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EMERGING ISSUES IN ANIMAL HUSBANDRY: EFFECTS OF REDUCED PROTEIN SUPPLY IN ANIMAL FARMS AND METHODOLOGIES FOR FATTY ACID ANALYSIS IN BIOLOGICAL SAMPLES

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GENERAL ABSTRACT

Aim of this thesis was to explore the effects of suboptimal N supplies on animals of different species on production performances to achieve an effective reduction of N excretion avoiding, or limiting, the economic losses due to impairment of performance or reduced quality of products. As aside different methods of fatty acid analysis were evaluated to identify an accurate and repeatable methodology of extraction of fatty acids that prevents isomerisation of PUFA and conjugated linoleic acid. All the contributions of this thesis have been published or submitted to international peer reviewed journals. This thesis was also reviewed by foreign referees and all their suggestions were accepted and included in the present version of this thesis. The first two contributions were aimed to verify the effect of suboptimal dietary crude protein supply on growing cattle and lactating cows. In lactating cows the effect of a supplementation with rumen protected conjugated linoleic acid (CLA) was also explored. The third contribute regards an experiment conducted to evaluate the effect of different fecal sample preparation procedures to determine the fatty acid profile of ruminants feces, including CLA. The fourth and the fifth contributions were aimed to evaluate the effects of reduced dietary crude protein and amino acid levels on growth performance of heavy pigs (4^{th} contribute) and on growth performance and intramuscular lipid deposition on fast growing pigs (5^{th} contribute). Overall, the results suggests that considerable reduction of dietary crude protein, compared to conventional standard, can be applied with small or null impairment of production response, but with a notable reduction of N excretion. Within the framework of the nitrate directive of the European Community, the strong reduction of N excretion achieved with the use of suboptimal protein supplies would result in the possibility of increasing the number of animals reared per unit of land available for manure disposal, and to reduce the feeding costs because of less use of expensive dietary protein sources. From the third contribute it resulted that fecal sample preparation method has an influence on fecal fatty acid profile, and in particular on the CLA component, stronger than that was previously evidenced in the literature that indicated the methylation as a critical step for an accurate analysis of fatty acids by gas chromatography. Analytical methods applicable on different biological substrates (feeds, feces, and animal products) are needed for a better knowledge of lipid digestion and metabolism of farm animals.

RIASSUNTO GENERALE

Scopo di questa tesi è stato quello di esplorare gli effetti conseguenti ad una somministrazione subottimale di proteina ad animali di diverse specie sulle performance produttive per poter ottenere una riduzione effettiva della escrezione di N ed evitare, o limitare, le perdite economiche dovute al peggioramento delle prestazioni o alla riduzione della qualità dei prodotti. Un capitolo a parte della tesi è stato di valutare diversi metodi di analisi degli acidi grassi per identificare una metodologia accurata e ripetibile di estrazione degli acidi grassi in grado di prevenire l'isomerizzazione degli acidi grassi polinsaturi in particolare dei coniugati dell'acido linoleico. Tutti i contributi presentati in questa tesi sono stati pubblicati o sottoposti riviste scientifiche internazionali. Questa tesi è stata valutata da due docenti stranieri e i loro suggerimenti sono stati accettati e inclusi nella versione finale di questa tesi. I primi due contributi sono stati finalizzati a verificare l'effetto di diete contenenti concentrazioni subottimali di proteina grezza, somministrate a bovini da carne e vacche in lattazione. Nelle vacche in lattazione è stato anche valutato l'effetto conseguente alla somministrazione di acido linoleico coniugato (CLA) rumino protetto. Il terzo contributo riguarda un esperimento condotto per valutare l' effetto di differenti procedure di preparazione dei campioni fecali per determinare il profilo degli acidi grassi, compresi i CLA, nelle feci dei ruminanti. Il quarto ed il quinto contributo sono finalizzati a valutare gli effetti della riduzione nella dieta dei livelli di proteina grezza e degli amminoacidi sulle performance di crescita dei suini pesanti (4° contributo) e sulle performance di crescita e di deposizione lipidica intramuscolare in suini selezionati per una crescita rapida e magra (5° contributo). Complessivamente, i risultati suggeriscono che una notevole riduzione del livello proteico della dieta, rispetto a standard tradizionali, può essere applicato compromettendo lievemente o per nulla la risposta produttiva, ma con una notevole riduzione di escrezione azotata. Nell'ambito della direttiva nitrati della Comunità Europea, la forte riduzione dell'escrezione di N ottenuto grazie all' impiego di diete ipoproteiche può comportare la possibilità di aumentare il numero di animali allevati per unità di superficie disponibile per lo smaltimento del letame, o di ridurre i costi di alimentazione a causa di un minor uso di costose fonti proteiche. I risultati del terzo contributo indicano che il metodo di preparazione del campione ha un'influenza sul profilo di acidi grassi delle feci, ed in particolare sulla componente dei CLA, maggiore rispetto a quella precedentemente evidenziata in letteratura che indicava la metilazione come tappa critica per un'analisi accurata degli acidi grassi mediante gascromatografia. L'individuazione di metodi di analisi applicabili a differenti substrati biologici (foraggi e alimenti, feci e prodotti di

origine animale) è necessaria per migliorare le conoscenze sulla digestione dei lipidi ed il metabolismo degli animali da allevamento.

GENERAL INTRODUCTION

Animal nutrition has always played a very important role in animal husbandry but in the last decadesit became even more crucial for the farm management. Nutrient requirements have been established to meet maintenance and production demand of nutrients from animal husbandry, but in the practice a slight excess of nutrients compared to the requirements is often applied to avoid performance impairment. To be competitive and to comply with the market needs, food products must be safe for the consumer, must be produced efficiently (competitively priced) and must reach the desired level of quality (Kyriazakis and Whittemore, 2006). An accurate definition and estimation of nutrient requirements is important to improve feed efficiency, to reduce waste of resources, limit the emission of nutrients in the environment and to achieve the production aims according to the reference market of the products.

Many energy and protein systems have been defined to estimate nutrient requirements and the nutritive values of feeds for swine, dairy and beef cattle and for many other species and categories of domestic animals. Even if the structure of the various models is similar, each energy and protein system reflects the prevailing conditions of rearing as the genetic resources, the feeding practices, the herd management and the climate conditions of the country where the system was developed. Inaccurate prediction of energy requirements or the nutritive value of feeds is expected when the use of a given system is extended to conditions different from those where the system was established. In addition, many of these models have been developed with the aim of maximizing the production, and less emphasis was put on studying the effects of suboptimal nutrients supply.

Following the Nitrate Directive (EEC, 1991) the European territory was divided into nitrate vulnerable or not vulnerable areas. In nitrate vulnerable zones a maximum load of 170 kg N/ha has been established as a maximum threshold, whereas in not vulnerable zones a threshold of 340 kg N/ha has beenindicated. As a consequence in many EU geographical areas the need for agricultural land is greater than the availability of land for manure disposal, with consequences on the number of animals which can be reared. A reduction dietary protein content is considered as one of the major way to reduce the excretion of N and this would increase the number of animals which can be reared per unit of agricultural land (Schiavon et al. 2012).

Another tendency has been observed in the last decade: the rise of the cost of feeds, protein sources in particular. This is mainly due to the international competition for raw materials caused by the impressively rate of growth of livestock sector, mainly in the developing countries which is also defined as “the livestock revolution” (Delgado et al., 1999). Soybean meal price increased from 194.48 euro per metric ton in September 2003 to 367.09 euro per metric ton in September 2013 (source: World Bank, 2013) with an increase of the 89%. During 1999 the value of 1 kg of pork meat was equal to the value of 6 kg of soybean meal while, nowadays, the price of 1 kg of pig meat is equivalent to the cost of 3 kg of soybean meal. This trend had a strong impact on farms economy, with an increase of feeding costs at a rate greater than therate of value increaseof the products.

The pressure exerted by environmental concerns and legislation as well as the increasing cost of protein sources is driving deep changes in the farm practices with a growing demand for establishing effective strategies to reduce the use of protein sources avoiding a quantitative or qualitative impairment of production outcomes.

Cattle protein and energy requirements

The theory that animal performances can be optimized by synchronizing the rumen availability of degradable protein and degradable energy substrates has been accepted and applied during the last 30 years (Hall and Huntington, 2008). It has been recently proposed that this concept of dietary synchrony should be revised as a variety of dietary and animal factors would determine the nutrient availability for the rumen microbes and the host. Many researches show how asynchronous diets gave results as good as or superior to synchronous diets, in ruminal and animal performances both in growing beef and dairy cows (Valkeners et al., 2004; Cole and Todd, 2008). This evidence the existence of physiological mechanisms, such as N recycling, that may mitigate the negative effects of dietary nutrient unbalance or N shortage. Current knowledges about the effect of a shortage of rumen degradable energy, protein or both suggests a reduction of feed digestibility, a decrease of dry matter intake (DMI) and a decrease of the production performance (Valkeners et al., 2008). However, recent evidences (Hall and Huntington, 2008; Schiavon et al., 2012) suggest that N recycling and other physiological adaptations can compensate for suboptimal N supply. From the economical point of view it could be interesting to evaluate the effect of suboptimal protein supply on individual and farm level. In fact, under the frame of the nitrate directive, a small reduction of individual performance would be largely compensated by the possibility of

increasing the number of animal reared per unit of land, because of the reduction of N excretion (Schiavon et al., 2012).

The lack of response when synchronised and unsynchronised diets were compared (Hall and Huntington, 2008) need to be better clarified also exploring the effects of less known factors such as variations of the rumination activity. Rumination is a cyclical behaviour characterized by regurgitation, re-mastication and re-swallowing of the feed with the function of facilitate digestion, reduce particle size and the subsequent passage from the reticulo-rumen. Ruminants require diets that must contain the adequate particle size to maintain healthy rumen function because long forage particles promote chewing and salivary buffer secretion, ultimately elevating rumen pH (Beauchemin and Yang, 2005). Adequate particle length can be difficult to ensure because most commercial cattle rations contain high levels of concentrate and high quality silages often finely chopped in a total mixed ration. These types of diets are highly fermentable in the rumen and encourage maximum production performances but would lead to subclinical ruminal acidosis, reduced digestive efficiency, reduced feed intake, milk production and quality, and induces lameness, or fat cow syndrome (NRC, 2001). The time spent ruminatingis an important parameter to identify metabolic disorders but also could be used for monitoring the ration composition and feeding practices. The main dietary factors affecting the rumination activity are: DMI, dietary composition, physical effective NDF (peNDF) intake, feed particle size, and forage chemical composition (Welch and Smith, 1970; Mertens, 1997, Tafaj et al., 2005). Rumination can affect the mechanical digestion of feed particles (Schirmann et al., 2012), it has beenb observed that as forage quality decrease more rumination time is required per gram of dry matter ingested (Welch and Smith, 1970) but less is known about the effect of suboptimal protein supply on rumination activity and its consequences on production performances.

Evidences shown how low protein diets in association with rumen protected conjugated linoleic acids (rpCLA) could improve feed and nitrogen efficiency in beef cattle (Schiavon and Bittante, 2012; Schiavon et al., 2012) but also increase meat CLA content (Gillis et al., 2007) and thus the potential economic value of the final product. It has been established that a supplementation of rpCLA can exert a protective effect against excessive body reserves use in early lactating primiparous Holstein Fresian cows and that a continuous rpCLA supplementation to the diet until 105 days in milk (DIM) increased protein accretion (von Soosten et al., 2012). Feeding of CLA supplements during early lactation could also improve reproduction performances of dairy cows (de Veth et al., 2009).

Analytical methods to determine fatty acid profile and CLA content in biological materials

Fatty acid composition of feed and digesta sample is routinely determined after an extraction step in order to concentrate the lipid fraction. But the solvent extracted content and the chemical composition of feedstuff is heterogeneous and not precisely related to the nutritive value of the analysed sample because not nutritive compounds such as waxes, unsaponifiable lipids and pigments could be coextracted. In addition, lipids with nutritional value present as salts of divalent cations could be incompletely extracted. Acid hydrolysis of the sample can releases the fatty acids present as insoluble salts but this could lead to an increase of analytic time and costs and to a decrease of precision (Palmquist and Jenkins, 2003).

Total and single fatty acid (FA) contents rather than the fat content, determined by solvent extraction, are preferred information in studying the digestive utilization of lipids (Palmquist and Jenkins, 2003) especially in the case of fat supplemented diets. Increasing advances in biotechnology and the increased understanding of nutrient utilisation by livestock caused a need for more sophisticated methodologies for measuring the composition and quality of the fat fraction of feedstuffs, digesta and faecal sample (Palmquist and Jenkins, 2003). Gas chromatography is a powerful and precise technique. The GC analysis to determine FA composition requires a methylation treatment to synthesize fatty acids methyl esters. This treatment can be conducted under acid or basic catalysis conditions. The methylation process is a critical step as acid catalysis esterifies all complex and simple forms of fatty acids but do not prevent from isomerisation of conjugated double bonds. On the contrary, base catalysis prevent isomerization of conjugated double-bounds, but does not esterify free fatty acids or soaps presents in feeds and faeces. Comparisons among methylation methods have been published but a major attention was paid on the methylation step with less attention about the effect of lipid extraction methods (Sukhija and Palmquist, 1988; Jenkins, 2010).

Notable beneficial effects also for human health have been associated to CLA thus research to enhance the content of these molecules in dairy and beef products could be interesting. In order to study with precision the digestive process of the lipid fraction of the feed it is necessary to identify and validate a suitable methodology to detect and quantify all the classes of fatty acids applicable to different matrixes, including faeces.

Swine energy and protein requirements

An important distinctionmust be done between pigs reared for fresh meat production, often realized with commercial genotypes selected for fast lean growth, and the so called 'heavy pigs' commonly used in the Italian pig industry with the use of genetic lines selected for high quality dry-cured ham production. In the case of fresh meat production, pigs are fed *ad libitum*high-protein dietsuntil the slaughter weight of 100-120 kg of live weight (LW). The aim of production is to achieve lean carcass to meet industry and consumers demand for low fat meat (Lenis, 1989).The 'heavy pig' system for high quality dry-cured ham production is typical of some areas of the Mediterranean, Italy in particular.This system is characterised by restricted feeding regimeswith pigs slaughtered when they are at least 9 months old and 165 ± 10% kg LW.This practice is aimed to obtain a subcutaneous fat cover thick,about 2 cm, that protect the internal lean part of the ham from excessive salt absorption during the dry-curing processing. Because of this aim of production the feeding practices are modulated to maintain a low rate of growth with an optimal degree of fatness (Mordenti et al., 1992). Current models to predict nutrient requirement of pigs have been not tested for heavy pig production. Practical diets for Italian heavy pigs are often formulated from tabulated values suggested by international research centres, with some adjustment based on empirical results or practical experience. The shortcoming of such empirical formulation is to use feeding plans irrespective to the pigs genetic line used, the climate conditions and the specific production aims. Thus result in an inefficient use of dietary nutrients, and to an increase of variability in carcass composition, quality of product and economic losses.

In pig, protein supply is oneof the major factors that influence daily weight gain, lean tissue accretion and feed efficiency. The proportion of body protein mass is about 0.15 to 0.18 of body weight. Dietary protein is a source of amino acids (AA) required for maintenance of body functions (including protein turnover, urine and gut losses) and protein retention (Kyriazakis and Whittemore, 2006). Due to the relatively low efficiency of N utilizationa large part of the consumed N is excreted with the urine and the faeces (Whittemore et al., 2001; Shirali et al., 2012).Evidence has been found that a reduction of the dietary protein levels by 1 to 2 percentage points reduce nitrogen excretion by up to 10 to 20% without compromising pig performance, when AA requirements are met (Latimier and Dourmad, 1993).Not all the consequences regarding the effect of a reduction of dietary protein supply are clear.In general it is expected that a reduction of dietary protein content would increase fat

deposition in the carcass, but remains unclear which fat deposits, external or internal, are influenced from such strategy(Wood et al., 2004).

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GENERAL AIMS

Aims of the current thesis were:

I. To evaluate the effect of suboptimal protein supply on the performance and the N balance of growing cattle and lactating dairy cows (contribute n. 1 and 2).

II. To evaluate the effect of different sample preparation techniques to assess the fatty acid content, including CLA, of faecal samples by using bi-dimensional gas chromatography (contribute n. 3).

III. To evaluate the effect of a reduction of the dietary crude protein and amino acids content on the growth performance, variation of backfat thickness and the energy balance in heavy pigs of different genetic lines used for dry-cured ham production (contribute n. 4).

IV. To evaluate the effect of a restriction of dietary crude protein and amino acid content on the growth performance and on the subcutaneous or the inter and intramuscular fat deposition in fast growing lean pigs (contribute n. 5).

CONTRIBUTE 1

Low crude protein diets and phase feeding for double-muscled crossbred young bulls and heifers

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Abstract

This study aimed to evaluate the effect of underfeeding dietary crude protein (CP) during the whole or part of the fattening period on growth performance, dry matter intake (DMI), carcass and meat quality traits of crossbred bulls and heifer calves obtained from double-muscled (DBM) Belgian Blue (BB) sires and dairy or dual purpose cows. Twenty-four crossbred BB sired young bulls and 30 BB sired heifer calves (236 ± 27.0 kg BW) were housed in 12 pens with males separated from heifers. They received a control diet (CP14) with 140 g CP/kg DM for the whole experiment (CP_{HH}) or a low-protein diet (CP10) with 102 g/kg CP/kg DM for the whole experiments (CP_{LL}) or CP14 for the first 90 days and CP10 for the remaining days (94 days on average) of finishing period till slaughtering (CP_{HL}), which occurred when heifers and bulls reached 485 and 535 kg BW, respectively. Compared to CP_{HH} and CP_{LL} calves, CP_{HL} animals evidenced faster ADG ($P < 0.05$), albeit the magnitude of this effect was limited (on average + 6.3%). During the whole trial no differences among groups were observed for DM intake (8.9 kg/d), gain:feed ratio (0.144), carcass weight (301 kg), carcass yield (589 g/kg BW), SEUROP carcass muscularity (U+) and for most of the meat quality traits. Only meat shear force was increased ($P = 0.04$) by CP_{LL} treatment compared to CP_{HH} and CP_{HL} (+ 17%). Bulls and heifers differed for growth performance and for many carcass and meat traits, but gender did not interact with dietary treatment for any trait. It was concluded that little benefits would be achieved using CP14 for the entire or part of the fattening period on these crossbreds. As aside, this paper covers a lack of information about quality traits of crossbred heifers and young bulls obtained from DBM BB sires and dairy and dual purpose dams showing that carcass and meat quality traits of these subjects are comparable or better than those achievable from non DBM beef breeds cattle.

Keywords: Beef production, Carcass quality, Double-muscled crossbreds, Low protein diets, Meat quality.

Introduction

The use of low-protein diets and phase feeding are gaining interest in beef fattening because of the costs of protein sources and the concerns about the environmental impact of farming (Sheppard and Bittman, 2011). The European Union, with the Nitrate Directive (EEC, 1991), fixed a maximum of 170 kg N/ha as a threshold for the disposal of N in areas defined as

vulnerable to nitrates. The reduction of dietary protein supply could reduce the production of the individual animal but it would largely increase farm beef production because of the possibility of reducing N excretion and increasing the number of animals fattened per unit of agricultural land (Schiavon et al., 2012). Beef cattle growth response to the amount and quality of protein supplied, has been tested on conventional breeds and some works regarded double-muscled (DBM) hypertrophic beef cattle breeds (Fiems et al., 1999; De Campeneere et al., 1999a; Schiavon and Bittante, 2012). Compared to other beef cattle, DBM calves have a greater potential for lean growth and a greater carcass yield due to the small proportion of the gastrointestinal tract on BW, so that higher dietary CP density, about 160 and 125 g CP/kg DM for early and late fattening period, respectively, have been suggested for these animals (Fiems et al., 1998). However, other studies found that a reduction from about 140 to 110 g CP/kg of diet impaired growth performance of DBM young bulls only during the initial fattening phase, without substantial effects on final BW, average daily gain (ADG), carcass and meat quality traits (De Campeneere et al., 1999b; Dal Maso et al., 2009; Schiavon et al., 2011). To our knowledge no information is available on crossbred calves obtained from DBM bulls mated to conventional cows. Thus, it remains unknown if or to what extent lowering the dietary CP density over the whole or a part of the finishing period would affect growth performance and carcass and meat quality traits of such kind of cattle.

Therefore, aim of this study was to evaluate the effects due to a suboptimal dietary CP content during the whole growing-finishing cycle or only during the last finishing phase on growth performance, DM intake and feed efficiency (gain:feed ratio), carcass and meat quality traits of crossbred bulls and heifers obtained from DBM Belgian Blue sires and dairy or dual purpose cows.

Materials and methods

Animals

The present study is part of a project aimed to evaluate the validity of using suboptimal dietary protein supply in purebred DBM calves or crossbred calves sired by DBM bulls for reducing N excretion (Dal Maso et al., 2009; Schiavon et al., 2010, 2011, 2012). Calves were treated following the Guideline for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Consortium, 1988) and the project was approved by the Ethical Committee for the care and the use of experimental animals of the University of Padova.

Dairy cows belonging to Brown Swiss (BS) breed, to dual purpose Simmental (Si) breed and to dual purpose autochthonous Rendena (Re) breed (Bittante, 2011), reared in the Italian alpine area in the province of Trento (Bazzoli et al., 2013), were artificially inseminated with semen of DBM Belgian Blues sires. Crossbred calves were housed and weaned in the same barn under the same feeding and housing conditions. Thirty-two DBM sired young bulls and 30 DBM sired heifers (236 ± 27.4 kg BW) were selected on the basis of their maternal breed, age and BW and moved to the experimental farm "Lucio Toniolo" of the University of Padova to be used in the current experiment.

At their arrival the calves were housed in 14 fully slatted floor pens with four bulls or five heifer calves each. The first and the last pen on the opposite sides of the barn, both with male calves, were considered as border pens, and were excluded from data analyses. These two pens were planned to be not included in the experiment first because they grouped animals with BW not homogeneous with those of the experimental pens, second to minimize possible border effects due to their position in the barn. Thus, 6 pens with 4 males each and 6 pens with 5 females each were used. In each pen for males, two Belgian Blue × BS, one Belgian Blue × Si and one Belgian Blue × Re young bulls were housed, whereas in the pens for females three Belgian Blue × BS, one Belgian Blue × Si and one Belgian Blue × Re heifers were housed. The calves were vaccinated against bovine rhinotracheitis, parainfluenza$_3$, and bovine respiratory syncytial viruses and injected with 2.5 mg/kg BW of Tulathromycin, and were fed a transition diet for 40 d in which meadow hay was progressively replaced by a diet containing 140 g CP/kg DM.

Feeding treatments

Three feeding treatments were compared in this experiment. In a first treatment (CP_{HH}) the calves were fed for the entire duration of the trial, till slaughter, a total mixed ration (TMR) with a conventional high 139 g/kg DM CP content (CP14). In a second treatment (CP_{LL}) the calves were fed for the entire duration of the trial a TMR containing only 102 g CP/kg DM (CP10). In the third treatment (CP_{HL}) the calves were kept for 90 days on CP14 and for the rest of finishing period till slaughter (94 days on average) on CP10. Each feeding treatment was tested on 4 pens, two of males and two of females. The CP14 and CP10 TMR had the same ingredient composition (Table 1) of the two diets used in a previous trial conducted on purebred DBM Piemontese young bulls (Schiavon et al., 2010, 2012). The

CP10 ration was formulated from the CP14 one by reducing the level of inclusion of soybean meal from 126 to 33 g/kg DM and accordingly increasing all the other ingredients.

In order to minimize differences of composition among treatments a common basal diet, corresponding to the composition of CP10 ration, was prepared daily using a mixer-wagon equipped with a computer assisted weighing scale, calibrated monthly. After the distribution of the CP10 ration to the each corresponding pens, the amount of soybean meal required to reach the 140 g CP/kg DM of CP14 ration was added to the mixer wagon, mixed with the other ingredients and distributed to each of the remaining CP14 pens.

The amount of each feed ingredient loaded into the mixer-wagon and the weight of the mixture uploaded in the manger of each pen were recorded daily. The orts remained in the mangers were weighed and sampled by pen weekly. As animals in pens were not fed individually, DMI and feed efficiency were computed on pen basis.

Samples of each feed ingredient of diets and of orts were analysed for their proximate composition (AOAC, 2000) and their NDF, ADF, ADL and AIA content (Van Soest et al., 1991). Metabolizable energy, net energy for maintenance (NEm), net energy for growth (NEg), rumen degradable (RDP) and undegradable (RUP) protein contents of the rations were computed from actual ration ingredient composition and from tabular values of each feed ingredient (NRC, 2000).

Table 1. Ingredients, chemical composition and nutritive value of diets.

