Interrelations between the rumen microbiota and production, behavioral, rumen fermentation, metabolic, and immunological attributes of dairy cows

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ABSTRACT

Different studies have shown a strong correlation between the rumen microbiome and a range of production traits (e.g., feed efficiency, milk yield and components) in dairy cows. Underlying dynamics concerning cause and effect are, however, still widely unknown and warrant further investigation. The aim of the current study was to describe possible functional interrelations and pathways using a large set of variables describing the production, the metabolic and immunological state, as well as the rumen microbiome and fermentation characteristics of dairy cows in early lactation ($n = 36$, 56 ± 3 d in milk). It was further hypothesized that the feed intake-associated behavior may influence the ruminal fermentation pattern, and a set of variables describing these individual animal attributes was included. Principal component analysis as well as Spearman's rank correlations were conducted including a total of 265 variables. The attained plots describe several well-known associations between metabolic, immunological, and production traits. Main drivers of variance within the data set included milk production and efficiency as well as rumen fermentation and microbiome diversity attributes, whereas behavioral, metabolic, and immunological variables did not exhibit any strong interrelations with the other variables. The previously well-documented strong correlation of production traits with distinct prokaryote groups was confirmed. This mainly included a negative correlation of operational taxonomic units ascribed to the *Prevotella* genus with milk and fat yield and feed efficiency. A central role of the animals' feed intake behavior in this context could not be affirmed. Furthermore, different methodological and interpretability aspects concerning the microbiome analysis by 16S rRNA gene sequencing, such as

the discrepancy between taxonomic classification and functional communality, as well as the comparability with other studies, are discussed. We concluded that, to further investigate the driving force that causes the difference between efficient and inefficient animals, studies including more sophisticated methods to describe phenotypical traits of the host (e.g., rumen physiology, metabolic and genetic aspects) as well as the rumen microbiome (e.g., metagenome, metatranscriptome, metaproteome, and metabolome analysis) are needed.

Key words: rumen microbiota, feed efficiency, behavior, PCA

INTRODUCTION

The cow, being a ruminant, lives in a symbiotic relationship with her rumen microbiota. By ingesting feed, she delivers new substrates to the microorganisms, which in return produce valuable nutrients through fermentation and form a nutrient source themselves (Mizrahi, 2013). Early studies showed that the rumen microbial composition is very much determined by the feed composition, as well as feed intake pattern of the host (Bryant and Burkey, 1953; Warner, 1962; Mackie et al., 1978; Leedle et al., 1982). It was further described that the rumen protozoal population especially exhibits a host individuality (Kofoid and MacLennan, 1933; Eadie, 1962). With the advent of DNA fingerprinting and sequencing techniques, this finding was extended to the rumen prokaryotes (Li et al., 2009; Kong et al., 2010; Welkie et al., 2010; Jami and Mizrahi, 2012). The current understanding of rumen microbial dynamics is that the rumen microbiota consists of a core and a variable microbiota (Wu et al., 2012; Creevey et al., 2014; Henderson et al., 2015). The core microbiota is found across a wide geographical range and consists of different taxa that increase or decrease in their abundance according to the diet fed (Henderson et al., 2015). It therefore constitutes a key element in the survival strategy of ruminants by

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allowing fast and appropriate adaptation to new diets (redundancy and resilience; Weimer, 2015; Dieho et al., 2017a; Schären et al., 2017b; Solden et al., 2017). It is thought that the variable or individual microbiota is a result of interanimal variation in behavioral and genetic attributes, as well as environmental influences (Mizrahi, 2013; McCann et al., 2014a; Henderson et al., 2015; Weimer, 2015; Malmuthuge and Guan, 2017). Different studies have shown interrelations between production variables, such as feed efficiency (Guan et al., 2008; Zhou et al., 2009; Hernandez-Sanabria et al., 2010, 2012; Zhou et al., 2010; Carberry et al., 2012; Rius et al., 2012; McCann et al., 2014b; Myer et al., 2015; Jewell et al., 2015; Shabat et al., 2016; Li and Guan, 2017) and milk production and composition (Jami et al., 2014; Lima et al., 2015), and the rumen microbiota. However, the underlying dynamics concerning cause and effect still need to be elucidated (Weimer, 2015; Malmuthuge and Guan, 2017).

Therefore, the aim of the current study was to investigate the associations between the rumen microbiota and a large set of variables describing the production, as well as the metabolic and immunological state of dairy cows in early lactation, plus behavioral attributes, in an attempt to describe possible functional interrelations and pathways.

