Plasma proteins $\delta^{15}N$ vs plasma urea as candidate biomarkers of between-animal variations of feed efficiency in beef cattle: phenotypic and genetic evaluation

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Abstract

Identifying animals that are superior in terms of feed efficiency may improve the profitability and sustainability of the beef cattle sector. However, measuring feed efficiency is costly and time-consuming. Biomarkers should thus be explored and validated to predict between-animal variation of feed efficiency for both genetic selection and precision feeding. In this work, we aimed to assess and validate two previously identified biomarkers of nitrogen (**N**) use efficiency in ruminants, plasma urea concentrations and the ^{15}N natural abundance in plasma proteins (**plasma $\delta^{15}N$**), to predict the between-animal variation in feed efficiency when animals were fed two contrasted diets (high-starch vs high-fibre diets). We used an experimental network design with a total of 588 young bulls tested for feed efficiency through two different traits (feed conversion efficiency [**FCE**] and residual feed intake [**RFI**]) during at least 6 months in 12 cohorts (farm × period combination). Animals reared in the same cohort, receiving the same diet and housed in the same pen were considered as a contemporary group (**CG**). To analyse between-animal variations and explore relationships between biomarkers and feed efficiency two statistical approaches, based either on mixed-effect models or regressions from residuals, were conducted to remove the between-CG variability. Between-animal variation of plasma $\delta^{15}N$ was significantly correlated with feed efficiency measured through the two criteria traits and regardless of the statistical approach. Conversely, plasma urea was not correlated to FCE and showed only a weak, although significant, correlation with RFI. The response of plasma $\delta^{15}N$ to FCE variations was higher when animals were fed a high-starch compared to a high-fibre diet. In addition, we identified two dietary factors, the metabolisable protein to net energy ratio and the rumen protein balance,

that influenced the relation between plasma $\delta^{15}N$ and FCE variations. Concerning the genetic evaluation, and despite the moderate heritability of the two biomarkers (0.28), the size of our experimental setup was insufficient to detect significant genetic correlations between feed efficiency and the biomarkers. However, we validated the potential of plasma $\delta^{15}N$ to phenotypically discriminate two animals reared in identical conditions in terms of feed efficiency as long as they differ by at least 0.049 g/g for FCE and 1.67 kg/d for RFI. Altogether, the study showed phenotypic, but non-genetic, relationships between plasma proteins $\delta^{15}N$ and feed efficiency, that varied according to the efficiency index and the diet utilised.

Keywords: feed conversion efficiency, biomarkers, individual variability, ^{15}N natural abundance, ruminants

Implications

Beef fattening cattle fed the same diet in the same contemporary group show individual variability in their ability to transform the feed into gain. We validated the potential of a new biomarker, the ^{15}N natural abundance in plasma, to identify most efficient cattle within contemporary groups. The use of such a biomarker may help farmers and producers to make decisions in the context of precision feeding and thus improve the profitability and sustainability of the beef cattle industry.

Introduction

Feed efficiency (**FE**) is defined as the animal ability to transform feed resources into animal products. In a context of increasing demand for protein resources, of necessities for the industry to lower production costs and to improve the sustainability of livestock systems, FE should be improved (Cantalapiedra-Hijar., 2018a). Feed efficiency is usually characterised through different traits, described as ratios between animal inputs and outputs (feed conversion efficiency [**FCE**]), or as residual regression traits (residual feed intake [**RFI**]) (Berry and Crowley, 2013). In the case of ruminants, especially beef cattle, feed efficiency is low compared to other livestock species (Tolkamp, 2010), but also highly variable between animals raised in the same conditions (17% CV, Arthur et al., 2001). In addition, feed efficiency evaluated by either FCE or RFI is moderately heritable (Taussat et al., 2019), which gives rise to the possibility of improving this animal trait by genetic selection (Archer et al., 1999). However, a major limit for the genetic selection remains the phenotypic measurement of feed efficiency itself, which requires at least 70 days of individual DM intake (**DMI**) and BW gain recording and which, as a result, is expensive, laborious and not always feasible on a large number of animals or in extensive conditions. It is thus necessary to find alternative tools that are accurate, phenotypically and genetically related to feed efficiency traits and that can be used to predict feed efficiency in field conditions and to select animals for this trait.

In this regard, biomarkers of between-animal variation of feed efficiency constitute interesting alternative tools. In animals fed the same diet, feed efficiency is by definition mathematically associated to the nitrogen (**N**) use efficiency, which reflects the partition of N intake between N excretion and N accretion. Nitrogen use efficiency

(**NUE**) depends on both dietary conditions and individual animal protein metabolism (Dijkstra et al., 2013). Therefore, biomarkers of feed efficiency could be searched among proxies related to NUE (Jonker et al., 1998; Cantalapiedra-Hijar et al., 2018b).

A classical biomarker of N partitioning and NUE in ruminants is the urea concentration in milk or blood (Kohn et al., 2005b). Although its ability to discriminate dietary treatments in terms of N utilization is unquestionable (McNamara et al., 2003), no conclusive results have been obtained about its potential to reflect between-animal variations in NUE (Hof et al., 1997; Huhtanen et al., 2015). An alternative potential biomarker to predict NUE is based on the difference in ^{15}N natural abundance between the diet and the animal proteins, the called isotopic N fractionation (**$\Delta^{15}N_{animal-diet}$**), which has the potential to reflect both dietary and individual effects on NUE (Cantalapiedra-Hijar et al., 2018b). Nitrogen naturally exists as two stable isotopes, ^{15}N and ^{14}N, and $\delta^{15}N$ notation reflects the relative abundance of the heaviest isotope. The $\delta^{15}N$ in animal proteins results from the $\delta^{15}N$ in the diet and the N isotopic fractionation occurring between N ingestion and N retention in animal tissues. The $\Delta^{15}N_{animal-diet}$ represents thus the difference between the isotopic signature of the product (animal proteins) and the substrate (feed consumed), and arises from rumen microbial and hepatic enzymatic activity (Silfer et al., 1992). Microbial and hepatic enzymes prefer substrates (ammonia and amino acids) containing the lighter N isotope (Macko et al., 1986; Wattiaux and Reed, 1995), which results in a greater excretion of ^{14}N in urine and greater retention of ^{15}N in animal proteins (Ganes et al., 1998). It is expected thus that efficient ruminants have lower $\Delta^{15}N_{animal-diet}$ values as a result of a lower amino acid catabolism (Cantalapiedra-Hijar et al., 2015) and higher ammonia uptake by rumen bacteria (Wattiaux and Reed, 1995). In this regard, $\Delta^{15}N_{animal-diet}$ was shown to be significantly

related to FCE (Wheadon et al., 2014; Cantalapiedra-Hijar et al., 2015) but not to RFI (Wheadon et al., 2014; Meale et al., 2017) in beef cattle. It is noteworthy that if this isotopic biomarker is used for discriminating individuals fed the same diet there is no need to know the $\delta^{15}N$ of diets (Wheadon et al., 2014) since all compared individuals share the same dietary $\delta^{15}N$ values. This may represent a considerable gain in the applicability of this biomarker in field conditions, since only $\delta^{15}N$ of animal proteins (as plasma proteins) rather than $\Delta^{15}N_{animal\text{-}diet}$ could capture between-animal differences in feed efficiency provided that animals are fed with identical diets.

Recent studies in beef cattle suggested that the relationships at the individual level between FE and isotopic N signatures could be diet-dependent (Nasrollahi et al., 2020), but this have not been evaluated experimentally on a large number of animals. We assumed that the relationships at the individual level between $\delta^{15}N$ in plasma proteins (**plasma $\delta^{15}N$)** or plasma urea concentration and FE traits (FCE, RFI) differ and that each relationship is affected by the diet fed to animals. Moreover, to be used for genetic selection, genetic correlation between biomarkers and animal traits should also be confirmed. Thus, the objectives of this study were to evaluate i) the phenotypic and genetic relationships between plasma $\delta^{15}N$ or plasma urea concentration and the most common traits of feed efficiency (FCE and RFI), ii) to which extent these relationships are diet-dependent, and iii) the applicability of these candidate biomarkers in field conditions.

Material and methods

Experimental network design

This study was conceived as an experimental network, defined as a set of experiments sharing the same experimental protocol but conducted in a set of different environments (Makowski et al., 2019). The experimental network (Fig. 1) was constituted of 12 cohorts defined by the combination of four experimental farms located at different places in France (F.1, F.2, F.3, F.4) and several experimental periods within each experimental farm (2, 3 or 4 depending on the farm). The study started in 2015 and finished in 2019. Within each cohort, the same two contrasted diets (a grass silage-based diet rich in fibre vs a corn silage-based diet rich in starch) were tested. For each cohort, each animal (on average 55 individuals per cohort) was assigned to one of the two experimental diets (on average 27 animals per diet within a cohort). Within each cohort and diet, animals were grouped in pens (5 to 10 individuals/pen) according to their initial BW. As a result, half of the pens (n = 3-4) in each cohort was assigned to the grass silage diet while the other half (n = 3-4) was assigned to the corn silage diet. Only two cohorts out of 12 used only one pen per diet with a greater number of animals per pen. Therefore, each of the 72 individual pens represented a contemporary group (**CG**), defined as those animals that were reared in the same cohort, that had a similar initial body weight, and that were fed the same diet and housed in the same pen (Pereira et al., 2018). The experimental unit is the individual animal because treatments and measurements were applied at the animal level. The environmental factors contributing to the between-CG variability were therefore i) the cohort (n = 12), ii) the diet within each cohort (n = 2) and iii) the pen within diet and within cohort (1≤ n ≤4 depending on the cohort).