	Experimental diets	
	CP14	CP10
Total mixed ration ingredient, g/kg of DM		
Corn grain, ground	360	400
Corn silage	250	276
Dried sugar beet pulp	102	113
Soybean meal (44% , sol. extr.)	126	33
Wheat bran	63	70
Wheat straw	60	66
Vitamin and mineral mixture[1]	24	26
Calcium soap	8	9
Hydrogenated soybean oil	7	7
Chemical composition[2], g/kg of DM		
CP	139	102
Starch	364	404
NDF	291	308
ADF	142	149
Ash	55	54
Lipids	39	40
Nutritional value[3]		
ME, MJ/kg of DM	13.0	13.0
NEm, MJ/kg of DM	8.0	8.1
NEg	5.3	5.4
RDP, g/kg of DM	91	66
RUP, g/kg of DM	48	36

[1] Content per kilogram: 120 g of Ca, 56 g of Na, 17 g of Mg, 16 g of P, 240,000 IU of vitamin A, and 15,000 IU of vitamin D_3.
[2] Values computed from the chemical analysis of each feed ingredient in triplicate and from the mean of the actual daily loads of each feed ingredient recorded by mixer wagon computer.
[3] Values computed according to the actual ingredient composition of diets and tabular values (NRC, 2000) where: ME, NEm, NEg, RDP and RUP are the dietary metabolizable energy, net energy for maintenance, net energy for growth, rumen degradable protein, and rumen undegradableprotein contents, respectively.

Individual controls and measurements

The bulls were individually weighed monthly. Monthly, a skilled operator, licensed for carcass evaluation according to the SEUROP grading system (Anonymous, 1991), evaluated *in vivo* the body conformation and condition of each bull according to the visual approach described by Schiavon et al. (2010) to evaluate the evolution of conformation and fatness during the growing period and to predict the time of commercial maturity. Body conformation

and condition were expressed in terms of expected carcass conformation and fat covering according to the SEUROP grid (Anonymous, 1991). Thus, conformation was linearly scored in 18 classes from S+ (all muscle profiles extremely convex; exceptional muscle development) to P- (all muscle profiles concave to very concave; very poor muscle development) considering the profiles of shoulders, loins, rump, tights and buttocks. Conformation was converted in numerical terms: S+ = 6.33, S = 6, S- = 5.66,.., P+ = 1.33, P = 1.00, P- = 0.66. Fat covering was linearly scored, by a combined visual and palpation approach (Schiavon et al., 2010), considering the presence and the thickness of subcutaneous fat depots at specific points of the body, from 1 (very lean: no palpable fat is detectable, the ribs, the bone structure and the head of the tail are very prominent) to 5 (very fat: thick fat depots are present over the shoulders, the ribs and around the head of the tail, bone structure is no longer visible).

Health status was monitored daily by a technician and three times per week by a veterinary, following the experimental protocol for animal care. After 105 days from the beginning of the trial the veterinarian indicated that the health status of one heifer of the CP_{HH} treatment was compromised. Therefore this animal was removed and all the data from this animal were excluded from the statistical analysis. Schiavon et al. (2010) evidenced as DBM bulls fed low-protein diets showed a better locomotion score and health status than DBM bulls fed conventional high-protein diets. Thus, to evidence possible influence of the dietary CP concentration on the health status of calves, the animals were monthly evaluated for their locomotion score as an index of health status. Locomotion score was individually assessed as proposed by Sprecher et al. (1997) which observes deviations from normal posture and walking using a 1 to 5 rating system. During the second part of the trial three bulls and one heifer exhibited only mild alteration of locomotion (locomotion score 2), one was receiving the CP_{LL} treatment and three were receiving the CP_{HH} one.

Carcass traits

At the end of the fattening trial, the calves were fasted for one day and then slaughtered. According to the reference meat market, heifers and bulls were slaughtered when they had reach an *in vivo* fatness score around 3 or 2 points, respectively. Slaughtering occurred after 159 d on trials for all heifers and after 204 and 222 d on trial for 16 and 8 young bulls, respectively (the bulls were randomly partitioned between the two slaughtering groups, taking care that all treatments, maternal breed and pens were represented in each group).

Carcasses were individually weighed and scored, for conformation (18 classes as described before) and fat covering (5 classes) according to the SEUROP system (Anonymous, 1991) modified only to accommodate for conformation + and - subclasses. Carcass yield was computed as the ratio between the carcass weight after 24 hours from slaughter and the BW.

Twenty-four hours after slaughter from the left half part of the carcass, the whole cut of the 5th rib was collected (from the cranial edge of the 5th rib to the cranial edge of the 6th rib). The entire rib was vacuum packed, moved to the laboratory, and aged at 4 °C in a chilling room for 10 days.

After ageing, drip losses were assessed as the ratio of the difference between the wet and the dried empty pack and the weight of the rib.

The rib cut was dissected into rib-eye muscles, and other muscles, fat, and bones. Each fraction was weighted. Muscles pH and temperature were measured in 4 points of L*ongissimusthoracis* muscle (LT) soon after its dissection from the rib cut using a Delta Ohm HI-8314 pH-meter (Delta Ohm, Padova, Italy) and the 4 data were averaged before statistical analyses. A slice of about 20-mm thick was dissected from *Longissimusthoracis* muscle (LT) and on its fresh cut surface color parameters were measured, after 1 hour of air exposure, using a Minolta CM-508c (illuminate: D65, Observer: 10°) on 5 anatomical positions. The mean of each color trait was taken as final value. Meat color was expressed according to the CIE-Lab color space by reporting *L**, a* and b* values (CIE, 1978).

The remaining part of the LT was taken for measuring cooking loss percentage, expressed as the ratio of the difference between the weight before and after cooking on the weight before cooking, using 2-cm thick steak sealed in a polyethylene bag and heated in a water bath to an internal temperature of 70 °C for 40 min (Pohlman et al., 1997). Shear force (WBSF) was assessed on the cooked LT samples, measures were obtained on five cylindrical cores of 1.13 cm in diameter taken parallel to muscle fibers. Shear force was measured by a TA-HDi Texture Analyser (Stable Macro System, London, Great Britain) with a Warner-Bratzler shear attachment (10 N load cell, crosshead speed of 2 mm/s) and interpreted using texture expert software (Joseph, 1979). The 5 data available for each sample were averaged before statistical analyses.

Statistical analysis

The number of calves to be used in the current experiment was quantified, as described by Lerman(1996), as the minimal number to detect significant differences (P = 0.05; power =

0.90) of ADG among groups of 0.100 kg/d, with an anticipated within-group standard deviation for ADG of 0.100 kg/d.

The pen data of DM intake and feed efficiency (ADG/DMI) were statistically analysed using the PROC GLM of SAS (2005) with a model considering the effects of dietary treatment (T), gender (G), G × T interaction, so that 4 observations for each of the three treatment levels were used and the error line coincided with the pen effect (pen was considered as experimental unit).

All individual data were statistically analyzed with the following linear model using the PROC GLM of SAS (2005), considering the animal as experimental unit:

$$y_{ijklm} = \mu + T_i + G_j + G{\times}T_{ij} + P(G{\times}T)_{ijk} + B_l + T{\times}B_{il} + e_{ijklm}$$

where y = the experimental observation, μ = overall mean, T = effect of treatment (i = 1,…, 3); G = effect of gender (j = 0, 1); G × T = interaction between treatment and gender; P = effect of pen nested within the G × T interaction (k = 1,..., 12); B = effect of the dam breed (i = 1,…, 3), T × B = interaction between treatment and dam breed; and *e* = residual error. Considering the hierarchical structure of the experimental design, the P effect was used as error line to test the effects of T, G, and T × G, while *e* was used as error line to test B and T × B. The effect of B and T × B interaction were included in the model to achieve a better confidence in the least square mean estimates for the two genders and to reduce the residual variance for traits influenced by B. In fact, among pens the number of males or females was almost balanced for type of crossbred, whereas irrespective by the crossbred the number of males (24) and females (30) was unbalanced. After a preliminary analysis the B × G interaction was excluded from the model as it resulted not significant for any trait. Data of BW were covariated with the corresponding values at the beginning of the trial, to correct for initial individual differences among and within pens. Orthogonal contrasts were used to evaluate the effects of T and of the T × G interaction.

As the T × B interaction never resulted significant, only the effects of T, G and G × T interaction are presented and discussed in the current study.

Results

Growth performance and feed efficiency

The dietary CP density influenced BW and ADG of Belgian Blue crossbred calves during the first period and over the whole trial (Table 2).

During the first period ADG of the CP_{HL} group was greater than the average of the other two groups ($P = 0.038$), even if CP_{HH} did not differed from CP_{LL} ($P = 0.62$). This difference was almost entirely due to the greater ADG of the bulls of CP_{HL} which grew more than those of the CP_{HH}, despite the fact that in this period these two groups received the same CP14 diet, whereas the ADG of heifers was very similar among treatments. Nevertheless, the G × T interaction was not significant.

During the second period of trial no significant differences among experimental treatments were observed, so that over the whole trial the differences in ADG between CP_{HL} and the average of the other groups were lower than those observed during the first period, but still significant ($P = 0.047$).

As expected, the ADG of young bulls was greater compared to that of heifers during both the first (+ 12.3%; $P = 0.032$), the second (+ 33.7%; $P = 0.001$) and hence over the whole (+ 20%; $P < 0.001$) experimental period.

The average DM intake was not affected by the dietary CP level during the first and the second growing period (Table 3). Over the whole trial the DM intake of young bulls tended to be +6.6% greater than heifers ($P = 0.07$). The average DM intake was not influenced by the G × T interaction.

Differences of feed efficiency due to the dietary treatment in the first period reflected those observed for ADG, however in the second period and over the whole experiment no significant differences among treatments were observed ($P = 0.51$). Feed efficiency was + 12.6% greater in males compared to females ($P = 0.024$).

Table 2. Body weight and average daily gain of Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

	Body weight, kg			Average daily gain, kg		
	Start	Intermediate	End	1st period	2nd period	Total
Diet[1]						
CP_{HH}	275	404	507	1.43	1.15	1.27
CP_{HL}	275	416	521	1.57	1.18	1.34
CP_{LL}	272	398	501	1.40	1.20	1.24
P-values						
CP_{HH} vs CP_{LL}	0.78	0.81	0.71	0.62	0.43	0.39
CP_{HL} vs (CP_{HH} +	0.71	0.07	0.012	0.038	0.94	0.047
Gender[2]						
Bulls	279	418	535	1.55	1.35	1.40
Heifers	269	394	485	1.38	1.01	1.17
P-value	0.33	0.23	0.008	0.032	0.001	<0.001
Gender × diet						
P-value	0.75	0.49	0.24	0.40	0.92	0.29
Model R^2	0.96	0.82	0.79	0.41	0.51	0.49
Pen RMSE	11.6	23.2	15.0	0.193	0.178	0.106
Residual RMSE	7.5	20.9	30.9	0.202	0.225	0.155

[1] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[2] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

Table 3. Dry matter intake and feed efficiency (gain:feed DM) of Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

	Dry matter intake, kg/d			Feed efficiency (gain:feed)		
	1st period	2nd period	Total	1st period	2nd period	Total
Diet[1]						
CP_{HH}	8.09	9.75	8.96	0.177	0.128	0.142
CP_{HL}	7.84	10.11	9.07	0.200	0.122	0.147
CP_{LL}	7.93	9.22	8.63	0.177	0.134	0.143
P-values						
CP_{HH} vs CP_{LL}	0.46	0.35	0.35	0.97	0.67	0.87
CP_{HL} vs (CP_{HH} +	0.38	0.22	0.37	0.035	0.49	0.51
Gender[2]:						
Bulls	8.13	10.07	9.17	0.191	0.141	0.152
Heifers	7.78	9.32	8.60	0.178	0.115	0.135
P-value	0.09	0.13	0.07	0.16	0.06	0.024
Gender × diet						
P-value	0.46	0.64	0.88	0.11	0.71	0.82
Model R^2	0.56	0.53	0.54	0.73	0.54	0.62
Residual (Pen) RMSE	0.298	0.747	0.456	0.014	0.019	0.010

[1] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[2] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

In vivo body conformation and fatness score

With increasing days on feed, the dietary treatment progressively affected muscular conformation and fatness of calves (Table 4). In particular at the end of experiment, animals that received the CP_{HH} diet showed greater muscular ($P = 0.025$) and fat ($P = 0.036$) scores than animals receiving the CP_{LL} treatment. Compared to heifers, bulls always exhibited a better conformation score ($P < 0.01$) and a lower fatness score ($P < 0.001$) over the growing period, except for fatness scores taken at the beginning of the trial.

Table 4. Conformation and fatness scores evaluated *in vivo*[1] on Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

	Conformation			Fatness		
	Start	Intermediate	End	Start	Intermediate	End
Diet[2]						
CP_{HH}	3.53	3.88	4.35	1.00	1.41	2.48
CP_{HL}	3.37	3.77	4.20	1.00	1.44	2.12
CP_{LL}	3.47	3.87	4.03	1.00	1.49	2.18
P-values						
CP_{HH} vs CP_{LL}	0.57	0.94	0.025	-	0.26	0.036
CP_{HL} vs (CP_{HH} +	0.22	0.25	0.73	-	0.79	0.07
Gender[3]:						
Bulls	3.78	4.15	4.37	1.00	1.06	1.77
Heifers	3.13	3.53	4.02	1.00	1.83	2.76
P-value	<0.001	<0.001	0.008	-	<0.001	<0.001
Gender × diet						
P-value	0.86	0.61	0.83	-	0.41	0.12
Model R^2	0.64	0.75	0.57	-	0.67	0.71
Pen RMSE	0.30	0.27	0.29	-	0.17	0.31
Residual RMSE	0.32	0.23	0.28	-	0.33	0.43

[1] According to Schiavon et al. (2010), body conformation was linearly scored *in vivo* in 18 classes from S+ (all muscle profiles extremely convex; exceptional muscle development) to P− (all muscle profiles concave to very concave; poor muscle development) considering the profiles of shoulders, loins, rump, thighs, and buttocks (S+ = 6.33; P− = 0.66) and fat covering in vivo was linearly scored in 5 classes (1 = very lean; 5 = very fat) by a combined visual and palpation approach considering the presence and thickness of subcutaneous fat depots at the base of the tail, ribs, and shoulders.
[2] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[3] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

Carcass quality

No influence of the dietary treatment on carcass weight and yield was observed at slaughter (Table 5). Gender influenced carcass yield, which was 21 g/kg greater in males compared to females ($P < 0.001$). No effect of the G × T interaction on carcass yield was observed.

No difference due to the dietary treatment was observed on SEUROP score, but the carcass of calves kept on CP_{HH} were fatter than those of the CP_{LL} group ($P = 0.01$). The carcasses of heifers were fatter than those of bulls ($P < 0.001$), and fat covering score of carcass was in agreement with the *in vivo* body fatness visually performed immediately before slaughtering. However, differently from what observed *in vivo,* any evidence of an influence

of diet or gender on carcass muscularity was observed, likely because the narrow range of variation.

Table 5. Carcass weight, carcass yield, SEUROP muscularity and fatness scores of Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

	Carcass weight	Carcass	Muscularity[1]	Fatness[1]
Diet[2]				
CP_{HH}	289	590	4.15	2.65
CP_{HL}	303	584	4.19	2.52
CP_{LL}	310	593	4.19	2.47
P-values				
CP_{HH} vs CP_{LL}	0.31	0.45	0.73	0.010
CP_{HL} vs (CP_{HH} +	0.51	0.06	0.85	0.34
Gender[3]:				
Bulls	313	600	4.17	2.10
Heifers	289	579	4.19	3.00
P-value	0.14	<0.001	0.88	<0.001
Gender × diet				
P-value	0.13	0.44	0.74	0.17
Model R^2	0.68	0.57	0.23	0.84
Pen RMSE	17.7	10.0	0.34	0.13
Residual RMSE	24.8	13.0	0.31	0.23

[1] SEUROP scoring system for carcass muscularity from S+ (all muscle profiles extremely convex; exceptional muscle development) to P− (all muscle profiles concave to very concave; poor muscle development) considering the profiles of shoulders, loins, rump, thighs, and buttocks (S+ = 6.33; P− = 0.66); and for carcass fat covering (1 = very lean; 5 = very fat).
[2] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[3] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

Meat quality

No effect of diet CP level was found for the weight of rib cut and on the incidence of rib-eye muscles on rib cut weight (Table 6), although there was a tendency ($P = 0.05$) toward a lighter rib weight for CP_{HL} compared to the other two diets. The rib cut tended to be heavier in young bulls than in heifers ($P = 0.07$), and bulls exhibited greater incidence of rib-eye muscles ($P = 0.023$) and bone ($P = 0.040$) and a lower incidence of dissectible fat ($P = 0.003$) on total rib weight. No influence of the G × T interaction on these variables was found.

Dietary treatment, gender and their interaction did not affect meat colour indices of the LT muscle (Table 7). No effect due to T were observed on drip losses and cooking losses, but the shear force of meat produced by the CP_{LL} group was greater than that obtained from the CP_{HH} one ($P = 0.040$), especially among young bulls. The drip and cooking losses and shear force were all affected by gender as heifers presented greater drip losses ($P = 0.015$), lower cooking losses ($P = 0.008$) and more tender meat ($P = 0.001$) compared to bulls.

Table 6. Fifth rib weight and composition of Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

		Composition:			
	Rib weight	Rib-eye muscles	Other muscles	Fat	Bone
Diet[1]					
CP_{HH}	956	464	230	187	110
CP_{HL}	919	475	222	171	120
CP_{LL}	956	473	235	166	113
P-values					
CP_{HH} vs CP_{LL}	0.99	0.67	0.70	0.64	0.72
CP_{HL} vs (CP_{HH} +	0.05	0.69	0.32	0.18	0.31
Gender[2]					
Bulls	986	495	227	144	124
Heifers	901	446	230	206	104
P-value	0.07	0.023	0.77	0.003	0.040
Gender × diet					
P-value	0.76	0.18	0.20	0.64	0.83
Model R^2	0.64	0.72	0.58	0.64	0.41
Pen RMSE	48	54	32	40	26
Residual RMSE	110	30	18	31	22

[1] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[2] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

Table 7. *Longissimusthoracis* muscle quality of Belgian Blue crossbred young bulls and heifers fed for the whole experiment diets containing 140 or 100 g CP/kg DM (CP_{HH} or CP_{LL}, respectively) or fed for 90 and 94 d diets containing 140 and 100 g CP/kg DM, respectively (CP_{HL}).

	pH	Lightness and			Drip	Cooking	Shear force
		L*	a*	b*	g/kg	g/kg	N/cm^2
Diet[2]							
CP_{HH}	5.43	36.3	10.9	14.2	53.1	278	29.0
CP_{HL}	5.45	35.9	10.1	13.7	55.3	278	29.5
CP_{LL}	5.50	35.6	10.1	13.8	51.5	281	34.0
P-values							
CP_{HH} vs CP_{LL}	0.14	0.54	0.26	0.49	0.77	0.69	0.040
CP_{HL} vs (CP_{HH} +	0.79	0.94	0.48	0.61	0.51	0.83	0.23
Gender[3]							
Bulls	5.48	35.4	10.1	13.5	46.0	291	35.5
Heifers	5.44	36.4	10.6	14.2	60.7	267	26.2
P-value	0.32	0.31	0.37	0.18	0.015	0.008	0.001
Gender × diet							
P-value	0.47	0.63	0.51	0.73	0.80	0.95	0.11
Model R^2	0.51	0.47	0.30	0.40	0.45	0.53	0.50
Pen RMSE	0.12	3.0	1.8	1.5	14.6	19.5	5.20
Residual RMSE	0.07	2.0	1.5	1.2	11.9	15.5	7.30

[1] L* = lightness; a* = redness; b* = yellowness (CIE, 1978).
[2] Least square mean of 4 pens (2 pens with 4 males, and 2 pens with 5 heifers).
[3] Least square means of 6 pens (4 or 5 heads/pen for bulls and heifers, respectively).

Discussion

Growth performance and carcass and meat quality of Belgian Blue crossbred calves

In the alpine area the DMB Belgian Blue is the most preferred sire breed because of the higher value of the calves at selling with respect to other beef breeds, and it is used on dairy (BS) as well as on dual purpose cows (Dal Zotto et al., 2009). Despite the interest for this kind of crossbred little or no information are available about growth performance, carcass traits and meat quality traits of DBM sired heifers and bulls when exposed to corn-silage and cereal based diets with conventional or lowered dietary CP content. Till about 450 kg BW, the BB crossbred young bulls in the present study showed ADG, DMI, feed efficiency and fatness score almost identical and conformation score even better than the purebred Piemontese young bulls of Schiavon et al. (2010) fed similar diets. With increasing days on feed, between 450 and about 535 kg, the crossbred calves showed a greater increase of DMI, a lower

decrease of ADG and feed efficiency, a lower improvement of muscularity and a faster increase of fatness score with respect to previous Piemontese data in the same range of BW (Schiavon et al., 2010). The crossbred young bulls were close to the target fatness value of 2.0 points at 535 kg slaughter BW after 210 days of fattening, whereas the purebred DBM Piemontese young bulls of Schiavon et al. (2010) reached two points of fat covering score at an average of 673 kg slaughter BW after 330 d of fattening. As expected, BB crossbred young bulls were characterized by lower carcass yield and muscularity score and their rib composition evidenced lower incidence of lean meat and higher incidence of dissectible fat when compared to purebred DBM Belgian Blue and Piemontese young bulls (Fiems et al., 1998; Schiavon et al., 2010, 2011). Gariépy et al. (1999) studied the effects of crossing normal or DBM sires with British or Continental dams on meat composition of the progeny and they observed that lean composition of Belgian Blue and Piemontese sired calves had less dissectable fat (170 g/kg rib) and more dissectable lean (660 g/kg rib) than Charolaise sired cattle (195 and 623 g/kg, respectively), while the origin of dams had no influences. The LTsamples of current experiment evidenced similar cooking losses, tenderness and lightness, but a smaller redness index of the muscle surface than the rib samples collected from purebred DBM bulls of Schiavon et al. (2011).

Overall, the growth rate, feed efficiency, carcass yield, carcass and meat quality of the crossbred young bulls of current study were comparable, or even better, than the corresponding values reported for young bulls of purebred European continental beef breeds, such as Charolais and Limousin (Gariépy et al. 1999; Cuvelier et al. 2006; Alberti et al., 2008; Allais et al., 2010). Differently from what commonly found in other beef breeds (Steen and Kilpatrick, 1995), and despite the fact that heifers evidenced a much greater aptitude for fat deposition than young bulls, little differences in carcass muscularity due to the gender were found in this experiment. Thus, the results of current experiment compared to those of literature suggest that DBM sired bulls and heifers would produce meat with less lean and more fat than purebred DBM calves, but leaner than calves with normal conformation.

Effects of different dietary CP supplies

A survey of Galyean (1996) evidenced as the dietary CP supply in the USA feedlot cattle system (steers and heifers) commonly ranged from 130 to 144 g CP/kg DM, a value considerably greater than the average of 120 g/kg predicted from factorial systems calculating

protein requirements of feedlot cattle (NRC, 1984, 1996, 2000). Similarly, Sturaro et al. (2005), in a survey conducted in one of the European areas (Veneto - North Italy) with the highest presence of beef fatteners (European Commission, 2011), concluded that for finishing intact young bulls of different genotypes (European continental breeds and crosses) an average dietary density from 140 to 144 g CP/kg DM is usually applied. Many evidences suggest that dietary CP densities for finishing cattle should decrease with increasing BW or age (NRC, 2000). However, Cole et al. (2006) on non-DBM beef steers found that the use of 130 or 115 g CP/kg DM during the whole fattening period yielded similar results of phase feeding treatments where in two consecutive periods the dietary CP density was decreased from 130 to 110 g/kg DM, or even from 110 to 100 g/kg DM, and evidenced impairments of ADG only in the case where the CP dietary density was reduced from 130 to 100 g/kg, as a consequence of a reduced DM intake. On DBM Piemontese young bulls, a strong reduction of dietary CP density from 145 to 108 g/kg DM impaired ADG of calves during the first phase of fattening only, but no effect on DMI, final BW, ADG, carcass weight, carcass yield, carcass conformation and fatness, meat quality traits were detected (Schiavon et al., 2010, 2011), and N excretion of young bulls fed low protein diets was strongly reduced (Schiavon et al., 2012). The results of the current experiment confirmed that the required dietary CP density of these crossbreds decreases with increasing BW. There were some signs that the amount of CP provided by the CP10 diet could be a limiting factor during the first 3 months of finishing when the calves, particularly males, were in the range of 275 to 400 kg BW. However, the greater ADG of the CP_{HL} compared to the other two treatments during the first 3 months of finishing periods had small influence on the overall ADG (+ 6.3%) and no differences among groups were observed for overall DM intake, feed efficiency, carcass weight, yield and SEUROP conformation and mostly for meat quality traits. The only differences among groups concerned carcass fatness score, increased in CP_{HH} (+ 7%) compared to CP_{LL} group, and meat shear force, increased by about 17% in CP_{LL} compared to the other groups.

