Effect of replacing organic grass-clover silage from primary growth with regrowth on N digestion in dairy cows

S.S. Naadland[a], H. Steinshamn[b], S. J. Krizsan[c], Å.T. Randby[a]

[a] *Department of Animal and Aquacultural Sciences, Norwegian University of Life Science, Norway, 1432 Ås, Norway*

[b] *NIBIO, Norwegian Institute of Bioeconomy Research, Department of Grassland and Forage, 6630 Tingvoll, Norway*

[c] *Swedish University of Agricultural Sciences, Department of Agricultural Research for Northern Sweden, 901 83 Umeå, Sweden*

[*] Corresponding author: sondre.naadland@nmbu.no

Abbreviations

AA, amino acid; AAT, amino acids to the intestine; BW, body weight; CP, crude protein; DM, dry matter; DMI, dry matter intake; EAA, essential amino acid; ECM, energy corrected milk; FP, fluid phase; iNDF, indigestible neutral detergent fiber; LP, large particle phase; ME, metabolizable energy; NAN, non-ammonia nitrogen; NDF, neutral detergent fiber; NDFom, neutral detergent fiber expressed exclusive of residual ash; OM, organic matter; PBV, protein balance in the rumen; PG, primary growth; RDP, Rumen degradable fiber; RG, regrowth; RUP, rumen undegradable protein; SP, small particle phase.

ABSTRACT

Clover proportions, and thereby chemical composition of herbage, differ between primary growth (PG) and regrowth (RG) in organic managed grass-clover fields. The characteristics of PG and RG silages suggest different supplementary feeding strategies to sustain an efficient milk production in dairy cows. Silage made of the RG generally offers more crude protein (CP) in the diet than silage made of the PG because of an increasing proportion of clover later in the season. Additionally, grass and clover have different amino acid (AA) profiles. His has been suggested to be the first limiting AA in grass silage, while Met has been suggested to be the primarily limiting AA in red clover silage. Eight rumen cannulated Norwegian Red cows were used in two replicated 4 × 4 Latin squares with 21-days periods. Organic PG and RG silages were fed *ad libitum* in four diets with RG replacing PG silage in ratios of 0, 0.33, 0.67 and 1 on dry matter (DM) basis. Changing RG silage proportions from 0 to 1 increased daily CP intake from 2.90 to 3.08 kg and rumen NH_3-concentrations from 4.9 to 8.4 mmol/L, but did not promote a better protein supply. Neither total ruminal outflow of AA nor the AA profile in the small intestine differed between dietary treatments. Met and His were probably the most limiting AA for a higher milk production. Limitations by His may be more related to diets based on PG, while production by cows fed diets based on more RG herbage were more likely limited by Met.

Keywords: dairy cows, nitrogen, grass-clover, organic milk production, regrowth

1. Introduction

Organic agriculture depends on legumes and their ability to fix atmospheric N_2 due to restrictions on the use of mineral fertilizers (Counc of the Eur Union, 2007). The main forage for dairy cows in Fennoscandia is grass-clover silage prepared from temporary grassland, due to the relatively long winter. Grassland legumes used in Fennoscandia have a higher optimal growth temperature than their companion grasses. Due to low spring temperatures the herbage legume proportion in mixed leys is usually lower in the primary growth (PG), i.e. the spring growth after the winter dormancy, than in the regrowth (RG), the growth after a cut (Steinshamn and Thuen, 2008; Eriksen et al., 2012). The organic PG has a relatively lower N concentration due to the higher proportion of grass than the corresponding legume-richer RG, as observed under both experimental and commercial farm conditions (Steinshamn et al., 2015; TINE Rådgivning, pers. commun.). It is desired to obtain a diet providing a high quantity of amino acids absorbed in the intestine (AAT), and a positive protein balance in the rumen (PBV), which depends on the amount of rumen digestible carbohydrates and N. Positive PBV-values describe sufficient amounts of carbohydrates for the rumen microbial protein synthesis. Low N concentrations and high concentrations of rumen digestible carbohydrates in PG might initiate a negative PBV value, whereas PBV usually increases in a legume-rich RG. In mixtures with grasses, legumes usually promote an increased dry matter intake (DMI) and a correspondingly increased milk production compared to grasses alone (Dewhurst et al., 2003; Vanhatalo et al., 2009). Thus, to high yielding cows, feeding a combination of silages prepared from PG and RG may provide a more optimal N supply than feeding the cuts separately.

The grass protein has a greater share of rumen degradable protein (RDP) compared to the legume protein, which potentially increase microbial protein synthesis (Halmemies-Beauchet-Filleau et al., 2014). Addition of the limiting essential amino acids (EAA) to an unbalanced forage amino acid (AA) profile might increase milk production (Korhonen et al., 2000; Vanhatalo et al., 2009; Lee et al., 2012). Red clover (*Trifolium pratense* L.) dominated diets are probably primarily limited by Met (Vanhatalo et al., 2009), and levels of Met can be assumed similar in red clover and white clover (Reverter et al., 1999). Studies with grass-based diets have shown His to be the most limiting AA (Vanhatalo et al., 1999; Korhonen et al., 2000). Omasal flow of Met and His should each constitute 25 g/kg of total omasal crude protein (CP) flow (National Research Council, 2001; Lee et al., 2012). Lys is recommended at 72 g/kg of CP in omasal flow and in a 3:1 relationship to Met (National Research Council, 2001). However, restricted dietary Lys or a generally negative PBV in early lactation is not expected to limit milk yield due to body tissue mobilization (Doepel et al., 2002; Mjoun et al., 2010).

To our knowledge, no previous studies have tested organic grass-clover silages made from PG and RG in the diets to lactating dairy cows with primary focus on the N metabolism. The objective of this study was to compare N metabolism with emphasis on qualitative as well as quantitative AA supply to the small intestine in lactating dairy cows fed diets based on PG and RG from grass-clover silages produced from the same field. We tested the hypotheses that increasing dietary RG proportions would increase AA flow to the small intestine, and that milk production from the RG with a large legume proportion is limited by a less balanced AA profile compared to PG.

2. Materials and methods

Laws and regulations controlling experiments with live animals by Norwegian University of Life Sciences Animal Care and Use Committee and the Norwegian Animal Research Authority were implemented in the experiment (Norwegian Ministry of Agriculture and Food, 2010).

