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Microbial signature inferred from genomic breeding selection on milk urea concentration and its relation to proxies of nitrogen-utilization efficiency in Holsteins

Hanne Honerlagen,[1] Henry Reyer,[1] Ibrahim Abou-Soliman,[1,2] Dierck Segelke,[3] Siriluck Ponsuksili,[1] Nares Trakooljul,[1] Norbert Reinsch,[4] Björn Kuhla,[5] and Klaus Wimmers[1,6]*

[1]Research Institute for Farm Animal Biology, Institute of Genome Biology, 18196 Dummerstorf, Germany
[2]Desert Research Center, Department of Animal and Poultry Breeding, Dokki, Giza Governorate 3751254, Egypt
[3]IT-Solutions for Animal Production, Vereinigte Informationssysteme Tierhaltung w.V. (vit), 27283 Verden, Germany
[4]Research Institute for Farm Animal Biology, Institute of Genetics and Biometry, 18196 Dummerstorf, Germany
[5]Research Institute for Farm Animal Biology, Institute of Nutritional Physiology "Oskar Kellner," 18196 Dummerstorf, Germany
[6]University of Rostock, Faculty of Agricultural and Environmental Sciences, 18059 Rostock, Germany

ABSTRACT

Increasing the nitrogen-utilization efficiency (NUE) of dairy cows by breeding selection would offer advantages from nutritional, environmental, and economic perspectives. Because data collection of NUE phenotypes is not feasible in large cow cohorts, the cow individual milk urea concentration (MU) has been suggested as an indicator trait. Considering the symbiotic interplay between dairy cows and their rumen microbiome, individual MU was thought to be influenced by host genetics and by the rumen microbiome, the latter in turn being partly attributed to host genetics. To enhance our knowledge of MU as an indicator trait for NUE, we aimed to identify differential abundant rumen microbial genera between Holstein cows with divergent genomic breeding values for MU (GBVMU; GBV_{HMU} vs. GBV_{LMU}, where H and L indicate high and low MU phenotypes, respectively). The microbial genera identified were further investigated for their correlations with MU and 7 additional NUE-associated traits in urine, milk, and feces in 358 lactating Holsteins. Statistical analysis of microbial 16S rRNA amplicon sequencing data revealed significantly higher abundances of the ureolytic genus *Succinivibrionaceae UCG-002* in GBV_{LMU} cows, whereas GBV_{HMU} animals hosted higher abundances of *Clostridia unclassified* and *Desulfovibrio*. The entire discriminating ruminal signature of 24 microbial taxa included a further 3 genera of the *Lachnospiraceae* family that revealed significant correlations to MU values and were therefore proposed as considerable players in the GBVMU–microbiome–MU axis. The significant correlations of *Prevotellaceae UCG-003*, *Anaerovibrio*, *Blautia*, and *Butyrivibrio* abundances with MU measurements, milk nitrogen, and N content in feces suggested their contribution to genetically determined N-utilization in Holstein cows. The microbial genera identified might be considered for future breeding programs to enhance NUE in dairy herds.

Key words: rumen microbiota, genomic breeding value milk urea, nitrogen-utilization efficiency

INTRODUCTION

The symbiotic relationship between the dairy cow and its rumen microbiome enables the utilization of nonprotein nitrogen (i.e., urea and ammonia) and increases the biological value of the dietary protein by rumen microbial protein synthesis (Bryant, 1970; Huber and Kung, 1981; Tan et al., 2021). Although ruminal nitrogen conversion in general increases the N availability for the host, the N-utilization efficiency (**NUE**) of dairy cows, defined as the ratio of grams of N in milk per grams of N intake, is on average only 25% (Calsamiglia et al., 2010). Nonutilized dietary N and N of endogenous origin is excreted via milk, urine, and feces (Abdoun et al., 2006; Reed et al., 2015; Tan et al., 2021). Whereas fecal N accounts quantitatively for the largest part of N excretion and is therefore most considerable for nutrient losses, urinary N excretion, with urea as the major N excretion metabolite, contributes remarkably to N emissions from dairy farms (Dijkstra et al., 2011; Spanghero and Kowalski, 2021). More efficient N utilization by the symbiotic interplay between dairy cows and their rumen microbiome would reduce the excretion of nonutilized N and provide higher nutrient availability, accompanied by economic and ecological advantages (Spanghero and Kowalski, 2021; Tan et al., 2021). Interestingly, NUE was found to vary widely between individual cows, with ranges from 16

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*Corresponding author: wimmers@fbn-dummerstorf.de

to 36% across diets, N intake levels, breeds, lactation numbers, and lactation stages (Calsamiglia et al., 2010; Powell et al., 2010). Recently, early lactating dairy cows were phenotyped with NUE values ranging from 9.7 to 81.7%, which let the authors assume breeding potential for NUE (Grelet et al., 2020; Bergen, 2021). However, individual NUE records are not available at a large scale, making NUE insufficient as a breeding trait (Jahnel et al., 2021; te Pas et al., 2021).

To overcome this problem, various studies have proposed individual milk urea concentration (**MU**) as an indicator trait (Nousiainen et al., 2004; Guliński et al., 2016; Bobbo et al., 2020; Lavery and Ferris, 2021). Milk urea concentration is known to be moderately correlated with urinary urea concentration (**UU**) and was therefore thought to depict N emissions, deriving from UU (Gonda and Lindberg, 1994; Burgos et al., 2010; Beatson et al., 2019). Moreover, MU has been attributed predictive power about cow individual NUE (Nousiainen et al., 2004; Huhtanen et al., 2015; Munyaneza et al., 2017; Bobbo et al., 2020; Lavery and Ferris, 2021). Because MU can be routinely obtained from monthly milk records, huge data sets of individual MU values are available. The moderate heritability of MU as well as the weak or absent genetic correlations with milk performance parameters further support the potential use of MU as an indicator trait for breeding programs (Wood et al., 2003; Miglior et al., 2007). However, breeding selection with MU to increase NUE would only succeed if MU and NUE are genetically linked. This cannot be proven due to the current lack of cow individual NUE data yet. Increasing the knowledge of the relationship between MU genetics and NUE phenotypes is a first step to evaluate whether MU can be considered as an indicator trait for breeding strategies to enhance NUE.

The rumen microbial community is known to be the major determinant of N metabolism in ruminants (Bach et al., 2005). Although the rumen microbiome is strongly influenced by the diet (Loor et al., 2016), recent studies have identified significant host genetic effects on rumen microbial abundances (Difford et al., 2018; Pérez-Enciso et al., 2021; Saborío-Montero et al., 2021). Genomic breeding selection for MU is therefore hypothesized to affect the host–trait axis and the triangular host–rumen microbiome–trait axis, which collectively influence individual NUE phenotypes.

To better understand the link between MU genetics and individual proxies of NUE, in the current study, we aimed to explore both axes by (1) identifying differential microbial genera abundances between dairy cows with divergent genomic breeding values for MU ($\mathbf{GBV_{LMU}}$ vs. $\mathbf{GBV_{HMU}}$), and (2) investigating these rumen microbial genera identified in (1) as potential microbial signature of genomic breeding values for MU (**GBVMU**) selection for their relationship to proxies of individual NUE.

MATERIALS AND METHODS

Ethics Statement

Animal housing and sampling were in accordance with the guidelines of the German Animal Protection Law. All protocols were approved by the Animal Welfare Commission of the Research Institute for Farm Animal Biology (FBN Dummerstorf). The sampling trial was conducted in strict compliance with the German Animal Welfare Legislation, has been approved by the Ethics Committee of the federal state of Mecklenburg-Western Pomerania, Germany (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V7221.3-2-019/19), and is in accordance with the ARRIVE guidelines (https://arriveguidelines.org/).

Cow Population and Sampling

The following description of data collection, statistical analyses, and the analysis strategy is shown in Figure 1. The cow population and the experimental trial have been described previously (Honerlagen et al., 2021). In brief, 371 lactating Holstein-Friesian cows were sampled for milk, urine, feces, and rumen fluid in a practice-operating dairy farm. All cows were fed a TMR that comprised 7.26 MJ of NE_L/kg of DM, 15.4% CP, and a ruminal nitrogen balance of −0.9 g of N/kg of DM. Milking and feeding were carried out twice daily. Urine, feces, and rumen fluids were obtained once from each cow in a maximum time interval of 24 h from milk samples, which were obtained by the monthly milk record procedure as pooled samples from morning and evening milkings. Monthly milk recording data for one entire lactation (14 mo), including protein, fat, lactose, and milk yield, were available for all individuals of the herd. To sample urine, feces, and rumen fluids, cows were fixed in a feeding fence directly after the morning or evening milking. Urine was collected after stimulating massage or spontaneous micturition, immediately cooled on ice, and stored at −20°C. Feces samples (~200 mg per cow) were collected by rectal removal and stored at −20°C. Rumen fluids were obtained by oral stomach tubing. Each cow was fixed with a rope in the feeding fence, a stomach tube (Hauptner-Herberholz GmbH & Co. KG) was warmed in water and inserted into the rumen. After discarding saliva, rumen fluids were transferred into 50-mL Falcon tubes and stored at ambient temperature (1–7°C) for

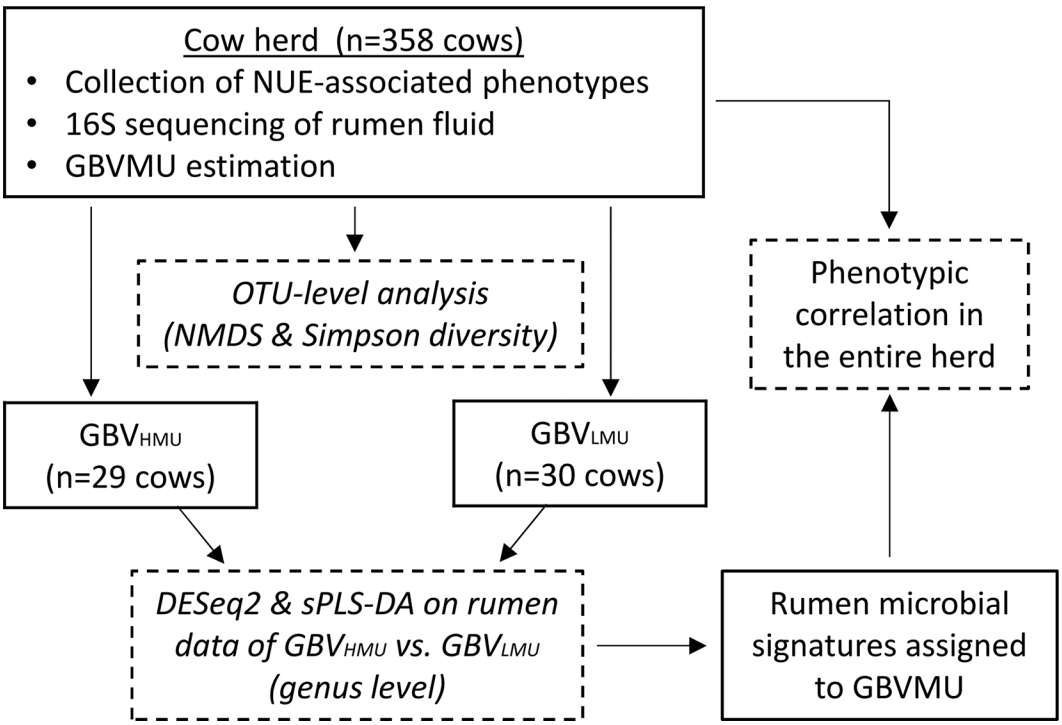

Figure 1. Flowchart of data collection and analysis. NUE = nitrogen-utilization efficiency; GBVMU = genomic breeding value for milk urea concentration (MU); GBV_{HMU} and GBV_{LMU} indicate groups of cows with high and low genomic breeding value for MU, respectively; NMDS = nonmetric multidimensional scaling; sPLS-DA = sparse partial least square-discriminant analysis; DESeq2 = Wald test implemented in the "DESeq2" R package; OTU = operational taxonomic unit.