Thus, except for meat tenderness, the magnitude of differences among groups were small, and in practice the preference toward one of the three feeding strategies proposed would depends in first instance by the feeding costs, the carcasses value and the costs related to N disposal according to the constraints imposed by law. Under the market conditions where this trial was conducted it was computed that for CP_{HH}, CP_{HL} or CP_{LL} the feeding costs were 0.82, 0.77 and 0.74 €/kg final BW, or 1.80, 1.63 or 1.61 €/kg ADG, respectively. The main

factor impacting production costs was the reduction of soybean meal consumption, which was lowered by 87 or 155 kg/finished calf in CP_{HL} and CP_{LL} compared to CP_{HH}, respectively.

Besides the reduction of feeding costs, the other important consequence of the different feeding strategies concerned the reduction of N excretion. Simple computations can help to show the magnitude of these reductions. Nitrogen excretion can be computed as a difference between N consumption and retention, by assuming that the N content in cattle body weight gain can be assumed to be rather constant around 26 g/kg gain (ERM, 2001). In the current experiment for CP_{HH}, CP_{HL} and CP_{LL} N consumption averaged 36.6, 31.2 and 25.8 kg/finished calf, respectively, N retention was in the order of 6.0, 6.4 and 5.9 kg/finished calf, and N excretion was in the order of 30.5, 24.8 and 19.8 kg/finished calf. Such a strong reduction of N excretion would have important consequences in terms of number of calves and meat which can be finished per unit of agricultural land in those areas where a fixed amount N/ha is stated by law. In relative terms, taking CP_{HH} as comparison, the use of CP_{HL} and CP_{LL} would approximately increase by + 19 and + 35%, respectively, the number of calves produced per unit of land.

Conclusions

Despite the fact that the DBM crossbred calves showed growth performance, carcass and meat quality comparable or better than purebred calves of beef breeds, the results of the current experiment evidenced that over the whole finishing period a reduction of the dietary CP density from 139 to 102 g CP/kg DM has little consequences on overall ADG and few or null influence on carcass and meat quality traits, except for a worsening of meat tenderness. However a proper meat aging could be used to correct this negative effect. Therefore it is suggested that the use of low-protein diets, with about 100 g CP/kg DM, is valid for this kind of beef production. This offers notable benefits in terms of spare protein resources and feeding costs, less N excretion per calf produced and a potential increasing the number of calf rearing per unit of agricultural land in those areas where the maximum N load per unit of agricultural land is restricted by law.

Conflicts of interest statement

The authors declare that there are no financial, commercial or other conflicts of interest related to our manuscript "Low crude protein diets and phase feeding for double-muscled crossbred young bulls and heifers" submitted to Livestock Science.

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CONTRIBUTE 2

Effects of suboptimal protein supply with or without rumen protected conjugated linoleic acid (CLA) on rumination activity, intake, digestibility, and milk yield and quality of Holstein cows

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Suboptimal protein supply in dairy cows.

In mid- late lactation cows, producing 28.8 kg/d of milk, a 6 wk-reduction of dietary crude protein content from 150 to 123 g/kg dry matter tended to decrease feed intake (1.7 kg/d), reduced milk (1.4 kg/d) and milk protein yield, but increased milk N efficiency (ENU) from 0.31 to 0.36 without apparent signs of body N mobilization. Long term investigations are required to evaluate the influence of labile body N reserves, but the results indicate that suboptimal N supplies would improve ENU and the environmental impact of dairy industry.

Abstract

The effects of N underfeeding and of rumen protected conjugated linoleic acid (rpCLA) supply on dry matter intake (DMI), rumination, rumen fluid parameters, milk yield (MY) , N partitioning and milk N/N intake ratio (ENU) were studied on Holstein cows. Twenty mid-late lactation cows housed in 4 pens in groups of 5, homogeneous for parity, days in milk (DIM), and MY, were fed 4 diets containing 150 (CP15) or 123 (CP12) g of crude protein (CP) per kilogram of dry matter (DM), supplied or not with rpCLA (6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12, respectively). The experimental design was represented by 4 repeated 2 × 2 Latin squares (with or without rpCLA) with periods of 3 wk, rotated according to a 2 × 2 Latin square (CP15 or CP12), so that the cows received the same treatment, CP15 or CP12, for 6 wk continuative. The CP12 diet was formulated from CP15 by replacing soybean meal with barley grain to maintain similar energy, fiber contents and feed particles size. Rumination activity, DMI and MY were recorded daily. Rumen fluid was analysed for volatile fatty acids (VFA), and ammonia N contents, and milk for quality traits. Nutrient digestibility was computed using lignin as a marker. Period, treatment and group (random) were included as sources of variation in the statistical analysis. The dietary CP restriction numerically reduced DMI (7.7%; $P = 0.09$), increased rumination from 16.8 to 19.8 min/kg DMI ($P = 0.009$), decreased rumen fluid ammonia N ($P < 0.001$), tended to reduce nutrient digestibility, reduced MY (28.8 to 27.0 kg/d; $P = 0.047$) and milk protein content (35.3 to 33.6 g/kg, $P = 0.026$), but did not influence MY/DMI. The dietary CP restriction, decreased N intake by 122 g/d and N in milk by 14 g/d, but did not influenced N in feces, so that ENU increased from 0.31 to 0.36 ($P<0.01$). The addition of rpCLA tended to reduce DMI ($P = 0.07$), decreased milk fat content from 36.7 to 31.2 g/kg milk ($P = 0.002$), and decreased N in milk only when supplied to CP12 (CP × rpCLA interaction, $P = 0.016$). Actual MY was

greater than that predictable on the basis of MP intake in the case of CP12 and $CP12_{rpCLA}$. It was found that over a short period of time (6 wk) a suboptimal N supply increases ENU without apparent alteration of BW, BCS and blood metabolites. Long term investigations are need to evaluate the role of body N reserves on N partitioning when suboptimal CP supply are applied.

Keywords: milk yield, nitrogen partition, low-protein diet, rumination

Introduction

Researches to evaluate the effects of suboptimal protein supply on production and quality of products are gaining interest in all domestic species because of the concerns about the environmental impact of farming and the increasing cost of protein sources (Steinfeld et al., 2006; Schiavon et al., 2012). Current energy and protein systems for ruminant were developed to establish nutrient requirements for optimizing production response under different circumstances (NRC, 2001; Ruiz et al., 2002; INRA, 2007). These models share a common structure, and account for N inputs provided by dietary, recycled, and endogenous N, but despite their availability, efforts to improve the efficiency of N utilization in ruminants (g N in product/g N intake; **ENU**) generally met with limited success with regards to production responses (Reynolds and Kristensen, 2008). Calsamiglia et al. (2010) found as both in the European Union and in the USA the overall average ENU is low, in the order of 0.25, but with a large range of variation. The European Union supported a project to develop approaches to improve ENU and reduce N excretion (RedNEx) in ruminants that involved 11 research centers across Europe and final reports are available in the web (www.rednex-fp7.eu/). Within the frame of this project Dijkstra et al. (2013) indicated that in a 600-kg cow producing 25 kg milk/d with 33 g protein/kg milk, and eating 18 kg/d DM the theoretical upper limit of ENU might be around or lower than 0.40 to 0.45 with a dietary CP content of 105 g/kg. A shortage of rumen degradable energy, protein or both would reduce feed digestibility, and in turn decreases DMI and production outcome (Tedeschi et al., 2000; Valkeners et al., 2008). However, Huhtanen and Hristov (2009) observed that increasing milk yield would increase ENU, provided that dietary CP concentration is not increased, and that this increase in ENU was considerably smaller than that obtainable by reducing CP intake. Strategies to increase ENU and to reduce the N excretion include reducing the amount fed in excess of requirements, improving the efficiency of capture of ammonia N and amino N as

absorbable microbial protein, and improving the efficiency of utilization of absorbed amino acids (Calsamiglia et al., 2010; Reynolds and Kristensen, 2008). Belanche et al. (2012) suggested that decreasing protein intake or incorporating easily fermentable carbohydrates in the diet, or both, may be a way to improve ENU, if negative effects on the rumen microbial population and animal performance are avoided.

A supplementation of rumen protected conjugated linoleic acids (**rpCLA**) to low protein diets was found to improve ENU in beef cattle (Schiavon and Bittante, 2012). Von Soosten et al. (2012) found that rpCLA exerted a protective effect against excessive use of body reserves in early lactating primiparous Holstein cows, and a continuous rpCLA supplementation until 105 DIM increased protein accretion. These results suggest that rpCLA might be involved in metabolic mechanisms of N partition among different body functions, which would be interesting to explore in lactating cows, too.

This experiment aimed to analyze and to quantify the effects of corn silage based-diets with optimal or suboptimal N content (obtained by replacing soybean meal with cereals), with or without the addition of rpCLA, on rumen fluid and blood metabolites contents, nutrient digestibility, rumination activity, DMI, milk yield and quality and N partitioning.

Materials and Methods

All the experimental procedures involving animals were approved by the "Ethical Committee for the Care and Use of Experimental Animals" of the University of Padova.

Animals, Rations, and Experimental Design

Two wk before the start of the experiment 20 lactating Holstein cows were divided in four experimental groups and housed in four pens, with straw bedded cubicles, with 5 cows per pen. The groups were balanced for milk yield (31.0 ± 1.4 kg/d), DIM (174 ± 6 d), parity (2.0 ± 0.36), BW (641 ± 26 kg) and BCS (2.9 ± 0.07). Cows had 2 wk of adaptation to the experimental conditions before the start of the experiment.

Four TMR were compared. The control ration (**CP15**) was formulated to be representative of the rations used in the north-east of Italy (Dal Maso et al., 2009), based on corn silage and cereal grains (Table 1). This ration was formulated according to NRC (2001) to provide, for an expected 21 kg/d of DMI, the amount of MP required for 29 kg/d milk yield with 35, 34, and 47 g/kg of protein, fat and lactose, respectively, and a slight excess NE_L, so

that MP was intend to be the first limiting factor. The low-protein ration (**CP12**) was formulated from CP15 diet by simply replacing 65 g/kg DM of soybean meal with 75 g/kg DM of barley grain and slightly reducing proportionately the other ingredients, so that the dietary CP decreased from 150 to 123 g/kg DM and dietary starch increased from 227 to 263 g/kg DM. To minimize differences in composition among treatments a common basal diet corresponding to the composition of the CP12 diet without barley grain was prepared daily using a mixer wagon equipped with a computer-assisted weighing scale, calibrated monthly. The amounts of barley grain or soybean meal required to complete the CP12 and CP15 rations were supplied by top dressing and mixing after the distribution of the common basal diet in the manger of each pen. The other two experimental diets, $CP15_{rpCLA}$ and $CP12_{rpCLA}$, differed from CP15 or CP12 only for the addition, by top dressing and mixing, of 80 g/d per cow of a rpCLA commercial mixture (SILA, Noale, Italy). The commercial mixture consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil. The lipid-coated rpCLA was composed of 800, 178, and 22 g/kg of lipid, ash, and moisture, respectively, and 456 g/kg of palmitic and stearic acids, 79.2 and 76.8 g/kg of C18:2*c*9,*t*11 and C18:2*t*10,*c*12, respectively, and 91 g/kg of other FA. A detailed description of the chemical composition of the rpCLA used is given in Schiavon et al. (2011). Cows were milked at 0530 a.m. and at 1700 p.m. and received their ration in a single daily distribution after the morning milking, approximately at 0630 a.m., but they had free access to the manger throughout the day.

The experimental design was organized as four repeated 2 × 2 rpCLA (0 vs 80 g/d) Latin squares with periods of 3 wk, the first 2 wk for adaptation and the third one for data collection. These four 2 × 2 Latin squares were rotated according to a 2 × 2 CP (CP15 vs CP12) Latin square, in such a way that the four groups of cows received the high- or the low-CP rations for 6 wk continuative and the addition or withdrawal of rpCLA was done without contemporary change in CP content of the diet (Table 2). This scheme was established to minimize sequence and carry over effects. To further minimizing carry over effects, 2 wk before the start of the experiment the groups of cows that were planned to receive during the first experimental period CP15 or CP12, with or without rpCLA, were fed CP12 or CP15, without or with rpCLA, respectively.

Table 1. Ingredient and chemical composition of the experimental diets[1].

	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$
Total mixed ration ingredients, g/kg DM				
Corn silage	313	313	309	309
Corn grain, ground	174	174	172	172
Soybean meal (solv. extr., 44)	63	63	63	63
Meadow hay	124	124	121	121
Sugar beet pulp, dried	103	103	105	105
Alfalfa hay	87	87	88	88
Wheat bran	54	54	50	50
Vitamin and mineral mixture[2]	17	17	17	17
Barley grain, ground (top dressed)	-	-	75	75
Soybean meal (top dressed)	65	65	-	-
rpCLA mixture, top dressed, g/d	-	80	-	80
Analyzed chemical composition, g/kgDM:				
CP	150 ± 4.4	150 ± 4.4	123 ± 2.6	123 ± 2.6
NDF	369 ± 3.5	369 ± 3.5	374 ± 2.4	374 ± 2.4
Starch	227 ± 4.4	227 ± 4.4	263 ± 3.7	263 ± 3.7
Ether extract	24 ± 0.2	28 ± 0.4	24 ± 0.2	28 ± 0.4
Computed chemical composition[3]				
ME, MJ/kgDM	10.46	10.46	10.46	10.46
NE_L, MJ/kgDM	6.53	6.69	6.61	6.69
NE_G, MJ/kgDM	4.52	4.56	4.52	4.56
CP, g/kgDM	152.6	152.8	124.1	123.6
RDP, g/kgDM [1]	97.8	99.9	81.1	81.1
RUP, g/kgDM	54.8	52.9	43.0	42.5
MP, g/kgDM	103	102	83	83

[1]CP15 or CP12: diets providing 150 or 123 g CP/kg DM, respectively; rpCLA: diets top dressed with 80 g/d of a commercial mixture of rumen protected conjugated linoleic acids (rpCLA) providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12, respectively (SILA, Noale, Italy).

[2] Quality Index Complete (Farmer Spa, Porto Mantovano, Mantova, Italy), contained per kg: 240,000 IU of vitamin A, 500 mg of vitamin E, 80 mg of vitamin B1, 0.4 mg of vitamin B12, 2040 mg of vitamin PP, 650 mg of Mn, 100 mg of copper (as copper sulphatepentahydrate), 12 mg of Co (as basic cobalt carbonate, monohydrate).

[3] Computed from dietary ingredient composition according to NRC (2001).

Table 2. Experimental design: 4 groups of cows (5 cows/group) fed during 4 periods (3 wk each[1)] according to four repeated 2 × 2 Latin squares (top dressing or not with 80 g/d of rumen protected CLA; rpCLA[2]) rotated according to a main 2 × 2 Latin square [diets with 150 (CP15) or 123 (CP12) g CP/kg DM].

	Group A.1	Group A.2	Group B.1	Group B.2
Pre-experimental period	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$
Period 1.1	CP12	$CP12_{rpCLA}$	CP15	$CP15_{rpCLA}$
Period 1.2	$CP12_{rpCLA}$	CP12	$CP15_{rpCLA}$	CP15
Period 2.1	$CP15_{rpCLA}$	CP15	$CP12_{rpCLA}$	CP12
Period 2.2	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$

[1] The first 2 wk were used for adapatation, the last for the experimental measurements.
[2] The rpCLA was a commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12, respectively (SILA, Noale, Italy).

Feed Control and Analysis

Over the course of the experiment, the amount of each feed ingredient loaded into the mixer-wagon, and the weight of the mixture uploaded into the manger of each pen were recorded daily. Orts remaining in the mangers during each experimental week were collected, weighted and sampled at the end of each week by pen. As cows were not fed individually, DMI and the MY/DMI ratio were computed on a pen basis. Feed ingredients used during the trial were sampled 2 wk before the start of the experiment and during the last week of each feeding period. Samples of each feed ingredient of rations and orts were analyzed in triplicate for DM (# 934.01; AOAC, 2003), N (# 976.05; AOAC, 2003), ether extract (EE: # 920.29; AOAC, 2003) and ash (# 942.05, AOAC, 2003). Neutral detergent fibre, expressed inclusive of residual ash, was determined (Mertens, 2002) with amylase and sodium sulphite in the neutral detergent solution using an Ankom220 Fibre Analyzer (Ankom Technology® Corporation, Macedon, NY). Acid detergent fibre, expressed inclusive of residual ash, and sulphuric acid lignin contents were determined sequentially after NDF determination (Robertson and Van Soest, 1981). Actual ingredient and chemical composition of the experimental diets were computed from the mean of the actual daily loads of feed ingredients recorded by mixer wagon computer and from the chemical analysis of each feed ingredient (Table 2). The dietary contents of ME, NE_L, RDP, RUP and MP were computed from ingredient composition of diets according to NRC (2001).

The TMRs were also tested for particle size using particle separator (NASCO®, Pennsylvania State University, USA) with two sieves retaining particle of 19 and 8 mm as

described by Heinrichs and Kononoff (2002), and no difference among diets were found with 0.13 DM retained in the first sieve (19 mm), 0.42 retained in the second sieve (8 mm) and the remaining 0.45 in the last collector.

Measurements of BW, BCS, Rumination Activity and Milk Yield

All cows were individually weighed and scored for BCS (Edmonson et al., 1989) by one expert operator at the start of each period and at the end of the last one. Each cow was equipped with RuminAct™ (Milkline®, Podenzano, Italy) a microphone-based rumination monitoring system. RuminAct™ use acoustic biotelemetry to automatically measure the sound of rumination, not that of eating, and to record the time spent in rumination during the day. The microphone of RuminAct™ was positioned on the left side of the neck of each cow and the equipment measured the minutes spent in rumination on a 2-h basis. Individual data were transmitted after each milking to a ID-unit positioned at the exit of the milking parlor and recorded in a personal computer. Time spent ruminating was recorded individually from the start to the end of the experiment but only the data regarding the last week of each period were analyzed. Rumination activity was expressed on daily basis and per kilogram of DMI, by summing the minutes spent in rumination every 2 h. Circadian evolution of rumination from 0 to 24 h with intervals of 2 hours, was also examined by expressing the proportion of time spent in rumination every 120 minutes.

Milk yield was automatically measured and the data stored in a personal computer at each milking throughout all the experiment.

Collection and Analysis of Biological Samples

Individual milk samples (2 per cow, without preservative) were collected during the morning and the afternoon milking of the second and the fifth days of the last week of each period and stored at 4°C to be analyzed for fat, protein, and lactose contents using a MilkoScan (Foss, Hillerød, Denmark). Milk urea N was measured by a conduct metric-enzymatic method (CL 10 micro analyser, Eurochem, Roma, Italy). Milk pH was measured using a pH-meter pH 25+ equipped with an electrode 5232 (Crison Instruments, Alella, Spain).

During the last week of each period, 3 hours after the distribution of the ration, samples of rumen fluid, blood and feces were collected from each cow. Blood samples were collected from the jugular vein and stored in heparinized tubes under vacuum (Venoject,

Terumo, Leuven, Belgium). Plasma was obtained by centrifugation (1500×*g*, 15 min, 4 °C) and analyzed as described by Cozzi et al. (2011) with an indirect potentiometric analyzer (Hitachi 911, Roche Boehringer, Mannheim, Germany) for NEFA, urea, total protein, glucose, triglycerides, CK, AST GGT, and creatinine at 37°C. Roche BM (Mannheim, Germany) commercial kits were used for these plasma constituents, except for the concentration of NEFA which was measured with the enzymatic colorimetric method (Randox Laboratories Ltd., Crumlin, UK). Rumen fluid was collected, as described by Tagliapietra et al. (2011), using a vacuum pump connected to a glass vacuum container in turn connected to a semi-flexible oro-ruminal probe equipped with a steel strainer fixed on the top. The steel strainer (15 cm of length and 3.5 cm of diameter, 600 g of weight) had 32 holes of 0.5 cm of width. The vacuum pump was activated when the top of the probe was positioned in the rumen and the first 100 to 200 mL of rumen sample collected from each cow was discarded to limit contamination with saliva. The rumen fluid, after being filtered through three layers of cheesecloth, was analyzed for pH and ammonia N using a Bench pH/ion meter (Oakton Instruments, Vernon Hills, IL, USA) equipped with a ion-selective electrode (pH meter 104 BASIC 20, Crison Instruments, Alella, Spain). The VFA profile was determined by HPLC using a Thermo Finnigan Spectra System AS3000 auto-sampler (Thermo Electron Corporation, Waltham, MA, USA) equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Richmond, CA, USA). The analysis was performed with a 0.00125 M H_2SO_4mobile phase with a flowrate of 0.6mL/min.

Grab fecal samples were collected from each cow at the fourth day of the third week of each experimental period. Fresh samples were scored for visual consistency as described by Stallings (1998) using a four points scale with 1 equal to dry appearance and 4 equal to liquid consistency; visual color was evaluated using a two points scale with 1 equal to pale and 2 equal to dark appearance. Fecal samples were then oven dried at 55 °C for 48 h and left at room temperature for 12 h, finely ground (1-mm screen) and stored at -18 °C before being analyzed in triplicate for NDF, ADF and lignin as described for feedstuffs. Total tract apparent digestibility of OM, CP and NDF was estimated using lignin as an internal marker (Schiavon et al., 2012).

Nitrogen Partitioning

Nitrogen intake was computed from DMI and analyzed N content of the diet. Milk N secretion was computed from actual milk yield and its N content. Milk N efficiency (Milk

N/N intake; **ENU**) was computed as a ratio between N in milk/N intake. Fecal N excretion was computed from N intake and apparent N digestibility. The amount of N intake not secreted with milk, or excreted with feces was computed as an index accounting both for N in urine and N retained or released from body tissues. Notwithstanding the shortcoming due to the short term nature of current experiment, an estimation of N retained or released from body tissues was done considering daily changes in BW, with BW measured at the end and the beginning of each 3-wk period, and assuming 22.1 g of N retained or released per kg BW change (Tedeschi et al., 2006). Nitrogen excretion with urine was estimated as N intake - (N in milk + N in feces + estimated N retained or released from body tissues).

Actual and Predicted Production Response

The NRC (2001) model predictions were compared with the actual results obtained in this experiment. Ingredient composition of diets were entered into the model. A general calcium soaps of FA was considered for rpCLA as this ingredient was not considered in the NRC feed database list. Differences between predicted and analyzed chemical composition of the diets were trivial. Animal data, averaged for each group of cows and period (DMI, BW, parity, DIM, milk yield and CP, casein, fat and lactose milk contents) and default environmental conditions were entered into the model. Among the various model outputs the following predictions were used for the comparison: NE_L and MP requirements to support all body functions, NE_L-allowable milk and MP-allowable milk. The following indexes of comparison were used: (i) the ratios between NE_L or MP supplied in the current experiment with the predicted NE_L or MP requirements; (ii) the ratios between actual milk yield and predicted NE_L-allowable milk or MP-allowable milk.

Statistical Analysis

Data were averaged by group and period and analyzed using the PROC MIXED procedure in SAS 9.2 (SAS Inst. Inc., Cary, NC) with the following model:

$$y_{ijklm} = \mu + P_i + G_j + D_k + e_{ijkl}$$

where y_{ijklm} is the dependent variable; μ is the overall intercept of the model, P_i is the fixed effect of the i_{th} period (i = 1, …, 4), G_j is the random effect of the j_{th} group of cows (j = 1, …, 4), D_k is the fixed effect of the dietary treatment (k = 1,…, 4) and e_{ijkl} is the random residual. Group was assumed to be independently and normally distributed with a mean of zero and

variance σ_j^2. The 3 degrees of freedom of D_k were used to fit orthogonal contrasts to test the effects of the dietary CP content (1 df), rpCLA addition (1 df), and of CP × rpCLA interaction (1 df). Based on this model, the effect of CP is the mean of the values measured at the end of the third and of the sixth week of each CP period.

Data about the proportions of time spent in rumination during the day were averaged by group, period and 2-hours intervals of rumination (H; 11 df) and analyzed with the following mixed model:

$$y_{ijklm} = \mu + P_i + G_j + D_k + P \times G \times D_{ijk} + H_l + P \times H_{il} + G \times H_{jl} + D \times H_{kl} + e_{ijklm}$$

where P_i, G_j, D_k and H_l have been defined above; $P \times G \times D_{ijk}$ was the triple interaction (random) among P_i, G_j, and D_k (6 df); $P \times H_{il}$ was the interaction between P_i and H_l; $G \times H_{jl}$ was the interaction between $G_j \times H_l$; $D \times H_{kl}$ was the interaction between $D \times H_{kl}$; and e_{ijklm} was the random residual (50 df). The $P \times G \times D_{ijk}$ (that correspond to the random residual of the first model) was used as line of error to test the effects of P_i, G_j, and D_k. As the D × H interaction was significant ($P < 0.001$), the 3 degrees of freedom of D_k available for each level of H were used to fit orthogonal contrasts to test the significance of the effect of CP, rpCLA and CP × rpCLA interaction.

