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PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	6, Figure 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	N/A
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	N/A
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	6, Appendix
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	N/A
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	N/A
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	6-7
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	8-10
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	8-10

PRISMA 2009 Checklist

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	N/A
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	N/A
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6, 11, Table 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	Table 1, 6, 10-11
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	N/A
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	12, Table 3
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	12, Table 4
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	N/A
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	N/A
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	13, 18-19
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	13, 18-19
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	2, 18-19
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	19

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Nitrogen isotopic discrimination as a biomarker of between-cow variation in the efficiency of nitrogen utilization for milk production: A meta-analysis

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INTERPRETIVE SUMMARY

Natural ^{15}N abundance as a biomarker of milk N use efficiency in dairy cows

The improvement of techniques to identify lactating dairy cows differing in its ability to convert dietary N into milk N, which is referred to as milk N efficiency (MNE), with accurate biomarkers would benefit both the dairy industry and the society, as MNE is related to farm profitability and environmental footprint. This research assessed the ability of natural ^{15}N enrichment of animal proteins over the diet (Δ^{15}N) to predict the between-animal variations in MNE in lactating dairy cows. Our database, including 20 experiments, confirm that Δ^{15}N permits to discriminate groups of dairy cows with contrasted MNE and thus could be used as a tool for precision feeding.

ABSTRACT

Estimating the efficiency of N utilization for milk production (MNE) of individual cows at a large-scale is difficult, particularly because of the cost of measuring feed intake. Nitrogen isotopic discrimination ($\Delta^{15}N$) between the animal (milk, plasma or tissues) and its diet was proposed as a biomarker of the efficiency of N utilization on a range of production systems and ruminant species. The aim of this study was to assess the ability of $\Delta^{15}N$ to predict the between-animal variability in MNE in dairy cows using an extensive database. For this, 20 independent experiments conducted as either changeover (n = 14) or continuous (n = 6) trials were available and comprised an initial data set of 1,300 observations. Between-animal variability was defined as the variation observed among cows sharing the same contemporary group (CG; individuals from the same experimental site, sampling period, and dietary treatment). Milk N efficiency was calculated as the ratio between mean milk N (MN, g of N in milk/d) and mean N intake (NI, g of N intake/d) obtained from each sampling period, which lasted 9.0 ± 9.9 d (mean ± SD). Samples of milk (n = 604) or plasma (n = 696) and feeds (74 dietary treatments) were analyzed for natural ^{15}N abundance ($\delta^{15}N$) and then the N isotopic discrimination between the animal and the dietary treatment was calculated ($\Delta^{15}N = \delta^{15}N_{animal} - \delta^{15}N_{diet}$). Data were analyzed through mixed-effect regression models considering the experiment, sampling period and dietary treatment as random effects. In addition, repeatability estimates were calculated for each experiment to test the hypothesis of improved predictions when MNE and $\Delta^{15}N$ measurements errors were lower. The considerable protein mobilization in early lactation artificially increased both MNE and $\Delta^{15}N$ leading to a positive rather than negative relationship and this limited the implementation of this biomarker in early lactating cows. When the experimental errors of $\Delta^{15}N$ and MNE decreased in a particular experiment (i.e., higher repeatability values) we observed a greater ability of $\Delta^{15}N$ to predict MNE at the individual level. The predominant negative and significant correlation between $\Delta^{15}N$ and MNE in mid and late lactation demonstrated that on average $\Delta^{15}N$ reflects MNE variations both across dietary treatments and between-animals. The root mean squared prediction error as a percentage of average observed value was 6.8% indicating that the model only allowed to differentiate 2 cows in terms of MNE within a CG if they differed by at least 0.112 g/g of MNE (95% confidence level) and this could represent a limitation to predict MNE at the individual level. However, the one-way ANOVA performed to test the ability of $\Delta^{15}N$ to differentiate within-CG the top 25% from the lowest 25% individuals in terms of MNE was significant indicating that it is possible to distinguish extreme animals in terms of MNE from their N isotopic signature, which could be useful to group animals for precision feeding.

Key words: meta-analysis, milk nitrogen efficiency, biomarker, individual variability, ^{15}N.

INTRODUCTION

Dairy products are important sources of food protein along with a range of other essential nutrients (FAOSTAT, 2017), and their increased consumption is driven by the growth of the world human population and their average incomes (Scott, 2017). Total food production is a significant contributor to global greenhouse gas emissions, which are undeniably related to climate change (Clark et al., 2020; Ocko et al., 2021). There are two main sources of environmental pollution in livestock systems: greenhouse gas emissions per se (carbon dioxide, methane, and nitrous oxide) (Uwizeye et al., 2020; Ocko et al., 2021) and the negative impact of excreta (mainly N and P) on the quality of surface and ground water (Castillo et al., 2000; Uwizeye et al., 2020). In this context, mitigation strategies for the livestock industry are highly needed (Uwizeye et al., 2020).

In the lactating cow, the efficiency of N utilization for milk production (MNE; g of milk N/g of N intake) is commonly used to describe the conversion of feed N inputs into dairy products (Cantalapiedra-Hijar et al., 2016) and also as an indicator of the N losses to the environment (Jonker et al., 1998; Castillo et al., 2000; Nousiainen et al., 2004). The main constrain to collect accurate estimations of MNE at the individual cow level is the determination of feed intake, which is costly and laborious (Hellwing et al., 2015). The identification and consolidation of techniques to predict MNE accurately from easy-to-collect samples will contribute to the design feed rations according to nutritional status and to increase the collection of records for breeding programs (Brito et al., 2021).

In the context of animal physiology, a biomarker can be defined as "a naturally occurring molecule, gene, or characteristic by which a particular pathological or physiological process, disease, etc. can be identified or referred to" (Oxford Dictionary; https://www.lexico.com). Ruminants have an effective internal N recycling system, where most of the excess dietary N is converted to urea in the liver through ureagenesis, designed to avoid toxic effects if ammonia enters the systemic circulation (Lapierre et al., 2005). In turn, urea is transported from the plasma to other body fluids such as saliva to be recycled as well as to the kidneys to be excreted. Because of its low molecular weight and neutral charge, urea easily diffuses across cellular membranes where it is incorporated to milk as MUN (Jonker et al., 1998). On this basis, MUN has been proposed as a biomarker for MNE and N excretion in dairy cows. However, the evidence regarding the potential of this biomarker to reflect the between-animal variation in MNE (Spek et al., 2013; Huhtanen et al., 2015) and its association with N partitioning at the individual animal level (Spek et al., 2013; Beatson et al., 2019) is inconclusive.

Alternatively, the natural ^{15}N abundance ($\delta^{15}N$; $^{15}N/^{14}N$ ratio relative to atmospheric N^2) in animal protein is a promising biomarker for predicting MNE because of its direct link with the ruminal microbial N metabolism (Wattiaux and Reed, 1995) and with the catabolism of AA in the liver (Cantalapiedra-Hijar et al., 2016). In short, it has been demonstrated across a variety of conditions and species, that ^{15}N natural abundance in animal proteins is higher than in the diet consumed (DeNiro and Epstein, 1981) and that N isotopic discrimination ($\Delta^{15}N = \delta^{15}N_{animal} - \delta^{15}N_{diet}$) is negatively correlated with the N use efficiency (NUE) estimated as g of milk N or retained body N/g of N intake (Cantalapiedra-Hijar et al., 2018). This discrimination phenomenon was confirmed to differ at the individual level which could be advantageous in the attempt to rank ruminants reared under similar conditions for NUE (Cheng et al., 2013; Cantalapiedra-Hijar et al., 2018) or for feed efficiency (Wheadon et al., 2014; Guarnido-Lopez et al., 2021). However, not all studies found a significant negative relationship between MNE and $\Delta^{15}N$ in lactating dairy cows (Cheng et al., 2011; Chen et al., 2020). In a recent study by Chen et al. (2020), the N isotopic signatures were strongly impacted by protein mobilization occurring during early lactation, and this resulted in positive, rather than negative associations with MNE. Another explanation for the disparity in the associations between MNE and $\Delta^{15}N$ could be related to a high experimental error associated to the measurements of N intake, milk N, or N isotopic signatures. This experimental error can be assessed statistically by analyzing the consistency of repeated measurements (Harper, 1994). Although guidelines and quality standards for measuring these traits exist, it was hypothesized that higher repeatability values (i.e., lower experimental errors) of both NUE and $\Delta^{15}N$ measurements could lead to improved model MNE prediction.

In the present study, we explored the ability of $\Delta^{15}N$ to predict the between-animal variability in terms of MNE in lactating dairy cows and potential factors affecting the prediction ability of $\Delta^{15}N$. In our previous meta-analysis (Cantalapiedra-Hijar et al., 2018), the association between NUE and $\Delta^{15}N$ was explored as the proof of concept from a range of ruminant species and production conditions employing a smaller dataset. The present study brings an update and refinement of the model, with a larger data set comprised only from lactating dairy cows.

MATERIALS AND METHODS

Experimental Data

A database including individual animal measurements was created from experiments proposed by the partners of the SmartCow project (grant agreement N°730924), a collaborative EU project aiming at the integration of the research infrastructures for the European cattle sector (https://www.smartcow.eu). Data originated from 20 dairy milk production experiments (ID1 to ID20) conducted in Belgium (n = 1), England (n = 1), Finland (n = 2), Denmark (n = 6), and France (n = 10). These experiments were conducted as either changeover (e.g., Latin-square; n = 14) or continuous (n = 6) experiments. The initial data set included multiple observations from 425 cows (i.e., different sampling period and dietary treatments) representing a total of 1,300 individual observations of N intake, milk N, MNE, and $\Delta^{15}N$. A summary of studies along with its corresponding design is presented in Table 1.