2.1. Experimental design and animals

An experiment consisting of two replicated 4 x 4 Latin squares, each with 4 Norwegian Red cows, and four 21-days periods consisting of 9 days of adaption and 12 days of sampling, was conducted in fall 2012 and spring 2013. Experimental treatments were four diets made of organic grass-clover silages from PG and RG harvested from the same field. Cows were equipped with rumen cannulae (Bar Diamond Inc., Parma, ID, USA) and entered the experiment at (mean ± SD) 56 ± 19 days in milk and BW 622 ± 83 kg. Indigestion excluded one cow from two experimental periods. Cows were housed in a tie-stall with continuous access to water and feed, and feed was assigned in equal shares three times daily at 0630, 1415 and 2200 h. Milking was conducted twice daily at 0700 and 1700 h.

2.2. Grass-clover silages and experimental diets

The PG and RG silages were prepared from organically managed fields in Ås, Norway (59°40’N, 10°46’E) in 2012 (Council of the European Union, 2007). The ley consisted mainly of timothy (*Phleum pratense* L. cv. ‘Grindstad’) and meadow fescue (*Festuca pratensis* Huds. cv. ‘Fure’), and the legumes white clover (*Trifolium repens* L. cv. ‘Hebe’) and red clover (‘Bjursele’). The PG and the RG contained 113 g/kg and 393 g/kg white clover and 65 g/kg and 14 g/kg red clover, respectively. Naadland et al. (2015) have reported a detailed description of silage production and quality. Experimental treatments comprised diets with replacement of PG and RG silage in the proportions 0, 0.33, 0.67 and 1 (treatments D1, D2, D3 and D4, respectively) on DM basis. Silages were chopped to a median length of 4.5 cm and hand mixed before feeding to minimize selection. Silages were offered *ad libitum* allowing 100 g refusals daily per kg silage fed. Cows were additionally fed 8 kg (on fresh basis) daily of a concentrate mixture containing peas (268 g/kg DM), oats (168 g/kg DM), wheat (165 g/kg DM), barley (150 g/kg DM), rapeseed cake (100 g/kg DM), molasses (55 g/kg DM), rapeseed seeds (50 g/kg DM) and a vitamins and mineral mixture (44 g/kg DM; Natura Minovit Drøv, Felleskjøpet Agri BA, Lillestrøm, Norway).

2.3. Sampling, recordings and chemical analyze

Daily samples of 1 kg PG and RG silage were collected separately every week in all periods. The samples were pooled within each period to a total of four samples of both silages. Milk samples were collected during six subsequent milkings day 11 to 14 and day 18 to 21. Milk samples were analyzed for fat, protein, lactose and urea with a MilkoScan 6000 (Foss Electric, Hillerød, Denmark). Digesta flow was estimated using the triple marker method described by France and Siddons (1986). Rumen marker infusion started on day 4 at 0800 h in each period with a priming dose of 2.80 g Cr (Cr-EDTA) and 2.46 g Yb (Yb-acetate). This was directly followed by the start of a continuous infusion using a peristaltic pump (Cenco Instruments MIJ N.V., Breda, the Netherlands) providing 2.80 g Cr/d and 2.46 g Yb /d. The infusion lasted until day 14 at 1500 h in all periods. The third marker was indigestible neutral detergent fiber (iNDF) that with Yb and Cr differentiated digesta into a large particle (LP), small particle (SP) and fluid phase (FP), respectively. Additionally, an aqueous solution with 100 g/L atom excess $(^{15}NH_4)_2SO_4$ (Sigma Aldrich (Isotec), Miamisburg, OH, USA) providing 200 mg/d of ^{15}N was infused from day 10 at 0600 h until day 14 at 1500 h. Samples of reticular digesta were collected using a 250 mL wide-necked plastic bottle with a rubber stopper according to Krizsan et al. (2010). The reticular sampling technique was used to collect nine digesta samples from the reticulum on day 12 to day 14 with 4.5 h interval between the three sampling occasions each day to cover sampling hourly during a complete 12-h feed cycle. On the last 2 days, sampling occasions were moved 1.5 h later than on previous day. Samples of 600 mL of each time point were pooled to a total of 5400 mL from each period. Pooled samples were frozen at -20ºC in the same container directly after sampling. After thawing the pooled samples were filtered and centrifuged at 1,000 × *g* for 10 minutes at 5ºC to separate the digesta into LP, SP and FP with the method described by Krizsan et al. (2010). Microbial mass was separated out of a 250 g sample from reticulum directly after each sampling time as described by Ahvenjarvi et al. (2000). The native rumen ^{15}N-content was measured in a rumen content sample on day six.

Rumen evacuations were conducted on day 19 and 21 at 0600 and 0930 h, at expected minimum and maximum rumen fill, respectively. From each Latin square, two cows were evacuated at 0600 h and two cows at 0930 h on day 19. On day 21, cows and times were changed. Organic matter (OM), DM, CP, neutral detergent fiber exclusive of ash (NDFom) and iNDF were analyzed. To assess ruminal fermentation, liquid samples of 250 ml were collected on day 17 at 0600, 0730, 0900, 1030, 1200, 1330, 1500 and 1630 h. From each sampling, 9.5 mL ruminal liquid was filled in a 15 mL test tube with 0.5 mL formic acid and kept at 4ºC until analysis of NH_3. Total collection of feces to measure total digestibility was conducted from day 10 to 12. Urine was separated from feces using a funnel device, bonded around vulva, leading urine in a hose ending into a container. To prevent NH_3 volatilization the container was daily added 1.5 L with 100 g/L H_2SO_4 solution.

Blood samples were collected on day 18 at 0600, 0900 and 1200 h from the coccygeal vessels, which were considered similar to arterial blood entering the mammary gland. The samples were collected using vacutainer tubes (Vacuette®, Greiner Bio-One) containing Li-heparin for AA and BHBA analyzes. Additionally a serum tube was used for urea analyzes. The Li-heparin tubes were immediately cooled and centrifuged (3000 × *g* for 10 min.).

Serum-tubes were stored for 2 h at room temperature to coagulate and before centrifuging (3000 × *g* for 10 min.). Plasma and serum were pooled across sampling times to provide one sample per cow per period.

