

Article

Nitrogen Utilization and Predictive Modeling of Nitrogen Efficiency and Excretion in Lactating Dairy Cows

Katharina Padberg ¹, Christian Visscher ², Marcus Peschel ¹, Christin Unruh ¹, Dirk von Soosten ¹, Erik Bannert ¹, Patricia Mertens ¹, Liane Hühner ¹, Susanne Kersten ¹, Sven Dänicke ¹ and Ulrich Meyer ^{1,*}

¹ Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI), Federal Research Institute for Animal Health, Bundesallee 37, 38116 Brunswick, Germany; katharina.padberg@fli.de (K.P.)

² Institute for Animal Nutrition, University of Veterinary Medicine Hanover—Foundation, 30173 Hanover, Germany

* Correspondence: ulrich.meyer@fli.de

Simple Summary

Efficient nitrogen use is essential for sustainable dairy production. This study investigates the relationships among nitrogen intake, nitrogen excretion, and nitrogen efficiency in dairy cows, given large variations in dietary ingredients and chemical composition, as a precondition for developing models to predict N utilization. The results show that balanced energy and protein supply improve N utilization, whereas N oversupply increases N losses. These general relationships can not only be reliably described by a model using variables that maximize explainable variance but also by a model based on variables available under practical feeding conditions.

Abstract

A key factor in reducing nitrogen (N) emissions from dairy farms is understanding the interdependencies between N intake (NI), N excretion (Nex), and N use efficiency (NEff) in cows. This study analyzed these parameters and their interrelationships to develop predictive models. Data were collected over two years, during six feeding periods with different rations (N: 20.5–25.6 g per kilogram dry matter [g/kg DM]; metabolizable energy (ME): 10.8–12.1 megajoules per kilogram dry matter [MJ/kg DM]; net energy for lactation (NEL): 6.5–7.5 MJ/kg DM). Each period included 48 German Holstein cows (620 ± 65 kg body weight [kg BW]; first to fifth lactation). Blood, urine, feces, milk, and feed samples were collected, and individual feed intake and milk yield were recorded. NI was positively related to nitrogen in milk (NM; $R^2 = 0.68^{**}$) and feces (NF; $R^2 = 0.49^{***}$). Several feeding, milk, and blood parameters showed significant correlations with NEff. These variables were used to develop two mixed-effects models. One model is based on variables available under practical feeding conditions. It includes dietary gross energy (GE), dietary crude protein (CP), milk yield, and milk protein concentration. This model shows good statistical performance in comparison to the calculated NEff (CCC = 0.78). The best explanatory model added blood parameters and further increased prediction accuracy (CCC = 0.90). In conclusion, NEff can be reliably predicted using moderately complex models. These models combine routine feeding and milk data with selected blood indicators.

Keywords: correlation; dairy cow; German Holstein; linear regression; mixed model; nitrogen balance; nitrogen efficiency; nitrogen excretion; prediction model



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1. Introduction

Nitrogen efficiency (NEff) in dairy cows indicates both production and environmental sustainability [1]. NEff is the ratio of nitrogen excreted in milk (NM) to total nitrogen intake (NI), usually given as a percentage. Inefficient nitrogen (N) utilization results in economic losses from unused protein and increased N emissions [2,3]. N is excreted through urine and feces [4–6]. Improving NEff is now a key objective in sustainable dairy cattle feeding, addressed through precise feeding strategies.

Accurate assessment of NEff requires reliable quantification of NI, nitrogen excretion (Nex), the sum of urinary (NU) and fecal (NF) losses, and NM. However, measuring individual animal parameters, especially total Nex in dairy cows, is often limited by methodological and practical constraints. These constraints are particularly notable under typical farm conditions. As a result, indirect or calculation-based approaches are used. In these, parameters such as fecal or urinary nitrogen are estimated rather than measured directly.

In our previous work [7], an individual animal approach to calculate urine excretion based on creatinine (Crea) excretion under controlled feeding conditions was evaluated. This approach has proven suitable and provides a basis for predicting daily urine volume (UV) and NU for individual cows. Urine volume can be estimated using the constant relationship between creatinine excretion and body weight, as shown earlier [8]. We validated this relationship for current Holstein genetics. We also included dry cows. This resulted in a factor of 29.5 mg [7]. This adjustment ensures a reliable estimate of UV as a function of BW.

Previous studies have described linear relationships between NI and Nex, as well as between protein or N-concentration in feed and N-metabolism indicators, such as blood urea nitrogen (BUN) and milk urea nitrogen (MUN) [9,10]. These parameters have proven useful as indirect markers of N status. However, their accuracy in predicting NEff under practical feeding conditions remains variable. The impact of other parameters on NEff still needs clarification [11].

2. Materials and Methods

2.1. Experimental Design and Animals

The NI and N excretions were recorded in 70 lactating German Holstein cows over six periods (P1–P6), using 48 cows per period. The 48 cows were used across all periods whenever possible. A total of 261 samples were collected to investigate N excretion rates under different diets. The experiment ran for three periods each year. Each lasted 42 days, starting with a 21-day adaptation phase to the respective experimental diet. This was followed by a 21-day sampling phase during which milk, urine, feces, and blood samples were collected weekly. To ensure equality, cows were evenly assigned to four groups based on BW, milk yield, feed intake, and parity. Except for P6, each period included four diets arranged in a 2×2 factorial design. The first factor was concentrate level (low concentrate = LC, high concentrate = HC). The second factor was different ration components (P1 control/fat (CON/FAT), P2 control/unsaturated fatty acids (CON/UFA), P3 control/hay (CON/HAY), P4 control/alfalfa silage (CON/ALF), P5 control/byproducts as brewers' grains silage and pressed pulp silage (CON/BYP), and P6 faba bean (FAV)). P6 used only two groups (LC and HC), with faba bean in both diets (Table 1). All cows were housed on a slatted floor in a free-stall barn with lying boxes.

Table 1. Ingredients and energy of feed in periods 1–6.

Period Supplement Concentrate % of Total Ration	1				2				3				4				5				6	
	FAT		CON		UFA		CON		HAY		CON		ALF		CON		BYP		CON		FAV	
	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC
	30	55	30	55	30	55	30	55	30	55	30	55	35	50	35	50	25	45	25	45	20	50
Ingredients (g/kg DM)																						
Crude ash	60	67	60	57	65	60	65	60	61	58	63	59	63	62	49	55	71	64	62	59	66	62
Crude protein	128	153	130	151	153	165	153	165	149	157	152	159	151	158	150	157	151	166	144	159	139	156
Ether extract	56	56	33	31	33	31	33	31	31	30	33	31	32	32	30	30	34	37	31	35	31	31
Crude fiber	187	147	188	144	209	157	209	157	212	162	201	155	187	162	188	163	225	206	206	186	216	164
aNDFom	376	320	380	321	411	340	411	340	415	347	392	333	356	323	368	333	469	416	399	359	416	339
ADFom	220	233	312	232	240	186	240	186	238	188	228	187	211	180	208	183	254	225	225	203	230	178
Starch	200	261	214	300	189	285	189	284	189	282	204	292	230	273	244	282	53	113	223	236	218	287
Sugars	51	56	52	54	47	51	43	51	53	54	46	50	28	36	33	40	46	43	38	45	42	49
Metabolizable Protein (MP)	144	155	147	156	146	155	146	155	145	154	148	155	153	159	150	156	155	162	146	157	141	157
RNB	−1	1	−1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	1	0	0	0	0
Energy (MJ/kg DM)																						
ME	11.6	12.1	11.2	11.7	10.9	11.5	10.9	11.5	10.8	11.0	11.0	11.5	11.1	11.4	11.1	11.5	10.9	11.5	10.8	11.4	10.8	11.5
NEL	7.1	7.5	6.9	7.3	6.6	7.1	6.6	7.1	6.5	7.0	6.7	7.1	6.7	7.0	6.8	7.1	6.7	7.0	6.5	7.0	6.5	7.1

Legend: LC = low concentrate, HC = high concentrate, CON = control, FAT = fat, UFA = unsaturated fatty acids, HAY = hay, ALF = alfalfa silage, BYP = byproducts (brewers' grains silage and pressed pulp silage), FAV = fava bean, RNB = Rumen Nitrogen Balance, ME = Metabolizable Energy, NEL = net energy for lactation, DM = dry matter, aNDFom = α -amylase-treated neutral detergent fibre, ADFom = acid detergent fibre.