Animals and experimental diets

A total of 588 Charolais bulls (303 ± 25 days old and 393 ± 58 kg of BW at the onset of the experiment) were recorded for feed efficiency during a 200 ± 27 day test. The experimental animals were offspring of 70 known sires and were homogenously distributed across cohorts and diets according to their sire origin. The two experimental diets had a forage to concentrate ratio close to 60:40, were distributed as total-mixed rations and based on either corn or grass silage. The grass silage was mainly composed from grasses such as English ray grass or dactyl and legumes as violet clover. The concentrate was always composed of wheat grain and soybean meal, and beet pulp was added in the grass silage diet (Table 1). Both diets were iso-CP but differed slightly in their net energy levels (1.50 [grass silage diet] vs 1.63 [corn silage diet] Mcal/kg of DM; INRA, 2018). Diets differed by the nature of the carbohydrate, showing very contrasted NDF (48% vs 34%) and starch concentrations (6% vs 32%), for grass and corn silage diets, respectively, on a DM basis. Because of the different geographical locations and climatic conditions of farms, the chemical composition of silages changed slightly across cohorts. Therefore, in order to keep feed values as similar as possible for each type of diet, the proportions of feed ingredients varied slightly among cohorts (Table 1). Animals were fed *ad libitum* once a day between 0900 and 1030 and had free access to water throughout the experiment.

Measurements for feed efficiency

Animals underwent an adaptation period of 4 weeks before the FE test to allow them to adapt to facilities and diets. Their age during the FE test varied slightly across cohorts, it ranged from 10 (minimum) to 17 (maximum) at the start of the test, but all

196 FE tests always covered the period between 12 and 15 months of age. Animals were
197 weighed on two consecutive days at the beginning and at the end of the test, and
198 every 28 days in between, always at 1400h. Individual DMI was recorded daily with
199 an automatic intake recording system based on mangers placed on weighing cells
200 (Biocontrol, Rakkestad, Norway). Representative samples from each silo were
201 collected between one and three times per cohort.

202

203 ***Chemical analyses of feeds and calculation of feed values***

204

205 Chemical composition of silages (n=24) was determined by four different laboratories
206 in France (Départemental d'Analyses du Morbihan [www.morbihan.fr/lda/laboratoire-
207 departemental], CESAR laboratory [http://www.labo-cesar.com], Inovalys
208 [www.inovalys.fr] and INRAE Site de Theix [www6.ara.inrae.fr]) using similar
209 laboratory protocols. Dry matter and organic matter concentration were determined
210 by oven-drying (103°C and 72h) and subsequent incineration in a muffle furnace at
211 550°C (NF V 18-101), respectively. CP concentration was analysed by the Dumas
212 method (Ebeling, 1968), NDF according to Van Soest et al. (1991) and cellulose
213 according to the Weende method (Nehring, 1966). In vitro organic matter digestibility
214 was determined according to Aufrère et al. (2007) and starch was analysed by an
215 enzymatic method (ISO 15914:2004). Feed values of silages, as defined by INRA
216 (2018), were calculated from their chemical composition using the Prevalim®
217 software (https://wwwdev.okteo.fr/).

218 The chemical composition and feed values of concentrate ingredients were obtained
219 from tabulated values (INRA, 2018). Thereafter, the feed values of the complete
220 diets, integrating the digestive interactions, were estimated through the Inration V5®

221 software (https://wwwdev.okteo.fr/) from the measured (silages) or estimated
222 (concentrates) chemical composition of ingredients, the ingredient composition of
223 diets, and the observed average feeding level (DMI as %BW). Estimated dietary feed
224 values included net energy (Mcal/kg DM), metabolisable protein (g/kg DM), rumen
225 protein balance (g/kg DM), microbial protein synthesis (g/kg DM) and the rumen
226 degradable protein (% CP). Finally, we estimated for each animal the fecal
227 endogenous protein losses (**FEPL**) from the observed DMI and the non-digestible
228 organic matter of the diet (INRA, 2018) as follows:
229 FEPL (g/d) = DMI (kg/d) x (0.5 x (5.7+0.074 x non-digestible organic matter (g/kg
230 DM))
231
232 ***Blood sampling and analyses***
233
234 Blood samples were obtained from each animal one month before the end of test at
235 an animal age of 477±27 days. Sampling was done before meal distribution between
236 0800 and 1100 h. Blood was obtained by coccygeal venipuncture and collected into
237 two tubes of 9 ml each (BD vacutainer, Playmouth, UK) containing either lithium
238 heparin or ethylenediaminetetraacetic acid. Tubes were centrifuged at 2 500 × *g*
239 during 10 min at room temperature. The tube containing lithium heparin was used for
240 the determination $\delta^{15}N$ in plasma proteins, while the other containing EDTA was used
241 for plasma urea analysis. Plasma was stored at -80° C until laboratory analyses.
242 Urea concentration was analysed in duplicate for each plasma sample (1.5 ml) by
243 spectrophotometry with an automated analyser (Arene 20XT, Thermo Scientific,
244 Vaanta, Finland). The accuracy profile (NF V03-110: 2010) of the method determined

for concentrations ranging between 0.05 and 0.90 g/l yielded an average accuracy of 101% and an average CV for replicates of 8%.

The $\delta^{15}N$ was determined in plasma proteins isolated by precipitation by adding 15 µL of a sulfosalicylic acid solution (1g/mL) into 300 µL of plasma. Supernatant and pellet were separated after 15 min of centrifugation (5 000 g at 4°C) followed by 1 h of storage at 4°C. The pellet was rinsed three times with MilliQ water and freeze-dried. The $\delta^{15}N$ values were determined using an isotope-ratio mass spectrometer (Isoprime Vision; Elementar France) coupled to an elemental analyser (Vario cube; Elementar France) as described in Cantalapiedra-Hijar et al. (2020a). International standards (glutamic acid) were included in each run every 10 samples to correct for possible time-variations in the analysis. Results were expressed using the delta notation according to the following equation:

$$\delta^{15}N = [(R_{sample} / R_{standard}) - 1] \times 1\,000,$$

where R_{sample} is the N isotope ratio between the heavier isotope and the lighter isotope ($^{15}N/^{14}N$) for the sample being analysed, $R_{standard}$ the N isotope ratio between the heavier isotope and the internationally defined standard (atmospheric N2, $R_{standard}$ = 0.0036765), and δ is the delta notation in parts per 1 000 (‰) relative to the standard. Samples were analysed in duplicates and measurements errors for the analysed internal standard were lower than 1.1%CV. Through the manuscript, the $\delta^{15}N$ of plasma proteins will be referred to as plasma $\delta^{15}N$.

Feed efficiency calculations

The average daily gain (**ADG**) was calculated for each animal by regressing its BW over the time on test. Mid-test BW was calculated from the intercept and slope of the regression equation and using the mean time between the start and the end of the FE test. Mid-test BW was then expressed as mid-test metabolic BW by raising the former to the power of 0.75 ($BW^{0.75}$). The individual daily DMI was calculated as the average daily DMI throughout the test period. The FCE was calculated as the ratio between ADG and average DMI.

RFI was determined as the difference between observed DMI and the DMI expected for a given mid-test metabolic BW (**$BW^{0.75}$**) and ADG. To adjust RFI for the effect of CG, the RFI model included it as a fixed effect (Arthur et al., 2001) as follows:

$$Y = \beta_0 + CG + \beta_1 (BW^{0.75}) + \beta_2 (ADG) + e \quad \text{(Eq. 1)}$$

where Y is the observed individual daily DMI, β_0 is the intercept, CG is the CG effect, β_1 is the regression coefficient for $BW^{0.75}$, β_2 is the regression coefficient for ADG, and e is the residual of the model or RFI.

Statistical analyses

Statistical analyses were performed in R (RStudio Core Team, version 1.1.463, 2018). All variables were tested for normality with the Lillie test and homoscedasticity within each environmental factor included in the CG effect (cohort, diet and pen) with the Levene test (Nortest package in R). Differences between treatments (diets) were tested through parametric (variables with normal distribution) or non-parametric (variables with non-normal distribution; Kruskal-Wallis test) analysis and declared significant when $P \leq 0.05$.

293 *Analysis of sources of variation*

294 First, the between-animal variability expressed as CV (%) was calculated dividing the
295 SD observed within-CG (SDr) by the average values of recorded traits (Bender et al.,
296 1989). In the case of RFI, with an average value equal to 0, we divided SDr by the
297 average value of DMI.

298 We also determined the influence of each environmental factor (cohort, diet nested
299 within cohort, and pen nested within diet and cohort) on animal performances and
300 candidate plasma biomarkers values. For this, variance-estimates component
301 analyses were conducted with mixed-effect models to obtain the contribution of each
302 random environmental factor:

303 $Y = \beta_1 + E_c + E_d + E_p + \varepsilon$ (Eq.2)

304 β_1 is the mean value of the parameters in the population of individuals. The
305 deviations from the mean values are represented by the random effects of the cohort
306 (E_c), the diet within cohort (E_d) and the pen within diet within cohort (E_p). Finally, ε
307 represents the residual error of the model, which includes the between-animal
308 variation and the experimental error (Fisher et al., 2017).

309 The Intra-class correlation coefficient, which represents the percentage of variance
310 explained by each random environmental factor, was calculated as follows:

311 Intra-class correlation coefficient = $\sigma^2_i / (\sigma^2_c + \sigma^2_d + \sigma^2_p + \sigma^2_r)$ (Eq.3)

312 where, σ^2_i refers to the between-class variability (i.e. variance ascribed to each
313 random experimental factor) and $\sigma^2_c + \sigma^2_d + \sigma^2_p + \sigma^2_r$ represents the total variance
314 including the within-CG variability ($\sigma^2 r$).

