cerevisiaen* increases DMI, milk yield and tended to increase milk fat content. However, there was no effect on protein degradation. The main effect of yeast on N metabolism is a decrease in NH_3 concentration caused through the stimulation of the growth of ruminal bacteria, which in turn use more NH_3 (Nagaraja et al., 1997).

Lately, the use of tannins has been extensively studied due to their ability to reduce ruminal proteolysis. Tannins exist primarily in condensed (CT) and hydrolysable (HT) forms; HT forms can be toxic for animals because their degradation products are absorbed from the small intestine (Min et al., 2003). Studies utilizing CT extracts from plants suggested reduced proteolysis (Aerts et al., 1999; Molan et al., 2000) and inhibition of the growth of proteolytic bacteria such as *Prevotella ruminicola* and *Streptococcus bovis* (Min et al., 2005). In *in vivo* studies, feeding sainfoin silage reduced ruminal protein degradation without affecting NH_3 concentration (Theodoridou et al., 2012). However, the concentration of CT in forages is not stable and the nutritional effect of CT depends on their concentration (Fraser et al., 2000).

(iv) Passive and Active Immunization as an Alternative Strategy

Another approach proposed by scientists is the use of immunization against main proteolytic or deaminating bacteria of the rumen (Walker et al., 2005; Calsamiglia et al., 2006). Specific immunity can result from either passive or active immunization, while both approaches have been investigated in dairy production.

Active immunization results from the vaccination of an animal with a specific antigen and the production of antibodies by the animal. When the target is ruminal bacteria, the produced antibodies enter in the rumen through saliva (Horacek et al., 1977; Walker et al., 2005). This strategy has been tested in the prevention of acidosis in cattle. Ruminal acidosis is a common digestive disorder in cattle fed high concentrate diets, where rumen pH drops below 6.0. Calsamiglia et al. (2008, 2012) demonstrated that effects of ruminal acidosis are due to a combination of rumen pH and type of diet, and proposed the use of the term "high concentrate syndrome" instead of ruminal acidosis. Strategies to prevent the syndrome include proper diet balancing and feeding management, control of ruminal pH, and control of the fermentation process (Calsamiglia et al., 2012). The main bacterium involved in the fermentation process is *Streptococcus bovis*, which is the main lactic acid producer in the rumen. Therefore, strategies that neutralize *S. bovis* might be a way to prevent acidosis.

Shu et al. (1999) investigated the efficacy of controlling ruminal acidosis through the vaccination of 1-year-old Hereford steers against lactic acid-producing bacteria, *Streptococcus bovis* and *Lactobacillus*, obtaining positive results. Immunized heifers had higher level of antibodies against against *S. bovis* and *Lactobacillus* in serum and saliva, and reduced counts of *S. bovis* and *Lactobacillus* in the rumen resulting in lower lactate concentration. Similarly, Gill et al. (2000) reported that sheep fed forage based diets immunized with live or killed *Streptococcus bovis* Sb-5 vaccine had higher feed intake, higher rumen pH, lower L-lactate concentrations, and less severe diarrhoea scores than non-vaccinated control sheep when they were challenged with a sudden switch to a grain-based diet. The induction of immune responses as a way to control rumen function has also been used to control methane emissions. Wright et al. (2004) immunized sheep with an anti-methanogen vaccine produced from an antigen specific ruminal methanogens.

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Methane emissions were reduced by 7.7% despite targeting probably less than 20% of the methanogen population in the rumen. Marini et al. (2003) immunized sheep against jackbean urease in order to decrease urease activity in the rumen and therefore to retain more urea-N for rumen microbial protein. Even though serum antibodies inhibited the jackbean urease, bacterial urease was not inhibited. Thus, urea kinetics of the animal were not affected.

An alternative approach is to administrate orally specific antibodies against bacteria. This approach has been tested in humans and animals, and it is becoming a rapidly growing therapeutic approach for a number of microbial infections (Keller and Stiehm, 2000; Mine and Kovacs-Nolan 2002; Bebbington and Yarraton, 2008).

This strategy was also used to prevent acidosis in cattle. DiLorenzo et al. (2006) fed preparations of polyclonal antibodies (PAb) against *Streptococcus bovis* or *Fusobacterium necrophorum* to crossbred steers. Steers fed PAb against *S. bovis* reduced *S. bovis* counts by 80% and increased ruminal pH compared with control. Feeding steers PAb against *F. necrophorum* did not affect ruminal pH, even though bacteria population was reduced. However, in a following study feeding PAb against *S. bovis* or *F. necrophorum* or a combination of them, increased mean daily ruminal pH compared with control (DiLorenzo et al., 2008). Similarly, Blanch et al. (2009) reported higher pH after 6, 8 and 9 days of acidosis induction, by increasing concentrate feeding by 2.5 kg/animal/day up to 12.5 kg/animal/day, of heifers fed PAbs preparation against *Streptococcus bovis*, *Fusobacterium necrophorum*, *Clostridium sticklandii*, *Clostridium aminophilum*, *Peptostreptococcus anaerobius* and *Escherichia coli* O157:H7. Marino et al. (2011), utilizing the same preparation of PAb, reported no effect on ruminal NH_3 concentration, but a significant increase of ruminal pH 4 h post feeding. Moreover, feedlot performance of yearling bulls supplied with PAbs (10 ml/day) was similar to those supplied with

monensin (300 mg/day; Pacheco et al., 2012). Up to today, there are no studies on the effects of oral administration of antibodies against proteolytic and deaminating ruminal bacteria to reduce ammonia losses in the rumen, but work on ruminal acidosis demonstrated the potential of PAbs to alter rumen microbial metabolism.

3.4. Conclusions

The N cycle in a dairy farm closes with the volatilization of NH_3 in manure, but the starting point is the degradation of protein in the rumen. The extent of ruminal protein degradation, the inefficient use of ammonia, AA and dipeptides by bacteria at the rate that they are produced in the rumen, and the presence of HAP bacteria that utilize AA as an energy source, result in an accumulation of ruminal NH_3, which results in substantial N losses in the rumen. Protein degradation and deamination can be controlled at the feed or microbial level. Monensin successfully suppresses some HAP bacteria, reducing NH_3 accumulation in the rumen, but its use is prohibited in the EU. The major alternatives to ionophores to control N metabolism in the rumen are EO compounds that have the potential to modulate ruminal degradation and deamination. The use of active and passive immunization to control rumen microbial metabolism is also being investigated as an alternative. However, most of the studies have been focused on ruminal acidosis and not on ruminal protein degradation and deamination.

4. Objectives

The general objective of this thesis was to use innovated and novel technologies to give answers and suggest solutions that may reduce the nitrogen excretion from ruminants in the environment.

Therefore, we planned four different studies with the specific objectives:

First study (chapter 2):

- To evaluate the potential of the near infrared spectroscopy technique for predicting degradation parameters and effective degradation of original feed samples utilizing a wide variety of feedstuffs commonly used in ruminant nutrition.

Second study (chapter 3):

- To evaluate the effects of the addition of essential oil compounds on ryegrass silage chemical composition and protein degradation.

Third study (chapter 4):

- To produce and test *in vitro* polyclonal antibodies against *Prevotella ruminicola* and hyper ammonia producing bacteria, to reduce ruminal protein degradation and deamination.

Fourth study (chapter 5):

- To investigate the effects of propyl-propylthiosulphonate, a garlic oil compound, addition on ruminal microbial fermentation in a dual flow continuous culture system.

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Chapter 2

Prediction of ruminal degradability parameters

by near infrared reflectance spectroscopy

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Abstract

A database of degradation parameters of CP, DM and NDF of 809 different feedstuffs frequently used in ruminant nutrition was created. Feedstuffs were grouped as forages (FF; n=256) and non-forages (NF; n=553). Degradability was described in terms of immediately rumen soluble fraction (a), the degradable but not soluble faction (b) and its rate of degradation (c). Overall effective degradability (ED) of DM and CP (5%/h passage rate), and NDF (2%/h passage rate) were calculated according to the equation of Ørskov and McDonald (1979). All samples were scanned from 1,100 to 2,500 nm using a NIRSystems 5000 scanning monochromator (FOSS, Hoganas, Sweden). Reflectance was recorded in 2 nm steps as log 1/Reflectance. Samples were scanned twice in duplicate using ring cup cells and mean spectrum was calculated for each sample. A WinISI III (v. 1.6) software was employed for spectra data analysis and development of chemometric models. Calibrations were developed by the modified partial least squares (MPLS) regression technique for all samples (ALL), FF and NF. The precision of the equations obtained was confirmed by an external validation set of 20% of total samples. The ED, a and b fractions of DM and CP were well predicted and improved by group separation. The rate of degradation of DM and CP were not satisfactorily predicted when all samples were included ($r^2 < 0.7$). However separating samples improved the prediction of DM ($r^2 > 0.7$) and of CP for FF. For NDF, the number of feedstuffs was lower and the majority was grouped in FF. Equations obtained satisfactorily predicted ED and fraction b of NDF and group separation further improved predictions. When all feedstuffs were included the rate of degradation was not well predicted, but when samples were grouped prediction for FF was acceptable. In conclusion, group separation into FF and NF improved NIRS equations especially for

prediction of degradation rate. Current equations are acceptable and allow to incorporate NIRS as a field tool for feed evaluation models.

Keywords: NIRS, *in situ*, effective degradation, degradation parameters.

1. Introduction

Feed evaluation for ruminants is changing from static to dynamic models. Dynamics of nutrient digestion in the reticulo-rumen is one major determinant of feedstuffs utilization by ruminants. Most current feed protein evaluation systems are based on the kinetics of protein degradation [e.g., Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992); Molly (Baldwin, 1995); Dairy NRC (NRC, 2001)]. The *in situ* method is a well-established method for determination of degradation kinetic parameters and the effective degradation (ED) of nutrients (Huhtanen et al., 2006). However, it is a costly and tedious method, and requires cannulated animals, making it very resource demanding and limiting its use for feed evaluation in practice.

Near infrared reflectance spectroscopy (NIRS) technique can predict chemical composition and several parameters of nutritional interest for different feeds and forages (Andres et al., 2005b). Moreover, NIRS offers a number of advantages over traditional chemical methods: it is a physical, non-destructive method, requiring minimal or no sample preparation, no reagents and produces no wastes. It is widely used to predict composition of feedstuffs with accuracy, and has been officially recognized for the analysis of dry matter (DM), crude protein (CP) and acid detergent fibre (ADF) by AOAC (AOAC, 2000). Recently, NIRS has been used as a tool to predict degradation parameters mainly of forages (Todorov et al., 1994; Andres et al., 2005a; Ohlsson et al.,

2007). However, most of these studies are feedstuff specific, predicting degradation parameters of a particular feedstuff or a specific category of feedstuffs, mainly forages.

Our objective was to evaluate the potential of the NIRS technique for predicting degradation parameters and effective degradation of original feed samples utilizing a wide variety of feedstuffs commonly used in ruminant nutrition.

2. Materials and Methods

2.1. Database

A database of chemical composition and degradation parameters of CP, DM and neutral detergent fibre (NDF) of 809 different feedstuffs frequently used in ruminant nutrition was created. Among them, 100 samples were used for NDF ruminal degradability analysis, and the rest for DM and / or CP ruminal degradability analysis.

2.1.1. Feedstuffs

Feedstuffs were grouped according to their use in ruminant nutrition in two main groups: forages (FF; n=256), and no forages (NF; n=553). Table 1 shows the exact grouping of different feedstuffs. The FF group included hays, straws, whole crops, pellets of forages and silages. The NF group included concentrates (n=234) and byproducts (n=319). Concentrates included different mixtures of concentrates, total mixed rations, seeds and grains. Byproducts included byproducts of distillery, oil production and of animal origin.

2.1.2. In Situ Analyses

In situ analyses were performed according to the Norfor procedure (Åkerlind et al., 2011). Feed samples were incubated in the rumen of three dry Holstein Frisian cows

in 11x8.5 cm (10x7.5 effective size) Dacron bags with 38 µm pore size [PES material 38/31 with 31% open bag area, Saatifil PES 38/31 (Saatitech S.p.A., 22070 Veniano, Como, Italy)]. Cows used for incubations were fed at maintenance level a ration containing (kg/d): 2.0 spring barley straw, 4.0 artificially dried grass hay, 0.15 vitamin-mix, and 2.8 concentrate (concentrate composition g/kg: 30 rapeseed meal, 100 soybean meal, 400 barley, 400 oats, 30 beet molasses and 40 mineral-mixture). Ration chemical composition (g/kg DM) was 139 crude protein (CP), 465 NDF and 137 starch. Feed samples were ground to pass a 1.5 mm screen and 1 g was weighed into each bag. Bags were mounted with plastic strips on rubber stoppers. The rubber stoppers (with hooks) were mounted on a plastic tube fitted with rings. The plastic tube had a sink (weight 200 g) in one end, and strings with a length of 40 cm in both ends, to ensure its mobility in relation to the rumen cannula. Bags were incubated in the rumen for 0, 2, 4, 8, 16, 24, 48, 96 and 168 h. Maximum incubation time was 48 h for protein degradation of concentrates and 96 h for forages. Incubations for indigestible NDF determination (iNDF) were performed in 12 µm pore size bags for 288 h. After retrieval from the rumen, bags were rinsed in cold tap water and washed in a washing machine with temperate water (25°C) using 2×22 L. Before transferring the sample to a filter paper, roughage's were treated in a stomacher to remove adhering microbes. The residue was transferred to a plastic bag with 60 ml demineralised water, and treated for 5 min in the stomacher before returned to the dacron bag and washed thoroughly with demineralised water. Residues in bags for dry matter (DM) and protein were transferred to tarred N free filter paper, dried at 103°C to determine the DM residue, and then analyzed for N content using the Kjeldahl procedure. Residues in bags for NDF were transferred directly to porosity 2 filter crucibles and ash free NDF residue was determined in a

Fibertec system using a heat stable amylase and sodium sulfite (aNDFom) (FOSS, Hillerød, Denmark) according to Mertens (2002).

Degradation parameters were fitted using PROC NLIN in SAS (version 9.2 SAS Institute, Inc., Cary, NC) according to the model of Ørskov and McDonald (1979). Effective degradation of DM, CP and NDF was calculated according to the following equation:

$$ED = a + b \ (c/(c + k)),$$

Where a= immediately rumen soluble fraction, b= degradable but not soluble faction, c= rate of degradation, k = fractional outflow rate from the rumen (0.05 h^{-1} for CP and DM; 0.02 h^{-1} for NDF). .

2.2. NIRS Analysis

All samples were scanned from 1,100 to 2,500 nm wavelength using a NIRSystems 5000 scanning monochromator (FOSS, Hilleröd, Denmark). Reflectance was recorded in 2 nm steps, which gave 692 data points for each sample, as log (1/R), where R represented reflected energy. Samples were scanned twice in duplicate using ring cup cells and the mean spectrum was calculated for each sample.

A WinISI III (v. 1.6) software program was employed for spectra data analysis and development of chemometric models. Prior to calibration, log 1/R spectra were corrected for the effects of scatter using the multiple scatter correction (MSC), standard normal variate (SNV) and/or detrend (D) transformed into first, second or third derivative using different gap size (nm) and smoothing interval. Different mathematical treatments (derivative number, subtraction gap and smoothing intervals) were tested. Modified partial least squares (MPLS) regression method was used for calibration development using different pre-treatments of spectral data to remove or reduce

disturbing effects not related to the chemical absorption of light, and cross-validation was applied to optimize calibration models and detect outliers. A total of twenty spectral models for each predicted parameter were developed, resulting from the evaluation of four scatter corrections techniques (MSC; SNV; D; SNV-D) and five math treatments (1,4,4,1; 1,10,10,1; 2,4,4,1; 3,4,4,1; 3,10,10,1; for derivative number, the gap over which the derivative is calculated, first smoothing and number of data points in the second smoothing second smooth, respectively).

Additional to cross-validation, an external validation was performed using a set of approximately 20% of the total population samples. Samples in the validation set were selected randomly from the total matrix and were balanced according to the previously mentioned grouping of feedstuffs to represent a wide range of composition. Samples in the validation set were not used in the calibration set or vice versa. The optimum calibration of the model was selected on the basis of minimum standard error of calibration (SEC) and standard error of prediction (SEP), and of highest coefficient of determination of calibration (R^2) and validation (r^2). The ratio of performance to deviation (RPD; >3) described as the ratio of standard deviation for the validation samples to the SEP, and the range error ratio (RER; >10) described as the ratio of the range in the reference data (validation set) to the SEP (Williams and Sobering, 1996), were used to evaluate the performance of calibrations. Equations were obtained using all feedstuffs (ALL), and within two major groups (FF and NF).

3. Results

3.1. Calibration and Validation Matrixes

The first attempt to obtain equations for the prediction of degradation parameters of DM, CP and NDF was performed by including all available feedstuffs, resulting in a wide range of values was obtained for each parameter. Table 2 presents the population statistics of the validation and calibration dataset, including the mean, minimum and maximum values of each parameter, the standard deviation and the total number of samples used. All parameters were well represented in both calibration and validation matrixes. For the DM calibration set the database included samples where the degradation at time 0 was 80.2% of total DM content and others where after 48 hours the degradation was only 16.5%, representing the existing variability in samples. The CP concentration of feedstuffs included in the calibration matrix ranged from 5.6% to 94.1% DM basis with an effective degradation ranging from 0.12 to 0.95. In the validation matrix, CP ranged from 7.8 to 90.0 % DM basis, while effective degradation ranged from 0.13 to 0.93. The NDF composition of samples ranged from 17.5 to 83.1 % DM basis in the calibration matrix and from 23.3 to 81.5 % DM basis in the validation matrix. Table 3 shows population statistics of FF and NF. Grouping samples in two major groups (FF and NF) reduced variation compared with ALL. However, a wide variation within parameters was still present.

3.2. Degradation Parameters of DM

Table 4 presents calibration and validation statistics of the degradation parameters of DM including ALL, FF and NF groups. The effective degradation, fraction a and fraction b were well predicted by NIRS equations and were improved for fraction a and b by separating samples into groups. Group separation did not improve the prediction of effective degradation for NF but improved it for FF. The asymptote of

the degradability equation and fraction c were not satisfactorily predicted when all samples were included (r^2<0.7). However separating samples in two main groups improved prediction in both cases: r_c^2>0.7 for cross validation for both groups and r^2 of 0.753 and 0.694 for the external calibration matrix of fraction c for FF and NF, respectively. The mathematical treatment that fitted best for DM was the 3,4,4,1, except for the asymptote where the 3,10,10,1 performed better. However, there was no correlation among the scatter correction that gave the best performance and groups or parameters of degradation.

3.3. Degradation Parameters of CP

Table 5 summarizes statistics of calibration and validation of the degradation parameters of CP. Similarly to DM, NIRS predicted satisfactorily effective degradation, fraction a and fraction b, but not the asymptote and the rate of degradation. Separating in groups improved the prediction of fraction a, b and effective degradation. Moreover, group separation improved the prediction of fraction c, especially in FF. The coefficient of calibration for prediction of fraction c of all samples was low when all samples were included (R^2=0.42), satisfactory for FF (R^2=0.82) and acceptable for NF (R^2=0.69). The concentration of CP was best predicted by using MSC as a scatter correction technique and 2,4,4,1 as math treatment, while most of the parameters were best predicted by 3,4,4,1. Like in DM, there was not a unique scatter correction that gave the best predictions of degradation parameters.

3.4. Degradation Parameters of NDF

Table 6 summarizes calibration and validation statistics of the degradation parameters of NDF. In this case, the number of available feedstuffs were lower (100 in total; Table 2), and the need for an external validation matrix reduced it further (N of calibration=84 and n of validation=16). Moreover, the majority of feedstuffs were

grouped as FF. Thus, separation in groups included only FF. As expected, the prediction of NDF content of feedstuffs was predicted satisfactorily when all samples were included. Degradation parameters of NDF do not include fraction a because the soluble fraction of NDF at 0 hours it is considered zero. As a consequence, fraction b is equal to the asymptote because the asymptote is equal to the sum of fraction a and b. Equations obtained predicted satisfactorily effective degradation and fraction b, and group separation improved predictions. When all feedstuffs were included the rate of degradation was not well predicted. However, when only FF samples were considered, the prediction of degradation rate was acceptable. Unlike DM and CP, there was variation on the best math treatment, although the 3,10,10,1 performed better in most cases.

4. Discussion

The main strength of the current work is the number and diversity of available samples incorporated into the database and its analysis with twenty different spectral models resulting from the evaluation of four scatter corrections techniques and five mathematical treatments. Results suggested the use of a different spectral model for each parameter instead of the use of a unique model for all parameters. This might explain the very poor predictions of degradation parameters obtained when only one spectral model was used (Lovett et al., 2004).

4.1. Fraction a, b and Effective Degradation

Global equations (ALL) provide accurate estimations of effective degradation and parameters a and b of DM, CP and NDF, while the prediction was improved in most cases by group separation. Other studies have shown that degradation parameters

of DM can be predicted better than those of CP and NDF (Andres et al., 2005a; Ohlsson et al., 2007). However, this was not the case in the current study with the exception of NDF degradation parameters that were indeed more difficult to predict. Some parameters where better predicted for DM and others for CP. As a secondary procedure, NIRS is not independent of the disadvantages arising from the reference method used for calibration. The analysis of NDF is more complex than CP or DM analysis. Thus, poorer prediction for NDF might be due to the reference method.

Group separation improved predictions, especially in FF, but also in some parameters of NF. Todorov et al. (1994) utilizing 34 forages reported lower values of R^2 and SEC for the prediction of fractions a, b and effective degradation of DM and CP than those of FF. Similarly, Mathison et al. (1999) using only barley straw reported lower values, compared with the current study, for calibration and validation statistics of degradation parameters of DM. Silages have better predictions of DM and CP degradation parameters than other forage sources (De la Roza et al., 1998). However, when silages are incorporated to forage sources predictions are improved (Hsu et al., 1998). Silages have a different degradation pattern than forages. Fraction a of CP is higher in silages (0.75 vs. 0.43 for silages vs. forages, respectively), making the degradable fraction (b) lower in silages than forages (0.18 vs. 0.51 for silages vs. forages, respectively; data not shown). Even though differences are not that strong for DM, still remain statistically significant. Thus, including silages in FF increased the range of values of degradation parameters improving overall prediction by NIRS. Most of the work on NIRS utilization for prediction of degradation parameters is focused on forages and not on concentrates or byproducts. De Boever et al. (2003) utilized NIRS in order to predict degradation parameters of different supplements used in dairy and beef

cattle. Unfortunately, no information on equations or other statistical values for calibration and validation were mentioned, making any comparison impossible.