2 h maximum before long-term storage at −20°C. The stomach tube was intensively rinsed with water before the next cow was sampled.

Genomic Breeding Value Estimation for MU

For all cows, a GBVMU was estimated by vit Verden (Vereinigte Informationssysteme Tierhaltung, Verden, Germany). The estimation was conducted by applying the established estimation model for SCC on MU phenotype data from monthly milk records (https://www.vit.de/fileadmin/DE/Zuchtwertschaetzung/Zws_Bes_deu.pdf; accessed January 20, 2020). Because of the normal distribution of MU data, log-transformation was not applied. Only cows with GBVMU and all phenotypes were included in the statistical analyses, resulting in a data set of n = 358 lactating Holsteins.

Based on GBVMU, the population was grouped into the following 2 extreme (high, HMU, and low, LMU) categories: $\mathbf{GBV_{HMU}}$ [GBVMU ≤85, n = 30, MU phenotype = 226.97 ± 19.88 mg/L (mean MU ± SD of the group)] and $\mathbf{GBV_{LMU}}$ (GBVMU ≥115, n = 29, MU phenotype = 161.54 ± 19.10 mg/L). The rest of the population was assigned to GBV_{MED} representing GBVMU between 86 and 115 (n = 299; MU phenotype = 191.95 ± 21.24 mg/L). The extreme cow groups (GBV_{HMU}, GBV_{LMU}) represented the top and bottom 15% for GBVMU of the population separated by 3 standard deviations (**SD**) between GBVMU values. The cow groups assigned to GBVMU were similar in parity (3, 3, 3; mean GBV_{HMU}, GBV_{LMU}, GBV_{MED}), lactation stages (198, 203, and 199 DIM) and BW (681, 686, and 684 kg).

Sample Analysis and Settings of NUE Proxies

Analyses of milk and urine samples were as previously described (Honerlagen et al., 2021). In brief, the State Control Federation of Mecklenburg-Western Pomerania provided the analyses of milk protein, milk fat, lactose, and milk urea, the latter being analyzed by mid-infrared spectroscopy (CombiFoss 7, Foss). Total milk N was determined by MQD (Qualitätsprüfungs- und Dienstleistungsgesellschaft Mecklenburg-Vorpommern, Güstrow, Germany) with the Kjeldahl method and converted into milk CP (g/100 g of milk), using the factor 6.38. Urine samples were analyzed photometrically for urea concentration using an ABX Pentra C400 clinical chemistry analyzer (Horiba Europe GmbH). Feces samples were dried, ground, and subsequently analyzed for

total N content, calculated as percentage of DM, using a Vario MAX element analyzer (Elementar). Rumen fluids were analyzed for microbial composition by 16S rRNA amplicon sequencing. Microbial DNA extraction, 16S rRNA amplicon sequencing, and data preparation followed the description in Honerlagen et al. (2022). Briefly, microbial DNA was extracted utilizing the PowerLyzer PowerSoil DNA isolation kit (Qiagen), the V4 region of the 16S rRNA gene was targeted via PCR, and the PCR products were subsequently sequenced on HiSeq2500 (Illumina Inc.) with 250-bp paired-end reads. After excluding 9 samples due to low read depth, the remaining data set was subsampled to 120,521 reads per sample (McMurdie and Holmes, 2014).

Proxies of NUE generated from milk, urine, and feces samples comprised repeatedly measured and one-time measured traits. Repeatedly measured traits were generated by averaging individual milk record data of a single lactation (14-mo duration). One-time measured traits were recorded as part of the collection procedure of rumen, urine, and fecal samples. Repeatedly measured proxies of NUE used in this study were milk urea concentration ($\mathbf{MU_{lac}}$), milk yield ($\mathbf{MY_{lac}}$), milk urea yield ($\mathbf{MUY_{lac}}$ = MU_{lac} × MY_{lac}), and milk protein yield [$\mathbf{MPY_{lac}}$ = milk protein percentage ($\mathbf{MP_{lac}}$) × MY_{lac}]. One-time measured traits were MU, UU, milk protein percentage (**Mp%**), milk CP as parameter for total milk nitrogen concentration (**MN**), and fecal N concentration (**FecN**).

Statistical Analyses

Milk, urine, and feces phenotypes were tested for significant differences between the extreme GBVMU groups (GBV_{HMU} vs. GBV_{LMU}) by utilizing Student's *t*-test and further investigated for phenotypic correlations among traits in the whole cow population (n = 358).

The microbial data were initially analyzed at the operational taxonomic unit (**OTU**) level in the whole population (n = 358) by nonmetric multidimensional scaling (**NMDS**) based on a Bray-Curtis dissimilarity matrix using the "vegan" R package (Oksanen et al., 2013). Moreover, permutational multivariate ANOVA (PERMANOVA) was performed based on the dissimilarity matrix to test differences between GBV_{HMU}, GBV_{MED}, and GBV_{LMU} groups. Inverse Simpson indices were calculated to assess α-diversity with the "agricolae" package in R (De Mendiburu, 2014).

Further analyses were conducted at the genus level considering genera with more than 30 counts in at least one-third of the entire cow population (remaining data set: 123 genera). Differences in taxa abundance between the extreme GBVMU groups were analyzed using Wald test implemented in the "DESeq2" R package (Love et al., 2014). Milk fat content was included as a fixed effect in the statistical model accounting for differences in feed intake between the cows. Genera were defined as significantly differentially abundant (**DAG**) at *P*-values <0.05. Furthermore, a sparse partial least squares discriminant analysis (**sPLS-DA**) was applied on the filtered and variance-stabilized transformed count data set (123 genera) utilizing the R package "mixOmics" (version 6.6.2; http://mixomics.org; Rohart et al., 2017). The sPLS-DA method was used to identify the most discriminative microbial genera that mainly distinguish the rumen profiles of extreme GBVMU groups. The analysis considered 2 components of microbial features, with the 10 most distinctive genera in each of the components.

The microbial genera identified by DESeq2 (DAG) and sPLS-DA were considered as a microbial signature of GBVMU and were further investigated for their correlation with the NUE-associated traits (milk, urine, feces) in the whole cow population (n = 358). Therefore, a Pearson correlation analysis between the sample-specific genus abundance and each trait was conducted and tested for significance by utilizing the "stats" package in R (https://www.r-project.org/). Significance was determined at adjusted *P*-values <0.05 (Benjamini-Hochberg). The most prominent microbe–trait correlations, considering correlation coefficients, significance, and literature research, were identified and visualized using "ggplot" in R.

RESULTS

Phenotypic Characterization of Cows Assigned to GBVMU

The NUE-associated traits are documented in Table 1, showing means and SD for the entire herd, as well as for GBV_{HMU} and GBV_{LMU} cow groups. The GBV_{LMU} cows showed significantly lower MU values than GBV_{HMU} cows, which was evident at the sampling time point (MU, $P < 0.001$) and across lactation (MU_{lac}, $P < 0.001$). The GBV_{LMU} cows also excreted significantly less absolute MU per lactation, as indicated by MUY_{lac} ($P < 0.001$) even though they yielded significantly more milk (MY_{lac}, $P < 0.05$) than GBV_{HMU} cows. Furthermore, GBV_{LMU} had significantly lower UU than GBV_{HMU} cows ($P < 0.001$), whereas MN, Mp%, MPY_{lac}, and FecN did not differ significantly between groups.

Correlations among the NUE-associated traits in the whole cow population are reported in Table 2. Traits MU_{lac} and MU were positively correlated (r = 0.63, $P < 0.05$), and UU revealed moderate positive correla-

Table 1. Means (± SD) of nitrogen-utilization efficiency (NUE)–associated traits in the whole cow population (n = 358) and in the cows belonging to the extreme groups defined by genomic breeding values for milk urea [GBV_{HMU} (n = 30), GBV_{LMU} (n = 29)]

NUE-associated trait	Acronym	Entire herd	GBV_{HMU}	GBV_{LMU}	*P*-value[1]
Milk urea[2] (mg/L)	MU_{lac}	192.27 ± 24.80	226.97 ± 19.88	161.54 ± 19.10	<0.001
Milk urea (mg/L)	MU	174.04 ± 40.40	204.07 ± 45.42	146.73 ± 29.11	<0.001
Milk urea yield[2] (g/d)	MUY_{lac}	6.82 ± 1.36	7.87 ± 1.31	6.13 ± 1.38	<0.001
Milk N (g of CP/100 g of milk)	MN	3.62 ± 0.38	3.64 ± 0.43	3.60 ± 0.40	0.355
Urinary urea (mmol/L)	UU	103.02 ± 50.52	131.12 ± 59.90	81.07 ± 54.69	<0.001
Fecal N (% in DM)	FecN	2.50 ± 0.30	2.53 ± 0.27	2.49 ± 0.42	0.335
Milk yield[2] (L/d)	MY_{lac}	35.40 ± 5.68	34.62 ± 6.04	37.85 ± 6.06	0.022
Milk protein (% in milk)	Mp%	3.61 ± 0.38	3.62 ± 0.40	3.59 ± 0.40	0.403
Milk protein yield[2] (kg/d)	MPY_{lac}	9.87 ± 2.49	10.05 ± 2.15	9.98 ± 2.86	0.909

[1]*P*-value <0.05 indicates significant differences between GBV_{HMU} and GBV_{LMU} cow groups.
[2]Averaged data of 1 lactation (lac; 14 mo).

tions with MU_{lac} (r = 0.25) and MU (r = 0.26) and a weak positive correlation with MN (r = 0.14). The FecN trait did not show any significant correlations with milk and urine traits. Although a moderate correlation between the repeatedly measured MPY_{lac} and MU_{lac} was observed (r = 0.25), there was no relationship between MPY_{lac} and one-time measured MU. However, one-time measured MU correlated positively with the one-time measured Mp% (r = 0.27).