Laboratory Analysis and Calculations

For both individual animal observations and dietary treatment (DT) means, values of MNE were calculated as the ratio between milk N (MN, g/d) and N intake (NI, g/d) considering all observations of the corresponding sampling period (SP) in order to account for daily variability in the observations. Nitrogen intake was calculated by multiplying dietary N content (g N/100 g DM) by the daily DMI corresponding to each SP for each cow. In the same manner for those experiments not including MN, this was calculated from average milk yield and the corresponding milk CP percentage reported for the same SP which ranged from 4 to 42 days and averaged 9 d (SD = 9.9). The large SD corresponds to the difference in the experimental setup between changeover and continuous experiments. It was assumed that milk CP contained on average 95% of protein N and thus total N was estimated with the following equation: [(milk yield × protein percentage)/6.38]/0.95 (DePeters and Ferguson, 1992). Milk composition including fat, protein, and lactose was provided from each independent experimental data set and determined by infrared spectroscopy.

Samples of plasma (696 samples from 13 experiments) or milk (604 samples from 7 experiments) provided by the SmartCow partners were processed and analyzed for N isotopic signatures at the INRAE laboratory (INRAE, Saint-Genès-Champanelle, France). Similar relationships with NUE were previously reported when analyzing $\Delta^{15}N$ in either plasma or milk samples (Cantalapiedra-Hijar et al., 2018). Thus, only 1 of the 2 matrices were analyzed in those occasions where samples of plasma and milk were available for a single observation. Because the within-sample repeatability of isotopic analysis is always greater when using plasma vs. milk samples, it was decided to prioritize analyzing samples of plasma over milk. Once thawed, milk and plasma samples were

vortex-mixed for homogenization and pipetted onto tin capsules and dried for 24h at room temperature before analysis. Samples were analyzed for the determination of N isotopic signatures ($\delta^{15}N$) using an isotope-ratio mass spectrometer (Isoprime Vision; Elementar, Manchester, UK) coupled to an elemental analyzer (EA Vario Cube; Elementar, Langenselbold, Germany) with glutamic acid used as the in-house standard. In the same manner, all dried feed ingredients or TMR samples received were analyzed in order to obtain their $\delta^{15}N$ values for each dietary treatment and measurement period. For this, subsamples of feed ingredients and TMR were weighed into the tin capsules (between 2 to 4 mg according to N content). In the case of diets comprised of separated ingredients, the average $\delta^{15}N$ of each ingredient was weighted by the percentage of N the ingredient represents in the diet in order to obtain a single value of $\delta^{15}N$ for each diet and period. To ensure reliable $\delta^{15}N$ determinations, 2 replicates for milk and plasma samples and between 3 to 4 replicates for the dietary ingredients were analyzed in order to obtain an average value with a SD < 0.2‰. Then, the isotopic discrimination between animal proteins and diet ($\Delta^{15}N$, ‰) was calculated for each animal as the $\delta^{15}N$ in animal proteins minus $\delta^{15}N$ of the corresponding diet.

Statistical Analysis

The primary objective of the present study was to assess the ability of $\Delta^{15}N$ to predict the between-animal variability in MNE of lactating dairy cows. Therefore, the notion of a contemporary group (CG) is defined here as a set of experimental animals sharing the same DT and SP within a particular experiment (i.e., animals fed the same diet, at the same time and place). According to this definition, in an experiment with a 4×4 Latin square design there are 16 CG unless the period effect was not observed significant in which case there would be only 4 CG (further explained). Between-animal variability will be then approached in the present study, through different statistical approaches, as the variance within-CG, also including the experimental error in addition to the true animal variance. Consequently, when discussing the between-animal variability or relationships between two variables at the individual level, we refer to the within-CG level. For the experiments containing CG with 3 or less observations (10 out of 20), a preliminary adjustment by SP was conducted on MN, NI, $\Delta^{15}N$, and MNE according to the methodology described by St-Pierre (2001). For this, data were adjusted by SP by using a simple linear model with SP (within experiment) as fixed factor and then the obtained residuals were added to the mean value (i.e., intercept) for that experiment. In situations where the period effect was not significant ($P > 0.05$), all animals sharing the same dietary treatment within-experiment were considered as a CG. This process allowed us to include those CG with a limited number of observations (e.g., in the case of experiments with unreplicated Latin-square design). Otherwise, it would not have been possible to calculate regressions for those conditions with a low number of observations. For continuous variables, the distribution of values was checked for normality and

analyzed for outliers (biologically impossible or unlikely values) using the boxplot function in R software (R Development Core Team, 2009). Observations with a residual beyond ±3 SD were rejected if biological reasons justified their elimination.

Sources of Variation for N Isotopic Discrimination and MNE

Estimates of variance components were evaluated using a random intercept model, through the 'nlme' package (version 3.1-153) using the R software with experiment, DT within experiment, and SP within experiment as grouping random factors. In this analysis, the source of variability for MN, NI, $\Delta^{15}N$, and MNE were separately analyzed using the following model:

$$Y_{ij} = \beta_0 + \beta_i + e_{ij} \quad [1]$$

where Y_{ij} is the observed variable (MN, NI, $\Delta^{15}N$, or MNE) for the observation j on the group i, β_0 is the mean value for the population, β_i is the random variable representing the deviation for the population mean for the i^{th} group, and e_{ij} is the random variable error for the observation j on the group i. The residual error of this model represented the within-CG variance and thus including both the between-animal variability and the experimental error.

The repeatability accounts for the contribution of individual animal variability to the total variance not explained by the known experimental factors. In other words, the repeatability provides an estimate of the correlation between values from consecutive measurements conducted on the same cow once the known experimental factors (dietary treatment and experimental period within the same experiment) have been accounted for. The repeatability of MN, NI, $\Delta^{15}N$, and MNE was calculated for each experiment separately with the following equation:

$$\text{Repeatability} = \sigma^2_{\text{Cow}} / (\sigma^2_{\text{Cow}} + \sigma^2_{\text{Residual}}) \quad [2]$$

where σ^2_{Cow} and $\sigma^2_{\text{Residual}}$ are the animal variance (between-animal variability) and experimental error (within-animal variability), respectively. Accordingly, we estimated σ^2_{Cow} and $\sigma^2_{\text{Residual}}$ for each experiment and variable by including the fixed effects of SP and DT and the random effect of the cow. In each case, the confidence intervals of estimates were checked after fitting the model in order to monitor for potential problems in model definition (i.e., abnormal wide intervals) (Pinheiro and Bates, 2000).

Analysis of the Relationship Between MNE and N Isotopic Discrimination

Initially, the 'lmList' function of the 'nlme' package (Pinheiro and Bates, 2000) was employed to fit linear regressions relating MNE to $\Delta^{15}N$ within-experiment and within-diet and experiment separately. The statistical

197 significance of the response of MNE to $\Delta^{15}N$ variations was also computed with Pearson correlation coefficients
198 and declared significant at $P \leq 0.05$.

199 The relationship between MNE and $\Delta^{15}N$ at the individual animal level was explored following different
200 statistical approaches. In the first approach, the between-animal variability in $\Delta^{15}N$ was assessed separately along
201 with that of MNE once the random effect of the experiment, SP and DT (i.e., between CG variability) were
202 removed from the actual values (i.e., MNE and $\Delta^{15}N$) according to equation [1] and thus to assess the ability of
203 $\Delta^{15}N$ to predict the between-animal variation. If a relationship between $\Delta^{15}N$ and MNE was still significant once
204 the between-CG variability was removed from actual values, their residuals, the ability of the biomarker to capture
205 between-animal variation in MNE would be demonstrated (Cantalapiedra-Hijar et al., 2018). In addition, a one-
206 way ANOVA on the $\Delta^{15}N$ residuals of the 25% highest and 25% lowest cows in terms of MNE within-CG was
207 conducted to test on half of the population whether the $\Delta^{15}N$ allowed us to differentiate these 2 contrasting groups
208 of animals.

209 The second approach involved fitting mixed-effects models (St-Pierre, 2001) using the 'nlme' package in the
210 R software to test the ability of $\Delta^{15}N$ to predict MNE variations at 2 levels. For this purpose, 2 tiers of equations
211 were developed: predictions of MNE variations across-dietary conditions within experiment by using mean
212 dietary values (Tier 1) and the prediction of the within-CG variability of MNE by using individual observations
213 (Tier 2). Whereas Tier 1 models were tested only at the superior grouping factor (i.e., experiment level), Tier 2
214 models were tested across all grouping factors proposed (i.e., experiment, period within experiment and CG
215 random effects). The random effects of these structures were tested on the intercept, slope or both. A general
216 positive-definite matrix was employed as variance-covariance structure. These variance-covariance structures
217 obtained from the candidate models were evaluated with the Akaike Information Criterion (AIC) in order to
218 identify the best random effect structure to predict MNE (lowest AIC and RMSPE). Random-effect structures
219 were always compared using the restricted maximum likelihood method. The general form of the mixed-effect
220 model was:

$$Y_{ij} = (\beta_0 + b_{0i}) + (\beta_1 + b_{1i}) X_{ij} + e_{ij} \quad [3]$$

221 where Y_{ij} is the MNE observed, X_{ij} corresponds to the observed values of $\Delta^{15}N$, β_0 and β_1 are the fixed effects for
222 the intercept and the slope, respectively; b_i are the random effects of experimental factors; and e_{ij} is the identically
223 distributed within-group error, assumed to be independent of the random effects. The coefficient of determination
224 (R^2) was determined for all candidate models via the 'r.squaredGLMM' function of the 'MuMIN' package
225 (version 1.43.17) in the R software. Residuals were checked for homoscedasticity (i.e., the dependent variable

exhibits similar variance across the range of values for an independent variable). The models derived from this section were evaluated against the same developmental database (observed vs. predicted). This evaluation focused on evaluating the performance of $\Delta^{15}N$ to capture the between-animal variation in MNE from the selected models based on their best random effect structure. For this evaluation, the concordance correlation coefficient (CCC; Lin, 1989) was employed which was calculated as:

$$CCC = r \times C_b \quad [4]$$

where r is the Pearson correlation coefficient and C_b is the bias correction factor. The CCC indicates how far the best fit line deviates from the concordance or unity line of the observed values predicted values plot. The CCC ranges from 0 to 1, with greater values indicating better model performance. While the r value provides a measure of precision, the CCC is an indicative of the model accuracy. In addition, the ratio of the root mean square prediction error (RMSPE) and standard deviation of observed values (RSR) was computed to compare the prediction performance of models.