MATERIALS AND METHODS

Experimental work was conducted at the experimental station of the Institute of Animal Nutrition (Friedrich-Loeffler-Institut) in Brunswick, Germany. The experiment was carried out in accordance with the German Animal Welfare Act (<http://www.gesetze-im-internet.de/tierschg/BJNR012770972.html>) approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

Experimental Design

The data were collected in a trial investigating the influence of monensin and a blend of essential oils on production, rumen fermentation, and metabolic variables (Drong et al., 2016), immunology (Drong et al., 2017b), and the rumen microbiome (Schären et al., 2017a) of transition dairy cows. Sixty German Holstein cows were divided into low- ($n = 15$) and high-condition groups ($n = 45$) at the beginning of the dry period according to their BCS. The animals in the high-condition group were further divided into a control group and 2 treatment groups (either receiving a blend of essential oils or a monensin controlled release bolus, $n = 15$). The animals of the low-condition group were then fed a nor-

mal transition ration [80% roughage (50% maize silage, 50% grass silage) and 20% concentrate based on DM content] during the dry period; after calving they were fed a TMR with an initial concentrate feed proportion of 30%, which was increased stepwise to 50% of the daily ration within 2 wk (details in Drong et al., 2016). The animals in the high-condition group were exposed to a ketogenic ration by an oversupply with energy during the dry period (concentrate feed proportion of 60%) and a subsequently decelerated increase in concentrate feed proportion postpartum (from 30 to 50% in 3 instead of 2 wk; animal model described in Schulz et al., 2014). Production data, blood, and liver and rumen fermentation samples were collected throughout the trial at different points in time and showed that monensin increased the energy availability in the animal by increasing the ruminal propionate production, whereas the blend of essential oils failed to elicit any positive effect (Drong et al., 2016, 2017b). At d 56 postpartum (**p.p.**), oral rumen liquid samples were collected from 48 animals ($n = 12$) to investigate the underlying microbial alterations. The PCR-single strand conformation polymorphism and 16S rRNA gene amplicon sequencing analysis revealed alterations in the rumen microbiota due to the monensin treatment and, corresponding to the results on an animal level, no effect of the blend of essential oils was observed (Schären et al., 2017a). At that stage of lactation (d 56 p.p.), no significant difference between the control and the essential oil groups on production, metabolic, and rumen fermentation level was observed (except higher serum protein concentrations in the high-condition control group), whereas the monensin-treated animals exhibited lower serum BHB as well as higher rumen propionate concentrations (Schären et al., 2017a). Because the present study aimed to investigate the interrelations between phenotypic traits of dairy cows and their rumen microbiota at a normal physiological state, it was decided to exclude the animals of the monensin group in the current analysis. The animals in the 2 control and the essential oils group ($n = 36$) were considered representative for dairy cows at this stage of lactation with an average milk production of 30.7 ± 6.0 kg/d (fat % = 4.3 ± 0.7 , protein % = 2.8 ± 0.2 ; means $\pm$ SD), DMI of 17.7 ± 2.9 kg/d, a BCS of 2.9 ± 0.4 , and all measured metabolic variables in physiological ranges (e.g., serum BHB of 0.86 ± 0.36 mmol/L and fatty acids of 0.48 ± 0.29 mmol/L; Schären et al., 2017a).

Sample Collection and Analysis

All variables presented in the manuscript were assessed in samples collected at d 56 p.p., a detailed

description of sample collection and analysis has been published in Drong et al. (2016, 2017b) and Schären et al. (2017a). The production data (milk yield, milk components, and BW) were summarized for the d 56 $\pm$ 3 p.p. and have previously been published in Schären et al. (2017a).

Production and Behavior Variables. The cows were milked twice per day (0530 and 1530 h) and the BW was recorded thereafter. The TMR was fed ad libitum (offered fresh daily at approximately 1100 h) and individual intakes were continuously recorded using electronic balance troughs (Insentec B.V., Marknesse, the Netherlands). Additionally, a small amount of concentrate (2.18 $\pm$ 0.73 kg of DM/cow per day, depending on the individual daily TMR intake to adjust to a concentrate feed proportion of 50%) was fed using automated feeding stations (Insentec B.V.). The cows were able to access the first half of their daily ration at midnight, the second half at 0800 h. The concentrate was fed in 100-g portions (a new portion was fed whenever the cow remained in the station until the ration limit was reached). The number and sizes of the ingested portions were recorded for TMR [number of meals per day (accessions when no feed was ingested excluded) = TMR Freq; average TMR intake per meal = TMR Size; variation in meal size = SD of TMR Size = TMR SD; average duration of a meal (time eating) = TMR TE; number of times cow accessed a weighing trough without eating (zero counts) = TMR ZC; average time spent at weighing trough when no feed was ingested (time zero) = TMR TZ], concentrate intake [CI; total CI from feeding station per day = CI Tot; number of meals per day at feeding station (accessions when no concentrate was ingested excluded) = CI Freq; average CI per meal = CI Size; variation in meal size = SD of CI Size = CI SD; average duration of meals = CI TE; how often the cow was standing in the feeding station without concentrate allowance/not eating = CI ZC; average time spent in feeding station when no feed was ingested = CI TZ], and water intake [WI; times cow accessed water trough to drink (accessions when no water was ingested excluded) = WI Freq; average WI per drinking = WI Size; variation in amount of water ingested = SD of TMR Size = WI SD; average duration of drinking = WI TD; number of times cow accessed a water trough without drinking = WI ZC; average time spent at water trough when no water was ingested = WI TZ]. The BCS was assessed according to a 5-point scale (Edmonson et al., 1989). Milk samples were collected twice per week (weighed means of Monday evening plus Tuesday morning and Thursday evening plus Friday morning, stored at 4°C until analysis). Milk component analysis included fat, protein, lactose, and urea con-

