Animal 16 (2022) 100471

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effect of decreasing dietary crude protein in fattening calves on the emission of ammonia and greenhouse gases from manure stored under aerobic and anaerobic conditions

M. Devant [a,*], A. Pérez [b], C. Medinyà [c], J. Riera [d], J. Grau [e], B. Fernández [a], F.X. Prenafeta-Boldú [a]

[a] IRTA – Institut de Recerca i Tecnologies Agroalimentàries, Torre Marimon, 08140 Caldes de Montbui, Spain
[b] Corporación Alimentaria Guissona, S.A., 25210 Guissona, Lleida, Spain
[c] Nutrición Animal S.L., 08650 Sallent, Barcelona, Spain
[d] Nanta S.A., 28760 Tres Cantos, Madrid, Spain
[e] Setna Nutrición Animal SAU, 28521 Rivas Vaciamadrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 7 March 2021
Revised 19 January 2022
Accepted 21 January 2022

Keywords:
Beef
Gaseous emissions
Nitrogen excretion
Performance
Protein concentration

ABSTRACT

Dietary strategies can potentially help to reduce nitrogen (**N**) emissions and decrease the environmental impact of beef production. This study aimed to evaluate the effects of dietary crude protein (**CP**) concentration on animal performance, N excretion, and manure N volatilisation of finishing Holstein animals. In a first study, 105 Holstein bulls (BW 344 ± 2.6 kg; age 252 ± 0.9 days) were allocated to eight pens to evaluate the effect of two treatments (medium (**M**) and low (**L**), which contained CP 14.5% and 12% on a DM basis, respectively) on performance, and results confirmed that dietary CP decrease did not impair animal growth. In a second study, N excretion study, 24 Holstein heifers (BW 310 ± 5.3 kg; age 251 ± 1.4 days) were distributed randomly depending on the initial BW to three treatments (high (**H**), M, and L, which contained CP 17%, 14.5% and 12% on a DM basis, respectively). Based on N excretion, urinary N excretion was greater ($P < 0.001$) in H than in M and L diets, but no differences in faecal N excretion were observed among treatments. A third study with *in vitro* assays under aerobic and anaerobic conditions was designed to analyse gaseous emissions (volatilisation of N and carbon, **C**) during the storage stage of manure. Manure, faecal and urine samples, mixed at a ratio of 1:1 (wet weight), were collected during the N excretion study (manure-H, manure-M, manure-L). Under aerobic conditions, manure-M and manure-L showed a delay of 4–5 days in manure ammonia emission compared with manure-H ($P < 0.01$). Total N content was lower ($P < 0.01$) in manure-L compared with manure-M and manure-H, but N volatilisation (percentage relative to initial N) in manure-L and manure-M was greater ($P < 0.01$) than in manure-H. In contrast, the anaerobic N volatilisation was 20 times greater in manure-M and 10 times greater in manure-H compared with manure-L. Under aerobic and anaerobic conditions, the emission of C, as C-CO_2 and C-CH_4, was greater in manure-L than in manure-H and manure-M. Therefore, the decrease of dietary CP concentration from 17% to 14.5% and 12% is an efficient strategy to reduce urinary N excretion by 40%, without impairing performance, and also to reduce manure N losses through ammonia volatilisation under anaerobic conditions. However, a dietary CP content of 14.5% resulted in less environmental impact than a CP content of 12.8% when also considering manure emissions under aerobic or anaerobic conditions.

Implications

The decrease of the environmental impact of beef production is a societal demand. The present study reinforces that there is still room for improvement and that dietary strategies such as dietary protein decrease can reduce nitrogen urinary excretion by 40% and the greenhouse emissions during manure storage without impairing animal performance. When considering the two main storage processes applied to manure (aerobic and anaerobic conditions) of beef cattle fed with high-concentrate diets, the optimum dietary crude protein concentration to reduce the undesirable carbon losses together with nitrogen losses is 14.5%.

* Corresponding author.
E-mail address: maria.devant@irta.cat (M. Devant).

https://doi.org/10.1016/j.animal.2022.100471
1751-7311/

Introduction

The environmental impact of beef production is becoming increasingly important to producers and consumers. The nitrogen (**N**) loss at the farm is related to proper animal feeding and manure management. The dietary composition of animal rations affects the content of N and carbon (**C**) in the ration itself, but also the excretion of N and C in faeces and urine, and, thereby, the losses of N and C to the environment. Emissions of greenhouse gases (**GHG**) from manure, such as carbon dioxyde (**CO_2**), methane (**CH_4**), and nitrous oxide (**N_2O**), are a major environmental concern. The N volatilisation through ammonia (**NH_3**) contributes to the formation of particulate matter that negatively affects human health, to the acidification and eutrophication of natural ecosystems when N is deposited from air to the ground and/or water bodies, and to the generation and leaching of nitrates into groundwaters (Erickson and Klopfenstein, 2010; Hristov et al., 2011). Moreover, although NH_3 is not a GHG, the N loss from animal manures may indirectly contribute to agricultural emissions of N_2O, a potent GHG with a global warming potential of 273 ± 130 times greater than CO_2 (Masson-Delmotte et al., 2021). Soil fertilisation and animal manure management contributed to about two-thirds of the 80% N_2O emission increase due to anthropogenic sources during the 1980–2016 period. These N_2O emissions are primarily released upon nitrification and partial denitrification of ammonia, which is a microbial process commonly encountered under partial aerobic/anaerobic conditions.

Animal feeding strategies are one of the first steps in reducing N loss from the farm. Reynolds and Kristensen (2008) summarised the most important nutritional strategies in ruminants to reduce N emissions, concluding that the decrease of the dietary crude protein (**CP**) concentration was one of the most effective methods because it improved the urea utilisation by microbes in the rumen. Also, as urine N is more prone to volatilise than faecal N, diminishing the N content in urine rather than in faeces is a better strategy to minimise N volatilisation (Bussink and Oenema, 1998). As far as the dietary N concentration does not affect the DM intake (**DMI**), Huhtanen et al. (2008) showed that a low N intake resulted in decreased urinary N excretion. However, these interventions must be balanced with the risk of production loss; therefore, reducing dietary CP might be particularly promising during the finishing phase where the animal N requirements are low and feed intake is high. However, few studies have evaluated the impact of dietary N content with an overall view on the impact of animal N excretion along with N and C manure emissions (James et al., 1999; Burgos et al., 2010; Koenig et al., 2018). The novelty of the present study is the evaluation of dietary CP concentrations below the common commercial concentrations on N excretion and animal performance, and also its impact on N and C manure potential emissions, in Holstein beef animals fed with high-concentrate diets during the finishing phase.

Material and methods

All experimental protocols were approved by the Institutional Animal Care Committee of the Institut de Recerca i Tecnologia Agroalimentàries (Barcelona, Spain, number FUE-2018-00702882-9970), and the study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013).

Experimental design

The present study was based on animal dietary trials that consisted of three different CP concentrations in the finishing concentrate: **H** (high, CP of 17.0% DM), above the reference CP commercial values; **M** or reference commercial (medium, CP values of 14.5% DM); **L** (low, CP of 12.0%DM), below the reference commercial values. Commercial reference CP values (from 15 to 13.5%) were the common values used in the finishing concentrate and were provided by the different companies involved in this project (Corporación Alimentaria Guissona S.A, Nutrición Animal S.L., and Nanta S.A). To achieve the different CP concentrations, soybean meal and urea inclusion rates were modified, and the soybean meal was replaced with wheat middling, beet pulp, and palm oil (Table 1). In consequence, the theoretical protein degradability (based on NRC 1996 tabular values) was modified: 67%, 65%, and 62% for H, M, and L treatments, respectively. Assuming a ratio barley straw to concentrate of 10:90 (Devant et al., 2000; Marti et al., 2010), the ingredient composition of the concentrate was formulated to meet FEDNA (2008) recommendations except for CP. All concentrate ingredients were grounded within a hammer mill (screen openings of 2.75 mm); in the case of the N excretion study, the concentrate was thoroughly mixed with chromic oxide (1 g/kg DM), which is an external marker to estimate total tract apparent digestibility, and then pelletised (pellets of 3.5 mm of uniform diameter and 10 mm of length), manufactured in one batch as Verdú et al. (2017).