4.2 Asymptote of Degradation

Equations obtained were not able to predict the asymptote of degradation in most cases. In one hand the weakest prediction was the asymptote of CP degradation. By using all samples, the coefficient of determination for calibration was as low as 0.476, while all statistical values used to assess the validity of prediction were below acceptable limits. Even though group separation improved R^2 in FF, it did not in NF, where the coefficient of determination for calibration was similar to ALL. On the other hand, the asymptote of NDF degradation was predicted satisfactorily in ALL and FF. Many concentrates and by-products had a complete CP degradation providing a value equal to 1.0 for the asymptote. In contrast, the asymptote of NDF degradation is rarely equal to 1. This is shown in Figure 1: Figure 1a shows the scatter plot of predicted and actual values of the asymptote of CP for ALL, where a particular group of feedstuffs creates a horizontal line because their actual value is 1.0. In contrast, Figure 1b shows the scatter plot of predicted and actual values of the asymptote of NDF for FF, where there is hardly any sample with value 1.0. Thus the calibration of different physical material with similar values is almost impossible. However, for practical reasons, the prediction of the asymptote is not of high importance because in any dynamic model the asymptote can be calculated from fractions a and b, which were well predicted by current equations.

4.3. Rate of Degradation

The prediction of the rate of degradation is more difficult. Most studies demonstrated the inability of NIRS to predict the rate of degradation. Hsu et al. (1998) reported an R^2 of 0.49 for the prediction of fraction c of DM utilizing barley silage and

straw as forage samples. Relatively poor relationship between NIRS spectra and the *in situ* rate of degradation of DM (R^2=0.57) and CP (R^2=0.51) of different forages (n=62) was reported by Andres et al. (2005a). Moreover, Ohlsson et al. (2007) reported low values of coefficients of determination for the rate of degradation of NDF (R^2=0.60, r_c^2=0.34). Nordheim et al. (2007) utilizing a large number of forage samples (n=382) also reported similar results concerning the prediction of rate of degradation (R^2=0.66, r_c^2=0.63). The higher coefficient of cross validation obtained by Nordheim et al. (2007) compared with other studies in the literature should be attributed to the higher number of samples used.

In the current study, the prediction of cDM of ALL was similar to that of other studies. However the separation into groups improved the prediction and were satisfactorily predicted for both FF and NF (R^2>0.75, r^2≥0.70, REP>10). The prediction of cCP was below acceptable limits in ALL, but it was satisfactorily predicted in FF. Similarly, cNDF prediction was improved in FF compared with ALL. However, it should be noted that most of the samples used belonged to FF group. Results suggest an improvement in the prediction of degradation rate of DM, CP and NDF by group separation and by utilizing a large calibration and validation set.

As discussed by Herrero et al. (1997), the difficulty of NIRS to predict such dynamic parameters as the degradation rate may be related to the non-linear and one-compartment nature of the models used in parameterization of the degradation rate. From a practical point of view, accuracy of degradation rate prediction depends on its impact on the calculation of effective degradation. Degradation rate have higher coefficient of variation compared with other degradation parameters (Hackmann et al., 2010). However, estimated ruminal availabilities are quite comparable despite the variation on degradation rate even when different mathematical models are used (Nocek

and English, 1986). Calsamiglia et al. (2000) reported that small errors in the estimation of rates of degradation may have a great impact on estimates of degradability of feedstuffs with of low degradation rate, while may have a smaller impact of feedstuffs with a higher degradation rate. Therefore the prediction of the rate of degradation is more important for DM and CP than NDF. This may explain the good predictions of ED in spite of less accurate prediction of the rate of degradation.

Many current feed evaluation systems [e.g., Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992); Molly (Baldwin, 1995); Dairy NRC (NRC, 2001); NorFor (Volden, 2011)] use simple averages of degradation parameters to estimate ruminal degradability of CP. However, a considerable variability exists in these mean values for forages and other feeds (von Keyserlingk et al., 1996; Hvelplund and Weisbjerg, 2000). Incorporating current equations and NIRS technology in feed evaluation models may improve the ability of these models to predict production performance.

5. Conclusions

The current work incorporated a large number of feeds varying from animal based concentrates to vegetable concentrates, and from silages to straws. Results indicate the potentials of NIRS technology to predict solubilities, effective and potential degradabilities either utilizing universal equations or by separating feeds into groups. Group separation of samples improved predictions in most cases, particularly those of the rates of degradation. Current equations are acceptable and allow the incorporation of NIRS as a field tool into dynamic feed evaluation models.

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Table 1. Feedstuffs used in the database.

Forages (FF)	*n*	No Forages (NF)	*n*		*n*
Forages:	*205*	*Byproducts*	*319*	*Concentrates*	*234*
Grass pellets	6	Amyfeed	1	Mix concentrate	123
Festulolium	1	Brewers grains	3	Barley grain	29
Galega	4	Citrus pulp	2	Grain mix	2
Grass	20	Coconut cake	1	Lupin grain	2
Grass clover	36	Corn distillers	28	Maize grain	6
Lucerne	17	Cottonseed byproduct	13	Oat grain	4
Red clover	12	Dry sugar-beet pulp	13	Rye grain	8
White clover	8	Elipe cake	1	Wheat grain	12
Grass hay	7	Feathermeal	1	Total mixed ratio	19
Barley straw	2	Fishmeal	3	Field beans seeds	1
Peas straw	1	Grain distillers	1	Peas seed	11
Red fescue straw	1	Guarmeal	7	Rapeseed seed	2
Ryegrass straw	2	Hair meal	10	Soybeans seed	10
Tropical forages	9	Maize gluten meal	14	Triticale grain	5
Barley whole crop	14	Malt dust	1		
Beans whole crop	4	Malt sprouts	4		
Lupin whole crop	12	Palm kernel cake	1		
Maize whole crop	34	Potato protein	2		
Peas whole crop	5	Rapeseed byproduct	107		
Wheat whole crop	10	Simsim cake	1		
		Soybean hulls	16		
		Soybean prod	39		
Silages:	*51*	Soypass	6		
Barley whole crop	4	Sunflower byproduct	24		
Grass clover	17	Treated soybean meal	2		
Maize	8	Wheat bran	2		
Maize pulp mix	2	Wheat gluten feed	4		
Peas whole crop	4	Wheat-barley distillers	1		
Ryegrass	7	Wheat distillers	5		
Winter wheat	8	Fodder beets roots	6		
Pea lucerne	1				
Total FF	**256**	**Total NF**			**553**

Table 2. Population statistics of calibration and validation matrixes of all samples together (values expressed on DM basis).

		Calibration set[1]					Validation set[1]			
		N	min	max	mean	sd	n	min	max	mean	sd
DM[2]	a	554	0.01	0.81	0.39	0.138	111	0.01	0.72	0.37	0.128
	b	554	0.06	1.00	0.51	0.152	111	0.13	0.99	0.54	0.147
	c	554	0.001	0.378	0.072	0.0550	111	0.023	0.297	0.069	0.0454
	ED	554	0.12	0.93	0.66	0.123	111	0.15	0.90	0.66	0.107
	asym	554	0.16	1.00	0.90	0.107	111	0.21	1.00	0.91	0.090
CP[2]	% CP	572	5.6	94.1	27.2	15.55	115	7.8	90.0	27.5	14.63
	a	569	0.02	0.92	0.39	0.201	113	0.01	0.90	0.39	0.196
	b	569	0.04	0.98	0.55	0.218	113	0.05	0.99	0.57	0.212
	c	568	0.004	0.372	0.073	0.0493	113	0.011	0.303	0.068	0.0425
	ED	569	0.12	0.95	0.69	0.151	113	0.13	0.93	0.69	0.160
	asym	569	0.21	1.00	0.95	0.099	113	0.19	1.00	0.96	0.088
NDF[2]	% NDF	84	17.5	83.1	41.1	12.91	16	23.3	81.5	42.7	12.79
	b	84	0.43	1.00	0.76	0.145	16	0.43	1.00	0.78	0.173
	c	84	0.009	0.417	0.055	0.0481	16	0.015	0.128	0.050	0.0317
	ED	84	0.23	0.78	0.51	0.138	16	0.28	0.82	0.52	0.162

[1]N: number of samples for calibration; n: number of samples for validation; min: minimum value of the data set; max: maximum value of the data set; mean: the mean of the data set; sd: standard deviation

[2] a: soluble fraction of DM and CP; b: degradable fraction of DM. CP and NDF; c: rate of degradation of DM. CP and NDF; ED: effective degradability of DM. CP and NDF; asym: asymptote of degradation (a+b)

Table 3. Population statistics of calibration and validation matrixes of groups forages (FF) and no forages (NF; values expressed on DM basis).

		Calibration set[1]					Validation set[1]			
		N	min	max	mean	sd	n	min	max	mean	sd
Forages (FF)										
DM[2]	a	111	0.12	0.62	0.41	0.100	23	0.28	0.72	0.45	0.117
	b	111	0.16	0.69	0.47	0.110	23	0.14	0.60	0.43	0.127
	c	111	0.01	0.15	0.06	0.027	23	0.02	0.15	0.07	0.036
	ED	111	0.33	0.82	0.65	0.098	23	0.50	0.83	0.68	0.092
	asym	111	0.59	0.98	0.87	0.075	23	0.70	0.99	0.88	0.079
CP[2]	% CP	112	6.6	30.6	17.0	5.41	22	9.2	27.6	17.0	5.04
	a	112	0.16	0.92	0.55	0.206	22	0.23	0.90	0.58	0.216
	b	112	0.04	0.74	0.40	0.217	22	0.05	0.76	0.36	0.221
	c	111	0.02	0.37	0.08	0.056	22	0.03	0.17	0.08	0.036
	ED	112	0.46	0.95	0.79	0.096	22	0.60	0.93	0.80	0.089
	asym	112	0.79	1.00	0.94	0.039	22	0.88	0.99	0.94	0.033
NDF[2]	% NDF	67	22.9	83.1	41.9	12.25	13	23.3	81.5	44.0	13.66
	b	67	0.43	1.00	0.75	0.136	13	0.43	0.94	0.76	0.152
	c	67	0.01	0.12	0.05	0.027	13	0.01	0.13	0.04	0.028
	ED	67	0.23	0.78	0.50	0.141	13	0.28	0.79	0.50	0.152
No Forages (NF)										
DM[2]	a	427	0.00	0.81	0.39	0.140	82	0.01	0.67	0.35	0.128
	b	427	0.13	1.00	0.53	0.149	82	0.26	0.99	0.56	0.148
	c	427	0.02	0.38	0.08	0.059	82	0.02	0.30	0.07	0.050
	ED	427	0.41	0.93	0.68	0.101	82	0.46	0.90	0.65	0.101
	asym	427	0.74	1.00	0.91	0.057	82	0.69	1.00	0.92	0.064
CP[2]	% DM	427	0.02	0.82	0.36	0.176	88	0.01	0.80	0.36	0.167
	a	427	0.15	0.98	0.61	0.195	88	0.20	0.99	0.62	0.180
	b	427	0.01	0.37	0.07	0.047	88	0.01	0.30	0.07	0.044
	c	427	0.26	0.94	0.07	0.134	88	0.27	0.93	0.67	0.150
	ED	427	0.52	1.00	0.97	0.060	88	0.59	1.00	0.97	0.053
	asym	427	0.02	0.82	0.36	0.176	88	0.01	0.80	0.36	0.167

[1]N: number of samples for calibration; n: number of samples for validation; min: minimum value of the data set; max: maximum value of the data set; mean: the mean of the data set; sd: standard deviation

[2] a: soluble fraction of DM and CP; b: degradable fraction of DM. CP and NDF; c: rate of degradation of DM. CP and NDF; ED: effective degradability of DM. CP and NDF; asym: asymptote of degradation (a+b)

Table 4. Calibration and validation statistics for determination of dry matter (DM) degradability parameters by near-infrared analysis.

Parameter[4]	Group[5]	Scatter correction[6]	Calibration[1]			Cross validation[2]			Validation[3]			
			N	R^2	SEC	r_c^2	SECV	n	r^2	SEP	RPD	REP
aDM	ALL	D	3.4.4.1	554	0.813	0.056	0.779	0.061	111	0.784	0.053	2.42	13.4
	FF	MSC	3.4.4.1	111	0.957	0.021	0.899	0.031	23	0.658	0.045	2.61	9.87
	NF	D	3.4.4.1	427	0.907	0.041	0.867	0.049	88	0.880	0.048	2.67	13.66
bDM	ALL	D	3.4.4.1	554	0.811	0.059	0.764	0.066	111	0.766	0.062	2.42	13.40
	FF	MSC	3.4.4.1	111	0.956	0.023	0.911	0.032	23	0.690	0.048	2.64	9.56
	NF	MSC	3.4.4.1	427	0.918	0.042	0.862	0.053	88	0.848	0.058	2.55	12.56
cDM	ALL	MSC	3.4.41	554	0.654	0.016	0.651	0.018	111	0.647	0.015	3.03	18.30
	FF	MSC	3.4.4.1	111	0.846	0.009	0.747	0.012	23	0.753	0.012	2.98	10.82
	NF	SNV-D	3.4.4.1	427	0.772	0.015	0.727	0.018	88	0.694	0.015	3.36	18.34
edDM	ALL	MSC	3.4.4.1	554	0.916	0.035	0.890	0.040	111	0.873	0.037	2.89	20.38
	FF	D	3.4.4.1	111	0.932	0.026	0.860	0.037	23	0.803	0.037	2.49	8.89
	NF	SNV-D	3.4.4.1	427	0.893	0.032	0.862	0.036	88	0.825	0.040	2.51	11.06
asymDM	ALL	MSC	3.4.4.1	554	0.748	0.031	0.745	0.039	111	0.632	0.032	2.82	24.55
	FF	SNV-D	3.10.10.1	111	0.932	0.017	0.878	0.021	23	0.803	0.025	3.16	11.68
	NF	MSC	3.10.10.1	427	0.811	0.023	0.778	0.024	88	0.709	0.028	2.28	11.00

[1] N: number of samples for calibration; R^2: coefficient of determination for calibration; SEC: Standard error of calibration

[2] r_c^2: coefficient of determination for cross validation; SECV: Standard error of cross validation

[3] n: number of samples for validation; r^2: coefficient of determination for external validation; SEP: Standard error of validation; RPD: Ratio of performance to deviation (= SD/SEP); RER: Range error ratio (=Range/SEP)

[4] aDM: soluble fraction of DM; bDM: degradable fraction of DM; cDM: rate of DM degradation; edDM: effective degradation of DM; asymDM: asymptote of degradation (a+b)

[5] ALL: all samples; FF: group of forages; NF: group of no forages

[6] MSC: multiple scatter correction; SNV: standard normal variate; D: detrend ; Math treatment: derivative number. subtraction gap. smooth. second smooth.

Table 5. Calibration and validation statistics for determination of crude protein (CP) degradability parameters by near-infrared analysis.

Parameter[4]	Group[5]	Scatter correction[6]		N	R^2	SEC	r_c^2	SECV	n	r^2	SEP	RPD	REP
				Calibration[1]			*Cross validation[2]*			*Validation[3]*			
% CP	ALL	MSC	2.4.4.1	572	0.993	1.205	0.991	1.341	115	0.991	0.995	10.64	59.77
	FF	MSC	2.4.4.1	112	0.981	0.743	0.964	0.965	22	0.956	1.023	4.38	16.03
aCP	ALL	SNV-D	3.4.4.1	569	0.846	0.076	0.81	0.085	113	0.774	0.956	2.26	10.20
	FF	SNV-D	3.4.4.1	112	0.974	0.034	0.943	0.049	22	0.956	1.035	4.59	14.23
	NF	D	3.4.4.1	427	0.89	0.056	0.834	0.068	88	0.815	0.988	2.57	12.15
bCP	ALL	MSC	3.4.4.1	569	0.831	0.09	0.76	0.106	113	0.767	0.992	2.09	9.30
	FF	MSC	3.4.4.1	112	0.965	0.042	0.936	0.056	22	0.938	1.016	3.94	12.64
	NF	SNV-D	3.4.4.1	427	0.836	0.074	0.78	0.086	88	0.729	0.963	2.04	8.98
cCP	ALL	SNV-D	3.10.10.1	569	0.424	0.021	0.354	0.023	113	0.475	1.001	2.02	13.91
	FF	D	3.4.4.1	112	0.824	0.017	0.646	0.025	22	0.837	0.957	2.11	7.94
	NF	SNV-D	3.4.4.1	427	0.687	0.017	0.579	0.021	88	0.554	0.772	2.22	14.61
edCP	ALL	MSC	3.4.4.1	569	0.853	0.057	0.825	0.062	113	0.802	0.906	2.53	12.78
	FF	SNV-D	3.4.4.1	112	0.891	0.027	0.801	0.038	22	0.859	1.012	2.63	9.76
	NF	SNV-D	3.4.4.1	427	0.881	0.045	0.795	0.058	88	0.766	0.952	2.30	10.16
asymCP	ALL	SNV-D	3.4.4.1	569	0.476	0.022	0.347	0.026	113	0.47	1.045	4.01	36.73
	FF	SNV-D	3.4.4.1	112	0.882	0.012	0.813	0.015	22	0.843	0.945	2.37	7.79
	NF	SNV-D	3.4.4.1	427	0.413	0.02	0.334	0.021	88	0.298	0.681	2.63	20.50

[1] N: number of samples for calibration; R^2: coefficient of determination for calibration; SEC: Standard error of calibration

[2] r_c^2: coefficient of determination for cross validation; SECV: Standard error of cross validation

[3] n: number of samples for validation; r^2: coefficient of determination for external validation; SEP: Standard error of validation; RPD: Ratio of performance to deviation (= SD/SEP); RER: Range error ratio (=Range/SEP)

[4] % CP: CP concentration as % of DM; aCP: soluble fraction of CP; bCP: degradable fraction of CP; cDM: rate of CP degradation; edCP: effective degradation of CP; asymDM: asymptote of degradation (a+b)

[5] ALL: all samples; FF: group of forages; NF: group of no forages

[6] MSC: multiple scatter correction; SNV: standard normal variate; D: detrend ; Math treatment: derivative number. subtraction gap. smooth. second smooth.

Table 6. Calibration and validation statistics for determination of neutral detergent fibre (NDF) degradability parameters by near-infrared analysis.

Parameter[4]	Group[5]	Scatter correction[6]		N	R^2	SEC	r_c^2	SECV	n	r^2	SEP	RPD	REP
			Calibration[1]				Cross validation[2]			Validation[3]			
% NDF	ALL	MSC	3.10.10.1	84	0.975	2.071	0.932	3.251	16	0.917	3.789	3.38	15.36
	FF	MSC	3.10.10.1	67	0.984	1.63	0.955	2.236	13	0.932	3.593	3.80	16.20
bNDF	ALL	D	3.10.10.1	84	0.818	0.062	0.63	0.084	16	0.701	0.095	1.82	5.94
	FF	D	3.4.4.1	67	0.854	0.071	0.728	0.053	13	0.736	0.082	1.86	6.20
cNDF	ALL	MSC	3.10.10.1	84	0.64	0.01	0.528	0.019	16	0.526	0.019	1.67	5.95
	FF	D	1.10.10.1	67	0.742	0.013	0.685	0.014	13	0.571	0.018	1.57	6.28
edNDF	ALL	MSC	3.10.10.1	84	0.836	0.054	0.747	0.066	16	0.765	0.072	2.25	7.48
	FF	SNV-D	3.4.4.1	67	0.974	0.024	0.891	0.046	13	0.866	0.058	2.63	8.66

[1]N: number of samples for calibration; R^2: coefficient of determination for calibration; SEC: Standard error of calibration

[2] r_c^2: coefficient of determination for cross validation; SECV: Standard error of cross validation

[3] n: number of samples for validation; r^2: coefficient of determination for external validation; SEP: Standard error of validation; RPD: Ratio of performance to deviation (= SD/SEP); RER: Range error ratio (=Range/SEP)

[4] % NDF : concentration of NDF as % of DM; bNDF: degradable fraction of NDF; cNDF: rate of NDF degradation; edNDF: effective degradation of NDF;

[5] ALL: all samples; FF: group of forages; NF: group of no forages

[6] MSC: multiple scatter correction; SNV: standard normal variate; D: detrend ; Math treatment: derivative number. subtraction gap. smooth. second smooth.