centrations using an infrared milk analyzer (Milkoscan FT 6000, Foss Electric A/S, Hillerød, Denmark) and weighted daily means were calculated.

From the different production variables, 3 feed efficiency estimators were calculated. For the first variable, the daily DMI was divided by the daily milk yield (**FE1** = inverse of gross feed efficiency = DMI/milk yield). For the second variable, the daily protein intake was divided by the daily milk protein production (**FE2** = protein intake/protein yield). The 2 variables exhibited a mean of 0.56 and 2.74 with a standard deviation of 0.09 and 0.42, respectively. The residual energy intake (**REI**) was also calculated for each animal according to Hurley et al. (2016). Therefore, a piecewise regression construction was applied including different possible energy sinks to estimate the estimated net energy intake ($\text{NEIntake}_{\text{Estimated}}$), resulting in the following equation ($R^2 = 0.614$):

$$\begin{aligned} \text{NEIntake}_{\text{Estimated}} = & -85.24 + (1.45 \times \text{milk, kg}) \\ & + (38.40 \times \text{milk protein, \%}) + (0.16 \times \text{BW}) \\ & + (-12.23 \times \text{BCS}). \end{aligned}$$

Thereafter, the actual net energy intake ($\text{NEIntake}_{\text{Actual}}$) was calculated from energy content of the diet multiplied by the individual DMI. The individual REI was then calculated by subtraction of the 2 latter variables: $\text{REI} = \text{NEIntake}_{\text{Estimated}} - \text{NEIntake}_{\text{Actual}}$. The REI exhibited a mean of -3.06 with a standard deviation of 13.04.

Blood Variables. Blood samples were collected from a *vena jugularis externa* in a serum-separating, an EDTA, and a heparin-containing blood tube. A complete blood count of each sample was performed within 2 h after sampling using an automated hematology analyzer [Celltac α MEK-6450, Nihon Kohden Corporation, Tokyo, Japan; including red blood cell count and associated variables (mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration), hemoglobin, hematocrit (**HCT**), white blood cell population (lymphocyte, monocyte, eosinophil, and granulocyte count and their respective proportions); Schären et al. (2016a)].

The serum was separated and stored at -80°C before chemical analysis using an automatic clinical chemistry analyzer (Eurolyser CCA180, Eurolyser Diagnostica GmbH, Salzburg, Austria; including serum glucose, BHB, fatty acids, urea, albumin, total protein, cholesterol, aspartate transaminase, γ -glutamyltransferase, total bilirubin, glutamate dehydrogenase and triglyceride; Schären et al., 2016a). Kynurenine and Trp concentrations were determined via HPLC (Shimadzu,

Kyoto, Japan; as described in detail in Drong et al., (2017a) and their ratio was calculated.