315

316 *Relationships between feed efficiency traits and candidate plasma biomarkers*

317 To evaluate the relationships between FE traits and candidate plasma biomarkers at
318 individual level, it was necessary to remove all environmental factors contributing to
319 the observed variability across-CG and only keep the observed variability within each
320 CG group (i.e between-animal variability). For this, two different statistical
321 approaches were applied.

322 *i) Removing the between-contemporary group variability before linear regression*
323 *analysis*

324 The random effect of the CG was removed from raw values of each variable (Phuong
325 et al., 2013; Cantalapiedra-Hijar et al., 2018b) by running the same model as shown
326 in Eq. 2 and storing the obtained residuals (ε). Eq. 3 was applied to both candidate
327 biomarkers and FCE. However, because RFI was already adjusted for the CG effect,
328 this initial step was not applied to this FE trait. Residuals of FE traits (FCE or RFI)
329 were then linearly regressed (as dependent Y variable) against the residuals of the
330 candidate plasma biomarkers (X) with the lm function in the R software.

331 In addition, we evaluated whether combining both candidate plasma biomarkers
332 (plasma $\delta^{15}N$ and urea concentrations) improved the explanation of the FE variance.
333 For this, we first checked absence of multicolinearity through a variance inflation
334 factor test by the Car package, where values below 5 were indicative of non-
335 correlated variables (Vatcheva et al., 2016). Then, we tested the multivariable linear
336 model as follows:

337 $$Y = (\beta1 \times X) + (\beta2 \times Z) + \varepsilon \quad \text{(Eq. 4)}$$

338 where Y, X, Z are FE values (one equation for each FE trait), plasma $\delta^{15}N$ values
339 and plasma urea values respectively, with β1 and β2 being the slopes of plasma $\delta^{15}N$

and plasma urea, respectively. Comparisons between models were conducted through Anova analysis.

ii) Accounting for the between-contemporary group variability through mixed effect models

The mixed-effect model analysis aimed to determine if biomarkers were significantly correlated to FE traits at individual level (within-CG) and to evaluate to what extent the two contrasted dietary conditions influenced this relationship. For this, we first attempted to consider the diet as a fixed effect. However, given the complex nested structure of our experimental network (Fig. 1), where the pen is nested within the diet, we decided to separate the database into two sub-databases, one for each diet (n = 294 for each one). This allowed the effect of pen to be considered as a random effect nested to the cohort. The plasma $\delta^{15}N$ values were zero-centered by subtracting the overall mean to each individual value to avoid the expected correlation between intercept and slope occurring when values from the independent variable are far from zero (Pinheiro and Bates, 2002).

Different structures of random effect, from simple (with only the cohort effect) to nested factors (including the pen effect within the cohort) were tested on both intercept and slope as here detailed:

$$Y = (\beta 0 + B0) + (\beta 1 + B1) \times X + \varepsilon \quad \text{(Eq.5)}$$

where Y and X are FCE and $\delta^{15}N$ values, respectively, β0 and β1 are the fixed coefficients for the intercept and the slope respectively; B0 and B1 are the random coefficients of environmental factors (cohort and pen within cohort) and ε are the distributed within-groups errors, assumed to be independent of random effects. The best random structure was identified from AIC and BIC criteria and using the restricted maximum likehood method in the nlme package.

To evaluate the diet effect on the potential of the candidate biomarker to reflect FE variations we compared the slope (β1) of both models through a t-test. For this, we took into account the coefficient of slopes and their associated errors as well as the degrees of freedom of both sub-databases (Andrade and Estévez-Pérez, 2014). We also extracted the proportion of model's variance explained by random and fixed effect through the package r.squaredGLMM in the library MuMin.

Animal performances of individuals presenting extreme high-vs-low plasma proteins $\delta^{15}N$ values

We ranked animals within-CG according to their plasma $\delta^{15}N$ values and then selected those above the 90th percentile (High plasma $\delta^{15}N$ values) and those below the 10th percentile (Low plasma $\delta^{15}N$ values). Differences in animal performances and plasma biomarkers between these two groups (High vs Low) were conducted by Anova by including the effect of diet, group and its interaction.

Influence of dietary characteristics on the relationships at cohort level

We aimed to evaluate whether some characteristics of the experimental diets could influence the ability of candidate plasma biomarkers to reflect the between-animal variation in FE. Therefore, we first obtained the 24 individual slopes (responses) from the FE trait - biomarker relationships determined for each combination of diet and cohort (i.e. 24 unique conditions sharing exactly the same diet x cohort). Half of these individual slopes (12) were obtained with grass-diets and the other half with corn-diets. The 24 slopes coefficients were regressed against their 24 corresponding feed values and diet chemical composition. An ANCOVA model was used to evaluate

these regressions, where the FE-biomarker slope represented the dependent variable (Y), the type of diet (n=2) was the categorical variable (X_1) and feed values or chemical composition were the quantitative variable (X_2). The *P*-values were obtained for both independent variables and their interaction. When significant relationships were obtained, the relevant feed characteristic was considered as having an impact on the response of the candidate plasma biomarker to FE variations.

Model's evaluation and cross-validation

We first compared the quality of the different FE trait - biomarker relationships. The simple linear models based on residuals were compared based on their *P* value, R^2, AIC and RMSE to SD ratio (**RSR**), which allows comparing residual variables as RFI with mean intercept values equals to 0. The mixed effects models were compared based on their RSR and CV, calculated as the RMSE divided by the average of the variable Y. Fit was considered good when $R^2 > 0.50$ and RSR < 0.70 as recommended by Moriasi et al. (2007). Finally, normality of residuals was tested with the Shapiro Wilk's test (function shapiro.test in R) for every model.

We then evaluated the conditions of use of the $\delta^{15}N$ biomarker relative to each FE trait using a Kfold cross-validation (James et al., 2013) performed from simple linear models based on residuals. The database was randomly split in 10 sub-database (package Caret) and each individual sub-database was treated as a validation set. The performance of the FE trait-$\delta^{15}N$ relationships obtained from the whole database was defined as the average performance of the FE trait-$\delta^{15}N$ relationships derived after the 10 random iterations from each sub-database for each diet, as in Benaouda et al. (2019). Parameters used to evaluate the FE trait - biomarker relationships were

414 the RMSE of predicted vs observed values, the correlation between observed and
415 predicted values, the concordance correlation coefficient, as a parameter indicating
416 precision and accuracy of obtained models (Tedeschi, 2006) and the percentage of
417 error in central tendency, or mean bias as the average tendency to under or
418 overestimate the predicted values according to the observed values (Gupta et al.,
419 1999). Finally, we calculated the interval of model's prediction at 95% confidence,
420 which reflects the prediction uncertainty around a single value, as ± 1.96 x RMSE of
421 the model (Bruce and Bruce, 2017).

422

423 *Genetic parameters estimations*

424 Heritability coefficients, environmental and genetic correlations were estimated for FE
425 traits and candidate plasma biomarkers using the restricted estimation of maximum
426 likelihood method of the Wombat software (Meyer, 2007). In addition to the random
427 additive genetic effect, the model included the contemporary group (72 CG) as fixed
428 effect, age at the start of the test as covariate and a farm origins × year random
429 effect. The relationship matrix among the additive genetic effects included up to the
430 5th generation from bulls included in the study.

431

432 **Results**

433

434 All variables followed a non-normal distribution, except DMI and RFI ($P > 0.05$). Also,
435 these two variables together with plasma urea concentrations did not fulfill
436 homoscedasticity within CG ($P < 0.05$) (Supplementary Table S1).

437

438 ***Animal performances, candidate biomarkers and their sources of variations***

439

440 The between-animal variability of DMI, ADG, FCE and RFI, expressed as CV, was
441 always below 12% (Table 2). The between-animal variability of the candidate
442 biomarkers however contrasted, 5.33% for plasma $\delta^{15}N$ and 20.3% for plasma urea
443 concentrations. Concerning the effect of diets (Table 2), animals fed the corn-based
444 diets had higher ADG (+16%; $P < 0.05$), DMI (+4%; $P < 0.05$), FCE (+6%; $P < 0.05$)
445 and plasma urea concentrations (+35%, $P < 0.05$) than animals fed the grass-based
446 diets. On the other hand, animals fed corn-based diets had lower (-13%, $P < 0.05$)
447 plasma $\delta^{15}N$ values than animals fed grass-based diets.

448 The cohort factor explained 29%, 19%, 13% and 7% of the total variance of FCE,
449 plasma urea, ADG and DMI, respectively, but had only a minor contribution to the
450 variability of plasma $\delta^{15}N$ (Table 2). The diet effect (within cohort) strongly
451 contributed to the variability of all variables explaining 70%, 46%, 45%, 33% and
452 21% of the total variance observed in plasma $\delta^{15}N$, plasma urea, ADG, FCE and
453 DMI, respectively. Finally, the pen effect (within diet and within cohort) only explained
454 less than 10% of the total variance of the variables except for DMI (16%). The
455 residual part of the variance, i.e. the between-animal variation, represented a large
456 and significant part of the total variance of DMI (55%), ADG (40%), FCE (33%),
457 plasma $\delta^{15}N$ (25%) and plasma urea (29%).