How the Repeatability of Evaluated Traits Impacts Model Fit

In the present study, the hypothesis that better repeatability values of both dependent and independent variables would enhance the model prediction performance was tested. The selected mixed-effects model of $\Delta^{15}N$ to predict MNE resulting from the mixed-effects meta-analysis in the Tier 2 was then evaluated for each experiment separately. Then, the coefficients of regression obtained during this model evaluation analysis were regressed on the repeatability values of MNE and of $\Delta^{15}N$ obtained separately for each experiment (according to equation [2]). These relationships were computed with Pearson correlation coefficients and declared significant at $P \leq 0.05$. If the repeatability of MNE and $\Delta^{15}N$ values significantly correlated with the model fitting, our hypothesis about the impact of the measurement precision on the ability of $\Delta^{15}N$ to predict MNE was accepted.

RESULTS

Description of the Data Set

Descriptive statistics for animal performances and diet composition are shown in Table 2. There was consistency in the number of observations across animal performance data, however, fewer records were available for some of the feed chemical composition variables. Only 13% of the data set (165 out of 1,300 observations) were from experiments conducted with cows in early lactation (< 50 DIM on average) and the remaining 87% observations corresponding to the mid and late lactation stages (DIM ≥ 50). Most of the experiments were conducted using Holstein Friesian cows and only ID11 and ID12 were conducted using Nordic Red cows. From

a total of 490 cows, 71% were multiparous. A wide range of DT (n = 74) was included in the initial data set. Corn silage, grass silage, and grass hay were the main forage ingredients used, but they were not present in all diets from all experiments. Feed chemical composition varied widely as a result of the heterogeneity of the experimental diets used in each independent experiment. Crude protein and NDF concentrations, measured in all experimental diets, averaged 157 and 379 g/kg of DM, respectively, and ranged from 110 to 268 and from 202 to 607 g/kg of DM, respectively. Large variation was also observed in ADF content, which ranged from 131 to 351 g/kg of DM. Based on the available information on chemical composition of diets, net energy content for lactation averaged 1.56 with a range of 1.44 to 1.70 Mcal/kg of DM.

Data Editing for Model Development

In the exploratory analysis of the initial data set, it was observed that the relationship between MNE and $\Delta^{15}N$ in early lactation (DIM < 50) was different compared to those observed in mid and late lactation (Figure 1). For instance, a strong and positive correlation (r = 0.88; $P < 0.01$) between $\Delta^{15}N$ and NUE was observed in 1 of the experiments (ID20) conducted with high producing dairy cows during the first 50 d of the lactation. Therefore, in the present meta-analysis it was decided to restrict the analysis of MNE to mid and late lactation stages (DIM ≥ 50) in order to improve modelling quality in terms of MNE prediction accuracy by using $\Delta^{15}N$. This resulted in the exclusion of experiment ID20 dedicated to examine the performance of 8 cows on 2 diets in the peripartum period (n = 32 observations), and the removal of some observations corresponding to cows in experiments transiting the declared early lactation period (Figure 2).

Table 3 describes statistics and repeatability values for MN, NI, MNE, and $\Delta^{15}N$ for the experiments included in this meta-analysis. A large variation in these traits was expected due to the contrasting experimental methods and designs. For instance, NI ranged from 271 (ID4) to 1,152 g of N/d (ID5), and average MN ranged from 19 (ID9) to 291 g of N/d (ID8). As a result of this, the data set covered a large range of MNE values (from 0.04 to 0.47 g/g) and showed a moderate variability (CV = 13%) in relation to its mean value (0.30 g/g). The difference in ^{15}N natural abundance between the cow and its diet ($\Delta^{15}N$) averaged 2.143‰ and ranged from 0.101‰ (ID1) to 4.457‰ (ID8) across diets and experiments.

The repeatability of all traits across experiments varied widely. For instance, repeatability values averaged from 36.1% (for NI in ID14) to 95.3% (for MN in ID14). The high overall repeatability values obtained for MNE (63.0%) was mainly due to the overall MN high mean repeatability observed across experiments. Removing observations from the early lactation (column 3 of Table 3) period led to greater repeatability estimates for all the variables analyzed when compared to the initial data set (columns 2 of Table 3).

285 ***Sources of Variation for Nitrogen Partitioning and N Isotopic Discrimination***

286 This analysis showed that the effect of experiment (ID) was the main grouping factor explaining the total
287 variance for all traits included (Table 4). For instance, more than one third of the variability observed in values
288 for MNE and $\Delta^{15}N$ was explained by the between-experiment variability. Around half of the variability observed
289 in MN was explained by the experiment effect and the largest source of variation for NI was captured by the
290 dietary treatment effect. Approximately 20% of the variance was captured by the dietary treatment (diets within
291 each sampling period and experiment) in MNE, and $\Delta^{15}N$. In the same manner, the random effect of experimental
292 period further captured around 13% of the variability in $\Delta^{15}N$ and in MNE. Furthermore, the resulting residuals
293 for the variables analyzed were mainly due to the between-animal variation and to the unidentified random sources
294 of error (within-animal variation).

295 ***Relationship Between MNE and N Isotopic Discrimination***

296 The response of MNE to $\Delta^{15}N$ variation (slope) was negative within-experiment for observations from 18
297 out of 19 experiments (Figure 3b and Table 3), and was different from 0 ($P < 0.05$) for 14 out of 19 experiments.
298 Likewise, although most slopes were negative within-diet (67 out of 72 diets; Figure 3c) only slopes for 29 out of
299 72 diets were different ($P < 0.05$) from 0, likely because the number of observations within diet was rather small
300 (mode = 4 observations per condition). A high variability in the response of MNE to $\Delta^{15}N$ variation among
301 experiments and dietary treatments was thus evident, suggesting the need for different response (slope)
302 coefficients across experimental conditions in our model.

303 ***Relationships Between MNE and N Isotopic Discrimination at the Individual Level***

304 When individual data for MNE and $\Delta^{15}N$ were independently adjusted by the random effects of experiment,
305 sampling period (within-experiment), and dietary treatment (within-period and experiment), their residuals were
306 still negatively correlated with each other ($P < 0.001$) with a moderate fit ($R^2 = 0.29$; Figure 4). Moreover, the
307 one-way ANOVA performed to test the ability of $\Delta^{15}N$ to differentiate the top 25% from the lowest 25%
308 individuals in terms of MNE within-CG was statistically significant ($P < 0.001$; Figure 5) indicating that it is
309 possible to distinguish extreme animals in terms of MNE from their N isotopic signature in a given CG.

310 ***Tier 1 and 2 Models.*** Table 5 presents the mixed-effect regression predictive models of MNE from $\Delta^{15}N$.
311 These models are displayed according the data employed for their development: on the dietary treatment means
312 (Tier 1) and on the individual observations (Tier 2). Whereas the overall slope obtained with the different models
313 of MNE prediction from $\Delta^{15}N$ were all negative and highly significant ($P < 0.001$), the slope of model 4 was more
314 pronounced and had a slightly lower error in comparison with the others (models 2 and 3). Additionally, model 4

had a better modelling fit than the model obtained from the dietary treatment mean observations (model 1). Based on the AIC criteria, the best mixed-effects model for $\Delta^{15}N$ included the random effects of all known experimental factors defining the CG level (i.e., experiment, sampling period, and dietary treatment) on both the intercept and the slope; that is the most complex model structure (model 4).

Models Evaluation. The overall fit statistics of the selected Tier 2 model for $\Delta^{15}N$ are presented for each experiment in Table 6. In line with the fluctuating overall correlation between MNE and $\Delta^{15}N$ observed form one experiment to another (Table 3), it was observed that the modelling performance, this time at the CG level, varied widely between experiments. Correlation coefficients (r) between actual and predicted MNE ranged between 0.20 and 0.91 but increases in these correlations did not necessarily result in lower RMSPE in a given experiment. For instance, ID9 had the highest r but also had the highest RMPSE. The inclusion of RSR, which includes the SD of the observed MNE at the CG level, allowed us to evaluate the fitness of the selected model on contrasting subsets (experiments).

Analysis of Repeatability for Explaining Variations in MNE Prediction Across Studies

A significant correlation was found between the coefficients of regression obtained during model evaluation analysis (observed vs. predicted correlation coefficient) and the repeatability values of MNE ($R^2 = 0.49$, $P = 0.06$; Figure 6a) or $\Delta^{15}N$ ($R^2 = 0.54$, $P = 0.03$; Figure 6b) obtained separately for each experiment. In other words, increases in repeatability of either MNE or $\Delta^{15}N$ enhanced the prediction fitness of the model.

DISCUSSION

The compilation of experiments conducted across 5 countries in Europe and resulting in a data set comprised of 1,300 individual observations of MNE in lactating dairy cows allowing us to explore the ability of $\Delta^{15}N$ as a candidate biomarker to predict the between-animal variability of MNE across a wide range of experimental conditions. In line with previous research, we observed that on average $\Delta^{15}N$ was negatively and significantly correlated with MNE at the individual level (Wheadon et al., 2014; Cantalapiedra-Hijar et al., 2018), but in agreement with the recent study by Chen et al. (2020) this association could not be confirmed in early lactation given the considerable body protein mobilization occurring at this stage. Finally, we identified that higher repeatability estimates for both dependent (MNE) and independent variables ($\Delta^{15}N$) resulted in models with better prediction fitness.