Peripheral blood mononuclear cells (**PBMC**) were isolated from whole-blood samples (heparinized blood) by gradient centrifugation following Renner et al. (2011) and stored at -80°C until analysis (Drong et al., 2017b). Cell metabolic activity and Concanavalin A (**ConA**)-stimulated proliferation of PBMC were evaluated using the Alamar Blue (**AB**) assay (described in detail in Drong et al. (2017b)). The proliferation of PBMC (stimulation index *ex vivo*) was then calculated by the ratio between fluorescence of ConA-stimulated PBMC (AB_{stim}) and nonstimulated PBMC ($\text{AB}_{\text{unstim}}$): stimulation index = (fluorescence of ConA – fluorescence of AB_{stim})/(fluorescence of $\text{AB}_{\text{unstim}}$) (Drong et al., 2017b). The T-cell phenotyping was performed on whole-blood samples by monoclonal antibodies staining (for CD4 and CD8 or isotype controls) and subsequent flow cytometry analysis (FACSCantoII, BD Bioscience, San Jose, CA). The lymphoid populations were then identified according to their side- and forward-scattering properties and an estimated number of T-cells of each phenotype as well as their ratio ($\text{CD4}^{+}/\text{CD8}^{+}$) were calculated using the percentages obtained by the flow cytometer (Drong et al., 2017b). The capacity of PMNL and leukocytes to release reactive oxygen species was measured using an assay based on the oxidation of the nonfluorescent dihydrorhodamine 123 to the fluorescent metabolite rhodamine 123 by quantifying the mean fluorescence intensity by oxidative species [flow cytometry (FACSCantoII)], resulting in the basal amount of PMNL and lymphocytes that oxidase dihydrorhodamine 123 and the amount of radicals that are produced per cell on average, detected by the mean fluorescence intensity. To elicit and quantify the maximum oxidative burst capacity, the PMNL were additionally stimulated in parallel with phorbol-12-myristate-13-acetate, resulting in the population of PMNL performing an oxidative burst and the mean capacity per cell for the oxidative burst (Drong et al., 2017b).

Rumen Fermentation Variables. Rumen liquid samples (~ 750 mL) were collected in the morning after milking (before feeding) orally using an oral rumen tube and a hand vacuum pump. Immediately after collection, pH was measured using a glass electrode (model pH 525; WTW, Weilheim, Germany). Until further processing (approximately 1–2 h after sample collection), samples for $\text{NH}_3\text{-N}$, VFA [total VFA concentration = VFA, acetate (**C2**), propionate (**C3**), butyrate (**C4**), isovalerate (**iC5**), valerate (**C5**), and their respective proportions (**C2%**, **C3%**, **C4%**, **iC5%**, **C5%**)], and LPS concentration were cooled to 4°C . Volatile fatty acids were determined according to Koch et al. (2006)

using a gas chromatograph (Gaschromatograph 5890 II, Hewlett Packard, Böblingen, Germany), and $\text{NH}_3\text{-N}$ was determined using steam distillation according to the Kjeldahl method (DIN38406-E5-2; Deutsche Institut für Normung e. V., 1998). Rumen LPS concentrations were measured spectrophotometrically after centrifugation at $1,500 \times g$ for 5 min, heating, and storage (-20°C), using the *Limulus* amoebocyte lysate assay (Kinetic-QCL, Lonza, Walkersville, MD; following the manufacturer's instructions; http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Kinetic-QCL_Product_Insert.pdf) as described in Schären et al. (2016b) and Gozho et al. (2005). Due to technical issues at the day of sample collection and storage, 11 rumen liquid samples for fermentation variable analysis were lost. Therefore, the data set includes fermentation variables of $n = 25$ instead of the total 36 animals.

Rumen Microbiome Analysis. Rumen liquid samples for protozoal density assessment were fixed 1:1 with methyl green-formalin solution (stored at 4°C) directly after sample collection, and protozoa were counted using a Fuchs-Rosenthal chamber under an optical microscope and differentiated into *Entodiniomorphida* and *Holotricha* (Ogimoto and Imai, 1981). The rumen liquid samples for microbial analysis were stored at -20°C and DNA extraction was performed as described in Schären et al. (2017a). Prokaryotes were separated from feed and debris through several centrifugation steps [1×5 min at $600 \times g$, 4×20 min at $27,000 \times g$ (4°C) with resuspension between centrifugation steps in 0.9% NaCl] and liquid shock frozen as little droplets. Extraction of DNA involved mechanical lysis by a bead beating method, incubation steps with lysozyme, SDS, proteinase K, and cetyltrimethylammonium bromide, a protein purification step with (phenol)-chloroform-isoamyl alcohol, and washing steps using the peqGold Tissue-Kit (peq lab, Erlangen, Germany). Samples were stored at 4°C until further analysis. For sequencing, genomic DNA samples were sent to Microsynth AG (Balgach, Switzerland). For 16S sequencing library preparation, the primers A519F (S-D-Arch-0519-a-S-15) and 802R (S-D-Bact-0785-b-A-18) were chosen (Klindworth et al., 2013) and amplified using the HiFi HotStart PCR Kit (Kapa Biosystems, Wilmington, MA). Illumina Nextera Libraries were prepared according to the manufacturer's instruction (Illumina, San Diego, CA; https://support.illumina.com/downloads/nextera_xt_sample_preparation_guide_15031942.html) and sequencing was performed on the Illumina MiSeq Sequencing System using the Illumina MiSeq reagent Kit v2 (2×250 bp). Demultiplexing (using the Illumina MiSeq v2.5.1.3. reporter and cutadapt v1.8.1 software package; Martin, 2011), read stitching