Figure 1. Scatter plots of predicted and actual asymptote of degradation of CP (group ALL; 1a) and NDF (group FF;1b)
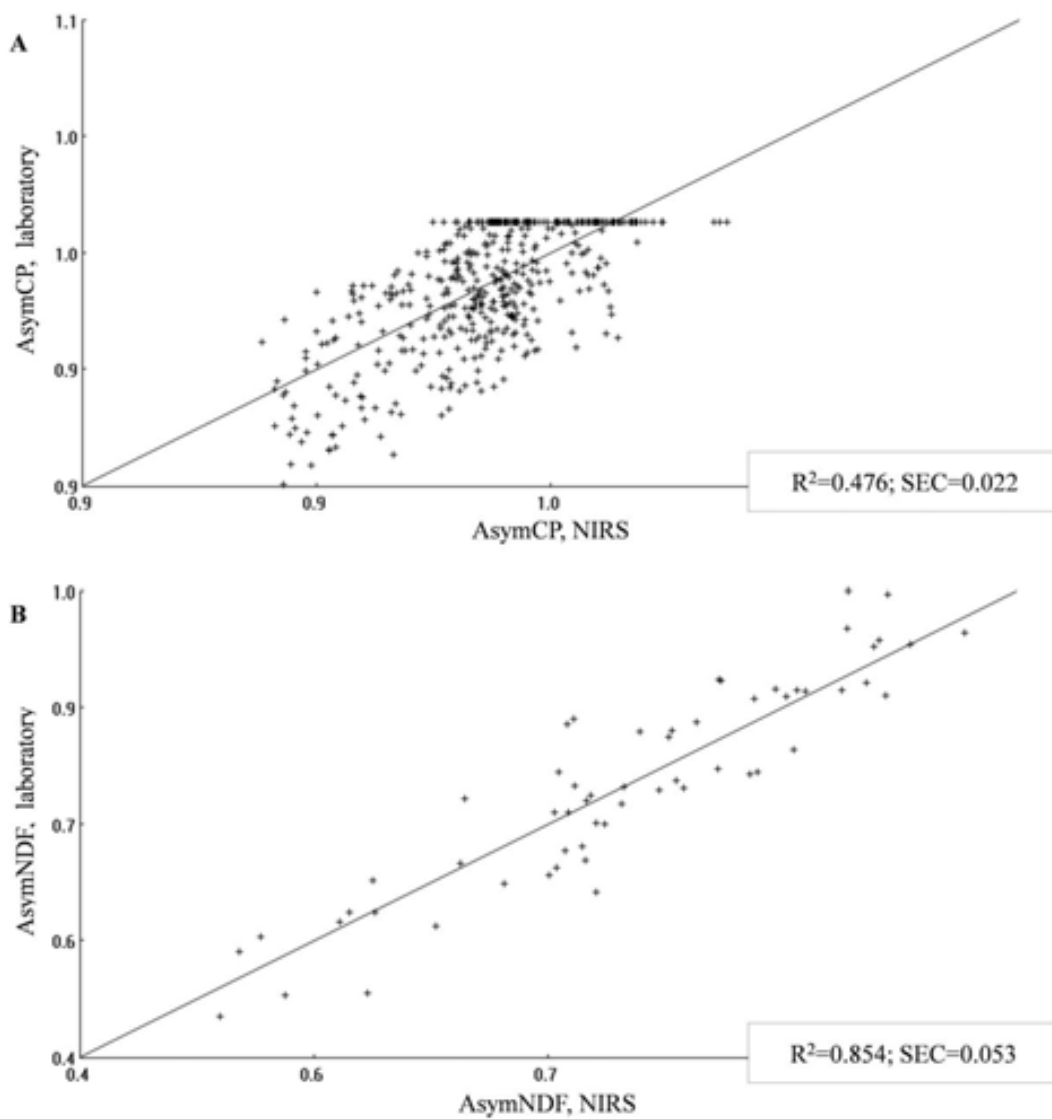
Chapter 3

Effects of essential oil compounds addition

on ryegrass silage protein degradation

Abstract

Measures to increased efficiency of nitrogen (N) use in crops and animal production are needed to reduce environmental impact of dairy production. During ensiling of forages an extensive degradation of protein has been documented. Some essential oils (EO) compounds alter protein metabolism through the inhibition of peptidolysis and deamination. The objective of this research was to evaluate the effect of the addition of EO compounds on ryegrass silage chemical composition and protein degradation. Microsilos (n=74) were prepared in polyester bags with 2.0 kg of fresh chopped ryegrass forage, sprayed according to treatments and sealed with an automated vacuum machine. The EO compounds tested were: thymol (THY), eugenol (EUG), cinnamaldehyde (CIN), capsaicin (CAP) and carvacrol (CAR), at 4 doses: 0, 50, 500 and 2,000 mg/kg of fresh forage. Silages were opened after 35 days and sampled. Samples were analyzed for pH, N fractions (large peptide-N, small peptide-N, and ammonia-N), dry matter (DM), lactic acid, lactic acid bacteria (LAB) and clostridia. Silages pH was higher than expected (5.5 to 6.6) and was attributed to the low DM content of the forage and the addition of EO. The addition of CAP did not affect any of the variables tested. The addition of THY, EUG and CAR in high dose (2,000 mg/kg of forage) inhibited deamination in ryegrass silages. Moreover, CAR inhibited deamination in the moderate dose (500 mg/kg of forage). The antimicrobial activity of these compounds reduced population of LAB, explaining the inhibition of deamination. The addition of CIN at 2,000 mg/kg of forage had an overall effect on protein degradation resulting in silages with 9.7% higher true protein N, but had no effect on LAB counts or lactic acid concentration of silages. These effects might be attributed to the inhibition of plant enzymatic activity, but the exact mechanism of action needs to be identified. Results suggest the contribution of LAB in the process of protein degradation

and deamination during ensiling. Tested EO compounds affected protein degradation and deamination of ryegrass forage during ensiling, but the effective dose was too high to be applied in practice.

1. Introduction

Nitrogen (N) is an essential element of food production determining the productivity of crops and animals (Jensen et al., 2011). However, its extensive utilization has led to the phenomenon described as N cascade. According to this theory, one atom of anthropogenic N, like the one used in fertilizers, circulates into the ecosystems causing multiple effects in the atmosphere, terrestrial ecosystems, freshwater and marine systems, and on human health (Galloway et al., 2003). Agriculture is the main contributor to this phenomenon and the increased efficiency of N use in crop and animal production were proposed as key actions for N management (Sutton et al., 2011).

Ryegrass is the predominant forage grass in Europe and fresh forage is usually ensiled (Akmal and Janssens, 2004). In the growing plant, approximately 75–90% of the N is in the form of true protein (TP; Slottner and Bertilsson, 2006). However, this figure is closer to 30 to 50% silage due to the extensive protein degradation to peptides, amino acids and ammonia (Ohshima and McDonald, 1978; Carpintero et al., 1979; Owens et al., 2002). Kemble (1956) suggested that the main cause of protein degradation in silages is the enzymatic activity in the plant before ensiling and not the microbial activity during ensiling. However, lactic acid producing bacteria (LAB), enterobacteria and clostridia present in silages have proteolytic activity and may contribute to the process (McDonald et al., 1991).

The role of essential oils (EO) as modifiers of the microbial fermentation in the rumen has been recently reviewed (Calsamiglia et al., 2007; Benchaar et al., 2008). Some EO alter protein metabolism through the inhibition of deamination, although the inhibition of peptidolysis has also been suggested (Calsamiglia et al., 2007; Benchaar et al., 2008). Among EO, eugenol ($C_{10}H_{12}O_2$; EUG), cinnamaldehyde (C_9H_8O; CIN), thymol ($C_{10}H_{14}0$; THY), capsaicin ($C_{18}H_{27}NO_3$; CAP) and carvacrol ($C_6H_3CH_3(OH)(C_3H_7)$; CAR) seem to be the more promising on altering microbial N metabolism in silages. The only attempt to use EO in silage preparation reported no effects (Kung et al., 2008). However, the low dose of a commercially available mixture of EO used (40 and 80 mg of EO / kg of fresh forage) and the selection of maize as the ensiling crop, limited the possibility of EO to affect protein degradation during ensiling.

The objective of this research was to evaluate the effect of the addition of EO compounds on ryegrass silage chemical composition and protein degradation.

2. Materials and Methods

2.1. Herbage

Fresh Italian ryegrass forage (*Lolium multiflorum*) was harvested as a first cut from a commercial farm in Vic (Barcelona, Spain) in 6 consecutive days during December 2008. The field was separated in 6 equal portions and each day a different part was used. The grass was chopped at 20 to 30 mm length using a machine (Sterwings, Germany), and duplicate samples from each part was frozen at –20°C for further analysis. The grass was ensiled within 2 h in order to avoid protein degradation during wilting.

2.2. Microsilos Preparation

Microsilos (n = 74) were prepared as follows: the fresh forage was manually chopped with paper cutters to 2 cm average particle size and 2 kg were weighted for each microsilo. Essential oils were dissolved in ethanol and sprayed on the fresh forage. The control microsilos were also sprayed with the equivalent amount of ethanol (25 ml/kg). The sprayed material was directly ensiled in polyester bags and sealed with an automated vacuum machine (EVA-9, Technotrip, Terrassa, Spain).

2.3. Experimental Treatments

The EO compounds tested were: THY, EUG, CIN, CAP and CAR; at three doses: 50, 500 and 2,000 mg/kg of forage. Treatment and the corresponded control microsilos were prepared in two consecutive days. Every day 2 microsilos of each treatment were prepared forming in total 4 replicates per treatment.

2.4. Sampling Process and Silage Juice Extraction

After 35 days of ensilage, each microsilo was opened, thoroughly mixed and sampled. Samples were divided in: (i) 25 g of silage for silage juice extraction, (ii) 250-300 g of silage for the analysis of dry matter (DM) and total nitrogen (TN), and (iii) 30 g of silage for microbial analysis.

Silage and forage juice was extracted after blending 25 g of material with 225 ml of distilled water for 1 min in a high speed blender and filtering the slurry through a Watman paper No 54. The pH of the filtrate was measured immediately. Samples were taken to determine ammonia-N, lactic acid concentration, trichloroacetic acid soluble N (TCA-N), and tungstic acid soluble N (TA-N).

2.5. Chemical and Microbial Analysis

For the determination of DM, 250 g of silage or forage were placed in duplicate in oven for 48 h at 60°C. The dried material was ground with a cutting mill (SM 2000, Retsch GmbH, Germany) to 1 mm for further analysis. The laboratory dry matter was determined by drying the grounded material for 24 h in a 103°C forced air oven. Dry samples were ashed for 5 h at 550°C in a muffle furnace, and organic matter (OM) was determined by difference. The Kjeldhal method (976.05; AOAC, 1990) was used to determine TN. Forage dry samples were analyzed for neutral detergent fibre assayed with a heat stable amylase, sodium sulfite and expressed exclusive of residual ash (aNDFom) and acid detergent fibre expressed exclusive of residual ash (ADFom).

Non protein nitrogen (NPN) was analyzed in silage juice after precipitation of true protein with TCA. To determine TCA-N, 4 ml of a 500 g/l TCA solution were added to 16 ml of filtered silage juice. After 4 h at 5°C, tubes were centrifuged at $9,000 \times g$ for 15 min. The supernatant was stored and frozen until analyzed for TCA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05). To determine TA-N, 4 mL of a 100 g/l sodium tungstate solution and 4 mL of 1.07 N sulfuric acid were added to a 16-ml sample of filtered silage juice. After 4 h at 5°C, tubes were centrifuged at $9,000 \times g$ for 15 min. The supernatant was stored frozen until analyzed for TA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05). Ammonia-N was analyzed in a 4 ml sample of filtered silage juice that was acidified with 4 ml of 0.2 N HCl and frozen. Samples were centrifuged at $15,000 \times g$ for 15 min, and the supernatant was analyzed by spectophotometry (Libra S21, Biochrom Technology, Cambridge, UK) for ammonia-N (Chaney and Marbach, 1962). Results were used to calculate large peptides (LPep = TCA-N − TA-N) and small peptides plus amino acids (SPep = TA-N − ammonia-N; Winter et al., 1964).

Samples for lactic acid analysis were prepared as described by Jouany (1982) and analyzed by gas chromatography: 1 ml of a solution made up of a 2 g/l solution of mercuric chloride, 2 g/l of 4-methylvaleric acid as an internal standard, and 20 g/l orthophosphoric acid, was added to 4 ml of silage juice and frozen. Before analysis, samples were defrosted and centrifuged at $15,000 \times g$ for 15 min. The supernatant was derivatized by N-methyl-trimethylsilyltrifluoroacetamide (MSTFA; Sigma Aldrich) and incubated for 30 min in a dry bath at 80°C. The vials were kept in ambient temperature for 72 h before being analyzed by gas chromatography (model 6890, Hewlett Packard, Palo Alto, CA, USA) using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Bucks, UK).

Microbial analysis was conducted immediately after sampling the microsilos for the determination of LAB according to ISO 15214 (1998) and clostridia according to Pascual Anderson and Calderon y Pascual (2000).

2.6. Statistical Analyses

All statistical analyses were conducted using SAS (version 9.2 SAS Institute, Inc., Cary, NC; SAS, 2009). Results were analyzed using the PROC MIXED procedure. The model accounted for the effects of treatments (fixed effect) and day was considered a random effect. The significance of differences between means of treatments and the control was declared at $P < 0.05$ using the Dunnett multiple comparison test.

3. Results

The addition of CAP had no effect on silage chemical characteristics and protein degradation and, therefore results are not shown.

3.1. Forage Composition and Silage Characteristics

Not wilted ryegrass forage had low DM content (Table 1), and high CP (220 g/kg of DM) with only 5.4% of TN in the form of NPN. Silages produced in this experiment had high pH, ranging from 5.5 to 6.6 (Table 2), and high CP (231-319 g/kg of DM). As intended, the overall proteolysis in silages was high, resulting in silages with 60% of TN in the form of NPN. The most important pool of NPN in silages was the SPep pool accounting for 39-57% of TN (Figure 1). The addition of CAR and THY increased silage pH in a dose response manner, with the largest effects occurring in the high dose (2,000 mg of EO compound/kg of forage).

Counts of LAB decreased with the addition of 2,000 mg/kg of forage of THY and CAR, resulting also in lower lactic acid concentration. Moreover, the addition of 2,000 mg of EUG/kg of forage reduced LAB counts without affecting the concentration of lactic acid. The addition of CIN did not affect either the LAB counts or the lactic acid concentration.

3.2. Effect of EO Compounds on Silage Protein Degradation

Table 3 shows results of the addition of EO compounds on N fractions of silages. The highest dose of EO compounds decreased the concentration of ammonia-N, suggesting an effect on amino acid deamination, while the strongest effect on ammonia-N concentration occurred with THY. The addition of CAR also decreased ammonia-N in a more moderate dose of 500 mg/kg of forage (1.8 vs. 1.2 g/kg of DM for CTR vs CAR500, respectively). Actually, the concentration of ammonia-N in this case was lower than that of CIN in the high dose.

The addition of CIN in a high dose (2,000 mg/kg of forage) was the only EO that decreased overall protein degradation resulting in silages with lower NPN (22.3 vs 18.4 g/kg of DM for CTR vs CIN2000, respectively), and lower SPep concentration (19.2 vs

15.9 g/kg of DM for CTR vs CIN2000, respectively), resulting in silages with 9.8% more TP (Figure 1).

Results suggested that EUG, THY and CAR influenced the deamination of amino acids causing a reduction of ammonia-N, and CIN affected overall protein degradation resulting in silages with lower NPN.

4. Discussion

4.1. Silage pH

Silage pH was affected by the addition of EO. However, control silages also had an unusually high pH (5.6 to 5.8). Both control and treated microsilos contained added ethanol that was used to dilute EO. In order to investigate potential effects of ethanol addition on silage pH, we conducted a short study where ryegrass silage was cut and ensiled as described previously. Silage (*n = 9*) treatments were: CTR (no addition), ETA (addition of 25 ml of ethanol/kg of fresh ryegrass forage), and CIN (addition of 2,000mg of cinemaldeydehyde / kg of fresh ryegrass forage diluted in ethanol). Results confirmed that silage pH increased by the addition of CIN (P<0.01), but there was no effect effect due to the addition of ethanol.

Similar pH in untreated silages have been observed in silages deficient in LAB produced from crops with low levels of fermentable carbohydrates or too wet. Schmidt et al. (2009) observed populations of 8-9 log cfu/g silage at 35-45 days of ensiling of alfalfa. The population of viable LAB in untreated alfalfa increased rapidly by d 2 of fermentation, peaked after 5 d and declined slowly at 45 and 90 d. In the current experiment, all silages had similar LAB populations to those observed in other studies (Whiter and Kung, 2001; Schmidt et al., 2009). Crops with low level of fermentable carbohydrates have reduced counts of LAB, but in the current experiment, LAB counts

were within normal ranges indicating that the level of water soluble carbohydrates did not influence silages pH. However, forage was ensiled immediately after harvest to reduce protein degradation during wilting. This resulted in silages with high moisture (forage DM was 125 g/kg of fresh forage). Moreover, the addition of EO compounds in high doses reduced LAB and lactic acid concentration, and resulted in the highest pH. Thus, the high pH values observed in this experiment are explained mainly by the low DM content of forages and the addition of EO.

4.2. Effect of EO Compounds on Silage Protein Degradation

The addition of THY2000 resulted in the largest reduction of ammonia-N concentration. In the rumen microbial environment, Borchers (1965) and Castillejos et al. (2006) reported that the addition of THY caused similar effects on protein fermentation, and these effects were attributed to the strong antimicrobial activity of THY. In the current study, counts of LAB and lactic acid concentration decreased suggesting the antimicrobial effect of THY as the mechanism of action.

The addition of EUG2000 caused a small reduction of LAB without affecting lactic acid concentration, but ammonia-N was reduced, suggesting reduced deamination in ryegrass silage. This is in accordance with the effects of EUG in the rumen microbial environment, where *in vitro* studies suggested decreased peptidolitic activity in the rumen by the addition of clove bud oil (85% of EUG; Busquet et al., 2005) and inhibition of deamination by reducing the concentration of ammonia-N (Castillejos et al., 2006).

The addition of CAR reduced ammonia-N of silages not only in the high dose but also in the moderate dose (500 mg/kg of forage). Busquet et al. (2005) reported that CAR decreased LPep concentrations and increased ammonia-N concentration in a rumen microbial environment, suggesting that CAR either inhibited proteolysis or

stimulated peptidolisis. Moreover, the antimicrobial activity of CAR2000 caused a numerical but non significant reduction of LAB, but lactic acid concentration was reduced.

Among the EO tested, CIN2000 was the only EO that had an overall effect on protein degradation resulting in silages with lower NPN and higher TPN, without affecting LAB counts or lactic acid concentration of silages. In a rumen microbial environment, Cardozo et al. (2004) were the first to suggest that cinnamon oil (0.22 mg/L of rumen fluid) modified the N metabolism of rumen microorganisms by inhibiting peptidolysis. Busquet et al. (2006), also in a ruminal microbial environment, reported deaminating effect of CIN, but in a high dose (3,000 mg/l), while moderate doses (31.2 and 312 mg/l) did not affected N metabolism (Busquet et al., 2005).

Silage protein degradation is mainly attributed to plant enzymatic activity (Oshima and McDonald, 1978; Owens et al., 2002; Lee et al., 2007). However, clostridia and LAB are capable to ferment protein (McDonald et al, 1991). Counts of clostridia were not affected by treatment and remained at low levels. McEniry et al. (2008) reported a clostridia population of 1.9-2.2 logcfu/g in grass silages produced by different techniques, while in the current experiment the population of clostridia did not exceed 1.45 logcfu/g, and it was not affected by treatment. However, counts of LAB were reduced by the addition of EO compounds in the high dose, with the exception of CIN, and ammonia-N concentrations were also reduced.

The strong antimicrobial activity of THY and CAR has been demonstrated against bacteria (Burt, 2004), mold (Daferera et al., 2003) and yeast (Arora and Kaur, 1999). Similarly, EUG also has a wide-spectrum antimicrobial activity against gram-positive and gram-negative bacteria (Davidson and Naidu, 2000). The reduction in the

population of LAB caused by the addition of THY, EUG and CAR may explain the inhibition of deamination observed in the current study.

However, the addition of CIN did not affect LAB or clostridia but inhibited both protein degradation and deamination. Several studies reported the capacity of phenol compounds of EO, such as CIN, to interact with proteins and enzymes through hydrogen bridges and ionic or hydrophobic interactions resulting in their inactivation (Juven et al., 1994). Wendakoon and Sakaguchi (1995) demonstrated that CIN could bind proteins and inhibit the enzymatic activity of *Enterobacter aerogenes,* and Fujita et al. (2006) that CIN inhibits phenylalanine ammonia-lyase, an enzyme responsible for the browning of vegetables. The exact mechanism by which CIN affects plant enzymatic activity or bacterial deamination during ensiling needs to be identified.

5. Conclusions

The antimicrobial properties of tested EO compounds affect protein degradation and deamination of ryegrass forage during ensiling. The current study demonstrates that microbes are also responsible for the high degradation of protein during ensiling, besides the already established effects of plant enzymes. For the majority of EO compounds, doses up to 2,000 mg/kg fresh silage are required to have these effects, with the exception of CAR at 500 mg/kg of forage. The necessity of a high dose makes it complicated and costly to be applied in practice.

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Table 1. Chemical composition of ryegrass forage (*n=6*)

Item[1]		SEM[2]
DM (g/kg)	125	0.2
OM (g/kg DM)	853	0.7
pH	6.60	0.024
aNDFom (g/kg DM)	459	38.7
ADFom (g/kg DM)	192	12.4
N fractions		
TN (g/kg of DM)	39.5	0.50
LPep (g/kg of DM)	0.08	0.176
SPep (g/kg of DM)	2.1	1.04
Ammonia N (g/kg of DM)	ND[3]	-
NPN (%TN)	5.16	0.440

[1] DM: dry matter; OM: organic matter; aNDFom: neutral detergent fibre; ADFom: acid detergent fibre; TN: total nitrogen; LPep: large peptides; SPep: small peptides; NPN: no protein nitrogen

[2] SEM: standard error of the mean.

[3] ND: not detectable

Table 2. The effect of essential oils compounds on ryegrass silage characteristics after 35 days of ensiling.[1]

	0	50	500	2000	SEM[2]	P-Value
EUG						
pH	5.81	5.37	5.95	6.48	0.317	NS
DM (g/kg)	120	118	118	122	2.6	NS
LAB (logcfu/g)	8.57^a	8.36^a	8.34^a	7.89^b	0.124	**
Clostridia (logcfu/g)	0.89	0.89	0.70	0.77	0.143	NS
Lactic Acid (mg/100ml)	52.0	55.4	41.8	40.0	7.24	NS
THY						
pH	5.60^a	5.44^a	6.31^{ab}	6.56^b	0.297	*
DM (g/kg)	115	118	116	114	2.4	NS
LAB (logcfu/g)	7.88^a	7.71^a	7.95^a	5.66^b	0.412	**
Clostridia (logcfu/g)	1.05	0.89	1.22	0.77	0.176	NS
Lactic Acid (mg/100ml)	45.9^a	47.2^a	41.4^a	3.54^b	7.36	***
CIN						
pH	5.63	5.59	5.81	6.02	0.279	NS
DM (g/kg)	116^a	112^{ab}	108^b	115^{ab}	1.8	*
LAB (logcfu/g)	8.58^a	8.43^{ab}	8.53^{ab}	8.21^b	0.097	NS
Clostridia (logcfu/g)	0.85	1.45	0.92	0.97	0.186	NS
Lactic Acid (mg/100ml)	50.7	62.9	60.7	41.5	6.4	NS
CAR						
pH	5.77^a	6.00^{ab}	6.46^{bc}	6.57^{bc}	0.171	*
DM (g/kg)	12.07	12.29	12.38	12.54	0.202	NS
LAB (logcfu/g)	8.04	8.29	8.25	7.11	0.395	NS
Clostridia (logcfu/g)	0.895	0.95	0.77	0.97	0.187	NS
Lactic Acid (mg/100ml)	58.7^a	43.8^b	36.4^b	12.2^c	3.90	***

[1]Concentration of eugenol (EUG), cinnamaldehyde (CIN), thymol (THY) and carvacrol (CAR): 0, 50, 500 and 2000 mg/kg of forage; DM: dry matter; LAB: lactic acid bacteria

[2]SEM: standard error of the mean.