Table 1
Ingredient and chemical composition of the feed concentrates with high (H), medium (M) or low (L) CP concentration fed to the Holstein bulls.

Ingredients and components	Concentrate		
	H	M	L
Ingredient (g/kg, as fed)			
Corn	400	399	399
Barley	81	81	81
Wheat	120	110	100
Wheat middling	137	160	182
Beet pulp	101	124	145
Soybean meal, 47% CP	108	75	42
Palm oil	26	28	31
Calcium carbonate	11.5	10.5	10.5
Salt	2	2	2
Urea	6	3	-
Sodium bicarbonate	4	4	4
Magnesium oxide	1.5	1.5	1.5
Premix (1)	2	2	2
Theoretical nutrients			
DM, g/kg	883	883	883
ME, Mcal/kg DM	3.27	3.27	3.27
CP, g/kg DM	170	145	120
Ether extract, g/kg	59	60	62
Starch, g/kg	486	486	486
NDF, g/kg	180	195	203
Ash, g/kg	53	53	52
Analysed Performance study			
DM, g/kg as fed		863	868
CP, g/kg DM		145	122
Ether extract, g/kg		60	62
Starch, g/kg		484	487
NDF, g/kg		196	200
Ash, g/kg		53	52
Analysed N excretion study			
DM, g/kg as fed		862	863
CP, g/kg DM	170	145	128
Ether extract, g/kg	58	62	62
Starch, g/kg	486	484	487
NDF, g/kg	180	195	204
Ash, g/kg	53	53	52

Abbreviation: ME: metabolisable energy.
[1]Composition: 3.54 10^6 IU/kg of vitamin A; 850 000 IU/kg of vitamin D3; 12 500 mg/kg of vitamin E; 2 500 mg/kg of vitamin B1; 29% of magnesium; 0.11% of sodium; 3% of sulphate; 150 mg/kg of selenium; 240 mg/kg of cobalt; 250 mg/kg of iodine; 15 500 mg/kg of manganese; 20 300 mg/kg of zinc; 2 500 mg/kg of copper; 7 150 mg/kg of iron.

Based on these dietary treatments, three separate experiments were carried out. The first study was a performance experiment with bull calves on a farm where calves were fed with two different concentrations of protein. The second study was an N excretion study with heifers fed with three different concentrations of protein. The third study was an *in vitro* manure storage test with faeces and urine from the N excretion study.

Experiment 1. Performance study

A total of 105 Holstein bulls (344 ± 2.6 kg of BW and 252 ± 0.9 days of age) were used in a randomised balanced complete design to evaluate the effect of dietary CP concentration for 70 days. Only treatments M and L were tested (14.5 and 12.2% on a DM basis, respectively; Table 1), since the main objective of the present study was to reduce the N excretion under commercial conditions. Before the start of the study, animals were weighed and allocated to eight pens, so that the average BW was similar across pens. Treatments were randomly distributed to each pen. Bulls were fed *ad libitum* with concentrate and unprocessed long barley straw (CP 3.5%, ether extract 1.6%, NDF 76.9%, and ash 6.1% on a DM basis), in separate troughs until day 70, when the target final BW was 450–470 kg.

Pens were deep bedded with straw (12 m × 6 m = 72 m^2 per pen): the space availability was 4–4.5 m^2 per animal. The feeding area consisted of one concentrate feeder (1.50 m × 0.40 m × 0.35 m), one separate straw feeder (3.60 m × 1.10 m × 0.32 m), and one water bowl (0.30 m × 0.30 m × 0.18 m). The concentrate feeders had three feeding spaces, equipped with a scale that consisted of four loading cells (Utilcell, Barcelona, Spain), where the feeder was suspended. The contained concentrate was continuously weighed and the weight was displayed in a digital screen reader, similarly to Verdú et al. (2017). The amount of straw offered to each pen was recorded weekly to estimate the total amount of straw consumed. However, these data were only an approximation of the straw intake because the straw was also used for bedding. Animals were weighed every 14 days throughout the study to calculate full BW data. From each concentrate manufacture (every 10–14 days), one sample of concentrate and one sample of straw were collected to be analysed (see Analyses section; DM, CP, ash, fat, NDF, and starch). Daily health incidences were recorded with special emphasis on issues related to digestive disturbances (laminitis, bloat, etc.). Faecal scoring was recorded every 2 weeks, based on Heinrichs et al. (2003). Bloat scoring was determined according to the scale defined by Johnson et al. (1958).

After 70 days, bulls were transported to a commercial slaughterhouse (La Closa, Guissona, Spain) by truck. The transport distance was less than 15 km, and animals were slaughtered upon arrival. Animal transport was organised in four different loads without mixing animals from different treatments or pens. Before each loading, each animal's BW was recorded (final BW). The hot carcass weight was recorded, and the dressing percentage was calculated from hot carcass weight. The carcass backfat and conformation were graded according to the EU classification system into 1.2.3.4.5 (EU Regulation no. 1208/81) and into (S)EUROP categories (EU Regulation no. 1208/81, 1026/91), respectively.

Experiment 2. Nitrogen excretion and total tract apparent digestibility study

That study used heifers instead of intact bulls (target animal of the production system studied) as urine sampling in 300 kg intact bulls is complicated and unsafe. A total of 24 Holstein heifers (310 ± 5.3 kg of BW and 251 ± 1.4 days of age at the start of the study) were housed in individual partially slatted pens (1.9 × 3.4 m) in a randomised balanced complete block with covariance adjustment design, at the experimental station of the Cooperativa Agrària de Guissona (Guissona, Spain). Heifers were weighed on day 0, stratified by mean BW, and randomly allocated to three treatments (H, M, L) to equalise initial BW among treatments. During a 28-day adaptation period, heifers were fed with their corresponding treatment diets; thereafter, the sample collection started. Concentrates and barley straw (long unprocessed straw with particle size around 20–30 cm and CP 3.5%, ether extract 1.6%, NDF 76.9%, and ash 6.1% on a DM basis) were both fed in separate troughs (0.6 × 1.2 × 0.3 m) *ad libitum* until day 67. Heifers were transported to the abattoir after 68 days of study. The transport distance was less than 1 km (La Closa, Guissona, Spain). As animals were fed *ad libitum*, they had concentrate and straw available during the whole day except the time needed to weigh the concentrate and straw offer and refusal (usually 10–15 min per animal); the intake was recorded daily and started at 08:00 am. The BW was recorded every 14 days. Faecal and bloat scoring were recorded every 2 weeks similarly to Experiment 1. Once every 2 weeks, one sample of concentrate and one sample of straw were collected to analyse their DM content, as well as for concentrate and straw intakes calculation.

To estimate the N excretion and the total tract apparent digestibility, two identical sampling periods were conducted on days 30 and 58 to reduce animal variability. In each sampling period, offered feed and refusals were collected during 7 consecutive days; these samples were later composited by animal to determine their nutrient (see Analyses section; CP, ash, ether extract, NDF, starch) and chromium content. Between days 34–36 and days 62–64, three faecal grab samples (1 h before, as well as 3 and 5 h after feeding) were collected from the rectum and dried at 55 °C for 48 h; these samples were later composited by the animal on an equal DM basis. In parallel with each faecal grab sample collection, a urine spot sample (100 mL) was obtained by perivaginal massage and frozen at −20 °C to determine creatinine and N contents. The total tract apparent digestibility was calculated by estimating total faecal output as the ratio of chromium concentration in the feed and faecal samples. The total urine volume was estimated assuming 883 μmol-creatinine/kg-metabolic BW/d (Chen et al., 1992).

On day 57, blood samples were collected by jugular venepuncture at 1, 5, 9, 13, and 24 h after feeding, and frozen to determine serum urea concentration. A 10 mL blood sample was collected within BD vacutainer serum tubes (Franklin Lakes, NJ) containing a spray-dried clot activator. Samples were centrifuged at 1 500*g* at 4 °C for 15 min to obtain a decanted serum that was stored at −20 °C until urea concentration analysis.