Results

Rumen Fluid, Blood Metabolites, Intakes and Digestibility

The reduction of dietary protein decreased ammonia N content ($P < 0.001$), increased pH ($P = 0.026$), and had null or small influences on total VFA concentrations and the relative proportions of individual VFA in the rumen fluid (Table 3). Protein restriction increased by 10% and 18% the time spent in rumination when expressed on daily basis ($P = 0.006$), or per kilogram of DMI ($P = 0.009$). Dietary CP content influenced the circadian rumination activity especially from 0800 to 2000 p.m. (Figure 1). No effect of rpCLA were observed on the rumen fluid characteristics. There was no effect of dietary CP content, rpCLA or their interaction on blood profile (Table 4), with the exception of BUN which was reduced in CP12 ($P < 0.001$) and in rpCLA addition ($P = 0.015$).

The dietary protein restriction reduced DMI by 7.7% (Table 5), even if the difference was not significant ($P = 0.09$). Crude protein restriction had small effect on total tract apparent digestibility of OM ($P = 0.11$), NDF ($P = 0.15$), ADF ($P = 0.06$) and NSC ($P = 0.66$), but reduced apparent digestibility of CP ($P < 0.001$). Fecal moisture, pH and color were

not influenced by dietary CP content, but cows receiving CP12 produced a drier consistency of feces (P = 0.004) than those produced by the CP15 groups. The addition of rpCLA tended to reduce DMI (P = 0.07), too. The dietary supply of rpCLA increased EE digestibility, but did not affect the digestibility of other nutrients and the physical characteristics of feces.

Figure 1. Effect of dietary CP density,123 (CP12) or 150 (CP15) g CP/kg DM, on the proportion of time spent in rumination during the day by lactating Holstein cows [within hour *** = P< 0.001].

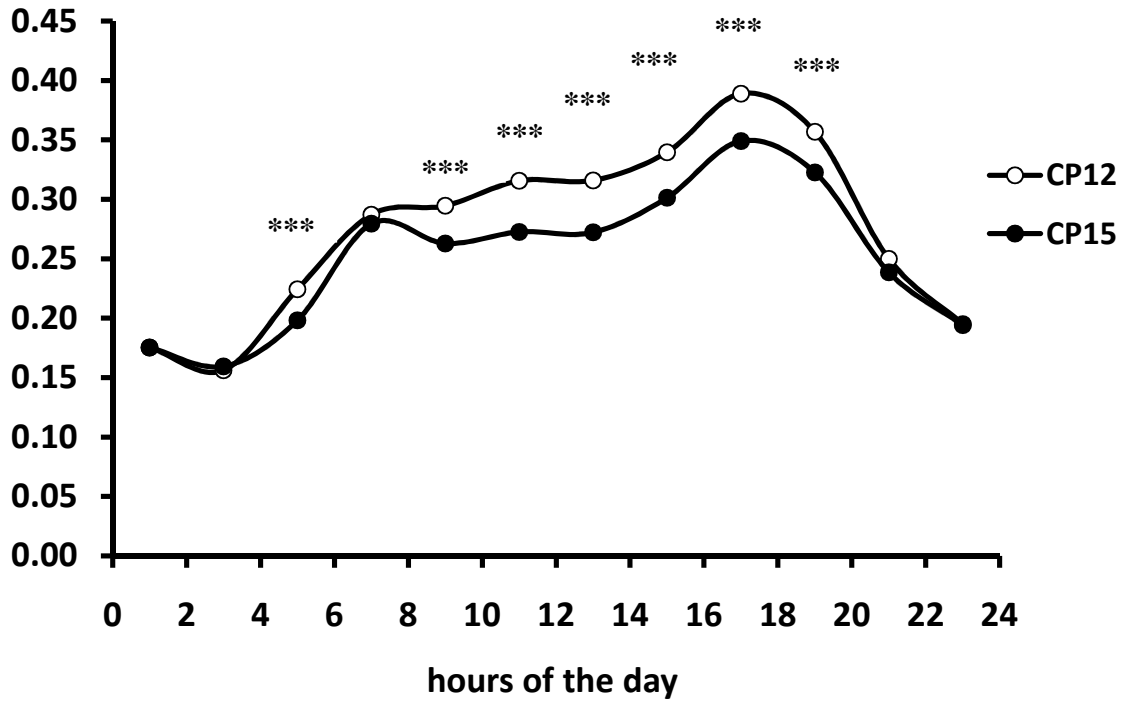

Table 3. Effects of diets containing 150 (CP15) or 123 (CP12) g CP/kg DM, top dressed or not with 80 g/d of rumen protected CLA (rpCLA)[1], on rumen parameters and rumination activity.

	Diet					*P*-values		
	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$	SEM	CP	rpCLA	CP ×
Rumen								
Ammonia	8.25	7.86	4.76	5.63	0.324	<0.001	0.50	0.10
pH	6.64	6.63	6.81	6.82	0.07	0.026	0.98	0.82
Total VFA,	79.4	82.9	78.1	74.6	3.01	0.16	0.99	0.29
VFA								
acetate	63.3	63.2	62.9	62.8	0.58	0.51	0.87	0.95
propionate	21.7	21.8	21.6	21.4	0.73	0.69	0.98	0.85
butyrate	11.1	11.2	11.8	11.7	0.28	0.042	0.98	0.81
iso-	0.57	0.57	0.52	0.51	0.03	0.12	0.85	0.91
valerate	1.46	1.45	1.36	1.38	0.05	0.06	0.99	0.67
Others[2]	2.05	2.00	1.75	2.00	0.05	0.36	0.49	0.23
Rumination[3],	357	359	389	398	11.7	0.006	0.52	0.69
Rumination[2],	15.6	18.0	19.2	20.3	0.96	0.009	0.06	0.43

[1] Commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12 isomers, respectively (SILA, Noale, Italy).
[2] Others VFA comprised *iso*-valerate, caproate and heptanoate.
[3] Measured with RuminAct™ (Milkline®, Podenzano, Italy).

Table 4. Effects of diets containing 150 (CP15) or 123 (CP12) g CP/kg DM, top dressed or not with 80 g/d of rumen protected CLA (rpCLA)[1], on blood parameters.

	Diet					*P*-values		
	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$	SEM	CP	rpCLA	CP ×
BUN, mg/100	12.84	11.78	7.16	6.42	0.369	<0.001	0.015	0.62
Total Protein,	75.2	73.4	74.05	74.12	1.07	0.77	0.24	0.26
Albumin, g/L	34.9	34.0	33.3	34.2	0.73	0.29	1.00	0.20
Globulin, g/L	40.2	39.4	40.8	39.9	1.66	0.69	0.48	0.97
AST[2], U/L	75.7	74.5	76.8	83.3	4.85	0.30	0.54	0.41
GGT[3], U/L	24.2	23.5	23.8	24.9	0.40	0.23	0.67	0.07
CK[4], U/L	127	183	229	181	42.1	0.11	0.88	0.10
Bilirubin,	1.56	1.79	1.68	1.83	0.123	0.49	0.14	0.75
NEFA, meq/L	0.115	0.104	0.118	0.118	0.007	0.22	0.45	0.41
Triglycerides,	97.0	92.0	93.0	94.0	1.87	0.61	0.33	0.16

[1] Commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12 isomers, respectively (SILA, Noale, Italy).
[2]AST = aspartateaminotransferase.
[3]GGT = gamma-glutamyltransferase.
[4]CK = creatine kinase.

Table 5. Effects of diets containing 150 (CP15) or 123 (CP12) g CP/kg DM, supplied or not with 80 g/d of rumen protected CLA (rpCLA)[1], on nutrient intakes, nutrient digestibility and fecal traits.

	Diet					*P*-values		
	CP15	$CP15_{rpCL}$	CP12	$CP12_{rpCL}$	SEM	CP	rpCL	CP ×
Intakes, kg/d:								
DM	23.0	20.1	20.2	19.6	0.81	0.09	0.07	0.20
OM	21.7	19.0	19.2	18.6	0.76	0.11	0.07	0.20
CP	3.45	3.01	2.50	2.43	0.113	<0.00	0.06	0.16
NDF	8.48	7.40	7.55	7.33	0.304	0.15	0.08	0.21
ADF	4.79	4.18	4.14	4.02	0.172	0.06	0.08	0.20
NSC	9.24	7.98	8.62	8.29	0.327	0.66	0.05	0.21
Ether extract	0.56	0.57	0.49	0.56	0.021	0.12	0.11	0.25
Digestibility								
OM	0.66	0.657	0.65	0.636	0.007	0.07	0.21	0.48
CP	0.62	0.619	0.54	0.531	0.009	<0.00	0.31	0.47
NDF	0.49	0.485	0.47	0.450	0.014	0.14	0.27	0.52
ADF	0.48	0.478	0.46	0.456	0.011	0.14	0.61	0.88
NSC	0.84	0.842	0.84	0.841	0.008	0.91	0.71	0.78
Ether extract	0.48	0.501	0.45	0.498	0.017	0.23	0.031	0.32
Fecal traits:								
moisture	0.86	0.863	0.85	0.862	0.002	0.15	0.97	0.25
pH	6.41	6.33	6.39	6.31	0.040	0.64	0.06	0.94
Colour[2]	1.38	1.48	1.59	1.46	0.144	0.53	0.93	0.44
Consistency[3]	2.40	2.38	1.98	2.02	0.158	0.004	0.90	0.77

[1] Commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2c9,t11 and C18:2t10,c12 isomers, respectively (SILA, Noale, Italy).
[2] Visual fecal color was evaluated using a two point scale with 1 equal to pale and 2 equal to dark appearance.
[3] Visual fecal consistency was evaluated according to Stallings (1998) using a four point scale with 1 equal to dry appearance and 4 equal to liquid consistency.

Milk Yield and Quality Traits

The CP restriction reduced milk yield (P = 0.047), milk protein yield (P< 0.001) and milk protein content (P = 0.026), milk fat yield (P = 0.048), lactose yield (P = 0.05) and milk lactose content (P = 0.020) (Table 6). The CP restriction reduced MUN by 48% (P< 0.001), consistently with the observed changes of BUN and of rumen ammonia N concentration. However, MY/DMI was not affected by CP, rpCLA or their interaction, and averaged 1.37 (P = 0.78). The dietary CP restriction had no significant influence on changes of BW (P = 0.35) or BCS (P = 0.34), despite its negative influence on DMI and despite the 6-wk duration of treatment. The addition of rpCLA did not influenced milk yield and BW change, but reduced milk fat yield (P< 0.001) and milk fat content (P = 0.002), milk protein yield (P = 0.041), but only with the CP12 diet (P = 0.016), and tended to reduce milk lactose contents (P = 0.05).

Nitrogen Partitioning

The dietary CP restriction influenced almost all measurements and estimates of N (Table 6). Nitrogen intake decreased by 24%, N secretion in milk decreased by about 9% (P< 0.001), so that ENU increased from 0.31 to 0.36 (P = 0.009); no difference was observed for fecal N excretion (P = 0.44). As a consequence of the dietary CP reduction, the difference between N intake and (N in milk + N in feces) decreased, on average, from 162 to 69 g/d (P< 0.001), and because the apparent contribution of N from body tissue was little, as estimated from BW changes, large part of this difference was attributed to a reduction of the estimated urinary N excretion.

The rpCLA supply tended to reduce N intake (P = 0.06), but there was a CP × rpCLA interaction (P = 0.016) on milk N, as the difference in N secretion in milk due to rpCLA addition was null when rpCLA was supplied to CP15 (158 vs 159 g/d, respectively), whereas it decreased from 150 to 138 g/d N when rpCLA was supplied to CP12. The value of ENU tended to increase when rpCLA was supplied to CP15, and to decrease when supplied to CP12. The CP × rpCLA interaction did not influenced the fecal N excretion. The difference between N intake and milk N plus fecal N tended to decrease (185 to 139 g/d) when rpCLA was supplied to CP15 but it was unaffected (70 to 68 g/d) when rpCLA was supplied to CP12. As the estimated apparent contribution of tissue N was apparently small, the trend observed for the estimated urinary N excretion was similar to that observed for the difference between N intake and milk N + fecal N.

Actual and Predicted Milk Yield

The ratio between intake and requirement of NE_L resulted slightly greater than the unity for all diets, confirming that NE_L was not the first limiting factor (Table 7). Conversely, the ratio between intake and requirement of MP was not different from the unity for CP15 diets, but decreased by 18% in CP12 diets ($P < 0.001$). Consistently, for the two CP15 diets the ratio between actual and predicted milk yield was slightly lower than the unity when the predictions were performed on NE_L basis, and closer to the unity when the predictions were done on the basis of available MP. On the contrary, the reduction of the dietary CP increased the ratio between actual and predicted MP-allowable milk yield by 35% ($P = 0.006$). No influence due to rpCLA supply was observed on these parameters.

Table 6. Effects of diets containing 150 (CP15) or 123 (CP12) g CP/kg DM, top dressed or not with 80 g/d of rumen protected CLA (rpCLA)[1], on milk yield and quality, BW and BCS changes and N partitioning (g/d).

	Diet					*P*-values		
	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$	SEM	CP	rpCLA	CP ×
Milk production:								
milk yield, kg/d	28.1	29.5	27.8	27.0	0.72	0.047	0.69	0.12
fat yield, kg/d	1.07	0.90	0.98	0.87	0.025	0.048	<0.001	0.26
protein yield, kg/d	1.01	1.02	0.96	0.88	0.016	<0.001	0.041	0.016
lactose yield, kg/d	1.38	1.37	1.28	1.31	0.040	0.05	0.77	0.56
Milk yield/DMI, kg/kg	1.26	1.46	1.38	1.37	0.073	0.78	0.21	0.17
Milk quality:								
fat, g/kg	36.6	31.2	36.9	31.2	1.23	0.90	0.002	0.88
CP, g/kg	35.2	35.4	34.2	33.1	0.65	0.026	0.41	0.29
lactose, g/kg	47.6	47.3	47.2	46.7	0.16	0.020	0.05	0.59
MUN, mg/100 mL	11.94	11.28	6.23	5.76	0.392	<0.001	0.19	0.81
Cows weight and								
Mean BW, kg	649	648	646	644	11.4	0.33	0.61	0.80
Mean BW	0.30	-0.02	0.26	-0.59	0.297	0.35	0.10	0.40
Mean BCS	2.91	2.92	2.93	2.91	0.024	0.47	0.47	0.17
Mean BCS	0.05	0.04	0.01	0.03	0.024	0.34	1.00	0.62
Nitrogen partitioning:								
N intake, g/d	552	482	400	389	18.1	<0.001	0.06	0.16
N in milk, g/d	158	159	150	138	2.6	<0.001	0.041	0.016
ENU (milk N/N	0.29	0.33	0.37	0.35	0.015	0.009	0.35	0.08
N in feces	210	184	181	182	10.6	0.20	0.29	0.25
N Intake - (N in	185	139	70	68	9.7	<0.001	0.049	0.06
Estimated N	6.6	-0.5	5.8	-13.0	6.56	0.35	0.10	0.40
Estimated N in	178	140	64	81	12.5	<0.001	0.43	0.06

[1] Commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12 isomers, respectively (SILA, Noale, Italy).

[2] Computed from BW or BCS changes over periods of 3 weeks.

[3] Estimated over periods of 3 weeks assuming a retention or a mobilization of 22.1 g N/kg of positive or negative changes of BW (Tedeschi et al., 2006), respectively. Caution should be taken when considering these data because of the short term nature of this experiment.

[4] EstimatedN in urine was computed as N intake - N in milk - N in feces - estimated N retention (or mobilization if negative values) in tissue. Caution should be taken when considering these data because of the short term nature of this experiment.

Table 7. Effects of diets containing 150 (CP15) or 123 (CP12) g CP/kg DM, supplemented or not with 80 g/d of rumen protected CLA (rpCLA)[1], on the ratios between the amounts of nutrients supplied and those required according to NRC (2001).

	Diet[2]					*P*-values		
	CP15	$CP15_{rpCLA}$	CP12	$CP12_{rpCLA}$	SEM	CP	rpCLA	CP ×
Ratio between								
DMI,	1.03	0.93	0.91	0.94	0.039	0.22	0.41	0.15
NE_L,	1.15*	1.08	1.05	1.13*	0.044	0.51	0.89	0.15
MP,	1.07	0.96	0.81*	0.83*	0.029	<0.001	0.22	0.08
RDP	0.99	0.99	0.80*	0.89	0.050	0.029	0.39	0.40
RUP,	1.18*	0.93	0.63*	0.68*	0.066	<0.001	0.17	0.07
Ratio between								
NE_L-	0.83*	0.90	0.93	0.84*	0.049	0.65	0.92	0.14
MP-	0.91	1.07	1.30*	1.41*	0.086	0.006	0.17	0.77

[1] Commercial mixture of conjugated linoleic acids providing 6.34 and 6.14 g/d of C18:2*c*9,*t*11 and C18:2*t*10,*c*12 isomers, respectively (SILA, Noale, Italy).
[2] Means with * superscripts differ from the expected value of 1.0 for $P < 0.05$, respectively.
[3] Values predicted using actual milk production, milk composition, BW, DIM and DMI for each group of cows and experimental period (NRC, 2001).

Discussion

General Considerations

Rumen metabolism is considered the most important factor that influences the efficient use of N in ruminants. As the manipulation of rumen fermentations is more feasible than interventions on animal metabolic processes, research efforts were mainly focused on optimizing rumen fermentation and N flow to the small intestine (Calsamiglia et al., 2010). Recommendations for optimizing the proportions of RDP and RUP, or supplying MP and fermentable energy, or modifying the AA profile in the small intestine have been consequently codified in many energy and protein systems. Despite the great increase in knowledge, Huhtanen and Hristov (2009) from a meta-analysis of a great number of data from the United States and European countries surprisingly found that little improvement in ENU was achieved in the last four decades. The need for a better understanding of the factors that influence the efficiency of capture of N as absorbable microbial protein, and the metabolic efficiency of utilization of absorbed amino acids is detailed in many reviews (Reynolds and Kristensen, 2008; Hall and Huntington, 2008; Calsamiglia et al., 2010). In their meta-analysis Huhtanen and Hristov (2009) observed that ENU was increased more by a decrease of the dietary CP level than by an increase of milk yield. Decreasing protein intake and

incorporating easily fermentable carbohydrates in the diet may be a way to improve ENU (Belanche et al., 2012). In this experiment the magnitude of the effects induced by a reduction of dietary CP below the recommended levels, obtained by replacing soybean with cereal grain, on DMI, rumination activity, milk production, change of BW and N balance of mid-late lactating cows was studied.

Rumen and Rumination, Digestibility and Blood

In the current paper the reduction of dietary CP decreased rumen ammonia N concentration from 8.1 to 5.2 mg/100 mL, BUN from 12.3 to 6.8 mg/100 mL, and MUN from 11.6 to 6.0 mg/100 mL, increased the time spent ruminating and increased ruminalpH. Despite the magnitude of these changes, there were only small changes in VFA content in ruminal fluid, and on blood protein fractions, liver enzymes, and other blood metabolites, but some numerical reductions of DMI and of nutrient digestibility, 1 to 3 percentage points, were observed. Within the frame of the RedNEx project, in a Latin square design experiment with period of 4 wk, Fanchone et al. (2013) compared high-starch rations containing 143 or 111 g/kg DM of CP and found that nutrient digestibility and DMI intake were not influenced. In agreement with current experiment, they also observed that the large reduction in dietary N intake did not affect N excretion in feces, but milk yield decreased by about 9%. However, cows used in the experiment of Fanchone et al. (2013) had lower average milk yield (24.1 kg/d) and milk protein content (30.7 g/L) compared with the present study.

Much research has been concentrated on the effect of ammonia N concentration on rumen fermentation and on microbial protein synthesis (Calsamiglia et al., 2010). With the low-CP diet used in the current experiment the ammonia N concentration in the rumen fluid averaged 5.2 mg/100 mL and with the high-CP diet the ammonia N concentration averaged 8.1 mg/mL. These values were likely to be near the peak of concentration as the rumen fluid was collected 3 h after the distribution of the ration, even if the cows had free access to the feed throughout the day. To optimize feed degradation, particularly of fibrous constituents, a minimum level of ammonia N is likely required (Schwab et al., 2005). To this regard Ruiz et al. (2002), using diets for dairy cows similar to those used in the current experiment, found that with dietary CP contents of 141, 111 and 94 g/kg DM the rumen ammonia N concentration decreased from 10.0, to 4.5 and to 2.6 mg/100 mL, respectively. They found that the *in situ* NDF degradability was higher for 10.0 mg/100 mL of ammonia N concentration compared to that of the other two ammonia N concentrations. However, total

tract NDF digestibility was 0.55 for both the diets with the highest CP contents and only 0.50 for the very low-CP diet. They concluded that the minimum ammonia N concentration to optimize NDF digestion in the rumen would depend by the kind and quality of the feed ingredients with variations from 4.5 to 10.0 mg/100 mL. They suggested that low NDF digestibility in the rumen found *in situ* was compensated by an increase of NDF digestion in the lower gut. From the data of the current experiment some compensation induced by an increase in the rumination activity and consequently on forage particle size and surface/volume ratio would be also proposed.

Several experiments conducted on dairy cows (Roffler and Satter, 1975; Clark et al., 2002; and others cited by Fanchone et al. (2013) suggested that increasing ammonia N concentration above 2.0 to 5.0 mg/100 mL had little or null effect on microbial protein production. Bach et al. (2005) observed from continuous culture system that the efficiency of microbial N utilization (g bacterial N/ g available N) increases linearly with decreasing rumen ammonia N concentration. Fanchone et al. (2013) also observed as the efficiency of microbial protein synthesis, expressed in term of g N/kg OM fermented, was not influenced by the dietary N level ($P = 0.29$) and tended to be greater with high-starch diets than with high-fiber diets ($P = 0.08$). In the experiment of Fanchone et al. (2013) the ruminal digestibility of NDF was depressed with the low-CP high-starch diet compared to the high-CP high-starch diet but the microbial growth was not impaired. This was in line with Hoover (1986), who reported that maximum microbial growth could be achieved with a lower ammonia concentration than that required to maximize cellulolysis. From experiments conducted on dairy cows (Belanche et al., 2012) it was observed as some non-cellulolytic bacteria have a relatively low constant of ammonia saturation, that allow them to scavenge and to incorporate ammonia even when ammonia is present at low concentrations. In agreement with others (Clark et al., 1992; Guadagnin et al., 2013), they indicated as a moderate N underfeeding does not affect the rumen concentration of non-cellulolytic microbes and microbial growth. They also suggested that the negative effect of N restriction would mainly reflect the sensitivity of fibrolytic microorganisms and of some minority microbial groups, and that an adaptation of the microbial populations would compensate for some deficit in N supply. In the current experiment the ammonia N concentration in the rumen fluid obtained with the low-CP diet was smaller than the maximum concentration above which little or null improvement of the amount of N captured by the microbes is achieved according to literature. Differently, with the CP15 treatment the rumen fluid ammonia N concentration was much greater than that

observed for CP12, but relative small benefits were observed in total VFA concentration, VFA profile, nutrient digestibility and blood metabolites content, and the amount of N excreted with feces was unaffected by the dietary CP restriction. This might suggest that in CP15 ammonia N concentration in the rumen fluid was in excess compared to the requirement, and that the N losses from rumen compartment were potentially increased compared with CP12.

Previous studies on chewing activity (eating + rumination) were principally focused to evaluate the effect of diets with different forage and concentrate contents and different feed particle size on milk yield and milk fat content, the relationships with feeding (Dado and Allen, 1994; Krause et al., 2002; Maekawa et al., 2002). These authors observed that duration of rumination is largely influenced by the amount of feed eaten together with ration composition and feed particle size and that cows spend in rumination about 344 to 496 min/d, 15.3 to 24.4 min/kg DMI or 47.7 to 79.9 min/kg NDF. Rumination increase feed particles fragmentation, increases the particle surface exposed to the rumen environment and would facilitate a further degradation of feed in the rumen and its digestion in the intestine (Krause et al., 2002). In addition, rumination reduces the size and increases the specific gravity of feed particles which would favor a sedimentation of feed particles within the rumen (Ramanzin et al., 1991 and 1994; Bailoni et al., 1998) and might accelerate the particle flow out of the rumen (Seo et al., 2009). In the current experiment it was considered that a reduction of dietary CP content would potentially reduce feed degradability, increase gut fill, and decrease DMI. Under such condition an increase of rumination activity would be expected, as a physiological response that ruminants would activate to compensate, at least in part, for reduced feed digestibility and DMI (Tafaj et al., 2005). In the current experiment cows spent on average 375 min/d, 18 min/kg DMI, or 49 min/kg NDF in rumination activity, and these figures fall within the ranges reported in literature. The rations compared in the current experiment were formulated and prepared to be almost identical for NDF content, feed particle size and feed ingredients, except for the addition of soybean meal or barley grain and rpCLA. In this way, interferences on rumination activity due to dietary factors other than the CP and rpCLA contents were minimized, except for dietary starch content which was greater in CP12 than in CP15 because of the replacement of soybean meal with barley grain. Therefore, the effects observed in this experiment would be attributable to the contemporary dietary change in CP and starch contents.