Differences in Rumen Microbiota Between Extreme GBVMU Groups

The filtered and subsampled microbial data set in the entire cow population comprised 1,064 genera, which were taxonomically assigned to 426 families. Analysis at the OTU level revealed neither a distinct clustering of the overall microbial communities (Figure 2A) nor differences in α-diversity between GBVMU groups (GBV_{HMU}, GBV_{MED}, GBV_{LMU}; Figure 2B).

Analyses at the genus level uncovered 13 DAG between GBV_{HMU} and GBV_{LMU} cows in the filtered data set (Table 3). The genera with the highest abundance among the DAG were *Lachnospiraceae NK3A20 group* and *Muribaculaceae ge*, both with a higher occurrence in GBV_{HMU} than in GBV_{LMU} animals. Two additional genera assigned to the *Lachnospiraceae* were identified as DAG. Furthermore, *Veillonellaceae unclassified*, *Succinivibrionaceae UCG-002*, and *Blautia* abundances indicated substantial differences between divergent GBVMU groups. Whereas *Veillonellaceae unclassified* was observed 2.5 times more in GBV_{HMU} than in GBV_{LMU}, *Succinivibrionaceae UCG-002* and *Blautia* were significantly more abundant in GBV_{LMU}, with fold changes of 2.7 and 2.1, respectively. The significantly higher abundance of *Desulfovibrio* in GBV_{HMU} was supported by the lowest *P*-value in the data set, whereas *Clostridia unclassified* might constitute a further prominent DAG with higher prevalence in the rumen of GBV_{HMU} cows.

Although sPLS-DA did not achieve a complete distinction of GBV_{HMU} and GBV_{LMU} cows' microbial profiles, the abundances of the selected microbial features partly separated the rumen profiles of the divergent GBVMU groups (Figure 3A). *Lachnospiraceae NK3A20 group*, which was identified as the most abundant DAG in the DeSeq2 analysis, was also selected as the most important driver of component 1, accounting for higher abundances in GBV_{HMU} (Figure 3B, Table 3). Eight

Table 2. Correlations of nitrogen-utilization efficiency (NUE)–associated traits in the whole cow population (n = 358)

Trait[1]	MU_{lac}	MU	MUY_{lac}	MN	UU	FecN	MY_{lac}	Mp%
MU_{lac}								
MU	0.63*							
MUY_{lac}	0.60*	0.31*						
MN	0.21*	0.25*	−0.11*					
UU	0.25*	0.26*	0.09	0.14*				
FecN	0.06	0.10	0.03	−0.03	−0.07			
MY_{lac}	−0.06	−0.09	0.74*	−0.34*	−0.11*	−0.01		
Mp%	0.19*	0.28*	−0.12*	0.96*	0.15*	−0.02	−0.33*	
MPY_{lac}	0.25*	0.07	0.68*	0.06	0.06	−0.08	0.63*	0.07

[1]MU = milk urea (mg/L); MUY = milk urea yield (g/d); MN = milk nitrogen (g of CP/100 g of milk); UU = urinary urea (mmol/L); FecN = fecal N (% in DM); MY = milk yield (L/d); Mp% = milk protein (% in milk); MPY = milk protein yield (kg/d); lac = averaged data of one lactation (14 mo).
*Significant correlation ($P < 0.05$).

Table 3. Significantly differentially abundant genera (DAG) between the rumen fluids of GBV_{HMU} and GBV_{LMU} cows

Genus	Relative abundance[1] (%)			Fold change[2]	*P*-value[3]
	Entire herd	GBV_{HMU}	GBV_{LMU}		
Anaerolineae unclassified[4]	0.0696	0.0943	0.0524	1.65	0.022
Blautia	0.0344	0.0243	0.0337	−2.15	0.008
Butyrivibrio[4]	0.3634	0.3037	0.3841	−1.36	0.018
Clostridia unclassified	0.3234	0.3579	0.2937	1.38	0.044
Desulfobulbus[4]	0.0424	0.0332	0.0438	−1.32	0.036
Desulfovibrio	0.0676	0.1113	0.0654	1.94	0.004
Lachnospiraceae AC2044 group[4]	0.1293	0.1253	0.1628	−1.27	0.047
Lachnospiraceae NK3A20 group[4]	2.5930	2.9075	2.2247	1.31	0.012
Lachnospiraceae XPB1014 group[4]	0.0907	0.0758	0.0909	−1.32	0.041
Muribaculaceae ge[4]	1.9545	2.1735	1.8869	1.14	0.015
Prevotellaceae NK3B31 group[4]	0.3637	0.3367	0.4309	−1.31	0.013
Succinivibrionaceae UCG-002[4]	0.2743	0.2731	0.2782	−2.68	0.043
Veillonellaceae unclassified	0.7186	0.9950	0.5936	2.48	0.043

[1]Calculated as mean of the respective group. GBV_{HMU} and GBV_{LMU} indicate groups of cows with high and low genomic breeding values for milk urea, respectively.

[2]Fold changes derive from DESeq2 analysis (n = 59 cows) and refer to GBV_{HMU} compared with GBV_{LMU}.

[3]*P*-values <0.05 indicate significance.

[4]Microbial genera that were additionally identified by sparse partial least square-discriminant analysis to distinguish GBV_{HMU} and GBV_{LMU} rumen profiles.

further DAG were identified by sPLS-DA, whereas *WCHB1–41 ge* and *Bacteroidales BS11 gut group ge* were uncovered exclusively by sPLS-DA and represented the 2 most important features of component 2 (Figure 3C). Both genera occupied higher abundances in GBV_{HMU} cows and covered mean relative abundances of 0.2378 and 0.1445% in the whole cow population.

Integration of a GBVMU-Derived Rumen Microbial Signature and Proxies of NUE

The correlation analysis between microbial abundances and NUE-associated traits (milk, urine, feces) in the entire cow population (n = 358) was conducted with the 24 microbial genera related to GBVMU obtained

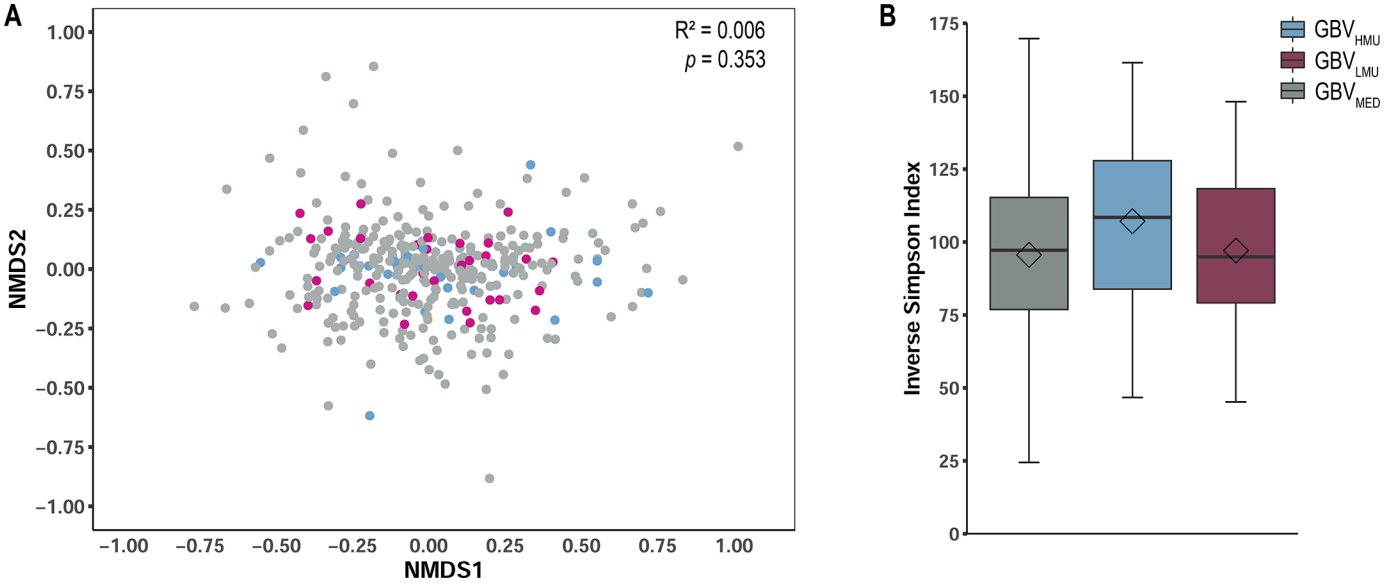

Figure 2. Analyses at the operational taxonomic unit (OTU) level did not indicate distinct clusters between the rumen profiles of cows grouped for different genomic breeding values for milk urea concentration (GBVMU) (A); α diversity did not differ significantly between groups (B). In the boxplot, the box ranges from the 25th percentile to the 75th percentile; the line, the diamond, and the whiskers indicate the median, the mean, and the minimum and maximum values, respectively. GBV_{HMU} and GBV_{LMU} indicate groups of cows with high and low GBVMU, respectively. NMDS = nonmetric multidimensional scaling.