Experiment 3. Volatilisation study during in vitro manure storage

Additional faecal and urine samples were collected on day 58 of Experiment 2 (N excretion study) and pooled by treatment (H, M, L) on equal fresh basis, and frozen at −20 °C. Then, separately preserved faecal and urine samples were mixed with a ratio of 1:1 (fresh basis) to produce three mixtures named H, M, and L, corresponding to each CP treatment of Experiment 2. These three manure mixtures were subjected to *in vitro* incubation assays by triplicate (60 g of manure per replicate), under aerobic and under anaerobic conditions, for 58 days to simulate extreme in-farm storage conditions (the removal of manure, stored inside calve pens, is done at 0.5–2.0 months). No inoculum was added, and therefore, the manure endogenous microorganisms were responsible for any biological process. No bedding materials were added to avoid the interference of these materials with the gaseous emission profile (Misselbrook & Powell, 2005). During storage under aerobic conditions, PVC buckets (30 L of total volume) were used as Külling et al. (2001), where manures were located into a tray (emission surface of 30 cm^2) to facilitate their handling. Each bucket headspace was periodically aerated with fresh air with an average flow rate of 3.5 L/d. For the anaerobic storage, sealed glass flasks (6.14 L of total volume) were used; once filled, all were

flushed with N_2 gas to ensure that no oxygen remained inside. The initial and final manures (days 0 and 58, respectively) were characterised for total carbon (**TC**), total nitrogen (**TN**), and DM. During the manure storage, headspace composition (CH_4, CO_2) was monitored once a week. The NH_3 concentration in the headspace was only measured under aerobic conditions, every 1–7 days depending on its evolution. However, the storage tests were done under room temperature (ranging from 18 to 25 °C).

Analytical methods

Samples of concentrate and straw were analysed for DM, organic matter (**OM**) and ash, CP (Kjeldahl method; AOAC, 1995), NDF (Van Soest et al., 1991), using sodium sulphite and heat-stable amylase, ether extract (Soxhlet with previous acid hydrolysis; AOAC, 1995) and starch (polarimetry method; EU Regulation for Feed Analyses no. 152/2009). The chromium concentrations of feed and faecal samples were determined as Devant et al. (2019). Urine samples were analysed for urinary creatinine (HPLC; Balcells et al., 1992) and N content (Kjeldahl method; AOAC, 1990). The measurement of urea N was performed by a Beckman Coulter® AU 480 analyser, using urea N OSR reagent (Olympus System Reagent®, Beckman Coulter®, Ireland), based on the enzymatic method of urease/glutamate dehydrogenase.

Samples from initial and final manures of the storage experiment were analysed for TC and TN content (TruSpec CN Analyser; LECO Corporation, St. Joseph, MI, USA), as well as total ammonia nitrogen, DM and volatile solid content (Standard Methods; APHA, AWA, WEF, 2005). Headspace samples from buckets and glass flasks were taken for CH_4 and CO_2 content determination using a gas-tight syringe and analysed with the aid of a gas chromatograph equipped with a thermal conductivity detector (Palatsi et al., 2010). The NH_3 concentration in the bucketś headspace was measured *in situ* using a multi-gas portable detector (model PGM 7840, RAE Systems Inc.) equipped with an ammonia sensor for a 0–150 mg/m concentration range. The CH_4, CO_2, and NH_3 concentrations in the headspace were standardised at 1 atm and 25 °C.

Calculations and statistical analysis

The normality of the data before the statistical analyses was evaluated by the frequency histogram distribution and the Shapiro-Wilk test. In the present paper, no data needed to be transformed to achieve normality of the data.

Data of Experiment 1 were analysed using a mixed-effects model with repeated measures (SAS Inst. Inc., Cary, NC). The model included initial BW as covariate, treatment, time (a 14-day period), and the interaction between treatment and time as fixed effects, and pen as random effect. Time was considered a repeated factor, and for each analysed variable, animal nested within treatment (the error term) was subjected to three variance–covariance structures: compound symmetry, autoregressive order one, and unstructured. The covariance structure that minimised Schwarz's Bayesian information criterion was considered the most desirable analysis. A chi-square test was conducted to evaluate the effects of treatment on carcass classification data (categorical variables).

In Experiment 2, intake and serum urea concentration data were analysed using a mixed-effects model with repeated measures (SAS Inst. Inc., Cary, NC). The model included initial BW as covariate, treatment, time (14-day period or sampling min), and the interaction between treatment and time as fixed effects, and animal as random effect. Time was considered a repeated factor, and for each analysed variable, animal nested within treatment (the error term) was subjected to three variance-covariance structures and analysed as described in Experiment 1.

Nitriogen excretion and digestibility data were analysed using a mixed-effects model (SAS Inst. Inc., Cary, NC). No interaction between time and treatment was analysed, as we were not expecting any changes within one month and the only reason to run to periods was to increase the amount of data. Therefore, the sampling periods were considered independent blocks. The model included initial BW as covariate, treatment and sampling period (block) as fixed effects, and animal as random effect.

For Experiment 3, three replicates per storage condition (aerobic, anaerobic) and manure (H, M, L) were prepared. The initial and final weights (wet and dry mass) and concentrations (total C, total N, total solids) per replicate (storage, manure) were registered (weights) or determined (concentrations). Individual (or per replicate) losses of wet and dry weight, total C, and total N were calculated as the difference between initial and final values and expressed as percentage with respect to the initial corresponding value [$100 \times ((in - fin)/in)$]. Then, the average value of losses per manure and storage condition was calculated. Based on the composition of the headspace, the emitted C was calculated as the sum of C-CH_4 and C-CO_2 and expressed as percentage with respect to the initial TC (%in-TC) of the corresponding manure. The cumulative emitted N-NH_3 was expressed as percentage with respect to the initial TN (%in-TN) of the corresponding manure. The experimental emitted N-NH_3 was adjusted to a logistic equation (see Supplementary Material S1, Supplementary Fig. S1, and Supple-

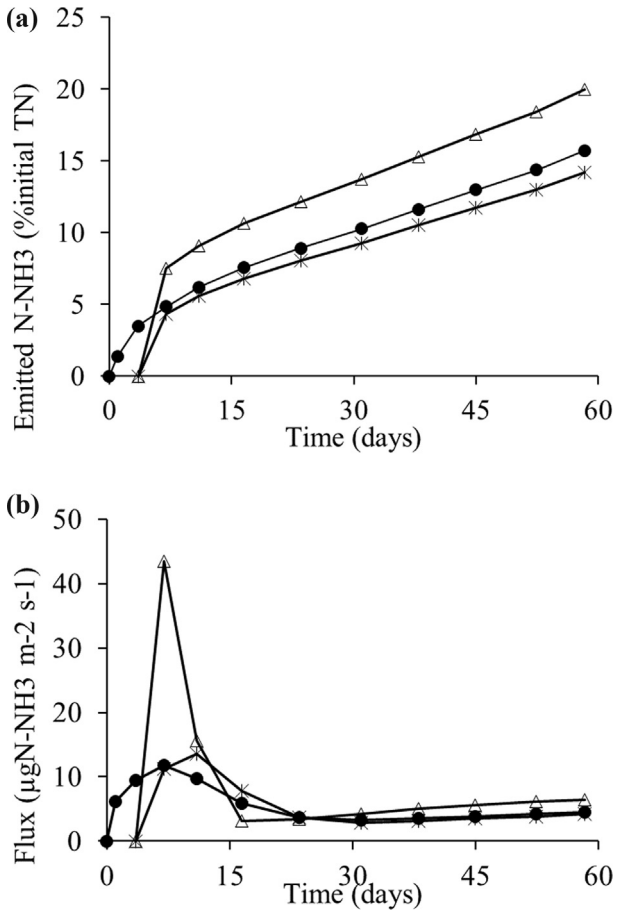

Fig. 1. N-NH3 emission from beef manure stored under aerobic conditions, obtained after the mathematical fitting (see Supplementary Material S1) (Volatilisation study). (a) Cumulative N-NH3 emission, expressed as percentage of initial TN of manure. (b) Emission flux (µgN-NH3 m^2/s). Symbols: triangles: manure-L; asterisks: manure-M; circles: manure-H. Abbreviations: N-NH_3 = ammonia nitrogen, TN= total nitrogen, manure-L= manure from calves fed low dietary CP concentration, manure-M= manure from calves fed medium dietary CP concentration, manure-H = manure from calves fed high dietary CP concentration

mentary Fig. S2). Once adjusted, this model was employed to calculate the N-NH_3 emission flux (emission flux; μN-NH_3 m/s), both represented in Fig. 1A and B.