[*] P < 0.05; ** P < 0.01; *** P< 0.001.

[a,b,c] Means in the same row with different superscript differ significantly (P<0. 05).

Table 3. The effect of essential oil compounds on ryegrass nitrogen fractions (g/kg DM) after 35 days of ensiling[1,2].

	TN	NPN	NH$_3$-N	SPep	LPep
CTR	38.86	21.82	1.74[a]	20.16	-0.09
EUG2000	37.87	21.07	0.97[b]	17.6	2.48
SEM[3]	1.201	1.507	0.108	1.990	1.005
P-value	NS	NS	***	NS	NS
CTR	42.01	22.38[a]	1.84[a]	19.19[a]	1.35
CIN2000	40.29	18.40[b]	1.23[b]	15.87[b]	1.29
SEM[3]	0.970	0.908	0.134	1.074	1.122
P-value	NS	**	**	*	NS
CTR	38.61	23.38	1.81[a]	21.17	0.40
CAR500	37.02	22.13	1.20[b]	19.88	1.05
CAR2000	37.83	21.96	1.08[c]	21.22	-0.34
SEM[3]	1.300	0.864	0.154	1.26	1.131
P-value	NS	NS	**	NS	NS
CTR	48.88[a]	23.17	1.59[a]	19.78	1.79
THY2000	42.29[b]	26.19	0.85[b]	24.14	1.19
SEM[3]	1.990	1.474	0.136	1.483	1.452
P-value	NS	NS	**	NS	NS

[1] CTR: control (addition of ethanol: 25ml/kg of fresh forage), EUG: eugenol, CIN: cinnamaldehyde, CAR: carvacrol, THY: thymol. Concentration of essential oils: 500 and 2,000 mg/kg of forage.

[2] TN: total nitrogen, NPN: non protein nitrogen, NH$_3$-N: ammonia nitrogen, SPep: small peptides, LPep: large peptides

[3] SEM: standard error of the mean.

[*] $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

[a,b,c] Means in the same column with different superscript differ significantly (P<0. 05).

Figure 1. The effect eugenol (EUG), cinnamaldehyde (CIN), thymol (THY) and carvacrol (CAR) on ryegrass silage nitrogen fractions at 35 ensiling days (TP: true protein (% total nitrogen), NH$_3$: ammonia nitrogen (% total nitrogen), SPep: small peptides nitrogen (% total nitrogen), LPep: large peptides nitrogen (% total nitrogen).
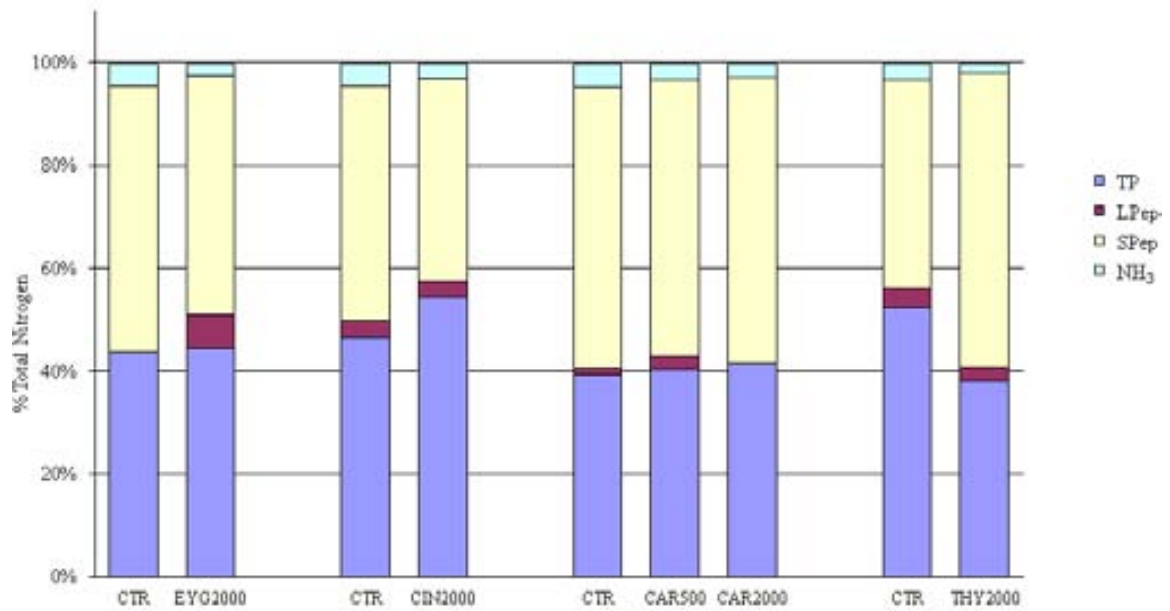
Chapter 4

Effects of polyclonal antibody preparation against *Prevotella ruminicola*,

Clostridium aminophilum and *Peptostreptococcus anaerobius* on rumen

microbial fermentation.

Abstract

Dairy cows are major contributors to environmental nitrogen (N) emissions to the environment. A significant amount of N is lost in the form of ammonia in the rumen, which is attributed to the imbalance between ammonia production and ammonia utilization by microbes for protein synthesis. Recently, polyclonal antibodies (PAbs) have been used to control specific bacteria responsible for ruminal acidosis, but no studies have been conducted with PAbs against proteolytic and deaminating bacteria. The objective of this study was to produce and evaluate PAbs against main bacteria involved in proteolysis and deamination as *Prevotella ruminicola, Clostridium aminophilum* and *Peptostreptococcus anaerobius*. Bacteria were grown according to recommendations, inactivated with formaldehyde, freeze dried, and used to immunize rabbits. Blood samples were collected after the 4th immunization and serum responses to the antigens were analyzed by ELISA. In the first experiment the modified gas production and the 24 h batch culture techniques were used to test the effects of PAbs in short term ruminal fermentation. Treatments were: control (CTR; serum of non-immunized animals), PAbs against *P. ruminicola* (APr), *C. aminophilum* (ACl), *P. anaerobius* (APa), and a mix of PAbs (1:1:1 of APr, ACl and APa, respectively; AMix). Treatments were tested at 0.005, 0.05 and 0.5 for gas production and at 0.005 and 0.05 ml of serum / 30 ml of medium for batch culture. Gas production was recorded for 24 h and selected tubes of the batch culture were withdrawn at 3, 12 and 24 h and sampled for ammonia-N and volatile fatty acids (VFA). In the second experiment, eight continuous culture fermenters were inoculated with ruminal liquid from a dairy cow fed a 50:50 concentrate:forage diet, in 2 replicated periods to test the effects of the same treatments, except the AMix, at 3.2 ml of serum/fermenter/day. During sampling days, fermenters were sampled at 0, 2, 4 and 6 h post feeding for N fractions and at 2 h for

VFA. Samples of the 24 h effluent were analyzed for N fractions, VFA and digestibility of nutrients. The addition of PAbs had no effect on ruminal fermentation in short term fermentation. In the fermenters study, ammonia-N in the effluents were not affected by treatments (average of 7.31 to 7.91 mg / 100 ml for CTR and APa, respectively). Nutrient digestibility and the hourly variation of N fractions did not differ among treatments. Tested PAbs did not affect ruminal protein degradation in the short or long term fermentation.

Keywords: polyclonal antibodies, *Prevotella ruminicola*, *Clostridium aminophilum*, *Peptostreptococcus anaerobius*,

1. Introduction

The extensive utilization of nitrogen (N) has led to the phenomenon described as N cascade with multiple effects in the atmosphere, terrestrial ecosystems, freshwater and marine systems, and human health (Galloway et al., 2003). Recently, the European Nitrogen Assessment project pointed out that agriculture, and particularly livestock production, is the main contributor to this phenomenon and suggested that the improvement of N utilization of farm animals is the key action to better manage the N cascade (Sutton et al., 2011). However, the dairy cow is characterized by low efficiency of N utilization. Tamminga (1992) calculated that dairy cows excrete 75-85% of the ingested N in faces and urine and Huhtanen and Hristov (2009), utilizing a large data base from North American and North European studies, verified this low N efficiency of dairy cows and reported an average milk N efficiency of 24.7 and 27.7 % for North American and North European studies, respectively.

Part of this low efficiency is attributed to the function of the rumen, and particularly the N losses in the form of ammonia (Tamminga, 1992, 1996). Bach et al. (2005) reported a strong relationship between the efficiency of N utilization (ENU) in the rumen and ammonia-N concentration in continuous culture studies (Ammonia-N = 43.6 − 0.469ENU; R^2 = 0.78; RMSE = 4.53). Therefore, the reduction of ruminal ammonia-N without affecting microbial protein synthesis might be an effective strategy to improve the efficiency of N utilization in the rumen (Calsamiglia et al., 2010). Ammonia in the rumen is produced via deamination of amino acids or from non protein nitrogen compounds, like urea and amides, which are converted to ammonia in the rumen and finally used by microbes for microbial protein synthesis (Bach *et al.*, 2005). Moreover, when the rate of ammonia production is higher than that of its use by the microbes, ammonia accumulates in the rumen leading to substantial losses of N (Walker et al., 2005).

Two main bacterial groups are involved in the deamination of amino acids: the hyper ammonia producing (HAP) bacteria, which are non-saccharolytic amino acid fermenters and rapid producers of ammonia from amino acids (Chen and Russell, 1988, 1989; Paster et al., 1993; Russell et al., 1988, 1991); and *Prevotella* spp., which can ferment amino acids at relatively slow rate, but due to their abundance in the rumen are consider major deaminating bacteria (Wallace, 1996; Rychlik and Russell, 2000; Walker et al., 2005). Therefore, targeting HAP and *Prevotella* spp. may be an effective strategy to reduce ammonia accumulation and improve N efficiency utilization in the rumen and therefore to reduce N excretion by ruminants.

Several strategies have been proposed that target proteolytic and deaminating ruminal bacteria. The addition of monensin successfully reduced ammonia-N concentration in pure and mixed cultures of ruminal bacteria (Russell and Martin, 1984;

Chen and Russell, 1989, 1990) and in continuous culture fermenters (Busquet et al., 2005; Castillejos et al., 2006), mainly due to the sensitivity of HAP bacteria to monensin (Paster et al., 1993; Rychlik et al., 2002). However, its use is not allowed in the European Union. Recently, the use immunization against main proteolytic and deaminating bacteria of the rumen has been suggested as an alternative for controlling bacteria populations (Walker et al., 2005; Calsamiglia et al., 2006). Active and passive immunization was successfully used to control ruminal bacteria responsible for acidosis, such as *Streptococcus bovis* and *Fusobacterium necrophorum* (Shu et al., 1999; DiLorenzo et al., 2006; Blanch et al., 2009) and methanogenesis (Wright et al., 2004.

No studies are available on the effects of the addition of polyclonal antibodies (PAbs) against proteolytic and deaminating ruminal bacteria on ammonia concentration in the rumen. The objective of this study was to produce and test *in vitro* PAbs against *Prevotella ruminicola* and HAP bacteria, in order to reduce ruminal protein degradation and deamination.

2. Materials and Methods

2.1. Polyclonal Antibody Production

2.1.1. Antigen Preparation

Bacterial strains were obtained from ATCC for the following species: *Prevotella ruminicola* (ATCC 19189), *Clostridium aminophilum* (ATCC 49906), *Peptostreptococcus anaerobius* (ATCC 27337) and *Clostridium sticlandii* (ATCC 49905). *Prevotella ruminicola* grew in a L-10 medium as proposed by Stewart et al. (1997) containing 15% of rumen liquid. Rumen liquid was obtained from a fistulated ewe, filtered through two layers of cheese cloth and centrifuged at 2,000 x g for 25 min.

The supernatant was autoclaved and centrifuged again at 2,000 x g for 20 min before its addition to the medium. *Clostridium aminophilum* and *Clostridium sticlandii* grew in a ATCC medium 1053 (Reinforced Clostridial medium; Oxoid CM149) and *Peptostreptococcus anaerobius* in a ATCC medium 1870 (Reinforced Clostridial medium with 1.5% casamino acids).

Bacteria were first cultured in 10 ml tubes containing the corresponding medium and then inoculated in anaerobic conditions in 50 ml vials of medium. All cultures were incubated at 39°C for 48 hours and the final bacteria concentration was determined microscopically. Bacteria were inactivated with 37% formaldehyde solution (1.5 ml/50 ml culture). Cells were resuspended in 40 ml sterile phosphate buffered saline (P4417, Sigma-Aldrich Chemical, St. Louis, MO): 0.01M phosphate, 0.138M NaCl-0.0027M KCl), transferred to sterile 200 ml bottles and centrifuged at 15,000 x g for 20 min at 10°C. Supernatant was removed and the pellet was resuspended in sterile PBS. This wash cycle was repeated three times. The pellet was collected and freeze dried.

2.1.2. Animal Immunization and Antibody Collection

The lyophilized bacteria were suspended in sterile saline solution, mixed for 10 min in a vortex and centrifuged at 10,000 x g for 10 min. The supernatant was collected and used for the immunization protocol. Each antigen was used to immunize 3 rabbits 4 times within a period of three months (days 0, 21, 42, and 63). Before the first immunization, blood samples were collected (bl1). Then 1 ml of the antigen mixed with Freund's complete adjuvant (1:1) was injected hypodermically in 5 different sites at the left shoulder of each rabbit. Then 1.5 ml of the antigen mixture, containing 1.5 mg of total protein was mixed with Freund's complete adjuvant (1:1; F5881, Sigma-Aldrich Chemical, St. Louis, MO) and injected hypodermically in 5 different sites at the shoulder of each rabbit (1 ml per rabbit). The following injections were conducted every

21 days using a Freund's incomplete adjuvant (1:1; F5506, Sigma-Aldrich Chemical, St. Louis, MO). Five days after the 3rd and 4th immunization, blood samples were collected (bl2 and bl3, respectively). Three animals were not immunized (rb1, rb2 and rb3) for the control serum; three were immunized against *Prevotella ruminicola* (rb4, rb5 and rb6; APr); three were immunized against *Peptostreptococus anaerobius* (rb7, rb8 and rb9; APa); and three were immunized against *Clostridium aminophilum* (rb10, rb11 and rb12; ACl). The level of production of antibodies and potential cross reactivity between produced PAbs were assessed using an enzyme linked immunosorbent assay (ELISA). When the level of antibodies in one bleeding did not increase compared with the previous bleeding, the immunization protocol was considered completed and a large volume of blood was collected to harvest the antibody.

2.1.3. Determination of Specific Antibodies in Serum by ELISA

Several 96-well plates (Maxisorp 442404, NUNC, Labclinics, Spain) were pre-coated with several protein extracts of each bacterial strain to determine the specific antibody production in rabbit's serum. Normal non-immune rabbit serum and phosphate buffer solution (AM9625, Ambion, Life Technologies, Madrid, Spain) were used as controls. The plate was maintained for 1 h at 37 °C and washed 3 times with PBST (PBS plus 0.05% Tween-20). Then the plate was blocked with 1% bovine albumin (10735078001, Roche, Manheim, Germany) in PBS for 45 minutes at 37 °C and washed 3 times with PBST. Immunized rabbit serum were serially diluted, added into 96-well plates and maintained for 2 hours at 37 °C and washed 3 times with PBST. The anti-rabbit IgG antibody conjugated with HRP (NB7162, Novus Biologicals, AntibodyBCN, Barcelona, Spain) was diluted 1/2000 with PBST, added into the wells, and incubated for 1 hour at 37 °C. After removal of the secondary antibody, OPD (P9187, Sigma-Aldrich Chemical, St. Louis, MO) was added into the wells for coloration. The optical

density value was measured at a 450 nm wavelength with an ELISA reader (Victor3, Perkin Elmer.).

2.2. Experiment 1

2.2.1. The Modified Gas Production Technique

The modified gas production technique described by Cone et al. (2009) was used to assess the effect of PAbs on ruminal protein degradation. Rumen fluid was collected from one rumen fistulated dairy cow 2 h before feeding to minimize the amount of available N and strained through two layers of cheesecloth. Rumen fluid was mixed with a N-free buffer/mineral solution (1:19) that contained (g/l) 10.03 $NaHCO_3$, 1.43 Na_2HPO_4, 1.55 KH_2PO_4, 0.15 $MgSO_4 \cdot 7H_2O$, 0.52 Na_2S, 0.017 $CaCl_2 \cdot 2H_2O$, 0.015 $MnCl_2 \cdot 4H_2O$, 0.002 $CoCl_3 \cdot 6H_2O$, 0.012 $FeCl_3 \cdot 6H_2O$ and 0.000125 resazurin. A mixture of rapidly fermented carbohydrates (glucose, xylose and soluble starch; 1:1:1 ratio) was added (10 g/l) and incubated for 4 h at 39°C to incorporate all available N into microbial protein and make N the limiting element. The pre-incubation was performed in 2 l bottles with continuous flushing of CO_2. At the same time, 3 bottles of the medium (50 ml) were incubated separately and gas production was recorded for 4 to 8 h to estimate the time of stable gas production, which indicates the time where all available N from the rumen fluid was incorporated into microbial protein. When gas production was stable, 50 ml of the N-free medium were added to the already prepared bottles according to treatments.

To verify that this technique can be used to assess the effect of additives on protein degradation, a preliminary trial was conducted using monensin as a positive control and two protein sources, soybean meal and tryptone. Each treatment had three (3) replicates and the experiment was repeated twice. Gas production was measured at 0, 0.5, 1, 2, 4, 6, 8, 12, 15, 18, 21 and 24 h. Samples of ruminal fluid, preincubation and

postincubation medium was analyzed for VFA and ammonia-N. At 24 h bottles were opened and pH measured.

For the preliminary trial treatments were: negative control with soybean meal as protein source (SNT; no addition of monensin), positive control with soybean meal as protein source (SMT; addition of 12.5 mg/l of monensin; Sigma-Aldrich Chemical, St. Louis, MO), negative control with tryptone (T9410 Fluka, Sigma-Aldrich Chemical, St. Louis, MO) as protein source (TNT; no addition of monensin), and positive control with tryptone as protein source (TMT; addition of 12.5 mg/l of monensin).

Experimental treatments consisted on control (CTR; serum addition of non-immunized animals), the addition of PAbs against *Prevotella ruminicola* (APr); *Clostridium aminophilum* (ACl); *Peptostreptococus anaerobius* (APa); and a mix of PAbs (1:1:1 of APr, ACl and APa, respectively; AMix). Three doses were tested: Low (0.005 ml serum / 50 ml of medium), Medium (0.05 ml serum / 50 ml of medium), and High (0.5 ml serum / 50 ml of medium). Each bottle was supplied with 12.5 mg of N from soybean or tryptone (for the monensin trial only).

2.2.2. In Vitro Batch Culture

The effects of PAbs against rumen bacteria were also evaluated in *in vitro* batch fermentation (Tilley and Terry, 1963). Experimental treatments consisted of control (CTR; serum addition of no immunized animals), the addition of PAbs against *Prevotella ruminicola* (APr), *Clostridium aminophilum* (ACl), *Peptostreptococus anaerobius* (APa), and a mix of PAbs (1:1:1 of APr, ACl and APa, respectively; AMix). Two doses of each treatment were tested: 0.005 and 0.05 ml serum / 50 ml of medium. Treatments were examined in triplicate, and fermentations were repeated in two periods. Incubations were conducted using rumen fluid from one fistulated dairy cow fed a 60:40

forage:concentrate diet. Rumen fluid was strained through 2 layers of cheesecloth and mixed in a 1:1 proportion with phosphate-bicarbonate buffer (McDougall, 1948) at an initial pH of 7.0. Incubations were conducted in 90 ml tubes containing 50 ml of diluted fluid and 0.5 g of soybean meal and tubes were placed in a water bath at 39°C. Tubes were gassed with CO_2 before sealing with rubber corks with a gas release valve. After 3, 12 and 24 h, samples were withdrawn from corresponding tubes to determine pH and analyze ammonia-N and VFA concentrations.

2.3. Experiment 2

2.3.1. Dual Flow Continuous Culture Fermenters

Eight 1,320 ml dual-flow continuous culture fermenters developed by Hoover et al. (1976) were used in 2 replicated periods. Each experimental period consisted of 5 d for adaptation and 3 d for sampling. Fermenters were inoculated with rumen liquid from a dairy cow fed a 50:50 forage:concentrate diet. Temperature (39°C), and liquid (0.10/h) and solid (0.05/h) dilution rates were maintained constant and pH was recorded automatically every 60 sec. Minimum and maximum pH limits were set at 5.8 and 6.5, respectively. All fermenters were fed 95 g/d of DM of a 50:50 forage:concentrate diet formulated to meet or exceed current nutrient recommendations for lactating dairy cows (18 % CP, 28% NDF, 20% ADF; NRC, 2001). The diet (DM basis) consisted of alfalfa pellets (200 g/kg), corn silage (300 g/kg), ground corn grain (320 g/kg), soybean meal (170 g/kg) and a vitamin and mineral mixture (10 g/kg). The vitamin and mineral mixture contained per kg of DM: 300 g of MgO; 267 g of urea; 33 g of sulphur; 67 g of NaCl; 4,660 mg Zn; 2,660 mg Mn; 167 mg Cu; 27 mg Se; 33 mg I; 7 mg Co; 1,000 KIU of vitamin A; 200 KIU of vitamin D3; and 1,330 mg of vitamin E.

During sampling days samples were collected at 0, 2, 4, and 6 hours after the morning feeding (07:00, 09:00, 11:00 and 13:00 respectively) and analyzed for VFA,

ammonia-N, tricloroacetic N (TCA-N) and tungstic acid N (TA-N). Moreover, samples for VFA analysis were collected 2 h after feeding. During sampling days, collection vessels were maintained at 4°C to prevent microbial activity. Solid and liquid effluents were mixed and homogenized for 1 min at 24,000 rpm (Diax900, Heidolph, Nurnberg, Germany), and a 600 ml sample was removed by aspiration and frozen at −20°C. Upon completion of each period, effluents from the 3 sampling days were composited, mixed within fermenter, and homogenized for 1 min. Subsamples were taken for total N, ammonia-N, TCA-N, TA-N and VFA analyses.