Chemical analyses of feeds are described in detail in our previous paper (Naadland et al., 2015). The same analyses as used for the feeds were used on digesta and fecal samples. Rumen fluid was analyzed for NH_3 using flow injection analyzer FIAstar 5010 (Tecator AB, Höganäs, Sweden). The concentration of Cr and Yb in reticular digesta and feces were analyzed in an atomic absorption spectrophotometer (GBC SavantAA Ser. No A6990, GBC Scientific Equipment, Hampshire, IL), as described by Njåstad et al. (2014). The ^{15}N isotope was analyzed in reconstituted reticular samples, microbial samples and ruminal background samples. Each sample contained 100 μg of N, and they were weighed into tin capsules (PDZ Europa, Cheshire, UK). Additionally, 50 µL of KCO_3 solution (10 g/L) was pipetted onto each sample. Samples were dried at 60°C overnight to remove NH_3 residues. The enrichment of ^{15}N in the samples was analyzed in duplicate using PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples for individual AA analyzes were freeze dried and ground to 0.5 mm before analyzing. The free AA were extracted with diluted HCl. Co-extracted N macromolecules were precipitated with sulfosalicylic acid and removed by filtration. The filtered solution was adjusted to pH 2.20. The AA were separated by ion chromatography and determined by ninhydrin reaction with photometric detection at 570 nm (Biochrom 30 Amino Acid Analyzer, Biochrom Ltd., Cambridge, UK).

2.4. Calculations and statistical analysis

Fecal recovery was used to correct the marker concentrations as described by Krizsan et al. (2010). The flow of OM was corrected for volatile fatty acids (Ahvenjarvi et al., 2002) and microbial OM.

The results of the rumen evacuations offered the basis of calculations for fractional rates of intake (k_i), passage (k_p) and digestion (k_d):

$$k_i = 1/24 \times (\text{intake, kg/d})/(\text{rumen pool size, kg});$$

$$k_p = 1/24 \times (\text{omasal canal flow, kg/d}) / (\text{rumen pool size, kg});$$

$$k_d = k_i - k_p.$$

Mean values of measurements from day 10-21 in each period were used for both feed intake and milk production results. All data were analyzed using the MIXED procedures of SAS software (SAS Institute Inc., 2012) with the following model:

$$Y_{ijkl} = \mu + c_i + D_j + P(S)_{kl} + S_l + e_{ijkl},$$

where μ is the overall mean, c is the random effect of cow (i = 1 through 8) and D (j = 1 through 4), P(S) (k = 1 through 4) and S (l = 1 and 2) are the fixed effects of diet, period within square and square. Period was calculated as a repeated week value for feed intake and milk production. Sum of squares were divided into orthogonal contrasts to assess linear and quadratic effects of the diets.

3. Results

3.1. Silage quality, feed intake and milk production

Chemical composition of the two grass-clover silages is given in Table 1. It shows higher concentrations of OM, water soluble carbohydrates and NDFom in the PG and higher concentrations of NH_3 and CP in the RG. The PG had higher concentration of Met but lower concentration of His compared to the RG. Both silages were well preserved, with restricted fermentation (low concentration of fermentation acids and no butyric acid; not presented) and low concentrations of NH_3 and pH. Intakes of DM and OM decreased whereas intakes of CP, NH_3, some AA (Asp, Cys, Glu, His, Phe, Ser, Thr and Tyr) and total non-essential AA increased with increasing proportions of RG (Table 2). The highest daily milk, milk fat, and milk protein yields were observed in D2 (Table 3). Accordingly, it was a quadratic effect of diet on energy corrected milk yield (ECM), with the lowest yield in D4. Milk urea concentrations increased with increasing proportions of RG.

3.2. Nitrogen metabolism, AA profile and blood metabolite

The omasal OM flow tended ($P = 0.09$) to decrease linearly with increasing RG proportions (Table 4). Similarly the share of microbial non-ammonia nitrogen (NAN) in total NAN flowing into the omasum decreased ($P = 0.01$) with increasing proportions of RG. There was no effect of diet on omasal flow of any individual AA or total AA (Table 5). The ruminal NH_3 concentration increased linearly with increasing proportions of RG (Table 6), whilst the N excretion through feces tended (P = 0.07) to decrease and urinal N excretion increased (P < 0.01) with increasing RG proportions. Total N excretion through feces and urine was highest for D4, measured as daily amount and as a proportion of ingested N (Table 6). Blood urea increased with increasing RG proportions (Table 7). Increasing PG proportions tended (P=0.07) to increase blood concentrations of Leu while Glu tended (P = 0.07) to be lower when mixed diets were fed.

4. Discussion

4.1. Feed intake and milk production

The purpose of the present study was to compare the effects of replacing primary growth of organic grass-clover silage with regrowth prepared from the same field of lactating dairy cows. Earlier studies have compared pure diets of grasses or legumes from the same cut or as mixtures of cuts (Bertilsson and Murphy, 2003; Dewhurst et al., 2003; Halmemies-Beauchet-Filleau et al., 2014). However, pure stands of grasses and legumes may have different chemical properties than when cultivated in mixtures. For instance, grasses are shown to have higher CP concentrations when grown in mixed leys with legumes and particularly with white clover (Gierus et al., 2012). The clover proportion increased from 0.18 in PG to 0.41 in RG, which is comparable to other studies (Steinshamn and Thuen, 2008; Steinshamn et al., 2015; Alstrup et al., 2016). Thus, the present results have applied relevance.

Silages were typical representatives of Fennoscandian organic silages with increasing CP concentration and decreasing metabolizable energy (ME) concentration from PG to RG (Steinshamn and Thuen, 2008). The decreasing DMI with increasing RG proportions was in line with studies on grass silages (Khalili et al., 2005; Kuoppala et al., 2008). The RG silage has usually a poorer digestibility than PG (Huhtanen et al., 2007), while feeding legumes generally increase DMI relative to grass (Dewhurst et al., 2003; Moorby et al., 2009). In the present study, the effect of legume was confounded with the effect of growth period, and the effect of growth period on DMI has likely been stronger than the effect of legume proportion.