The cows spent a greater proportion of time in ruminating during the diurnal hours (6:00 to 20:00 h), with a peak at 1600 to 1800 p.m., than during the night (Figure 1). Previous research conducted on dairy cows found an opposite pattern (Adin et al., 2009; Soriani et al., 2013) with rumination activity more pronounced during the night than during the day. The pattern of eating and rumination behavior over the day is greatly influenced by a number of factors including feeding frequency and ration composition, feeding time, milking times and frequency, photoperiod, housing systems and social interactions. In the present study the rations were distributed once per day at about 0600 a.m.. However, the sinusoidal pattern of rumination observed by Jaster and Murphy (1983) was confirmed by the result of current work.

With decreasing the dietary CP and increasing the starch contents rumination activity increased from about 358 to 394 min/d (+9.9%), and the proportion of time spent in rumination was consistently greater with CP12 compared to CP15 throughout the day. In agreement with Tafaj et al. (2005) such increase of rumination would partially explain the small influences that the dietary CP restriction had on nutrient digestibility, on total VFA concentration and VFA profile of the rumen fluid, despite the different ammonia N concentration and the increase of dietary starch content. However, such increase of rumination appeared to be not sufficient to fully prevent the reduction of DMI caused by the dietary CP content restriction applied in this experiment.

Nitrogen Partitioning

As a consequence of the restriction of dietary CP, N intake decreased by 122 g/d, but N in milk decreased by only 14 g/d and fecal N excretion remained constant, so that ENU increased from 0.31 to 0.36. The difference between N intake and N in milk and in feces accounts for N in urine and N retained in body tissues or released from labile protein reserves to provide amino N to the mammary gland. The value of this difference averaged 162 and 69 g/d for CP15 and CP12, respectively. Signs of N mobilization from body tissues as a consequence of CP restriction were weak, as both with CP12 and CP15 little changes of BW were observed, although each treatment lasted 6 consecutive weeks. However, a change in BW does not necessarily indicate changes in tissue reserves (Ruiz et al., 2002), particularly in short term experiment. This has been primarily explained by considering that gut fill would varies from 2.5 to 4.0 kg/kg of increase in DMI (Tedeschi et al., 2006). In the current experiment the dietary CP reduction nominally decreased by 1.7 kg/d DMI, so that a decrease

of gut fill, and hence a reduction of BW, in the order 4.3 to 6.8 kg might be expected. But on the opposite in current experiment the measurements of BW for CP12 as well as for CP15 without rpCLA supply, suggested an average increase of BW by 0.28 kg/d. This might exclude the possibility that mobilization of body reserves have been masked by changes in gut fill. Responses to a protein deficiency can take long time to manifest due to the buffering effect of labile body protein reserves. Paquay et al. (1972) suggested there are two labile N pools in early lactating dairy cow, a very labile N pool that is mobilized readily and another one that is available for extended times. The first pool corresponds to N compounds in blood and possibly protein from internal organs such as liver while the second pool may represent skeletal muscle. When cows begin to deplete body pools of N, small, or minimal change of BW are expected as it is the change in the first labile N pool that largely contributes to the initial change in N status of the animal (Paquay et al., 1972). This was observed by Biddle et al. (1975) in growing cattle fed diets containing only 80 g/kg DM of dietary CP for 5 wk. These authors observed as during the first 3 wk from the beginning of the treatment a progressive reduction of blood total protein and albumin, together with a reduction in BUN occurred, whereas deviation from linearity of BW gain started only after 3 wk from the beginning of treatment. In lactating cows few information are available about relationships between changes in protein body reserves and changes in blood protein and albumin content. In the current experiment the dietary CP reduction caused not significant change in BW and BCS, with some reduction in DMI, and null influences in the blood profile except for BUN. On the basis of these data it might be suggested that N mobilization from body tissues was small. Thus the increase of ENU caused by the dietary CP reduction observed in this experiment would be mainly attributed to a decrease of the urinary N. Moreover, a large percentage error in the estimation of N from tissue mobilization correspond to a few grams of N per day and consequently to a small error in the prediction of urinary N excretion. However, the short term nature of this experiment cannot allow to draw conclusive considerations about mobilization of body protein in support to lactation or possible reduction in body protein deposition.

The magnitude of the difference in N partitioning and the notable improvement of ENU observed in this experiment when the dietary CP content was reduced can likely be explained by three reasons, the first is that CP15 likely provided N in excess with respect to the requirement; the second is that CP12 likely provided amounts of N slightly lower than the requirements, so that diets with a slightly greater dietary N content than that used in the

present trial would presumably result in less accentuated reduction in milk yield and quality; the third is that decreasing protein intake and incorporating starch in the diet might cause an adaptation of the microbial population with increased efficiency of microbial N capture.

Actual and Predicted Milk Yield

In the current experiment we intentionally tested dietary N content well below the recommendation to test the magnitude of the differences with respect to a diet considered adequate in N content. Current models have been developed on the basis of current knowledge to offer general recommendations for a variety of different dietary, animal and environmental conditions, and their outputs do not necessarily agree with the results of a single experiment, particularly if characterized by short periods of treatment. Nevertheless, in the current work the MP-allowable milk yield predicted by NRC (2001) with the CP15 diets indicated that the model provides accurate estimates of milk yield when conventional diets are used. This was reflected by the fact that the ratios between supply and simulated requirements for MP were close to the unity. However, the NRC (2001) model was unable to predict accurately milk production in the case of N underfeeding and the observed ratio between actual and predicted MP-allowable milk, averaging 1.35, indicates that NRC (2001) would underestimate milk yield or overestimate N requirements, with respect to the conditions of this experiment. Cyriac et al. (2008) also suggested that NRC (2001) would overestimate the RDP requirement in mid-lactation cows. Despite the short duration of this experiment, the magnitude of the differences observed between actual and predicted values suggests that some reduction of the dietary CP content with respect to recommendations might be applied with success, at least in short periods and in mid- late lactation cows fed corn-silage and cereal based diets with milk production around 28 kg/d of milk.

Effect of rpCLA and its Interaction with CP content of the Diet

Conjugated linoleic acid isomers affect an large range of biological processes, including the ability to inhibit milk FA synthesis (Pariza et al., 2001). Although this has been demonstrated in several mammals, research has been most extensive with dairy cows and small ruminants. The isomer able to primarily affect milk fat synthesis during lactation was the C18:2*t*10,*c*12, and its effects have been well characterized, including dose-response relationships (Bauman et al., 2008). Regulation by CLA occurs naturally in dairy cows when specific CLA isomers

produced as intermediates in rumen bio-hydrogenation act to inhibit milk fat synthesis, referred to as diet-induced milk fat depression (Bauman et al., 2008). In dairy cows, milk fat synthesis is progressively reduced by increasing supplemental amounts of the C18:2*t*10,*c*12 isomer in the diet in rumen protected forms, and the commercial rpCLA used in current study was found to be effective in reducing milk fat content in previous studies on dairy cows (Dal Maso et al., 2008) and sheep (Bittante et al., 2014), and to increase the content of CLA isomers in milk (Bauman et al., 2008), with a recovery in ripened cheese in the order of 0.90 (Cattani et al., 2014). Few studies have been conducted to investigate effects of rpCLA on total tract digestibility of feed constituents. However, in sheep, Huang et al. (2009) found that diets supplemented with 10 g/kg DM of rpCLA, as free acids or calcium salts, did not influence total VFA, VFA profile of rumen fluid and digestibility of DM and of CP compared to control. Results of the current experiment are in agreement with the previously cited literature, as no effects of rpCLA or rpCLA × CP interaction were found on rumen fluid and blood profile (with the only exception of a reduction of BUN), and on the apparent digestibility coefficient of nutrients, whereas milk fat content was reduced.

Besides CLA effects on fat metabolism, much less is known about CLA effects on protein metabolism in the dairy cow. The CLA effects on daily milk protein synthesis in dairy cows are inconsistent; most studies observed no effect on milk protein yield (Pappritz et al., 2011; von Soosten et al., 2011), whereby de Veth et al. (2006) and Medeiros et al. (2010) reported that CLA-fed cows with restricted energy supply showed an increased milk protein yield. Research conducted on growing cattle suggested that rpCLA increased N efficiency of young bulls fed low-CP diets suggesting that CLA affects protein turnover and compensated for deficiency of dietary CP (Schiavon et al., 2010, 2011). The experiment of von Soosten et al. (2012) suggested a protective effect of CLA supplementation against excessive use of body reserves within 42 DIM and a continuous CLA supplementation until 105 DIM increased protein accretion in primiparous Holstein cows. The current experiment explored the possibility that rpCLA could exert some sparing effects on protein metabolism. However, result obtained are unclear, as rpCLA negatively interacted with the dietary CP level on the amount of N retained in milk, but the protein content of milk was unaffected. Milk N efficiency tended to increase when rpCLA was supplied to CP15 and to decrease when rpCLA was added to the CP12 diet. These effects were apparently caused by some influences of rpCLA on DMI and consequently on MP intakes. Further researches aimed to evaluate the

effects of rpCLA on protein metabolism should consider possible interplays with energy metabolism and feed intake regulation.

Practical Implications

From an environmental point of view a reduction of 122 g/d of N intake that only marginally decreased milk N secretion (14 g/d), notably increased ENU (0.31 to 0.36) and reduced the estimated urinary N is important for at least two reasons. The first regards the estimated reduction of urinary N excretion, close to 54%, and the second concerns the total N excretion which was estimated to be reduced by 29% with CP12 compared to CP15. Many studies have shown that urea and other urinary N compounds can be easily converted in volatile compounds and lost in the air, and it is expected that N losses in the air will decrease with decreasing urinary N excretion (Hristov et al., 2011). It is desirable that tools for predicting emission of N compounds in the air applicable at farm level will be developed in the future. To achieve this goal, besides the cited effects of feeding, housing, slurry management and climate conditions, another key variable to be considered is the volume of the slurry in which the excreted fecal and urinary N compounds are diluted, as demonstrated by mathematical models to predict fresh and mature volumes of slurry of other species (Schiavon et al., 2009). Environmental constraints are becoming limiting because the agricultural surface available for manure spreading is no longer sufficient in many areas, especially in the EU (Xiccato et al., 2005), and the cost of protein sources is increasing. In ruminants the use of low-protein rations with minimal losses in milk yield would be a possible strategy to overcoming these constraints, as a strong reduction of N excretion would allow a notable reduction of agricultural land needed, and the cost of feeding and for manure disposal, according to the production circumstances (Schiavon et al. 2013). Further researches are needed to evaluate the effect of low-protein diets over extended period of lactation, as protein mobilization or reduction in protein deposition can compensate for dietary protein deficiencies in short period, but this would be not sustainable over an entire lactation. A further element which should be considered regards the effect of N underfeeding on the cheese making properties of milk, as these are influenced by milk composition and milk protein characteristics (Bailoni et al., 2004; Bittante et al., 2012; Macciotta et al., 2012).

Conclusions

In this experiment a good agreement between actual and predicted milk yield was achieved using a current model for dairy cows in mid-late lactation fed a corn-silage and cereal based diet with 150 g CP/kg DM that consumed 21.6 kg/d of DM and produced 28 kg/d of milk. Such agreement disappeared when the dietary CP content was reduced to 123 g/kg. Such reduction had no effect on milk yield/DMI ratio and increased ENU from 0.31 to 0.36, with some reduction of DMI, milk yield and milk quality. The dietary CP reduction decreased N intake of 122 g/d and N in milk by only 14 g/d, did not influenced fecal N excretion, and in appearance did not increase the estimated N mobilization from body tissues, so that the estimated urinary N excretion were reduced. Nitrogen supply was likely in excess with the conventional CP diet, and slightly below the requirements with the low-protein diet, so that use of an intermediate protein diets would presumably yield favorable results both from the environmental and, hopefully, from the economical points of view.

Because the short term nature of this experiment no conclusive affirmations can be drawn about the effects suboptimal CP supply on the mobilization of labile body protein reserves in support to lactation or on the reduction in body protein deposition. The use of rpCLA to enhance N efficiency for milk production remains controversial as with rpCLA ENU tended to increase with CP15 and to decrease with CP12. Results of this study would be useful to improve the efficient use of protein sources and to reduce the environmental impact of dairy industry, but need long term confirmation.

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CONTRIBUTE 3

Fecal sample preparation methods for gas chromatography analysis of fatty acids of ruminants fed different amounts of rumen protected conjugated linoleic acids (CLA)

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Abstract

Aim of this study was to compare three methods for determining fatty acid (FA) profiles in ruminant feces by GC. The first method (J) was based on a mild acid-base treatment directly performed on the dry fecal samples, completing in one step hydrolysis, extraction and methylation of FA. The second method (J_{EE}) was based on acid hydrolysis followed by an accelerated solvent extraction (ASE) of ether extract (EE) and by a mild acid-base catalyzed methylation of FA. The third method (C_{EE}) was based on an acid hydrolysis followed by ASE of EE and by an acid catalyzed methylation of FA (C_{EE}). The experimental design involved the fecal samples of 9 bulls fed a total mixed ration supplemented with 0, 8 or 80 g/d of rumen protected CLA (rpCLA; 3 bulls/dose). Feces collected from these bulls were analyzed, by GC, in triplicates by each method expressing FA contents as mg/g DM. The repeatability of FA and CLA measurements of each method was determined. For the content of CLA isomers, the methods presented heteroscedastic residual variances and, thus, were compared by linear regression. Within method, fecal contents of CLA were regressed against the rpCLA dose. The F-testwas employed to test the significance of any slope that deviated from unity and any intercept that was different from zero. There were no differences among methods for the total amount of FA extracted, which averaged 24.55 mg/g DM. The J method was the most repeatable method for most single FA, and for the sums of SFA, MUFA and PUFA. The two EE-methods evidenced for C18:2*c*9,*t*11 CLA and C18:2*t*10,*c*12 CLA linear relationships with slopes and intercepts close to 1 and 0, respectively, whereas the relationships of J_{EE} and C_{EE} with J had slopes lower than unity. With increasing rpCLA dosage the EE-based methods provided lower increase of fecal contents of C18:2*c*9,*t*11 CLA and of C18:2*t*10,*c*12 CLA isomers and a higher C18:2*t*9,*t*11 CLA content than J, probably as a result of a modification of cis-trans isomerism caused by acid hydrolysis and ASE. The J method should be preferred as it provides more repeatable measures of the fecal FA profiles and because it causes a lower shift in CLA isomer composition with respect to procedures based on acid hydrolysis and ASE.

Keywords: conjugated linoleic acids, fatty acid methyl esters, feces,gas chromatography, repeatability, ruminants

Abbreviations: ASE, accelerated solvent extraction; CLA, conjugated linoleic acid; CP, crude protein; DF, degrees of freedom; DM, dry matter; EE, ether extract; FA, fatty acids; FAME, fatty acids methyl esters GC, gas chromatography; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; rpCLA, rumen protected CLA; RT, coefficient of repeatability; SFA, saturated fatty acids.

Introduction

Ether extraction is the classical method for lipid determinationof feeds and feces (Palmquist and Jenkins, 2003). Cold extraction methods, using different organic solvents to quantify the ether extract (EE) contents of feeds and digesta samples, are time consuming and often uneconomical (Sukhija and Palmquist, 1988). As feces are rich in soaps an acid hydrolysis need to be done before the solvent extraction step (Palmquist and Jenkins, 2003). To reduce the analytical times, the labor, the use of solvent and the analytical cost the solvent extraction can be automatically completed with an accelerated solvent extraction technique (ASE). Schafer (1998) evidenced as the content of FA extracted with ASE from muscle matrices was similar or better in comparison to the conventional extraction according to Folch et al. (1957).

Lipid content estimated by solvent extraction procedures is not precisely related to the nutritive value of the analyzed sample because non-nutritive waxes and pigments are also extracted, while soap are not extracted without an acid digestion, leading to inaccurate estimation of the nutrients content of feed and feces. Total and single FA contents, rather than the EE content of the sample are preferred information in studying the digestive utilization of lipids (Palmquist and Jenkins, 2003), particularly in the case of fat supplemented diets. With GC analysis the determination of the FA contents requires a methylation treatment to convert FA into fatty acid methyl esters (FAME), which can be conducted either on the collected EE or directly on the dried sample by *in situ* techniques (Sukhija and Palmquist, 1988; Carrapiso and Garcia, 2000).

The use of rumen protected conjugated linoleic acids (rpCLA) as additive for ruminant and the analytical quantification of CLA in biological samples gained interest in the last decade (Medeiros et al., 2010). In dairy cattle a supplementation of rpCLA markedly reduces milk fat and increase milk CLA contents (Bauman et al., 2008). Von Soosten et al. (2012) also suggested that rpCLA supplementation could exert a protective effect against excessive use of protein and fat body reserves of dairy cows within 42 days in milk. Feeding of CLA

supplements during early lactation may also improve the reproductive performance of dairy cows (de Veth et al., 2009). In beef cattle a supplementation of rpCLA was found to increase meat CLA content (Gillis et al., 2007; Schiavon et al., 2010), feed efficiency (Dal Maso et al., 2009; Schiavon et al., 2010; Schiavon and Bittante, 2012) and N efficiency (Schiavon et al., 2012).

The CLA isomers have considerable effects in biological systems at low concentrations (Pariza et al., 2001; Pariza, 2004; Bhattacharya et al., 2006) and the quantification of CLA isomers is also complicated by their unstable nature, due to the presence of unsaturated double bonds which makes these isomers easily subjected to epimerization and isomerisation(Park et al., 2002; Jenkins and Lee, 2007; Nuernberg et al., 2007). Methylation was found to be a critical step for a contextual determination of FA and CLA contents in various lipid samples (Park et al., 2002). Base catalysts avoid migration and isomerisation of double bonds but do not esterify free FA (Kramer et al., 1997). On the opposite, acid catalysis esterifies all complex and simple forms of FA but may cause isomerisation of conjugated double bonds (Kramer et al., 1997; Christie et al., 2007; Jenkins, 2010).

A number of comparisons among GC methods for measuring FA profiles have been published, but these very rarely regarded feces (Outen et al., 1976; Sukhija and Palmquist, 1988, Cesaro et al., 2010). To date, most comparisons performed on tissues, blood, milk and meat products (Kramer et al. 1997;Yamasaky et al. 1999; Fritsche et al. 2000; Park et al. 2002; Aldai et al., 2005,Ficarra et al., 2010) were focused to evaluate the effects of different methylation procedures and reagents, but these treatments were done after that fat fractions were extracted from the matrixes. Thus, a major emphasis was put on methylation process and less on the extraction step. Fecal matrix differs substantially from other biological matrixes. As feces contains soaps, the acid hydrolysis commonly used for the extraction of FA, and physical conditions applied to accelerate the extraction such as ASE, would be responsible of an alteration of the FA profile even before the methylation step, irrespectively of the acid or alkaline nature of the reagents involved in the methylation step. This hypothesis could be tested against the method recently proposed by Jenkins (2010) that was specifically developed to operate a contextual hydrolysis extraction and methylation of FA with a mild acid-base treatment that prevent the isomerization of CLA.

Thus, the aims of this work were: i) to evaluate if and to what extent, an extraction of FA from faeces conducted under acid-ASE conditions influences the FA profiles with respect

to the one step mild acid-base treatment proposed by Jenkins (2010); ii) to verify if after the acid-ASE extraction of EE from the fecal matrix the acid or the mild acid-base methylation treatment influence the resulting FA profiles of feces; and iii) to compare these procedures in terms of repeatability.

Materials and Methods

All experimental procedures were approved by the Ethical Committee for the Care and Use of Experimental Animals of the University of Padova (CEASA, Legnaro, Italy).

Animals, diets and fecal collection

Fifty-four crossbred young bulls and heifers were fed *ad libitum* a total mixed ration containing 108 g/kg DM of CP, 35 g/kg of FA and supplemented with 0, 8 or 80 g/d of rumen protected CLA (rpCLA) from 5 to 16 months of age (18 animals for each rpCLA dose) and they consumed 9.3 kg/d of DM on average. The total mixed ration was composed, on DM basis, of corn meal (400 g/kg), corn silage (276 g/kg), soybean meal (33 g/kg), dried sugar beet pulp (113 g/kg), wheat bran (70 g/kg), wheat straw (66 g/kg), vitamin and mineral mixture (26 g/kg), calcium soap (9 g/kg), and hydrogenated soybean oil (7 g/kg). The rpCLA supplement consisted of methyl esters of CLA bound to a silica matrix and coated with hydrogenated soybean oil. The lipid-coated rpCLA was composed of 800, 178, and 22 g/kg of lipid, ash, and moisture, respectively, and 456 g/kg of palmitic and stearic acids, 79.2 and 76.8 g/kg of C18:2*c*9,*t*11 and C18:2*t*10,*c*12, respectively, and 91 g/kg of other FA. A detailed description of the chemical composition of the rpCLA used is given in (Schiavon et al., 2010). Fecal grab samples were collected from all the bulls after 180 days on feed. The feces collected from each bull were oven dried at 55°C for 48 h and left at room temperature for 12 h, finely ground (1 mm screen) and stored at 4°C.

Sample preparation methods

Before GC analysis the individual fecal samples were processed with three different methods with three independent replications (3 rpCLA doses × 3 bulls × 3 methods × 3 replications).

The first method (J) was based on a one step mild acid-base methylation treatment performed on 500 mg of dried fecal samples according to (Jenkins, 2010). Two mL of sodium methoxide (0.5 *M* in methanol) and 2 mL of toluene, containing 2 mg/mL of methyl 12-

tridecenoate as internal standard (# U-35M, Nu-chek prep inc., MN, USA), were added to the sample placed in a culture tube. The sample was incubated in a 50°C water bath for 10 min, removed from the bath and cooled for 5 min. After the addition of 3 mL of freshly prepared methanolicHCl (1.37 *M*) the sample was incubated again in an 80°C water bath for 10 min, removed from the bath and cooled for 7 min. Thereafter, 5 mL of K_2CO_3 (0.43 *M*) and 2 mL of toluene were added to each tube. The tube was vortexed for 30 seconds and centrifugated for 5 min at 400 *g* and 4°C. The organic phase (the upper layer) of the tube was transferred into a screw-capped tube, and 0.5 g of anhydrous sodium sulphate and 0.5 g of active charcoal (Sigma-Aldrich, MO, USA) were added. The solution was vortexed for 5 min and rested for 1 h. After centrifugation for 5 min at 400 *g* and 4°C the clear upper layer containing the FAME was transferred in a GC vial and stored at -20°C prior the GC analysis.

The second method (J_{EE}) involved an acid hydrolysis, followed by a petroleum ether extraction performed with ASE, and a mild acid-base methylation performed as described for J. Acid hydrolysis was performed according to the official method, procedure B, of the European Communities for the determination of fats in feedstuffs (EC, 1998). Three grams of oven dried feces were accurately weighed and transferred into 250 mL round-bottomed Kjeldahl tubes with 2.5 g of diatomaceous earth celite (#545, Thermo Fisher Scientific Inc., MA, USA), boiled in 150 mL of HCl (3N) for 60 min using an hydrolyzing unit (Soxtec System Tecator, Thermo Fisher Scientific Inc., MA, USA). The sample was filtrated into extraction thimbles with a pore size of 16 to 40 µm (#20693, RobuGlasfilter-Geräte GmbH, Hattert, Germany), washed with de-ionized water until neutral reaction revealed by a litmus paper, and oven dried at 60°C for one night. The hydrolyzed feces were transferred into 10 mL stainless steel extraction cells for ASE (Dionex ASE 350, Thermo Fisher Scientific Inc., MA, USA) with petroleum ether as solvent as described by (Schafer, 1998). The conditions of extraction were: 105°C of temperature, 10 MPa of pressure, 1 min of static time, 3 static cycles, with about 8 mL/sample of re-flushing volume of fresh solvent (giving a total solvent consumption of <20 mL/sample). Thereafter, the solution was heated at 50°C under N_2 stream for about 20 min to complete solvent evaporation, placed in an oven at 60°C for 15 min, cooled in a desiccator and the resulting EE was weighted. The methylation was performed on 40 mg of EE according to (Jenkins, 2010), as previously described. The resulting FAME solution was stored GC vials at -20°C prior the analysis.

With the third method (C_{EE}) each sample was subjected to an acid-ether extraction, completed as described for J_{EE}, and to an acid methylation completed according to Christie

(1993) with slight modifications. The methylation was performed using 40 mg of EE placed into culture tubes with 1 mL of methanolic H_2SO_4 (0.20 *M*). The tubes were placed in a oven (Verderi, Padova, Italy) at 105°C for 60 min and agitated by hand every 10 min. To each sample, after 10 min of cooling at ambient temperature, 2 mL of *n*-heptane, and containing 0.6 mg/mL of methyl 12-tridecenoate as internal standard, was added. After the addition of 2 mL of deionized water the sample was centrifugated for 5 min at 400 *g*, the clear upper layer containing the FAME was separated and transferred into a vial for the GC analysis.