Cows were three to six years old. They were in their first to fifth lactation and, on average, 168 ± 35 days in milk. BW of all cows was measured twice daily throughout the experiment and averaged 620 ± 65 kg.

Cows were milked at 6:00 AM and 4:00 PM daily. They had ad libitum access to fresh water in drinking bowls. Composition of the diets varied according to the period. The proportions of PMR and concentrate feed in the rations were calculated according to the nutrient supply recommendations of the GfE [12,13] (Table 1).

More details on the diets and experimental setup are available in a superior study, which was designed to quantify methane emissions (unpublished). The samples in this study are a subset of those from that experiment and focus exclusively on nitrogen metabolism. All animal procedures followed national animal welfare legislation. Approval was granted by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; approval no. 33.19-42502-04-20/3467).

2.2. Data Recording and Sample Collection

Feed intake was recorded continuously throughout the experiment for each animal, with weighing troughs (Hokofarm Group, Emmeloord, The Netherlands). Every week, samples of Partial Mixed Ration (PMR) and concentrate were collected. Feed samples were dried at 60°C .

Milk yield was recorded daily using the GEA Group Aktiengesellschaft milking system (Düsseldorf, Germany). Each week, one milk sample was taken in the morning and one in the evening on two consecutive days to represent the daily yield. Samples from both days were pooled for each cow based on yield, giving one weekly sample. Each 35–50 mL pool sample was preserved with Bronopol, kept at 4°C , and shipped for milk component analysis at Labor und Dienstleistungs GmbH & Co. KG, Krefeld, Germany.

In each sampling phase (weeks 4, 5, and 6 of each period), feces samples were taken twice: once in the morning and once in the evening, rectal or spontaneous. This resulted in six 300 g samples. They were pooled into 5 L containers and stored at -20°C . Before analysis, samples were unfrozen, homogenized, and then freeze-dried. Afterwards, they were ground through a 1 mm sieve.

Urine samples were taken every sampling week, once in the morning and once in the evening, during spontaneous urination or stimulation. Each day, 15 mL was pooled into 100 mL Kautex tubes throughout the sampling period and stored directly at -20°C in a fridge.

Blood was collected by needle puncture of a Vena jugularis externa in serum tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) collected in the morning on one day per sample week. After collection, the samples were allowed to clot for 60 min at room temperature and centrifuged at $1940 \times g$ for 15 min (Varifuge 3.0, Heraeus, Hanau, Germany) to separate serum. Aliquots were then stored till analysis at -80°C .

2.3. Analyses

Feed samples were dried at 60°C and analyzed according to the standard methods of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2012) [14]. The following parameters were determined: dry matter (DM; method 3.1), crude ash (CA; 8.1), crude fibre (CF; 6.1), crude protein (CP; 4.1.2), ether extract (EE; 5.1), acid detergent fibre (ADFom; 6.5.2), and α -amylase-treated neutral detergent fibre (aNDFom; 6.5.1), both expressed ash-free, as well as starch (7.2.1), sugars (7.1), and net energy for lactation (NEL; 25.1). Acid-insoluble ash (AIA) was determined gravimetrically, following method 8.2, and gross energy (GE) was measured using a bomb calorimeter. All results are expressed per kilogram of dry matter (see Table 2).

Table 2. Nitrogen intake and nitrogen excretion determined for period 1–6 (values represent means $\pm$ SD, $n = 261$ total, 48 per period).

Period Supplement Concentrate	1				2				3				4				5				6	
	FAT		CON		UFA		CON		HAY		CON		ALF		CON		BYP		CON		FAV	
	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC
NI (g/d)	399	519	457	562	461	596	454	529	437	501	478	511	461	517	489	534	435	535	425	525	413	516
SD of NI (g/d)	± 48	± 64	± 35	± 71	± 49	± 83	± 53	± 83	± 51	± 58	± 58	± 59	± 35	± 61	± 70	± 66	± 63	± 59	± 45	± 49	± 62	± 48
NM (g/d)	156	195	167	189	166	188	151	184	141	183	162	179	161	178	161	179	157	176	145	171	136	173
SD of NM (g/d)	± 21	± 31	± 24	± 32	± 24	± 36	± 22	± 35	± 16	± 28	± 23	± 26	± 26	± 32	± 27	± 32	± 21	± 27	± 18	± 23	± 21	± 21
NF (g/d)	119	145	133	160	153	173	156	155	141	144	168	162	166	174	172	188	154	173	167	192	163	190
SD of NF (g/d)	± 9	± 22	± 18	± 37	± 25	± 41	± 25	± 27	± 14	± 21	± 27	± 32	± 18	± 27	± 30	± 26	± 19	± 28	± 20	± 23	± 27	± 19
NU (g/d)	104	66	97	77	79	84	66	56	77	82	99	93	109	105	121	113	101	133	102	119	111	88
SD of NU (g/d)	± 55	± 16	± 29	± 28	± 18	± 32	± 30	± 24	± 24	± 22	± 36	± 44	± 20	± 16	± 24	± 20	± 19	± 21	± 18	± 22	± 22	± 13
NB (g/d) [†]	20	113	60	136	62	150	81	135	78	92	50	77	55	88	51	60	30	55	18	49	0	62
SD of NB (g/d)	± 74	± 33	± 41	± 35	± 27	± 23	± 33	± 43	± 31	± 30	± 43	± 50	± 26	± 33	± 26	± 25	± 20	± 26	± 18	± 22	± 50	± 31
NEff (%)	39	38	37	34	36	31	33	35	32	36	34	35	35	34	33	34	36	33	34	32	33	34
SD of NEff (%)	± 5	± 3	± 4	± 4	± 3	± 3	± 3	± 3	± 3	± 2	± 2	± 4	± 4	± 3	± 3	± 4	± 2	± 3	± 2	± 2	± 4	± 3

Legend: LC = low concentrate, HC = high concentrate, CON = control, FAT = fat, UFA = unsaturated fatty acids, HAY = hay, ALF = alfalfa silage, BYP = byproducts (brewers' grains silage and pressed pulp silage), FAV = faba bean, SD = Standard Deviation, NI = Nitrogen intake, NM = Nitrogen in milk, NF = Nitrogen in feces, NU = Nitrogen in urine, NB = Nitrogen balance ($NI - (NM + NF + NU)$), NEff = Nitrogen Efficiency (NM/NI), [†] = $NB \neq NI - (NM + NF + NU)$ due to different sample sizes for individual measurements; NI stands for the mean value of individual nitrogen intakes/d/period, NF and NU for a value/animal/period, and NM for 3 values/animal/period.

Milk samples were analyzed enzymatically using the international reference method (ISO 14637:2004, enzymatic assay) for milk urea. Milk components, such as milk protein and milk fat, were determined with an infrared milk analyzer (Milkoscan FT+, combined with a Fossomatic 7 DC 600; Foss Electric, Hillerød, Denmark).