Concentrate increased dietary CP concentrations in all diets. Still, CP concentrations in diets were below 165 g/kg DM. Calculated N-efficiency does usually not decrease significantly with increasing dietary CP concentrations below this level (Castillo et al., 2001;

Colmenero and Broderick, 2006). In the current experiment, highest milk production and lowest excretion of non-protein N in urine and milk was found when the pure PG diet with lowest CP content was fed. Moreover, highest energy utilization was observed on the RG dominated diets with highest CP concentration. Together, this suggests that the dietary ME concentration was too low in the pure RG diet for an optimal rumen microbial protein synthesis. The PBV was above recommended levels (Madsen et al., 1995). Legumes contain more RUP than grasses, which might offer insufficient N substrate for rumen microbial protein synthesis and a less ideal AA profile to the intestine (Vanhatalo et al., 2009). This shows the advantage of mixing PG and RG as they together complement each other in energy- and protein concentrations.

4.2. Total N supply

Increasing proportions of PG and decreasing N intake increased the rumen N outflow rate (k_p) in line with Vanhatalo et al. (2009). This was likely due to an improved microbial protein synthesis caused by more rumen digestible feed energy.

Origin of CP in omasal flow differed between diets. Similar to previous studies, the PG promoted a higher share of microbial NAN in total NAN compared to RG (Merry et al., 2006; Vanhatalo et al., 2009; Halmemies-Beauchet-Filleau et al., 2014). The larger RUP concentrations in legumes can explain this. However, no dietary effect was found in omasal flow of total NAN, which confirmed a proportionally greater microbial activity with greater intakes of ME and increasing proportions of PG in line with Halmemies-Beauchet-Filleau et al. (2014).

Milk protein was produced in similar quantities in diets containing PG, while milk protein production was slightly lower in the pure RG diet. The surplus N was converted into urea, displayed as increasing blood and milk concentrations with increasing RG proportions. Increasing RG proportions was related to higher NH_3 concentrations in rumen, and underpins that energy supply limited microbial protein synthesis. The low rumen NH_3 concentrations in the pure PG diet appeared to limit the neutral detergent fiber (NDF) digestion (Broderick et al., 2010). Higher NH_3 concentrations in the two mixed diets improved NDF digestibility, relatively to pure PG, in the present study. Urea concentrations in milk and blood were in the lower reference range (Kraft, 2005), in line with the low to moderate dietary N levels. Dietary CP concentrations were below 16.5 g/kg, and increasing levels of urea are not expected with sufficient quantities of ME (Castillo et al., 2001; Broderick, 2003; Colmenero and Broderick, 2006).

4.3. AA profile

Histidine has been recognized as the first limiting AA in grass silages (Vanhatalo et al., 1999; Korhonen et al., 2000), and Met has been proposed to be the first limiting AA in red clover (Vanhatalo et al., 2009). In the present study, the concentration of His increased from the PG to the RG and Met decreased from the PG and the RG, and concentrations were similar to silages from other studies (Vanhatalo et al., 2009; Halmemies-Beauchet-Filleau et al., 2014). Lee et al. (2012) found that the ideal proportion of both Met and His should be at a 0.022 proportion of MP. In the current study, His concentrations were slightly lower in both silages. However, the His concentrations were greater than the Met concentrations. Vyas and Erdman (2009) predicted that an intake at 40 g/d of Met and 130 g/d of Lys would be sufficient for a 1000 g of daily milk protein yield, which is comparable to the present study.

Intakes of Lys were higher than 130 g/d while Met were around 40 g/d, making Met possibly more limiting than Lys. The increasing intakes of His with increasing proportions of RG may confirm a possible limitation in grass silages (Vanhatalo et al., 1999; Korhonen et al., 2000). All the observed differences in intakes of AA disappeared when the digesta entered the intestine. Increasing RG proportions offered a greater total AA intake but all diets provided similar quantities of AA to the intestine due to greater microbial protein synthesis in rumen and lower N-intake in diets with increasing PG proportions.

Leucine is proposed to be the first limiting AA in rapeseed meal (Boisen et al., 2000). The proportions of Leu in the omasal flows in this study were around 0.19 of EAA and slightly lower than recommendations (National Research Council, 2001). The concentrate contained 150 g/kg DM rapeseed meal and had a lower Leu concentration than both experimental silages. Ideally, animal feeding in organic farming should be based on local produced feedstuff, and rapeseed is a useful protein source that can be grown in temperate climates (Huhtanen et al., 2011). Rapeseed has shown a better production potential in diets based on organic grass-clover silages compared to peas in cold-temperate climate (Khalili et al., 2002).

We hypothesized that increasing dietary RG proportions would increase AA flow to the small intestine. However, the flows were similar for all diets and the hypothesis was rejected. In addition, the second hypothesis was rejected, as this study could not support that milk protein synthesis in the pure RG diet was limited by a less balanced AA profile compared to diets including PG.

5. Conclusion

Increasing dietary proportions of RG silage increased daily intakes of CP, total AA and some individual AA, including His, but neither the total AA flow to the intestine nor the flow of any individual AA differed between diets. Higher daily yields of milk and milk solids were observed for cows on the mixed diets than on the pure PG or RG diets. A more complete NDF digestion caused by higher rumen NH_3 concentrations with the mixed diets might have provided those cows with more energy than the pure PG diet, and therefore increased milk yield. Intakes of CP from these grass-clover silages were not the most limiting factor for milk production. Energy intake seemed to be more important. Met seemed to be the first limiting AA in the grass-clover silages with His as a possible second limiting AA. Low level of Leu in the concentrate mixture probably related to rapeseed meal inclusion might also have been a potential limiting AA.

Acknowledgements

The project was funded by the Norwegian Agricultural Agreement Research Fund (Project number 207755 in The Research Council of Norway), the County Governors of Sør- and Nord-Trøndelag, the Sør- and Nord-Trøndelag County Authorities, TINE SA and the Norwegian Agricultural Extension Service. The authors have no financial or other conflict of interest in the manuscript. Further, the authors acknowledge Torstein Garmo for his help with botanical composition and the always helpful staff at the experimental unit led by Dag Kristoffer Forberg.

References

Ahvenjarvi, S., Vanhatalo, A., Huhtanen, P., 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: I. Rumen degradability and microbial flow. J Anim Sci 80, 2176-2187, doi:/2002.8082176x.