(using FLASH v1.2.11; Magoč and Salzberg, 2011), de novo chimera detection, identification and removal [using the Uchime v4.2 (Edgar et al., 2011) and Usearch v8.1.1861 (Edgar, 2010)], operational taxonomic units (OTU) clustering (based on 97% sequence similarity, using Uclust; Edgar, 2010), and taxonomic assignment (using QIIME v1.9.1 (Caporaso et al., 2010) and the SILVA rRNA database v111 (Quast et al., 2013)) were performed. Singletons were removed from the data set to reduce bias introduced by sequencing errors. For downstream analysis, only OTU with a relative abundance of at least 0.1% were considered to guarantee a solid differentiation between artifact and true organism. The amplicon sequencing resulted in $12,206 \pm 3,424$ reads (after filtering; mean $\pm$ SD) per sample. In total, several 177 different species-level OTU were identified (167 ± 6 different OTU per sample). A list of the detected OTU, their corresponding taxonomic classification, and proportional abundance is given in Table 1. Most OTU could be taxonomically classified to the family level, whereas their genus or species level affiliation were “uncultured bacterium or archaeon” in many cases.

Statistical Analysis

All statistical analyses were performed in Statistica 12.0 (StatSoft Inc. 2014, Tulsa, OK). If variables were recorded more than once a week, means were calculated per cow and week ($d 56 \pm 3$ p.p.) before statistical evaluation. To obtain a normal distribution, rumen LPS concentrations were logarithmically transformed before statistical analysis. Correlation coefficients between different variables were estimated using Spearman's rank correlation and results were considered significant at $P < 0.05$. Principal component analysis (PCA) was performed including all measured variables. Thereafter, for some variables (which clustered due to their close biological interrelation) only 1 representative variable was chosen to obtain a clearly arranged graph. This was the case for (representative variable chosen, followed by deleted variables in brackets) DMI (Starch intake, concentrate intake, protein intake, NEIntake_{Actual}), HCT (hemoglobin, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration), and lymphocyte, monocyte, and granulocyte (and their respective proportions).

RESULTS AND DISCUSSION

All data points were summarized in one master table and a Spearman's rank correlation with all 265 variables was conducted, resulting in a table with 70,225 corre-

lations (all data shown in Supplemental Files S1–S10, master table in Supplemental File S10; <https://doi.org/10.3168/jds.2017-13736>). Because the interpretation and the deduction of biological dynamics from such a table is difficult, one main PCA plot (master plot) was created to gain a better overview of the data and the different interrelations (Figure 1 and Supplemental File S9). The master plot is interpreted with each dot representing 1 variable (grouped by color). The closer 2 variables are, the higher they are positively associated. The farther away 2 variables are, the more negatively they are associated. The more variables are located toward the middle of the plot, the less they contribute to the variation within the data and the smaller is their association with other variables (in this particular PCA plot). The master plot was then divided into subjective clusters based on the spatial similarity and biological interpretations of the variables captured within them (Figure 1, cluster 1–7). Their significance and interpretation are discussed herein. The master plot was further used as a base to plot the significant correlations detected by the Spearman's rank correlations. For every single variable, a copy of the master plot was created in which only the labels of those variables that correlate significantly with the respective main variable are shown (positive correlations in green, negative correlations in orange; see Figures 2–7; Supplemental Files S1–S8, <https://doi.org/10.3168/jds.2017-13736>). To systematically guide the reader through the data within the master plot, the production variables and the different associated (well-known) biological interrelations are used as a starting point. From there, associated data and thematic clusters (cluster 1–7) will be discussed in an integrative manner in the following sections.

Production Variables and Feed Efficiency

In the lower left-hand corner of Figure 1, a cluster containing several variables related to milk production quantity (milk, protein and fat yield, and FCM) are observed (cluster 1). These variables are also closely associated with the metabolic variables cholesterol and albumin, most likely due to their role in milk protein synthesis (example of plot for fat yield in Figure 2; Busato et al., 2002; Kida, 2003). The protein percentage and protein yield are further associated with the blood Trp concentrations, which can be ascribed to an increased protein metabolism in animals with higher feed intake and milk production (Supplemental File S2; <https://doi.org/10.3168/jds.2017-13736>), rather than an altered immune status (alterations in the kynurenine: Trp ratio may indicate an influence on immune reactivity; Laich et al., 2002). Furthermore, as expected, milk

Table 1. Summary of detected operational taxonomic units (OUT) and their proportional abundance