458

459 ***Relationships between candidate plasma biomarkers and feed efficiency traits***
460 ***at the individual level***

461

All the simple linear models on residuals ($P > 0.58$) and the mixed effect models ($P > 0.37$) showed normal residuals. Models obtained for FCE were consistent, whatever the statistical methodology considered [removal (Table 3 and Fig. 2) or inclusion (Table 4 and Fig. 3) of the factors that significantly contributed to the GC in the model]. Plasma $\delta^{15}N$ was negatively correlated to the between-animal variability of FCE with both approaches ($P < 0.001$; Table 3 [Eq. 1] and Table 4 [Eq. 10 and 11]). The negative slope of the FCE-plasma $\delta^{15}N$ relationship was however greater (+35%, $P < 0.05$) with the corn than with the grass based diets in the mixed effects models analysis. Concerning plasma urea concentrations, they were non-significantly related to FCE whatever the approach (Table 3 [Eq. 2]; Supplementary Figure S1). In the case of RFI, the simple linear regression showed significant positive correlations with both plasma $\delta^{15}N$ ($P < 0.001$; Table 3) and plasma urea concentrations ($P = 0.04$), despite the poor fitting ($R^2 = 0.00$) obtained with the latter biomarker.

When the variability due to contemporary group was removed before linear regression, the best models (based on R^2, AIC and RSR) were obtained for plasma $\delta^{15}N$ whatever the FE trait. Combining both candidate biomarkers in the same model (Eq. 3 and 6 in Table 3) did not further improve the fit ($P > 0.20$). It had first been verified that plasma $\delta^{15}N$ and urea presented no-multicolinearity (Variance inflation factor =1.02). The FCE- $\delta^{15}N$ mixed-effect model (Table 4) was better in terms of RSR and error (CV) when animals were fed the corn as compared to the grass-based diets. In addition, the biomarker explained a higher percentage (17% vs 12%) of model's variance with the corn vs grass-based diets.

Performances and candidate plasma biomarkers of extreme animals

Animals identified as having the 10% highest and lowest plasma $\delta^{15}N$ values were selected as extreme individuals (Table 5), and showed differences in $\delta^{15}N$ averaging 1.02‰ (18%CV). Compared to animals with the highest plasma $\delta^{15}N$ values, those showing lowest values had lower DMI, FEPL and plasma urea concentrations ($P < 0.001$), but greater feed efficiency ($P < 0.001$). No interactions were observed between extreme $\delta^{15}N$ groups (highest vs lowest) and diet ($P > 0.05$) on these analyzed parameters.

Influence of feed characteristics on the relationship between plasma proteins $\delta^{15}N$ and feed efficiency

For this analysis, we removed two grass-based diets out of the 12 from our database because of 1) they were considered as outliers in most of the relationships (> 2.5 SD) and 2) biologically, the net energy concentrations of silages were low resulting in a lower average net energy intake (-10%) per average $BW^{0.75}$. Therefore, the number of observations used in this analysis was 22 rather than 24. In addition, because in the plasma urea-FCE relationship the diet effect was not significant ($P > 0.05$), the influence of diet characteristics was only analysed for the plasma $\delta^{15}N$-FE trait relationships. Graphs showing relationships at cohort level between plasma $\delta^{15}N$ and FE traits are shown in Supplementary Figure S2 (FCE in panel A and RFI in panel B).

We found that the higher the metabolisable protein to net energy ratio of the diet the greater the slope of the plasma $\delta^{15}N$ - FCE relationship ($P = 0.002$; Fig. 4, panel 1) and a similar trend for the rumen protein balance ($P = 0.10$; Fig. 4, panel 2). Concerning RFI, the higher the rumen N degradability of diets the greater the slope of the plasma $\delta^{15}N$ –RFI relationship ($P= 0.004$; Supplementary table S2). Details of the

511 influence of the other dietary characteristics on the slope of plasma δ^{15}N-FCE and
512 RFI relationships are presented in Supplementary Table S2.

513

514 ***Ability of plasma proteins δ^{15}N to discriminate between-animal variation of feed***
515 ***efficiency***

516 The cross-validation was only carried out for the best-fitted plasma δ^{15}N models (Eq:
517 1 and 4 from table 3). The plasma δ^{15}N was found to capture the difference in FE
518 between two individuals sharing the same diet as long as the difference in FE is
519 higher than 0.049 g/g for FCE and 1.67 kg/d for RFI (interval of prediction in Table 6).
520 However, the concordance correlation coefficient of both models was low to
521 moderate, accounting 0.16 and 0.33 for RFI and FCE, respectively. In addition the
522 two FE traits- δ^{15}N models presented a slightly under (4%) and overestimation (-3%),
523 for FCE and RFI respectively, and the accuracy (r and concordance correlation
524 coefficient) to predict FCE was better than to predict RFI from δ^{15}N.

525

526 ***Genetic and environmental correlations***

527

528 We determined if phenotypic relationships between FE traits and candidate
529 biomarkers involved genetic or environmental factors (Table 7). First, the heritability
530 values were moderate for FE traits (h2 = 0.18 for FCE and h2 = 0.22 for RFI) and
531 plasma biomarkers (h2 = 0.27 for plasma urea and 0.28 for δ^{15}N).

532 Concerning genetic correlations, RFI showed no-correlation with FCE (r_g < 0.05).
533 Both candidate plasma biomarkers showed no genetic correlations with FCE
534 (r_g<0.20) and moderate genetic correlation with RFI (r_g =0.38 and 0.27 for plasma

535 $\delta^{15}N$ and urea concentration, respectively), however the associated errors were large
536 to consider significant these relationships. Concerning the environmental
537 correlations, they were high between FCE and RFI (r_e = -0.61) and between plasma
538 $\delta^{15}N$ and FCE (r_e = -0.55), but moderate between $\delta^{15}N$ and RFI (r_e = 0.26), and low
539 between plasma urea concentrations and both FE traits (r_e < 0.15). Also, moderate
540 genetic correlations (r_e= 0.28) and low environmental correlations (r_e=0.14) were
541 found between both biomarkers.

542

543 **Discussion**

544

545 Moderate between-animal variability exists for FE (Berry and Crowley, 2012). Genetic
546 selection programs may exploit this moderate between-animal variability by selecting
547 the FE superior animals for breeding, and biomarker-assisted genetic selection may
548 contribute to accelerate the genetic progress through rapid identification of superior
549 animals. Today, however, no validated biomarker, genetically linked to FE, has been
550 proposed for beef cattle. Part of the difficulty to identify biomarkers of the between-
551 animal variability of FE is due to a methodological limitation. Indeed, a non-negligible
552 proportion of the measured between-animal variation, within a pen, is not ascribed to
553 the true between-animal variability but to the experimental error, which can be as
554 high as 50% (Fischer et al., 2018).

555 In this study, we tested two NUE biomarkers in ruminants (i.e plasma urea
556 concentration [Kohn et al., 2005] and plasma proteins $\delta^{15}N$ [Cantalapiedra-Hijar et
557 al., 2018b]) and their ability to reflect FCE and RFI, using a large experimental setup
558 of young bulls (n = 588) raised under similar conditions. As further discussed in the
559 following sections our results highlight that plasma $\delta^{15}N$ is significantly and

560 phenotypically correlated to the between-animal variations of FE, regardless of the
561 FE trait used, but with diet-dependent responses. Also these significant phenotypic
562 correlations might be mostly due to non-identified environmental factors rather than
563 to genetic ones. In contrast, plasma urea concentrations were only weakly, though
564 significantly, correlated with RFI and not significantly with FCE.

565

566 ***Plasma proteins $\delta^{15}N$ vs plasma urea as candidate biomarkers***

567

568 Both the plasma $\delta^{15}N$ and plasma urea concentration are recognised biomarkers of
569 NUE. They are both strongly impacted by the balance between ammonia release
570 from dietary CP degradation and ammonia uptake by rumen bacteria (i.e. rumen
571 protein balance) and the hepatic amino acid catabolism (Wattiaux and Reed, 1995;
572 Cantalapiedra-Hijar et al., 2015; INRA, 2018). These two mechanisms determine
573 most of the variations in NUE in ruminants (INRA, 2018) and explain the relationship
574 between both candidate biomarkers and NUE in ruminants (Huhtanen et al., 2015;
575 Cantalapiedra-Hijar et al., 2018b). Also given the biological link that exists between
576 NUE and FE (Nasrollahi et al., 2020; Liu and VandeHaar, 2020), we anticipated
577 significant relationships between both candidate biomarkers and FE traits.

578 Our results confirm that both biomarkers are significantly related to between-animal
579 variations in FE, but not to the same extent. Plasma $\delta^{15}N$ is a better biomarker than
580 plasma urea concentration. If the potential of blood or milk urea concentration to
581 predict the mean NUE is well established for groups of ruminants (Kohn et al., 2005),
582 reports highlighted its inability to phenotypically (Hof et al., 1997; Dechow et al.,
583 2017; Huhtanen et al., 2015) or genetically (Sebek et al., 2007; Vallimont et al., 2011;
584 Beatson et al., 2019) reflect the between-animal variation in NUE. In the present

585 study, both plasma biomarkers were significantly and positively correlated, but only
586 weakly (r = 0.21; $P < 0.001$). This suggests that their contrasted potential as
587 biomarkers may originate from some specific metabolic features differing between
588 them. We argue that the different ability of plasma $\delta^{15}N$ and plasma urea
589 concentration to reflect between-animal variations in FE is probably due to
590 mechanisms such as post-absorptive kinetics, urea recycling and endogenous N as
591 discussed below.

592 First, the most likely and prominent factor that explains the within-CG variability of
593 plasma urea concentration, is related to its postprandial variation. A considerable
594 diurnal variation in plasma urea concentration has been widely reported in the
595 literature associated to the daily pattern and frequency of feed intake (Gustafsson
596 and Palmquist, 1993; Hwang et al., 2001). Because blood sampling lasted 3-4h per
597 cohort, and because strictly speaking animals were not completely fasted (leftovers
598 remained in the automatic feeder before sampling), we could argue that plasma urea
599 was not determined exactly at the same postprandial moment for all animals. In
600 contrast, isotopic signatures remain much more stable in plasma proteins because
601 the short-term fluctuations of plasma $\delta^{15}N$ values depend on the slow turnover of this
602 protein pool (4.3%/d; Cantalapiedra-Hijar et al., 2019). This may explain why the
603 within-CG variation was much higher for plasma urea vs plasma $\delta^{15}N$ in our study
604 (20% vs 5% of CV) and elsewhere (25-29% CV for urea [Jonker et al., 1998; Krogh et
605 al., 2020] vs 13% CV for $\delta^{15}N$ [Cantalapiedra-Hijar et al., 2018b]).