N in feces and urine was analyzed using the Dumas method (according to the general principle described in ISO (International Organization for Standardization) 16634-1:2008, Geneva, Switzerland, 2008) [15] with the automated analyzer (rapid MAX N exceed, Elementar Analysensysteme GmbH, Langenselbold, Germany).

AIA content in feces was determined gravimetrically according to VDLUFA 2012 (method 8.2) [14]. DM in feces was measured by drying the samples at 103 °C until a constant weight was reached, following standard gravimetric procedures (method 3.1) [14].

Blood serum concentrations of urea, aspartate aminotransferase (AST), cholesterol, and glucose were determined using kits (981954, 981771, 981957 and 981779, Thermo Fisher Scientific, Vantaa, Finland) and free fatty acids (FFA) with the kit NEFA-HR(2) (Fujifilm Wako Chemicals Europe GmbH, Neuss, Germany) according to the manufacturers protocol on the automated analyzer Indiko™Plus (Thermo Fischer Diagnostics GmbH, Henningsdorf, Germany). Urinary Crea concentrations (kit 981954, Thermo Fisher Scientific, Vantaa, Finland) were measured on the same device.

2.4. Calculations and Statistics

Calculations

Each of the individual animal data was standardized to daily values and used for further calculations.

NI for each cow for every period was determined as:

$$\text{NI [g/d]} = (\text{CP [g/kg DM]}/6.25) \times \text{dry matter intake (DMI) [kg/d]}, \quad (1)$$

NM for every period was estimated using:

$$\text{NM [g/d]} = (\text{milk protein [g/L]}/6.38 \times \text{milk yield [L/d]}), \quad (2)$$

UV was calculated according to [7], where 29.5 represents the average Crea excretion (mg/kg BW/d) for cows:

$$\text{UV [L/d]} = \text{BW [kg]} \times (29.5/\text{Crea concentration [mg/L]}), \quad (3)$$

NU was determined as:

$$\text{NU [g/d]} = \text{NU in g/L} \times \text{UV [L/d]}, \quad (4)$$

By using the AIA marker method for the daily fecal DM output [16], the daily NF was calculated using the formula:

$$\text{NF [g/d]} = \text{NF [g/kg DM]} \times \text{fecal DM output [kg/d]}, \quad (5)$$

The N-balance (NB) was estimated with the following equation:

$$\text{NB [g/d]} = \text{NI [g/d]} - (\text{NM [g/d]} + \text{NF [g/d]} + \text{NU [g/d]}), \quad (6)$$

N-efficiency (NEff) was determined as:

$$\text{NEff in \%} = (\text{NM [g/d]}/\text{NI [g/d]}) \times 100, \quad (7)$$

Statistical analyses were performed using RStudio with R (version 4.4.2), where graphical presentations were created using the ggplot2 package [17].

The relationships between variables were analyzed using linear regression analyses as well as pairwise Pearson correlation coefficients. Procedure `lm()` of base R was used for linear regressions, while function `cor()` of base R was used for estimation of Pearson correlation coefficients. For evaluating agreement between different methods for the same target variable, Lin's Concordance Correlation Coefficient (CCC) [18] was estimated using the procedure `ccc()` of package DescTools, version 0.99.48 [19].

Linear regressions were used to assess relationships between NI and NU, NF, NM, and NB; between dietary N concentration (g/kg DM) and blood urea (BU), MUN, and milk protein; and between NEff and milk (yield, protein, fat, urea), blood (AST, cholesterol, glucose, free fatty acids), and feed (GE, NEL, EE, CP) parameters.

Pairwise Pearson correlation coefficients were applied to all variables and to the significant variables ($p > 0.05$) related to NEff, accounting for multicollinearity among themselves, and were used in the following models.

Two linear mixed models were fitted using `lmer()` from the lmerTest R package [20]. One model with practically available parameters and one model with scientifically usable parameters. The fact that the cows were used across several periods for different treatments was accounted for by including "animal" and "week" as random factors. Model selection was carried out using a top-down approach, whereby fixed effects were eliminated based on their p -values until only significant variables remained in the model. The Akaike Information Criterion [21,22] with `AIC()` and the Bayesian Information Criterion [23] with `BIC()` in base R showed the best performance of the model.

Developed models were tested for normality of residuals, homoscedasticity, outliers, and autocorrelation.

If autocorrelation was observed, autoregressive moving-average (ARMA) correlation structures were fitted using the R package nlme [24]. Correction was assessed through autocorrelation plots (`acf`, `pacf`) and the Ljung-Box test (`Box.test()`).

Linear regressions with NEff (%) calculated by the mixed-model with practically available parameters or the mixed-model with scientifically available parameters as the dependent variable and actual NEff (%) as the independent variable were performed. The package BlandAltmanLeh (version 0.3.1) [25] was used to create Bland–Altman plots to visualize method differences and evaluate systematic faults between NEff estimated by a mixed-model with practically available parameters or a mixed-model with scientifically available parameters and the actual NEff.

For both models, random intercepts were included for animal, and an ARMA(1,1) correlation structure was specified within animals over weeks (parameters model with practically available parameters: $\Phi_1 = 0.912$, $\Theta_1 = -0.614$; parameters model with scientifically available parameters: $\Phi_1 = 0.789$, $\Theta_1 = -0.535$). The models were fitted using ML. For the final model, REML was used.

Predicted NEff values from both mixed-effects models were calibrated to correct proportional bias using a linear regression of the differences between observed and predicted values on their mean. Bias, limits of agreement, and CCC were computed.

3. Results

3.1. Relationship of NI with Different N Output Variables

The NI is positively related to NM (see Figure 1A) and NF (see Figure 1B). In these two contexts, the data show moderate to strong linear relationships with relatively high coefficients of determination. In comparison, no apparent association is observed between NI and NU excretion (see Figure 1C). The relationship between NI and NB (see Figure 1D)

is positive, with a similar degree of variability as seen for NM and NF. NM, NU and NF tend to increase with higher ME concentration. The low ME diet shows the lowest excretion values, the medium ME diet the intermediate values, and the high ME diet has the highest excretion. For statistical parameters see Table 3.