Ahvenjarvi, S., Vanhatalo, A., Huhtanen, P., Varvikko, T., 2000. Determination of reticulo-rumen and whole-stomach digestion in lactating cows by omasal canal or duodenal sampling. Brit J Nutr 83, 67-77, DOI: http://dx.doi.org/10.1017/S0007114500000106.

Alstrup, L., Søegaard, K., Weisbjerg, M., 2016. Effects of maturity and harvest season of grass-clover silage and of forage-to-concentrate ratio on milk production of dairy cows. J Dairy Sci 99, 328-340, doi:10.3168/jds.2015-9802.

Bertilsson, J., Murphy, M., 2003. Effects of feeding clover silages on feed intake, milk production and digestion in dairy cows. Grass and Forage Sci 58, 309-322, DOI: 10.1046/j.1365-2494.2003.00383.x.

Boisen, S., Hvelplund, T., Weisbjerg, M.R., 2000. Ideal amino acid profiles as a basis for feed protein evaluation. Livest Prod Sci 64, 239-251, doi:10.1016/S0301-6226(99)00146-3.

Broderick, G.A., 2003. Effects of varying dietary protein and energy levels on the production of lactating dairy cows. J Dairy Sci 86, 1370-1381, doi:10.3168/jds.S0022-0302(03)73721-7.

Broderick, G.A., Huhtanen, P., Ahvenjarvi, S., Reynal, S.M., Shingfield, K.J., 2010. Quantifying ruminal nitrogen metabolism using the omasal sampling technique in cattle--a meta-analysis. J Dairy Sci 93, 3216-3230 doi:10.3168/jds.2009-2989.

Castillo, A., Kebreab, E., Beever, D., Barbi, J., Sutton, J., Kirby, H., France, J., 2001. The effect of protein supplementation on nitrogen utilization in lactating dairy cows fed grass silage diets. J Animal Sci 79, 247-253, doi:/2001.791247x.

Colmenero, J.J.O., Broderick, G.A., 2006. Effect of Dietary Crude Protein Concentration on Milk Production and Nitrogen Utilization in Lactating Dairy Cows. J Dairy Sci 89, 1704-1712, doi:10.3168/jds.S0022-0302(06)72238-X.

Counc. of the Eur. Union, 2007. Council Regulation (EC) No. 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No 2092/91. Off. J. Eur. Communities 189, 20.7.2007, 1-23, http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32007R0834.

Dewhurst, R., Fisher, W., Tweed, J., Wilkins, R., 2003. Comparison of grass and legume silages for milk production. 1. Production responses with different levels of concentrate. J Dairy Sci 86, 2598-2611, doi:10.3168/jds.S0022-0302(03)73855-7.

Doepel, L., Lapierre, H., Kennelly, J., 2002. Peripartum performance and metabolism of dairy cows in response to prepartum energy and protein intake. J Dairy Sci 85, 2315-2334, doi:10.3168/jds.S0022-0302(02)74312-9.

Eriksen, J., Askegaard, M., Søegaard, K., 2012. Complementary effects of red clover inclusion in ryegrass–white clover swards for grazing and cutting. Grass and Forage Sci 69, 241-250, DOI: 10.1111/gfs.12025.

France, J., Siddons, R.C., 1986. Determination of digesta flow by continuous marker infusion. J Theor Biol 121, 105-119, doi:10.1016/S0022-5193(86)80031-5.

Gierus, M., Kleen, J., Loges, R., Taube, F., 2012. Forage legume species determine the nutritional quality of binary mixtures with perennial ryegrass in the first production year. Anim Feed Sci Tech 172, 150-161, doi:10.1016/j.anifeedsci.2011.12.026.

Halmemies-Beauchet-Filleau, A., Vanhatalo, A., Toivonen, V., Heikkilä, T., Lee, M.R.F., Shingfield, K.J., 2014. Effect of replacing grass silage with red clover silage on nutrient digestion, nitrogen metabolism, and milk fat composition in lactating cows fed diets containing a 60:40 forage-to-concentrate ratio. J Dairy Sci 97, 3761-3776, doi:10.3168/jds.2013-7358.

Huhtanen, P., Hetta, M., Swensson, C., 2011. Evaluation of canola meal as a protein supplement for dairy cows: A review and a meta-analysis. Can J Anim Sci 91, 529-543, http://dx.doi.org/10.4141/cjas2011-029.

Huhtanen, P., Rinne, M., Nousiainen, J., 2007. Evaluation of the factors affecting silage intake of dairy cows: a revision of the relative silage dry-matter intake index. Int J Anim Biosci 1, 758-770, DOI: http://dx.doi.org/10.1017/S175173110773673X.

Khalili, H., Kuusela, E., Suvitie, M., Huhtanen, P., 2002. Effect of protein and energy supplements on milk production in organic farming. Anim Feed Sci Tech 98, 103-119, doi:10.1016/S0377-8401(02)00005-6.

Khalili, H., Sairanen, A., Nousiainen, J., Huhtanen, P., 2005. Effects of silage made from primary or regrowth grass and protein supplementation on dairy cow performance. Livest Prod Sci 96, 269-278, doi:10.1016/j.livprodsci.2005.02.007.

Korhonen, M., Vanhatalo, A., Varvikko, T., Huhtanen, P., 2000. Responses to graded postruminal doses of histidine in dairy cows fed grass silage diets. J Dairy Sci 83, 2596-2608, doi:10.3168/jds.S0022-0302(00)75153-8.

Kraft, W., 2005. Klinische Labordiagnostik in der Tiermedizin, sixth ed. Schattauer Verlag, Stuttgart.

Krizsan, S.J., Ahvenjärvi, S., Volden, H., Broderick, G.A., 2010. Estimation of rumen outflow in dairy cows fed grass silage-based diets by use of reticular sampling as an alternative to sampling from the omasal canal. J Dairy Sci 93, 1138-1147, doi:10.3168/jds.2009-2661.

Kuoppala, K., Rinne, M., Nousiainen, J., Huhtanen, P., 2008. The effect of cutting time of grass silage in primary growth and regrowth and the interactions between silage quality and concentrate level on milk production of dairy cows. Livest Sci 116, 171-182, doi:10.1016/j.livsci.2007.10.002.