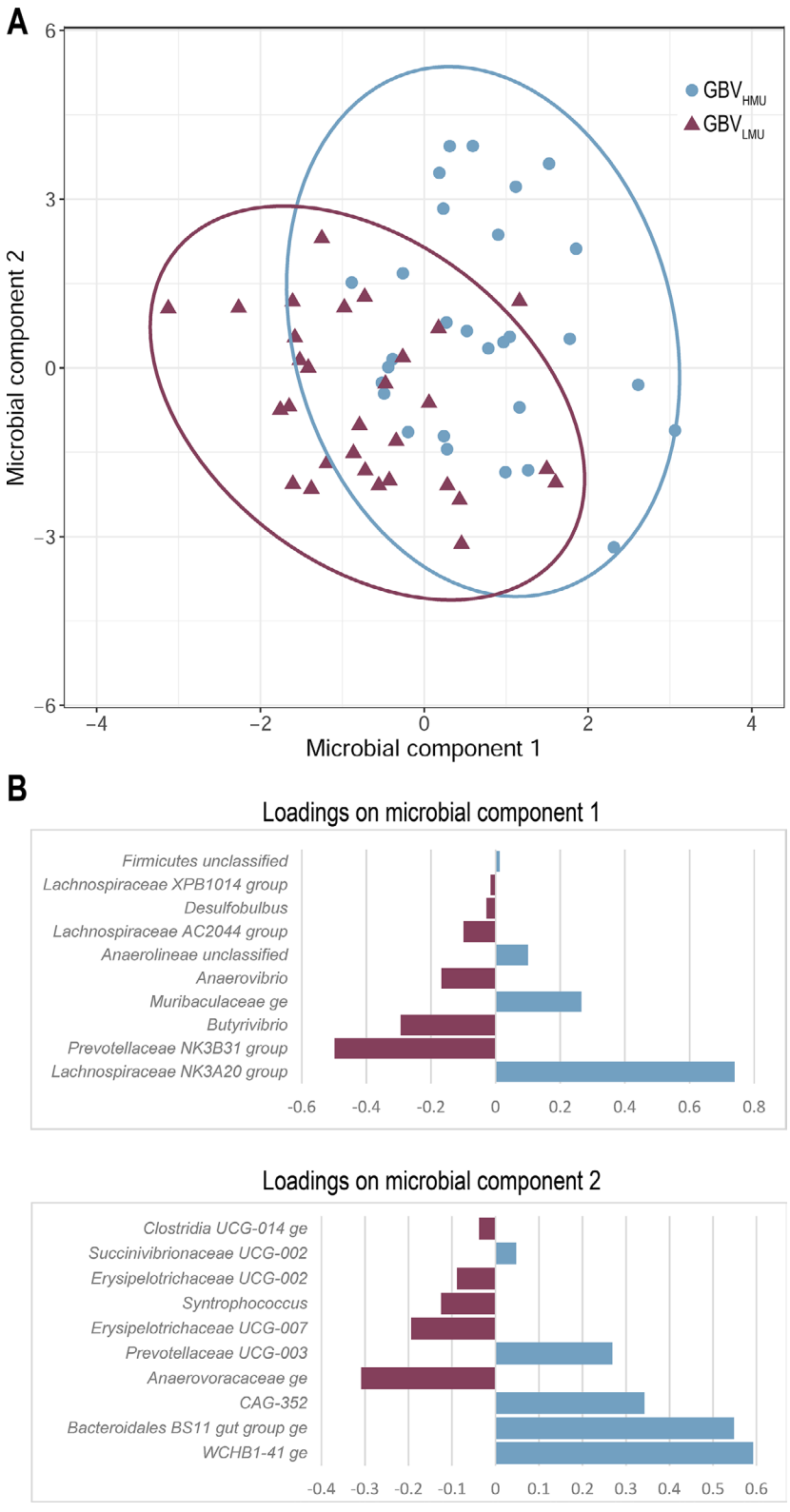

Figure 3. Sparse partial least square-discriminant analysis (sPLS-DA) in dependence on extreme genomic breeding value (GBV) for milk urea concentration (GBV_{HMU} vs. GBV_{LMU}), generated by the loading vectors (i.e., selected microbial genera) of component 1 and component 2. GBV_{HMU} and GBV_{LMU} indicate groups of cows with extreme high and low genomic breeding value for milk urea, respectively. The ellipses (A) account for the 0.95 confidence interval. The bar lengths of the microbial vectors (B) correspond to the importance of the microbial feature driving the respective component. The color of the vectors corresponds to the cow group with higher median value of the respective microbe.

Table 4. Correlation between the abundances of potential microbial signature, derived by selecting for extreme genomic breeding values of milk urea concentration (GBV_{HMU} vs. GBV_{LMU}) to proxies of nitrogen utilization efficiency (NUE) in milk, urine, and feces in the entire cow population (n = 358 cows)

	Proxy trait[1]								
Genus	MU_{lac}	MU	MUY_{lac}	MY_{lac}	MN	UU	FecN	Mp%	MPY_{lac}
Anaerolineae unclassified[2]	−0.09	−0.05	−0.14*	−0.10	0.03	−0.14	0.09	0.01	−0.21*
Anaerovibrio	0.00	0.16*	−0.05	−0.08	0.16*	0.07	0.25*	0.17*	−0.03
Anaerovoracaceae ge	0.00	−0.14	0.12	0.17*	−0.11	0.04	−0.20*	−0.11	0.13
Bacteroidales BS11 gut group ge	−0.07	0.07	−0.23*	−0.23*	0.03	−0.11	0.22*	0.04	−0.30*
Blautia	−0.03	0.15*	−0.11	−0.13	0.20*	0.02	0.29*	0.21*	0.00
Butyrivibrio[2]	−0.15*	0.06	−0.13	−0.06	0.07	0.01	0.24*	0.09	−0.17*
CAG-352	−0.11	0.06	−0.21*	−0.20*	0.08	−0.08	0.23*	0.10	−0.30*
Clostridia UCG-014 ge	−0.05	−0.11	0.04	0.12	−0.08	−0.03	−0.08	−0.08	0.01
Clostridia unclassified	0.00	−0.18*	0.04	0.07	−0.17*	0.08	−0.21*	−0.16*	0.07
Desulfobulbus[2]	−0.04	0.03	0.08	0.14	0.00	0.04	0.00	0.00	0.09
Desulfovibrio	0.02	−0.06	0.04	0.05	−0.14*	−0.02	−0.16*	−0.17*	−0.05
Erysipelotrichaceae UCG-002	−0.03	−0.10	0.05	0.09	−0.07	0.06	−0.21*	−0.08	0.10
Erysipelotrichaceae UCG-007	−0.02	−0.17*	0.02	0.06	−0.08	0.06	−0.21*	−0.09	0.10
Firmicutes unclassified	−0.03	−0.03	−0.12	−0.12	0.03	−0.08	0.03	0.03	−0.22*
Lachnospiraceae AC2044 group[2]	−0.13	−0.10	0.00	0.12	−0.11	−0.05	−0.07	−0.10	−0.04
Lachnospiraceae NK3A20 group[2]	0.13	0.05	0.09	−0.01	0.15*	0.01	−0.07	0.15*	0.10
Lachnospiraceae XPB1014 group[2]	−0.15*	0.03	−0.14*	−0.06	0.03	−0.09	0.21*	0.05	−0.21*
Muribaculaceae ge[2]	−0.01	−0.03	−0.14	−0.17*	0.02	−0.05	0.21*	0.01	−0.20*
Prevotellaceae NK3B31 group[2]	−0.16*	−0.06	−0.08	0.02	0.00	−0.08	0.12	0.00	−0.05
Prevotellaceae UCG-003	0.00	0.15*	−0.10	−0.13	0.10	−0.04	0.24*	0.11	−0.11
Succinivibrionaceae UCG-002[2]	−0.06	0.10	−0.06	−0.05	0.05	0.00	0.11	0.07	−0.04
Syntrophococcus	0.03	−0.16*	0.11	0.12	−0.12	0.08	−0.22*	−0.12	0.14
Veillonellaceae unclassified	0.07	−0.10	0.09	0.09	−0.15*	0.07	−0.25*	−0.17*	0.09
WCHB1–41 ge	−0.04	0.13	−0.18*	−0.21*	0.12	−0.07	0.23*	0.12	−0.18*

[1]MU = milk urea (mg/L); MUY = milk urea yield (g/d); MY = milk yield (L/d); MN = milk nitrogen (g of CP/100 g of milk); UU = urinary urea (mmol/L); FecN = fecal N (% in DM); Mp% = milk protein (% in milk); MPY = milk protein yield (kg/d); lac = averaged data of one lactation (14 mo).

[2]Microbial genera were identified by both DeSeq2 and sparse partial least square-discriminant analysis.

*Significant correlation (adjusted $P < 0.05$).

from the joint lists of DESeq2 and sPLS-DA (Table 4; Supplemental File S1, https://doi.org/10.6084/m9.figshare.22643767.v2; Honerlagen, 2023). The most prominent genus abundance–trait correlations are shown in Figure 4A–F.

The abundances of 2 genera of *Lachnospiraceae* family: the *XPB1014 group* and *AC2044 group*, as well as *Prevotellaceae NK3B31 group* and *Butyrivibrio* revealed considerable negative correlation coefficients with MU_{lac} (r = −0.16 to −0.13). *Butyrivibrio* and *Lachnospiraceae XPB1014 group* abundances showed further notable negative correlations with MUY_{lac} (r = −0.13 and r = −0.14, respectively), but positive correlations with FecN (r = 0.24; r = 0.21). Another 6 genera were significantly correlated with MU ($|r| \geq 0.15$; adjusted $P < 0.05$). Whereas abundances of *Erysipelotrichaceae UCG-007* and *Clostridia unclassified* correlated negatively with MU, higher abundances of *Prevotellaceae UCG-003*, *Blautia*, and *Anaerovibrio* were observed with higher MU values. The latter 3 genera were further found to be positively correlated with MN and FecN. *Blautia* abundances revealed the strongest relationships (MN: r = 0.2; FecN: r = 0.29), but was only barely observed (0.0344% mean relative abundance). Moreover, *Muribaculaceae ge*, which was identified as DAG, as well as the abundances of 3 genera (*WCHB1–41 ge*, *CAG-352*, and *Bacteroidales BS11 gut group ge*) that contributed to the discrimination of extreme GBVMU groups in sPLS-DA, were accompanied by higher FecN excretion and lower MUY_{lac} and MY_{lac} values. Furthermore, the abundances of *Desulfovibrio* and *Veillonellaceae unclassified* were negatively correlated with MN, Mp%, and FecN. Although both genera were identified as DAG, their abundances were not correlated with MU_{lac} or MU phenotypes considering the entire cow herd. *Anaerolineae unclassified* abundances revealed the only considerable correlation with UU values.

DISCUSSION

This study was conducted under the hypothesis that because of the symbiotic interplay between dairy cows and their rumen microbiome, individual MU is affected by host genetics and the rumen microbiome, which in turn is partly attributed to host genetics. Thus, differences in GBVMU should be displayed by different abundances of rumen microbes playing a role for individual N utilization and N excretion. Specific microbial