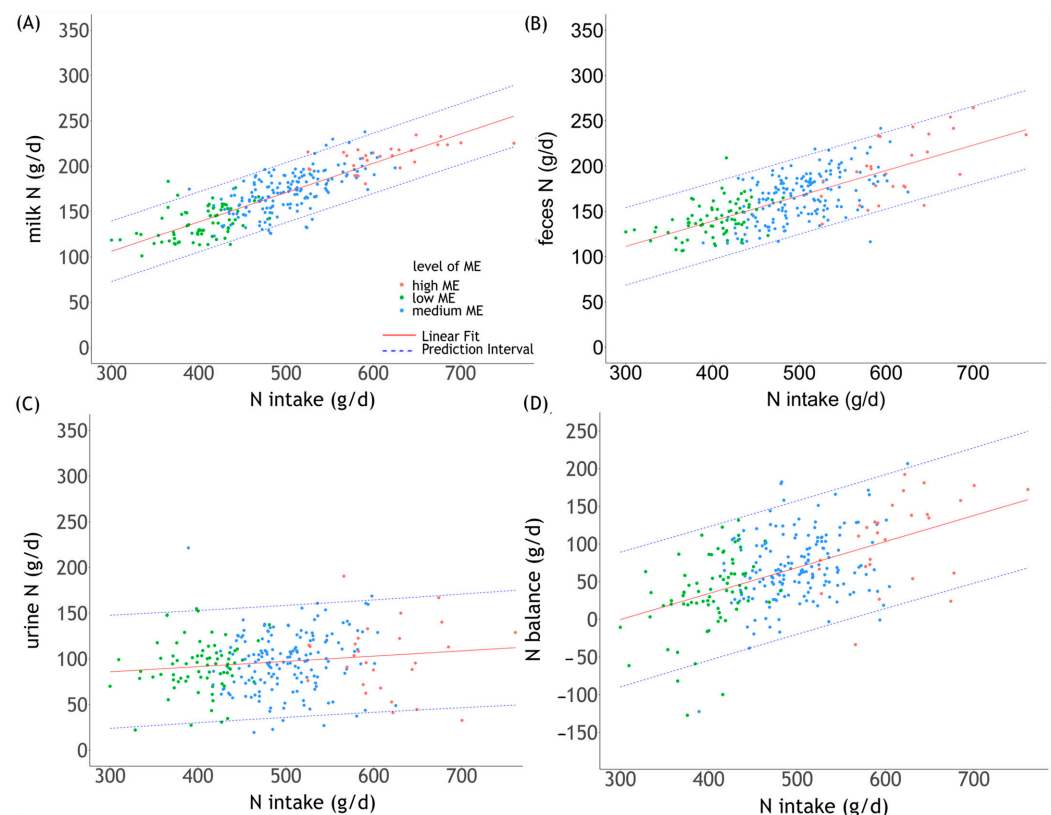


Figure 1. Relationships between nitrogen (N) intake and milk N (A), fecal N (B), urinary N (C), and N balance (D) in dairy cows. Each point represents an individual observation. Colors indicate the dietary Metabolizable Energy (ME) level: red = high ME diet (292–360 MJ/kg DM), blue = medium ME diet (216–292 MJ/kg DM), green = low ME diet (140–216 MJ/kg DM). Solid red lines show the linear regression fits, and dashed blue lines represent the 95% prediction intervals.

Table 3. This table shows the statistical parameters for Figure 1.

Parameter	Intercept	SE Intercept	Slope	SE Slope	RSE	R ²	p-Value
NM	9	6.7	0.32 ***	0.01	16.7	0.677	***
NF	27 **	8.6	0.28 ***	0.02	21.4	0.490	***
NU	69 ***	12.4	0.06 *	0.03	31.1	0.019	-
NB	−104 ***	17.9	0.35 ***	0.04	44.9	0.251	***

Legend: SE = Standard Error, RSE = Residual Standard Error, asterisks indicate the level of statistical significance of the parameter estimates: no asterisk = not significant, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, NM = nitrogen excreted in milk, NF = nitrogen excreted in feces, NU = nitrogen excreted in urine, NB = nitrogen balance (NI − (NM + NF + NU)).

3.2. Relationships Between Dietary N-Concentration on BU, MUN and Milk Protein

BU increases significantly with dietary nitrogen concentration (see Figure 2E), showing a statistically significant linear relationship with relatively high residual variation. Similarly, milk urea nitrogen (MUN) is positively associated with dietary nitrogen concentration (see Figure 2B), exhibiting a stronger relationship than BU and explaining a larger proportion of the total variance. In contrast, milk protein concentration responds slightly to increasing dietary nitrogen levels (see Figure 2C), indicating a low positive association and considerable individual variation among cows. For statistical parameters see Table 4.

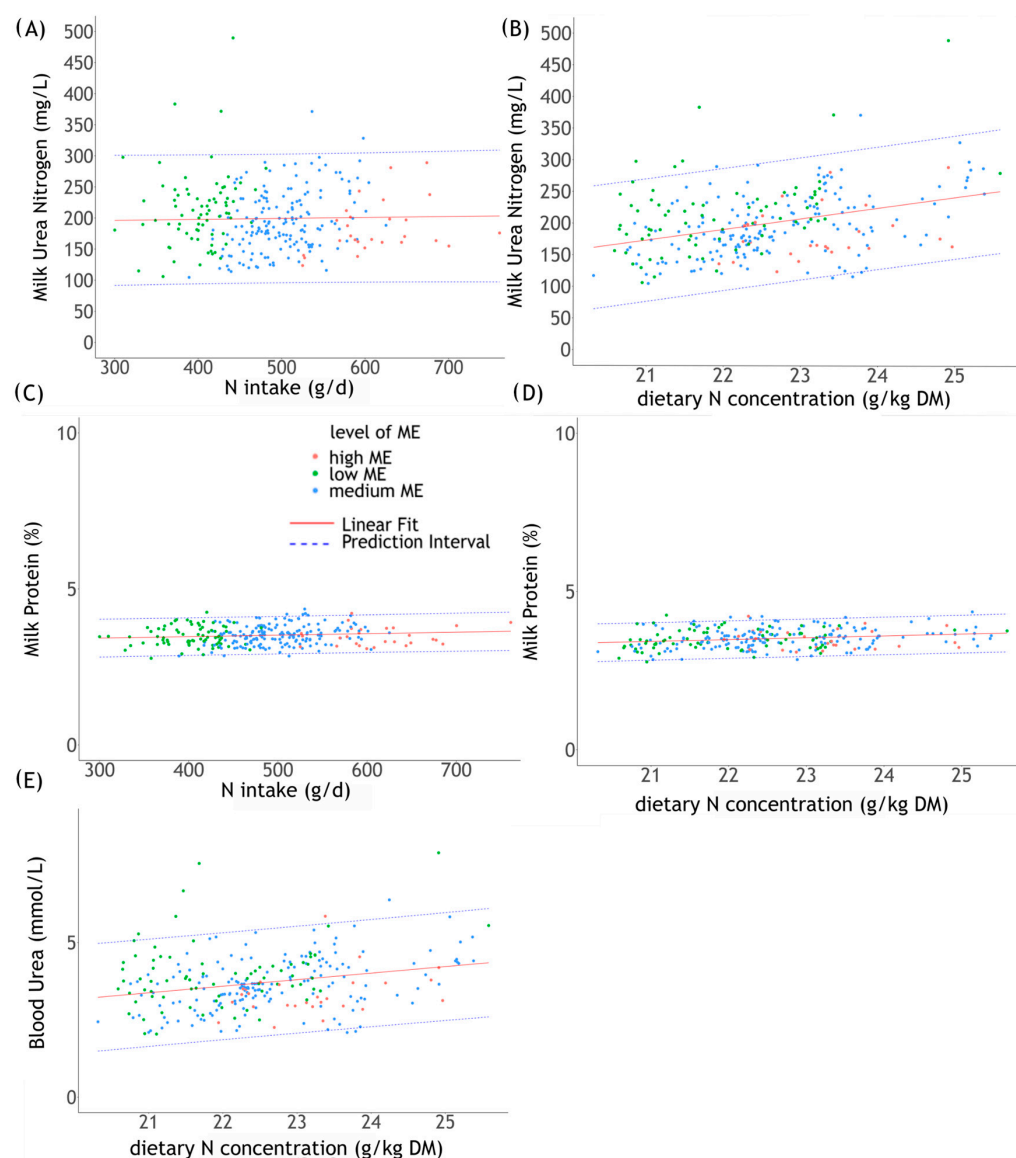


Figure 2. Correlation between dietary N concentration (dN) and N intake on milk urea nitrogen (MUN) (A,B), milk protein (C,D) and blood urea (BU) (E). Each point represents an individual observation. Colors indicate the dietary Metabolizable Energy (ME) level: red = high ME diet (292–360 MJ/kg DM), blue = medium ME diet (216–292 MJ/kg DM), green = low ME diet (140–216 MJ/kg DM). Solid red lines show the linear regression fits, and dashed blue lines represent the 95% prediction intervals.

Table 4. This table shows the statistical parameters for Figure 2.