Lee, C., Hristov, A., Cassidy, T., Heyler, K., Lapierre, H., Varga, G., de Veth, M., Patton, R., Parys, C., 2012. Rumen-protected lysine, methionine, and histidine increase milk protein yield in dairy cows fed a metabolizable protein-deficient diet. J Dairy Sci 95, 6042-6056, doi:10.3168/jds.2012-5581.

Madsen, J., Hvelplund, T., Weisbjerg, M.R., Bertilsson, J., Olsson, I., Spörndly, R., Harstad, O.M., Volden, H., Tuori, M., Varvikko, T., Huhtanen, P., Olafsson, B.L., 1995. The AAT/PBV protein evaluation system for ruminants - a revision. Norweg J Agric Sci Suppl, Vol 19, 3-37.

Merry, R.J., Lee, M.R., Davies, D.R., Dewhurst, R.J., Moorby, J.M., Scollan, N.D., Theodorou, M.K., 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. J Anim Sci 84, 3049-3060, doi:10.2527/jas.2005-735.

Mjoun, K., Kalscheur, K.F., Hippen, A.R., Schingoethe, D.J., 2010. Performance and amino acid utilization of early lactation dairy cows fed regular or reduced-fat dried distillers grains with solubles. J Dairy Sci 93, 3176-3191, doi:10.3168/jds.2009-2974.

Moorby, J., Lee, M.R., Davies, D., Kim, E.J., Nute, G., Ellis, N., Scollan, N.D., 2009. Assessment of dietary ratios of red clover and grass silages on milk production and milk quality in dairy cows. J Dairy Sci 92, 1148-1160, doi:10.3168/jds.2008-1771.

Naadland, S.S., Steinshamn, H., Randby, Å.T., 2015. Effect of replacing organic grass-clover silage from primary growth with regrowth on feed intake and milk yield of dairy cows, Org Agr, Springer, http://link.springer.com/article/10.1007/s13165-015-0144-0, pp. 1-11, DOI 10.1007/s13165-015-0144-0.

National Research Council, 2001. Nutrient Requirements of Dairy Cattle: Seventh Revised Edition, 2001. The Natl Acad Press, Wash. DC, DOI: 10.17226/9825.

Norwegian Ministry of Agriculture and Food, 2010. FOR-2010-07-08-108, Forskrift om bruk av dyr i forsøk, 01.07.2015, https://lovdata.no/dokument/SFO/forskrift/2010-07-08-1085.

Njåstad, K.M., Adler, S.A., Hansen-Møller, J., Thuen, E., Gustavsson, A.-M., Steinshamn, H., 2014. Gastrointestinal metabolism of phytoestrogens in lactating dairy cows fed silages with different botanical composition. J Dairy Sci 97, 7735-7750, doi:10.3168/jds.2014-8208.

Reverter, M., Lundh, T., Lindberg, J.E., 1999. Ileal amino acid digestibilities in pigs of barley-based diets with inclusion of lucerne (Medicago sativa), white clover (Trifolium repens), red clover (Trifolium pratense) or perennial ryegrass (Lolium perenne). Brit J Nutr 82, 139-147, http://dx.doi.org/10.1017/S0007114599001294.

SAS Institute Inc., 2012. SAS/STAT® User's Guide. Cary, NC: SAS Institute Inc. .

Steinshamn, H., Adler, S., Frøseth, R., Lunnan, T., Torp, T., Bakken, A., 2015. Yield and herbage quality from organic grass clover leys—a meta-analysis of Norwegian field trials. Org Agr, 1-16, DOI 10.1007/s13165-015-0137-z.

Steinshamn, H., Thuen, E., 2008. White or red clover-grass silage in organic dairy milk production: Grassland productivity and milk production responses with different levels of concentrate. Livest Sci 119, 202-215, doi:10.1016/j.livsci.2008.04.004.

Vanhatalo, A., Huhtanen, P., Toivonen, V., Varvikko, T., 1999. Response of dairy cows fed grass silage diets to abomasal infusions of histidine alone or in combinations with methionine and lysine. J Dairy Sci 82, 2674-2685, doi:10.3168/jds.S0022-0302(99)75524-4.

Vanhatalo, A., Kuoppala, K., Ahvenjärvi, S., Rinne, M., 2009. Effects of feeding grass or red clover silage cut at two maturity stages in dairy cows. 1. Nitrogen metabolism and supply of amino acids. J Dairy Sci 92, 5620-5633, doi:10.3168/jds.2009-2249.

Vyas, D., Erdman, R.A., 2009. Meta-analysis of milk protein yield responses to lysine and methionine supplementation. J Dairy Sci 92, 5011-5018, doi:10.3168/jds.2008-1769.

Table 1

The chemical composition of organic grass-clover silages (n = 16) and concentrate (n = 4) offered dairy cows

	Primary growth		Regrowth		Concentrate	
Item	Mean	SE	Mean	SE	Mean	SE
Dry matter, g/kg	369	0.5	336	0.4	876	3.9
pH	4.43	0.012	4.31	0.010		
g/kg dry matter						
Organic matter	932	0.47	915	0.48	922	0.69
CP [a]	116	1.00	138	0.90	165	0.25
NH_3	0.212	0.0269	0.309	0.0269		
Water soluble carbohydrates	39.3	1.99	26.0	0.64	63.6	0.86
NDF [b]	501	3.4	473	2.0	154	2.8
ADL [c]	39.0	2.61	37.5	0.52	33.0	3.41
AA [d] g/100 g CP						
Cys	0.83	0.019	0.82	0.013	1.78	0.123
Met	1.49	0.035	1.35	0.039	1.18	0.106
Asp	9.14	0.169	10.30	0.206	8.43	0.813
Thr	4.49	0.117	4.51	0.199	3.33	0.258
Ser	4.12	0.109	4.19	0.183	3.71	0.315
Glu	10.19	0.171	10.13	0.270	17.10	1.350
Pro	4.90	0.089	4.75	0.083	4.95	0.354
Gly	4.77	0.111	4.70	0.148	3.47	0.264
Ala	6.42	0.159	6.03	0.174	3.43	0.369
Val	5.83	0.158	5.54	0.223	3.82	0.297
Ile	4.80	0.139	4.61	0.199	3.42	0.314
Leu	8.31	0.218	7.95	0.276	5.98	0.520
Tyr	2.91	0.087	2.46	0.105	2.15	0.198
Phe	5.27	0.125	5.25	0.164	3.94	0.343
His	1.83	0.031	2.00	0.069	2.20	0.185
Lys	5.27	0.074	5.05	0.165	5.19	0.449
Arg	3.65	0.062	3.43	0.083	5.68	0.464
BCAA [e]	18.9	0.51	18.1	0.70	13.2	1.13
NEAA [f]	43.3	0.86	43.4	1.06	45.0	3.63
EAA [g]	40.9	0.95	39.7	1.40	34.7	2.92

[a] Crude protein.
[b] Neutral detergent fiber.
[c] Acid detergent lignin.
[d] Amino acid
[e] Branched-chain amino acids (Val, Ile and Leu).
[f] Non-essential amino acids (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr).
[g] Essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val).