606 Second, we considered whether renal urea reabsorption and clearance rate,
607 hydration status and animal BW (reviewed by Spek et al. 2013) could explain why the
608 between-animal variation are greater for plasma urea concentration than for NUE or
609 $\delta^{15}N$ values, but we had no data to support it. Instead and although somehow

speculative and counter-intuitive, we hypothesise that urea-N recycling and endogenous urinary N excretion (INRA, 2018) might have a greater impact on plasma $\delta^{15}N$ than on plasma urea concentration, and thus may further explain the better ability of the plasma $\delta^{15}N$ to detect the between-animal variability of FE. Urea recycling in beef cattle can be high and may contribute to increase the digestible N inflow from 43% to 85% (Lapierre and Lobley, 2001). But plasma urea is depleted in ^{15}N relative to the diet (Sutoh et al., 1993), and thus, this extra N entering the rumen could, once degraded into ammonia and assimilated, decrease the naturally enriched rumen bacteria (Cantalapiedra-Hijar et al., 2016). This N conservation mechanism in ruminants would further deplete animal proteins in ^{15}N and thus strengthen the negative relationship between NUE and plasma $\delta^{15}N$ usually found in ruminants (Cantalapiedra-Hijar et al., 2018b). Conversely, plasma urea concentration is only weakly correlated to urea transfer into the rumen through the epithelium (i.e. portal drained viscera net flux) according to the review by Lapierre and Lobley (2001), which may partly explain the weak relationship between plasma urea concentration and FE.

Finally we speculate that animal variation in endogenous urinary N losses, which represent on average 20-30% of total urinary N excretion (INRA, 2018) and an estimated 13% of the total N intake in our database (INRA, 2018; data not shown), would mostly affect plasma $\delta^{15}N$ and FE but not plasma urea concentration. While the urinary urea excretion is highly related to the hepatic urea synthesis and plasma urea concentration (Kohn et al., 2005), other non-ureic N compounds (e.g. creatine, creatinine, endogenous purine derivatives, methylhistidine and other free amino acids) are rather related to the endogenous protein renewal (Da Silva Braga et al., 2012). Because protein mobilization in ruminants highly affect both NUE and $\delta^{15}N$

635 values of animal proteins (Chen et al., 2020), it can be hypothesized that FE
636 variations associated to protein turnover and metabolism would affect plasma $\delta^{15}N$ in
637 a higher extent than plasma urea concentration.

638 Taken together we consider that animal factors affecting NUE and thus FE variation
639 are better signed by plasma $\delta^{15}N$ than by plasma urea because the former is more
640 stable in time and likely reflects some specific metabolic pathways related to N
641 partitioning. Therefore, the following sections will only discuss the limits and potential
642 of plasma $\delta^{15}N$ to reflect the between-animal variation of FE.

643

644 ***The potential of plasma proteins $\delta^{15}N$ depends on the feed efficiency trait***

645

646 This is the first study showing by regression a significant relationship between
647 plasma $\delta^{15}N$ and RFI in contrast to previous studies (Wheadon et al., 2014; Meale et
648 al., 2017). This relationship was however much weaker than that obtained between
649 plasma $\delta^{15}N$ and FCE. Although FCE and RFI were statistically related in this work (r
650 = -0.29; $P < 0.001$) and share common biological processes related to FE, their
651 biological determinants are not exactly the same (Taussat et al., 2020). While FCE
652 favors the selection of growth traits with inconsistent intake responses (Arthur and
653 Herd, 2012), selection based on RFI will downward feed intake without any impact on
654 animal performances (Arthur et al., 2004). This would have implications in the way
655 the energy and protein are allocated with both criteria (Rauw, 2012), and may explain
656 why plasma $\delta^{15}N$ correlated better with FCE than with RFI. Here, we discuss some
657 statistical and biological reasons that may explain why the correlation between
658 plasma $\delta^{15}N$ and RFI found in the present study or previously reported is weak or null
659 (Wheadon et al., 2014; Meale et al., 2017).

First, from a statistical point of view, it can be expected that biomarkers correlate better with phenotypes presenting higher variability. In the present study, the within-CG variance was twofold higher for FCE (9.37% CV) than for RFI (4.54% CV) in agreement with previous studies (Wheadon et al., 2014; Meale et al., 2017). Thus, the difficulty of plasma $\delta^{15}N$ to detect RFI variations could stem in part from the lower between-animal variability observed with RFI compared to FCE. Moreover, RFI being a residual it includes measurement errors (Fischer et al., 2018), which likely hampers the ability of plasma $\delta^{15}N$ to detect the true between-animal variability in FE when measured as RFI.

Second, while from a biological point of view plasma $\delta^{15}N$ is primarily a biomarker of NUE (Cantalapiedra-Hijar et al. 2018b), it can be simply argued that FCE and RFI just differ in the way they are related to NUE. At the individual level, FCE and NUE share a similar calculation principle based on the ratio between performances and intake, and thus correlations between both of them is mathematically expected and have been experimentally proven (Cantalapiedra-Hijar et al., 2015; Nasrollahi et al., 2020). Conversely, the relationship between NUE and RFI has not been experimentally observed (Lines et al., 2014; Carmona et al., 2020; Da Silva et al., 2020) despite the fact that efficient RFI animals are supposed to eat less CP while retaining an equal (or a greater) amount of body protein. More work is warranted to confirm that efficient RFI cattle upregulate N conservation mechanisms (Cantalapiedra-Hijar et al., 2020b) to the same extent as efficient FCE animals do (Arndt et al., 2015).

Third, because RFI is highly and consistently related to feed intake (Taussat et al., 2019), efficient RFI animals usually present higher rumen retention time and DM digestibility than inefficient RFI animals (Fitzsimons et al., 2014; Bonilha et al., 2017).

This would lead to a proportionally greater availability of substrates for rumen bacteria in efficient RFI animals, with the consequently greater N isotope fractionation occurring in the rumen (Wattiaux and Reeds, 1995) by unit intake. This can counterbalance the expected positive relationship between FE and plasma $\delta^{15}N$. We termed this phenomenon a "rumen interference" in the ability of plasma $\delta^{15}N$ to detect FE variations. In this regard, a high contribution of the rumen efficiency to the overall FE was suggested as the reason for the weak relationship observed between milk $\delta^{15}N$ and NUE in dairy cows (Cabrita et al., 2014). Likewise, Nasrollahi et al. (2020) identified in some conditions a yet unknown rumen effect lowering the ability of isotopic N discrimination to reflect between-animal variation in FE. Finally, FEPL could also contribute to the low correlation of plasma $\delta^{15}N$ with RFI. Because the higher the DMI the greater the FEPL (INRA, 2018), the positive correlation between RFI and DMI found in our study ($r = 0.57$; $P < 0.001$) may suggest differences in FEPL between RFI efficient and non-efficient animals. Although FEPL represent a net N loss from the animal, and so may impact FE, these losses does not involve enzymatic reactions (i.e. desquamated epithelium and intestinal secretions) nor isotopic fractionation. Therefore, the estimated FEPL were positively correlated to RFI ($r = 0.28$; $P < 0.001$), which could have lowered FE of high RFI phenotypes without increasing plasma $\delta^{15}N$ values. Because FCE, in contrast to RFI, presented no correlations with DMI in our study ($r = -0.06$; $P > 0.05$) no changes at the rumen or digestive level were expected across the FCE extreme cattle.

The potential of plasma proteins $\delta^{15}N$ to capture variations in feed conversion efficiency also depends on the type of diet

710 The present study confirmed preliminary results obtained from a sub-set of animals of
711 the present experimental setup (Nasrollahi et al., 2020), where the slope of the FCE-
712 plasma $\delta^{15}N$ relationship was significantly higher with diets high in starch vs high in
713 fibre (Eq. 10 vs 11 in Table 4). It also supported our previous meta-analysis, which
714 suggested that the relationship between the isotopic N discrimination and N use
715 efficiency could be diet-dependent (Cantalapiedra-Hijar et al., 2018b). More
716 specifically, the slope of the FCE-plasma $\delta^{15}N$ relationship was affected by the farm-
717 to-farm and year-to-year variations in feed composition (Supplementary Figure S2).
718 To precisely assess which variables could explain the diet-to-diet variability, we
719 characterised the experimental diets according to INRA (2018) (Supplementary Table
720 S2).

721 The rumen protein balance, representing the difference between the protein intake
722 and the protein flowing at duodenum (INRA, 2018), was an important dietary factor
723 that explained the influence of dietary conditions on the ability of plasma $\delta^{15}N$ to
724 reflect FCE variations (Fig. 4B). The higher the rumen protein balance (i.e. higher
725 rumen ammonia absorption compared to the urea recycled into the rumen), the
726 smaller the slope of the FCE-plasma $\delta^{15}N$ relationship. In other words, when rumen
727 protein balance was above requirements (between 0 and -12 for 300 - 600 kg BW;
728 INRA, 2018), the ability of plasma $\delta^{15}N$ to reflect FCE variations decreased. This
729 analysis reinforces our hypothesis about a “rumen interference” in the relationship
730 between plasma $\delta^{15}N$ and FE variation. In this sense, diets with high rumen
731 degradable N concentration and leading to high rumen protein balance showed low
732 or no correlations between $\delta^{15}N$ and FE or NUE in several previous studies in
733 ruminants (Cheng et al., 2013; Cabrita et al., 2014; Nasrollahi et al., 2020).
734 Furthermore, in the present study, high-fibre diets presented a higher proportion of

735 metabolisable protein coming from microbial vs dietary origin (+7%; $P = 0.02$), which
736 may increase the N isotopic fractionation at rumen level (Wattiaux and Reed, 1995)
737 and consequently interfering in a greater extent on the expected relationship between
738 plasma $\delta^{15}N$ and FE.