Label ¹	OTU ID ²	Taxonomy ³					% ⁴	% ⁵
		Kingdom	Phylum	Class	Order	Family		
A	EF112194	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	0.857	
B1	EU779121	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	0.439	3.70
B2	AB559503	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	2.716	
B3	AM277978	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	0.139	
B4	New.Ref.OTU	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	0.144	
B5	HQ842703	Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	0.266	
C1	New.Ref.OTU	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.239	1.54
C2	AB270068	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.196	
C3	EU469015	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.613	
C4	AB270014	Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	0.494	
D1	AB185544	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BS11 gut group	0.193	1.30
D2	EU773647	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BS11 gut group	0.475	
D3	EF686531	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BS11 gut group	0.263	
D4	AY244965	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	BS11 gut group	0.369	
E1	EF445210	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.169	30.11
E2	AB009187	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.363	
E3	EU845138	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.180	
E4	EU259377	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	2.339	
E5	EF445293	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	1.282	
E6	AY850504	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.249	
E7	AB009192	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	4.933	
E8	EU381847	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.326	
E9	AB269981	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	3.810	
E10	EU472961	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.215	
E11	GQ326951	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.332	
E12	GQ326973	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.294	
E13	GQ327024	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.273	
E14	EF436359	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.953	
E15	AB185608	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.960	
E16	AY244948	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.183	
E17	AY244946	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	1.312	
E18	AF018469	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.205	
E19	GQ327306	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.484	
E20	AF018482	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.204	
E21	AB270138	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	2.485	
E22	AY244916	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.368	
E23	GQ327214	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.212	
E24	EU179226	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.962	
E25	EU381791	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.156	
E26	EU381948	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.177	
E27	AB034102	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	1.330	
E28	AB269968	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	3.065	
E29	GU302536	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.681	
E30	New.Ref.OTU	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.270	
E31	New.Ref.OTU	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.293	
E32	New.Ref.OTU	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.286	
E33	New.Ref.OTU	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.156	
E34	EU381920	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.176	
E35	EU461494	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	0.427	
F	AY244938	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	RF16	0.618	

Continued

Table 1 (Continued). Summary of detected operational taxonomic units (OUT) and their proportional abundance

Label ¹	OTU ID ²	Taxonomy ³					% ⁴	% ⁵
		Kingdom	Phylum	Class	Order	Family		
G1	AB494890	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.299
G2	DQ394621	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.845
G3	FJ028738	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.172
G4	EU42535	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.855
G5	GU304085	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.369
G6	HQ008599	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.197
G7	AY244944	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.239
G8	AB494915	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.886
G9	EU719222	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.174
G10	GU302529	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.564
G11	EU719287	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.197
G12	GU302534	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.236
G13	New.Ref.OTU	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	RC9 gut group	0.336
H	EU470196	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	u.b.	0.886
J1	GQ327541	Bacteria	Candidate division	u.b.	u.b.	u.b.	u.b.	0.193
J2	EU474584	Bacteria	Candidate division	u.b.	u.b.	u.b.	u.b.	0.266
J3	EU381496	Bacteria	Candidate division	u.b.	u.b.	u.b.	u.b.	0.224
K	GU303955	Bacteria	Cyanobacteria	SHA-109	u.b.	u.b.	u.b.	0.184
L1	EF436353	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.657
L2	AB270057	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.924
L3	AB185717	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.277
L4	EU468616	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.201
L5	AY854343	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.280
L6	AB185553	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	3.642
L7	AB494899	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.643
L8	AB270004	Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	u.b.	0.785
M1	EU842291	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	Mogibacterium	0.706
M2	AY854273	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	Mogibacterium	0.465
M3	FJ682205	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	Mogibacterium	0.438
M4	GU303061	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	Mogibacterium	0.204
M5	JF797395	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	u.b.	0.235
M6	GU120128	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	u.b.	0.200
M7	AB185628	Bacteria	Firmicutes	Clostridia	Clostridiales	Family XIII Incertae Sedis	u.b.	0.195
N1	AB494806	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.807
N2	New.Ref.OTU	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.235
N3	EU773612	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.227
N4	EU381579	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.205
N5	AB494866	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.208
N6	EU466207	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.159
N7	AY854272	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.287

Continued

Table 1 (Continued). Summary of detected operational taxonomic units (OUT) and their proportional abundance