Associations Between MNE and N Isotopic Discrimination in Periparturient Dairy Cows

In the peripartum, dairy cows often undergo a period of negative energy balance because of the inability to increase energy intake at the same rate as the energy requirements for milk production increase (de Vries and Veerkamp, 2000; Xu et al., 2018). Body reserves are used (mobilized) to compensate for the resulting energy deficit and this could alter the estimations of MNE if this phenomenon is not properly accounted for (McNamara et al., 2016; Daniel et al., 2017). Unless body mobilizations is adequately measured (Friggens and Newbold, 2007), it is difficult to ascertain how much of the feed N intake is actually contributing to the total N supply for milk synthesis.

In the same way as MNE measurements, N isotopic signatures are affected by protein mobilization occurring during early lactation. Recently, a study by Chen et al. (2020) tested the ability of milk isotopic signatures (^{15}N and ^{13}C) to predict MNE, energy balance, and milk production of early lactation cows. Our results support theirs conclusions and suggest that the natural ^{15}N enrichment of animal proteins relative to the diet ($\Delta^{15}N$) could have some drawbacks and limitations when dairy cows are experiencing net protein mobilization. Indeed, it is well demonstrated from ecophysiological research and human longitudinal studies that protein mobilization and body weight loss may lead to a greater ^{15}N enrichment of animal pools relative to the diets received (Fuller et al., 2005; Barboza and Parker, 2006). This is because organisms are using their own already ^{15}N enriched proteins in addition to N from the diet for maintenance or functional purposes. The study by Chen et al. (2020) observed a positive and linear correlation of 0.55 between $\Delta^{15}N$ and MNE in healthy cows from 4 to 11 wk postpartum. In the present study, a strong and positive correlation between $\Delta^{15}N$ and MNE was observed in one of the experiments (ID20) conducted with high producing dairy cows in early lactation stage (Correa-Luna et al., 2021). Moreover, the coefficient of determination (R^2) between MNE and $\Delta^{15}N$ in the present study for the observations across all experiments in the first 50 d of lactation was lower ($R^2 = 0.02$) and non-significant when compared to the R^2 obtained from the mid and late lactation stages (Figure 1). In early lactation, body protein mobilization contributes to alterations in natural ^{15}N enrichment of milk (or plasma) over the diet, which in turn affects the response in $\Delta^{15}N$ due to MNE variation.

Mobilization of body reserves has been associated with dairy cow milk production and reproduction performance (Buckley et al., 2003), and with health status (de Vries and Veerkamp, 2000; Xu et al., 2018). The alteration of the isotopic signatures due to body reserves mobilization might provide additional evidence towards indirect or proximal detection for health events. More studies ideally based on larger databases generated from real-world farming conditions are required to confirm if $\Delta^{15}N$ is suitable for these purposes.

N Isotopic Discrimination as a Predictive Biomarker of MNE

In line with the results by Cantalapiedra-Hijar et al. (2018), our analysis confirmed that the most important variance-component for MNE (NUE in their study) was the between experiment variation. The contribution of experiment to the variance of MNE in the present study was around a third lower when compared to Cantalapiedra-Hijar et al. (2018), probably due to the differences between diets and production systems employed in both meta-analyses (dairy cows vs. multiple ruminant systems). Similarly, experiment was the main grouping factor for the total variance of $\Delta^{15}N$ and it was observed that the reduced mean MNE was associated with higher mean $\Delta^{15}N$ (Cantalapiedra-Hijar et al., 2018). Although on average, a negative association between MNE and $\Delta^{15}N$ was observed in the present study the responses were not the same across experiments and diets. The use of mixed-effects models on individual observations allowed the effects of experiment, sampling period, and diet to be removed, and thus, to evaluate the overall association between this biomarker and MNE at the individual level.

Residual standard error of models obtained in this present study and those reported by Cantalapiedra-Hijar et al. (2018) are comparable and ranged from 0.020 to 0.030 g/g of NUE or MNE, respectively. The differences in the slopes obtained between this study and those obtained by Cantalapiedra-Hijar et al. (2018), could be due to the contrasting set of diets employed by them. In their study, diets corresponded to different production systems including beef cattle, dairy goats, and non-lactating sheep and in this present study, diets were only from dairy production systems. Also, the larger intercepts obtained for the models of this present study are probably related to employing observations from only lactating cows specifically in mid-late lactation. A meta-analysis to evaluate the ability of MUN to predict MNE at the individual level was conducted by Huhtanen et al. (2015). In their study, the model residual error reported as residuals was in the range of what was obtained in this study and represented a slightly larger RMSPE% (8.1% vs. 6.8%). In the same way, the error obtained by Jonker et al. (1998) when using MUN for predicting MNE at the individual level was higher than ours (14.7% vs. 6.8%). In the study conducted by Huhtanen et al. (2015), they showed that employing MUN was not robust enough as a predictive biomarker of N partitioning at the individual level, and that the systematic addition of animal factors such as milk yield, BW, stage of lactation, dietary CP, and DMI had to be considered in order to achieve better characterizations of the between-animal variability in N partitioning. The lack of response of MUN to predict MNE at the between-cow variations could be due to the diurnal variation in MUN (Spek et al., 2013), and that some of this variation depends on the time of feeding and on the milking time with respect to the milk sampling (Gustafsson and Palmquist, 1993; Broderick and Clayton, 1997). Another factor of variation in MUN could depend on the method of analysis. A recent study by Portnoy et al. (2021) identified the need to perform regular calibrations for the mid-infrared spectroscopy method as there is considerable within- and between-laboratory variation in the reference

values for MUN that can improve the precision of its determination. Alternatively, mid-infrared spectra of milk has been proposed as a proxy to predict animal variation in MNE in early-lactation dairy cows (Grelet et al., 2020), but this methodology could be also conditioned to calibrations in order to achieve precise determinations, especially when the determination of mid-infrared spectra is done in different laboratories. Compared to the selected model at the individual level in this study, the RMSPE% of the predictive model by Grelet et al. (2020) was more than two-fold larger (6.8 vs. 17%). The large error obtained by Grelet et al. (2020) was considered not suitable to discriminate between low- and high-NUE cows and, in this case, this was attributed to the artificially high MNE observed in early lactation related to the severe mobilization of body reserves in this period of the lactation. In our case, the significant association between MNE and $\Delta^{15}N$ across different experiments and dietary treatments confirmed the suitability of this biomarker to significantly discriminate two cows randomly selected from the same CG if they differ by at least 0.112 g/g of MNE (±1.96 × RMSEP at 95% confidence level). At this stage, even though ^{15}N signature in plasma has been proven to be a moderate heritable trait in ruminants (Guarnido-Lopez et al., 2021), the minimum detectable difference of MNE here found (0.112 g/g) is considered too high for being proposed as a tool to assist genetic selection on MNE. Further studies are warranted to confirm this point.

Model Evaluation and Trait Repeatability

Based on different criteria employed to evaluate $\Delta^{15}N$ as a biomarker of MNE at the within-CG, we observed a contrasting performance across experiments (Table 6). The different modelling data set sizes from one experiment to another could have influenced some of these results (Fuentes-Pila et al., 2003). The RSR is a useful tool to compare the performance of models when different data is employed. Ideally this indicator should be less than 0.70 for satisfactory prediction models (Moriasi et al., 2007). Moreover, the different prediction fitness between experiments may also be a consequence of the diets employed in each experiment. For instance, Cantalapiedra-Hijar et al. (2016) identified that the association between $\Delta^{15}N$ and NUE could be compromised when diets are high in rumen degradable N. If the parallel between NUE and feed efficiency is permitted, Guarnido-Lopez et al. (2021) observed that feed conversion efficiency was poorly correlated to $\Delta^{15}N$ when employing diets high in fiber relative to diets high in starch, and attributed this to the rumen protein balance. Greater rumen ammonia concentration will increase fractionation of N isotopes at the rumen level (Wattiaux and Reed, 1995). Although beyond the objectives of this present study, mean increases in grass silage at the experiment

433 level resulted in poorer prediction fitness (lower r and higher RSR; data not shown) due to the increased RDP in
434 diets with higher proportion of grass silage relative to more starchy diets (Cantalapiedra-Hijar et al., 2018).
435 The consistency of a trait or phenotype across time (i.e., repeatability) is of upmost importance for genetic
436 studies (Friggens and Newbold, 2007). For instance, in genetic evaluations repeatability models based on test-
437 days are used for production traits in order to differentiate the genetic from the phenotypic variance (Berry et al.,
438 2014). In the present study, the mean repeatability estimate for $\Delta^{15}N$ across experiments was higher than that
439 observed by Wheadon et al. (2014) in growing heifers over a 3-month period (0.62 vs. 0.56). Across experiments,
440 the mean repeatability estimate for MNE was higher in the present study when compared to another study
441 (Ariyarathne et al., 2020) in two grazing herds with contrasting farming management in New Zealand. In their
442 study, they observed that the repeatability for efficiency of crude protein utilization (CP in milk divided by CP
443 intake) fluctuated from 0.60 to 0.13 according to the stage of the lactation throughout the grazing season, but their
444 mean repeatability was still lower than our mean repeatability estimate for MNE across studies (0.38 vs. 0.65).
445 While both studies had access to individual records of milk N (often generated from calibrated milk-meters), the
446 observations of the present study were generated in housed conditions with individual records of N intake, and in
447 the study by Ariyarathne et al. (2020), the repeatability was computed based on estimations of N intake on herd
448 level calculated from pasture disappearance and this might resulted in lower figures (Berry et al., 2014).
449 Moreover, the repeatability can also be referred as the consistency of repeated measurements (Harper, 1994). In
450 other words, a repeatability of 1 indicates that the measurement is perfectly consistent with no experimental error.
451 The present study confirmed the hypothesis that better repeatability values of both dependent (MNE) and
452 independent variables ($\Delta^{15}N$) would enhance model prediction performance as we observed a positive and strong
453 correlation along with the fitness prediction of the selected model (Figure 6a and 6b). This strong association
454 observed highlights the importance of measurement precision for the identification of proxies for phenotyping
455 animals.
456 Although in the present study we managed to establish and confirm the negative association of MNE with
457 $\Delta^{15}N$ over a range of experimental conditions, some potential limitations of the predictive ability for MNE of this
458 biomarker must be highlighted. The fact that in some particular CG the negative association of MNE with $\Delta^{15}N$
459 was not observed could be attributable to the uncertainly of reaching a new isotopic equilibrium when animals
460 shifted from one dietary treatment to another, especially for those experiments with a changeover design.
461 Nonetheless, in this study strong correlations were observed in two changeover studies (ID9 and ID15) which
462 means that even if the isotopic equilibrium had not reached at 100%, the biomarker is still working to predict