Parameter	Intercept	SE Intercept	Slope	SE Slope	RSE	R ²	p-Value
MUN/NI	191.7 ***	21.0	0.02	0.4	52.4	<0.01	
MUN/dN	−183.3 **	60.6	17 ***	2.7	48.9	0.13	***
Milk protein/NI	3.3 ***	0.12	<0.01	<0.01	0.3	0.01	
Milk protein/dN	2.2 ***	0.37	0.05 ***	0.02	0.3	0.04	***
BU	−1.1	1.1	0.21 ***	0.05	0.9	0.07	***

Legend: SE = Standard Error, RSE = Residual Standard Error, asterisks indicate the level of statistical significance of the parameter estimates: no asterisk = not significant, ** = $p < 0.01$, and *** = $p < 0.001$, BU = blood urea, MUN = milk urea nitrogen, dN = dietary nitrogen concentration.

3.3. Relationships of Dietary, Milk and Blood Parameters with NEff

3.3.1. Dietary Parameters (Figure 3A–D)

Dietary components show generally weak to moderate associations with NEff. CP in g/kg DM is negatively related to NEff (see Figure 3C), indicating decreasing efficiency with higher dietary protein supply. In contrast, energy-related parameters, such as NEL and EE, are positively associated with NEff (see Figure 3A,D), suggesting that greater energy availability supports more efficient nitrogen utilization. GE also shows a moderate positive relationship (see Figure 3B).

3.3.2. Milk Parameters (Figure 3E–H)

Milk yield shows the strongest positive association with NEff (see Figure 3E), indicating that higher-producing cows utilized dietary nitrogen more efficiently. In contrast, milk protein and fat concentrations, as well as milk urea nitrogen (MUN), are negatively related to NEff (see Figure 3F–H). Overall, these relationships are weak to moderate, with limited agreement between predicted and observed values.

3.3.3. Blood Parameters (Figure 3I–M)

For blood metabolites, cholesterol concentration exhibits the strongest positive association with NEff (see Figure 3K), followed by aspartate aminotransferase (AST) and free fatty acids (FFA) (see Figure 3I,L). In contrast, glucose concentration is negatively associated with NEff (see Figure 3M), showing potential metabolic exchanges between carbohydrate and nitrogen utilization. While several relationships are significant, overall concordance remained low, highlighting considerable biological variation among individuals. For statistical parameters see Table 5.

Table 5. This table shows the statistical parameters for Figure 3.

Parameter	Intercept	SE Intercept	Slope	SE Slope	RSE	R ²	p-Value	Pearson r
Feed parameters								
NEL	29 ***	4.9	0.78	0.720	3.6	<0.001	-	0.07
GE	-72 *	27.9	5.72 ***	1.510	3.5	0.05	***	0.23 ***
CP	46 ***	4.4	-0.10 *	0.030	3.6	0.02	*	-0.15 ***
EE	28 ***	1.2	0.20 ***	0.400	3.4	0.09	***	0.3 ***
Milk parameters								
Milk yield	25 ***	1.0	0.30 ***	0.030	3.1	0.26	***	0.51 ***
Milk protein	43 ***	2.5	-2.40 ***	0.710	3.5	0.41	***	-0.20 ***
MUN	37 ***	0.8	-0.01 ***	0.004	3.5	0.04	***	-0.30 ***
Milk fat	42 ***	1.4	-1.70 ***	0.320	3.4	0.09	***	-0.20 ***
Blood parameters								
AST	33 ***	0.7	0.02 **	0.006	3.6	0.03	**	0.16 ***
FFA	33 ***	0.5	0.008 **	0.003	3.5	0.04	**	0.19
Glucose	41 ***	2.6	-1.80 *	0.720	3.6	0.02	*	-0.15 ***
Cholesterol	28 ***	0.9	1.20 ***	0.170	3.3	0.16	***	0.40 ***

Legend: SE = Standard Error, RSE = Residual Standard Error, asterisks indicate the level of statistical significance of the parameter estimates: no asterisk = not significant, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$, NEL = net energy for lactation, GE = gross energy, CP = crude protein, EE = ether extract, MUN = milk urea nitrogen, AST = aspartate aminotransferase, FFA = free fatty acids.

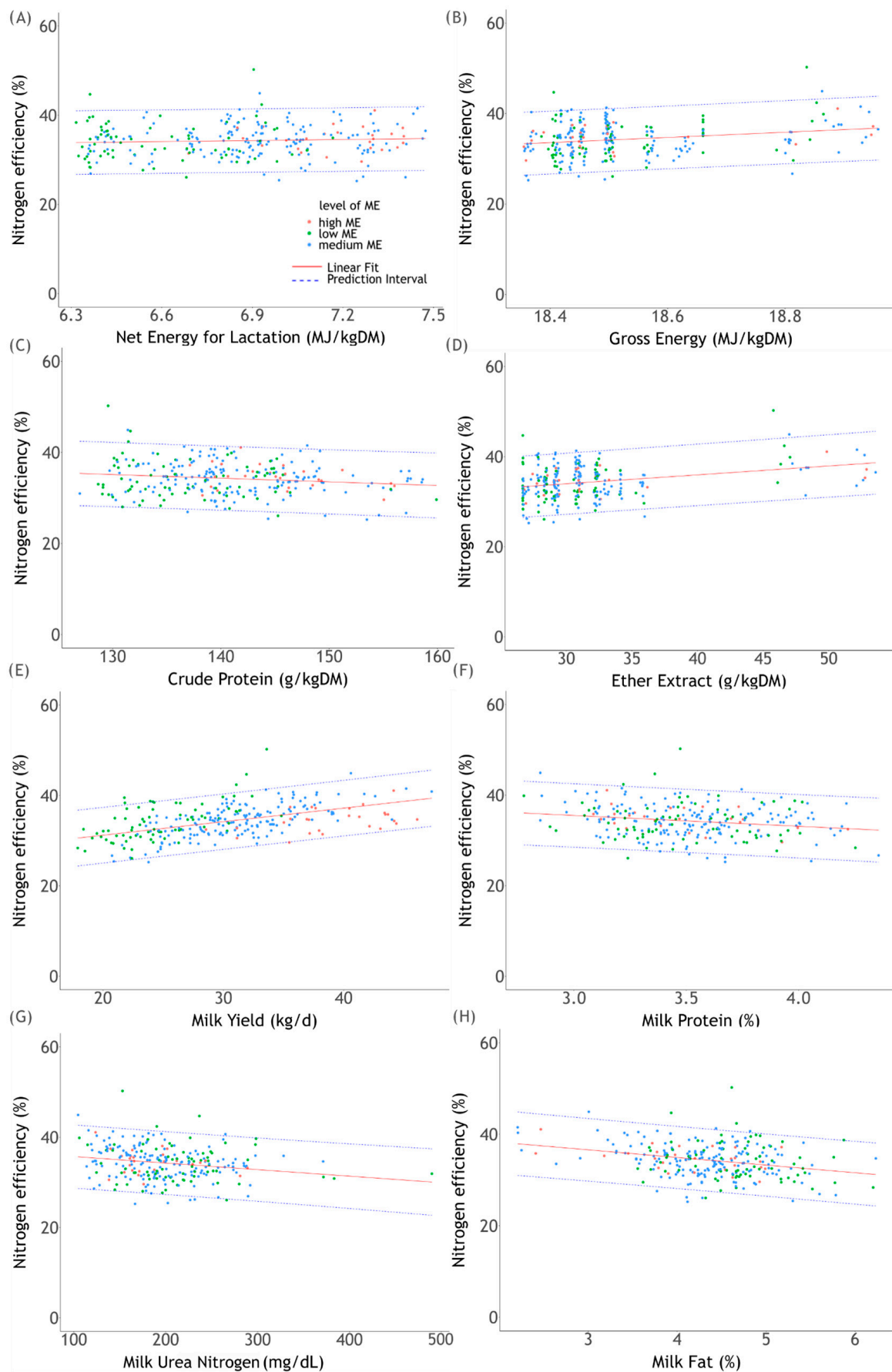


Figure 3. Cont.