Statistical analysis of the data was carried out using a mixed-effects model with repeated measures (SAS Inst. Inc., Cary, NC) for a randomised balanced complete design. The model included treatment as fixed effect and sampling bottle as random effect. The same model was used for the evolution of ammonia emissions in the aerobic assay, but it included treatment, time (in days), and the interaction between treatment and time as fixed effects and replicate as random effect. Time was considered a repeated factor and analysed as described in previous studies.

For all statistical analyses, significance was declared at $P \leq 0.05$ and tendencies were discussed at $0.05 < P \leq 0.10$.

Results

Performance experiment

Two animals were removed from the study: one bull from the M treatment was removed on day 21 because of a leg injury; one bull from the L treatment was removed on day 55 because it had fallen down with splayed legs. The only bloat score recorded throughout the study was "0" (no bloat, data not shown). Faecal scores did not differ among treatments, and the most commonly registered values were "1" (normal; data not shown).

The effects of dietary treatments on the final BW, ADG, concentrate and straw intakes, total DMI, and total efficiency are summarised in Table 2. No interaction between treatment and time (14-day periods) was statistically significant, and no treatment effect on performance or concentrate intake was observed. Daily straw intake was 1.1 ± 0.08 kg per bull, which is within the range of the data observed in the previous N excretion study, and therefore, it is not affected by treatment. The final BW, carcass weight, and carcass conformation were not affected by the CP concentration decrease, whereas L carcasses tended ($P = 0.10$) to have greater carcass fatness score compared with M carcasses (94.52% vs 86.67% scored "3", respectively).

Nitrogen excretion and digestibility study

The target CP content was not achieved in the L treatment, being 6% greater than expected (experimental and theoretical values of 12.8% vs 12.0% DM, respectively). The CP content of raw materials was analysed before the manufacture in order to avoid these deviations in the CP concentrations; however, the CP content of wheat middling may vary significantly. The nitrogen excretion and digestibility experiment showed that concentrate, straw, and total DMI were not affected by the CP decrease (Tables 3 and 4), while DM and OM total apparent digestibility decreased ($P < 0.05$) in heifers fed with L compared with heifers fed H and M concentrates (Table 4). Starch total apparent digestibility was lower in L fed heifers compared with M fed heifers, being the values in H fed heifers at the intermediate range ($P < 0.05$). The reduction of CP concentration decreased the total CP apparent digestibility ($P < 0.001$): H heifers had the highest CP digestibility, followed by M and L fed heifers. The volume (kg DM per day) of faeces tended ($P = 0.06$) to be lower in M fed heifers. Moreover, the reduction of CP decreased ($P < 0.001$) the starch content of M faeces, compared with H and L faeces, and reduced ($P < 0.001$) the CP content of L faeces when compared with H and M treatments.

Despite the numerical differences in the N intake among L and M heifers, these values were not statistically significant (169.6 and 185.2 g/d, respectively), probably as a consequence of the unexpected greater CP concentration in the L concentrate (Table 4), as mentioned above. However, the N intake was greater ($P < 0.001$) in H heifers compared with M and L heifers. Whereas the CP concentration did not affect the faecal N content, urinary N excretion was reduced ($P < 0.001$) by 40% when lowering the CP concentra-

Table 2
Performance of Holstein bulls fed concentrate with medium (M) or low (L) CP concentration (Performance study).

	Treatment			*P*-value[1]		
Parameter	M	L	SEM	T	P	T × P
Initial age, d	252	251	0.16	0.12		
Initial BW, kg	343	344	0.15	0.10		
Final BW at 70 d of study, kg	462	460	3.5	0.77		
ADG, kg/d	1.68	1.65	0.041	0.63	< 0.001	0.12
Concentrate DMI, kg/d	7.1	7.1	0.05	0.95	< 0.001	0.69
Straw DMI, kg/d	1.2	1.1	0.08	0.32	0.57	0.95
Total DMI, kg/d	8.3	8.2	0.12	0.50	< 0.05	0.74
Concentrate to total DMI ratio, %	85.4	86.7	0.81	0.29	0.43	0.96
Efficiency, kg/kg[2]	0.20	0.20	0.005	0.87	< 0.001	0.46
Carcass						
Days in study	90	91	0.47	0.29		
Slaughter age, d	342	342	1.12	0.96		
Slaughter BW, kg	494	490	2.4	0.21		
Carcass weight, kg	259	256	1.7	0.23		
Dressing percentage, %	52.4	52.2	0.30	0.65		
Conformation[3]				0.42		
R	5.3	4.7				
O	58.7	54.0				
P	36.0	41.2				
Fatness[4]	0.10					
2	13.33	5.48				
3	86.67	94.52				

Abbreviation: ADG = average daily gain, DMI = DM intake.
[1] T = treatment effect; P = period effect (14 days); T × P = treatment by period interaction.
[2] kg of ADG divided by kg of DM intake.
[3] Carcass conformation classification: "E" (excellent), "U" (very good), "R" (good), "O" (fair), "P" (poor).
[4] Fatness classification: "1" (low) to "5" (high).

Table 3
Intake of Holstein heifers fed concentrate with high (H), medium (M) or low (L) CP concentration (N excretion study).

	Treatment				*P*-value[1]		
Parameter	H	M	L	SEM	T	P	T × P
Initial age, days	250	249	252	1.1	0.22		
Initial BW, kg	311	310	310	4.1	0.97		
Final BW after 67 days, kg	411	417	410	6.1	0.65		
Concentrate DMI, kg/d	7.3	7.3	7.3	0.30	0.98	< 0.001	0.85
Straw DMI, kg/d	0.52	0.51	0.52	0.025	0.92	< 0.001	0.89
Total DMI, kg/d	7.8	7.8	7.8	0.30	0.98	< 0.001	0.82
Concentrate to straw ratio, %	93.3	93.3	93.3	0.40	0.98	< 0.001	0.73

Abbreviation: DMI = DM intake.
[1] T = treatment effect; P = period effect (14 days); T × P = treatment by period interaction.

Table 4
Total tract apparent digestibility and nitrogen excretion of Holstein heifers fed concentrate with high (H), medium (M) or low (L) CP concentration (nitrogen excretion study).

	Treatment				*P*-value[1]	
Parameter	H	M	L	SEM	T	B
Intake, kg/d						
Concentrate DMI	7.9	7.6	7.9	0.28	0.59	0.85
Straw DMI	0.71	0.70	0.70	0.040	0.96	0.01
Total DMI	8.6	8.3	8.6	0.28	0.61	0.56
Faeces, kg/d as fed	13.0	12.0	14.0	0.07	0.06	0.57
Faeces, kg DM/d	2.6	2.4	2.8	0.14	0.06	0.57
Urine, l/d	9.8	10.2	10.4	2.10	0.96	0.81
Total tract apparent digestibility, %						
DM	70.3[a]	71.4[a]	66.6[b]	1.34	< 0.05	0.93
OM	72.3[a]	73.5[a]	68.9[b]	1.27	< 0.05	0.33
Starch	94.8[ab]	96.4[a]	93.8[b]	0.72	< 0.05	0.84
CP	69.3[a]	64.0[b]	58.3[c]	1.23	< 0.001	0.97
NDF	38.6	43.5	35.0	3.95	0.28	0.33
EE	70.9	70.6	70.9	2.07	0.98	0.95
Faeces analyses, %DM						
OM	88.1	87.3	88.1	0.35	0.07	0.35
Starch	7.5[a]	5.6[b]	7.9[a]	0.75	< 0.05	0.40
CP	16.8[a]	17.5[a]	15.4[b]	0.43	< 0.05	0.13
NDF	47.1	47.7	48.2	1.30	0.79	0.39
EE	5.3	5.9	5.1	0.49	0.42	0.60
N excretion						
N intake, g/d	224.9[a]	185.2[b]	169.6[b]	6.83	< 0.001	0.71
Faecal N excretion, g/d	69.2	66.6	70.5	2.87	0.54	0.91
Urinary N excretion, g/d	91.5[a]	63.1[b]	50.4[b]	5.72	< 0.001	0.69

Abbreviation: DMI = DM intake, OM = organic matter, EE = ether extract.
[1] T = treatment effect; B = sampling period (block) effect.
[a,b,c] Means within a row with different superscripts are statistically different ($P < 0.05$).