Gas chromatography analysis

The samples obtained with the 3 different methods were analyzed for their FA profile using a GC (Agilent Technologies 7890 A, CA, USA) with two columns in series, equipped with a modulator (Agilent G3486A CFT, CA, USA), an automatic sampler (Agilent 7693, CA, USA) and a flame ionization detector connected with a chromatography data system software (Agilent ChemStation, CA, USA). This instrument was used because the use of a double column allows a much better separation and identification of fatty acids, on a three-dimensional basis, compared to the traditional one column GC. Between the two columns the modulator unit collects in a fixed volume channel the analyte bands of the first column and these are successively launched into the short second column in narrow bands. The operative conditions of the GC apparatus were: first column of 60 m × 180 µm (internal diameter) × 0.2 µm of film thickness (Agilent custom HP88, CA, USA), H_2 carrier flow of 0.2 mL/min increased to 0.3 mL/min at a rate of 0.003 mL/min;; second column of 5 m × 250 µm of internal diameter × 0.15 µm of film thickness (Agilent HP-50$^+$, CA, USA), H_2 carrier flow of 24 mL/min held for 58 min and then increased to 25 mL/min at a rate of 0.1 mL/min. Planned oven temperature variation: increase from 120°C (held for 5 min) to 150°C (held for 20 min) at 8°C/min and then increased to 240°C (held for 20 min) at 2°C/min. Valves: modulation delay, 1 min; modulation period, 3 sec; sample time, 2.85 sec. Gas flows: hydrogen, 20 mL/min; air, 450 mL/min. Sample injection: 0.8 µL (pulsed split mode, injection pressure 0.172 MPa × 0.3 min, split ratio 150:1). The resulting three-dimensional chromatograms were analyzed with the comprensive GC × GC software (Zoex Corp., TX, USA) to evaluate the volumes of each fatty acid cone. Fatty acids were identified by comparison of the cone position in the chromatogram with the cone position of FA presents in a GC reference standard (#674, Nu-chek prep inc., MN, USA), which was a mixture of 52 pure FA, and in C18:2*c*9,*t*11 (#UC-60M, Nu-chek prep inc., MN, USA) and C18:2*t*10,*c*12 CLA standards

(#UC-61M, Nu-Chek prep, inc. MN, USA). Identification of the C18:2*t*9,*t*11 isomer was made from the elution order compared with those of previous published FA profile (Kramer et al., 1997; Park et al., 2002).The proportion of each single FA present in the sample was computed as the ratio between each single FA cone volume and the total FA cones volume. Thereafter, the fecal content in mg/g DM of each FA present in the feces was computed as the ratio between the cone volume of the FA of interest and that of the internal standard multiplied by the ratio between the internal standard weight (mg) and DM sample weight (g).

Saturated fatty acids (SFA) category were the sum of: C6:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0 (C14:0 *iso*), C15:0 (C15:0 *iso*, C15:0 *anteiso*), C16:0 (C16:0 *iso*), C17:0 (C17:0 *iso*, C17:0 *anteiso*), C18:0 (C18:0 *iso*), C19:0, C20:0, C21:0, C22:0, C23:0 and C24:0. Monounsaturated fatty acids (MUFA) were the sum of C14:1, C15:1, C16:1 (C16:1 n-7, C16:1 n-9), C17:1 (C17:1 n-7), C18:1 (C18:1 n-3, C18:1 n-7, C18:1 n-9), C19:1 (C19:1 n-2, C19:1 n-9), C20:1 (C20:1 n-7, C20:1 n-9, C20:1 n-11), C22:1 (C22:1 n-9, C22:1 n-11), C24:1. Polyunsaturated fatty acids (PUFA) were the sum of C16:2, C18:2 (C18:2 n-6), C20:2 (C20:2 n-6), C18:3 (C18:3 n-3, C18:3 n-6), C20:3 (C20:3 n-3, C20:3 n-6), C16:4, C18:4 (C18:4 n-3), C20:4 (C20:4 n-6); the identified CLA isomers were: C18:2*c*9,*t*11C18:2*t*10,*c*12 and C18:2*t*9,*t*11. The sum of the identified CLA isomers was indicated as ∑CLA. This work paid particular attention to the determination of only the major mentioned CLA isomers which were included in increasing amounts in the bull rations. No attempts were done to separate and to identify other CLA isomers, such as the C18:2*t*7,*c*9 and the C18:2*t*11,*c*13 which have been found in beef meat (Nuernberg et al., 2007).

Statistical analysis

A prospective power analysis was conducted to evaluate the sample size required to detect a treatment effect at a predetermined level of significance (Richardson et al., 2004). In the case of the CLA isomers, assuming a coefficient of variation within method of 0.25, a level of significance P of 0.05 and a power of 0.90 it was computed that to detect relative differences of ±17% in the analytical measurements 27 observations for each method were required (Lerman, 1996). Therefore, 27 fecal samples from 9 randomly selected bulls, with 3 bulls for each rpCLA dose, were used in the current test.

Estimation of Repeatability of FA and FA category

Estimation of variance components was accomplished, separately for FA or FA category determined by different methods, using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA), with data previously tested for normality by using the Shapiro-Wilk statistic (Shapiro and Wilk, 1965). The rpCLA dose (CLA) and the bull within rpCLA dose (Bull), as well as residuals, were found to be independently and normally distributed with a mean zero and variance σ^2_{CLA}, σ^2_{Bull}, and σ^2_e, respectively. To evaluate the repeatability of measures of FA and FA category (n=27), the coefficient of repeatability (RT), which is an indicator of the degree of agreement between repeated measures performed with the same method by the same operator on the same sample within a short period of time, was determined according to the International Organization for Standardization (1994 a, b).

This parameter was computed as: $RT = \frac{\sigma^2_{CLA} + \sigma^2_{Bull}}{\sigma^2_{CLA} + \sigma^2_{Bull} + \sigma^2_e}$

where: σ^2_{CLA} is the variance due to the rpCLA dose, σ^2_{Bull} is the variance due to the bull (within rpCLA dose) and σ^2_e is the residual error.

Sources of variation

The FA measurements and FA sum categories obtained from the three methods for each FA (n=81) were tested for variances homogeneity using Levene's test (Milliken and Johnson, 1984). As for the large majority of FA the residual variances among methods were homoscedastic, the analytical results of three methods were analyzed together with a hierarchical linear mixed model, using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA),which considered method (2 df), rpCLA dose (2 df) and method × rpCLA dose interaction (4 df) as fixed effects, and bull within rpCLA dose (2 df) as random effect. The effects of rpCLA dose and of method were tested using as error line the bull within rpCLA dose and the residual error of the model, respectively. Orthogonal contrasts were run to test: the effects due to the solvent extraction method (J vs. J_{EE} and C_{EE}), and to the kind of methylation (J_{EE} vs. C_{EE}), both tested on the residual variance, and the linear effect of rpCLA dose (80 vs. 0 g/d) and the deviation from linearity due to rpCLA dose, both tested on bull within CLA dose variance. Deviation from linearity was computed by comparing the analytical value obtained with 8 g/d of CLA against a weighted value resulting from the following relationship: 0.9 × 0 g/d of rpCLA + 0.1 × 80 g/d of rpCLA.

For those FA where the variances among methods were heteroscedastic, CLA isomers in particular, methods were compared by linear regression (SAS Institute Inc., Cary, NC, USA) by averaging the three replications available for each bull (n=9) and testing the differences of the intercept and of the slope from zero and unity, respectively.

Results

In the current experiment the three methods were analyzed for their variance components and repeatability and they were compared for their mean analytical responses. The number of observations was adequate to evidence differences due to the methods and to the rpCLA dose, particularly for the CLA isomers.

Repeatability of the methods

Overall, the J method showed, for almost all FA and their sums, higher repeatability compared to J_{EE} and C_{EE} (Table 1). The J_{EE} method showed the highest repeatability for the branched isomers of C16:0 and C17:0, for the SFA with a high number of C (C20:0, C22:0, and C24:0) and for the C18:3 n-3. The C_{EE} method yielded the highest repeatability in the case of the C18:2*c*9,*t*11 and C18:2*t*9,*t*11 CLA isomers.

The residual variances of the three methods were homoscedastic for almost all the FA, with some exceptions, notably for C18:2*c*9,*t*11 and C18:2*t*10,*c*12 CLA isomers and ∑CLA. For these isomers all the variances of the random factors included in the model of analysis (dose of CLA, animal within dose, and the error) were influenced by the method (data not shown) and their sum was always greater for J compared to J_{EE} and C_{EE} (i.e. for ∑CLA the values of this sum were 0.0254, 0.0046 and 0.0059 for J, J_{EE} and C_{EE}, respectively), so that differences of repeatability were caused not only by variation in the residual variance but also in the other variances.

Table 1. Coefficient of repeatability of measurements (mg/g DM) obtained by 3 different methods[1,2] of fatty acids analysis and homogeneity of the variances among methods evaluated by the Levene's test.

	Method of Analysis			Levene Test
Fatty Acids (FA)	J	J_{EE}	C_{EE}	P
∑ FA content	0.983	0.975	0.899	0.24
C14:0	0.992	0.988	0.955	0.40
C15:0 *iso*	0.892	0.453	0.823	0.019
C15:0 *anteiso*	0.976	0.533	0.868	0.64
C15:0	0.996	0.843	0.960	0.24
C16:0 *iso*	0.987	0.999	0.943	0.62
C16:0	0.975	0.957	0.807	0.40
C17:0 *iso*	0.988	0.989	0.967	0.34
C17:0 *anteiso*	0.987	0.971	0.934	0.80
C17:0	0.995	0.991	0.971	0.34
C18:0	0.989	0.984	0.946	0.23
C20:0	0.836	0.919	0.897	0.11
C22:0	0.696	0.957	0.894	<0.001
C24:0	0.739	0.890	0.782	0.25
C18:1 n-7	0.982	0.946	0.903	0.08
C18:1 n-9	0.913	0.872	0.783	0.98
C18:2 n-6	0.972	0.972	0.903	0.42
C18:2*c*9,*t*11	0.948	0.909	0.968	<0.001
C18:2*t*10,*c*12	0.993	0.950	0.980	<0.001
C18:2*t*9,*t*11	0.902	0.944	0.947	0.16
∑ CLA	0.988	0.970	0.987	<0.001
C18:3 n-3	0.436	0.897	0.782	0.24
∑ identified FA	0.982	0.979	0.903	0.22
∑ others FA	0.957	0.303	0.769	0.38
SFA	0.986	0.979	0.919	0.24
MUFA	0.964	0.887	0.802	0.57
PUFA	0.966	0.963	0.873	0.87

[1]J = method based on a direct acid-base hydrolysis and on methylation of fecal fatty acids (FA) according to Jenkins (2010); J_{EE} = method based on an acid hydrolysis, on accelerated solvent extraction (ASE) and on a mild acid-base methylation of FA according to Jenkins (2010); C_{EE} = method based on acid hydrolysis, on accelerated solvent extraction (ASE) and on acid methylation of FA according to Christie (1993).
[2] For each method number of analyses were 3 doses of rpCLA (0, 8 and 80 g/d) × 3 bulls × 3 replications (n=27).

Sources of variations of fecal FA profile and CLA contents

With J_{EE} and C_{EE} the total FA content of the EE extracted from the feces did not differed being on average 512±49.9 and 490±60.5 mg/g (data not shown), but the individual values ranged from 382 to 685 mg/g. Total fecal FA content expressed as gram of fecal DM was not influenced by method, rpCLA dose and their interaction (Table 2). Fatty acids content was not influenced by the solvent extraction (J vs. J_{EE} and C_{EE}), by the kind of methylation (J_{EE} vs.C_{EE}) and by the linear and not linear effects of the rpCLA dose. The same was observed for the main FA categories, namely SFA and MUFA. However, method influenced almost all the single FA and PUFA. For almost all the single FA the differences among methods were explained especially by the effect due to the acid solvent extraction step (J vs. J_{EE} + C_{EE}) and not to the methylation one (J_{EE}vs. C_{EE}).

The effects due to the rpCLA dose were in general not significant, with exception for single CLA isomers and total CLA. The effect of rpCLA dose was linear (80 g/d vs. 0 g/d), without deviation from linearity. The interaction method × rpCLA dose was significant for CLA isomers and their sum, but also for the C17:0 *anteiso*, the unidentified FA and the PUFA.

In general, despite the significant effect of extraction and methylation methods, the average fecal content of the various FA achieved with the three methods was not much different, as the maximum difference remained lower than 17% for almost all FA and the three FA categories (Table 3). Slightly higher differences were found for C14:0 and C18:1n-7, and much higher differences (between 42 and 90%) were found for all isomers of CLA, for C15:0 *iso*, C24:0 and the sum of non-identified FA. However, the sum of the various CLA isomers was similar among methods. With the J method the mean contents of C18:2*c*9,*t*11 CLA and of C18:2*t*10,*c*12 CLA were much greater than those achieved with J_{EE} and C_{EE}, whereas the opposite was observed for C18:2*t*9,*t*11 CLA.

Table 2. Effects (P-values) on fecal fatty acids contents (FA) due to: method (M)[1] of analysis, rumen protected CLA (rpCLA) dose[2], M × rpCLA interaction, and of the contrasts between methods based on a prior solvent extraction step or not (EE vs. J)[3], between different methylation methods after acid hydrolysis and ASE (J_{EE} vs.C_{EE}), between the highest and the lowest rpCLA dose (L: linear contrast between 0 vs. 80 g/d) and between the weighed intermediate dose respect the other two doses (Δ L: deviation from linearity).

Fatty Acids, mg/g DM	Main factors			Contrasts			
	M	rpCLA	M×rpCLA	M		rpCLA dose[4]	
				EEvs. J	J_{EE} vs.C_{EE}	L	Δ L
∑ FA content	0.33	0.41	0.14	0.87	0.15	0.20	0.67
C14:0	0.002	0.72	0.10	0.002	0.015	0.66	0.67
C15:0 *iso*	0.06	0.52	0.19	0.037	0.20	0.42	0.69
C15:0 *anteiso*	0.011	0.55	0.13	0.015	0.037	0.58	0.30
C15:0	0.005	0.44	0.39	0.001	0.86	0.27	0.31
C16:0 *iso*	0.011	0.57	0.16	0.004	0.33	0.49	0.67
C16:0	<0.001	0.82	0.08	<0.001	0.89	0.58	0.98
C17:0 *iso*	0.16	0.66	0.15	0.06	0.66	0.73	0.39
C17:0 *anteiso*	0.46	0.37	0.031	0.30	0.49	0.52	0.18
C17:0	0.003	0.71	0.20	<0.001	0.53	0.84	0.43
C18:0	0.010	0.29	0.13	0.036	0.014	0.13	0.54
C20:0	0.002	0.49	0.13	0.001	0.07	0.25	0.69
C22:0	0.034	0.32	0.07	0.75	0.011	0.15	0.52
C24:0	<0.001	0.86	0.31	<0.001	0.15	0.60	0.74
C18:1 n-7	<0.001	0.59	0.20	<0.001	0.83	0.34	0.86
C18:1 n-9	0.005	0.51	0.10	0.003	0.15	0.33	0.35
C18:2 n-6	<0.001	0.82	0.16	<0.001	0.99	0.79	0.55
C18:2*c*9,*t*11	<0.001	0.015	<0.001	<0.001	0.93	0.008	1.00
C18:2*t*10,*c*12	0.002	0.007	<0.001	<0.001	0.52	0.004	0.93
C18:2*t*9,*t*11	<0.001	0.004	0.048	<0.001	0.89	0.002	0.88
∑ CLA	0.31	0.004	<0.001	0.16	0.60	0.002	0.97
C18:3 n-3	0.003	0.79	0.15	<0.001	0.72	0.66	0.53
∑ identified FA	0.26	0.39	0.14	0.73	0.11	0.19	0.66
∑ others FA	<0.001	0.96	0.009	<0.001	0.007	0.82	0.83
SFA	0.21	0.43	0.15	0.81	0.09	0.22	0.72
MUFA	0.49	0.53	0.14	0.73	0.26	0.29	0.50
PUFA	0.001	0.78	0.023	<0.001	0.77	0.54	0.59

[1]J = method based on a direct acid-base hydrolysis and methylation of fecal fatty acids (FA) according to Jenkins (2010); J_{EE} = method based on an acid hydrolysis, accelerated solvent extraction (ASE) and a mild acid-base methylation of FA according to Jenkins (2010); C_{EE} = method based on acid hydrolysis, accelerated solvent extraction (ASE) and an acid methylation of FA according to Christie (1993). Data were about 3 methods × 3 doses of rpCLA × 3 bulls, with 3 replications (n=81). The effects of method (2 df) and rpCLA (2 df) were tested on animal within rpCLA as error term (6 df).
[2]Doses of rpCLA were 0, 8 and 80 g/d.
[3]The method J was compared against the 2 methods based on acid hydrolysis and ASE (J_{EE} and C_{EE}).
[4]Deviation from linearity was computed by comparing the analytical result obtained with 8 g/d of CLA versus a weighted value resulting from the following relationship: 0.9 × 0 g/d of rpCLA + 0.1 × 80 g/d of rpCLA.

Table 3. Least square means and SEM due to the 3 different methods[1,2] of FA analysis and to three increasing doses of rumen protected CLA (rpCLA).

	Method[2]				rpCLA			
Fatty Acids, mg/g	J[3]	J_{EE}[4]	C_{EE}	SEM	0 g/d[5]	8 g/d[6]	80 g/d	SEM
∑ FA content	24.490	25.130	24.050	2.5004	20.5077	23.9675	29.1948	4.2745
C14:0	0.352**	0.387*	0.437	0.0607	0.388	0.332	0.455	0.1036
C15:0 *iso*	0.107*	0.168	0.137	0.0198	0.131	0.119	0.163	0.0262
C15:0 *anteiso*	0.463*	0.493*	0.550	0.0457	0.564	0.441	0.501	0.0754
C15:0	0.567**	0.482	0.486	0.0687	0.642	0.449	0.443	0.1168
C16:0 *iso*	0.146**	0.161	0.167	0.0266	0.166	0.189	0.119	0.0457
C16:0	3.675**	4.124	4.138	0.3190	3.821	3.851	4.265	0.5435
C17:0 *iso*	0.214	0.199	0.202	0.0323	0.239	0.166	0.210	0.0554
C17:0 *anteiso*	0.284	0.288	0.293	0.0301	0.342	0.231	0.292	0.0517
C17:0	0.356**	0.311	0.302	0.0565	0.370	0.257	0.341	0.0971
C18:0	13.224*	12.965*	11.710	1.8265	8.894	12.398	16.607	3.1336
C20:0	0.215**	0.195	0.180	0.0216	0.166	0.193	0.231	0.0366
C22:0	0.144	0.156*	0.136	0.0120	0.122	0.145	0.169	0.0198
C24:0	0.127**	0.196	0.185	0.0198	0.155	0.173	0.181	0.0334
C18:1 n-7	1.017**	0.812	0.805	0.0941	0.779	0.841	1.014	0.1599
C18:1 n-9	1.576**	1.664	1.717	0.0931	1.495	1.731	1.730	0.1574
C18:2 n-6	1.369**	1.627	1.628	0.1525	1.430	1.661	1.534	0.2597
C18:2*c*9,*t*11	0.064**	0.046	0.045	0.0048	0.036**	0.041	0.079	0.0077
C18:2*t*10,*c*12	0.048**	0.030	0.026	0.0082	0.004**	0.010	0.090	0.0133
C18:2*t*9,*t*11	0.031**	0.059	0.059	0.0037	0.034**	0.039	0.076	0.0057
∑ CLA	0.143	0.135	0.131	0.0145	0.074**	0.090	0.245	0.0240
C18:3 n-3	0.108**	0.097	0.096	0.0060	0.095	0.105	0.102	0.0100
∑ identified FA	24.089	24.459	23.300	2.4371	19.872	23.374	28.601	4.1659
∑ others FA	0.401**	0.672**	0.750	0.0742	0.635	0.593	0.594	0.1263
SFA	20.012	20.456	19.312	2.3830	16.324	19.205	24.252	4.0820
MUFA	2.756	2.737	2.816	0.1860	2.479	2.834	2.997	0.3153
PUFA	1.721**	1.937	1.922	0.1554	1.704	1.929	1.946	0.2646

[1] J = method based on a direct acid-base hydrolysis and methylation of fecal fatty acids (FA) according to Jenkins (2010); J_{EE} = method based on an acid hydrolysis, accelerated solvent extraction (ASE) and a mild acid-base methylation of FA according to Jenkins (2010); C_{EE} = method based on acid hydrolysis, accelerated solvent extraction (ASE) and an acid methylation of FA according to Christie (1993).

[2] For each method data were about 3 bulls × 3 doses of rpCLA (0, 8 and 80 g/d) × 3 replications (n=27).

[3] *P<0.05 J vs. (J_{EE} + C_{EE}); **P<0.01 J vs. (J_{EE} + C_{EE});

[4] *P<0.05 J_{EE} vs. C_{EE}; **P<0.01 J_{EE} vs. C_{EE};

[5] *P<0.05 0 g/d vs. 80 g/d of rpCLA; **P<0.01 for 0 g/d vs. 80 g/d of rpCLA;

[6] *P<0.05 8 g/d vs. (0.9 × 0 g/d + 0.1 × 80 g/d) of rpCLA; **P<0.01 8 g/d vs. (0.9 × 0 g/d + 0.1 × 80 g/d) of rpCLA.

Relationships among sample preparation methods

The regression analysis (Table 4) was carried out for the various CLA isomers and their sum because of the heteroscedasticity of their residual variances across methods. In general, there were close relationships between J_{EE} and C_{EE}, methods with slopes and intercepts close to the expected values of 1 and zero, respectively. Differently, when the J_{EE} and the C_{EE} values were regressed against those of J, intercepts greater than zero and slopes lower than one were observed.

Analytical responses with increasing rpCLA dose

The three methods responded differently to increasing rpCLA doses. Increasing rpCLA intakes by young bulls where better reflected by increasing fecal concentrations of C18:2*c*9,*t*11 CLA and C18:2*t*10,*c*12 CLA when measured with J than with J_{EE} and C_{EE} (Figure 1a, b), whereas the fecal content of C18:2*t*9,*t*11 CLA, that was not supplemented to animals, was more constant and lower with J than with J_{EE} and C_{EE} (Figure 1c).

Table 4. Intercepts (significance of being different from 0.00), slopes (significance of being different from 1.00) and R^2 of the regressions between methods[1] of analysis of CLA isomers contents in feces[2, 3].

	J vs.J_{EE}			J vs. C_{EE}			J_{EE}vs. C_{EE}		
Fatty	Interc	Slope	R^2	Interc	Slope	R^2	Interc	Slop	R^2
C18:2*c*9,*t*	0.029*	0.264*	0.847*	0.027*	0.284*	0.918*	-0.001	1.01	0.962*
C18:2*t*10,	0.009*	0.420*	0.989*	0.004	0.469*	0.988*	-	1.11	0.988*
C18:2*t*9,*t*	0.040*	0.635*	0.724*	0.035*	0.782	0.747*	-	1.19	0.971*
∑ CLA	0.075*	0.422*	0.954*	0.063*	0.478*	0.964*	-0.020	1.11	0.982*

[1]J = method based on a direct acid-base hydrolysis and methylation of fecal fatty acids (FA) according to Jenkins (2010); J_{EE} = method based on an acid hydrolysis, accelerated solvent extraction (ASE) and a mild acid-base methylation of FA according to Jenkins (2010); C_{EE} = method based on acid hydrolysis, accelerated solvent extraction (ASE) and an acid methylation of FA according to Christie (1993).

[2] For each method data regarded 3 bulls × 3 doses of rpCLA (0, 8 and 80 g/d), the 3 replications available for each bull were averaged (n=9).

[3] Statistical differences of intercept and slopes from zero and unity, respectively, and of the R^2 of the regression model are evidenced as *** = P<0.001; ** = P<0.01; * = P<0.05.