Table 2

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on feed intake (n = 8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
Dry matter intake, kg/d							
Grass-clover silage	15.1	14.9	14.4	14.1	0.70	<0.01	0.55
Total	22.1	21.9	21.4	21.0	0.70	<0.01	0.56
Intake							
Organic matter, kg/d	20.5	20.3	19.7	19.3	0.64	<0.01	0.51
NDF [a], kg/d	8.64	8.40	7.96	7.72	0.382	<0.01	1.00
Water soluble carbohydrates, g/d	1057	1048	1001	987	30.2	0.02	0.92
N, g/d	464	475	480	492	15.5	<0.01	0.79
AAT [b], g/d	1584	1549	1484	1439	55.5	<0.01	0.76
PBV [c], g/d	139	257	392	541	10.6	<0.01	0.15
MJ ME/d [d]	239	235	224	217	5.1	<0.01	0.38
Intake g/d							
Cys	35.1	35.5	35.7	36.1	0.87	0.05	0.96
Met	39.6	39.6	39.2	38.9	1.57	0.32	0.76
Asp	257.0	269.2	278.8	292.7	10.09	<0.01	0.82
Thr	117.0	119.5	120.7	122.6	4.93	0.03	0.84
Ser	114.9	117.7	119.1	121.3	4.53	0.01	0.89
Glu	375.5	381.3	383.2	388.7	10.61	0.03	0.97
Pro	142.9	144.7	145.3	146.9	4.95	0.17	0.98
Gly	123.4	125.6	126.4	128.1	5.10	0.08	0.88
Ala	151.9	153.1	152.3	152.4	6.67	0.94	0.78
Val	146.0	147.6	147.1	147.6	6.14	0.65	0.77
Ile	123.4	125.0	124.9	125.5	5.14	0.43	0.77
Leu	214.4	216.8	216.7	217.6	8.80	0.47	0.80
Tyr	75.7	74.6	72.6	70.3	3.00	<0.01	0.54
Phe	137.6	140.4	141.6	144.0	5.64	0.04	0.92
His	57.4	59.5	61.0	63.3	5.03	<0.01	0.89
Lys	152.1	153.9	153.7	154.9	5.49	0.38	0.88
Arg	129.5	130.3	129.9	130.3	3.72	0.73	0.87
BCAA [e]	484	489	489	491	20.1	0.51	0.78
NEAA [f]	1277	1302	1313	1336	45.7	0.02	0.95
EAA [g]	1117	1133	1135	1144	43.4	0.21	0.84
Total AA [h]	2393	2434	2448	2481	89.0	0.06	0.90

[a] Neutral detergent fiber.
[b] Amino acid to the intestine.
[c] Protein balance in rumen.
[d] ME, calculated according to Van Es (1978).
[e] Branched chain amino acid (Val, Ile and Leu).
[f] Non-essential amino acid (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr).

[g] Essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val).[h] Amino acid

Table 3

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on milk production (n = 8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
Milk, kg/d	30.5	30.9	30.8	29.9	1.53	0.14	0.05
ECM [a], kg/d	30.6	31.0	30.4	29.3	1.97	0.01	0.03
Milk composition							
Fat, g/kg	40.7	40.2	39.3	38.8	1.43	<0.01	0.99
Protein, g/kg	31.5	31.9	31.5	31.6	0.81	0.79	0.42
Lactose, g/kg	47.9	47.4	47.9	47.9	0.49	0.76	0.36
Urea, mmol/L	2.23	2.50	2.92	3.57	0.155	<0.01	0.02
Yield of milk components, g/d							
Fat	1248	1286	1228	1175	113	0.01	0.04
Protein	959	978	964	940	39.4	0.10	0.02
Lactose	1445	1430	1455	1409	66.9	0.71	0.39
ECM/MJ ME [b]	0.126	0.127	0.134	0.134	0.0034	<0.01	0.55
Milk N/Feed N	0.324	0.324	0.317	0.300	0.0099	<0.01	0.09

[a] Energy corrected milk.

[b] Metabolizable energy.

Table 4

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on daily omasal flow and digestibilities (n = 8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
Omasal canal flow, g/d							
OM [a]	11373	11052	10152	10651	524	0.09	0.29
MNAN [b]	339	335	310	333	17.9	0.49	0.37
DNAN [c]	203	215	195	242	17.2	0.12	0.22
TNAN [d]	541	549	506	573	32.0	0.62	0.22
CP	3142	3183	2971	3287	183	0.73	0.34
MNAN/TNAN g/kg	630	611	613	577	14.0	0.01	0.51
AA/CP g/kg [e]	86.7	85.4	86.3	86.4	0.74	0.79	0.61
Digestibility in rumen							
OM, true	0.62	0.65	0.67	0.63	0.013	0.40	0.04
NDF	0.58	0.64	0.61	0.59	0.021	0.99	0.02
CP, true	0.65	0.64	0.66	0.61	0.025	0.36	0.35
Digestibility in total tract							
OM, apparent	0.74	0.76	0.76	0.75	0.006	0.11	0.15
NDF	0.64	0.66	0.66	0.67	0.011	0.06	0.54
CP	0.69	0.71	0.72	0.73	0.0061	0.00	0.22

[a] Organic matter

[b] Microbial non-ammonia nitrogen.

[c] Dietary non-ammonia nitrogen.

[d] Total non-ammonia nitrogen.