739 The metabolisable protein to net energy ratio, which represents the amount of
740 metabolisable protein for a given net energy level, was also identified as a dietary
741 factor explaining differences in the slope of the plasma $\delta^{15}N$ - FCE relationship (Fig.
742 4A). Indeed, when this ratio was above requirements (between 48 and 53,
743 irrespective of the diet for 300 - 600 kg BW; INRA, 2018) the slope approached zero
744 and reflected the inability of plasma $\delta^{15}N$ to catch FCE variations at high
745 metabolisable protein to net energy levels. It is known that amino acid catabolism
746 increases when the metabolisable protein to net energy ratio increases (Hanigan et
747 al., 1998), and thus our results may suggest that between-animal variability would be
748 lower when diets promoted greater amino acid catabolism.

749

750 ***Potential and limits of plasma proteins $\delta^{15}N$ to predict feed efficiency***

751

752 Unfortunately, the size of our experimental setup although important (n = 588) was
753 too restricted to detect any significant genetic correlations between plasma $\delta^{15}N$ and
754 FE (FCE and RFI). This implies that the significant phenotypic relationships found
755 between the plasma $\delta^{15}N$ and FE were mostly due to non-identified environmental
756 factors. Because diet conditions were controlled in our study, we speculate that other
757 uncontrolled environmental factors could be involved such as the previous
758 background of animals (pre-weaning and cow-calf period), feeding behavior
759 (preferential ingestion of specific low $\delta^{15}N$ ingredients from the total-mixed ration

760 promoting higher FE) and mild subacute diseases not detected during fattening
761 (lameness, rumen acidosis, liver abscess). We are aware that the size of our
762 experimental setup may be a limitation for concluding about genetic correlation. In
763 this regard, Lozano-Jaramillo et al. (2020) established that a population of 2 000
764 individuals minimum per environment was necessary to minimize the SE of genetic
765 correlations and obtain robust genetic correlations. To the best of our knowledge,
766 studies reporting genetic correlations between biomarkers and FE in beef cattle are
767 very scarce in the literature and none has demonstrated a significant correlation.
768 Nkrumah et al. (2007) reported a genetic correlation of -0.44 and -0.24 between
769 serum leptin concentration and feed conversion ratio (the inverse of FCE) and RFI,
770 respectively, from 813 steers. However, the large SE associated with their
771 estimations (± 0.24 and ± 0.38 for feed conversion ratio and RFI, respectively) made
772 it difficult to conclude to a true genetic association. Similarly, IGF-1 was shown to be
773 genetically correlated to RFI but also with a large associated error (Johnston et al.,
774 2002; Moore et al., 2005). Thus, more studies are warranted to establish genetic
775 relations between FE and plasma $\delta^{15}N$ with a greater population size.

776 Concerning the potential of this biomarker for phenotyping animals, our cross-
777 validation model confirmed that plasma $\delta^{15}N$ could be used as a biomarker to
778 discriminate the FE of two animals from the same CG providing they differ by at least
779 0.049 g/g in FCE and 1.67 kg/d in RFI (Interval of model's prediction in Table 6). The
780 number of animals within the same CG showing at least that difference was
781 calculated to be 30% (198 out of 588) and 6% (35 out of 588) for FCE and RFI,
782 respectively, highlighting once more the better ability of plasma $\delta^{15}N$ to stablish
783 between-animal difference in FCE than in RFI. These are the percentages of animals
784 that can be discriminated in terms of FE when comparisons are done from two

785 animals randomly selected from the same CG. Logically, when comparisons are
786 done from a group of animals rather than from two randomly selected individuals the
787 statistical power increases. Indeed, animals with the 10% lowest plasma $\delta^{15}N$ values
788 within-CG had on average significantly higher feed efficiency (+0.021 FCE and -0.47
789 RFI) than their counterparts having 10% highest plasma $\delta^{15}N$ values (Table 5).
790 Because of the size of our experimental setup, the biomarker may still significantly
791 discriminate feed efficiency when all experimental animals (n = 588) were assigned
792 to either low or high plasma $\delta^{15}N$ group ($P < 0.001$; data not shown).

793 Overall, we showed that plasma $\delta^{15}N$ is capable of capture the between-animal
794 variations of FE in animals fed two contrasting diets. Nevertheless, better results may
795 be expected when using FCE than RFI, and when animals are fed with high-starch vs
796 high-fibre diets.

797

798 **Conclusion**

799

800 We demonstrated through an experimental network design and two different
801 statistical approaches, significant phenotypic correlations between plasma proteins
802 $\delta^{15}N$ and the between-animal variation of FE (FCE and RFI) in fattening cattle. In our
803 conditions, we validated the capability of the isotopic biomarker to discriminate
804 animals in terms of FE when they differed by at least 0.049 g/g in FCE or 1.67 kg/d in
805 RFI. However, the size of our experimental setup (n = 588) was insufficient to show
806 significant genetic correlations between $\delta^{15}N$ and FE. In addition, because $\delta^{15}N$ is a
807 well stablished biomarker of N use efficiency in ruminants, we suggest that efficient
808 FCE and RFI animals may present better N utilization that their less efficient
809 counterparts. Finally, relationships between FE and biomarkers of N use efficiency

may depend on the diet and the feed efficiency trait since better statistical correlations were obtained with high-energy diets and when using FCE over RFI.

Ethics approval

This study was carried out in compliance with the French legislation on animal care. All procedures were approved by the regional ethics committee (Auvergne-Rhône-Alpes, France) and subsequently validated by the French Ministry of Agriculture under the authorization number APAFIS#2930-2015111814299194v3.

Data and model availability statement

The data was not deposited in an official repository. Data are confidential but available to reviewers upon request.

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Pablo Guarnido-Lopez: data curation, formal analysis, writing-original draft. All authors were involved in writing, reviewing & editing the final manuscript.

Declaration of interest

None

Acknowledgements

Authors wish to thank APIS-GENE for their financial support of this project, which forms part of the larger national program BEEFALIM 2020. We also thank Marine Gauthier and Céline Chantelauze (INRAE-UMRH) for their help with the laboratory analyses and the “Chambres d’Agriculture” from Pays de Loire, Bretagne and Saône-et-Loire (France) and the “Herbipôle” staff from INRAE experimental facilities at Theix for their great support in conducting the feed efficiency tests, blood samplings and measurements. In addition, we thank Mohammed Benaouda for its contribution and support in the statistical analysis.

Financial support statement

The project was financially supported by APIS-GENE. The first author received a PhD scholarship from APIS-GENE and INRAE-Phase.

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Tables

Table 1. Ingredients, chemical composition and feed values of the corn and grass based diets fed to Charolais bulls (average ± SD of the 12 experimental cohorts)

	Diet	
	Corn	Grass
Ingredients[1] (% DM)		
Forages		
Corn silage	61.4 ± 2.2	-
Grass silage	-	60.2 ± 6.0
Wheat straw	5.8 ± 1.6	5.5 ± 1.2
Concentrate		
Wheat grain	20.4 ± 5.5	8.05 ± 1.2
Beet pulp	-	20.9 ± 2.4
Soybean meal	12.4 ± 2.0	5.25 ± 1.4
Chemical composition (g/kg DM)		
Organic matter	958 ± 3.88	909 ± 13.4
CP	144 ± 9.69	142 ± 6.25
NDF	338 ± 25.3	479 ± 21.7
Starch	319 ± 36.2	56.4 ± 31.0
Starch/NDF (g/g)	0.95 ± 0.18	0.11 ± 0.07
Feed Values[2]		
Net Energy (Mcal/kg DM)	1.63 ± 0.04	1.50 ± 0.06
MP (g/kg DM)	85.6 ± 2.53	81.4 ± 3.72
MP/Net energy (g/Mcal)	52.5 ± 1.12	53.4 ± 1.88
RPB (g/kg DM)	7.75 ± 7.22	6.51 ± 6.81

Abbreviations: MP, metabolisable protein; RPB, rumen protein balance calculated as CP intake minus non-ammonia N at the duodenum (INRA, 2018).