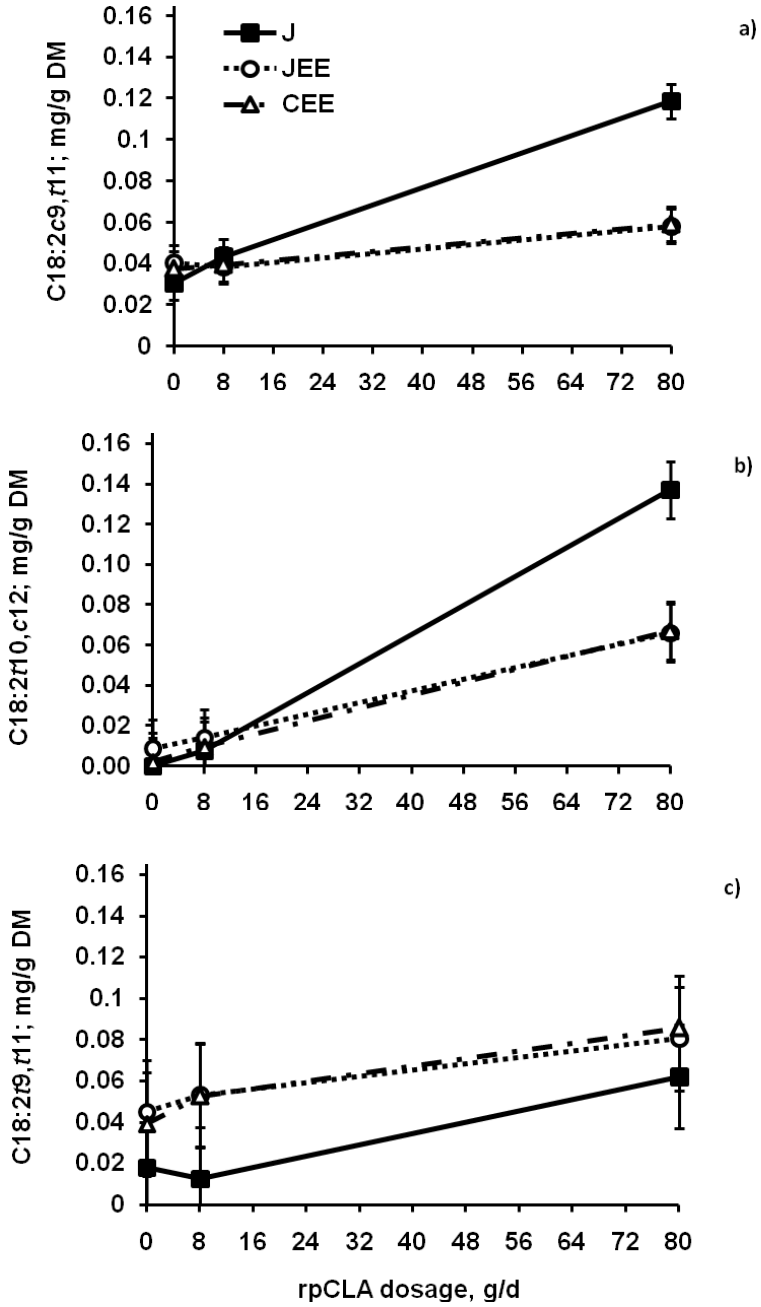

Figure 1. Relationships between the fecal content of C18:2*c*9,*t*11 (**a**), C18:2*t*10,*c*12 (**b**) and C18:2*t*9,*t*11 (**c**) CLA (mg/g DM) measured with different methods: J) based on a direct acid-base extraction and methylation of fecal fatty acids (FA) according to Jenkins (2010); J_{EE}) based on an acid hydrolysis, accelerated solvent extraction (ASE) and an acid-base methylation of FA according to Jenkins (2010), and, C_{EE}) based on acid-hydrolysis, extraction with ASE and an acid methylation of FA according to Christie (1993). [For each methoddata regarded 3 doses of rpCLA (0, 8 and 80 g/d) × 3 bulls × 3 replications, each point is the mean of 3 bulls × 3 replications (n=9).]

Discussion

Fecal fatty acid profiles

In the current experiment the 3 analytical methods compared did not differ significantly for the amount of total FA determined in the feces, whose FA content represented 0.50 of collected EE. The result of the current paper evidences that the Jenkins's mild acid-base treatment does not impair the total quantity of FA extracted with respect to the stronger treatments of acid hydrolysis followed by ASE. The J method is a modification of the Sukhija and Palmquist (1988) one, where the sample incubation in 3 mL of methanolicHCl (1.37 *M*) at 70°C for 2 h is replaced by a digestion with sodium methoxide in methanol for 10 minutes at 50°C, and after cooling and addition of methanolicHCl, for 10 minutes at 80°C. Thus, the major difference between these two methods is a shorter exposure of the feed sample to the acid digestion. Haan et al. (1979) recommended lower temperature during digestion because they anticipated some oxidation of PUFA at high temperature. However, Sukhija and Palmquist (1988) evidenced that no oxidation occurs with purified solvents in the absence of air and that some PUFA may be lost only if the solvent evaporates during the esterification process, so that precautions must be taken to ensure air-tight reaction tubes. When the Sukhija and Palmquist (1988) method was compared with a standard procedure involving acid digestion, extraction and methylation (AOAC# 7.058, 1980) no differences in the total and single FA contents (C14:0, C16:0, C18:0, C18:1, C18:2; C18:3) of calcium soaps where observed (Sukhija and Palmquist, 1988). Similarly, but except for the CLA isomers and C18:1, no differences in the total and single FA contents (C14:0, C16:0, C16:1, C18:0, *trans* C18:1, C18:2, C18:3) were detected when the original Sukhija and Palmquist (1988) and the Jenkins (2010) modified procedures were compared using different fat sources (Jenkins, 2010). Differently, with ASE the non polar fraction is extracted at temperature above the boiling point of the solvent because of the elevated pressure used. Under elevated temperature and pressure dissolved lipids diffuse rapidly from the core to the surface of the sample particles and then are transferred to the extraction solvent. Compressed gas then purge the solubilized fat into a vessel and the collected fat is quantified gravimetrically (Shahidi and Wanasundara, 2002). In agreement with Schafer (1998), Toschi et al. (2003) found a high correlation between the Folch et al. (1957) method and the ASE performed at 120°C and 20 MPa, they also found that the methods were equivalent for the measurements of the FA profile of poultry meat, even if the CLA isomers were not considered.

In the current experiment the three methods compared were not completely equivalent in terms of fatty acid profiles, as they differed for almost all the single FA and for PUFA concentrations and because the measurements provided by J were often more repeatable than those obtained with J_{EE} and C_{EE}. The two EE-based methods, despite their different methylation treatments applied, had small or negligible differences of FA profile among them, but their FA profile differed, to some extent, from that of J. This last method, with respect to the other two, showed higher contents of C18:1n-7 (+26%) and lower contents of C15:0 *iso*(-30%), C24:0 (-33%), and sum of not identified FA (-44%). Anyway, the most notable differences were about the CLA isomers, whereas for all the other FA the differences among methods were lower than 17%.

Fecal CLA content

Oxidation of polyunsaturated FA may be of concern with oven drying of sample, however, as Sukhija and Palmquist (1988) found that freeze dried or oven-dried (55°C) samples gave reproducible results, and because the comparative nature of this research, the use of such samples was considered acceptable.

The two EE-based procedures yielded about the same total CLA content (0.136 mg/g DM) but showed a major shift in isomer composition with respect to J. With both the EE based methods, the C18:2*t*9,*t*11 was the primary CLA isomer at the expense of both the C18:2*c*9,*t*11 and C18:2*t*10,*c*12 CLA isomers, even if the former isomer was not supplemented to the diet. Moreover, the amount of the supplemented CLA isomers (C18:2*c*9,*t*11 and C18:2*t*10,*c*12) detected with J_{EE} and C_{EE} were less rpCLA dose dependent and, on average, about half than those measured with J method, while the opposite occurred for the C18:2*t*9,*t*11 CLA. The lower C18:2*c*9,*t*11 and C18:2*t*10,*c*12 and the higher C18:2*t*9,*t*11 contents found both with J_{EE} and C_{EE} compared to J are likely due the combined effects of the acid hydrolysis and ASE, a set of conditions which have likely promoted a greater isomerization of the bioactive CLA molecules before the methylation step. Increased concentrations of C18:2*t*9,*t*11 are considered, even not exclusively (McIntosh et al., 2009), an artifact due to the isomerisation of CLA which occurs when the samples are subjected to an acid treatment (Kramer et al., 1997; Park et al., 2002).

Although with J the concentration of C18:2*t*9,*t*11 was on average much smaller than that found with the other methods, some degree of CLA isomerisation cannot be excluded, as even with this method the concentration of C18:2*t*9,*t*11 increased linearly with increasing

rpCLA. On the other hand, as FA extraction from feces requires at least a mild acid treatment, some losses of bioactive CLA isomers appear to be unavoidable. However, because the comparative nature of the results of this paper, it cannot be concluded that the absolute values of the various CLA isomers were the true values, as in this case it would have been necessary to use certified standard materials with known contents for all the CLA isomers, which are not available for feces, or to spiking samples with a known CLA isomer concentration and determine recovery rates across the sample preparation and analytical process. Major confirms will be achieved by the use of high resolution GC in combination with a silver ion HPLC technique, to determine all FA including all CLA isomers.

Conclusions

The result of the current paper evidences as the total amount of FA extracted with the Jenkins's mild acid-base treatment does not impair the total amount of FA extracted with respect to the stronger treatments of acid hydrolysis and ASE. The application of a mild acid-base treatment for a contextual hydrolysis, extraction and methylation of FA is recommended for measuring the FA profile and the CLA contents of ruminant feces because, besides its simplicity and the lower labor required, it provides repeatable measures of the FA profile of ruminant feces and because it causes a lower shift in CLA isomer composition with respect to treatments of acid hydrolysis and ASE.

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CONTRIBUTE 4

Energy balance estimated from individual measurements of body weight and backfat thickness of heavy pigs of four genetic lines fed different diets

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Summary

Pigs of four genetic lines (GL): Anas (A), DanBred (D), Goland (G) and Topigs received either a conventional (140 g CP/kg and 46 g lysine/kg CP; C-CP) or a low protein diet (106 g CP/kg and 46 g lysine/kg CP; L-CP). Body weight (BW) and backfat depth (P2) were individually measured at the start and the end of two growing periods and individual feed intake (FI) was recorded daily. Body protein and lipid mass at the start and at the end of each period were estimated from BW and P2, and hence protein (Pr) and lipid (Lr) retention were computed. Energy requirement for maintenance (ME_m), and growth (ME_g) were estimated according to National Research Council guidelines, while ME intake (MEI) was computed from measured FI and ME content of the diets. The MEI/(ME_m + ME_g) ratio was used as index of efficiency. Differences among GL ($P<0.001$) were observed for Pr, which averaged 103, 113, 108 and 101 g/d for A, D, G and T, respectively, and Lr which averaged 204, 186, 194 and 172 g/d for A, D, G, and T, respectively. The L-CP diet reduced ($P = 0.014$) Pr by 8% compared to C-CP, but not Lr. The MEI/(ME_m+ME_g) index was influenced by GL ($P<0.001$) being 0.99, 0.96, 0.99 and 1.03 for A, D, G and T, respectively.

Measurements of BW and P2 permits to achieve acceptable quantification of Pr and Lr. In this range of BW (90 to 165kg), gain composition is influenced more by GL than by the substantial reduction of CP and essential amino acids dietary density used in this trial.

Keywords: Heavy pig, Genetic line, Growth performance, Low-protein diets.

Aim

This work aimed to test the effects of a 25% reduction of dietary crude protein and essential amino acids, with respect to 145 g CP/kg of conventional diets, on growth performance of pigs from 90 to 165 kg BW belonging to four genetic lines and to evaluate the use of simple body measurements (BW and backfat depth) as predictors of body composition, body status changes and energy balance.

Materials and Methods

All experimental procedures were reviewed and approved by the Ethical Committee for the Care and Use of Experimental Animals (CEASA) of Padua University.

Pigs, diets and experimental design

In two batches a total of 184 gilts and barrows were reared at the experimental farm of the University of Padova. The pigs belonged to three genetic lines commonly used in the Italian pig industry for high quality dry-cured ham production (DOP), Anas (A), Goland (G) and Topigs (T), and an additional line the DanBred (D) with an higher potential lean growth compared to the others. Within each batch, the piglets, barrows and gilts equally represented in each genotype and born in the same week, were moved to the experimental farm at an average weight of 30 kg. The pigs were housed in 8 pens (10 to 12 pigs/pen) equipped with a feeding station (Compident Pig – MLP, SchauerAgrotronic, Austria) recording individual daily FI, and raised on a conventional feeding regime till about 80 kg BW. Starting from 80 kg BW, the experimental diets were introduced and all pigs received a restricted feeding regime adjusted every 2 weeks. The amount of pellet diet per pig increased from 2.4 to 3.2 kg/d from the first to the last week on trial, when the pigs were about 90 and 165 kg BW, respectively. During early (90 to 120 kg BW) and late (120 to 165 kg BW) finishing the conventional diet (C-CP) contained 147 and 132 g CP/kg, respectively, whereas the low-protein and low-essential amino acids diet (L-CP) contained 112 and 100 g CP/kg, respectively. Both C-CP and L-CP diets contained 50 and 41 g lysine/kg CP in early and late finishing, respectively. The L-CP diets were formulated from C-CP by replacing soybean with wheat meal, and diet compositions are given in Table 1. Water was freely available from a nipple drinker. Every 3 weeks pigs were individually weighed and backfat thickness was measured at the P2 point (last rib, 6 to 8 cm from midline, left side) with an ultrasound device (Renco Lean-Meater series 12, Renco corporation, Minneapolis, USA). The ME intake (MEI) was computed from measured feed intake and ME content of dietary ingredients (Sauvant et al., 2004).

Body and gain composition and ME requirements

According to Schiavon et al. (2007), at the start and the end of each finishing period body lipid mass (L) was estimated as $L = (9.17 + 0.70 \times P2) \times BW/100$, fat free empty BW (FFEBW) was estimated as $FFEBW = (BW \times 0.95 - L)$ and protein mass (P) was estimated as $P = 0.1277 \times FFEBW^{1.11}$. Within period, the mean daily protein (Pr) and lipid (Lr) retentions were computed from changes of body chemical status and period duration. The mean protein mass was computed within period and the ME need for maintenance (ME_m) was calculated as ME_m

= $1.85 \times P^{0.78}$ (NRC, 1998). The ME need for growth (ME_g), were computed assuming to be necessary 44.4 and 52.3 MJ ME/kg of Pr and Lr, respectively (NRC, 1998). The MEI/(ME_m+ME_g) ratio was considered as an index of energy efficiency.

Chemical analysis

During manufacturing, 10 samples of each diet were collected on line, pooled and sampled to achieve 1 kg of feed sample and sub-sampled. Sub-samples were analysed in 3 independent replications for their proximate composition (AOAC, 2003) and NDF content (Van Soest et al., 1991). Starch content was determined, after its hydrolysis to glucose (AOAC, 2003), by liquid chromatography (Bouchard et al., 1988). The ME and amino acid contents were assessed from dietary ingredient composition and tabled values of ingredients (Sauvant et al., 2004).

Table 1. Chemical composition (g/kg as fed, unless otherwise indicated) of early (90 to 120 kg average BW) and late finisher diets (over 120 kg average BW)[1].

	Earlyfinisherdiets		Late finisherdiets	
	C-CP	L-CP	C-CP	L-CP
Analysednutrient composition[2]:				
DM	881	882	885	889
CP (N × 6.25)	147	112	132	100
Starch	423	478	450	501
NDF	162	163	151	152
Etherextract	39	38	38	35
Computednutrientcomposition				
ME[3], MJ/kg	13.0	13.0	13.1	13.1
Lysine[4]	7.3	5.6	5.6	4.1
Methionine[4]	2.3	1.8	2.1	1.6
Threonine[4]	5.2	4.0	4.6	3.7
Tryptophan[4]	1.8	1.4	1.6	1.2

[1] C-CP: conventional feeds; L-CP: low protein feeds.[2] Analytical results (AOAC, 2003) obtained fromthree independent replications.[3] Computed from chemical composition of feeds according to NRC (1998). [4] Computed from tabulated values of dietary feed ingredients (Sauvant et al., 2004).

Statistical analysis

Statistical analysis of performance and simulated N balance traits was based on the following linear model using the statistical package of SAS (SAS Inst. Inc., Cary, NC, USA):

$$Y_{ijklmn} = \mu + batch_i + GL_J + diet_k + sex_l + pen\ (batch \times diet)_{ikm} + (GL \times diet)_{jk} + (GL \times sex)_{jl} + (diet \times sex)_{kl} + (batch \times GL)_{ij} + (batch \times diet)_{ik} + e_{ijklmn}$$

Where y_{ijklmn} is the single observation; μ is the intercept, batch is the fixed effect of round (i = 1, 2); GL is the fixed effect of the genetic line (j = 1,…,4); diet is the fixed effect of the feeding treatment (k = 1, 2); sex is the effect of gender (l = 1, 2); pen is the effect of pen (m = 1,…, 8) within batch×diet; GL×diet is the interaction effect between GL and diet, GL×sex is the interaction effect between GL and gender; diet×sex is the interaction effect between diet and gender, batch×GL is the interaction effect between batch and GL; batch×diet is the interaction effect between round and diet and is the random residual. The effect of the diet was tested using the pen (batch×diet) as error line.

Results and Discussion

Significant GL effects were observed for almost all the growth traits considered, except for MEI intake as the pigs received restricted amounts of feed (Table 2).

The greater ability for lean growth of the D pigs was reflected by greater initial and final BW, and thinner backfat thickness, so that the resulting Pr was greater and Lr was smaller compared to the pigs of other GL. Across GL, in early and late finishing periods Pr averaged 120 and 98 g/d, respectively, and Lr averaged 157 and 210 g/d, respectively. The effects of feed were much smaller than those observed for GL, and indicated that the reduction of dietary CP only slightly decreased Pr in the late finishing period (P = 0.022), and consequently over the entire trial (P = 0.014). The MEI/(ME_m + ME_g) ratio averaged 0.993, and it was lower for D (0.96), greater for T (1.03) and intermediate for the A and G pigs (0.99). The reduction of the dietary CP level slightly worsened energy efficiency from 0.98 to 1.00 (P = 0.044).

Overall, the growth performance obtained in this trial was in agreement with that commonly observed in the heavy pig production system and described elsewhere (e.g. Xiccato et al. 2005; Ceolin et al., 2005; Tagliapietra et al., 2005). Energy requirements indirectly computed from individual body measurements, using the NRC (1998) relationship to quantify the needs of ME for maintenance and growth, were in good agreement with the values of ME intake based on measured feed intake and ME dietary contents. Even though such approach can be biased both on the side of the prediction of body chemical status and on the dietary ME content, it offers the advantage to be easily and cheaply applied *in vivo* on a large number of pigs. Slaughter experiments are certainly accurate in the determination of

body chemical status, but they cannot be repeated under different production circumstances. Thus, the approach proposed would be useful for a proximate estimation of protein and lipid retentions of heavy pigs, and offer the opportunity to modulate the diet characteristics according to the production aim of this production system.

Protein restriction permits to achieve a strong reduction of N excretion (37%) which would increase the pig production/ha in areas where the N load is fixed by law (Schiavon et al., 2012). Evaluations about the slurry volume in which N excreted is diluted are desirable (Schiavon et al., 2009).

Table 2. Body composition and energy balance of heavy pigs of four genetic lines[1] fed conventional (C-CP) or low-protein diets (L-CP)[2].

	Genetic Line (GL)				SEM	Diet		SEM	*P*	
	A	D	G	T		C-	L-		GL	Diet
Body weight (kg)										
Initial	86	92	87	87	0.9	88	88	0.9	<0.001	0.82
Intermediate	118	125	119	116	1.2	119	119	1.2	<0.001	0.98
Final	164	172	166	159	1.9	167	163	1.9	<0.001	0.14
Backfatdepth (mm)										
Initial	10.0	8.3	9.2	8.5	0.22	9.1	8.9	0.18	<0.001	0.60
Intermediate	12.9	10.3	11.9	10.7	0.28	11.2	11.8	0.26	<0.001	0.13
Final	19.4	15.9	17.7	16.2	0.44	17.1	17.5	0.32	<0.001	0.33
Body protein (kg)										
Initial	13.7	15.1	14.0	14.1	0.16	14.2	14.3	0.16	<0.001	0.73
Intermediate	18.9	20.7	19.4	19.0	0.21	19.6	19.5	0.19	<0.001	0.68
Final	25.5	28.0	26.4	25.6	0.31	26.8	25.9	0.30	<0.001	0.05
Body lipid (kg)										
Initial	14.0	13.8	13.5	13.1	0.23	13.6	13.6	0.22	0.015	0.90
Intermediate	21.4	20.5	20.9	19.3	0.36	20.3	20.8	0.40	<0.001	0.39
Final	37.3	35.1	35.7	32.8	0.79	35.4	35.1	0.69	<0.001	0.75
Proteinretention (g/d)										
0 to 44 d on feed	119	128	123	111	3.2	122	118	3.1	<0.001	0.35
45 to 115 d on	93	103	99	95	3.1	103	92	2.9	0.032	0.022
0 to 115 d on feed	103	113	108	101	2.3	111	102	2.1	<0.001	0.014
Lipidretention (g/d)										
0 to 44 d on feed	170	151	166	140	6.1	151	163	5.9	<0.001	0.19
45 to 115 d on	228	207	213	192	8.6	215	204	6.9	0.022	0.28
0 to 115 d on feed	204	186	194	172	6.2	190	188	5.5	0.001	0.79
ME_m requirement[4],	18.8	20.3	19.3	19.0	0.16	19.5	19.2	0.15	<0.001	0.17
ME_g requirement[4],	15.3	14.7	15.0	13.5	0.39	14.8	14.4	0.36	0.002	0.36
ME_m+ME_g[4], MJ/d	34.1	35.0	34.2	32.5	0.50	34.4	33.6	0.47	<0.001	0.25
ME intake (MEI)[5],	33.7	33.6	33.8	33.6	0.46	33.7	33.6	0.45	0.99	0.88
MEI/(ME_m+ME_g)	0.99	0.96	0.99	1.03	0.77	0.98	1.00	0.71	<0.001	0.044

[1] Data are from pigs (281 ± 3.6 old at slaughter) of 4 GL, Anas (A), DanBred (D), Goland (G) and Topigs (T) fed C-CP diets [147 and 132 g CP/kg in early and late finishing, respectively] or L-CP diets [112 and 100 g CP/kg in early and late finishing, respectively]; [2] Presented are the least square mean based on 46 and 92 observations for GL and feed, respectively. [3] Body composition and nutrient retentions were estimated from BW and backfat depth measurements (Schiavon et al., 2007). [4] ME_m and ME_g are requirement for maintenance and growth according to NRC (1998); [5] MEI = measured feed intake× dietary ME content.

Conclusions

During this trial the reduction of dietary CP did not influenced the growth performance of the pigs and reduced slightly the protein retention and the efficiency of ME use. These results confirm the opportunity for farmers to reduce the N excretion respecting the actual legislation without compromising production efficiency.

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CONTRIBUTE 5

Effects of low protein diets on performance of pigs with a lean genotype between 40 and 115 kg live weight

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Aim

The aim of the work was to assess the effects of three dietary protein regimes on pig performance and nitrogen (N) excretion,in particular, whether performance can be maintained in lean, fast growing pigs when protein levels are reduced to limit N excretion. Entire male pigs of a lean genotype (Pietrain × Large White × Landrace), 192 in total in four batches, were grown from 40 to 115 kg in pens with four pigs per pen. The diets were: (i) a high-protein control regime; (ii) a low-protein regime in which protein was reduced by ~2 percentage units in each growth stage, but with levels of five essential amino acids the same as in the control (LP1); (iii) an even lower protein regime in which levels of essential amino acids were not maintained beyond 60 kg (LP2). The LP2 regime was designed to promote intramuscular fat deposition rather than efficient growth. Excretion of N was reduced by 17% and 19% in LP1 and LP2, respectively, compared with the control. Average daily gain was lower and feed conversion ratio higher in LP2 than the other regimes, as expected. The control and LP1, which differed in protein but not essential amino acid levels, produced broadly similar results for performance, but pigs in LP1 had poorer feed conversion than control pigs, which could be due to slightly greater fat deposition. The results show the difficulty in maintaining consistently high levels of performance in fast-growing, lean pigs when dietary protein levels are reduced.

Keywords: feeding regime, growth performance, lean genotype, low protein, N pollution, pigs.

Introduction

Protein levels have historically been high in the diets of pigs in the UK compared with those in the rest of Europe, according to a survey conducted by Ajinomoto Eurolysine S.A.S. (Paris)in 2002 (M Overend, pers. comm.). This is probably because of the belief that high-protein diets increase lean growth and performance (growth rate and feed conversion). Early research indeed showed that as protein levels declined, fat deposition increased and performance fell (Davey and Morgan 1969; Cromwell et al. 1978).

It is now known that dietary protein levels can be reduced considerably with limited effects on performance, if levels of essential amino acids are kept at recommended levels (Canh et al. 1998;Lee and Kay 2003). This reduction in protein has benefits for the

environment because it leads to lower excretion of nitrogen (N) from pigs and pig units. Nitrous oxide and ammonia are potent greenhouse gases whose production in agriculture is under increasing scrutiny from legislators (e.g.Gill et al. 2010).