MNE at the individual level if cows differed in at least 0.112 g/g. It was suggested that the transitioning period between a diet shift should be no less than 27 d in order to reach a new isotopic equilibrium to ensure appropriate analyzing of $\Delta^{15}N$ data (Cantalapiedra-Hijar et al., 2015). Moreover, the determination of the isotopic signatures of diets could also be a limitation. Even though it is more feasible to pipette liquid subsamples (milk or plasma) to a higher level of accuracy and consistency onto the tin capsules, it is difficult to accurately collect minuscule portions of homogenous dried feed ingredients. Although samples were ground after drying, those feed ingredients represent a combination of large and small particles, such as silages of pasture or corn, represented a major challenge considering that the tiny fraction subsampled could substantially change from one portion to another. To avoid this, several repetitions had to be undertaken in order to reduce the CV aiming to achieve reliable $\delta^{15}N_{diet}$ determinations. Nonetheless, our results show that $\Delta^{15}N$ is still a powerful biomarker for discriminating within-CG a group of extreme cows in terms of MNE (Figure 5). This approach was recently employed to distinguish Brahman steers in terms of feed efficiency from isotopic signatures measured from tail hair and fed a low quality senesced C4 grasses (Costa et al., 2021). In line with our results, the steers with lower $\delta^{15}N$ had higher feed efficiency, less N excreted in the urine, and higher NUE in comparison with steers having higher $\delta^{15}N$. Also, $\delta^{15}N$ of the 20% highest feed efficiency steers resulted statistically different from the $\delta^{15}N$ of the 20% lowest feed efficiency steers indicating that N isotopic signatures could be used as a tool to identify animals with contrasting NUE. In our case N isotopic signatures of milk or plasma could not differentiate all cows in terms of MNE in a given CG, but this biomarker permitted to significantly differentiate the highest from the lowest quartile of lactating cows fed the same diet at the same place and time in terms of MNE without the necessity of measuring feed intake. In the context of precision feeding, the implementation of nutritional grouping aims at providing different diets to different groups of animals to better fulfill their nutrient requirements. For instance, N isotopic signatures could be used as a tool to sub-groups cows with the highest $\Delta^{15}N$ values (recognized as of having less MNE) and assigned diets with enzyme and/or inoculant additives to protect CP from rumen degradation or fed restricted-CP diets aiming to increase their MNE and to reduce their N excreta. Hence, cluster sub-groupings towards more precise feeding without compromising the farm management would improve the overall feed efficiency while reducing the environmental footprint which should be translated into economic and social benefits (Cabrera and Kalantari, 2016).

CONCLUSIONS

In the present study, we have confirmed the negative and significant correlation between $\Delta^{15}N$ and MNE in lactating dairy cows regardless of experimental site, sampling period, and dietary treatment in mid and late lactation stages. However, the obtained prediction error of the developed model (0.028 g/g) exposes that $\Delta^{15}N$ only allows to differentiate extreme cows in terms of MNE. Hence, $\Delta^{15}N$ can be implemented as a tool to group animals (25% highest vs. 25% lowest MNE) for precision feeding. In early lactation both MNE and $\Delta^{15}N$ values might be artificially increased because of the considerable protein mobilization of body reserves. This was confirmed by observing a positive (rather than negative) association of $\Delta^{15}N$ along with MNE in early lactation. Increases in repeatability of either MNE and $\Delta^{15}N$ improved the prediction fitness of the model to differentiate cows in terms of MNE when fed the same diet at the same time. This emphasizes the need to identify best sampling protocols and to monitor the accuracy of measurements towards the identification and improvement of proxies to phenotype animals.

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APPENDIX

References of experiments used in the meta-analysis

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698 **Table 1.** Description of experiments included in the present meta-analysis study

Experiment	Design	Sampling periods	Dietary treatments	Reference[1]
ID1	Changeover	4	2	Saro et al., 2019
ID2	Changeover	2	2	Herremans et al., 2020
ID3	Continuous	36	3	Reynolds et al., 2021
ID4	Continuous	3	4	Pourazad et al., 2021
ID5	Changeover	4	8	Johansen et al., 2017
ID6	Changeover	4	6	Damborg et al., 2019
ID7	Changeover	4	4	Unpublished data
ID8	Changeover	4	6	Giagnoni et al., 2021
ID9	Changeover	4	4	Unpublished data
ID10	Changeover	4	4	Martin et al., 2019
ID11	Continuous	4	3	Unpublished data
ID12	Continuous	13	1	Wallace et al., 2019
ID13	Changeover	2	2	Guyader et al., 2016
ID14	Changeover	2	2	Guyader et al., 2017
ID15	Changeover	4	8	Mendowski et al., 2019
ID16	Changeover	4	4	Mendowski et al., 2020
ID17	Changeover	4	4	Edouard et al., 2018
ID18	Changeover	4	2	Unpublished data
ID19	Continuous	1	3	Coppa et al., 2020
ID20	Continuous	4	2	Bahloul et al., 2021

699 [1]Detail of references included in the Appendix.

700 **Table 2.** Description of animal performances and diets from experimental studies

Item	n	Mean	SD	Minimum	Maximum
Production data					
Days in milk	1,300	139	83	4	1,074
DMI, kg/d	1,300	22.4	3.5	12.0	35.4
BW, kg	1,300	663	79	394	1,033
Milk yield, kg/d	1,300	32.8	8.05	3.5	60.1
Milk fat concentration, g/kg	1,300	39.8	5.9	21.1	61.9
Milk protein concentration, g/kg	1,300	33.5	3.6	25.2	50.6
Milk lactose concentration, g/kg	1,219	48.5	2.6	37.4	57.1
Fat:Protein ratio	1,300	1.19	0.16	0.70	1.93
Fat yield, kg/d	1,300	1.30	0.35	0.13	2.79
Protein yield, kg/d	1,300	1.09	0.28	0.12	1.96
Lactose yield, kg/d	1,219	1.58	0.40	0.13	3.05
Feed composition data					
Diet composition[1]					
Concentrate, % of DM	74	37.28	15.27	8.79	82.64
Corn silage, % of DM	55	30.77	17.66	4.63	75.76
Grass silage, % of DM	45	42.90	17.17	16.50	70.00
Grass hay, % of DM	28	22.44	8.88	3.03	35.78
Urea, % of DM	6	1.40	0.19	0.78	1.78
Chemical composition[2]					
CP, g/kg of DM	74	157	25	110	268
NDF, g/kg of DM	74	379	94	202	607
ADF, g/kg of DM	36	241	66	131	351
Starch, g/kg of DM	52	186	56	92	293
Net energy for lactation, Mcal/kg DM	48	1.56	0.06	1.44	1.70
OM, g/kg of DM	62	861	94	653	955

701 [1]According to diet formulation.
702 [2]According to data availability.

Table 3. Mean, variability and repeatability values for milk N, N intake, N isotopic discrimination ($\Delta^{15}N$), and milk N efficiency (MNE) from experimental studies used in the mixed-effect model analysis