Bacterial cells were obtained from fermenter flasks the last day of each experimental period. Solid and liquid associated bacteria were isolated using a combination of several detachment procedures (Whitehouse et al., 1994) selected to obtain the maximum detachment without affecting cell integrity. One hundred milliliters of a 2 g/l methylcellulose solution and small marbles (30 of 2 mm and 15 of 4 mm of diameter) were added to each fermenter and incubated in the same fermenter flasks at 39ºC, and mixed for 1 h to remove attached bacteria. After incubation, fermenter flasks were refrigerated for 24 h at 4ºC and fermenter contents were agitated for 1 h to dislodge loosely attached bacteria. Finally, the fermenter content was filtered through cheesecloth and washed with saline solution (8.5 g/l NaCl). Bacterial cells were isolated within 4 h by differential centrifugation at 1,000 x g for 10 min to separate feed particles, and the supernatant was centrifuged at 20,000 x g for 20 min to isolate bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 x g for 20 min. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N, and purine contents. Digestion of OM, neutral detergent fibre and acid detergent fibre were calculated as described by Stern and Hoover (1990).

2.4. Chemical Analyses

Effluent DM was determined by lyophilizing 300 ml aliquots in triplicate. The DM content of diets and bacterial samples was determined by drying samples for 24 h in a 103°C forced air oven (AOAC, 1990; method 950.01). Dry samples of diets, effluents and bacteria were ashed overnight at 550°C in a muffle furnace (AOAC, 1990; method 942.05), and OM was determined by difference. Neutral detergent fibre components of diets and effluents were analyzed sequentially (Van Soest et al., 1991) using a heat stable alpha-amylase and sodium sulfite, and expressed without residual ash (aNDFom), and acid detergent fibre expressed exclusive of residual ash (ADFom). Total N of diets, effluents and bacterial samples was determined by the Kjeldhal method (AOAC, 1990; method 976.05). Sample CP was calculated as N x 6.25.

To determine TCA-N, 4 ml of a 500 g/l TCA solution were added to 16 ml of filtered fermenter fluid. After 4 h at 5ºC, tubes were centrifuged at 9,000 x *g* for 15 min. The supernatant was stored and frozen until analysed for TCA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05). To determine TA-N, 4 ml of a 100 g/l sodium tungstate solution and 4 ml of 1.07 N sulphuric acid were added to 16 ml sample of filtered fermenter fluid. After 4 h at 5ºC, tubes were centrifuged at 9,000 x *g* for 15 min. The supernatant was stored and frozen until it was analysed for TA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05). Ammonia-N was analyzed by colorimetry (Chaney and Marbach, 1962), where 4 ml of a 0.2 N HCl solution were added to 4 ml of filtered rumen fluid and frozen. Samples were centrifuged at 3,000 x *g* for 20 min, and the supernatant was used to determine ammonia-N by spectophotometry (Libra S21, Biochrom Technology, Cambridge, UK). Results were used to calculate large peptides (LPep = TCA-N − TA-N) and small peptides plus amino acids (SPep = TA-N − ammonia-N; Winter et al., 1964).

Samples for VFA analysis were prepared as described by Jouany (1982) and analyzed by gas chromatography: 1 ml of a solution made up of a 2 g/l solution of mercuric chloride, 2 g/l of 4-methylvaleric acid as an internal standard, and 20 g/l orthophosphoric acid, was added to 4 ml of filtered rumen fluid and frozen. Samples were centrifuged at 15,000 x g for 15 min, and the supernatant was analyzed by gas chromatography (model 6890, Hewlett Packard, Palo Alto, CA, USA) using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Bucks, UK).

2.5. Statistical Analyses

All statistical analyses were conducted using SAS (version 9.2 SAS Institute, Inc., Cary, NC). For the batch culture and the dual flow continuous culture, results of VFA concentration, N fractions, nutrient digestion and flows were analyzed using the PROC MIXED procedure. The model accounted for the effects of treatments (fixed) and the period was considered a random effect. The significance of differences between means of treatments and CTR was tested using the Dunnett option and declared at $P < 0.05$.

3. Results

3.1. Polyclonal Antibodies Production

Among bacteria, *Clostridium sticlandii* did not grow successfully and the immunization protocol was not conducted for this bacteria. Concentrations of bacteria in the culture medium were: *Prevotella ruminicola*: 5.1×10^8 cells/ml, *Clostridium aminophilum*: 2.7×10^9 cells/ml, *Peptostreptococcus anaerobius*: 7.6×10^8 cells/ml.

Figures 1-3 present results of the ELISA analysis. For each antibody are presented results of the hyper-immunized serum (bl3; a). Moreover, the responses to the particular antigen of hyper-immunized serum of rabbits immunized with the other two antigens (b) are presented to detect cross reactivity. All PAbs were evaluates also in pre-immunized blood samples, and the responses were below detectable limits; thus, the results are not shown. For APr (Figure 1a) the responses of the corresponding rabbits (rb4, rb5 and rb6) were elevated compared with pre-immunized serum, indicating a strong reaction against the antigen. The rest of the rabbits did not recognize the antigen, which demonstrates that there were no cross reactivity between APr and APa or ACl (Figure 1, b). For APa (Figure 2a) the responses of the corresponding rabbits were not uniform. The best response was of rb8 and rb9, followed by rb7. However, in the following studies a mix of the three animals was used. Two of the rabbits immunized against *C. aminophilum* (rb11 and rb12) recognized the antigen (Figure 2, b), indicating cross reactivity for ACl and APa. For ACl (Figure 3), corresponding rabbits (rb10, rb11 and rb12) had a strong response against the antigen. However, rabbits immunized against *P. ruminicola* and *P. anaerobius* recognized the antigen, indicating cross reactivity between ACl and APr, and ACl and APa.

3.2. Experiment 1

The addition of monensin decreased gas production in the soybean and tryptone incubations (Figure 4). Performing the incubations in a N-free media, and with an excess of rapidly fermentable carbohydrates, N becomes the limiting factor to microbial growth, and so gas production reflects the availability of N from feed (Cone *et al.*, 2009). Therefore, the reduced gas production indicates a reduced protein degradation caused by monensin addition. The reduction of gas produced due to the addition of monensin reflects the well-known effect of this ionophore on deamination (Chen and

Russell, 1989, 1990). Therefore, this method is suitable to assess the effect of different additives on protein degradation and deamination of feedstuffs.

Figure 5 presents results of PAbs addition in three doses on gas production using soybean meal as protein source. The addition of antibodies did not affect gas production at the end of the fermentation or at any of the individual hours of fermentation.

In the batch culture, the addition of PAbs caused minor effects on pH, total VFA or molar proportions of VFA but the effect was not always consisted within hours or doses (Tables 1 and 2). For example, the molar proportion of propionate was lower compared with control at 12 h of incubation when a low dose was tested, but at 24 h of incubation the effect was not present. Moreover, the same treatment at the high dose caused the reverse effect at 2 and 12 h of incubation, where propionate molar proportion was higher than control, but also at 24 h the effect was not present. The small standard error observed in the current study made statistically significant small numerical differences of the variables tested.

3.3. Experiment 2

Table 3 shows effects of PAbs addition on hour by hour concentration of N fractions in a dual flow continuous culture system measured after the morning feeding. The addition of PAbs did not affect concentrations of LPep, SPep and ammonia-N in any of the hours tested. The concentration of ammonia-N in the 24 h effluents varied from 7.31 (CTR) to 7.91 (APa) mg/100 ml without any significant differences among treatments. Similarly, true digestibility of OM and CP, aNDFom and ADFom digestibility and EMPS were not affected by treatments. Total VFA concentrations and VFA profile at 2h post feeding and at the 24 h effluent were not affected by treatments (Table 4).

4. Discussion

Results of ELISA analysis demonstrated the successful antigen-antibody complex of PAbs produced. Cross reactions occurred for ACl and APe, where immunized rabbits recognized slightly each other antigen. These two bacteria, *C. aminophilum* and *P. anaerobius*, belong to the same group of rumen bacteria, the HAP (Chen & Russell, 1988, 1989; Paster *et al.*, 1993; Russell *et al.*, 1988, 1991). The phylogeny analysis indicated 81.5% similarity between these two bacteria (Paster *et al.*, 1993) which may explain the observed cross reactions.

Polyclonal antibodies did not affect microbial fermentation in short term fermentations as demonstrated by results of the 24 h batch culture and the modified gas production technique. Similarly, results of the dual flow continuous culture study indicated that PAbs did not affect microbial fermentation in long term fermentation.

Few studies have been conducted using PAbs as a strategy to manipulate rumen microbial population (DiLorenzo et al., 2006; Blanch et al., 2009). In the current study, the lack of effects may be attributed to several factors, including the dose used. It is difficult to determine the adequate doses because the exact concentration of PAbs in serum or egg yolk is difficult to define. DiLorenzo et al. (2006) obtained PAbs against *Streptococcus bovis* and *Fusobacterium necrophorum* from chicken egg yolk. The same PAbs preparation was tested by DiLorenzo et al. (2006, 2008) and Blanch et al. (2009). There are two potential was to calculate the concentration of PAbs administrated:

(i) Preparations of PAbs contained 2 ml of egg protein in 2.5 ml of the preparation. The concentration of PAbs in the preparation was 10^{18} molecules / ml of egg protein (DiLorenzo et al., 2006). Taking into account that 1 mole equals $6.02*10^{23}$ molecules

(Avogadro constant) and that the molecular weight of IgY is around 180 kDa the following formula was used to estimates the amount of antibodies per ml of the preparation:

$$g \text{ PAbs / ml preparation} = (\text{molecules/AvogadroConstant}) * MW,$$

$$g \text{ PAbs / ml preparation} = [10^{18}/(6.02*10^{23})]*180*10^3 = 29.9 * 10^{-2},$$

$$mg \text{ PAbs / ml preparation} = 299$$

DiLorenzo et al. (2006, 2008) administrated 7.5 ml/d of the preparation to steers of 505 $\pm$ 85 kg of BW, and Blanch et al. (2009) administrated 10 ml of the same PAbs preparation to heifers of 452 $\pm$ 20 kg of BW. Considering a rumen volume of 100 l and a dilution rate at 0.10 h^{-1}, the estimated rumen fluid flow through the rumen is around 240 l/day. Therefore, the estimated rumen concentration of PAbs preparation was approximately 7.5 to 9.9 mg / l for Dilorenzo et al. (2006) and Blanch et al. (2009), respectively.

(ii) An alternative method of calculating the concentration of antibodies is to consider that one egg contains 100 to 150 mg of antibodies (Mine and Kovacs-Nolan, 2002) and an egg contains approximately 3.3 g of yolk protein; then the concentration of antibodies is 30-45 mg / g of egg protein. A mean density of proteins is 1.35 g / ml (Fischer et al., 2004). Thus, 2.5 ml of PAbs preparations contain 81 − 121 mg of PAbs, or 32.4 - 48.4 mg of PAbs / ml of preparation. Considering a rumen volume of 100 l and a dilution rate at 0.10 h^{-1}, the estimated rumen fluid flow through the rumen is around 240 l/day. Therefore, the estimated rumen concentration of PAbs preparation was 1.5 and 2.0 mg/l for Dilorenzo et al. (2006) and Blanch et al. (2009), respectively.

In the current study PAbs were produced in rabbit's serum. The concentration of IgG in rabbit serum is 5 – 10 mg / ml (Farrell et al., 2004). Doses in the current study were: 0.5- 1.0, 5.0-10.0 and 50-100 mg/l for Low, Medium and High doses, respectively, in the gas production; 0.5- 1.0 and 5.0-10.0 mg/l for Low and High dose, respectively, in the batch culture; and 5-10 mg/l in the dual flow continuous culture. The exact amount of PAbs against target bacteria in not known because purification of the specific PAbs was not performed. However, the estimated doses were higher than the studies of Dilorenzo et al. (2006) and Blanch et al. (2009). Therefore, inadequate dosage should be excluded as a limiting factor.

In the current study PAbs were produced utilizing the entire bacteria as antigens. Similar strategy is frequently followed for the immunization against bacteria (Shimizu et al., 1988; Wright et al., 2004). Moreover, formaldehyde was used to inactivate bacteria. Formaldehyde is a strong bactericidal that pass bacterial cell wall, causing structural and functional changes with resultant lysis of the cytoplasmatic membrane and release of cellular contents (Deyner 1995; McDonell and Russell, 1999). The PAbs are produced when different lymphocytes are activated by the same antigen producing different antibodies against it (Lipman et al., 2005). Selecting the entire bacteria as an antigen and utilizing formalin for inactivation increased further the complexity of the antigen and provides numerous epitopes for antibody production. Produced PAbs may bind with a variety of epitopes that their function might not be fundamental for the blockage or the death of the bacteria itself or could be targeting intracellular components that in a real situation, where the bacteria is alive, would be impossible to block. Therefore, even though the antigen-antibody complex was successful, the corresponding PAbs may not cause the neutralization of the targeting bacteria.

Another factor that might explain the ineffectiveness of PAbs is the interaction between the effects of PAbs and the rumen microbial ecosystem. The diversity of bacteria in the rumen is well documented (Stewart et al., 1997). Molecular technologies improved our knowledge on rumen microbiome and demonstrated the DNA similarities among species and strains (McSweeney et al., 2007; Wright and Klieve, 2011). Paster et al. (1993) and Attwood et al. (1998) utilizing analyses of 16S rRNA sequences of HAP bacteria reported similarities not only among HAP, but also among isolated HAP and other ruminal bacteria that do not utilize amino acids as energy source and, therefore, are not considered ammonia producers. One of the main functions of PAbs is that they can bind and neutralize multiple epitopes. Moreover this function is considered a benefit compared with monoclonal antibodies (Newcombe and Newcombe, 2007). However, the rumen microbial environment is very diverse and similarity among bacteria may make this property of multi-targeting a disadvantage. As demonstrated by ELISA, weak cross-reactions were observed for *C. aminophilum* and *P. anaerobius*. Perhaps, PAbs bind also other bacteria similar to our targets minimizing the potential effects on target populations of bacteria, or the action of the target bacteria may be taken over by others, minimizing the effect. However, the use of PAbs as tested in the present experiment did not affect microbial fermentation.

It remains to be demonstrated if the observed lack of results should be attributed to the lack of effectiveness of the produced PAbs or to the reaction of the microbial ecosystem in counterbalancing the effects of these PAbs. The addition of PAbs in pure cultures of the specific bacteria would validate their effectiveness, and PCR analysis of the fermenters microbial population study would provide more information of their effect on microbial populations.

4. Conclusions

Although the production of specific polyclonal antibodies against major bacteria involved in proteolysis and deamination was successful, the supplementation of these antibodies to in vitro rumen microbial fermentation of protein was not modified. Lack of effect can be attributed to the lack of effectiveness of the produced PAbs or to the reaction of the microbial ecosystem in counterbalancing the effects of these PAbs.

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Table 1. The effect of polyclonal antibody preparations against *Prevotella ruminicola* (APr), *Clostridium aminophilum* (ACl) and *Peptostreptococcus anaerobius* (APa) and a mix of them (1:1:1; AMix) compared with control (CTR; serum of no immunized animals) at a low dose (0.005 ml serum / 50 ml of medium) on ruminal fermentation in *in vitro* short term fermentation.

	CTR	APr	ACl	APa	AMix	SEM[4]	P-value[5]
3 hours							
pH	6.93	6.88^y	6.88^y	6.91	6.89^y	0.036	0.03
NH$_3$ (mg/100ml)	7.2	7. 0	7.4	8.7	8.4	2.33	NS
Total VFA[1] (mM)	53.9	55.1	57.8	54.3	56.1	9.96	NS
VFA (mol/100mol):							
Acetate	67.0	67.2	67.0	67.0	67.0	0.63	NS
Propionate	17.9	18.0	18.0	17.8	17.9	0.14	NS
Butyrate	9.1	9.0	9.1	9.1	9.1	0.57	NS
Valerate	1.63	1.60	1.60	1.59	1.62	0.026	NS
BCVFA[2]	4.25	4.18	4.23	4.14	4.15	0.313	NS
A:P[3]	3.68	3.68	3.64	3.68	3.65	0.0517	NS
12 hours							
pH	6.97	6.88	6.88	6.95	6.94	0.071	NS
NH$_3$ (mg/100ml)	25.5	25.8	25.4	25.9	27.8	2.51	NS
Total VFA[1] (mM)	75.9	80.2	78.5	78.5	80.2	10.07	NS
VFA (mol/100mol):							
Acetate	62.3	62.5	62.5	62.4	63.0	0.38	NS
Propionate	20.0	20.1	20.0	20.0	19.0^y	0.28	0.01
Butyrate	9.4	9.3	9.4	9.5	9.7	0.2	NS
Valerate	2.43	2.37	2.38	2.4	2.43	0.053	NS
BCVFA[2]	5.81	5.61	5.67^y	5.73	5.9	0.267	0.01
A:P[3]	3.11	3.1	3.13	3.13	3.32^y	0.067	0.02
24 hours							
pH	7.08	6.9	6.94	6.94	7	0.067	NS
NH$_3$ (mg/100ml)	40.5	46.9^y	44.8^y	44.2^y	44.3^y	2.91	0.03
Total VFA[1] (mM)	86.9	83.9	91.6	91.7	90.5	14.68	NS
VFA (mol/100mol):							
Acetate	60.8	62.0	60.8	61.5	61.1	0.60	NS
Propionate	19.8	19.2	19.7	19.3	19.7	0.50	NS
Butyrate	9.6	9.1	9.8	9.6	9.6	0.49	NS
Valerate	2.75	2.63	2.79	2.73	2.76	0.063	NS
BCVFA[2]	6.88	6.91	6.83	6.78	6.83	0.413	NS
A:P[3]	3.06	3.23	3.07	3.18	3.10	0.110	NS

[1] VFA: volatile fatty acids

[2] BCVFA: branched chain volatile fatty acids

[3] A:P: acetate to propionate ratio

[4] SEM: standard error of the mean

[5] NS: no significant

[y] Means in the same row with different superscript differ significantly from control (P<0. 05).

Table 2. The effect of polyclonal antibody preparations against *Prevotella Ruminicola* (APr), *Clostridium aminophilum* (ACl) and *Peptostreptococcus anaerobius* (APa) and a mix of them (1:1:1; AMix) compared with control (CTR; serum of no immunized animals) at a high dose (0.05 ml serum / 50 ml of medium) on ruminal fermentation in *in vitro* short term fermentation.

	CTR	APr	ACl	APa	AMix	SEM[4]	P-value[5]
3 hours							
pH	6.88	6.89	6.88	6.87	6.84	0.039	NS
NH_3 (mg/100ml)	6.1	6.2	6.1	6.3	6.9	0.21	NS
Total VFA[1] (mM)	52.0	53.6	50.3	54.7	54.5	8.85	NS
VFA (mol/100mol):							
Acetate	66.8	66.9	66.7	67.0	66.8	0.31	NS
Propionate	18.1	18.2	18.3[y]	18.2	18.3[y]	0.19	0.02
Butyrate	9.2	9.1	9.1	9.2	9.1	0.41	NS
Valerate	1.63	1.60	1.60	1.59	1.62	0.026	NS
BCVFA[2]	4.25	4.18	4.23	4.14	4.15	0.313	NS
A:P[3]	3.68	3.68	3.64	3.68	3.65	0.052	NS
12 hours							
pH	6.93	6.92	6.86	6.9	6.89	0.053	NS
NH_3 (mg/100ml)	28.3	26.9	27.0	27.3	27.7	2.52	NS
Total VFA[1] (mM)	81.4	79.9	81.1	79.4	79.5	9.52	NS
VFA (mol/100mol):							
Acetate	63.2	62.9	62.9	62.8[y]	62.5[y]	0.59	0.03
Propionate	19.0	19.3	19.2[y]	19.3[y]	19.5[y]	0.57	0.01
Butyrate	9.6	9.5	9.6	9.6	9.6	0.26	NS
Valerate	2.38	2.44	2.41	2.44	2.47	0.036	NS
BCVFA[2]	5.78	5.82	5.75	5.81	5.88	0.233	NS
A:P[3]	3.33	3.27[y]	3.27[y]	3.25[y]	3.21[y]	0.127	0.01
24 hours							
pH	7	7.02	7.02	7.1	7.2	0.099	NS
NH_3 (mg/100ml)	41.4	41.3	44.6	39.9	38.1	3.17	NS
Total VFA[1] (mM)	90.0	89.2	85.0	90.6	89.4	11.70	NS
VFA (mol/100mol):							
Acetate	60.7	60.5	62.2	60.6	60.6	0.74	NS
Propionate	20.0	20.2	19.5	20.1	20.1	0.30	NS
Butyrate	9.5	9.6	9.4	9.7	9.7	0.40	NS
Valerate	2.75	2.75	2.44	2.77	2.79	0.126	NS
BCVFA[2]	6.89	6.92	6.42	6.78	6.81	0.321	NS
A:P[3]	3.00	3.00	3.20	3.01	3.01	0.090	NS

[1] VFA: volatile fatty acids

[2] BCVFA: branched chain volatile fatty acids

[3] A:P: acetate to propionate ratio

[4] SEM: standard error of the mean

[5] NS: no significant

[y] Means in the same row with different superscript differ significantly from control (P<0. 05).

Table 3. The effect of the addition of polyclonal antibody preparations[1] on ammonia nitrogen (NH_3; mg / 100 ml), small peptide nitrogen (SPep; mg / 100 ml), and large peptide nitrogen (LPep; mg / 100 ml) at 0, 2, 4 and 6 hours post feeding in a dual flow continuous culture system.

	CTR	APr	ACl	APa	SEM[2]	P-value[3]
0 hours post feeding						
NH_3	4.7	6.1	5.3	6.3	0.74	NS
SPep	5.6	7.0	3.6	5.1	1.09	NS
LPep	4.4	4.6	7.1	5.6	1.02	NS
2 hours post feeding						
NH_3	6.5	4.8	6.5	7.6	1.22	NS
SPep	8.4	11.9	7.4	7.2	1.49	NS
LPep	5.7	1.7	4.2	5.7	1.59	NS
4 hours post feeding						
NH_3	3.5	3.0	4.0	4.2	0.95	NS
SPep	6.8	8.2	5.5	7.0	0.88	NS
LPep	5.8	5.3	5.2	4.5	1.16	NS
6 hours post feeding						
NH_3	3.2	3.8	3.8	3.7	0.79	NS
SPep	5.9	5.8	4.9	5.8	0.96	NS
LPep	5.8	6.1	5.3	4.8	1.22	NS

[1] CTR: addition of serum from no immunized animals; APr: addition of polyclonal antibodies against *Prevotella ruminicola*; ACl: addition of polyclonal antibodies against *Clostridium aminophilum*; APa: addition of polyclonal antibodies against *Peptostreptococcus anaerobius*.