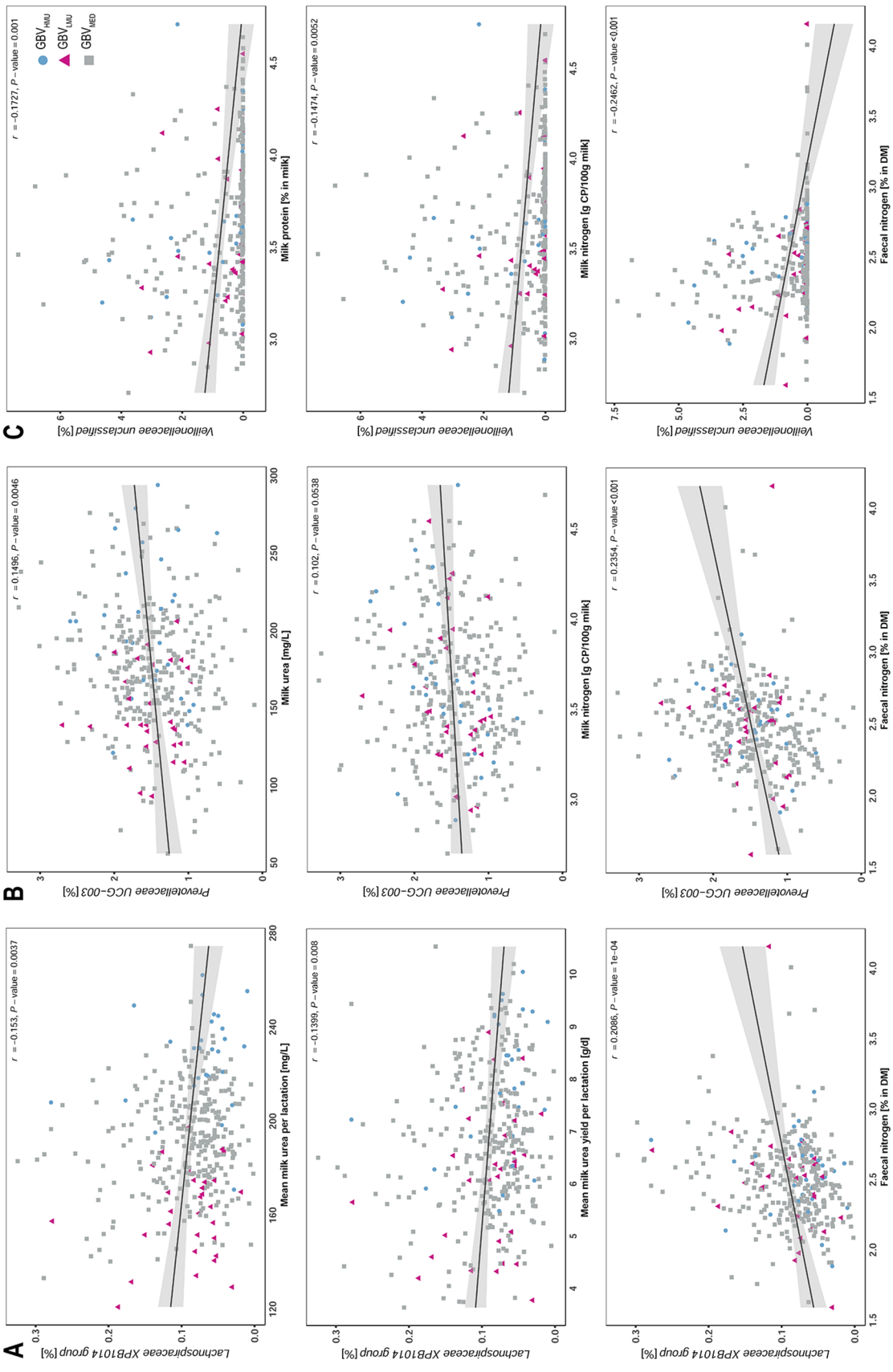

Figure 4. Most prominent relationships of a rumen microbial signature associated to a genomic breeding value of milk urea concentration (GBVMU) and specific proxies of nitrogen-utilization efficiency (NUE) identified in the entire cow population (n = 358). Dots display the microbial abundance–trait correlation colored in correspondence to the respective extreme GBVMU group of individual cows: GBV_{HMU} (blue) or GBV_{LMU} (red), where GBV_{HMU} and GBV_{LMU} indicate groups of cows with high and low genomic breeding value for milk urea concentration, respectively. Cows that belong to the nonextreme, remaining population are portrayed by gray dots (GBV_{MED}). The shaded area displays the 0.95 confidence interval.

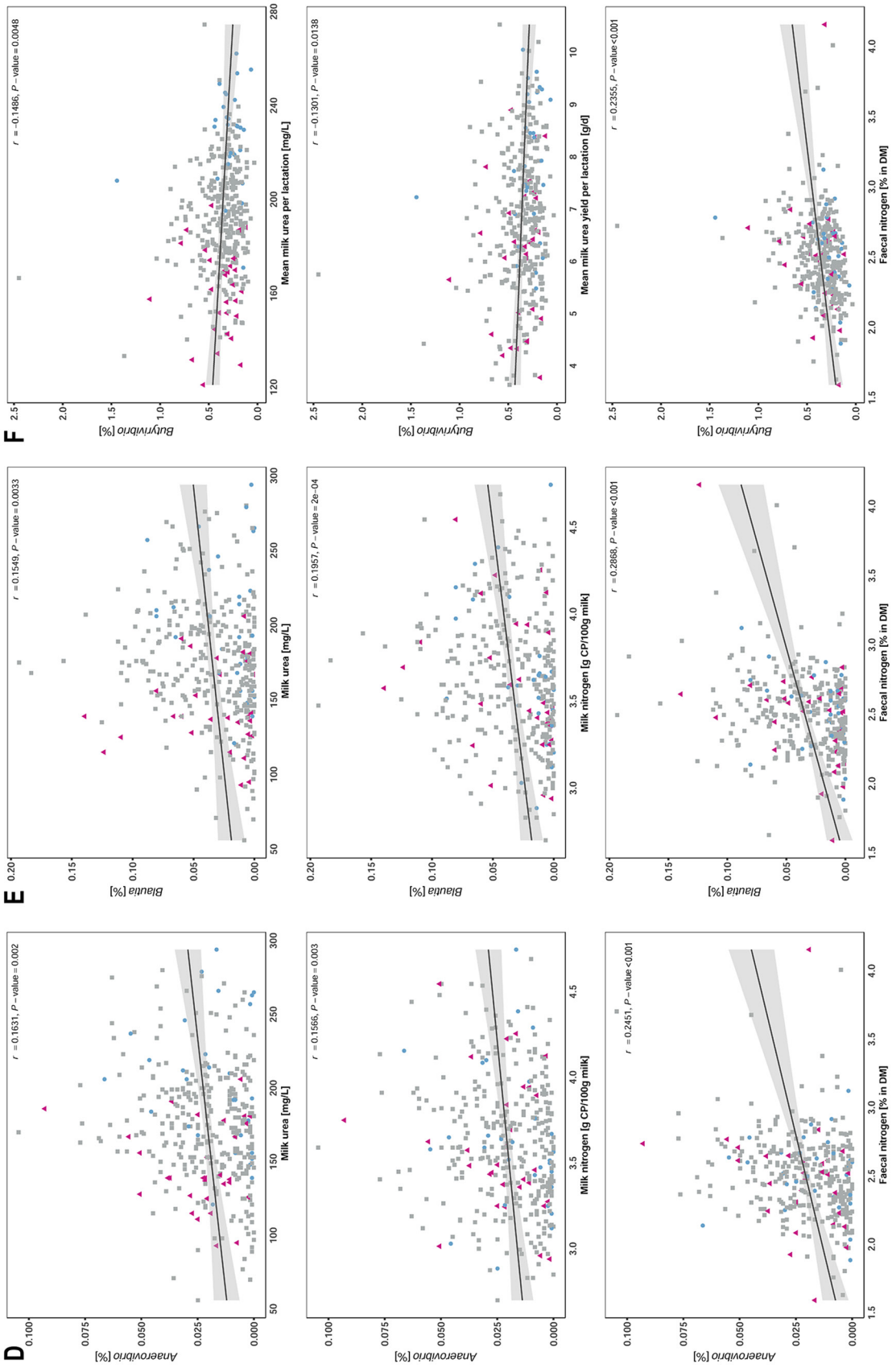

Figure 4 (Continued). Most prominent relationships of a rumen microbial signature associated to a genomic breeding value of milk urea concentration (GBVMU) and specific proxies of nitrogen-utilization efficiency (NUE) identified in the entire cow population (n = 358). Dots display the microbial abundance–trait correlation colored in correspondence to the respective extreme GBVMU group of individual cows: GBV_{HMU} (blue) or GBV_{LMU} (red), where GBV_{HMU} and GBV_{LMU} indicate groups of cows with high and low genomic breeding value for milk urea concentration, respectively. Cows that belong to the nonextreme, remaining population are portrayed by gray dots (GBV_{MED}). The shaded area displays the 0.95 confidence interval.

genera were identified as a potential rumen microbial signature related to GBVMU. These genera were further investigated for their relationship to proxies of NUE in milk, urine, and feces to assess their potential effect on cow individual NUE phenotypes.

All NUE-associated phenotypes ranged in standard norms, confirming suitable dietary N and energy supply in a high-producing herd (Gonda and Lindberg, 1994; Sørensen et al., 2003; Ruska and Jonkus, 2014). The phenotypic characterization of milk parameters revealed that GBV_{LMU} cows excreted significantly less urea via milk (MU_{lac}, MU, and MUY_{lac}), but yielded more milk (MY_{lac}) than GBV_{HMU}. However, in accordance with various studies that confirmed the absence of genetic and phenotypic correlations between MU and milk performance parameters in large cow populations (Wood et al., 2003; Miglior et al., 2007), no phenotypic correlations between MU_{lac} and MU with MY_{lac} were identified in the present study. Thus, higher MY_{lac} in GBV_{LMU} cows does not indicate that GBVMU selection would concurrently enhance MY_{lac}. However, our results strengthen the hypothesis that GBVMU selection would at least not negatively influence MY_{lac}. Moreover, even though GBV_{LMU} cows were phenotyped with higher MY_{lac} than GBV_{HMU}, they excreted significantly less absolute MU per lactation ($MUY_{lac} = MU_{lac} \times MY_{lac}$). These findings indicate the high potential to substantially reduce MU_{lac} by GBVMU breeding selection.

Urea, as the quantitatively most abundant N metabolite in urine, is also known as the most variable urinary N fraction (Bristow et al., 1992), which might explain the high standard deviations in our study. Nonetheless, lower UU in GBV_{LMU} compared with GBV_{HMU} was significantly evident. The moderate correlations between UU and MU (MU_{lac} and MU) are concordant with various studies and strengthen the general assumption that selection on GBV_{LMU} cows would reduce UU and thus N emissions (Burgos et al., 2010; Guliński et al., 2016; Bergen, 2021; te Pas et al., 2021).

Although GBV_{LMU} cows had significantly lower urea concentrations in milk and urine, they did not occupy significantly different FecN phenotypes compared with GBV_{HMU} cows. This observation is in accordance with Arunvipas et al. (2008), who confirmed the lack of associations between MU and fecal N concentration by multiple measurements in 79 lactating dairy herds. These findings suggest the independency of GBVMU selection on the excretion of undigested N in feces. However, it should be considered that data collection of absolute urinary and fecal N excretion was not possible in the present study due to the freestall housing of the herd, the large sample size, and obtaining samples within the daily farming routine. Hence, some NUE-associated phenotypes were depicted relative to the normalized sampling volume (i.e., UU and FecN). The determination of absolute N losses by exact calculations of N input, N deposit (i.e., milk and muscle protein), and N excretion would enhance our knowledge of phenotypes deriving from GBVMU selection.

Nitrogen compounds in milk and urine originate from the cows' N pool as a result of N absorption in the rumen and intestine, whereas fecal N is mainly determined by nonabsorbed N. Consequently, a potential effect of specific rumen microbial genera on proxies of NUE demands differentiation between milk and urine traits compared with fecal N excretion.