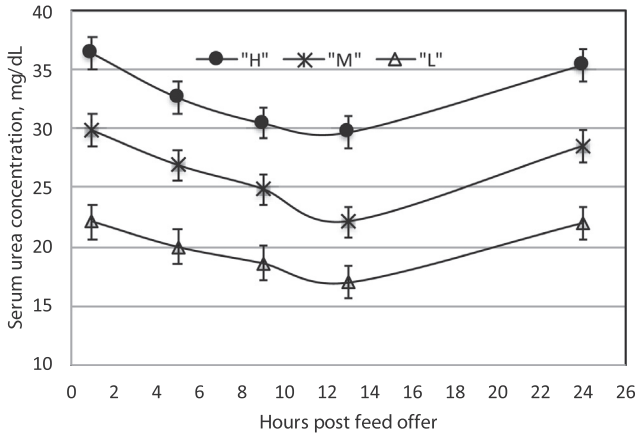

Fig. 2. Serum urea concentration evolution after feed offer in Holstein heifers fed concentrate with high (H), medium (M) or low (L) dietary CP concentration (Nitrogen excretion study).

tion from H to M or L. Serum urea concentration varied with time during postfeeding (Fig. 2), and the lowest concentrations were found after 12 h postfeeding ($P < 0.001$). Moreover, the serum urea concentration was greater ($P < 0.001$) in H heifers than in M heifers and in L heifers. No treatment by time interaction was observed in this parameter.

Finally, there were no bloat incidences, all records were "0" scores. The faecal score did not differ among treatments, and the most common register was "1" (normal), except for one heifer at H and another one at M, which scored "2" (soft to lose) on days 14 and 35, respectively (data not shown).

Manure storage in vitro experiment

The DM content of initial manures ranged from 10.5 to 9.6% (Table 5). The TN content was affected by the treatment: manure-L had the lowest ($P < 0.05$) initial TN and tended to have the lowest TC content ($P = 0.07$) as well when compared with H and M manures.

After 8.3 weeks of storage in aerobic conditions, the TN content decreased due to biological transformation and volatilisation.

Table 5
Nitrogen and carbon emissions of manure from Holstein bulls fed concentrate with high (H), medium (M) or low (L) CP concentration during the *in vitro* aerobic and anaerobic storage. Results obtained before and after 8.3 weeks of storage (Volatilisation study).

	Treatment				*P*-value[1]
Item	H	M	L	SEM	T
Wet weight					
Initial manure, g	60.0	60.0	60.0		
Final manure, g					
Aerobic	55.1	55.4	53.1		
Anaerobic	58.7	59.5	59.2		
Wet weight loss after storage, % initial wet weight					
Aerobic	8.1[a]	7.6[b]	11.4[a]	0.59	< 0.05
Anaerobic	2.2	0.9	1.3	0.79	0.52
DM concentration					
Initial manure, g/kg	105.3	97.7	96.5	4.33	0.41
Final manure, g/kg					
Aerobic	84.7	82.0	86.3	1.27	0.12
Anaerobic	84.3[a]	79.0[b]	77.7[b]	1.32	0.03
DM					
Initial dry mass, g	6.3	5.9	5.8		
Final dry mass, g					
Aerobic	4.7	4.5	4.6		
Anaerobic	4.9	4.7	4.6		
Dry weight loss after storage, % initial dry weight					
Aerobic	26.1[a]	22.5[ab]	20.8[a]	1.10	< 0.05
Anaerobic	21.7	19.8	20.5	1.48	0.69
Total nitrogen (TN)					
Initial manure, g/kg	8.6[b]	9.5[a]	7.5[c]	0.08	< 0.01
Final manure, g/kg					
Aerobic	5.7[a]	5.4[a]	4.2[b]	0.16	< 0.05
Anaerobic	7.8[a]	7.7[a]	7.6[b]	0.03	< 0.01
TN Loss after storage, % initial TN					
Aerobic	39.7[b]	47.9[a]	50.1[a]	2.00	< 0.05
Anaerobic	11.6[b]	19.2[a]	0.6[c]	0.10	< 0.001
N-NH_3 emitted, % initial TN					
Aerobic	15.7	14.2	20.0	0.07	0.09
N-NH_3 emitted, % TN loss					
Aerobic	39.6	29.7	39.9		
Total carbon (TC)					
Initial manure, g/kg	39.2	37.1	30.8	1.71	0.07
Final manure, g/kg					
Aerobic	24.0	27.7	21.3	2.27	0.22
Anaerobic	35.7	34.3	32.0	1.51	0.09
TC Loss after storage, % initial TC					
Aerobic	43.8	31.1	38.7	5.9	0.37
Anaerobic	11.0	6.2	2.2	4.7	0.17
Emitted C-CH_4+C-CO_2, % initial TC					
Aerobic	14.7[b]	15.0[b]	21.3[a]	0.75	< 0.05
Anaerobic	8.4[b]	8.3[b]	9.1[a]	1.59	< 0.05
Carbon/Nitrogen ratio					
Initial manure, g/g	4.5	3.9	4.1	0.17	0.15
Final manure, g/g					
Aerobic	4.2	5.2	5.0	0.57	0.50
Anaerobic	4.6	4.4	4.2	0.19	0.14

[1] T = treatment effect.
[a,b,c] Means within a row with different superscripts are statistically different ($P < 0.05$).

Manure-L still had the lowest ($P < 0.05$) TN content at the end of the assay, compared with H and M manures. However, the percentage of aerobic N loss was greater ($P < 0.05$) in L and M manures (50.1% and 47.9% initial TN, respectively) than in manure-H (39.7% initial TN). The total aerobic emitted N-NH_3 tended ($P = 0.09$) to comprise the total N loss, and was 15.7%, 14.2%, and 20.0% initial TN for manures H, M, and L, respectively (Table 5, Fig. 1A). Regarding the emission rate, the greatest cumulative N-NH_3 volatilisation was recorded for manure-L (Fig. 1B) and occurred primarily during the first 7 days of the aerobic assay, with a maximum peak near 43 μgN-NH_3/m^2/s. This emission was greater during the first 60 days of storage of manure-H; however, the greatest N loss was recorded in manure-L and this value was slightly greater than in manure-M (Fig. 1B). This different behaviour was also registered as a time delay in ammonia emission of 4.0–4.5 days in manure-M and manure-L. The TN losses during storage were inversely correlated with the TC to TN ratio of the manure (Fig. 3A) and increased with urine N (Fig. 3B).