[2] SEM: standard error of the mean

[3] NS: no significant

Table 4. The effect of the addition of polyclonal antibody preparations on total volatile fatty acid (VFA) and VFA profile at 2 hours post feeding and at the effluent and ammonia (NH^3) concentration of the effluent in a dual flow continuous culture system.

	CTR	APr	ACl	APa	SEM[2]	P-value[3]
2h post feeding						
Total VFA(mM)	75.3	81.3	76.6	85.5	10.86	NS
VFA (mol/100 mol)						
Acetate	47.4	35.8	35.0	34.6	8.17	NS
Propionate	22.5	24.2	25.5	31.4	3.56	NS
Butyrate	24.2	31.0	31.5	28.5	7.12	NS
Valerate	4.37	7.52	5.98	3.60	1.729	NS
i-butyrate	0.50	0.52	0.64	0.68	0.119	NS
i- valerate	1.00	0.89	1.17	1.21	0.274	NS
BCVFA[4]	1.50	1.42	1.81	1.90	0.335	NS
24 h effluent						
NH_3 (g/100ml)	6.8	7.0	6.9	7.4	1.15	NS
Total VFA(mM)	70.6	75.4	78.1	81.4	5.09	NS
VFA (mol/100 mol)						
Acetate	41.3	42.4	43.2	43.2	5.55	NS
Propionate	24.6	22.7	22.0	26.1	2.53	NS
Butyrate	26.7	26.7	27.2	25.0	5.65	NS
Valerate	5.04	6.77	5.45	3.41	1.632	NS
i-butyrate	0.78	0.52	0.74	0.83	0.086	NS
i- valerate	1.42	0.91	1.26	1.34	0.262	NS
BCVFA[4]	2.21	1.43	1.99	2.17	0.300	NS

[1] CTR: addition of serum from no immunized animals; APr: addition of polyclonal antibodies against *Prevotella ruminicola*; ACl: addition of polyclonal antibodies against *Clostridium aminophilum*; APa: addition of polyclonal antibodies against *Peptostreptococcus anaerobius*.

[2] SEM: standard error of the mean

[3] NS: no significant

[4] BCVFA: branched chain volatile fatty acids

Figure 1. Polyclonal antibodies response against *Prevotella ruminicola*: (a) specific response of rabbits 4, 5 and 6 (rb4, rb5 and rb6, respectively) in hyper-immune serum (bl3); (b) cross reactivity in serum from rabbits immunized against *P. anaerobius* (rb7, rb8 and rb9) and *C. aminophilum* (rb10, rb11 and rb12).
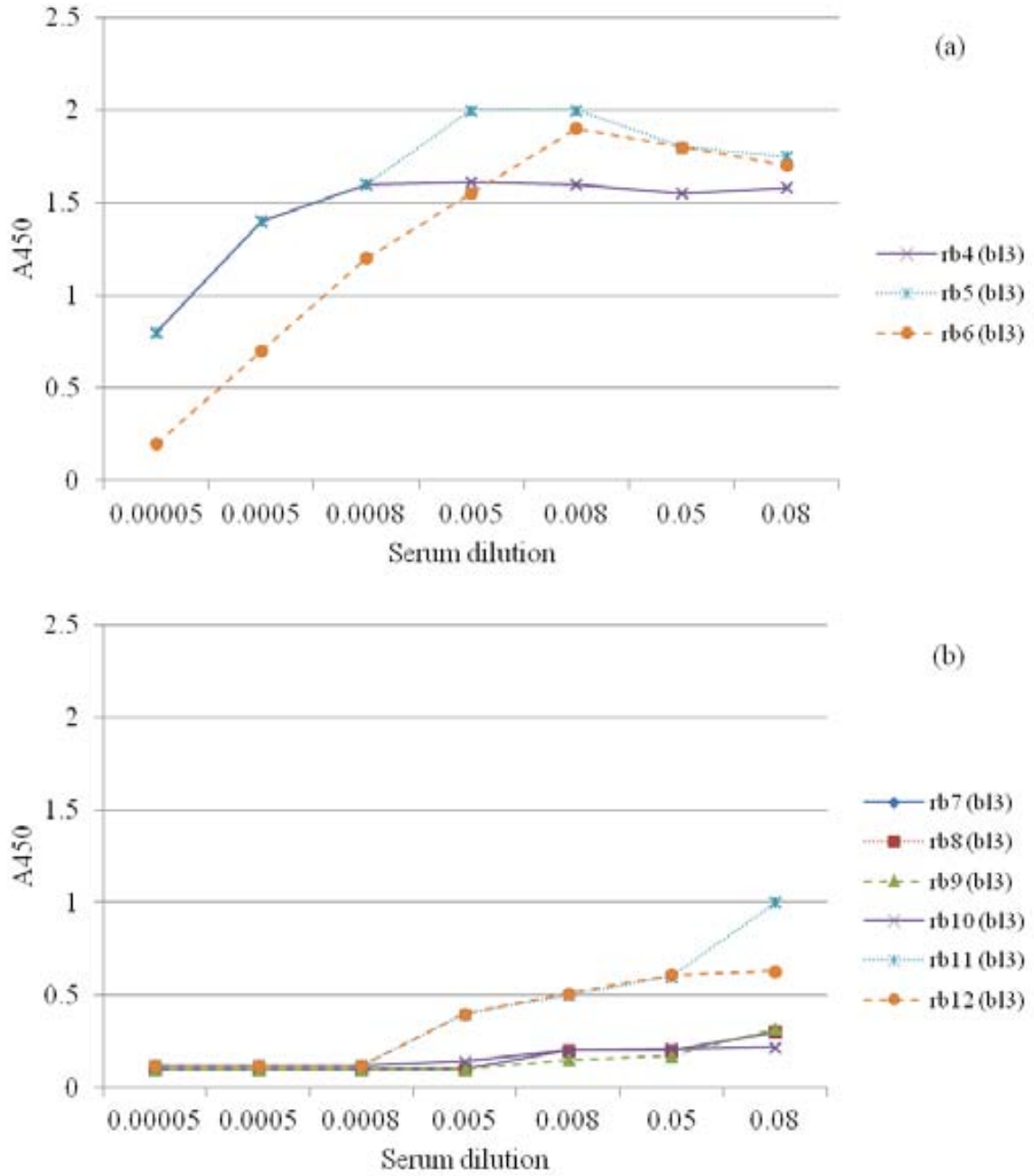
Figure 2. Polyclonal antibodies response against *Peptostreptococcus anaerobius*: (a) specific response of rabbits 7, 8 and 9 (rb7, rb8 and rb9, respectively) in hyper-immune serum (bl3); (b) Cross reactivity in serum from rabbits immunized against *P. ruminicola* (rb4, rb5 and rb6) and *C. aminophilum* (rb10, rb11 and rb12).
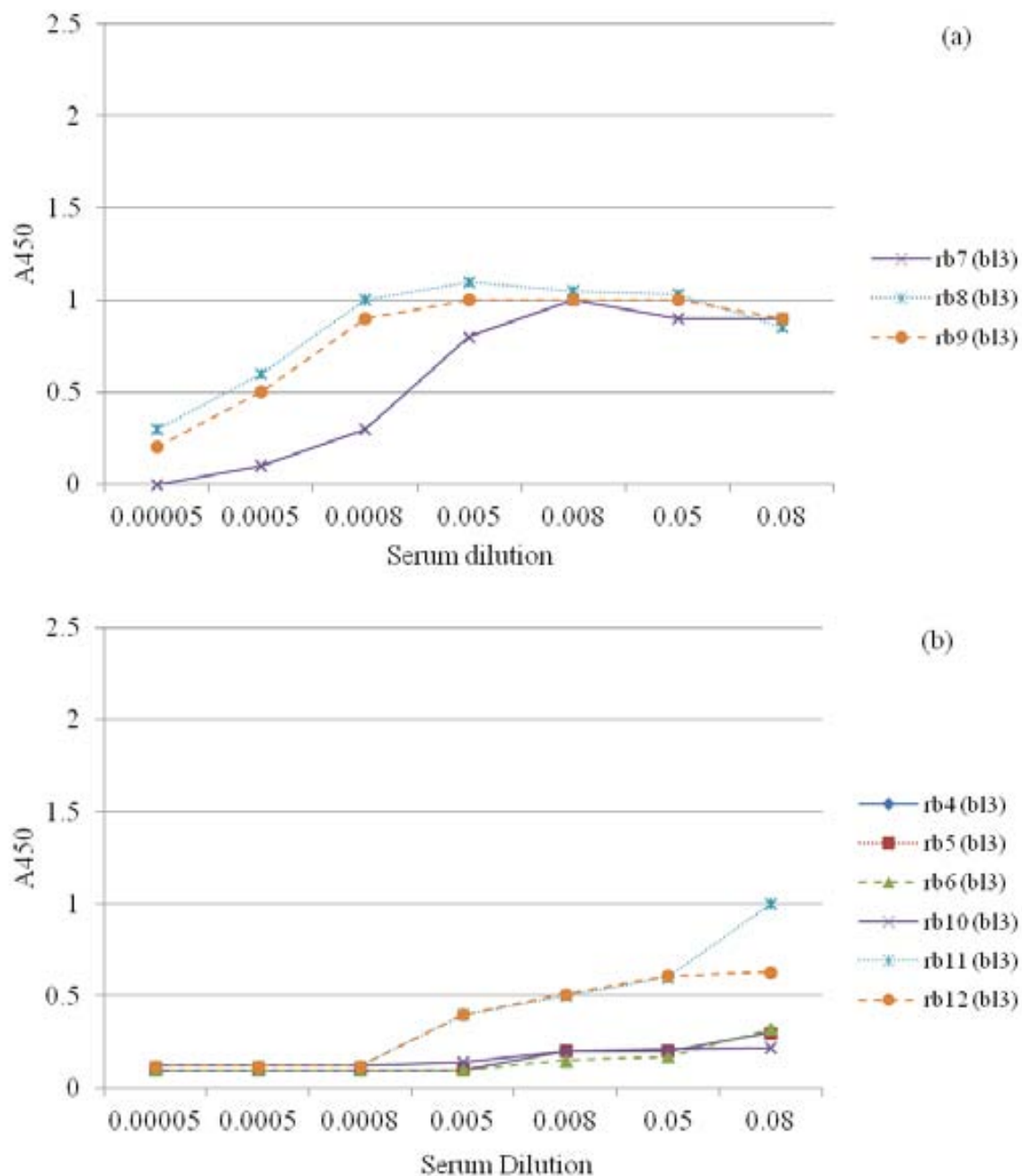
Figure 3. Polyclonal antibodies response against *Clostridium aminophilums*: (a) specific response of rabbits 10, 11 and 12 (rb10, rb11 and rb12, respectively) in hyper-immune serum (bl3); (b) Cross reactivity in serum from rabbits immunized against *P. ruminicola* (rb4, rb5 and rb6) and *P. anaerobius* (rb7, rb8 and rb9).
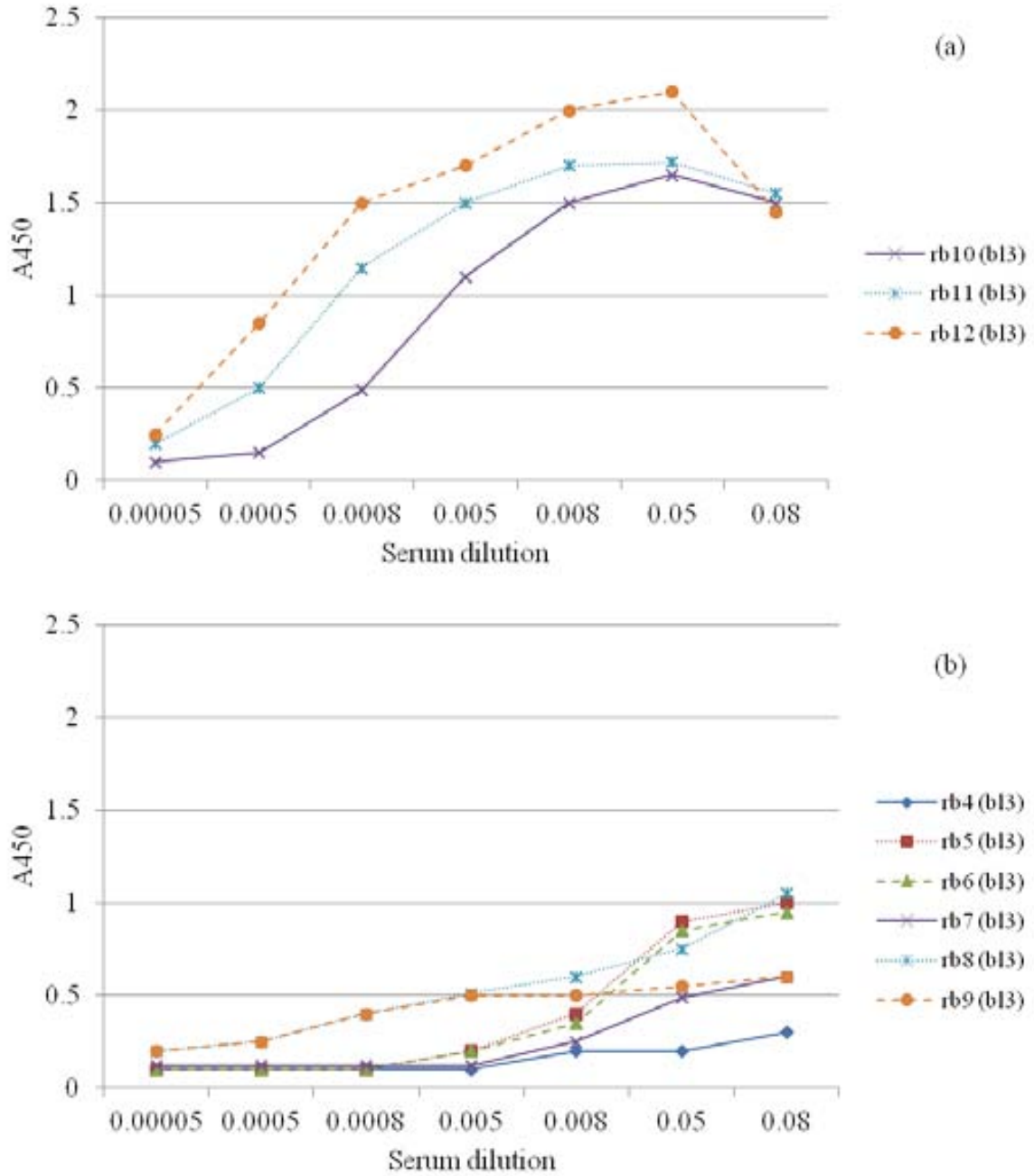
Figure 4. The effect of monensin addition on protein degradation of soybean meal and tryptone (SNT: soybean meal no treated, SM: soybean meal with monensin, TNT: no treated, TM: tryptone with monensin).
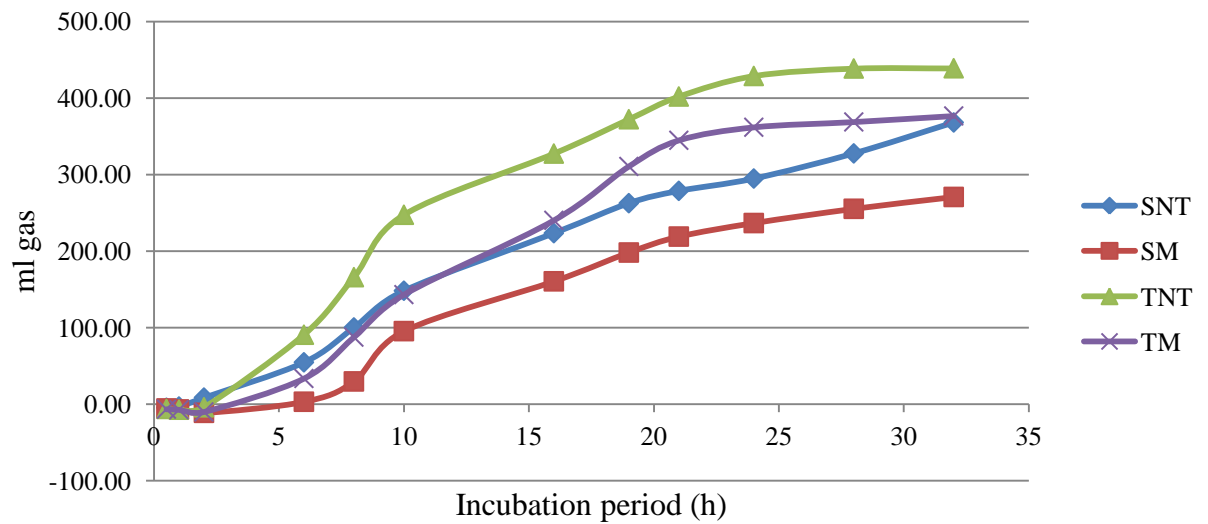
Figure 5. The effect of antibody addition in three doses on gas production profiles of soybean (CTR0: no addition, CTRL, CTRM, CTRH: addition of serum of no immunized rabbits in a low, medium and high dose; APrL, APrM, APrH: addition of serum of immunized rabbit against *P. ruminicola* in a low, medium and high dose; AClL, AClM, AClH: addition of serum of immunized rabbit against *C. aminiphilum* in a low, medium and high dose; APaL, APaM, APaH: addition of serum of immunized rabbit against *P. anaerobius* in a low, medium and high dose; Doses: 0.005, 0.05 and 0.5 ml serum / 50 ml of medium for low, medium and high, respectively)
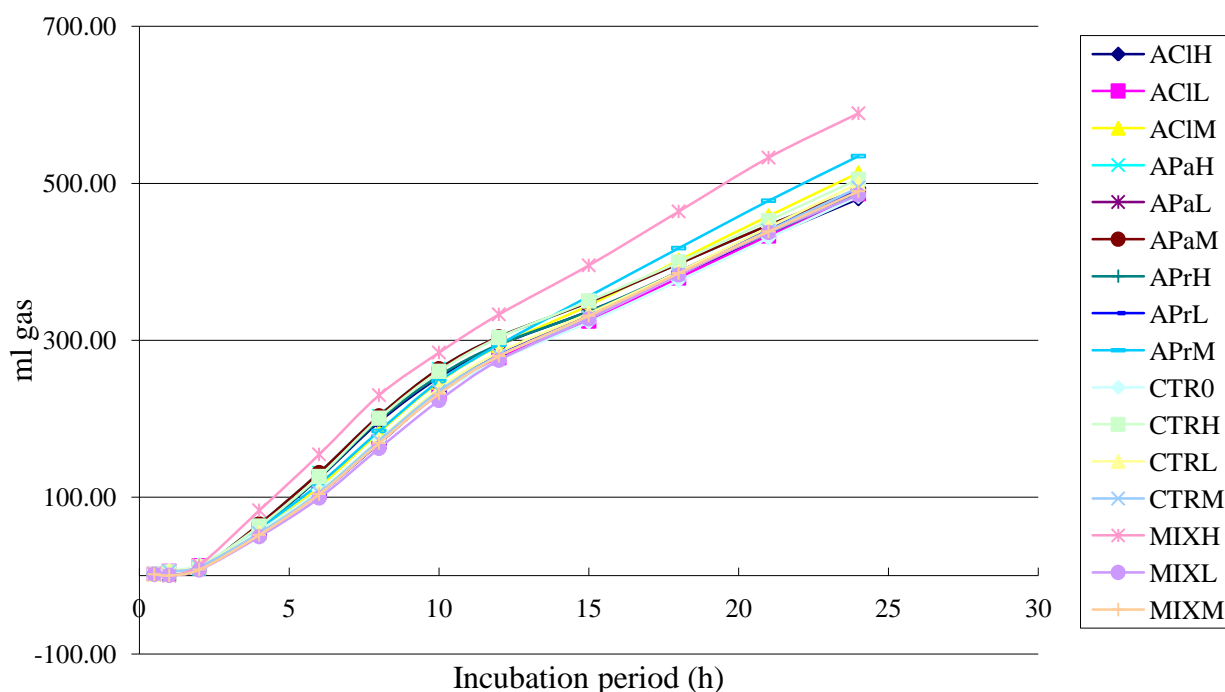
Chapter 5

Effects of a garlic oil chemical compound,

propyl-propylthiosulphonate (PTSO), on rumen microbial

fermentation in a dual flow continuous culture system.

Abstract

The ban on the use of antibiotics as growth promoters in animal feeds in the European Union has stimulated the research on potential alternatives. Garlic oil and its active components reduced the concentrations of acetate, branch-chained volatile fatty acid (BCVFA) and ammonia-N, and increased that of propionate and butyrate. Recently, propyl-propylthiosulfonate (PTSO), a stable organosulfurate compound of garlic, was purified and its antimicrobial activity was tested on the gastrointestinal microbiota of pigs. The objective of the current study was to investigate the potential effects of PTSO on ruminal microbial fermentation and to define effective doses. Two experiments were conducted using dual flow continuous culture fermenters in two replicated periods for each experiment. Each experimental period consisted of 5 d for adaptation of the ruminal fluid to treatments and 3 d for sampling. Temperature (39ºC), pH (6.4), and liquid (0.10 h^{-1}) and solid (0.05 h^{-1}) dilution rates were maintained constant. During the last 3 days, samples were taken at 2 h after the morning feeding and from the 24 h effluent. Samples were analyzed for VFA, ammonia-N, large peptide (LPep), small peptides (SPep) and digestibility of organic matter (OM), crude protein (CP), neutral detergent fibre (aNDFom) and acid detergent fibre (ADFom). In experiment 1 treatments included a negative control without additive (CTR), a positive control with monensin at 12 mg/l (MON) and two doses of PTSO at 30 mg/L (PTSO30) and 300 mg/L (PTSO300). The addition of PTSO30 did not affect any of the measurements. The PTSO300 decreased dramatically the concentration of total VFA in the effluent, reduced true digestibility OM and digestibility of aNDFom and ADFom, indicating a strong antimicrobial activity and the inhibition of microbial fermentation. Experiment 2 was conducted to test increasing doses of PTSO (0, 50, 100 and 150 mg/l) on rumen microbial fermentation. Total VFA and propionate molar proportion responded

quadratically with higher values in the intermediate doses. Butyrate increased and BCVFA decreased linearly with increasing doses of PTSO, and concentrations of ammonia-N, LPep and SPep were not affected by treatments. In the samples from the 24-h effluents, only the total VFA and BCVFA concentrations responded quadratically and linearly with increasing dose of PTSO, respectively. Digestibilities of OM, CP, aNDFom and ADFom were not affected by treatments. Results suggest the potential of PTSO to modify rumen fermentation in a direction consistent with better energy utilization in a effective dose between 50 and 100 mg/l.