Label ¹	OTU ID ²	Taxonomy ³					% ⁴	% ⁵
		Kingdom	Phylum	Class	Order	Family	Genus	
N8	AF001717	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.254
N9	AB494778	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	u.b.	0.082
O1	AB494822	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Acetitomaculum	0.412
O2	AB185642	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Acetitomaculum	1.18
P1	AB494935	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.224
P2	AM039826	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	5.32
P3	EU469259	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.234
P4	AB494792	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.267
P5	AB494765	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.216
P6	AB494805	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.201
P7	AB034052	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	1.630
P8	GQ327740	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	1.558
P9	GU303299	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.255
P10	FJ172809	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.157
P11	AB494833	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.209
P12	EU483345	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Butyrivibrio	0.176
Q	AY854354	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Howardella	0.197
R1	GU124460	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Howardella	0.296
R2	AF001722	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.340
R3	AB185771	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.444
R4	EU845624	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.192
R5	EU381578	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.223
R6	New.Ref.OTU	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.194
R7	DQ237938	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.259
R8	EF436345	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	1.027
R9	EF436445	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.317
S	EU843661	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Incertae Sedis	0.282
T1	DQ085079	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Oribacterium	0.176
T2	AB494919	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Pseudobutyrvibrio	0.221
U1	FJ032427	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Pseudobutyrvibrio	0.957
U2	AB034119	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	0.921
U3	EU842536	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	0.254
U4	AF371623	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	0.350
V	EF445237	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia	1.158
W	AB270001	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Syntrophococcus	0.299
X1	EF686593	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Incertae Sedis	0.316
X2	EF436321	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.658
X3	EU469842	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.951
X4	DQ673486	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.169
X5	EU468942	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.305
X6	EU381458	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.176
X7	EU474863	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.279
X8	AAQK01009861	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	0.174
Y1	EF686527	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus	2.132
Y2	GQ327231	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Saccharofermentans	0.313
Y3	AB494824	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Saccharofermentans	0.340
Y4	EU381703	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Saccharofermentans	0.780
Y5	AB034038	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Saccharofermentans	0.258
Z1	AF371808	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Saccharofermentans	0.214
Z2	EU468242	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.153
							u.b.	0.237

Continued

Table 1 (Continued). Summary of detected operational taxonomic units (OUT) and their proportional abundance

Label ¹	OTU ID ²	Taxonomy ³					% ⁴	% ⁵
		Kingdom	Phylum	Class	Order	Family	Genus	
Z3	EU344218	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.297
Z4	AB494879	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	1.830
Z5	EU381706	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.164
Z6	AB270093	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.227
Z7	EU381964	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.172
Z8	EU381950	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.503
Z9	FJ508236	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.266
Z10	AB185556	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	1.909
Z11	AY854363	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.336
Z12	AB185810	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.169
Z13	AJ863538	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.125
Z14	AB009189	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.257
Z15	EU842742	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	u.b.	0.214
AA	AB009216	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Anaerovibrio	0.233
AB	AB034139	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Selenomonas	0.678
AC	AY244976	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Succinilacticum	2.629
AD	AB210825	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Catenibacterium	0.569
AE1	AB009179	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	u.b.	0.236
AE2	EU475554	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	u.b.	0.555
AE3	New.Ref.OTU	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	u.b.	0.209
AE4	EU458717	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	u.b.	1.186
AE5	EU381583	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	u.b.	0.418
AF	AB682235	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acetobacter	0.170
AG1	AB239483	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Ruminobacter	0.269
AG2	EF445274	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	u.b.	0.592
AG3	GQ327554	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	u.b.	0.624
AG4	EU381934	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	u.b.	2.322
AH1	AB270123	Bacteria	Spirochaetes	Spirochaetales	Spirochaetales	Treponema	u.b.	0.440
AH2	AF001693	Bacteria	Spirochaetes	Spirochaetales	Spirochaetales	Treponema	u.b.	1.035
AH3	HM049812	Bacteria	Spirochaetes	Spirochaetales	Spirochaetales	Treponema	Treponema sp.	0.231
AH4	GQ402096	Bacteria	Spirochaetes	Spirochaetales	Spirochaetales	Treponema	Treponema sp.	0.216
AI1	EU871422	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	0.286
AI2	New.Ref.OTU	Bacteria	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	Anaeroplasma	0.196
AJ	EF445251	Bacteria	Tenericutes	Mollicutes	RF9	u.b.	u.b.	0.219
AK	New.Ref.OTU	Unassigned						0.174

¹Label used to group and label OTU in principal component analysis plots.²New.Ref.OTU = new reference OTU.³u.b. = unculturable bacterium; all OTU were classified as “unculturable bacterium or archaeon” at species level, and therefore only the taxonomic classification up to the genus level is shown.⁴Proportional abundance in % of OTU.⁵Proportional abundance in % of OTU group (grouping is indicated by common letter in label).