Item	Initial data set	Data set for $\Delta^{15}N$ modelling	ID1	ID2	ID3	ID4	ID5	ID6	ID7	ID8	ID9	ID10	ID11	ID12	ID13	ID14	ID15	ID16	ID17	ID18	ID19
n	1,300	1,135	52	12	71	167	140	128	138	87	17	32	22	100	16	10	31	32	12	23	45
Milk N, g/d																					
Average	173.4	168.5	175.3	103.5	163	116.1	174.8	203.3	188.5	180	128.3	152.8	213.6	188.4	144.8	125.8	140.9	157.4	153.1	178.4	182.8
SD	42.4	40.1	26.4	7.7	29	17.2	24.2	34.4	38.1	31	54	18.4	21	28.8	27.6	31.8	17.5	17.4	15.6	14.9	30.2
Minimum	19	19	94	93.8	112	70.7	120.7	133.1	46.6	116.6	19	117.8	176.3	115.6	105.3	73.6	105.9	127	122.2	139.5	112.8
Maximum	323.8	291.4	222.1	116.8	240	154.8	233.2	276.3	251.5	291.4	216.2	189.3	254.9	247.8	189.3	198	174.8	183.7	170.2	203.6	230
Repeatability[1], %	70	72.4	69.8	79.8	58.6	87.2	82.1	80.1	93.5	81.4	88.8	69.6	68.9	-	-	95.3	79.7	60.3	87.6	71.6	-
N intake, g/d																					
Average	565.8	570.3	489.3	412.5	558.6	423.1	646.6	615	627.1	666.5	533.7	541.5	630.8	615.8	486.6	481.2	490.5	653.5	463.5	499.5	559.9
SD	122.9	123.4	53	18.5	101	59.4	168.5	92.9	81.6	73.7	56.8	49.2	108.7	76.7	86.7	79.7	59.2	39.1	72.3	22.1	74
Minimum	271.1	271.1	397.8	390.6	369	271.1	397.1	412.9	445.4	493.2	442.8	449.5	496.9	464.1	347.3	399.1	410.6	538.6	372.9	457.1	362.1
Maximum	1151.8	1151.8	623.5	437	819	689.6	1151.8	856.4	801.8	826.9	672.1	645.1	851.3	822.3	635	672.5	618.6	734.6	564.1	545.5	724.2
Repeatability, %	67.6	74.8	46.9	87.2	56.1	85.7	76.9	65.7	86.8	85.8	56.5	61.1	68.7	-	-	36.1	80.9	50	91.8	60.1	-
$\Delta^{15}N$, ‰																					
Sample type[2]			Milk	Milk	Plasma	Milk	Milk	Milk	Plasma	Plasma	Milk	Milk	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma	Plasma
Average	2.209	2.239	1.104	2.928	1.983	2.041	2.713	1.999	2.112	3.164	2.286	2.023	2.653	2.818	1.558	2.018	2.298	2.775	1.384	1.362	1.497
SD	0.747	0.667	0.502	0.189	0.511	0.31	0.406	0.38	0.55	0.441	0.663	0.397	0.369	0.226	0.382	0.357	0.215	0.276	0.565	0.115	0.691
Minimum	0.038	0.101	0.101	2.626	1.122	1.297	1.82	0.943	0.763	2.208	1.562	1.307	2.079	2.303	1.06	1.503	1.843	2.15	0.584	1.131	0.2
Maximum	4.553	4.457	2.55	3.257	2.941	2.984	3.556	3.055	3.82	4.457	4.048	2.936	3.464	3.364	2.103	2.475	2.654	3.335	2.193	1.544	2.269
Repeatability, %	37.2	56.1	56.8	65.3	59.6	60	40.1	36.5	79.2	55.9	56.1	62.6	69.8	-	-	93.7	75.8	63.7	59.1	41.8	-
MNE, g/g																					
Average	0.31	0.3	0.36	0.25	0.31	0.28	0.28	0.33	0.3	0.27	0.24	0.28	0.36	0.31	0.3	0.26	0.29	0.24	0.34	0.36	0.33
SD	0.06	0.05	0.05	0.01	0.04	0.03	0.05	0.04	0.05	0.04	0.09	0.03	0.03	0.03	0.03	0.04	0.04	0.02	0.05	0.02	0.02
Minimum	0.04	0.04	0.2	0.23	0.24	0.14	0.16	0.23	0.09	0.18	0.04	0.23	0.3	0.21	0.24	0.17	0.24	0.2	0.24	0.32	0.28
Maximum	0.69	0.47	0.47	0.28	0.39	0.36	0.39	0.44	0.38	0.38	0.32	0.35	0.43	0.36	0.35	0.31	0.35	0.27	0.41	0.39	0.39
Repeatability, %	51.7	67.5	69.8	45.4	37.4	84.3	65.7	65.9	89.5	73.1	89.9	52.8	54.4	-	-	45.6	87.9	58.8	37.5	49.7	-
Correlation[3] of MNE with $\Delta^{15}N$	-	-	-0.55*	-0.40NS	-0.81*	-0.48*	-0.47*	-0.41*	-0.61*	-0.29*	-0.87*	-0.45*	-0.50**	-0.33*	-0.24NS	-0.43NS	-0.76*	-0.07NS	-0.84*	-0.48**	0.44*

[1]Repeatability values were calculated as $\sigma^2_{Cow} / (\sigma^2_{Cow} + \sigma^2_{Residual})$, where σ^2_{Cow} and $\sigma^2_{Residual}$ are cow within a single experiment and residual variances, respectively.
[2]The N isotopic signatures ($\delta^{15}N$) of cows was determined either from milk or plasma samples.
[3]Within-study linear regressions between milk N efficiency and $\Delta^{15}N$: *$P \leq 0.001$; **$P \leq 0.05$; NSnon-significant.

707 **Table 4.** Variance-component estimates of animal performances, N isotopic discrimination ($\Delta^{15}N$), and milk N efficiency
708 (MNE) from experimental studies used in the mixed-effect model analysis

Item[1]	Mean value ± SD	Estimate	95% CI[2]	ICC[3] (%)
Milk N, g/d (n = 1,135)	168.5 ± 40.1			
ID		29.1	20.7 - 41.0	45.3%
SP		0.9	0.1 - 5.8	1.3%
DT		6.8	4.6 - 10.1	10.6%
Residual		27.5	26.3 - 28.7	42.7%
N intake, g/d (n = 1,135)	560.7 ± 128.4			
ID		75.8	48.7 - 117.9	34.3%
SP		5.8	0.3 - 98.9	2.6%
DT		73.8	60.1 - 90.5	33.4%
Residual		65.6	62.6 - 68.7	29.7%
$\Delta^{15}N$, ‰ (n = 1,135)	2.239 ± 0.667			
ID		0.558	0.386 - 0.806	42.6%
SP		0.177	0.150 - 0.209	13.3%
DT		0.286	0.225 - 0.364	21.8%
Residual		0.289	0.276 - 0.303	22.1%
MNE, g/g (n = 1,135)	0.30 ± 0.05			
ID		0.067	0.039 - 0.083	41.0%
SP		0.019	0.015 - 0.024	13.7%
DT		0.024	0.019 - 0.032	17.5%
Residual		0.039	0.037 - 0.041	27.8%

709 [1]ID = experiment; SP = sampling period within experiment; DT = dietary treatment within period and experiment.
710 [2]Confidence interval.
711 [3]Intra-class correlation coefficient = total variance explained by the corresponding random variable. For instance, for the nested
712 random variables of DT it refers to the proportion of variance explained only by the dietary treatment from the total variance.

713 **Table 5.** Mixed-effect regression models of milk N efficiency (g/g) on the N isotopic discrimination ($\Delta^{15}N$) using either dietary treatment means or individual observations in mid and late lactating
714 dairy cows

Item[§]	Model no.	Intercept	Slope	AIC[2]	Fit statistics variables[1]				
					RMSPE (g/g)	RMSPE (%)	R^2	CCC	RSR
Tier 1: dietary treatment means									
$\Delta^{15}N$, ‰ (n = 72)									
Experiment random effects	1	0.378* ± 0.017	-0.037* ± 0.007	-289	0.034	8.9	0.28	0.478	0.822
Tier 2: individual observations									
$\Delta^{15}N$, ‰ (n = 1,135)									
Experiment random effects	2	0.403* ± 0.014	-0.049* ± 0.007	-4,313	0.036	8.8	0.30	0.380	0.882
Experiment and period random effects	3	0.407* ± 0.013	-0.050* ± 0.007	-4,316	0.035	8.7	0.32	0.381	0.883
Contemporary group random effects[$]	4	0.417* ± 0.013	-0.056* ± 0.007	-4,498	0.028	6.8	0.36	0.400	0.851

715 [1]RMSPE = square root of the mean square prediction error expressed in g/g and RMSPE% as a percentage of mean observed MNE; R^2 = coefficient of determination calculated for equations
716 according to the experimental factor nesting level included in each case; CCC = concordance correlation coefficient; RSR = square root of the mean square prediction error to standard deviation
717 of observed values ratio.
718 [§]At the treatment means level, the model was tested with random effects on the intercept and at the individual observations level, all models were tested with random effects on the intercept, slope
719 or both.
720 [2]AIC = Akaike information criterion.
721 [$]Best random structure model based on the AIC criterion.
722 *$P \leq 0.001$; **$P \leq 0.05$; [NS]non-significant.

723 **Table 6.** Fit statistics of the models obtained to predict milk N efficiency (MNE) from N isotopic discrimination ($\Delta^{15}N$) at the individual level for each experiment used in the mixed-effect model
724 analysis

Item	Modelling data set	ID1	ID2	ID3	ID4	ID5	ID6	ID7	ID8	ID9	ID10	ID11	ID12	ID13	ID14	ID15	ID16	ID17	ID18	ID19
Fit statistics variables[1]																				
n	1,135	52	12	71	167	140	128	138	87	17	32	22	100	16	10	31	32	12	23	45
RMSPE, g/g	0.026	0.044	0.011	0.021	0.028	0.019	0.028	0.035	0.032	0.056	0.025	0.023	0.027	0.025	0.026	0.026	0.021	0.026	0.018	0.018
RMSPE, %	8.8	12.2	4.4	6.7	10.2	6.8	8.4	11.5	11.6	23.6	8.8	6.4	8.8	8.3	9.8	8.9	8.7	7.7	4.9	5.6
CCC	0.391	0.433	0.510	0.389	0.340	0.172	0.262	0.483	0.394	0.604	0.453	0.345	0.245	0.322	0.541	0.452	0.227	0.283	0.237	0.339
r	0.52	0.55	0.61	0.43	0.45	0.20	0.40	0.72	0.49	0.91	0.51	0.40	0.33	0.45	0.71	0.80	0.25	0.45	0.43	0.41
RSR	0.857	0.829	0.764	0.922	0.891	1.044	0.914	0.767	0.866	0.653	0.855	0.906	0.945	0.867	0.710	0.762	1.035	0.860	0.893	0.911

725 [1]Fit statistics variables: n = observations; RMSPE = square root of the mean square prediction error expressed in g/g and RMSPE% as a percentage of mean observed MNE; CCC = concordance
726 correlation coefficient; r = correlation coefficient; RSR = square root of the mean square prediction error to standard deviation of observed values ratio.
727 [$]Selected mixed-effect regression models obtained based on best random structure model based on AIC criteria (Table 5).
728 Calculations of observed and predicted for modelling evaluation at the highest contemporary group reached in the modelling stage:
729 Observed between-animal variability MNE = $MNE_{contemporary\ group} - MNE_{individual}$.
730 Predicted MNE = $(\Delta^{15}N_{contemporary\ group} - \Delta^{15}N_{individual}) \times -0.056$ [Model 4].