In general, a high ruminal fermentation rate of feed-N compounds yielding high amounts of absorbable N metabolites (i.e., peptides and AA) in the small intestine enhances N usability for the cow, decreases the ruminal and the cow's (blood) NPN pool, and thus reduces N losses via milk and urine. A specific rumen microbial effect on milk and urinary N excretion would therefore be conceivable, if a genus (1) influences the ruminal amount of NH_3 or NH^{4+} by its deamination activity (effect on ruminal NPN pool), (2) determines the diffusion and utilization of blood urea in the rumen by its ureolytic activity (effect on cow's NPN pool), or (3) influences the transport of NH_3, NH^{4+}, and urea across the rumen epithelium (effect on ruminal and cow's NPN pool).

Fecal N excretion originates from undigested dietary N, nonabsorbed microbial protein, and, to a small extent, from endogenous N (Stallcup et al., 1975). Fecal N content is therefore mainly determined by the total amount of N absorbed by the cow, independent of the N absorption form (NH_3 or NH^{4+} via rumen epithelia; AA and peptides via small intestine membrane). Microbial influence on FecN phenotypes might therefore be more due to (1) the initial breakdown of dietary protein N by proteolytic activity in the rumen; (2) ruminal N fermentation activity, which determines the amount of N incorporation into microbial protein; or (3) an effect on the dietary passage rate, which affects the cow's intestinal barrier absorption capacity of peptides and AA.

The results of the overall microbial composition indicated that GBVMU selection is not accompanied by specific ruminotypes nor would microbial diversity have been substantially increased or decreased. These findings are in accordance with low to moderate heritability estimations of microbial features in dairy cattle (Difford et al., 2018) and might be explained by the fact that rumen microbial composition is mainly influenced by environmental factors (i.e., feed components) and only to a certain extent by host genetics (Yáñez-Ruiz et al., 2015). Nonetheless, 14 DAG were identified between

the extreme GBVMU groups. Interestingly, 7 of these were also identified to distinguish the rumen profiles of another cow population grouped for high and low MU predisposition in our previous study (Honerlagen et al., 2022). Specifically, a higher abundance of the ureolytic genus *Succinivibrionaceae UCG-002* was identified in GBV_{LMU} cows, whereas GBV_{HMU} cows hosted significantly greater abundances of *Clostridia unclassified* and *Desulfovibrio*, which became prominent as hyper-ammonia-producing bacteria (**HAB**) species (Paster et al., 1993; Bento et al., 2015; Hartinger et al., 2018; Honerlagen et al., 2021; Libera et al., 2021). Ureolytic bacteria are known to enhance the diffusion of blood urea into the rumen, which reduces the cow's blood NPN pool, facilitates the ruminal N incorporation into microbial protein, and thus reduces N losses (Hartinger et al., 2018). Accordingly, ureolytic genera were identified to facilitate the ruminal N utilization (Jin et al., 2016). In contrast, HAB species are known to massively increase NH_3 levels in the rumen fluid by their rapid AA deamination activity (Patra and Aschenbach, 2018). The subsequent steep increases of the ruminal NH_3 pool enhance NH_3 effluxes into the blood, increase the blood NPN pool, and promote urea synthesis in the hepatocytes. Accordingly, HAB are thought to negatively influence the NUE and enhance N losses (Hartinger et al., 2018).

Furthermore, in this study, we identified 3 genera of *Lachnospiraceae* family—*Lachnospiraceae NK3A20 group*, *Lachnospiraceae AC2044 group*, and *Lachnospiraceae XPB1014 group*—as part of the microbial signature that might influence MU phenotypes. The *Lachnospiraceae* family has been associated with low-N-utilizing phenotypes in beef steers and goats (Wang et al., 2019; Alves et al., 2021). However, this family hosts a large variety of genera that adapt to individual ecological niches and might therefore contribute to N metabolism in ruminants in a different manner (Meehan and Beiko, 2014). *Lachnospiraceae NK3A20 group* has been described as a major genus of the *Lachnospiraceae* family in the rumen (Anderson et al., 2021) and was significantly more abundant in GBV_{HMU} cows. Interestingly, Huang et al. (2021) reported a positive correlation between ruminal *Lachnospiraceae NK3A20 group* abundances and rumen papillae length in yaks that quantitatively enhanced the absorption capacity of the tissue. Regarding higher occurrences of *Lachnospiraceae NK3A20 group* in cows with HMU phenotypes and correspondingly higher blood N pools (Müller et al., 2021), it might be speculated whether *Lachnospiraceae NK3A20 group* enhanced NH_3 and NH^{4+} absorption by stimulating rumen papillae growth and thus promote N effluxes into the blood. *Lachnospiraceae AC2044 group* and *Lachnospiraceae XPB1014 group* were more abundant in GBV_{HMU} cows. Both genera were negatively correlated with MU_{lac}, and the *XPB1014 group* showed further considerable negative correlations with MUY_{lac} and positive correlations with FecN. Interestingly, *Lachnospiraceae XPB1014 group* abundances were found to be increased under N scarcity in the hindgut of pigs and contributed to enhanced NUE (Zhao et al., 2020). Furthermore, increased *Lachnospiraceae XPB1014 group* abundance has been observed along with high carbohydrate fermentation levels in cows (Hendawy et al., 2021) and pigs (Zhao et al., 2020). In general, a high fermentation rate of carbohydrates stimulates feed intake, accelerates the feed passage rate of the diet, reduces the absorption time of AA and peptides in the intestine, and thus increases fecal nutrient losses (McCarthy et al., 1989; Schuba et al., 2017).

A further considerable positive correlation was found between FecN and *Prevotellaceae UCG-003* abundances. Interestingly, high abundances of *Prevotellaceae UCG-003* were observed along with low ruminal fermentation degrees in yaks and steers (Liu et al., 2019; Qiu et al., 2020). Moreover, Huang et al. (2021) identified a negative correlation between *Prevotellaceae UCG-003* and the length of the rumen papilla in yaks. Short papilla generally decrease the nutrient absorption capacity of the rumen tissue and might subsequently lead to enhanced fecal nutrient losses (Huang et al., 2021). The *Prevotellaceae* family is a major player in ruminal fermentation processes and hosts various genera that are known to massively affect ruminal AA metabolism (Liu et al., 2019; Qiu et al., 2020; Zhao et al., 2020). The present study revealed numerically higher abundances of *Prevotellaceae UCG-003* in GBV_{HMU} cows and further positive correlations with MU and MN. *Prevotellaceae UCG-003* might therefore constitute a genus in the microbial signature of GBVMU selection, which is proposed for independent effect estimation on fecal and milk N losses from dairy cows.

A significant positive correlation with FecN was further observed for *Muribaculaceae ge*, which had a significantly higher abundance in GBV_{HMU} compared with GBV_{LMU} cows. The negative correlations between *Muribaculaceae ge* abundance and MY in our study are in accordance with the findings of Dong et al. (2023), who investigated the physiology of different MY in Holsteins. The *Muribaculaceae* family has been attributed a role in the N metabolism of Holstein calves (Zhang et al., 2021) and was found to have varying abundance in response to seasonally differing diets in adult cattle (Martinez-Fernandez et al., 2020). The *Muribaculaceae* family occupies a huge variety of genus- and strain-specific functions, which were initially explored (Lagk-

ouvardos et al., 2019). However, the specific role of *Muribaculaceae ge* abundance in the rumen has not yet been verified.

A high relative abundance was identified for *Veillonellaceae unclassified*, which correlated negatively with MN, Mp%, and FecN and accounted for higher abundance in GBV_{HMU} than in GBV_{LMU} cows. The *Veillonellaceae* are one of the most dominant microbial communities in the rumen of goats (Giger-Reverdin et al., 2020) and in the feces of pigs (Spring et al., 2020). Lower abundance of *Veillonellaceae* was observed in the rumen of lambs when the animals were fed diets with a high urea level (Li et al., 2020).

Anaerovibrio and *Blautia* abundances were positively correlated with FecN, MU, and MN. Although both genera were, in accordance with other studies, only barely abundant (Ramos et al., 2018; Xie et al., 2022), *Anaerovibrio* and *Blautia* have been identified as differentially abundant in the digestive tract of goats grouped for high and low NUE phenotypes (Wang et al., 2019). *Anaerovibrio* was found to be significantly more abundant in low-NUE goats, whereas *Blautia* was detected with higher presence in the high-NUE phenotype. Although these findings are in accordance with significantly higher abundance of *Blautia* in GBV_{LMU} cows in the present study, the positive correlation between *Blautia* abundances and MU, FecN, and MN excretion would attribute *Blautia* a disadvantageous role in ruminal N metabolism. Because *Blautia* has recently been defined as a single genus (Liu et al., 2021), further research on the biological contribution of *Blautia* to the N metabolism in ruminants is proposed.

The considerable positive correlation between *Butyrivibrio* abundances and FecN is of interest. *Butyrivibrio* is a dominant genus in the rumen (Henderson et al., 2015) and accounted for considerable abundances in the present study. *Butyrivibrio* was identified with significantly higher occurrence in predisposed HMU cows in our previous study (Honerlagen et al., 2022) and correlated with lower N-recycling efficiency phenotypes in beef cattle (Alves et al., 2021). However, in the present study, *Butyrivibrio* accounted for significantly greater abundances in GBV_{LMU} cows and correlated accordingly negatively with MU_{lac}. Considering the major occurrence of this genus together with the findings of Derakhshani et al. (2018), who suggested *Butyrivibrio* as a major fibrolytic rumen dweller in Holsteins with a major effect on ruminal fermentation, we might speculate whether *Butyrivibrio* affects dietary N digestibility by its fermentation activity and therefore promotes FecN losses. At this time, the causality between host genetics, *Butyrivibrio* abundances, and N utilization and N excretion remains unclear but warrants further research.

CONCLUSIONS

The results of this study implied that selection for GBV_{LMU} cows would reduce MU and UU but would not affect FecN. Although GBVMU selection would potentially not lead to fundamental changes in the rumen microbial composition, specific genera abundances distinguished GBV_{LMU} and GBV_{HMU} cows. Considering their relationship to MU and further NUE-associated traits, *Succinivibrionaceae UCG-002*, *Clostridia unclassified*, *Desulfovibrio*, the *Lachnospiraceae* family, *Prevotellaceae UCG-003*, and *Butyrivibrio* are proposed as the most important microbial genera linked to GBVMU, possibly affecting proxies of NUE in Holsteins. These genera are suggested for quantitative effect estimations, to determine their potential as a microbial signature of future breeding selection on enhanced NUE in dairy cows.

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REFERENCES

Abdoun, K., F. Stumpff, and H. Martens. 2006. Ammonia and urea transport across the rumen epithelium: A review. Anim. Health Res. Rev. 7:43–59. https://doi.org/10.1017/S1466252307001156.