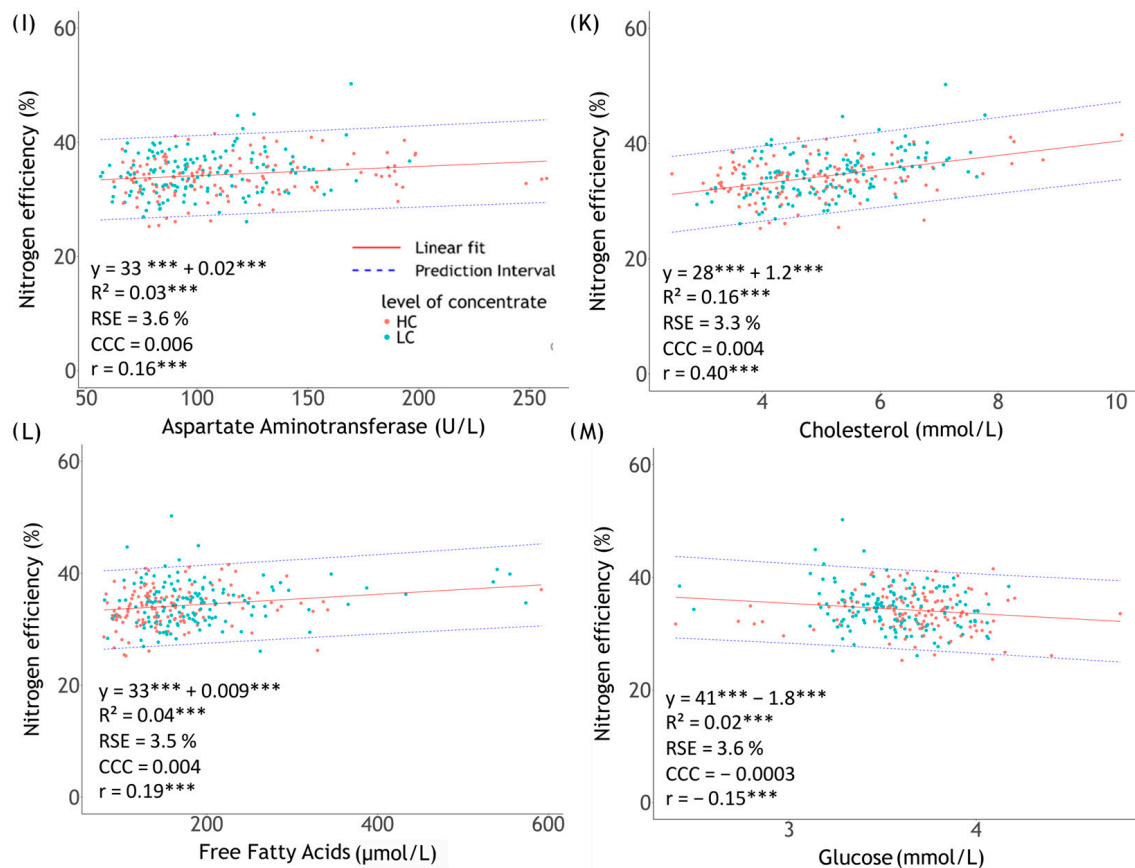


Figure 3. Relationship of significant dietary (A–D), milk (E–H) and blood (I–M) parameters on N-efficiency. Each point represents an individual observation. Colors indicate the dietary Metabolizable Energy (ME) level: red = low ME diet (140–216 MJ/kg DM), blue = medium ME diet (216–292 MJ/kg DM), green = high ME diet (292–360 MJ/kg DM). Solid red lines show the linear regression fits, and dashed blue lines represent the 95% prediction intervals. Asterisks indicate the level of statistical significance of the parameter estimates: no asterisk = not significant.

3.4. Relationships Between the Observed NEff with a Model with Practically Available Parameters and a Model with Scientifically Available Parameters That Predicted NEff

The final mixed-effects model for NEff, developed for practical use, included the fixed effects of milk protein, milk yield, and their interaction, as well as the interaction between GE and CP concentrations of the diet.

The corresponding model for scientific applications additionally incorporated metabolic blood indicators (AST, cholesterol, free fatty acids, and glucose) as fixed effects, alongside the same feeding and milk parameters.

Both models show good agreement between the estimated NEff and the calculated NEff. In both linear regressions (see Figure 4A,B), the points scatter closely along the regression lines, with those in the model with scientifically available parameters lying closer to the line. The regression line deviates less from the bisector in the model with scientifically available parameters, although the deviation in the model for practical use is also moderate.

Neither model shows a systematic deviation (bias = 0), but the limits of agreement are narrower in the model for scientific applications, and R² and CCC are higher (see Table 6).