[1] The whole ration was complemented (approximately 1% of total DM) with a vitamin-mineral supplement, non-reflected in the table

[2] Feed values of diets calculated from the measured chemical composition of diets and taking into account digestive interactions (INRA, 2018)

Table 2. Animal performances and candidate plasma biomarkers for Charolais bulls fed a corn or a grass based diet and their variance-component analysis

	Diet		Variance-component analysis								
	Corn	Grass	Cohort[1]		Diet within cohort		Pen within diet and within cohort		Residuals		
			SDc	ICC (%)	SDd	ICC (%)	SDp	ICC (%)	SDr	ICC (%)	CV[2] (%)
Animal performances											
DMI (kg/d)	9.82 ± 1.02[a]	9.44 ± 1.19[b]	0.30	7.19	0.52	21.6	0.45	16.2	0.83	55.1	8.64
ADG (kg/d)	1.62 ± 0.21[a]	1.39 ± 0.30[b]	0.10	12.5	0.19	45.1	0.04	2.00	0.18	40.4	11.8
FCE (g/g)	0.166 ± 0.02[a]	0.147 ± 0.02[b]	0.01	28.7	0.02	33.0	0.01	5.28	15.0	33	9.37
RFI (kg/d)	0.00 ± 0.44	0.00 ± 0.47	-	-	-	-	-	-	0.45	100	4.54
Biomarkers											
Plasma proteins $\delta^{15}N$ (‰)	5.13 ± 0.40[b]	5.82 ± 0.52[a]	0.01	0.03	0.49	70.4	0.13	4.95	0.29	24.6	5.33
Plasma urea (g/l)	0.195 ± 0.06[a]	0.145 ± 0.05[b]	0.03	18.7	0.04	46.1	0.016	6.09	0.04	29.2	20.3

Abbreviations: DMI, DM intake; ADG, average daily gain; FCE, feed conversion efficiency; ICC, intra-class correlation coefficient (calculated as the percentage of total variance explained by each environmental factor; RFI, residual feed intake; SDc, SDd, SDp and SDr, square root values of the cohort, diet, pen and residual variances respectively

[a,b] Averages with different letters within the same row are significantly different ($P < 0.05$)

[1] Cohort refers to the farm x period combination

[2] Coefficient of variation calculated as within-contemporary group SD divided by the average value of each variable (except for RFI where the DM intake was used as its average)

Table 3: Linear regression models between feed efficiency traits and candidate plasma biomarkers for Charolais bulls when variability across contemporary groups was previously removed.

FE traits (Y)	N° Eq	Plasma biomarker (X)	Slope $\delta^{15}N$	Slope Urea	*P*-value	R^2	AIC	RSR
FCE	1	Plasma proteins $\delta^{15}N^{a}$	-0.021 ±0.001***	-	<0.001	0.20	-3280	0.47
FCE	2	Plasma urea[b]	-	-0.037 ± 0.018*	0.053	0.00	-3044	0.55
FCE	3	Both[a]	-0.023 ±0.002***	0.006 ± 0.017^{ns}	<0.001	0.20	-3031	0.47
RFI	4	Plasma protein $\delta^{15}N^{a}$	0.468 ±0.064***	-	<0.001	0.10	501	0.95
RFI	5	Plasma urea[b]	-	1.330 ± 0.588*	0.04	0.00	669	1.00
RFI	6	Both[a]	0.489 ±0.069***	0.419 ± 0.577^{ns}	<0.001	0.09	584	0.94

Abbreviations: FE, feed efficiency; FCE, feed conversion efficiency; RFI, residual feed intake; AIC, akaike information criterion; Eq, N° of equation; RSR, RMSE-observations standard deviation ratio, calculated as the ratio of the RMSE and the SD of the efficiency trait.

Statistics: *** $P < 0.001$, * $P < 0.05$, ns = non-significant ($P > 0.05$).

[a,b] For a given feed efficiency trait, models with different letters are significantly different ($P < 0.05$).

Table 4: Mixed effect regression models between feed conversion efficiency (Y) and plasma proteins $\delta^{15}N$ for Charolais bulls fed a corn or a grass based diet when the random variability across contemporary groups was accounted

							Explained variance[1] (%)		
Diet	N° Eq	Intercept $\delta^{15}N$	Slope $\delta^{15}N$ (X)	*P*-value	CV%	RSR	Ran	Biom	Res
Corn based diets	10	0.159*** ± 0.008	-0.024*** ± 0.002[a]	<0.001	7.95	0.66	0.55	0.17	0.28
Grass based diets	11	0.153*** ± 0.009	-0.018*** ± 0.002[b]	<0.001	15.2	0.77	0.63	0.12	0.25

Abbreviations: FCE, feed conversion efficiency; CV, coefficient of variation calculated as the ratio between RMSE and the average value of FCE by diet; Eq, N° of equation; RSR, RMSE-observations standard deviation ratio calculated as the ratio of the RMSE and the SD of FCE by diet.

Statistics: *** $P<0.001$, [ab] Slopes with different letters are significantly different ($P<0.05$)

[1] Total variance (across-contemporary group variability) was partitioned into random (Ran), biomarker (Biom) and residual (Res).

Table 5: Animal performances and candidate plasma biomarkers from high and low extreme Charolais bulls in terms of plasma proteins $\delta^{15}N$ values (90^{th} and 10^{th} percentile) and fed a corn or a grass based diet

Diet	Grass-based diets		Corn-based diets			*P*-value		
Extreme animals	High $\delta^{15}N$	Low $\delta^{15}N$	High $\delta^{15}N$	Low $\delta^{15}N$	SEM	Diet	$\delta^{15}N$	INT
Observed animal performances								
DMI (kg/d)	9.90	9.13	10.3	9.31	0.105	0.14	<0.001	0.55
ADG (kg/d)	1.38	1.45	1.63	1.68	0.022	<0.001	0.22	0.83
FCE (g/g)	0.139	0.160	0.159	0.181	0.0020	<0.001	<0.001	0.80
RFI (kg/d	0.26	-0.19	0.27	-0.21	0.040	0.97	<0.001	0.87
Candidate biomarkers								
Plasm urea (g/L)	0.140	0.121	0.217	0.158	0.0058	<0.001	0.001	0.12
Plasma proteins $\delta^{15}N$ (‰)	6.38	5.40	5.65	4.60	0.042	<0.001	<0.001	0.71
FEPL (g/d)	171	162	182	165	1.6	0.03	<0.001	0.26

Abbreviations: DMI, DM intake; ADG, average daily gain; FCE, feed conversion efficiency; RFI, residual feed intake; FEPL, Fecal endogenous protein losses; INT, effect of the interaction (Diet × $\delta^{15}N$)

Table 6: Statistical parameters from the K-fold cross validation with the best-fitted models for feed conversion efficiency (FCE) and residual feed intake (RFI) in Charolais bulls evaluated in the Table 3.

Model[1]	N° Eq	RMSE	r	CCC	ECT	IP
FCE ~ plasma proteins $\delta^{15}N$	1	0.012	0.44	0.33	4%	0.049 g/g
RFI ~ plasma proteins $\delta^{15}N$	4	0.422	0.29	0.16	-3%	1.67 kg/d

Abbreviations: CCC, concordance correlation coefficient; ECT, percentage of error in central tendency; IP, interval of model's prediction at 95%; Eq, N° of equation; r, correlation coefficient between observed and predicted values; RMSE, root mean squared error of predicted vs observed values (expressed in g/g for FCE and kg/d for RFI).

[1]All statistical parameters presented are the average results of the 10 random iterations between observed and predicted values.

Table 7: Heritability (Diagonal, ±SE), genetic (Above diagonal ±SE), and environmental (Below diagonal ±SE) correlations between animal performance traits and candidate plasma biomarkers in Charolais bulls.

Traits	FCE (g/g)	RFI (kg/d)	Plasma proteins $\delta^{15}N$ (‰)	Plasma urea (g/L)
FCE (g/g)	0.18 ± 0.10	-0.05 ± 0.36	0.00 ± 0.39	0.19 ± 0.37
RFI (kg/d)	-0.61 ± 0.09	0.22 ± 0.10	0.38 ± 0.32	0.27 ± 0.33
Plasma proteins $\delta^{15}N$ (‰)	-0.55 ± 0.11	0.26 ± 0.12	0.28 ± 0.14	0.28 ± 0.33
Plasma urea (g/L)	-0.13 ± 0.11	0.07 ± 0.11	0.14 ± 0.13	0.27 ± 0.12

Abbreviations: FCE, Feed conversion efficiency; RFI, residual feed intake.

Figures

Figure 1. Experimental network design. Squares and circles below periods represent the experimental pens; half assigned to a corn-based diet (□) and half to a grass-based diet (○). Charolais bulls within each pen were considered the contemporary group (CG) unit. The number of cohorts (farm × period combination) and animals within cohort are shown at the bottom.

Figure 2. Relationships between the within-contemporary group (CG) variability of feed efficiency traits (feed conversion efficiency [FCE; Panels 1], residual feed intake [RFI; Panels 2]) and $\delta^{15}N$ of plasma proteins (Panels A) or plasma urea concentration (Panels B) for Charolais bulls fed a corn or a grass based diet. Symbols represent individual values adjusted for the CG effect while lines represent the linear regression from models shown in Table 3.