The overall TC losses under aerobic conditions were caused by the biodegradation of organic matter and by the emission of CO_2 (no CH_4 was detected). After 8.3 weeks of storage under anaerobic conditions, the TN loss was lesser than when incubated under aerobic conditions. This phenomenon was exemplified in manure-L, where there were almost no N losses. The decrease of the initial TN in this *in vitro* anaerobic assay must be regarded primarily as the result of the biological activity rather than a volatilisation process, since manure samples were incubated in airtight vials. In this anaerobic assay, the initial TC was converted to CH_4 and CO_2, which indicates that methanogenic microbial communities were active. In this regard, manure-H losses were numerically greater

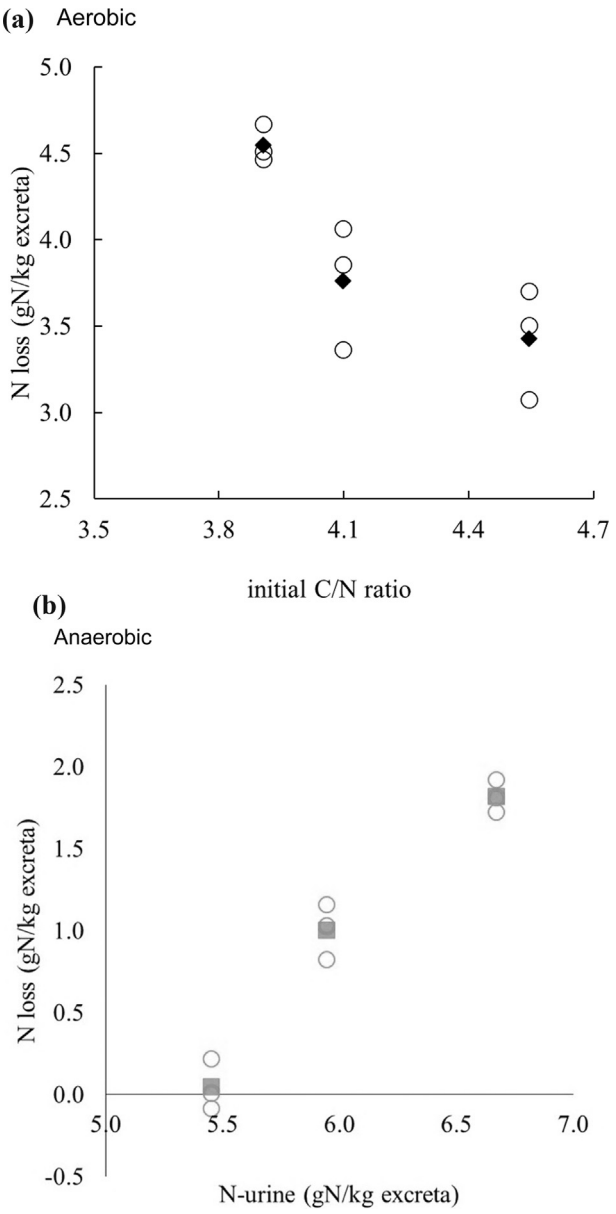

Fig. 3. Total N loss expressed per kg of beef manure, at the end of storage (Volatilisation study). (a) Relationship with total C to total N ratio of the manure. (b) Relationship with the N-urine content of the manure. Symbols: diamonds, average value of triplicates; circles, value per replicate. Abbreviations: C = carbon, N= nitrogen, N-urine= urine nitrogen.

when expressed as percentage with respect to the initial TC (11% initial TC). Yet, the TC to TN ratio was not affected, neither by treatment nor by storage conditions (Table 5).

The initial DM did not differ among treatments (Table 5), but the percentage of wet weight loss after the aerobic storage was around 10%, being significantly greater ($P < 0.05$) in manure-L than in H and M manures. However, dry weight loss during aerobic storage was significantly lesser ($P < 0.05$) in manure-L, followed by M and H manures. The percentage of wet weight loss after the anaerobic storage was lesser than that of aerobic storage because of the confinement of the former. The total solids after 8.3 weeks of anaerobic storage were also lesser ($P < 0.05$) in L and M manures than in H-manure.

Discussion

Performance and nitrogen excretion study

Based on results from the performance study (Table 2) and the N excretion study (Table 4), the decrease of dietary CP concentration did not affect concentrate, straw, or total DMI. This agrees with previous studies conducted with Holstein heifers with high-concentrate diets and similar N dietary sources (Devant et al., 2000 and 2001). When feeding the lowest dietary CP concentration, the DM, OM, and starch apparent total digestibility decreased. However, the magnitude of the decrease in starch digestibility was rather low (4 to 1 points); in these diets, starch digestibility was high (above 92%) and therefore, it may not have had an impact on performance as it was supported by the absence of differences in growth in the performance study. Other feeding strategies rather than dietary CP concentration, such as a concentrate presentation form (Secrist et al., 1995; Devant et al., 2018) or the addition of enzymes (Salem et al., 2013), have a greater impact on apparent total tract starch digestibility and, consequently, on performance. Moreover, the mechanisms whereby the decrease in dietary CP intake may have decreased starch total apparent digestibility are unknown. Generally, the literature describes how starch digestibility may limit microbial growth and, in turn, N use in the rumen (Theurer, 1986; Huntington, 1997), rather than how N availability may limit starch digestibility. In the present study, a relatively low N availability in the rumen (less N intake and no urea intake in L treatment) may have limited rumen microbial growth, which is essential to lyse the protein structures that protect starch granules (Svihus et al., 2005). Moreover, the different ingredient composition among treatments (wheat, wheat middling, beet pulp, and palm oil) may have also caused the differences observed in starch digestibility among treatments. As mentioned above, the effect of dietary CP on starch digestibility was small compared with the great impact that the decrease of dietary CP had on CP digestibility reduction (from 69.3% in H diet to 64.0% and 58.3% in M and L diets, respectively) and this effect has been previously described (Devant et al., 2000).

The N urinary excretion diminished as CP was reduced, although there were no statistical differences in excreted N urinary between M and L treatments, probably because N intake did not differ that much. Nitrogen intake did not differ as dietary CP content in the concentrates in these two treatments was more similar than expected, and during the N excretion, the DMI of M and L heifers was numerically close. Moreover, when analysing all study data together (linear regression, data not shown), N intake explained 66% of the variation of the urinary N excretion ($P < 0.001$). As shown in Fig. 3B, as urinary N content in manure increased, the manure N loss (emission) also increased; so, the reduction of urinary N excretion when decreasing dietary CP is a successful strategy to reduce the N pollution in beef production. In summary, analysing experimental results 1 and 2 together, the present study results indicate that reducing dietary CP concentration from 14.0% to 12.0% (when modifying soybean meal and urea inclusion levels), CP digestion, serum urea concentration, and N urinary excretion decrease without having detrimental effects on the major economic performance indicators and carcass quality. So, the results of the present study demonstrate that there are still opportunities to reduce N excretion in beef cattle. Our data might provide further evidence on the fact that current feeding systems for ruminants in the United States (NRC, 1996 and 2000) and in Spain (FEDNA, 2008) should review their recommendations. These systems probably overestimate the dietary CP requirements of the

animals as they do not consider urea recycling, and the presented serum urea and N excretion data support this hypothesis.

Manure greenhouse gas emissions

In a recent review, Sajeev et al. (2018) performed a meta-analysis of scientific literature on NH_3 reductions following a dietary CP decrease in cattle and pigs. These authors concluded that mean NH_3 emissions in cattle can be reduced by 17% for each 1% of dietary CP decrease. In their estimation, the nutritional factors N source and dietary concentration, among other aspects, such as manure management systems, which may differ greatly from farm to farm, are considered. The present study supports Sajeev et al. (2018) results if only animal excretion data are analysed (Table 3). However, when considering the effect of manure storage (Table 5) and depending on the type of biological degradation (aerobic or anaerobic), the decrease of dietary CP does not always have a positive effect on preventing N loss (emissions), as indicated by the storage experiment.

In Spanish beef commercial farms, the manure management practice usually begins with the bedding removal, under aerobic conditions, followed by outdoors manure storage, under intermingled aerobic and anaerobic conditions, usually with no further handling until the field application. *In vitro* storage tests from the present study simulated aerobic and anaerobic processes that take place during the first 8.3 weeks of manure storage. The results of the present study indicate that N and C losses are significantly greater under aerobic conditions than under anaerobic conditions.

Surprisingly, the percentage of aerobic N loss was greater in manure from heifers fed with a relatively low CP concentration (manure-L) than with a medium and high CP diet (M and H manures). This result is explained by the rate of NH_3 loss (Fig. 1B), which was greater in manure-L than in manure-H and manure-M during the first 20 days. Moreover, at the end of the aerobic storage process, the N content in manure decreased along with constraints in the CP dietary content. Interestingly, there was a delay of 4 days in the emission of NH_3 in M and L manures compared with manure-H (Fig. 1A). Burgos et al. (2010) found that urea N was almost completely lost within 24 h at 22.5 °C and that initial TN in urine in manure slurry was directly proportional to the final N-NH_3, which is consistent with the hydrolysis of urea as the determining factor for N-NH_3 concentration. Maeda and Matsuda (1997) related N losses to initial manure N-NH_3, and the urine N was hydrolysed to N-NH_3 within 1 week of manure storage at 20 °C. Therefore, decreasing dietary CP may be a good strategy to reduce the NH_3 emissions during the first days of a composting process. Burgos et al. (2010) found that daily cow NH_3 emissions from manure slurries ranged from 57 to 149 g of N and, in disagreement to the present study, NH_3 emissions were proportional to the dietary CP content.