Alves, K. L. G. C., Y. T. Granja-Salcedo, J. D. Messana, V. C. de Souza, M. J. G. Ganga, P. H. D. Colovate, L. T. Kishi, and T. T. Berchielli. 2021. Rumen bacterial diversity in relation to nitrogen retention in beef cattle. Anaerobe 67:102316. https://doi.org/10.1016/j.anaerobe.2020.102316.

Anderson, C. J., L. R. Koester, and S. Schmitz-Esser. 2021. Rumen epithelial communities share a core bacterial microbiota: A meta-analysis of 16S rRNA gene Illumina MiSeq sequencing datasets. Front. Microbiol. 12:625400. https://doi.org/10.3389/fmicb.2021.625400.

Arunvipas, P., J. A. VanLeeuwen, I. R. Dohoo, G. P. Keefe, S. A. Burton, and K. D. Lissemore. 2008. Relationships among milk urea-nitrogen, dietary parameters, and fecal nitrogen in commercial dairy herds. Can. J. Vet. Res. 72:449.

Bach, A., S. Calsamiglia, and M. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9–E21. https://doi.org/10.3168/jds.S0022-0302(05)73133-7.

Beatson, P. R., S. Meier, N. Cullen, and H. Eding. 2019. Genetic variation in milk urea nitrogen concentration of dairy cattle and its implications for reducing urinary nitrogen excretion. Animal 13:2164–2171. https://doi.org/10.1017/S1751731119000235.

Bento, C. B. P., A. C. de Azevedo, E. Detmann, and H. C. Mantovani. 2015. Biochemical and genetic diversity of carbohydrate-fermenting and obligate amino acid-fermenting hyper-ammonia-producing bacteria from Nellore steers fed tropical forages and supplemented with casein. BMC Microbiol. 15:28. https://doi.org/10.1186/s12866-015-0369-9.

Bergen, W. G. 2021. Amino acids in beef cattle nutrition and production. Adv. Exp. Med. Biol. 1285:29–42. https://doi.org/10.1007/978-3-030-54462-1_3.

Bobbo, T., M. Penasa, A. Rossoni, and M. Cassandro. 2020. Genetic aspects of milk urea nitrogen and new indicators of nitrogen efficiency in dairy cows. J. Dairy Sci. 103:9207–9212. https://doi.org/10.3168/jds.2020-18445.

Bristow, A. W., D. C. Whitehead, and J. E. Cockburn. 1992. Nitrogenous constituents in the urine of cattle, sheep and goats. J. Sci. Food Agric. 59:387–394. https://doi.org/10.1002/jsfa.2740590316.

Bryant, M. P. 1970. Normal flora—Rumen bacteria. Am. J. Clin. Nutr. 23:1440–1450. https://doi.org/10.1093/ajcn/23.11.1440.

Burgos, S. A., N. M. Embertson, Y. Zhao, F. M. Mitloehner, E. J. DePeters, and J. G. Fadel. 2010. Prediction of ammonia emission from dairy cattle manure based on milk urea nitrogen: relation of milk urea nitrogen to ammonia emissions. J. Dairy Sci. 93:2377–2386. https://doi.org/10.3168/jds.2009-2415.

Calsamiglia, S., A. Ferret, C. K. Reynolds, N. B. Kristensen, and A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. Animal 4:1184–1196. https://doi.org/10.1017/S1751731110000911.

De Mendiburu, F. 2014. Agricolae: Statistical procedures for agricultural research. R package. https://cran.r-project.org/web/packages/agricolae/index.html.

Derakhshani, H., J. C. Plaizier, J. De Buck, H. W. Barkema, and E. Khafipour. 2018. Association of bovine major histocompatibility complex (BoLA) gene polymorphism with colostrum and milk microbiota of dairy cows during the first week of lactation. Microbiome 6:203. https://doi.org/10.1186/s40168-018-0586-1.

Difford, G. F., D. R. Plichta, P. Løvendahl, J. Lassen, S. J. Noel, O. Højberg, A.-D. G. Wright, Z. Zhu, L. Kristensen, H. B. Nielsen, B. Guldbrandtsen, and G. Sahana. 2018. Host genetics and the rumen microbiome jointly associate with methane emissions in dairy cows. PLoS Genet. 14:e1007580. https://doi.org/10.1371/journal.pgen.1007580.

Dijkstra, J., O. Oenema, and A. Bannink. 2011. Dietary strategies to reducing N excretion from cattle: Implications for methane emissions. Curr. Opin. Environ. Sustain. 3:414–422. https://doi.org/10.1016/j.cosust.2011.07.008.

Giger-Reverdin, S., C. Domange, L. P. Broudiscou, D. Sauvant, and V. Berthelot. 2020. Rumen function in goats, an example of adaptive capacity. J. Dairy Res. 87:45–51. https://doi.org/10.1017/S0022029920000060.

Gonda, H. L., and J. E. Lindberg. 1994. Evaluation of dietary nitrogen utilization in dairy cows based on urea concentrations in blood, urine and milk, and on urinary concentration of purine derivatives. Acta Agric. Scand. A Anim. Sci. 44:236–245. https://doi.org/10.1080/09064709409410904.

Grelet, C., E. Froidmont, L. Foldager, M. Salavati, M. Hostens, C. P. Ferris, K. L. Ingvartsen, M. A. Crowe, M. T. Sorensen, J. A. Fernandez Pierna, A. Vanlierde, N. Gengler, and F. Dehareng. 2020. Potential of milk mid-infrared spectra to predict nitrogen use efficiency of individual dairy cows in early lactation. J. Dairy Sci. 103:4435–4445. https://doi.org/10.3168/jds.2019-17910.

Guliński, P., E. Salamończyk, and K. Młynek. 2016. Improving nitrogen use efficiency of dairy cows in relation to urea in milk—A review. Anim. Sci. Pap. Rep. 34:5–24.

Hartinger, T., N. Gresner, and K.-H. Südekum. 2018. Does intra-ruminal nitrogen recycling waste valuable resources? A review of major players and their manipulation. J. Anim. Sci. Biotechnol. 9:33. https://doi.org/10.1186/s40104-018-0249-x.

Hendawy, A. O., S. Sugimura, K. Sato, M. M. Mansour, A. H. Abd El-Aziz, H. Samir, M. A. Islam, A. R. Bostami, A. S. Mandour, A. Elfadadny, R. F. Ragab, H. A. Abdelmageed, and A. M. Ali. 2021. Effects of selenium supplementation on rumen microbiota, rumen fermentation, and apparent nutrient digestibility of ruminant animals: A review. Fermentation (Basel) 8:4. https://doi.org/10.3390/fermentation8010004.

Henderson, G., F. Cox, S. Ganesh, A. Jonker, W. Young, Global Rumen Census Collaborators, and P. H. Janssen. 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Sci. Rep. 5:14567. https://doi.org/10.1038/srep14567.

Honerlagen, H. 2023. Supplemental File S1. figshare. Dataset. https://doi.org/10.6084/m9.figshare.22643767.v2.

Honerlagen, H., H. Reyer, M. Oster, S. Ponsuksili, N. Trakooljul, B. Kuhla, N. Reinsch, and K. Wimmers. 2021. Identification of genomic regions influencing N-metabolism and N-excretion in lactating Holstein-Friesians. Front. Genet. 12:699550. https://doi.org/10.3389/fgene.2021.699550.

Honerlagen, H., H. Reyer, D. Segelke, C. B. Müller, M. C. Prahl, S. Ponsuksili, N. Trakooljul, N. Reinsch, B. Kuhla, and K. Wimmers. 2022. Ruminal background of predisposed milk urea (MU) concentration in Holsteins. Front. Microbiol. 13:939711. https://doi.org/10.3389/fmicb.2022.939711.

Huang, C., F. Ge, X. Yao, X. Guo, P. Bao, X. Ma, X. Wu, M. Chu, P. Yan, and C. Liang. 2021. Microbiome and metabolomics reveal the effects of different feeding systems on the growth and ruminal development of yaks. Front. Microbiol. 12:682989. https://doi.org/10.3389/fmicb.2021.682989.

Huber, J. T., and L. Kung Jr.. 1981. Protein and nonprotein nitrogen utilization in dairy cattle. J. Dairy Sci. 64:1170–1195. https://doi.org/10.3168/jds.S0022-0302(81)82695-1.

Huhtanen, P., E. Cabezas-Garcia, S. Krizsan, and K. Shingfield. 2015. Evaluation of between-cow variation in milk urea and rumen ammonia nitrogen concentrations and the association with nitrogen utilization and diet digestibility in lactating cows. J. Dairy Sci. 98:3182–3196. https://doi.org/10.3168/jds.2014-8215.

Jahnel, R. E., D. Wittenburg, M. Mayer, H. Taeubert, and N. Reinsch. 2021. Estimation of genetic parameters of milk urea content. Zuchtungskunde 93:157–168.

Jin, D., S. Zhao, P. Wang, N. Zheng, D. Bu, Y. Beckers, and J. Wang. 2016. Insights into abundant rumen ureolytic bacterial community using rumen simulation system. Front. Microbiol. 7:1006. https://doi.org/10.3389/fmicb.2016.01006.

Lagkouvardos, I., T. R. Lesker, T. C. Hitch, E. J. Gálvez, N. Smit, K. Neuhaus, J. Wang, J. F. Baines, B. Abt, B. Stecher, J. Overmann, T. Strowig, and T. Clavel. 2019. Sequence and cultivation study of Muribaculaceae reveals novel species, host preference, and functional potential of this yet undescribed family. Microbiome 7:28. https://doi.org/10.1186/s40168-019-0637-2.

Lavery, A., and C. P. Ferris. 2021. Proxy measures and novel strategies for estimating nitrogen utilisation efficiency in dairy cattle. Animals (Basel) 11:343. https://doi.org/10.3390/ani11020343.

Li, Z., C. Mu, Y. Xu, J. Shen, and W. Zhu. 2020. Changes in the solid-, liquid-, and epithelium-associated bacterial communities in the rumen of hu lambs in response to dietary urea supplementation. Front. Microbiol. 11:244. https://doi.org/10.3389/fmicb.2020.00244.

Libera, K., M. Szumacher-Strabel, M. Vazirigohar, W. Zieliński, R. Lukow, K. Wysocka, P. Kołodziejski, D. Lechniak, Z. Varadyova, A. K. Patra, and A. Cieslak. 2021. Effects of feeding urea-treated triticale and oat grain mixtures on ruminal fermentation, microbial population, and milk production performance of midlactation dairy cows. Ann. Anim. Sci. 21:1007–1025. https://doi.org/10.2478/aoas-2020-0108.

Liu, C., H. Wu, S. Liu, S. Chai, Q. Meng, and Z. Zhou. 2019. Dynamic alterations in yak rumen bacteria community and metabolome characteristics in response to feed type. Front. Microbiol. 10:1116. https://doi.org/10.3389/fmicb.2019.01116.