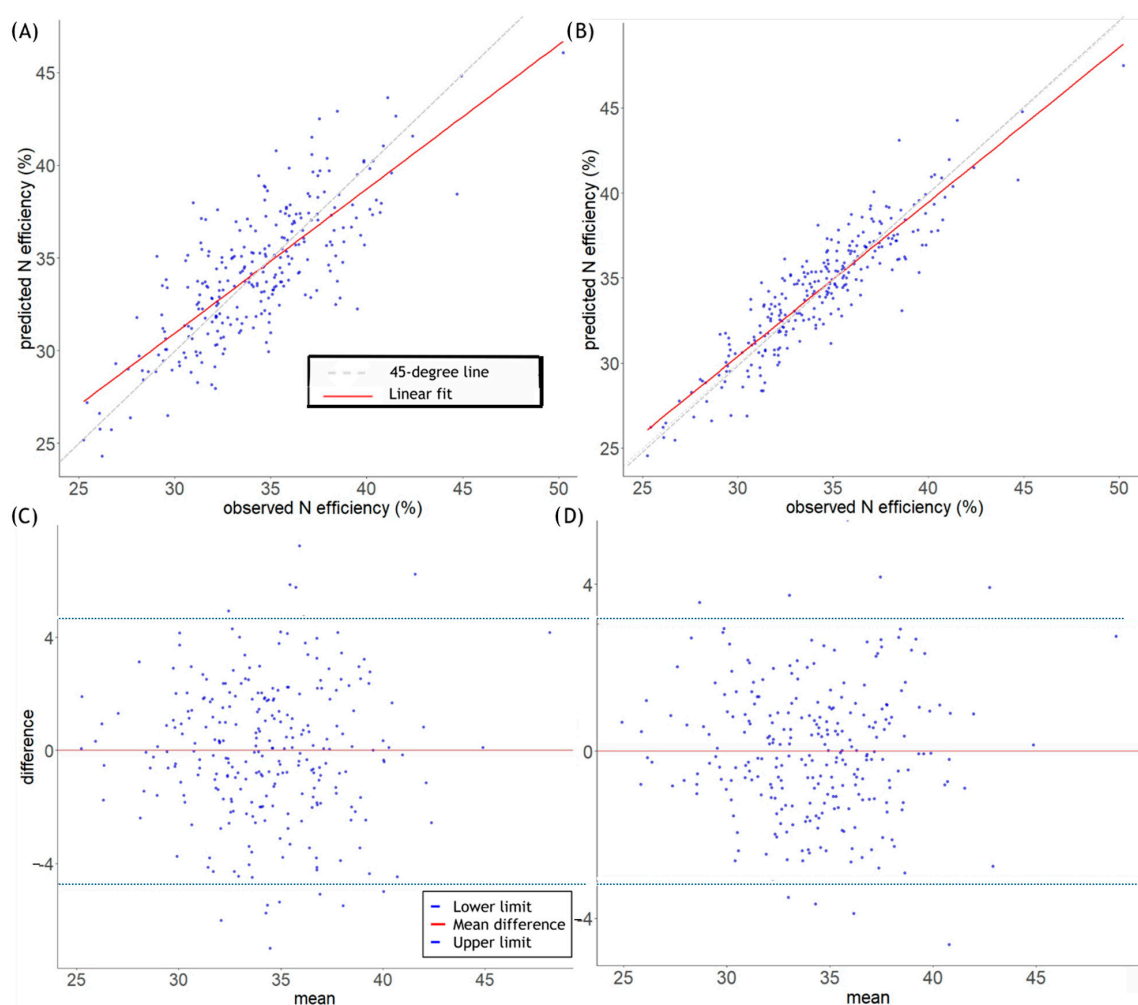


Figure 4. Relationship between observed and predicted N-efficiency with a practical mixed model (A,C) and a scientific mixed model (B,D). Each point represents an individual observation. Solid red lines in (A,C) show the linear regression fits. Grey dashed line is the 45-degree line. Bland–Altman plot: the x-axis displays mean values of predicted and observed N efficiency, and the y-axis displays the related differences in the distribution. The red solid line in (B,D) shows the average difference between the two methods. Blue dashed lines mark the 95% limits of agreement.

Table 6. This table shows the statistical parameters for Figure 4.

Parameter	Model for Practical Use	Model for Scientific Applications
Linear Regression		
Intercept	7.6 ***	3.2 ***
SE Intercept	1.3	0.9
Slope	0.8 ***	0.9 ***
SE Slope	0.04	0.03
RSE	2.3	1.5
R ²	0.6	0.8
p-value	***	***
CCC	0.8	0.9
Bland–Altman Plot		
Bias	0	0
Lower Limit of Agreement	−4.7	−3.1
Higher Limit of Agreement	4.7	3.1

Legend: SE = Standard Error, RSE = Residual Standard Error, CCC = Concordance Correlation Coefficient, asterisks indicate the level of statistical significance of the parameter estimates: no asterisk = not significant, *** = $p < 0.001$.

4. Discussion

Accurate prediction of NEff under practical feeding conditions in dairy cows demands accurate measurement of both NI and Nex. In this study, the essential data were collected to examine the relationship between these two factors.

The results were compared with previous research, and predictive models for NEff were developed, applicable in both practical and scientific settings. While earlier studies have established linear relationships between NI, Nex, and nitrogen concentrations in feed, as well as nitrogen metabolism indicators like BU and MUN, their accuracy at predicting NEff under practical conditions stay variable. Additionally, the influence of other factors on NEff is still an area requiring further explanation.

The relationship between NI (g/d) and Nex (g/d) is shown in Figure 1. As expected from the literature, regressions show increases in NF, NM, and NB with rising NI [9,26]. Specifically, the slopes of the regression lines are 0.28 for NF, 0.32 for NM, and 0.35 for NB. These values show that as NI increases, Nex in feces and milk, as well as NB, increase proportionally.

It can be assumed that the NM data are highly accurate, since milk yield was quantitatively recorded twice a day in the milking parlor using technology and subsequently analyzed from a pooled sample of daily milk yield applying standardized methods. In contrast, NF was estimated using a calculation approach based on AIA in feed and feces rather than by direct total collection [16]. Compared with the literature data, where feces were collected quantitatively, the values obtained from the calculation are consistent and physiologically plausible, with a few outliers.

NU does not appear to be affected by higher NI (g/d) in this data set. This could be the case because cows received less N overall in their feed during this experiment than the average N in feed in the experiments evaluated by Spanghero and Kowalski [26]. The literature frequently refers to a significant increase in NU when protein and urea are fed in excess of requirements. This suggests that the lack of increased NU may be due to the RNB = 0-adjusted feeding regime, thereby avoiding protein oversupply. This is supported by the observation that NEff in cows is relatively high, averaging $34 \pm 4\%$ [27,28].

With regard to NU, Spanghero and Kowalski [26] also showed that spot urine samples often underestimate total UV compared to total samples. As a result, the calculated NB would tend to be overestimated, and the UN would tend to be underestimated. Padberg et al. showed that Crea concentrations in spot and pooled samples were the same, and that one can therefore assume the same basis for calculating UV [7]. Although the calculation does not fully correspond to a quantitative measurement, the research in this literature provides practical, usable calculations with a high degree of statistical consistency, based on a data set that reflects the genetics and husbandry practices of the experimental cows used at the same location.

Comparing the calculated results with the literature, one can identify a few outliers, possibly due to the calculation, but with an average of 24.6 ± 9.4 L/d for UV and 0.8 ± 0.2 g/L for Crea concentration, these values are physiologically appropriate [8,29–31]. The measured NU are, on average, 4.1 ± 1.25 g/L. These N values are low, but physiologically possible [32,33].

Chowdhury et al. measured NU in low-protein diets (<150 g/kg DM) and reported lower NU, averaging 90 g/d [34]. In the present study, CP content of the rations ranged from 126 to 166 g/kg DM, resulting in NU comparable to that observed by Chowdhury. The NEff measured by Chowdhury et al. for low-protein diets is also as high as in this data set, with a mean of 34.5% (see Table 7).

Table 7. Nitrogen intake and nitrogen excretion were determined for Spanghero and Kowalski and the current experiment.

Parameter in g/d	Spanghero and Kowalski (2021) [26]	This Data Set	Derived from Slope (Figure 1)
NI	572 ± 114	489 ± 51	
NM	155 ± 34 (27%)	168 ± 16 (34%)	32%
NU	186 ± 61 (33%)	95 ± 20 (19%)	6%
NF	192 ± 42 (34%)	161 ± 18 (33%)	28%
NB	38.5 ± 43.2	69 ± 39	35%

Legend: % in () = percentage of total nitrogen excreted, NI = Nitrogen intake, NM = Nitrogen excreted in milk, NF = Nitrogen excreted in feces, NU = Nitrogen excreted in urine, NB = Nitrogen balance (NI–(NM + NF + NU)).

In this data set, NB is not equal to 0 and is also higher than the values for Spanghero and Kowalski [26]. It should be noted that the cows exhibited different feeding behaviors, mainly regarding the proportion of concentrate feed and actual PMR intake, resulting in slight variations from the optimal nutrient composition due to differences between the formulated ration and the cows' actual intake in their group-housing environment. It therefore remains unclear whether these variations in lower protein intake are due to the average or individual ration design, or both.

Differences in calculations are to be expected, as different methods rely on different assumptions and are noted by distinct sources of uncertainty and variation. However, all parameters of N metabolism in dairy cows observed in the present experiment are within the physiological range, although the absolute values may be influenced by the methods applied. In our view, there is therefore nothing to prevent this data from being used for statistical purposes.

The N concentration in the ingested rations (g/kg DM) shows positive relationships with BU, MUN, and milk protein concentrations, even though the coefficients of determination were relatively low (Figure 2). BU increases linearly with increasing dietary N concentration, indicating that higher dietary NI is reflected in higher circulating urea levels. This result is consistent with the physiological mechanism: excess dietary N, when not utilized for microbial or tissue protein synthesis, is converted to urea in the liver and excreted in urine and milk. Although part of the urea is recycled to the rumen, this does not offset the increase in circulating urea at higher dietary N levels [35]. The relatively low R^2 suggests that factors beyond dietary N—such as diurnal variation, energy supply, and individual variation in hepatic urea recycling appear to influence BU concentrations under practical feeding conditions [36].