Moreover, as mentioned above, the total N loss during the storage was greater under aerobic than under anaerobic conditions (Table 5). Manure volatile solid is a key driver of N transformations and losses (Petersen and Sommer, 2011). Readily biodegradable organic components serve as an O_2 sink, as well as a C and energy source for heterotrophic denitrification under aerobic-anaerobic environmental gradients, while particulate volatile solid adds structure to manure. The manure type (solid or liquid) may thus affect the storage conditions (aerobic or anaerobic). Solid manure rich in high fibre bedding material has relatively high porosity that promotes aerobic biodegradation and the generation of metabolic heat, which may result in NH_3 and N_2O emissions. In contrast, liquid manure (slurry) contains buoyant particulate matter that might constitute a physical barrier to gas exchange if a surface crust is formed. Therefore, if possible, the anaerobic processing of manure should be encouraged to reduce N losses.

However, slight differences in total aerobic or anaerobic C losses were observed among treatments, and the emitted carbon as CH_4 and CO_2 was always greater in manure-L than in M and H manures. Studies that describe the effects of varying the dietary CP levels on C emissions from manures are still scarce and inconsistent. In agreement with the present study, Külling et al. (2001) found that methane emissions from cow manure were not reduced and even slightly increased by dietary protein decrease. Yet, further research is needed to understand such an apparent inverse correlation between dietary CP and C emissions from manures. Perhaps not only the C content is important, and the C nature may influence C emissions as well and so, the rather large content of starch in manure-L may have enhanced C emissions. Microbial consortia of autotrophic and heterotrophic bacteria and archaea utilise the energy and N contained in urine and faeces as substrate, thereby transforming the original compounds into various chemical species, including CO_2, CH_4, NH_4^+, NO_3^-, N_2O and N_2, in varying amounts (Dijkstra et al., 2013). Moreover, in urine-rich slurry, where almost no fibre is present, CH_4 formation can be inhibited by NH_4^+ (Hansen et al., 1998). Thus, the considerable decrease of N-NH_3 content in manures originated from low CP diets might explain the observed increase in CH_4 emissions.

The present study provides a broader view beyond the effect of nutrition on animal excretions and analyses possible scenarios of the effect of nutrition at farm level including also manure emissions. Reducing N and C losses from the farm must begin with proper animal feeding and, subsequently, by implementing efficient manure management practices to reduce N and C emissions. The loss of volatile compounds starts soon after the excretion and continues through all manure handling processes until the manure nutrients are incorporated into the soil. This volatilisation is linked to biological processes evolving under aerobic or anaerobic conditions, depending on the manure management practices.

In summary, the decrease of dietary CP concentration from 17% to 14.5% and 12% was an efficient strategy to reduce urinary N excretion up to 40%, without impairing performance or carcass quality. Manure storage under anaerobic conditions was highly efficient in preventing N losses. Under such anaerobic conditions, N volatilisation was 20 times greater in manure from animals fed 14.5% CP and 10 times greater in animals fed 17% CP, compared to the N losses of manure from animals fed 12% CP. However, a dietary CP content of 14.5% resulted in less environmental impact than a dietary CP content of 12.8% when also considering manure emissions under aerobic or anaerobic conditions. But if CH_4 is collected and valorised through the anaerobic digestion process (biogas production), then dietary protein can still be reduced down to 12% CP for obtaining additional environmental benefits.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2022.100471.

Ethics approval

All experimental protocols were approved by the Institutional Animal Care Committee of the Institut de Recerca i Tecnologia Agroalimentàries (Barcelona, Spain), and the study was conducted in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes; Boletín Oficial del Estado, 2013).

Data and model availability statement

Data analyses were performed with SAS (SAS Inst. Inc., Cary, NC). None of the data were deposited in an official repository. The data that support the study findings are available upon reasonable request.

Authors ORCIDs

Maria Devant: https://orcid.org/0000-0001-8474-5058
Belen Fernández: https://orcid.org/0000-0001-6854-2349.
Francesc Xavier Prenafeta-Boldú: https://orcid.org/0000-0001-9514-7029

Authors contribution

Maria Devant: Conceptualization, methodology, investigation, data curation resources, writing: original draft & review&editing, project administration, funding acquisition
Armando Pérez: conceptualization, methodology, investigation
Carles Medinyà: conceptualization, methodology, investigation
Joan Riera: conceptualization, methodology, investigation
Joan Grau: conceptualization, methodology, investigation
Belen Fernández: conceptualization, methodology, investigation, data curation resources, writing: original draft & review
Francesc Xavier Prenafeta-Boldú: conceptualization, methodology, investigation, writing: original draft & review

Declaration of interest

There is no conflict of interest.

Acknowledgements

The authors thank the collaboration of the personnel of the Cooperativa Agropecuaria de Guissona (Lleida, Spain).

Financial support

This research was funded by the Operational Group "Estratègies per reduir l'excreció de nitrogen en vedells d'engreix durant la fase d'acabat", financially supported by DARP (Generalitat de Catalunya) and FEADER (European Union). IRTA also thanks the support of the Generalitat de Catalunya through the Consolidated Research Group TERRA (ref. 2017 SGR 1290) and the CERCA Programme.