Liu, X., B. Mao, J. Gu, J. Wu, S. Cui, G. Wang, J. Zhao, H. Zhang, and W. Chen. 2021. *Blautia*—A new functional genus with potential probiotic properties? Gut Microbes 13:1875796. https://doi.org/10.1080/19490976.2021.1875796.

Loor, J. J., A. A. Elolimy, and J. C. McCann. 2016. Dietary impacts on rumen microbiota in beef and dairy production. Anim. Front. 6:22–29. https://doi.org/10.2527/af.2016-0030.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15:550. https://doi.org/10.1186/s13059-014-0550-8.

Martinez-Fernandez, G., J. Jiao, J. Padmanabha, S. E. Denman, and C. S. McSweeney. 2020. Seasonal and nutrient supplement responses in rumen microbiota structure and metabolites of tropical rangeland cattle. Microorganisms 8:1550. https://doi.org/10.3390/microorganisms8101550.

McCarthy, R. D. Jr., T. Klusmeyer, J. Vicini, J. Clark, and D. Nelson. 1989. Effects of source of protein and carbohydrate on ruminal fermentation and passage of nutrients to the small intestine of lactating cows. J. Dairy Sci. 72:2002–2016. https://doi.org/10.3168/jds.S0022-0302(89)79324-3.

McMurdie, P. J., and S. Holmes. 2014. Waste not, want not: Why rarefying microbiome data is inadmissible. PLOS Comput. Biol. 10:e1003531. https://doi.org/10.1371/journal.pcbi.1003531.

Meehan, C. J., and R. G. Beiko. 2014. A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. Genome Biol. Evol. 6:703–713. https://doi.org/10.1093/gbe/evu050.

Miglior, F., A. Sewalem, J. Jamrozik, J. Bohmanova, D. M. Lefebvre, and R. K. Moore. 2007. Genetic analysis of milk urea nitrogen and lactose and their relationships with other production traits in Canadian Holstein cattle. J. Dairy Sci. 90:2468–2479. https://doi.org/10.3168/jds.2006-487.

Müller, C. B. M., S. Görs, M. Derno, A. Tuchscherer, K. Wimmers, A. Zeyner, and B. Kuhla. 2021. Differences between Holstein dairy cows in renal clearance rate of urea affect milk urea concentration and the relationship between milk urea and urinary nitrogen excretion. Sci. Total Environ. 755:143198. https://doi.org/10.1016/j.scitotenv.2020.143198.

Munyaneza, N., J. Niyukuri, and Y. E. Hachimi. 2017. Milk urea nitrogen as an indicator of nitrogen metabolism efficiency in dairy cows: A review. Theriogenology Insight 7:145–159. https://doi.org/10.5958/2277-3371.2017.00032.8.

Nousiainen, J., K. Shingfield, and P. Huhtanen. 2004. Evaluation of milk urea nitrogen as a diagnostic of protein feeding. J. Dairy Sci. 87:386–398. https://doi.org/10.3168/jds.S0022-0302(04)73178-1.

Oksanen, J., F. G. Blanchet, R. Kindt, P. Legendre, P. R. Minchin, R. O'hara, G. L. Simpson, P. Solymos, M. H. H. Stevens, and H. Wagner. 2013. Package 'vegan'. Community ecology package, version 2(9):1–295. CRAN.Rproject.org/package=vegan.

Paster, B. J., J. B. Russell, C. Yang, J. Chow, C. R. Woese, and R. Tanner. 1993. Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and *Clostridium aminophilum* sp. nov. Int. J. Syst. Bacteriol. 43:107–110. https://doi.org/10.1099/00207713-43-1-107.

Patra, A. K., and J. R. Aschenbach. 2018. Ureases in the gastrointestinal tracts of ruminant and monogastric animals and their implication in urea-N/ammonia metabolism: A review. J. Adv. Res. 13:39–50. https://doi.org/10.1016/j.jare.2018.02.005.

Pérez-Enciso, M., L. M. Zingaretti, Y. Ramayo-Caldas, and G. de Los Campos. 2021. Opportunities and limits of combining microbiome and genome data for complex trait prediction. Genet. Sel. Evol. 53:65.

Powell, J., C. Gourley, C. Rotz, and D. M. Weaver. 2010. Nitrogen use efficiency: A potential performance indicator and policy tool for dairy farms. Environ. Sci. Policy 13:217–228. https://doi.org/10.1016/j.envsci.2010.03.007.

Qiu, Q., C. Gao, M. Aziz ur Rahman, B. Cao, and H. Su. 2020. Digestive ability, physiological characteristics, and rumen bacterial community of Holstein finishing steers in response to three nutrient density diets as fattening phases advanced. Microorganisms 8:335. https://doi.org/10.3390/microorganisms8030335.

Ramos, A. F., S. A. Terry, D. B. Holman, G. Breves, L. G. Pereira, A. G. Silva, and A. V. Chaves. 2018. Tucumã oil shifted ruminal fermentation, reducing methane production and altering the microbiome but decreased substrate digestibility within a RUSITEC fed a mixed hay–concentrate diet. Front. Microbiol. 9:1647. https://doi.org/10.3389/fmicb.2018.01647.

Reed, K. F., L. Moraes, D. Casper, and E. Kebreab. 2015. Predicting nitrogen excretion from cattle. J. Dairy Sci. 98:3025–3035. https://doi.org/10.3168/jds.2014-8397.

Rohart, F., B. Gautier, A. Singh, and K.-A. Lê Cao. 2017. mixOmics: An R package for 'omics feature selection and multiple data integration. PLOS Comput. Biol. 13:e1005752. https://doi.org/10.1371/journal.pcbi.1005752.

Ruska, D., and D. Jonkus. 2014. Crude protein and non-protein nitrogen content in dairy cow milk. Proc. Latv. Univ. Agric. 32:36–40. https://doi.org/10.2478/plua-2014-0011.

Saborío-Montero, A., M. Gutiérrez-Rivas, A. López-García, A. García-Rodríguez, R. Atxaerandio, I. Goiri, J. A. Jiménez-Montero, and O. González-Recio. 2021. Holobiont effect accounts for more methane emission variance than the additive and microbiome effects on dairy cattle. Livest. Sci. 250:104538. https://doi.org/10.1016/j.livsci.2021.104538.

Schuba, J., K.-H. Südekum, E. Pfeffer, and A. Jayanegara. 2017. Excretion of faecal, urinary urea and urinary non-urea nitrogen by four ruminant species as influenced by dietary nitrogen intake: A meta-analysis. Livest. Sci. 198:82–88. https://doi.org/10.1016/j.livsci.2017.01.017.

Sørensen, P., M. Weisbjerg, and P. Lund. 2003. Dietary effects on the composition and plant utilization of nitrogen in dairy cattle manure. J. Agric. Sci. 141:79–91. https://doi.org/10.1017/S0021859603003368.

Spanghero, M., and Z. Kowalski. 2021. Updating analysis of nitrogen balance experiments in dairy cows. J. Dairy Sci. 104:7725–7737. https://doi.org/10.3168/jds.2020-19656.

Spring, S., H. Premathilake, C. Bradway, C. Shili, U. DeSilva, S. Carter, and A. Pezeshki. 2020. Effect of very low-protein diets supplemented with branched-chain amino acids on energy balance, plasma metabolomics and fecal microbiome of pigs. Sci. Rep. 10:15859. https://doi.org/10.1038/s41598-020-72816-8.

Stallcup, O. T., G. Davis, and L. Shields. 1975. Influence of dry matter and nitrogen intakes on fecal nitrogen losses in cattle. J. Dairy Sci. 58:1301–1307. https://doi.org/10.3168/jds.S0022-0302(75)84710-2.

Tan, P., H. Liu, J. Zhao, X. Gu, X. Wei, X. Zhang, N. Ma, L. J. Johnston, Y. Bai, W. Zhang, C. Nie, and X. Ma. 2021. Amino acids metabolism by rumen microorganisms: Nutrition and ecology strategies to reduce nitrogen emissions from the inside to the outside. Sci. Total Environ. 800:149596. https://doi.org/10.1016/j.scitotenv.2021.149596.

te Pas, M. F., T. Veldkamp, Y. de Haas, A. Bannink, and E. D. Ellen. 2021. Adaptation of livestock to new diets using feed components without competition with human edible protein sources—A review of the possibilities and recommendations. Animals (Basel) 11:2293. https://doi.org/10.3390/ani11082293.

Wang, L., K. Liu, Z. Wang, X. Bai, Q. Peng, and L. Jin. 2019. Bacterial community diversity associated with different utilization efficiencies of nitrogen in the gastrointestinal tract of goats. Front. Microbiol. 10:239. https://doi.org/10.3389/fmicb.2019.00239.

Wood, G. M., P. Boettcher, J. Jamrozik, G. Jansen, and D. Kelton. 2003. Estimation of genetic parameters for concentrations of milk urea nitrogen. J. Dairy Sci. 86:2462–2469. https://doi.org/10.3168/jds.S0022-0302(03)73840-5.

Xie, Y., H. Sun, M. Xue, and J. Liu. 2022. Metagenomics reveals differences in microbial composition and metabolic functions in the rumen of dairy cows with different residual feed intake. Anim. Microbiome 4:19. https://doi.org/10.1186/s42523-022-00170-3.

Yáñez-Ruiz, D. R., L. Abecia, and C. J. Newbold. 2015. Manipulating rumen microbiome and fermentation through interventions during early life: a review. Front. Microbiol. 6:1133. https://doi.org/10.3389/fmicb.2015.01133.

Zhang, N., Z. Teng, P. Li, T. Fu, H. Lian, L. Wang, and T. Gao. 2021. Oscillating dietary crude protein concentrations increase N retention of calves by affecting urea-N recycling and nitrogen metabolism of rumen bacteria and epithelium. PLoS One 16:e0257417. https://doi.org/10.1371/journal.pone.0257417.

Zhao, Y., G. Tian, D. Chen, P. Zheng, J. Yu, J. He, X. Mao, Z. Huang, Y. Luo, J. Luo, and B. Yu. 2020. Dietary protein levels and amino acid supplementation patterns alter the composition and functions of colonic microbiota in pigs. Anim. Nutr. 6:143–151. https://doi.org/10.1016/j.aninu.2020.02.005.

ORCIDS

Hanne Honerlagen https://orcid.org/0000-0001-6860-0131
Henry Reyer https://orcid.org/0000-0001-6470-0434
Siriluck Ponsuksili https://orcid.org/0000-0003-4714-1057
Nares Trakooljul https://orcid.org/0000-0002-3214-2498
Björn Kuhla https://orcid.org/0000-0002-2032-5502
Klaus Wimmers https://orcid.org/0000-0002-9523-6790