MUN also increases significantly with increasing dietary N concentration, showing a somewhat stronger association than BU. This supports previous findings that MUN reflects systemic urea levels and thus provides an indirect indicator of N status [37,38]. However, the moderate correlation ($r = 0.36$, $p < 0.001$) indicates that MUN, while directionally consistent, may be influenced by additional factors, such as milk yield, water balance, and the timing of sample collection relative to feeding. These findings agree with those of Nousiainen et al. [38], who showed that MUN varies not only with dietary protein content but also with dietary energy, carbohydrate fermentability, and other management factors.

Milk urea nitrogen (MUN) concentrations were determined using a reference enzymatic colorimetric method (ISO 14637:2004; IDF 195:2004 [39]), which is considered the gold standard due to its high analytical precision and low susceptibility to variations in milk composition, somatic cell count, or fat and protein content [40,41]. Milk protein concentration is also positively associated with dietary N (milk protein = $2.2 + 0.5 \times N$; $R^2 = 0.04$; $p < 0.001$), though the relationship is weak. This is expected, as milk protein synthesis depends not only on the amount of N supplied, but also on the balance of energy and

amino acids available for microbial protein synthesis in the rumen. Under a non-limiting energy supply, increased dietary N is able to enhance microbial protein yield and thereby milk protein concentration [42].

Among feed parameters, the positive association between NEL, EE, and NEff suggests that energy-rich, highly digestible fiber fractions support more efficient nitrogen utilization [43]. This is consistent with the observed effects on BU and MUN. The availability of sufficient energy allows nitrogen to be more effectively utilized in productive pathways, such as milk protein synthesis, rather than being excreted as waste. Therefore, energy availability influences nitrogen utilization efficiency, as reflected in both BU and MUN.

Overall, these results confirm that increasing dietary N concentration can cause higher BU and MUN, and a slight rise in milk protein content. Nevertheless, the weak to moderate relationships demonstrate the complexity of N metabolism and the influence of animal- and management-related variability under practical feeding conditions. For a potential model development, the results show the need to include factors beyond dietary N concentration, such as energy balance and individual animal characteristics, to improve the prediction of NEff in dairy cows.

This multifactorial nature of NEff in dairy cows reflects complex interactions between feed composition, milk production traits, and metabolic status (Figure 3).

Milk production traits exhibit strong physiological correlations with NEff. Daily milk yield is positively associated with NEff, indicating that cows producing more milk efficiently convert dietary N into milk protein. Conversely, higher milk protein, fat, and urea concentrations were negatively associated with NEff.

The observed bilateral relationships may only partially show the underlying multifactorial interactions that govern NEff.

However, the obtained relationships between all significant parameters provide a firm foundation for exact and practical predictive modelling of NEff.

An integrated analytical approach comprises implementing mixed-effects models to demonstrate and apply these effects [44].

By accounting for random animal effects and time correlation, such models capture repeated-measures structures and allow correlated predictors to be analyzed together. This approach also directly addresses multicollinearity between nutritional and physiological variables and more precisely captures the multifactorial nature of nitrogen metabolism.

Several approaches to modelling Nex [45,46] in dairy cows have already been described in the literature. Modelling for estimating NEff is less common in the literature than modelling for NB. The present study, therefore, focuses on NEff as an integrative outcome variable. The few existing model approaches for NEff are based on milk parameters, such as MUN [47] or mid-infrared milk spectra [48]. Other models primarily rely on dietary CP and NI, as well as milk production parameters [46]. Our results confirm these conclusions and demonstrate the importance of CP, NI, and milk components for simultaneously predicting NEff. The inclusion of blood metabolites (AST, Cholesterol, FFA, Glucose) as additional predictors extends this approach and improves explanatory power beyond what dietary and milk parameters alone can achieve. However, we acknowledge that additional separate models for fecal and urinary nitrogen excretion would facilitate a more direct comparison with the current literature and provide additional physiological insights.

The models developed in this study use metabolic biomarkers to make reliable calculations. The model with practically available parameters, based on feeding and milk parameters, already achieved a high degree of explanatory performance ($R^2 = 0.60$; CCC = 0.78). The model with scientifically available parameters, which additionally included metabolic blood indicators (AST, cholesterol, FFA, and glucose), further improved the model adjust-

ment ($R^2 = 0.82$; CCC = 0.90) and reduced residual variation from 2.3 in the model for practical use to 1.5 in the model for scientific applications.

Blood metabolites, including FFAs, glucose, and cholesterol, are shown in Figure 3 and provide insight into systemic metabolic status, which influences NEff. The strong positive association of NEff with cholesterol probably reflects adequate energy and fat metabolism, which support anabolic processes, milk synthesis, and, consequently, a more efficient partitioning of nitrogen towards productive pathways [49,50]. AST and FFAs are also positively related to NEff, indicating that moderate mobilization of body reserves and active hepatic metabolism provide energy that supports nitrogen metabolism, consequently contributing to efficient N utilization [51]. In contrast, the negative association with glucose may indicate that temporary reductions in blood glucose levels, possibly due to high milk yield or dietary imbalance, limit the energy available for effective N incorporation into milk protein [52,53].

It can be assumed that the inclusion of these blood metabolites probably improved the model performance, as they reflect short-term metabolic adjustments that cannot be directly observed from feed or milk data alone.

Both models also show a high agreement between the calculated and observed NEff values, as confirmed once again by the Bland–Altman plots (bias = 0). The tighter agreement limits of the model with scientifically available parameters confirm its higher precision. These results demonstrate the potential to combine routinely measurable production data with selected metabolic biomarkers to improve the prediction of NEff in dairy cows. Future work could aim to validate these models with, for example, cross-validation and investigate non-linear or machine-learning approaches to capture the remaining biological variability beyond the limits of linear mixed modelling.

5. Conclusions

The relationships between NI or NEff and various parameters associated with N metabolism show physiological relationships that can be included in the development of the predictive models. Based on the preselected parameter sets, a mixed model with practically available parameters and a mixed model with scientifically available parameters for NEff were developed. The best-fitting model for practical use and for scientific applications was conducted using statistical evaluations that combined AIC, CCC, and Bland–Altman plots, while also accounting for physiological relevance.

As a result, the model for practical use included parameters that are routinely available under practical feeding conditions. The model for scientific applications added parameters that are usually not available under practical feeding conditions. Both models performed reasonably well, although the model for scientific applications exhibits slightly better statistical parameters, with a slope of 0.9, R^2 of 0.8, Residual Standard Error of 1.5, and CCC of 0.9, compared to a slope of 0.8, R^2 of 0.6, Residual Standard Error of 2.3, and CCC of 0.8 for the model for practical use.

Nevertheless, the models were developed and evaluated using the same data source, and no independent validation was performed. Therefore, the predictive performance and generalizability of the models should be interpreted with caution. Future studies should validate the equations using independent data sets to assess their reliability.

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Abbreviations

The following abbreviations are used in this manuscript:

AIA	Acid Insoluble Ash
AST	Aspartate Aminotransferase
BU	Blood Urea
BUN	Blood Urea Nitrogen
BW	Body Weight
CCC	Concordance Correlation Coefficient
CP	Crude Protein
Crea	Creatinine
DM	Dry Matter
DMI	Dry Matter
dN	Dietary Nitrogen Concentration
EE	Ether Extract
FFA	Free Fatty Acids
GE	Gross Energy
ME	Metabolizable Energy
MUN	Milk Urea Nitrogen
N	Nitrogen
NB	Nitrogen Balance
NEff	Nitrogen Efficiency
Nex	Nitrogen Excretion
NF	Nitrogen Excretion in Feces
NI	Nitrogen Intake
NM	Nitrogen Excretion in Milk
NU	Nitrogen Excretion in Urine
RSE	Residual Standard Error
SD	Standard deviation
SE	Standard Error
UV	Urine Volume

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