[e] Amino acids in total CP.

Table 5

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on omasal flow of amino acids (n = 8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
Omasal canal flow, g/d							
Cys	39.9	39.8	37.5	41.8	2.31	0.67	0.24
Met	57.8	57.5	53.8	60.0	3.29	0.84	0.26
Asp	331	335	310	350	21.1	0.66	0.28
Thr	146	147	136	153	8.7	0.78	0.27
Ser	121	122	114	126	7.2	0.78	0.36
Gln	426	425	394	437	25.1	0.99	0.31
Pro	119	116	111	124	7.2	0.73	0.24
Gly	134	135	127	141	7.4	0.65	0.34
Ala	170	170	155	171	8.7	0.77	0.31
Val	165	166	154	171	9.2	0.85	0.31
Ile	169	170	157	177	10.9	0.77	0.29
Leu	232	234	219	244	14.0	0.68	0.31
Tyr	90.2	89.4	87.6	95.5	6.75	0.60	0.47
Phe	154	155	146	164	9.8	0.57	0.26
His	52.2	52.8	49.9	55.5	3.05	0.53	0.33
Lys	182	189	173	198	13.9	0.50	0.38
Arg	140	141	137	148	9.0	0.56	0.49
BCAA [a]	566	570	530	592	34.0	0.75	0.30
EAA [b]	1298	1312	1225	1370	81.0	0.66	0.31
NEAA [c]	1431	1433	1337	1485	84.4	0.83	0.30
Total amino acids	2730	2745	2562	2855	165.2	0.75	0.31

[a] Branched-chain amino acids (Val, Ile and Leu).
[b] Non-essential amino acids (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr).
[c] Essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val).

Table 6

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on rumen pool size, passage, digestion kinetics and excretion (n=8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
Rumen content, kg	87.6	87.6	85.2	89.0	3.51	0.81	0.28
Rumen contents, kg							
Dry matter	10.9	11.0	10.7	11.0	0.46	1.00	0.58
Organic matter	10.0	10.1	9.7	10.0	0.43	0.73	0.64
N	0.268	0.284	0.292	0.313	0.0129	<0.01	0.64
NH3-N, mmol/L	4.90	6.37	6.97	8.43	0.520	<0.01	0.99
g/kg / h							
Organic matter, k_p [a]	63	62	59	58	3.7	0.15	0.89
Organic matter, k_d [b]	23	24	27	24	2.5	0.64	0.33
NDF, kp	24	20	22	22	1.4	0.46	0.07
NDF, kd	32	36	34	33	2.4	0.97	0.31
N, k_p	79	75	68	67	4.5	0.02	0.68
N, k_d	-5.2	-3.1	1.4	0.5	3.27	0.11	0.60
Feces							
Dry matter, kg/d	5.86	5.66	5.41	5.42	0.169	<0.01	0.25
N, g/d	152.4	146.8	145.0	145.3	4.60	0.07	0.28
Urine N, g/d	87.5	98.8	106.1	125.9	3.62	<0.01	0.18
N in feces and urine, g/d	239.9	245.0	251.1	270.7	7.41	<0.01	0.15
N balance g/d [c]	71.5	85.4	80.2	78.0	8.00	0.67	0.29

[a] Rate of passage.

[b] Rate of digestion.

[c] N balance = N intake − (N in milk + N in feces + N in urine).

Table 7

Effect of replacing silages prepared from primary growth with regrowth in the DM ratio 0, 0.33, 0.67 and 1 (Diet D1, D2, D3, and D4, respectively) in the diet of lactating dairy cows on blood metabolites and amino acids from a coccygial blood vessel (n=8)

	Diet					Orthogonal contrasts	
Item	D1	D2	D3	D4	SEM	Linear	Quadratic
mMol/L							
BHBA [1]	1.11	1.06	1.04	0.98	0.109	0.14	0.94
Urea	1.85	2.47	2.81	3.65	0.224	<0.01	0.59
Total amino acids	3.81	3.34	2.51	3.53	0.166	0.38	0.15
μMol/L							
Cys	13.3	10.8	11.5	11.8	3.18	0.77	0.64
Met	29.7	28.8	33.3	28.4	2.50	0.97	0.42
Asp	11.1	10.0	10.6	11.2	1.02	0.77	0.30
Thr	140.8	133.3	142.8	120.5	12.54	0.36	0.55
Ser	131.9	123.1	137.0	132.2	9.41	0.72	0.83
Glu	76.7	61.7	73.4	76.9	4.91	0.57	0.07
Pro	104.7	90.2	103.5	103.5	8.19	0.79	0.38
Gly	427.5	371.1	413.9	447.7	31.07	0.45	0.15
Ala	338.7	296.6	299.8	290.3	19.42	0.11	0.40
Val	333.2	266.3	291.0	281.2	21.11	0.17	0.18
Ile	185.3	158.3	176.7	164.5	13.11	0.45	0.57
Leu	147.3	115.4	123.4	112.7	11.33	0.07	0.35
Tyr	57.6	50.2	55.3	51.2	4.08	0.44	0.68
Phe	53.5	46.9	52.8	50.0	3.32	0.75	0.57
His	41.2	28.7	28.1	30.7	7.18	0.32	0.29
Lys	131.5	105.5	110.8	112.8	9.43	0.23	0.15
Arg	98.4	75.3	84.0	80.6	7.84	0.17	0.18
Gln	267.9	241.5	280.2	267.0	18.16	0.65	0.71
Trp	50.0	42.9	55.8	52.2	4.53	0.33	0.70
BCAA [2]	666	540	591	558	43.4	0.17	0.29
EAA [3]	1310	1086	1194	1115	78.9	0.18	0.36
NEAA [4]	1515	1328	1468	1460	73.7	0.94	0.23
EAA g/kg of TAA [5]	464	452	446	443	11.5	0.20	0.75
NEAA g/kg of TAA	536	548	554	557	11.5	0.20	0.75

[1] Betahydroxy butyric acid.
[2] Branched-chain amino acids (Val, Ile and Leu).
[3] essential amino acids (Ala, Asn, Asp, Cys, Gln, Glu, Gly, Pro, Ser, and Tyr).
[4] Non-Essential amino acids (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val).
[5] Total amino acid.