However, some studies have shown that even when low-protein diets are supplemented with essential amino acids, a tendency towards increased fatness is seen, with a consequent reduction in performance (Figueroa et al. 2002;Gomez et al. 2002). This tendency is likely to be greater in lean than fat genotypes (Wood et al. 2004). Because of the importance of modern lean genotypes in producing meat at low cost, further examination of the effects of low-protein diets is needed in high-performing pigs.

The present study was therefore conducted to determine whether performance could be maintained in pigs of a lean commercial genotype when sizeable reductions in dietary protein levels were applied. A high-protein control regime was compared with a low-protein regime in which essential amino acid levels were the same as in the controlthroughout (LP1), and an even lower protein regime in which essential amino acids were not maintained in the two final growth stages (LP2). The LP2 regime was designed to promote fat deposition in muscle for possible improvement in pork eating quality. It served in this study to illustrate the importance of balancing diets with amino acids when protein levels are reduced. Body and carcass composition are examined in two companion papers: Wood et al. (2013) and Lambe et al. (2013).

Materials and methods

Animals, diet regimes and diets

Four batches of 48 entire male pigs, male progeny of Genepacker 90 (Large White × Landrace) sows and Geneconverter 600 (a synthetic Piétrain-type) boars, 192 in total, were used. Pigs arrived at the growing/finishing unit weighing ~36 kgliveweight (LW_st) and were allocated at random to the threedietaryregimes;they began the growth trial at 40 kg. Within each regime, a different diet was fed at different stages of growth, i.e. at 40–60, 60–85 and 85–115 kg average LW.

Within each batch of pigs, one-third (16 pigs) were fed according to a commercial control regime.One-third were fed according to the LP1 regime, in which protein levels were reduced by ~2 percentage units compared with the control in each of the threeliveweight ranges, but concentrations of lysine and other essential amino acids were maintained.The

other 16 pigs were fed according to the LP2 regime, in which protein was reduced to its lowest level in the final stage of growth and lysine and other essential amino acid levels were not maintained after 60 kg.This regime was designed to increase intramuscular fat and pork eating quality rather than to promote efficient growth.

The main ingredients of all the diets, which were pelleted, were barley, wheat, maize, wheat feed and soybean meal. Diets were formulated on an ileal digestible amino acid basis (British Society of Animal Science2003) and were formulated to contain the same net energy value, 9.7 MJ/kg. The concentrations of protein, lysine, net energy and four essential amino acids after lysine in the control were those suggested for lean and fast-growing pigs by British Society of Animal Science (2003).The concentrations of protein, lysine, methionine, threonine, tryptophan, valine (amino acids expressed as total, not digestible) and predicted digestible energy (DE) in the feeds used are shown in Table 1. Values are the means and standard deviations of the feeds after milling fed to the four batches of pigs. In regimes LP1 and LP2, the four amino acids after lysine were maintained at the same proportions relative to lysine as were present in the control diets.

Crude protein was measured using the Kjeldahl procedure as described by British Standards Institution (2005). Lysine and other amino acids were measured using the method described in EC Directive 98/64/EC (1998). Predicted DE was calculated using the method of HM Government (2005).

Pigs were reared in pens of four on straw and fed ad libitum. The location of the pen in the shed (row) was used as a factor in the data analysis. Twelve of 16 pigs in the last three batches (108 in total) were repeatedly passed through the CT scanner at Scotland's Rural College (SRUC) at the target liveweights of 60, 85 and 115 kg to visualise and quantify tissue deposition during growth (Lambe et al. 2013). The CT data were used herein to predict N retention. When the average weight of the batch was ~115 kg, all of the pigs of a batch were transported to the Tulip abattoir in Spalding, Lincolnshire. They were kept in a lairage with feed overnight and slaughtered the following day using a CO2 stun–kill unit. Their carcass composition is described by Wood et al. (2013).

Table 1. Values for crude protein (CP), lysine, methionine, threonine, tryptophan and valine (% of fresh wt, amino acids expressed as totals), and predicted digestible energy (DE, MJ/kg) in diets in the three dietary regimes.

Weight range	Control			LP1			LP2		
	40–60	60–85	85–115	40–60	60–85	85–115	40–60	60–85	85–115
CP	18.9	17.1	15.2	16.6	14.7	12.7	16.7	14.5	11.3
s.d.	0.58	0.29	0.12	0.86	0.96	0.45	0.96	0.77	0.82
Lysine	1.11	0.98	0.81	1.13	1.00	0.84	1.13	0.69	0.54
s.d.	0.060	0.047	0.034	0.077	0.042	0.038	0.024	0.029	0.036
Methionine	0.34	0.28	0.23	0.38	0.32	0.26	0.37	0.22	0.17
s.d.	0.021	0.010	0.008	0.045	0.015	0.017	0.013	0.012	0.010
Threonine	0.74	0.66	0.55	0.75	0.65	0.54	0.74	0.50	0.36
s.d.	0.022	0.017	0.015	0.050	0.036	0.015	0.029	0.026	0.026
Tryptophan	0.27	0.24	0.22	0.24	0.21	0.18	0.23	0.21	0.16
s.d.	0.009	0.010	0.005	0.018	0.010	0.001	0.013	0.008	0.008
Valine	0.90	0.79	0.69	0.81	0.70	0.59	0.81	0.67	0.50
s.d.	0.033	0.022	0.014	0.044	0.040	0.021	0.024	0.036	0.033
DE	14.4	14.2	14.0	13.7	13.7	13.6	14.1	13.9	13.5
s.d.	0.27	0.25	0.12	0.12	0.13	0.08	0.33	0.28	0.27

LP1, LP2: Low protein diets. Mean and standard deviation (s.d.) for the four batches.

Average daily gain, feed intake, feed conversion ratio, and intake, retention and excretion of nitrogen

From 40 kg LW, pigs were weighed weekly and average daily gain (ADG) was calculated. Feed intake (FI) was recorded on a pen basis, and by dividing by the number of pigs per pen, transformed to an individual basis.The FI and feed conversion ratio (FCR) are expressed as a pen average, reducing the total sample size (n =192) to the number of pens (n =48). Approximately 3 weeks after 40 kg, the pigs reached the target weight of 60 kg LW, on average, when the second diet was introduced (Table 1). After about another 4 weeks, at an average of 85 kg LW, the third diet was introduced, and at the target weight for the batch of 115 kg (LW_sl), the pigs were sent for slaughter. Results were calculated for the periods LW_st–LW40, LW40–LW60, LW60–LW85, LW85–LW_sl, and finally LW40–LW_sl.

Each pig had ~15 weekly LW measurements taken. Gompertz growth curves, found to fit well the postnatal growth of various mammalian species (Renne et al. 2003;Lambe et al.

2006), were fitted to the LW data in order to estimate growth parameters for individual animals and to condense ≥15 LW measurements into three parameters of a Gompertz growth curve. These biologically meaningful parameters are: estimated mature LW (A), maximum ADG (B), and the days from the start when maximum ADG is achieved (C). The half-life (HL), the days from the start at which the pig achieved half its mature weight, was also calculated.

Nitrogen intake (NI) was calculated from feed intake and dietaryN content. Nitrogen retention (NR) was estimated from carcass lean content, assessed by CT at 60, 85 and 115 kg by Lambe et al. (2013), using the prediction model described by Arthur et al. (2011). Nitrogen excretion (NE) was calculated from NI minus NR.

Statistical analysis

Data were analysed using the general linear model (GLM) procedure in SAS 9.1 (SAS Institute Inc., Cary, NC). The model applied was:

$$Yijkl = \mu + LW\_sti + Bj + Rk + DRl + eijkl$$

where μ is overall mean, LW_stiis LW at start, B is batch,Risrow (location of pen in shed), DR is diet regime, and e is residual error.

It was originally thought that a term for whether or not the pig had been CT scanned (the process includes feed withdrawal and administration of an anaesthetic) should also be included in the models used for data analysis. However, there were no consistent effects of the scanning procedure on any aspect of performance (results not shown), so these factors were not considered.

Results

Effects of diet regime on LW, FI, ADG and FCR

Results are presented in Table 2. The stage from 36 to 40 kg was considered an adjustment period. Dietary regime had nosignificant effect on any trait in the 40–60 kg stage. In the 60–85 kg stage, significant effects of dietary regime occurred, with LW and ADG being lowest in LP2 ($P < 0.001$) and FCR highest in LP2 ($P < 0.001$). Liveweight and ADG were not different between the control and LP1, but FCR was higher in LP1 than in the control ($P < 0.05$).

In the 85–115 kg stage, the same trends were apparent, but FCR was not significantly different between control and LP1 treatments ($P > 0.05$). At slaughter, pigs in LP2 were 8.9 kg

lighter than those in the controltreatment and 7.6 kg lighter than those in LP1. Over the whole period 40–115 kg, ADG was lowest in LP2 ($P < 0.05$) and FCR was highest in LP2 ($P < 0.05$). Control and LP1 treatments were not different for ADG, but LP1 had a higher FCR than the control ($P < 0.05$). Values for FCR were 2.469 (100%), 2.608 (106%) and 2.857 (116%) in control, LP1 and LP2, respectively. In only one growth stage, 60–85 kg, was there a difference in FI due to dietary regime, intake being higher in LP2 than in the control ($P < 0.05$). For the whole period 40–115 kg, FI was numerically higher in both LP regimes than in the control, but the differences were not significant.

Liveweight at the beginning of each stage was affected by LW_st, as expected. Batch was an important factor in the analysis at every stage, but there were no interactions between dietary regime and batch, showing that diet exerted its effects in a consistent way in each batch. Position in the shed (row) had minor effects.

Table 2. Least-squares means for the dietary regimes (DR) for liveweights (LW), feed intake (FI), average daily gain (ADG), and feed conversion ratio (FCR) in the different periods.

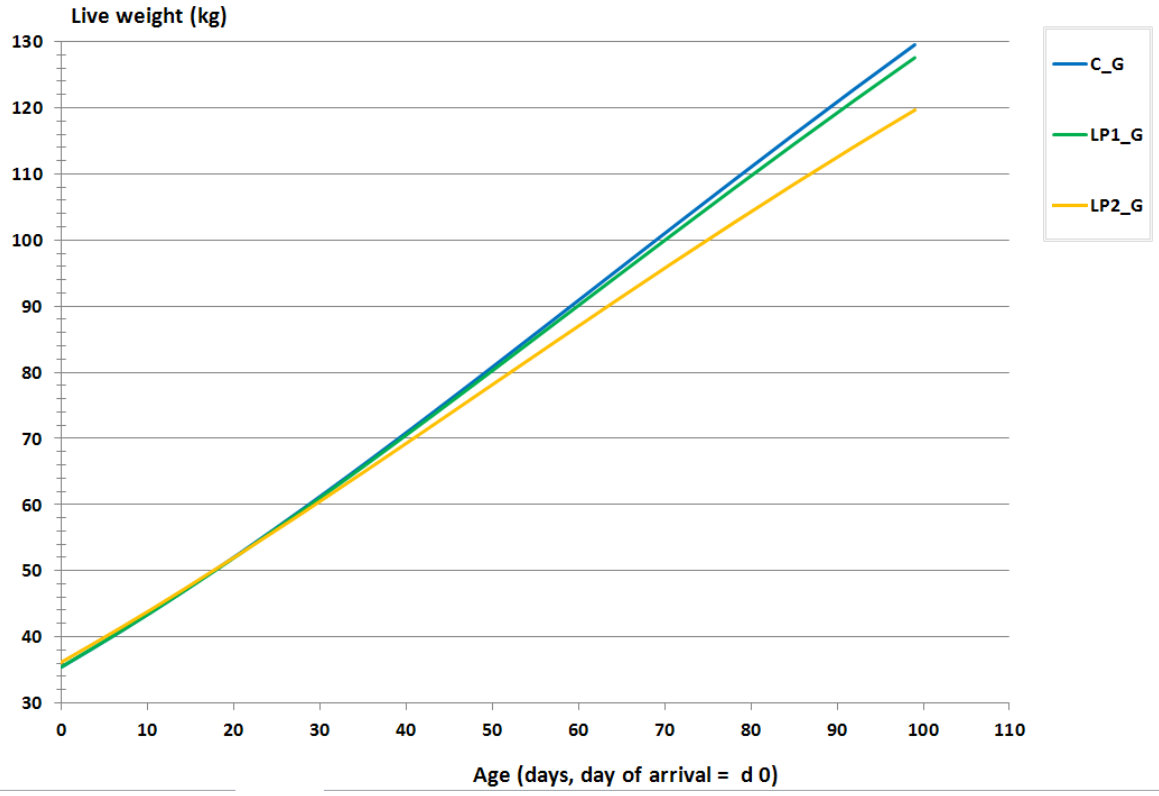
	Control	LP1	LP2	Av. SE	P-value			
					DR	LW_st	Batch	Row
Start to 40 kg								
LW_st	36.18	36.18	36.49	0.336	0.746	n.e.	0.012	0.366
LW 40	40.52	40.73	40.53	0.189	0.689	<0.001	<0.001	0.271
FI st–40	9.35	9.24	9.34	0.122	0.799	<0.001	<0.001	0.082
ADG st–40	0.764	0.797	0.779	0.029	0.720	0.982	<0.001	0.140
FCR st–40	2.334	2.233	1.689	0.382	0.449	0.685	0.816	0.482
40–60 kg								
LW 60	59.54	59.04	58.57	0.410	0.264	<0.001	<0.001	0.345
FI 40–60	38.28	39.16	38.16	0.454	0.247	0.001	<0.001	0.629
ADG 40–60	0.874	0.852	0.808	0.019	0.061	0.720	<0.001	0.672
FCR 40–60	2.059	2.158	2.224	0.047	0.056	0.040	0.792	0.833
60–85 kg								
LW 85	87.82a	87.35a	84.54b	0.590	<0.001	<0.001	<0.001	0.039
FI 60–85	68.44a	71.25ab	71.90b	1.090	0.070	0.162	0.005	0.194
ADG 60–85	1.002a	1.000a	0.895b	0.017	<0.001	0.357	<0.001	0.156
FCR 60–85	2.430a	2.540b	2.788c	0.026	<0.001	0.439	<0.001	0.488
85 kg to slaughter								
LW_sl	117.81a	116.50a	108.88b	1.065	<0.001	0.029	0.036	0.010
FI 85-sl	83.62	87.11	84.9	1.802	0.394	0.970	<0.001	0.657
ADG 85–sl	0.975a	0.9430a	0.799b	0.027	<0.001	0.720	<0.001	0.086
FCR 85–sl	2.844a	3.015a	3.559b	0.0732	<0.001	0.959	0.0013	0.849
Totals								
FI total 40–sl	190.34	197.52	195.05	2.530	0.138	0.277	0.011	0.428
ADG 40–sl	0.979a	0.959a	0.8680b	0.013	<0.001	0.629	0.017	0.021
FCR 40–sl	2.469a	2.608b	2.857c	0.029	<0.001	0.506	0.272	0.094

Data expressed in kg.LP1, LP2: Low protein diets; SE, standard error; st,start of recording period (on arrival in Edinburgh); sl, slaughter (at ~115 kg on average for batch).Within a row, means followed by the same letter are not significantly different (P >0.05).

Gompertz analysis of LW over time

The Gompertz model used to fit the individual LW data over time produced the growth curves shown in Fig. 1. These are the average curves for the four batches. The curves for control and LP1 treatments were similar, with LP2 showing lower growth rate. The parameters of the growth curves are presented in Table 3. The close fit of the data is shown by the high R2 values. Significantly lower mature weights (A) and a lower maximum growth rate (B) were predicted for pigs in dietary regime LP2, with no significant differences between the control and LP1. The days from the start when maximum growth rate (C) and half the mature weight (HL) were achieved tended to be lowest in LP2, but these differences were not significant.

Fig. 1. Growth from arrival at the farm (day 0) until slaughter in the three feeding groups (Control, LP1, LP2) across batches 1–4.



For illustration purposes, the Gompertz model has been fitted to the mean live weight of each dietary regime group in each batch. These values were then averaged over the batches and the fitted curves presented.

Table 3. Least-squares means for liveweights (LW) at the start (st) and at the different target weights of 40, 60, 85 and 115 (slaughter, sl) kg, and the parameters of the modified Gompertz equations.

	Control	LP1	LP2	Av. SE	P-value			
					DR	LW_st	Batch	Row
LW_st	36.21	36.28	36.55	0.345	0.7636		0.022	0.317
LW 40	40.60	40.86	40.60	0.161	0.404	<0.001	<0.001	0.14
LW 60	59.58	59.07	58.86	0.351	0.323	<0.001	<0.001	0.515
LW 85	87.86a	87.28a	84.51b	0.586	<0.001	<0.001	<0.001	0.037
LW_sl	117.92a	116.45a	108.79b	1.002	<0.001	<0.001	0.014	0.002
A (Gompertz)	308.9a	287.2ab	238.4b	19.14	0.030	0.590	0.020	0.059
B (Gompertz)	1.102a	1.053 a	0.933b	0.025	<0.001	0.104	0.022	0.003
C (Gompertz)	73.71	70.55	57.63	5.460	0.089	0.553	0.002	0.101
HL (Gompertz)	140.1	137.8	130.9	4.596	0.337	0.331	0.003	0.136
R2 (Gompertz)	0.998	0.998	0.997					

LP1, LP2: Low protein diets; DR, Dietary regime; SE, standard error.Gompertz parameters: A, mature weight; B, maximum growth rate; C, days after start when maximum average daily gain achieved.HL, Number of days after start at half of mature weight.Within a row, meansfollowed by the same letter are not significantly different(P >0.05).

Nitrogen intake, retention and excretion

The results (Table 4) relate to the 108 pigs that were CT-scanned. Nitrogen intake was higher in the control than in LP1 and LP2 in each of the growth stages (P <0.05). In the first two stages, 40–60 and 60–85 kg, LP1 and LP2 had a similar NI, but in 85–115 kg, when the main diet change was made, NI was lower in LP2 than LP1 (P <0.05). For the whole period, NI was 11% lower in LP1 than the control and 16% lower in LP2 than the control.

Nitrogen retention was lower in LP2 than the control and LP1 in each growth stage from 60 kg, when the first CT measurements were made (P <0.05). Nitrogen excretion, the difference between NI and NR, was lower in LP1 and LP2 than the control in each stage. In the period over which CT measurements were made (60 kg to slaughter), NE was 2.892 kg (100%) in the control, 2.411 kg (83%) in LP1 and 2.351 kg (81%) in LP2. Thus, regimes LP1 and LP2 resulted in 17% and 19% lower NE, respectively, than the control regime.

Table 4. Least-squares means for the different diet regimes for nitrogen intake (NI), retention (NR) and excretion (NE).

	Control	LP1	LP2	Av. SE	P-value			
					DR	LW_st	Batch	Row
NI 40–60	1.157a	1.040b	1.038b	0.013	<0.001	<0.001	<0.001	0.665
NI 60–85	1.872a	1.676b	1.725b	0.027	<0.001	0.149	<0.01	0.227
NI 85–sl	2.033a	1.770b	1.497c	0.039	<0.001	0.545	<0.001	0.578
NI 40–sl	5.063a	4.486b	4.260c	0.059	<0.001	0.239	0.071	0.347
NR 60–85	0.624a	0.604a	0.440b	0.012	<0.001	0.977	<0.001	0.400
NR 60–sl	1.150a	1.120a	0.848b	0.023	<0.001	0.658	<0.001	0.221
NR 85–sl	0.527a	0.516a	0.408b	0.017	<0.001	0.546	<0.001	0.360
NE 60–85	1.297a	1.101b	1.226a	0.033	<0.01	0.259	0.040	0.770
NE 60–sl	2.892a	2.411a	2.351b	0.061	<0.001	0.365	0.116	0.829
NE 85–sl	1.595a	1.311b	1.125c	0.040	<0.001	0.657	<0.001	0.781

Data in kg. NR calculations based on Arthur et al. (2011) (see Materials and methods for details). LP1, LP2: Low protein diets; SE, standard error; DR, Dietary regime; LW, liveweight. Within a row, means followed by the same letter are not significantly different (P >0.05).

Discussion

Both of the low-protein regimes caused NE to be reduced by ~18%, which is approximately the amount expected from the conclusion of Kerr and Easter (1995) that each percentage reduction in dietary protein results in 8% less N excreted. In this study, the reduction in dietary protein was from a higher control level (19% in the 40–60 kg stage) than in most other studies. This high level of protein is often assumed necessary to promote optimum performance in modern lean genotypes.

Several papers have shown that growth rate and feed conversion can be retained at the level of high-protein diets when protein is reduced but essential amino acid levels are maintained and diets have the same NE value. However, many authors have stated that diets with very low concentrations of protein compared with the control diet, designed to obtain the maximum reduction in NE, lead to reduced NR and greater fatness. For example, Lee and Kay (2003) fed low-protein diets that contained similar levels of 11 digestible essential amino acids as controls, but found a significant decline in NR when the greatest protein reduction was made. Canh et al. (1998) found that carcass lean content declined when protein was

reduced from 16.5% to 12.5% in finisher diets. Similar results were found by Kerr et al. (1995), Figueroa et al. (2002) and Gomez et al. (2002).

Nitrogen retention was greatly reduced when pigs in the present study were fed dietary regime LP2, which had low protein concentrations compared with the control (11.3% v. 15.2% in the final growth stage), but lower levels of essential amino acids in the second and third growth stages. This regime retarded the overall growth of the pigs, as shown by the estimated lower mature weight in the Gompertz analysis and the days from the start when maximum growth rate was achieved. This type of regime has been used to promote fat deposition in muscle for the sake of eating quality (e.g.Teye et al. 2006). For the pigs in the present study, the effects on fatness are reported by Wood et al. (2013). Total fat in the carcass was higher in LP2 than the control (25.3 v. 21.5%), as was intramuscular fat in the longissimus muscle (1543 v. 1055 mg/100 g).

Maintaining essential amino acid intakes at the level of the control regime, but reducing protein by ~2 percentage units in each growth stage in regime LP1, produced NRsimilar to the control, but there were indications that pig performance was reduced. Thus, FCR in the 60–85 kg stage and in the whole period 40–15 kg was significantly higher in LP1 than in the control. In the period 40–15 kg, FCR was 2.47, 2.61 and 2.86 in control, LP1 and LP2 regimes, respectively. There was also an indication from the Gompertz analysis that the overall growth of these pigs was retarded, as indicated by a lower mature weight. The carcass data (Wood et al. 2013) showed that the control and LP1 pigs had a statistically similar total fat content, although numerically, the value was higher in LP1 than control pigs (22.45 v. 21.54%). Intramuscular fat in longissimus was 11% higher in LP1 ($P < 0.001$). Values were 1055 and 1177 mg/100 g in control and LP1 treatments, respectively.

These results show the difficulty of retaining similar levels of performance and carcass composition when protein levels in diets are reduced. Great effort was made to provide the same levels of five essential amino acids in control and LP1 diets. The results in Table 1 show that this aim was largely achieved. Values of valine were slightly lower in LP1 than control diets in each growth stage, but they were higher than the value of 0.7% assumed necessary for optimum performance in growing pigs (British Society of Animal Science 2003).

In conclusion, the results show that protein levels in pig feeds given to very lean genotypes can be reduced to obtain a meaningful decreased excretion of N into the environment. Maintaining levels of essential amino acids in the reduced-protein diets goes a

long way to maintaining performance levels, but there is a possibility that feed conversion and fatness may be compromised.

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GENERAL CONCLUSIONS

In DBM crossbreed beef cattle a dietary CP density of about 100 g CP/kg DM diets during the finishing period has little consequences on overall ADG and few influence on carcass and meat quality, only tenderness resulted negatively affected with respect to a diet with 140 g/kg of crude protein. Thus, it is suggested that,for this kind of beef production the use of low protein diets during the finishing period is valid and applicable. Similarly, in mid-late lactation Holstein cows a diet with about 120 g CP/kg DM had small consequence on DMI, nutrient digestibility ad milk yield and quality, but strongly reduced the urinary excretion of N compared to a ration with 150 g/kg of crude protein. In both contributions 1 and 2 theresults evidenced the magnitude of effect of low protein diets on the amount of N excretion, and it was evidenced that in some conditions a reduction of the feeding costs is also achievable.

For measuring the FA profile and the CLA contents of ruminant feces the Jenkins' method based on a mild acid-base one-step treatment is recommended as, besides its simplicity and the low labor required, it does not impair the total amount of FA extracted with respect to other analytical procedures, it provides repeatable measures of the FA profile and causes lower shifts in CLA isomer composition of ruminant feces.

The reduction of dietary CP did not influenced the growth performance of the heavy pigs but reduced slightly the protein retention and the efficiency of ME use. The results confirmsthat a reduction of the dietary crude protein level is one of the major strategies to reduce N excretionwith small influences on growth performance. In fast growing lean pig the dietary protein level can be reduced to decrease the excretion of N, but feed conversion and fatness may be compromised if levels of essential amino acids are unsufficient to maintain performance levels, and an increase of intramuscular fat deposition likely occurs.

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