1. Introduction

The ban on the use of antibiotics as growth promoters in animal feeds in the European Union has stimulated the research on potential alternatives. Among them, essential oils (EO) seem promising because of their antimicrobial properties (Cowan, 1999). Garlic oil is a complex mix of many different compounds present in the plant or derived from processing with antimicrobial activity against a wide spectrum of bacteria (Calsamiglia et al, 2007). However, most of the active compounds of garlic oil are not in the whole plant, like most other essentials oils, but are produced from thiosulfates during the steam treatment of the plant (Pentz and Siegers, 1996). Several in vitro fermentation trials with rumen fluid reported that garlic oil reduced the concentrations of acetate, branch-chained volatile fatty acid (BCVFA), and ammonia-N, and increased concentrations of propionate and butyrate (Cardozo et al., 2004; Busquet et al. 2005a,b,c, 2006).

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Busquet et al. (2005b) investigated the effects of garlic oil and four of its main active compounds and found that diallyl disulphide and allyl mercaptan were the major responsible for its action. Recently, two stable organosulfurate compounds of garlic was have been purified: propyl-propylthiosulfinate (PTS) and propyl-propylthiosulfonate (PTSO). Both compounds are structurally similar and only differ in the presence of one more oxygen function in PTSO. The main difference between these two compounds is the lower polarity and volatily of PTSO (patent number: US2010/0035984 A1). Their antimicrobial effects were tested in the gastrointestinal microbiota of pigs and PTSO demonstrated a stronger antimicrobial activity than PTS against main microbial groups as well as against *Eschericichia coli* and *Salmonella typhimurium* (Ruiz et al., 2010). However, their effects on ruminal microbial environment remain to be demonstrated. The objective of the current study was to investigate potential effects of PTSO addition on ruminal microbial fermentation in a dual flow continuous culture system.

2. Materials and Methods

2.1. The Dual Flow Continuous Culture Fermenter

Eight 1,320 ml dual flow continuous culture fermenters developed by Hoover et al. (1976) were used in two replicated periods. Each experimental period consisted of 5 d for adaptation of the ruminal fluid to treatments and 3 d for sampling.

On the first day of each period, undiluted ruminal fluid taken from a cow fed a 600 g/kg forage and 400 g/kg concentrate diet and filtered through two layers of cheesecloth to remove large feed particles was inoculated into fermenters. Fermentation conditions were maintained constant with a temperature of 39ºC, and pH at 6.4 ± 0.05 controlled by infusions of 3 N HCl or 5 N NaOH, and monitored by a computer and a Programmable Linear Controller (FieldPoint, National Instruments, TX). Anaerobic

conditions were maintained by the infusion of N_2 gas at a rate of 40 ml/min. Artificial saliva (Weller and Pilgrim, 1974) was continuously infused into flasks and contained 0.4 g/l of urea to simulate recycled N. Liquid and solid dilution rates were set at 0.10 and 0.05 h^{-1}, respectively.

2.2. Experimental Diets and Treatments

2.2.1. Experiment 1

All fermenters were fed 95 g/d of DM of a diet formulated to meet or exceed current nutrient recommendations for lactating dairy cows (170 g/kg CP, 270 g/kg aNDFom, 154 g/kg ADFom; NRC, 2001) in three equal portions at 0700, 1500 and 2300 h. The diet (DM basis) consisted of alfalfa hay (237 g/kg), corn silage (305 g/kg), ground corn grain (296 g/kg), soybean meal (154 g/kg) and a vitamin and mineral mixture (8 g/kg). The vitamin and mineral mixture contained per kg of DM: 300 g of MgO; 267 g of urea; 33 g of sulphur; 67 g of NaCl; 4,660 mg Zn; 2,660 mg Mn; 167 mg Cu; 27 mg Se; 33 mg I; 7 mg Co; 1,000 KIU of vitamin A; 200 KIU of vitamin D3; and 1,330 mg of vitamin E.

Treatments included a negative control without additive (CTR), a positive control with monensin at 12 mg/l (MON; Sigma-Aldrich Chemical, St. Louis, MO) and two doses of PTSO at 30 mg/l (PTSO30) and 300 mg/l (PTSO300). Treatments were incorporated directly into the fermenter fluid 1 min before each feeding. Daily doses of PTSO30, PTSO300 and MON were dissolved in 1.2 ml of ethanol and fermenters with the CTR treatment were also supplied with 1.2 ml of ethanol in 3 doses per day.

2.2.2. Experiment 2

All fermenters were fed 95 g/d of DM of a diet formulated to meet or exceed current nutrient recommendations for lactating dairy cows (174 g/kg CP, 302 g/kg

aNDFom, 191 g/kg ADFom; NRC, 2001) in three equal portions at 0700, 1500 and 2300 h. The diet (DM basis) consisted of alfalfa hay (346 g/kg), corn silage (213 g/kg), ground corn grain (315 g/kg), soybean meal (120 g/kg) and the same vitamin and mineral mixture (8 g/kg) as in experiment 1.

Treatments included a control without additive (PTSO0), and PTSO at 50 mg/l (PTSO50), 100 mg/l (PTSO100), and 150 mg/l (PTSO150). Treatments were incorporated directly into the fermenter fluid 1 min before each feeding. Daily doses of PTSO50, PTSO100 and PTSO150 were dissolved in 1.2 ml of ethanol and fermenters with the CTR treatment were also supplied with 1.2 ml of ethanol in 3 doses per day..

2.3. Sample Collection

During the last three days, 40 ml of filtered fermenter fluid were taken 2 h after the morning feeding to determine ammonia-N and VFA concentration, trichloroacetic acid soluble N (TCA-N), and tungstic acid soluble N (TA-N). Results were used to calculate large peptides (LPep = TCA-N − TA-N), small peptides plus amino acids (SPep = TA-N − ammonia N), and ammonia-N concentrations in fermenters (Winter et al., 1964).

During sampling days, effluent collection vessels were maintained at $4^{o}C$ to prevent microbial activity. Solid and liquid effluents were mixed and homogenized for 1 min at 24000 rpm (Diax900, Heidolph, Nurnberg, Germany), and a 500 ml sample was removed by aspiration and frozen at -20ºC. Upon completion of each period, effluents from the three sampling days were composited and mixed within fermenter, and homogenized for 1 min. Subsamples were taken for total N, ammonia-N, VFA, TCA-N, and TA-N analyses. The remainder of the sample was lyophilized. Dry samples were analyzed for dry matter (DM), ash, neutral detergent fibre (aNDFom), acid detergent fibre (ADFom), and purine contents.

Bacterial cells were obtained from fermenter flasks the last day of each experimental period. Solid and liquid associated bacteria were isolated using a combination of several detachment procedures (Whitehouse et al., 1994) selected to obtain the maximum detachment without affecting cell integrity. One hundred milliliters of a 2 g/l methylcellulose solution and small marbles (30 of 2 mm and 15 of 4 mm of diameter) were added to each fermenter and incubated in the same fermenter flasks at 39ºC, and mixed for 1 h to remove attached bacteria. After incubation, fermenter flasks were refrigerated for 24 h at 4ºC and fermenter contents were agitated for 1 h to dislodge loosely attached bacteria. Finally, the fermenter content was filtered through cheesecloth and washed with saline solution (8.5 g/l NaCl). Bacterial cells were isolated within 4 h by differential centrifugation at 1,000 x *g* for 10 min to separate feed particles, and the supernatant was centrifuged at 20,000 x *g* for 20 min to isolate bacterial cells. Pellets were rinsed twice with saline solution and recentrifuged at 20,000 x *g* for 20 min. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N, and purine contents. Digestion of DM, OM, aNDFom, ADFom and crude protein (CP), and flows of total, non-ammonia, microbial, and dietary N were calculated as described by Stern and Hoover (1990).

2.4. Chemical Analyses

Effluent DM was determined by lyophilizing 300 ml aliquots in triplicate. The DM content of diets and bacterial samples was determined by drying samples for 24 h in a 103ºC forced air oven (AOAC, 1990; method 950.01). Dry samples of diets, effluents and bacteria were ashed overnight at 550ºC in a muffle furnace (AOAC, 1990; method 942.05), and OM was determined by difference. Neutral detergent fibre components of diets and effluents were analyzed sequentially (Van Soest et al., 1991)

using a heat stable alpha-amylase and sodium sulfite, and expressed without residual ash (aNDFom), and acid detergent fibre expressed exclusive of residual ash (ADFom). Total N of diets, effluents and bacterial samples was determined by a Kjeldhal method (AOAC, 1990; method 976.05). Sample CP was calculated as N x 6.25.

Peptide and amino acid N were determined as described by Winter et al. (1964). To determine TCA-N, 4 ml of a 500 g/l TCA solution were added to 16 ml of filtered fermenter fluid. After 4 h at 5ºC, tubes were centrifuged at 9,000 x g for 15 min. The supernatant was stored and frozen until analysed for TCA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05). To determine TA-N, 4 ml of a 100 g/l sodium tungstate solution and 4 ml of 1.07 N sulphuric acid were added to 16 ml sample of filtered fermenter fluid. After 4 h at 5ºC, tubes were centrifuged at 9,000 x g for 15 min. The supernatant was stored and frozen until it was analysed for TA-N by the Kjeldahl procedure (AOAC, 1990; method 976.05).

Ammonia-N was analyzed by colorimetry as described by Chaney and Marbach (1962), where 4 ml of a 0.2 N HCl solution were added to 4 ml of filtered rumen fluid and frozen. Samples were centrifuged at 3,000 x g for 20 min, and the supernatant was used to determine ammonia-N by spectophotometry (Libra S21, Biochrom Technology, Cambridge, UK).

Samples for VFA analysis were prepared as described by Jouany (1982) and analyzed by gas chromatography: 1 ml of a solution made up of a 2 g/l solution of mercuric chloride, 2 g/l of 4-methylvaleric acid as an internal standard, and 20 g/l orthophosphoric acid, was added to 4 ml of filtered rumen fluid and frozen. Samples were centrifuged at 15,000 x g for 15 min, and the supernatant was analyzed by gas chromatography (model 6890, Hewlett Packard, Palo Alto, CA, USA) using a

polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, SGE, Europe Ltd., Bucks, UK).

Samples of lyophilized effluent and bacterial cells were analyzed for purine content (adenine and guanine) by HPLC as described by Balcells et al. (1992), using allopurinol as the internal standard.

2.5. Statistical Analyses

All statistical analyses were conducted using SAS (version 9.2 SAS Institute, Inc., Cary, NC). Results of VFA concentration, N fractions, nutrient digestion and flows were analyzed using the PROC MIXED procedure. The model accounted for the effects of treatment (fixed effect) and period was considered a random effect.

In experiment 1, differences between means of treatments were tested using the Tukey option, and significance was declared at $P < 0.05$. In experiment 2, orthogonal contrasts were used to analyze for linear (L), quadratic (Q) and cubic (C) responses. Differences were declared significant at $P < 0.05$.

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3. Results

3.1. Experiment 1

Table 1 shows results of total concentration and molar proportions of VFA in the effluent. As expected, MON reduced the acetate to propionate ratio, increased the molar proportion of acetate and decreased that of propionate. Moreover, MON reduced ammonia-N concentration and increased the concentration of LPep (Table 2), without affecting overall CP degradability (Table 3). However, it reduced true digestibility of OM, and digestibility of aNDFom and ADFom.

The addition of PTSO30 did not affect any of the measurements. The PTSO300 decreased dramatically the concentration of total VFA in the effluent, indicating an inhibition of microbial fermentation, and it increased the molar proportion of acetate and decreased those of propionate and butyrate. Moreover, PTSO300 increased LPep concentration 2h post feeding suggesting that it reduced peptidolysis, and increased SPep pool in the 24h effluent (Table 2). However, like MON, PTSO300 also reduced true digestibility OM and digestibility of aNDFom and ADFom (Table 3).

3.2. Experiment 2

Increasing doses of PTSO caused a quadratic response in total VFA and propionate molar proportion 2 h after feeding, with higher values in the intermediate doses. Moreover, PTSO increased linearly the butyrate molar proportion and decreased linearly that of BCVFA (Figure 1). In the samples from the 24-h effluents, only the total VFA and BCVFA concentrations responded quadratically (highest in the intermediate doses; $P < 0.02$) and linearly (decreasing with higher doses; $P < 0.01$), respectively (Table 4).

Ammonia-N concentration of the 24-h effluent ranged from 7.47 to 9.05 mg/100ml for PTSO0 and PTSO300, respectively, without any significant difference among treatments. Similarly, concentrations of SPep and LPep were not affected by the addition of PTSO neither in the effluent nor at 2 h post feeding. The addition of PTSO did not affect true digestibility of OM and CP, aNDFom and ADFom digestibility.

4. Discussion

4.1. Experiment 1

The addition of MON was chosen to provide a positive control due to its demonstrated effects on ruminal nitrogen metabolism (Russell and Strobel, 1989). As expected, MON reduced ammonia-N and increased LPep concentration. However, it reduced the digestibility of OM, mainly due to the reduction of aNDFom and ADFom digestibility. Similar dual flow continuous culture system studies observed that supplementation of the same amount of MON also reduced aNDFom and ADFom digestion, but lower doses (1.25 mg/l) had no effect on ruminal metabolism (Busquet et al., 2005a; Castillejos et al., 2006). Other *in vitro* studies also demonstrated similar negative effects of MON on fiber digestion (Wallace et al., 1981; Russell and Strobel, 1988). However, *in vivo* studies had not had these negative effects. Russell and Strobel (1989) explained this phenomenon of the *in vitro* studies attributing it to the high sensitivity of some cellulolytic bacteria and the longer adaptation time of the *in vivo* experiments: some cellololytic bacteria, like cellulotytic ruminococci and *Butyrivibrio fibrisolvens,* were very sensitive in vitro, while others, like *Fibrobacter succinogenes*, did not demonstrate the same sensitivity. Probably the longer adaptation time of the *in vivo* experiments allows MON resistant cellulolytic bacteria to replace MON sensitive bacteria avoiding the overall decreased digestion of aNDFom and ADFom.

Busquet et al. (2005a,b) investigated the effect of garlic oil and four of its active components in *in vitro* studies. They demonstrated that a dose of 300 mg/L of garlic oil or the main active components tested was adequate to alter ruminal fermentation without affecting digestibility of nutrients. However, this was not the case in the current study. The addition of PTSO300 decreased digestibility of OM, NDF and ADF and caused a dramatic decrease of total VFA concentration. Ruiz et al. (2010) reported that

PTSO had stronger antimicrobial activity than PTS. Current results suggest that the antimicrobial activity of PTSO is stronger than garlic oil or other of its active components, and more moderate doses are required.

4.2. Experiment 2

The second experiment was conducted to define effective doses of PTSO. Results of PTSO addition on VFA molar proportions are in agreement with main effects of garlic oil and its compounds. Busquet et al. (2005a,b,c, 2006) showed in several *in vitro* fermentation trials with rumen fluid that garlic oil reduced the proportions of acetate and BCVFA, and increased the proportions of propionate and butyrate. The quadratic responces on total VFA, propionate and butyrate indicate that the most effective dose of PTSO might be between 50 and 100 mg/l. Lower concentrations had small or no effect, and higher concentrations seem to overkill ruminal bacteria. The increased propionate concentration in the intermediate doses suggests the potential of PTSO to modify rumen fermentation in a direction consistent with better energy utilization and lower methane production in the rumen. Moreover, propionate is primarily used by the animal as a precursor for glucose. Because glucose absorption from the gastrointestinal tract of ruminants is low, the role of propionate as a glucose precursor is of particular importance (Dijkstra, 1994; van Soest, 1994).

In contrast, the addition of PTSO had no effects on protein degradation. Cardozo et al. (2004) reported that garlic oil reduced ammonia-N and increased SPep concentrations in dual flow continuous culture, suggesting that deamination was inhibited. Ferme et al. (2004) using samples of Cardozo et al. (2004) reported that garlic modified the microbial population profile, reducing the population of *Prevotella* spp. (mainly *P. ruminicola* and *P. bryantii*), the most abundant proteolytic and deaminating bacterium (Falconer and Wallace, 1998). However, Busquet et al. (2005a,b) reported

only small and variable effects. In the current study, BCVFA decreased linearly in both the 24 h effluent and 2 h post feeding. The early work of Annison and Bryant (Annison, 1954; Bryant and Doestch, 1955; Allison and Bryant, 1963) indicated that the accumulation of BCVFA in the rumen was due to the oxidative deamination of branch chain amino acids, and namely of iso-valine and iso-leucine. The observed reduction of BCVFA suggests an inhibition of deamination, in spite of the lack of effect on ammonia-N and SPep.

5. Conclusions

In the first experiment a similar dose to that used in garlic oil and its main components was used, but it reduced microbial fermentation and digestion of OM, aNDFom and ADFom. Results suggested that PTSO had a stronger antimicrobial activity than garlic oil. The second experiment was conducted to identify the effective dose of PTSO. Results suggested that the most effective dose of PTSO may be between 50 and 100 mg/l, when rumen fermentation changed in a direction consistent with better energy utilization.

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Table 1. Effect of PTSO addition[1] on total VFA concentration and VFA profile of effluents in a dual flow continuous culture (Experiment 1).

	CTR	MON	PTSO30	PTSO300	SEM[2]	P value[3]
Total VFA[4] (mM)	83.5[a]	84.8[a]	84.5[a]	18.0[b]	4.72	<0.0001
VFA (mol/100mol) :						
Acetate	56.8[b]	49.6[c]	56.2[b]	79.5[a]	1.66	<0.0001
Propionate	19.5[b]	40.6[a]	20.3[b]	8.9[c]	1.91	<0.0001
Butyrate	16.0[a]	4.7[b]	16.6[a]	6.8[b]	1.86	<0.0001
Iso-butyrate	1.23	0.74	1.10	0.46	0.237	NS
Valerate	2.86	3.71	3.04	2.31	0.391	0.06
Iso-valerate	3.59[a]	0.68[c]	2.74[a]	1.96[b]	0.535	<0.001
A:P[5]	2.97[b]	1.23[b]	2.92[b]	9.53[a]	0.750	<0.0001

[1] CTR =0 mg/l; PTSO30 = 30 mg/l; PTSO300= 300 mg/l; MON= 12 mg/l

[2] SEM: standard error of the mean;

[3] NS: not significant

[4] VFA: volatile fatty acids

[5] A:P: acetate to propionate ratio

[a,b,c] Means in the same row with different superscript differ significantly (P<0. 05).

Table 2. Effect of PTSO addition[1] on ammonia-N, small peptide (SPep) and large peptide (LPep) concentration of effluents and 2 h post feeding in a dual flow continuous culture (Experiment 1).

	CTR	MON	PTSO30	PTSO300	SE	P value[3]
Effluent (g/100ml)						
Ammonia-N	7.35	2.62	6.34	7.34	1.551	NS
LPep-N	5.96^{ab}	3.30^b	4.96^{ab}	10.00^a	2.336	< 0.05
SPep-N	13.7^b	10.2^b	13.7^b	20.5^a	1.40	< 0.05
2 h post feeding (g/100ml)						
Ammonia-N	6.82^a	1.42^b	6.71^a	5.97^a	0.904	< 0.0001
LPep-N	0.84^b	3.76^b	3.28^b	11.47^a	1.99	< 0.001
SPep-N	11.0^{ab}	11.9^{ab}	8.0^b	15.6^a	1.47	< 0.01

[1] CTR = 0 mg/l; PTSO30 = 30 mg/l; PTSO300 = 300 mg/l; MON = 12 mg/l;

[2] SEM: standard error of the mean; NS: not significant

[3] NS: not significant

[a,b,c] Means in the same row with different superscript differ significantly (P < 0. 05)

Table 3. Effect of PTSO addition[1] on true organic matter (OM), neutral detergent fibre (aNDFom), acid detergent fibre (ADFom) and crude protein (CP) digestion in a dual flow continuous culture (Experiment 1).

	CTR	MON	PTSO30	PTSO300	SEM[2]	P-value[3]
True digestibility (g/kg)						
OM	568^a	444^b	505^a	382^c	19	0.001
aNDFom digestibility (g/kg)	222^a	13^b	153^a	195^a	53	0.07
ADFom digestibility (g/kg)	625^a	229^b	427^c	237^b	56	0.01
CP degradation (g/kg)	509	255	401	326	83	NS
EMPS[4]	24.8	18.3	27.2	20.7	3.29	NS

[1] Concentration of PTSO: CTR = 0 mg/l; PTSO30 = 30 mg/l; PTSO300 = 300 mg/l

[2] SEM: standard error of the mean

[3] NS: not significant

[4] EMPS: Efficiency of microbial protein synthesis (g bacterial N/Kg organic matter truly digested)

[a,b,c] Means in the same column with different superscript differ significantly (P<0. 05)

Table 4. Effects of increasing doses of PTSO (0, 50, 100 and 150 mg/l) on ammonia-N concentration, total volatile fatty acid (VFA) and VFA profile of the 24 h effluent in a dual flow continuous culture (Experiment 2).