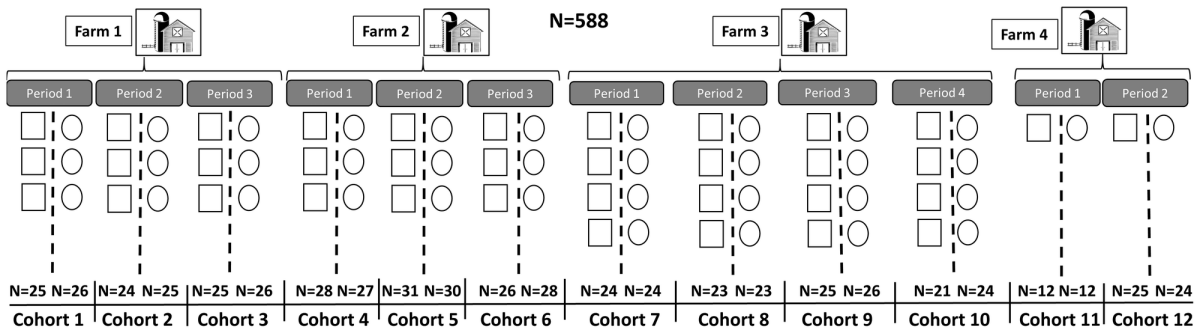
Figure 3. Relationships between feed conversion efficiency (FCE) and plasma proteins $\delta^{15}N$ (zero-centered values) in Charolais bulls. Panel A) Within-contemporary group regressions (n = 78) of FCE against plasma proteins $\delta^{15}N$ obtained by simple linear model. Dotted grey lines represent corn-based diets and thick black line grass-based diets. Panel B) Overall within-diet regressions (n = 2) of FCE against plasma proteins $\delta^{15}N$ obtained through mixed-effect model. Symbols are individual raw values for FCE and zero-centered values for plasma $\delta^{15}N$. Dotted grey

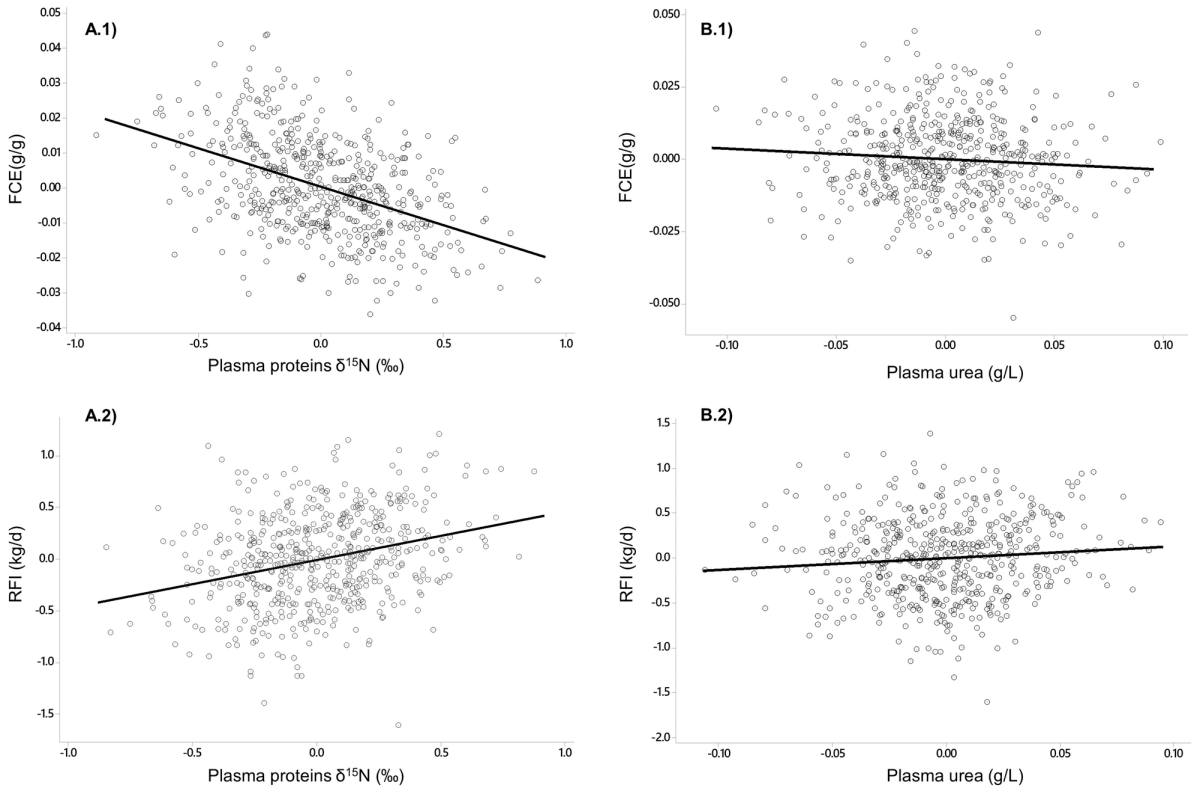
line and triangles represented animals fed corn-based diets whereas the thick continuous line and black points represent animals fed grass-based diets.

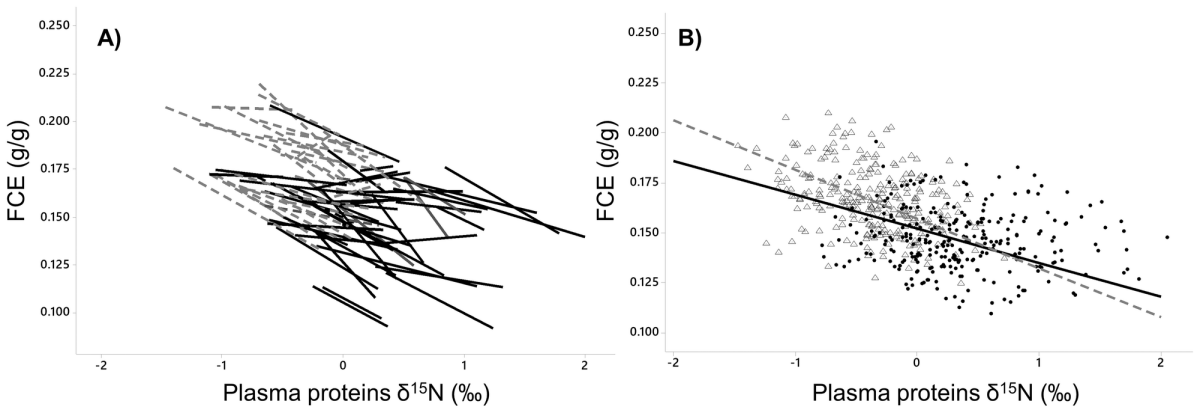
Figure 4. Relationships between within-cohort slopes of plasma proteins $\delta^{15}N$ vs. feed conversion efficiency (FCE) in Charolais bulls and significant feed characteristics. Panel 1; Metabolisable protein to Net energy ratio, Panel 2; Rumen protein balance. Dotted grey lines and continuous black lines represent relationship for corn and grass-based diets, respectively. Each observation within-diet correspond to one of the 12 cohorts.

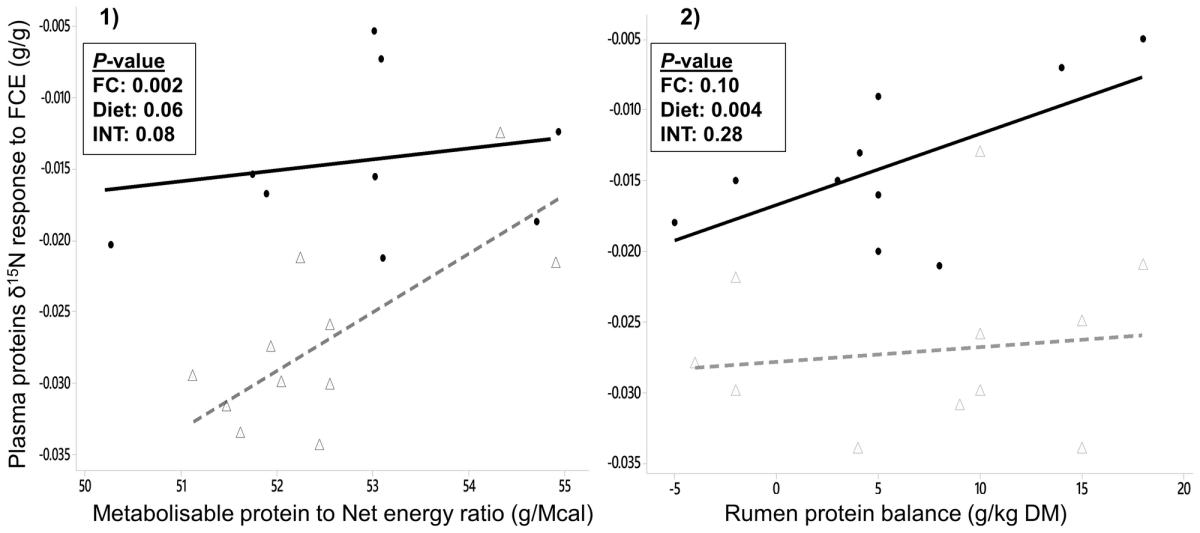
Abbreviations: FC, feed characteristic; INT, interaction.

(Δ) Represent corn-based diets, while (●) represents grass-based diets.


N=588
Farm 1
Farm 2
Farm 3
Farm 4
Period 1
Period 2
Period 3
Period 4
N=25 N=26
N=24 N=25
N=25 N=26
N=28 N=27
N=31 N=30
N=26 N=28
N=24 N=24
N=23 N=23
N=25 N=26
N=21 N=24
N=12 N=12
N=25 N=24
Cohort 1
Cohort 2
Cohort 3
Cohort 4
Cohort 5
Cohort 6
Cohort 7
Cohort 8
Cohort 9
Cohort 10
Cohort 11
Cohort 12


A.1)
FCE(g/g)
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
-0.04
-1.0
-0.5
0.0
0.5
1.0
Plasma proteins δ15N (‰)
B.1)
FCE(g/g)
0.050
0.025
0.000
-0.025
-0.050
-0.10
-0.05
0.00
0.05
0.10
Plasma urea (g/L)
A.2)
RFI (kg/d)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
Plasma proteins δ15N (‰)
B.2)
RFI (kg/d)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-0.10
-0.05
0.00
0.05
0.10
Plasma urea (g/L)


A)
B)
FCE (g/g)
0.250
0.225
0.200
0.175
0.150
0.125
0.100
-2
-1
0
1
2
Plasma proteins δ15N (‰)


1)
P-value
FC: 0.002
Diet: 0.06
INT: 0.08
Plasma proteins δ15N response to FCE (g/g)
-0.005
-0.010
-0.015
-0.020
-0.025
-0.030
-0.035
50
51
52
53
54
55
Metabolisable protein to Net energy ratio (g/Mcal)
2)
P-value
FC: 0.10
Diet: 0.004
INT: 0.28
-5
0
5
10
15
20
Rumen protein balance (g/kg DM)