BIOMARKERS FOR MILK N USE EFFICIENCY IN DAIRY COWS

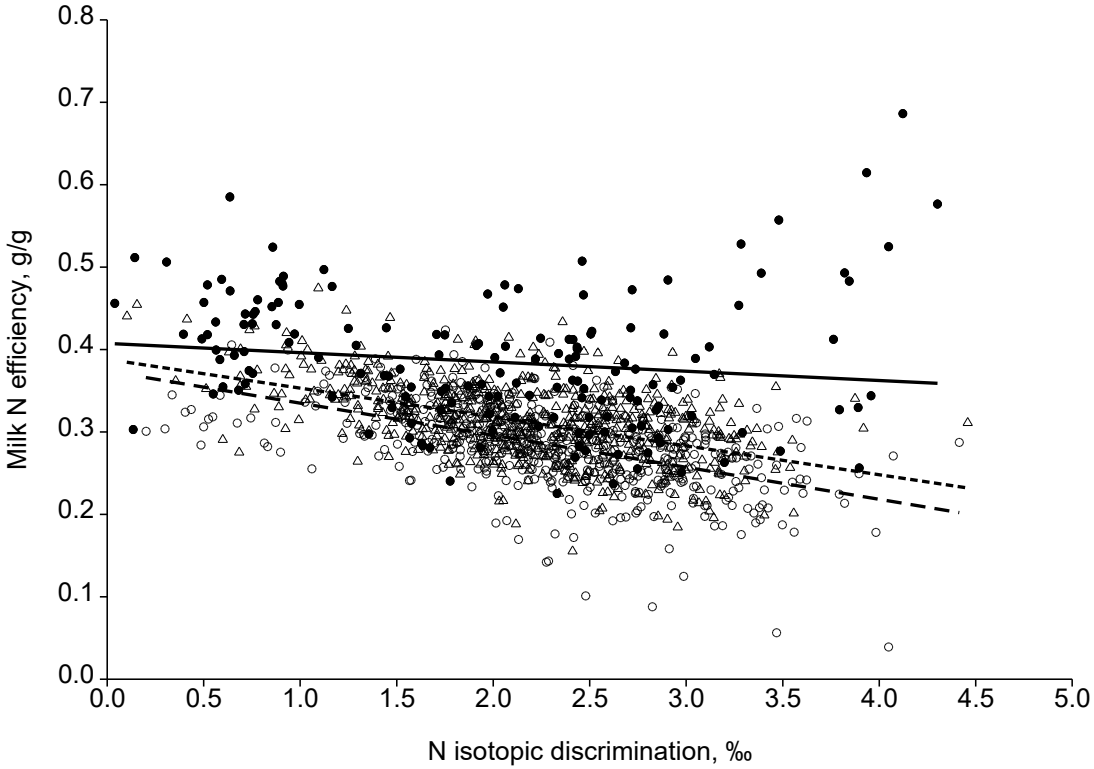

Figure 1. Relationship between milk N efficiency (MNE, g/g) and N isotopic discrimination ($\Delta^{15}N$, ‰) in lactating cows using individual values (n = 1,300) for early (solid line and closed circles), mid (dashed line and open circles), and late lactation (dotted line and open triangles). Overall relationships:

$MNE_{EARLY} = 0.408 - 0.011 \times \Delta^{15}N$ (n = 165; $R^2 = 0.02$; RSE = 0.08; P = 0.08);

$MNE_{MID} = 0.388 - 0.035 \times \Delta^{15}N$ (n = 610; $R^2 = 0.25$; RSE = 0.04; $P < 0.001$);

$MNE_{LATE} = 0.374 - 0.039 \times \Delta 15N$ (n =525; $R^2 = 0.28$; RSE = 0.04; $P < 0.001$).

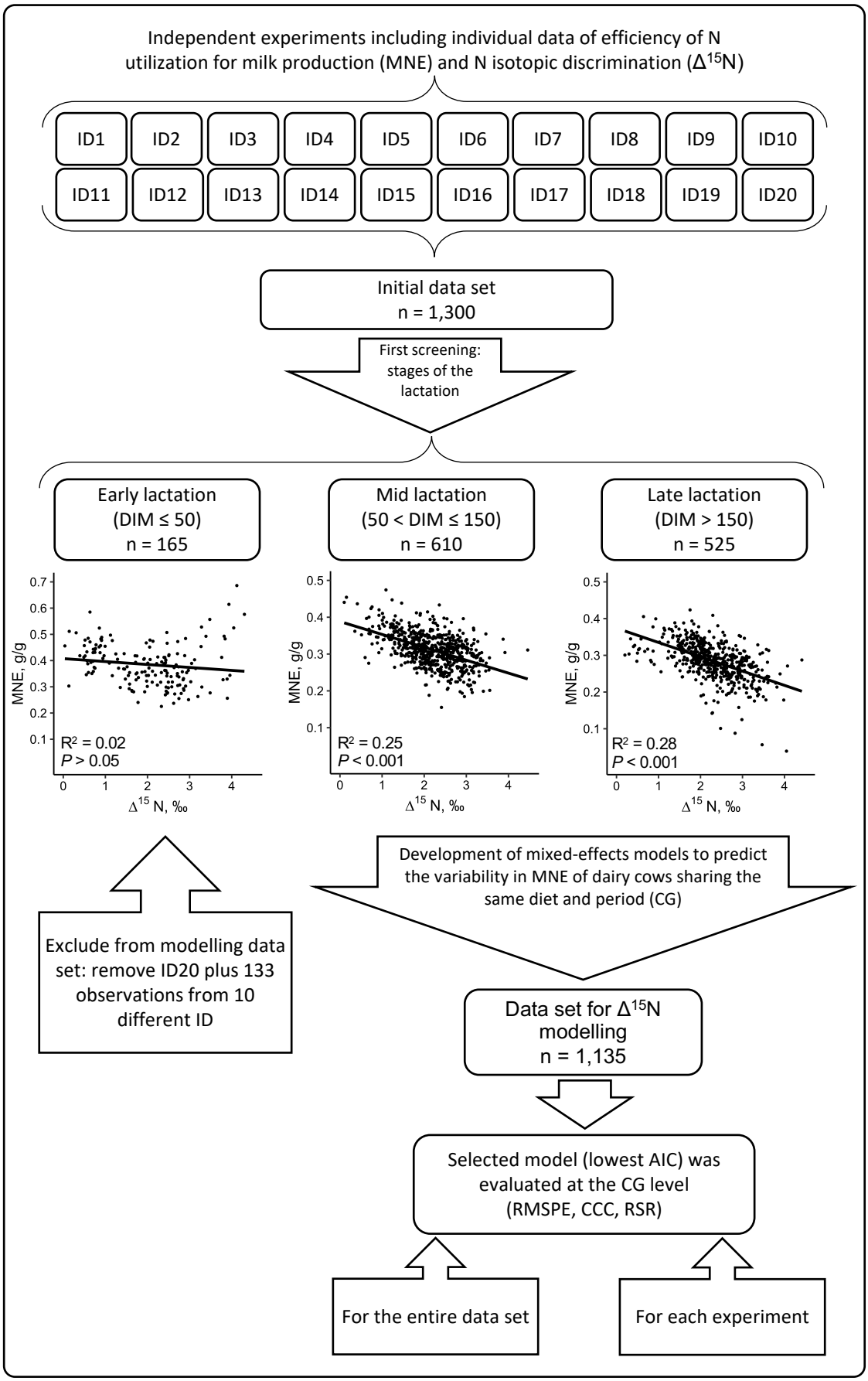

Figure 2. Diagram illustrating the experiment (ID) compilation, data screening, and model development with its evaluation. AIC = Akaike Information Criterion; RMSPE = root mean square prediction error; CCC = concordance correlation coefficient; RSR = square root of the mean square prediction error to standard deviation of observed values ratio.

BIOMARKERS FOR MILK N USE EFFICIENCY IN DAIRY COWS

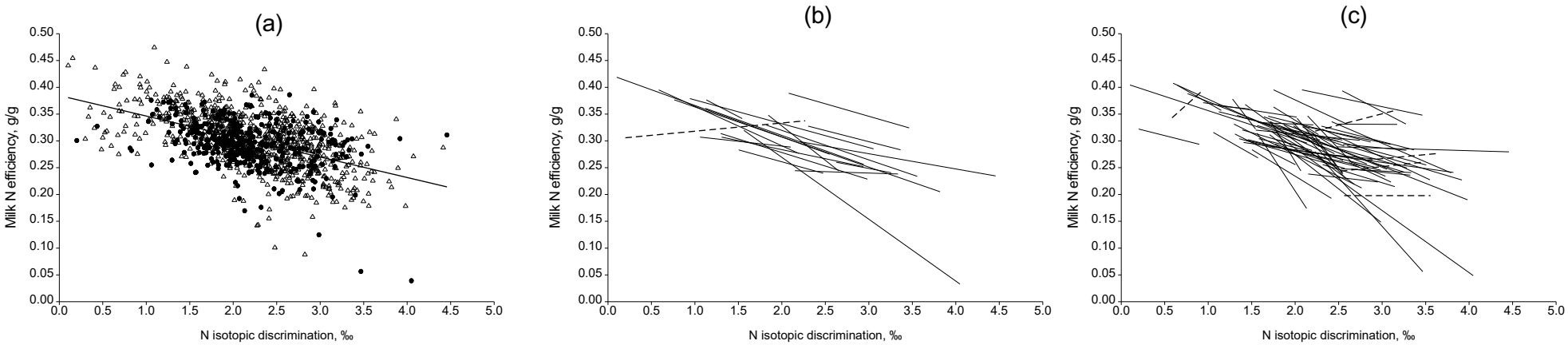

Figure 3. Relationship between milk N efficiency (MNE) and N isotopic discrimination ($\Delta^{15}N$) in lactating cows using individual values (n = 1,135): (a) Simple linear regression analysis [overall relationship: MNE = 0.385 − 0.038 × $\Delta^{15}N$ (n = 1,135; R^2 = 0.26; RSE = 0.04; $P < 0.001$)] where open triangles represent multiparous cows and closed circles represent primiparous cows; (b) simple linear regression for each independent study (n = 19; within-study regression) (c) simple linear regression analysis for each independent diet (n = 72; within-diet regression). In (b) and (c) solid lines represents negative slopes and dashed lines represents positive slopes. Correlations coefficients (and statistical significances) between MNE and $\Delta^{15}N$ are presented in Table 3.