	0	50	100	150	SEM[1]	P- value[2]		
						L	Q	C
NH$_3$ (mg/100ml)	7.89	8.95	9.85	11.36	1.573	NS	NS	NS
Total VFA (mM)	102.0	98.4	98.7	67.1	7.31	0.001	NS	NS
VFA (mol/100mol):								
Acetate	60.2	57.3	55.0	62.5	3.44	NS	NS	NS
Propionate	19.7	19.3	22.9	16.2	1.90	NS	NS	NS
Butyrate	11.7	15.5	15.6	14.7	2.82	NS	NS	NS
Valerate	3.22	4.26	3.78	4.70	0.956	NS	NS	NS
Iso-Butyrate	0.87	0.73	0.57	0.53	0.098	0.01	NS	NS
Iso-Valerate	4.20	2.74	2.08	1.25	0.538	0.001	NS	NS
BCFA[3]	5.41	3.49	2.67	1.81	0.613	0.001	NS	NS
A:P[4]	3.09	2.98	2.53	4.23	0.557	NS	NS	NS

[1] SEM: standard error of the mean

[2] L: linear, Q: quadratic, C: cubic responses, declared at $P < 0.05$; NS: not significant

[3] BCVFA: branched chain volatile fatty acids

[4] A:P: acetate to propionate ratio

Figure 1. Quadratic (P < 0.05) responses of total VFA (A) and propionate molar proportion (B) and linear (P < 0.05) responses of butyrate molar proportion (C) and branch-chained volatile fatty acid (BCVFA; D) to increasing doses of PTSO (0, 50, 100 and 150 mg/L) at 2 h post feeding in a dual flow continuous culture (Experiment 2).
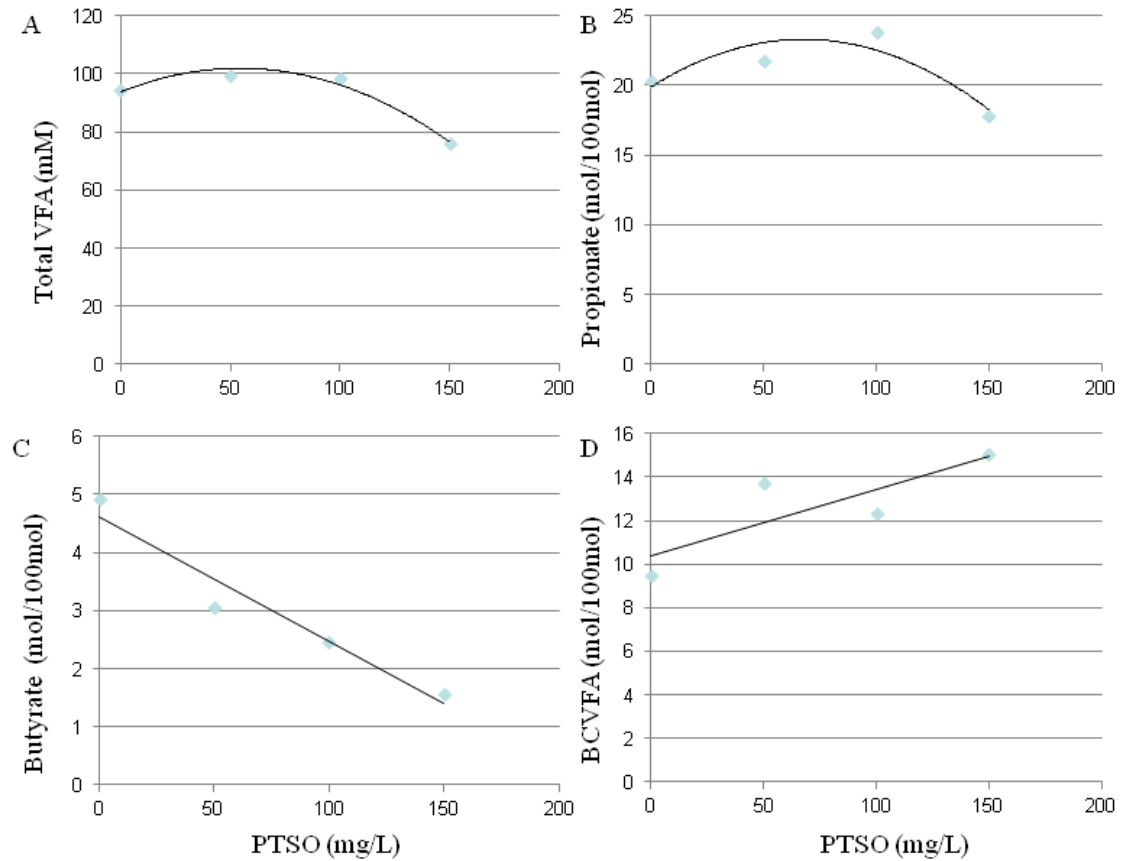
Chapter 6

General Discussion

1. Introduction

The last decades the institutionalization of environmentalism has progressed rapidly in the European Union (EU; Rootes, 2003). As a consequence, the EU dedicates scientific efforts to solve animal food production problems in an animal and environmentally "friendly" way. The N cascade is among the main environmental issues due to its impact on different ecosystems (Galloway et al., 2003) and, recently, the European Nitrogen Assessment project pointed out that agriculture, and particularly livestock production, is the main contributor to this phenomenon (Sutton et al., 2011). RedNex is an EU funded project focused on the environmental contamination with N from dairy cattle production. The current work was developed within the RedNex project; therefore our objective was to evaluate strategies that reduce N excretion from dairy cattle.

2. Theoretical Approach of the Thesis

The first step was to review the literature (chapter 1) and discuss main proposed strategies to reduce N excretion from ruminants. One of the main proposed strategies is the organic production (Halberg et al., 1995; Dalgaard et al., 1998; Khalili et al., 2002). However, a comparative analysis of organic and intensive system suggested that organic systems excrete 30% more N than intensive system for the same milk production (Jarvis et al., 2011). We should have in mind that milk is among the most important foods for human consumption and the European dairy sector one of the most important sectors of European agriculture (EC, 2010). The American dietary guidelines recommend the daily intake of 3 cups of low fat milk (1 cup = 237 ml; thus, daily intake of approximately 711 ml; USDA, 2010). Taking into account that the EU-27 population is 503 million people (Eurostat, 2011), the annual consumption of dairy products would be 130.4 million tones. The EU-27 collected 136.1 million tones of milk in 2010 (Eurostat, 2011)

suggesting a self-sufficiency of 104 %. Therefore, maintaining the high milk production of the EU is beneficial for both human health and EU economy, and in order to do so the intensive system is the most adequate way of production. However, the dairy cow is characterized by low efficiency of N utilization. Huhtanen and Hristov (2009) reported an average milk N efficiency of 24.7 and 27.7 % for North America and North Europe intensive farming, respectively, suggesting that a high portion of the dietary N (70 - 75%; Tamminga, 1992) is excreted in feces and urine. On the other hand the improvement of N efficiency has been proposed as the main key action for the reduction of N excretion from animals (Sutton et al., 2011), and the low N efficiency of dairy cattle provides room for improvement.

We investigated two different strategies to do so: the use of technology that will provide better management tools of nutrient formulation at the farm level to contribute to the so called "precision feeding", and the manipulation of ruminal protein metabolism to improve efficiency of N utilization in the rumen.

2.1. Tools to Better Manage Nutrition at the Farm Level

One of the issues identified in the literature review was that CP is overfed in dairy herds (Colmenero and Broderick, 2006; Huhtanen and Hristov, 2009) to provide a safety margin for changes in forage CP concentration (Satter et al., 2002; Firkins and Reynolds, 2005). A more precise feeding that will adjust CP diet level to animal requirements would have substantial effects on milk N efficiency (MNE; Schwab et al., 2005). Colmenero and Broderick (2006) demonstrated that an adjustment of CP concentration to animal requirements would improve MNE by 21.2%. Moreover, Jonker et al. (2002) reported that utilizing monthly milk yield and feed component analysis to reformulate diets increased MNE by 4.2% without adjusting CP concentration. Therefore, tools that facilitate feed management and formulation at the farm level are

needed. The most promising modern technology towards this direction is near infrared reflectance spectroscopy (NIRS). Its main advantage is that it can provide a cheap and fast tool to assess nutrient concentration of feedstuffs in practice. It is widely used to predict chemical composition and several parameters of nutritional interest for different feeds and forages (Andres et al., 2005), but in order to be incorporated in nutritional strategies and feed formulation models it should predict satisfactorily degradation parameters and effective degradation.

Few studies have been conducted to investigate the potential of NIRS to predict degradation kinetics of feedstuffs, and most of them have studied the degradation parameters of a particular feedstuff or a specific category of feedstuffs, mainly forages (Todorov et al., 1994; Andres et al., 2005; Ohlsson et al., 2007). Within the network of the RedNex project, it become available a large number of feedstuffs derived from different partners, making possible the utilization of a large database of diverse feedstuffs used in ruminant nutrition. In the first study of the current thesis, we investigate the potential of NIRS to predict degradation parameters and effective degradation (chapter 3).

2.2. The Manipulation of Ruminal Protein Metabolism

The rumen, and particularly the N losses in the form of ammonia, was proposed to be the most appropriate step for modification in the metabolism of proteins in dairy cattle (Tamminga, 1992, 1996). The accumulation of ammonia-N in the rumen in rates higher than that the microbes can utilize for their growth, leads to substantial losses of N from the rumen (Walker et al., 2005). Bach et al. (2005) reported a strong relationship between efficiency of N utilization in the rumen (ENU-R) and ammonia-N concentration in continuous culture studies (Ammonia-N = 43.6 − 0.469ENU; R^2 = 0.78; RMSE = 4.53). Therefore, the reduction of ruminal ammonia-N without affecting

microbial protein synthesis, and the increase of dipeptide and amino acid outflow from the rumen might be an effective strategy to improve ENU-R (Calsamiglia et al., 2010). We examined two approaches to do so: one focused on the protein fractions of silages fed to dairy cows and the other on the ruminal microbes responsible for protein degradation and deamination.

Forages have extensive protein degradation during ensiling; about 50-70% of N in silages is in the form of non-protein N (Owens et al., 2002; Slottner and Bertilsson, 2006). Therefore, feeding dairy cows with silages increases the non-protein sources in the rumen, resulting in higher accumulation of ammonia-N. Even though the enzymatic activity in the plant during ensiling is considered the main cause of protein degradation (Kemble, 1956), lactic acid producing bacteria, enterobacteria and clostridia present in silages have proteolytic activity and may contribute to the process (McDonald et al., 1991). In the current study, we conducted an experiment utilizing essential oils (EO) that inhibit proteolysis and deamination in a rumen microbial environment, to modify silages protein degradation (chapter 4).

The second approach was to target specific proteolytic and deaminating ruminal bacteria. The oral administration of polyclonal antibodies (PAbs) against bacteria involved in acidosis (*S. bovis*) reduced ruminal populations of target bacteria and increased ruminal pH of steers and heifers (DiLorenzo et al., 2006, 2008; Blanch et al., 2009). Within our group, Blanch et al. (2009) tested a PAbs preparation against *Streptococcus bovis*, *Fusobacterium necrophorum*, *Clostridium sticklandii*, *Clostridium aminophilum*, *Peptostreptococcus anaerobius* and *Escherichia coli* O157:H7 in heifers, and reported higher pH after 6, 8 and 9 days of acidosis induction of heifers fed the PAbs preparation. In the literature review, we identified main bacteria involved in ruminal protein degradation and deamination. The genus of *Prevotella* spp. is among

the most abundant bacteria in the rumen and participates in most parts of ruminal protein degradation (Walker et al., 2005). In the deamination of amino acids are mainly involved the hyper ammonia producing (HAP) bacteria and *Prevotella* spp. are the main contributors to this process (Wallace, 1996; Rychlik and Russell, 2000; Walker et al., 2005). Therefore, we produced and evaluated three PAbs against: *Prevotella ruminicola, Clostridium aminophilum* and *Peptostreptococcus anaerobius*. However, the addition of these PAbs did not affect ruminal fermentation in short or in long term *in vitro* fermentation studies (chapter 5).

As an alternative to modulate ruminal proteolytic and deaminating bacteria, several EO compounds have been tested. Within our group, a considerable research has been conducted on EO that could affect ruminal protein degradation (Calsamiglia et al., 2007), including eugenol (EUG), cinnamaldehyde (CIN), thymol (THY) and garlic oil (GAR). Recently, propyl-propylthiosulfonate, a stable organosulfurate compound of GAR, was purified and its antimicrobial effects were tested on the gastrointestinal microbiota of pigs (Ruiz et al., 2010). Therefore, we conducted an experiment to investigate its effects on ruminal fermentation utilizing a dual flow continuous culture system (chapter 6).

3. General Discussion

Within the RedNex project a integrated cow-oriented approach was followed to give answers and suggest solutions to a modern problem: the N contamination of the environment from dairy production. Our work was mainly focused in the rumen and tested three technologies and innovations: near infrared reflectance spectroscopy, polyclonal antibodies, and essential oils in both rumen and silage.

3.1. NIRS Could be Incorporated in Feed Formulation Models to Predict Degradation Kinetics of Feedstuffs

In this study we created a database of 809 different feedstuffs frequently used in ruminant nutrition. Part of these samples was analyzed for CP and DM degradation, and a smaller sample was analyzed for aNDFom degradation with the *in situ* method. Unfortunately, in our database starch degradability was not included due to the small number of available samples (n = 25). Starch forms an important element of feed evaluation and should be included in a future application of NIRS. Predictions of degradability parameters (soluble fraction, degradable fraction and the rate of degradation) and effective degradation were obtained for all samples (ALL) and by dividing feedstuffs in two main groups: forages (FF; n=256) and non-forages (NF; n=553). Most of the studies in the literature utilized small data set (n ≈100; Todorov et al., 1994; Andres et al., 2005; Ohlsson et al., 2007). In contrast, Nordheim et al. (2007) utilized a large number of samples (n=382) to predict degradation parameters, but it was feedstuff specific including only forages. Therefore, the large sample size and the diversity of feedstuffs utilized in the current study are unique in the literature and strengthen the value of this study. Results indicated that soluble and degradable fraction, and effective degradation of DM, CP and NDF of feedstuffs can be predicted satisfactorily ($R^2 > 0.7$) for ALL providing universal equations for all feedstuffs; however the rate of degradation was not satisfactorily predicted for ALL ($R^2 < 0.7$). Low prediction of the rate of degradation of DM and CP of forages (Andres et al., 2005) and NDF of forages (Ohlsson et al., 2007) has been reported previously. However, in the current study group separation improved predictions of the rate of degradation, and obtained equations were considered satisfactory for a practical application of NIRS. It should be considered that many current feed evaluation systems [e.g., Cornell Net

Carbohydrate and Protein System (Sniffen et al., 1992); Molly (Baldwin, 1995); Dairy NRC (NRC, 2001); NorFor (Volden, 2011)] use simple averages of degradation parameters to estimate ruminal degradability of CP. Therefore, the incorporation of NIRS technology into feed evaluation protocols with the equations provided may improve diet formulation in practice.

One positive criticism of the current study is that all forages and silages samples were dry and milled before NIRS scanning, which it is not a real situation at a farm level. Drying and grounding forages can take up to 24 - 48 h. Even considering this limitation, NIRS will speed up considerably the feed evaluation process, especially when degradation parameters are needed. On the other hand, Gordon et al. (1998) demonstrated that similar predictions of feedstuffs chemical composition can be obtained for dried and fresh silages. Further research may incorporate the prediction of degradation parameters and effective degradability in fresh feedstuffs.

3.2. Polyclonal Antibodies Against Proteolytic and Deaminating Ruminal Bacteria did not Alter Ruminal Protein Degradation and Deamination

The use of passive immunization through the administration of PAbs as an approach to alter microbial populations in the rumen was investigated recently. DiLorenzo et al. (2006, 2008) and Blanch et al. (2009) administrated PAbs against *S. bovis* and a group of bacteria involved in ruminal acidosis, and their results suggested that it is possible to modify ruminal microbial populations and prevent acidosis with this approach. The current study was the first to produce and evaluate PAbs against proteolytic and deaminating bacteria. However, our hypothesis that PAbs preparations against proteolytic and deaminating bacteria could neutralize target bacteria populations causing a decrease of ammonia-N concentration was not verified. The addition of

produced PAbs did not affect protein degradation and deamination in short or long term *in vitro* ruminal fermentation. However, the reason for this is not clear.

The ineffectiveness PAbs may be attributed to the failure of PAbs to block and neutralize the bacteria or to the complexity of rumen microbial environment. Initially, we considered two main strategies to select a proper antigen: (i) to isolate a specific protein sequence of the external membrane of the bacteria, and (ii) to use the bacteria itself as an antigen. Research in protein databases did not result in any protein sequence of the external membrane of our target bacteria, probably because most of these bacteria are located mainly in the rumen of ruminants and the HAP bacteria were relatively recently described (Chen and Russell, 1988, 1989). Thus, the entire bacteria were selected as antigens. Similar strategy is frequently followed for the production of vaccines against ruminal microbes, such as *S. bovis* (Shimizu et al., 1988) or methanogenic microbes (Wright et al., 2004). Our results of ELISA suggested that the antigen-antibody complex was successful, but it is not known if this complex could lead to the neutralization of the bacteria in pure cultures or the rumen ecosystem. If this strategy resulted in PAbs that cannot neutralize the selected bacteria, a more sophisticated research should be developed that will identify protein sequences of the bacterial external membrane. These protein sequences should be specific for each bacterium and their blockage should lead to the neutralization of it. Then, PAbs may be produced against these sequences. In contrast, if our strategy resulted to PAbs that successfully blocked and neutralized target bacteria, their ineffectiveness should be attributed to the complexity of the ruminal bacterial ecosystem.

Wright and Klieve (2011) discussed the microbial diversity of the rumen as a factor for the poor effects of strategies against specific methanogenic ruminal microbes. They suggested that the accumulating knowledge derived from new technologies would

improve our understanding of the rumen microbes and this could lead to a modification towards the desired direction. Similar to ruminal methanogenesis, many different microbes are involved in ruminal protein degradation (Walker et al., 2005). In the current work, we targeted three bacterial species: *P. ruminicola*, *C. aminophilum* and *P. anaerobius*; however, the genus of *Prevotella* contains three more species (*P. brevis*, *P. bryantii* and *P. albensis*; Avgustin et al., 1997), more than 20 different HAP bacteria have been identified (Walker et al., 2005), and other microbes are involved in protein degradation and deamination, such as *Streptococcus bovis*, *Ruminobacter amylophilus*, *Fibrobacter succinogenes, Megashaera elsedenii*, *Lachnospira multipara,* protozoa etc (Wallace, 1996; Rychlik and Russell, 2000; Walker et al., 2005). Therefore, the neutralization of these targeted bacteria may be counterbalanced by the proteolytic and deaminating activity of other bacteria. The diversity of microbes involved in protein degradation and deamination is a crucial factor that should be considered in a future application of immunization. A potential alternative would be the production of PAbs against all Prvotella spp. or /and most HAP bacteria.

3.2. Essential Oils as Modifiers of Ruminal Protein Degradation.

Our first approach was to use EO compounds to modify protein fractions of ryegrass silages. The only attempt to use EO in silage preparation reported no effects (Kung et al., 2008). However, the low dose of a commercially available mixture of EO used (40 and 80 mg of EO / kg of fresh forage) and the selection of maize as the ensiling crop, limited the possibility of EO to affect protein degradation during ensiling. In the current study, all EO compounds tested, with the exception of carvacrol, resulted in silages with reduced ammonia-N suggesting the inhibition of deamination. These effects were attributed to the reduced counts of lactic acid bacteria, with the exception of cinnamaldehyde, indicating that microbes present in silages contribute to the process

of degradation during ensiling, and especially to the deamination process. Moreover, the addition of cinnamldehyde resulted in silages with 9.7% higher true protein N. However, its addition did not affect microbial population, suggesting an action through the inhibition of plant enzymatic activity, but the exact mechanism of action needs to be identified. This is the first study where the addition of EO compounds affected forage protein degradation during ensiling. However, this occurred when high doses of EO compounds were used, making complicated and costly a potential application in practice.

For the second approach we tested propyl-propylthiosulphonate (PTSO), a recently purified EO compound from garlic oil, to modulate ruminal microbial populations. Garlic oil is a mix of a large number of different molecules (Lawson, 1996). The effects of GAR and its main active components on N metabolism have been variable. Cardozo et al. (2004) reported that GAR in continuous culture reduced ammonia-N and increased peptide and AA N concentrations, suggesting that deamination was inhibited, but Busquet et al. (2005a, b) reported only small and variable effects of GAR on N metabolism in the rumen. Moreover, a substantial instability has been documented for garlic oil compounds (Fujisawa et al., 2008). In contrast, PTSO is a stable compound (Ruiz et al., 2010). In the first experiment we tested doses similar to those that were indentified for other compounds of garlic oil (effective dose of 300 mg/l; Busquet et al., 2005b). However, the addition of 300 mg/l of PTSO in a dual flow continuous culture system resulted in reduced digestibility of nutrients and volatile fatty acid (VFA) concentration. In a following experiment, increasing doses of PTSO were evaluated (0, 50, 100 and 150 mg/l). The addition of PTSO caused quadratic responses of total VFA and propionate molar proportion with higher values in the intermediate doses 2 h after feeding. Butyrate increased and

BCVFA decreased linearly with increasing doses of PTSO, but concentrations of ammonia-N, small peptide-N and large peptide-N were not affected by treatments. Results suggest the potential of PTSO to modify rumen fermentation in a direction consistent with better energy utilization and not N metabolism.

Results indicate that PTSO has a higher antimicrobial activity than other GAR compounds and, as a consequence, low doses are required. The effective dose of PTSO was between 50 – 100 mg/l, which is less that the 1/3 of the effective dose of other compounds of GAR (300 mg/l; Busquet et al., 2005b). This strong antimicrobial activity may be attributed to the chemical stability of PTSO, and that benefits its potential for commercialization.

4. Conclusions

This PhD thesis was developed within the RedNex project, which followed an integrated, cow-oriented approach to suggest solutions that reduce N excretion from dairy farming. Our contribution was mainly targeting the rumen. The main conclusions are:

(i) Obtained equations to predict feedstuff degradation parameters and effective degradability with NIRS technology were satisfactory for the incorporation of NIRS technology into feed evaluation protocols.

(ii) The addition of essential oils compounds to ryegrass silages reduced protein degradation and deamination during ensiling, resulting in silages with higher true protein nitrogen. However, the effective doses required were too high to be applied in practice.

(iii) The antigen-antibody complexes of the produced polyclonal antibodies against proteolytic and deaminating bacteria were successful, but the corresponding polyclonal antibodies were not effective in a rumen microbial environment. However, the reason for this is not clear and remains to be clarified.

(iv) Propyl-propylthiosulphinate, a garlic oil compound, has strong antimicrobial activity and modifies ruminal fermentation towards a direction of improved energy utilization and not N metabolism.

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