References

Association of Official Analytical Chemists (AOAC), 1995. Official methods of analysis, 16th edition. AOAC, Arlington, VA, USA.
American Public Health Association, American Water Works Association and Water Pollution Control Federation (APHA, AWWA and WPCF), 2005. Standard Methods for the Examination of Water and Wastewater. 21st Edition. APHA, AWWA and WPCF, Washington DC, USA.
Balcells, J., Guada, J.A., Peiro, J.M., Parker, D.S., 1992. Simultaneous determination of allantoin and oxypurines in biological fluids by high-performance liquid chromatography. Journal of Chromatography 575, 153–157.
Burgos, S.A., Emberston, N.M., Zahor, Y., Mitloehner, F.M., DePeters, E.J., Fadel, J.G., 2010. Prediction of ammonia emission from dairy cattle manure based on milk urea nitrogen: relation of milk urea nitrogen to ammonia emissions. Journal of Dairy Science 93, 2377–2386. https://doi.org/10.3168/jds.2009-2415.
Bussink, D.W., Oenema, O., 1998. Ammonia volatilization from dairy farming systems in temperate areas: a review. Nutrient Cycling in Agroecosystems 51, 19–33.
Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance). Retrieved 9 January 2022 from http://data.europa.eu/eli/reg/2009/152/oj.
Chen, X.B., Grubic, G., Ørskov, E.R., Osuji, P., 1992. Effect of feeding frequency on diurnal variation and urinary derivatives in steers. Animal Production 55, 185–191.
Devant, M., Solé, A., Quintana, B., Pérez, A., Ribó, J., Bach, A., 2019. Effect of particle size of a mash concentrate on behavior, digestibility, and macroscopic and microscopic integrity of the digestive tract in Holstein bulls fed intensively. Translational Animal Science 3, 473–484. https://doi.org/10.1093/tas/txy116.
Devant, M., Ferret, A., Gasa, J., Calsamiglia, S., Casals, R., 2000. Effects of protein concentration and protein degradability on performance, ruminal fermentation and nitrogen metabolism in crossbred heifers fed concentrate and barley straw from 100 to 230 kg body weight. Journal of Animal Science 78, 1667–1676.
Devant, M., Ferret, A., Calsamiglia, S., Casals, R., Gasa, J., 2001. Effect of nitrogen source in high-concentrate diets on microbial fermentation studied in vivo and in vitro. Journal of Animal Science 79, 1944–1953.
Devant, M., Solé, A., Quintana, B., Pérez, A., Ribó, J., Bach, A., 2018. Effect of particle size of concentrate in meal form on behavior, digestibility, and macroscopic and microscopic integrity of the digestive tract in Holstein bulls fed intensively. Translational Animal Science 3, 473–484. https://doi.org/10.1093/tas/txy116.
Dijkstra, J., Oenema, O., van Groenigen, J.W., Spek, J.W., van Vuuren, A.M., Bannink, A., 2013. Diet effects on urine composition of cattle and N_2O emissions. Animal 7 (Suppl 2), 292–302. https://doi.org/10.1017/S1751731113000578.
Erickson, G., Klopfenstein, T., 2010. Nutritional and management methods to decrease nitrogen losses from beef feedlots. Journal of Animal Science 88 (E. Suppl.), E172–E180. https://doi.org/10.2527/jas.2009-2358.
Ferret, A., Calsamiglia, S., Bach, A., Devant, M., Fernández, C., García-Rebollar, P., 2008. Necesidades nutricionales para rumiantes de cebo. FEDNA. Retrieved on 9 January 2021 from http://www.fundacionfedna.org/sites/default/files/NORMAS_RUMIANTES_2008.pdf.
Hansen, K.H., Angelidaki, I., Ahring, B.K., 1998. Anaerobic digestion of swine manure: inhibition by ammonia. Water Research 32, 5–12.
Heinrichs, A.J., Jones, C.M., Bolt, B.M., Diaz, D.E., Brownie, C., Brown, T., Hungtington, G.B., 2003. Effects of mannan oligosaccharide or antibiotics in neonatal diets on health and growth of dairy calves. Journal of Dairy Science 86, 4064–4069. https://doi.org/10.3168/jds.S0022-0302(03)74018-1.
Hristov, A.N., Hanigan, M., Cole, A., Todd, R., McAllister, T.A., Ndegwa, P.M., Rotz, A., 2011. Review: Ammonia emissions from dairy farms and beef feedlots. Canadian Journal of Animal Science 91, 1–35. https://doi.org/10.4141/CJAS10034.
Huhtanen, P., Nousiainen, J.I., Rinne, M., Kytölä, K., Khalili, H., 2008. Utilization and partition of dietary nitrogen in dairy cows fed grass silage-based diets. Journal of Dairy Science 91, 3589–3599. https://doi.org/10.3168/jds.2008-1181.
Huntington, G.B., 1997. Starch utilization by ruminants: from basics to the bunk. Journal of Animal Science 75, 852–867.
James, T., Meyer, D., Esparza, E., Depeters, E.K., Perez-Monti, H., 1999. Effects of dietary nitrogen manipulation on ammonia volatilization from manure of Holstein heifers. Journal of Dairy Science 82, 2430–2439. https://doi.org/10.3168/jds.S0022-0302(99)75494-9.
Johnson, R.H., Brown, L.R., Jacobson, N.L., Homeyer, P.G., 1958. Effectiveness and practicability of some oils, penicillin, *n*-decyl alcohol, and lecithin in the control of alfalfa bloat. Journal of Animal Science 17, 893–902. https://doi.org/10.2134/jas1958.173893x.
Koenig, K.M., Beauchemin, K.A., McGinn, S.M., 2018. Feeding condensed tannins to mitigate ammonia emissions from beef feedlot cattle fed high-protein finishing diets containing distillers grains. Journal of Animal Science 96, 4414–4430. https://doi.org/10.1093/jas/sky274.
Külling, D.R., Menzi, H., Kröber, T.F., Neftel, A., Sutter, F., Lischer, P., Kreuzer, M., 2001. Emissions of ammonia, nitrous oxide and methane from different types of dairy manure during storage as affected by dietary protein content. Journal of Agricultural Science 137, 235–250.
Maeda, T., Matsuda, J., 1997. Ammonia emissions from composting livestock manure. In: Voermans, J.A.M., Monteny, G.J. (Eds.), Ammonia and Odour Emissions from Animal Production Facilities, International Conference Proceedings. Dutch Society of Agricultural Engineering, Vinkeloord, The Netherlands, pp. 145–153.
Marti, S., Velarde, A., de la Torre, J.L., Bach, A., Aris, A., Serrano, A., Manteca, X., Devant, M., 2010. Effects of ring castration with local anesthesia and analgesia in Holstein bulls at three months of age on welfare indicators. Journal of Animal Science 88, 2789–2796. https://doi.org/10.2527/jas.2009-2408.
Masson-Delmotte, V., Zhai, P., Pirani, A., Connors, S. L., Péan, C., Berger, S., Caud, N., Chen, Y., Goldfarb, L., Gomis, M. I., Huang, M., Leitzell, K., Lonnoy, E., Matthews, J.B.R., Maycock, T. K., Waterfield, T., Yelekçi, O., Yu, R., Zhou, B., 2021. Climate Change 2021: The Physical Science Basis. Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press. Cambridge, UK (in press).
Misselbrook, T.H., Powell, J.M., 2005. Influence of bedding material on ammonia emissions from cattle excreta. Journal of Dairy Science 88, 4304–4312. https://doi.org/10.3168/jds.S0022-0302(05)73116-7.
National Research Council (NRC), 1996. Nutrient requirements of beef cattle. National Academies Press, Washington, DC, USA.
National Research Council (NRC), 2000. Nutrient requirements of beef cattle. National Academies Press, Washington, DC, USA.
Palatsi, J., Illa, J., Prenafeta-Boldú, F.X., Laureni, M., Fernandez, B., Angelidaki, I., Flotats, X., 2010. Long-chain fatty acids inhibition and adaptation process in

anaerobic thermophilic digestion: batch tests, microbial community structure and mathematical modelling. Bioresource Technology 101, 2243–2251. https://doi.org/10.1016/j.biortech.2009.11.069.

Petersen, S., Sommer, S., 2011. Ammonia and nitrous oxide interactions: Roles of manure organic matter management. Animal Feed Science and Technology 166–167, 503–513. https://doi.org/10.1016/j.anifeedsci.2011.04.077.

Reynolds, C.K., Kristensen, N.B., 2008. Nitrogen recycling through the gut and the nitrogen economy of ruminants: Anasynchronous symbiosis. Journal of Animal Science 86 (E. Suppl.), E293–E305. https://doi.org/10.2527/jas.2007-0475.

Royal Decree 53/2013 of February 1st on the protection of animals used for experimentation or other scientific purposes. 2013. Boletín Oficial del Estado. Retrieved 9 January 2022 from https://www.boe.es/eli/es/rd/2013/02/01/53.

Sajeev, E.P.M., Amon, B., Ammon, C., Zollitsch, W., Winiwarter, W., 2018. Evaluating the potential of dietary crude protein manipulation in reducing ammonia emissions from cattle and pig manure: a meta-analyses. Nutrient Cycling in Agroecosystems 110, 161–175. https://doi.org/10.1007/s10705-017-9893-3.

Salem, A.Z.M., Gado, H.M., Colombatto, D., Elghandour, M.M.Y., 2013. Effects of exogenous enzymes on nutrient digestibility, ruminal fermentation and growth performance in beef steers. Livestock Science 154, 69–73. https://doi.org/10.1016/j.livsci.2013.02.014.

Svihus, B., Uhlen, A.K., Harstad, O.M., 2005. Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. Animal Feed Science and Technology 122, 303–320. https://doi.org/10.1016/j.anifeedsci.2005.02.025.

Secrist, D.S., Hill, W.J., Owens, F.N., Welty, S.D., 1995. Effects of corn particle size on feedlot steer performance and carcass characteristics. Animal Science Research Report 1995, 99–104 (http://www.ansi.okstate.edu/research/researchreport).

Theurer, C.B., 1986. Grain processing effects on starch utilization by ruminants. Journal of Animal Science 63, 1649–1662. https://doi.org/10.2527/jas1986.6351649x.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral fiber and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3588–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Verdú, M., Bach, A., Devant, M., 2017. Effect of feeder design and concentrate presentation form on performance, carcass characteristics, and behavior of fattening Holstein bulls fed high-concentrate diets. Animal Feed Science and Technology 232, 148–159.