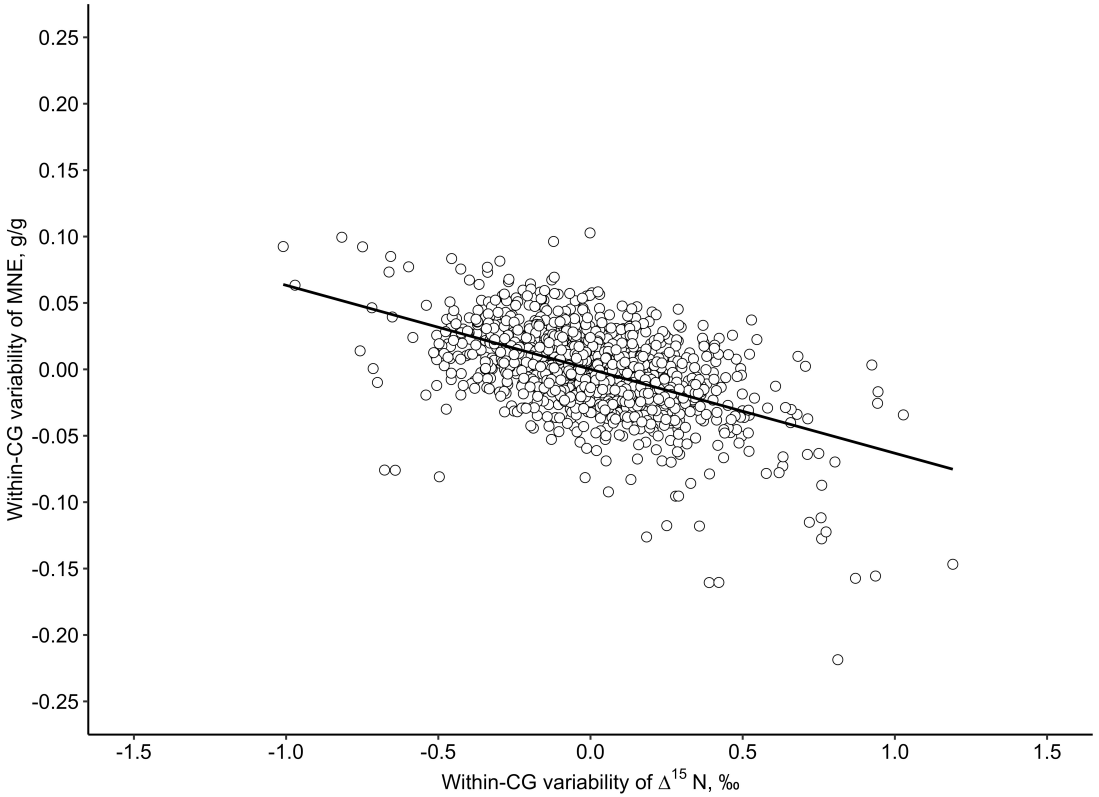

Figure 4. Simple linear regression between residuals of milk N efficiency (MNE) in lactating dairy cows and N isotopic discrimination ($\Delta^{15}N$). Residuals were obtained when variables were independently adjusted for the random effects of the study, period (within-study), and diet (within-period and study). Equation: MNE = −0.067 (± 0.003) × $\Delta^{15}N$, ‰ (n = 1,135; R^2 = 0.29; RSE = 0.028; $P < 0.001$).

BIOMARKERS FOR MILK N USE EFFICIENCY IN DAIRY COWS

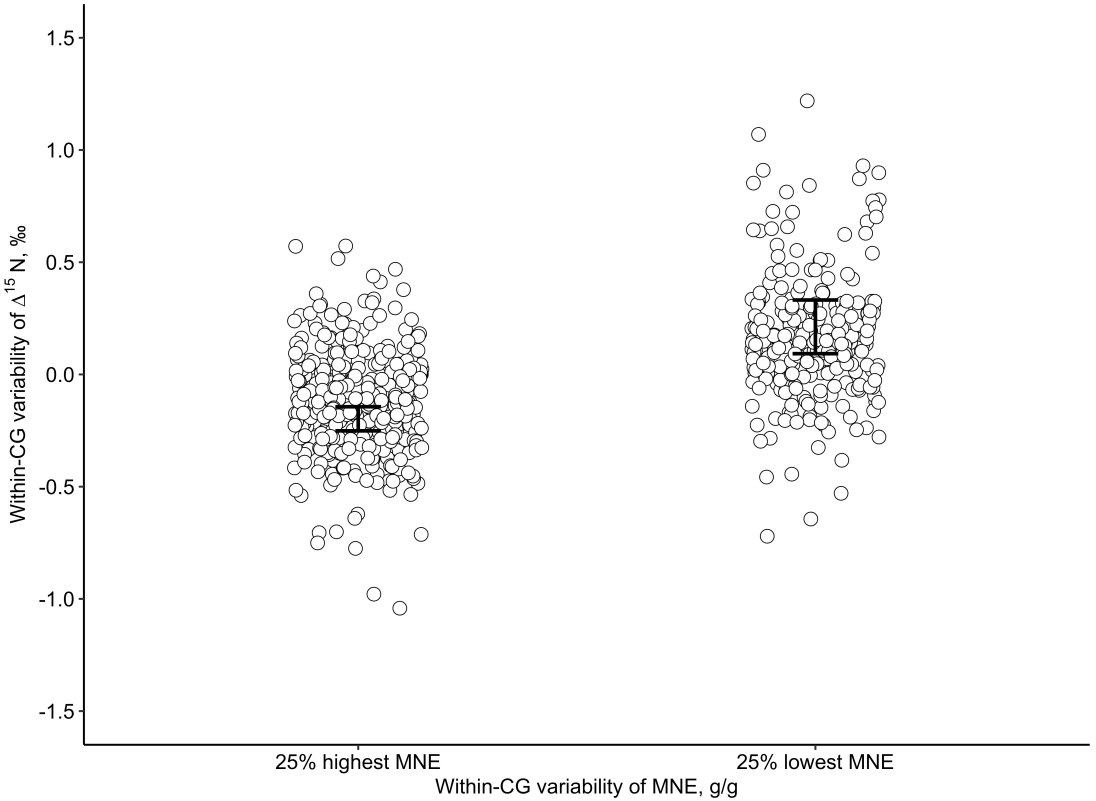

Figure 5. Within-contemporary group (CG) values for N isotopic discrimination ($\Delta^{15}N$) in the top 25% highest and lowest efficient animals within-CG according to milk N efficiency (MNE).

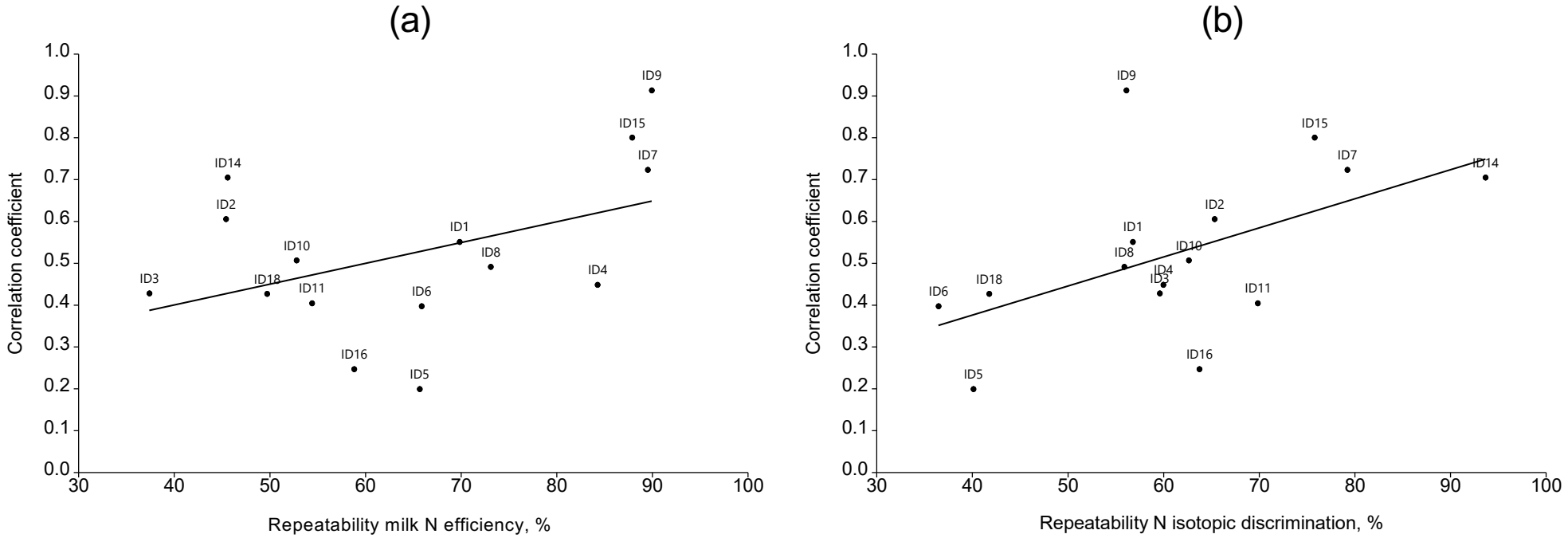

Figure 6. Relationship between mixed-effects model of milk N efficiency (MNE) from N isotopic discrimination ($\Delta^{15}N$) (MNE = 0.415 − 0.052 × $\Delta^{15}N$) model evaluation (correlation coefficient between observed vs. predicted MNE) at the within-study level (Table 5) and repeatability of either (a) MNE (R^2 = 0.49; *P* = 0.06) or (b) $\Delta^{15}N$ (R^2 = 0.54; *P* = 0.03).