(cluster 2) with the efficiency variables FE1 and FE2 in its center. The allocation of clusters 1 and 2 in one axis, but in an opposite manner, can most likely be attributed to the fact that animals with high milk production are generally more efficient (lower FE1 and FE2), as

Figure 2. Principal component (PC) analysis plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with fat yield (dark red label; dark gray). Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. Color version available online.

they need proportionally less energy for maintenance (Vandehaar, 1998). Both of these efficiency variables correlate significantly with the third one, the REI, which by itself correlates with only a few other variables and prokaryotes, and, due to its location within the plot (within cluster 7, closer to the center), only accounts for a small part of the variation in the data set. Aside from the efficiency variables FE1 and FE2, cluster 2 is mainly composed of OTU belonging to the genus *Prevotella* (Label: E) and the variable F/B (the *Firmicutes/Bacteroidetes* ratio), due to the dominant abundance of *Prevotella* within the *Bacteroidetes* phylum. Also in the Spearman's rank correlations, the 2 efficiency variables FE1 and FE2 show a strong association with several *Prevotella* species (example for FE1

in Figure 3). These observations are very much in line with the data presented by Jami et al. (2014), showing a strong positive correlation between the F/B and fat yield, and Myer et al. (2015), who observed a significant positive correlation between the abundance of *Firmicutes* and the feed efficiency of steers. Jewell et al. (2015), Lima et al. (2015), Carberry et al. (2012), and McCann et al. (2014b) showed a significant negative correlation between the abundance of *Prevotellaceae* or *Prevotella* and feed efficiency or milk production. Additionally, Jewell et al. (2015) illustrated that the association of the individual OTU (belonging to the *Prevotella* genus) may be positive or negative, illustrating possible differences in functional properties between members of a common genus (discussed in the section

Behavior Variables and Lactation Effect

As Myer et al. (2015) and Malmuthuge and Guan (2017) stated, it is not clear whether the observed patterns in the previously described microbial groups are actively contributing to differences in feed efficiency and production, or if other host factors are the driving cause for the observed alterations in the microbiome. Therefore, we hypothesized that the animal feed intake behavior (e.g., meal size, duration, and frequency) could play a central role in this context by influencing the ruminal fermentation pattern and included several behavior variables in the PCA analysis. All of these variables were, however, located in cluster 7 and did not exhibit any clear association with the feed efficiency

Figure 3. Principal component (PC) analysis plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with the feed efficiency variable FE1 = DMI-to-milk yield ratio (dark red label; dark gray). Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. Color version available online.

traits or microbial groups. Even variables that may predict dominance or social rank, such as the time spent at the concentrate feeding station or water bunk without any feed or water intake (Wierenga, 1990; Grant and Albright, 1995; Olofsson, 1999), only correlate with a few other variables (Supplemental File S4; <https://doi.org/10.3168/jds.2017-13736>). However, the recorded behavioral attributes only included aspects of feed and water intake. Because the rumination and resting time may also have a large influence on the rumen environment (Gao and Oba, 2014), these aspects should be included in future studies.

The number of meals per day decreased and the time spent eating and size of the meals increased with the lactation number, which indicates that older cows seemed to spend less time at the feed bunk while consuming larger quantities in 1 meal and highlights their social dominance (Grant and Albright, 1995). At this point it should be noted that our study did not include any first-lactation animals (heifers), and the observed effects can be attributed to age-dependent alterations observed from the second lactation onwards. The lactation number also correlated negatively with several immunological variables, such as haptoglobin, stimulation index, and rhodamine 123 mean capacity per cell for the oxidative burst, and positively with serum protein concentrations and HCT (Figure 4). We suggest this can be attributed to age-dependent alterations in the immune system, such as a lower reactivity and higher immunoglobulin content with increasing age (Stoop et al., 1969; Weiskopf et al., 2009; Tienken et al., 2015; Bühler et al., 2018). A small cluster of bacteria species (at the line between the squares B1 and B2 in Figure 4) are also positively associated with the lactation number. These bacteria are part of a larger cluster (formed by cluster 4 and 6 illustrated in Figure 1), which represents an increase in species diversity, as they include the largest part of prokaryotes and the 3 diversity indices (number of species and the Shannon and Chao1 index, detailed in the next section). This is in line with earlier studies describing an increase in microbiome diversity with age (Jami et al., 2013; Jewell et al., 2015; Lima et al., 2015).

In sum, our data set does not provide any conclusive pattern explaining the clearly emerging correlation between certain microbial species and production traits. We suggest that future studies involve data collection concerning rumen fermentation variables (e.g., continuous fermentation variable recording using rumen sensor technique; Duffield et al., 2004; Zebeli et al., 2008) and concomitant bacterial abundance and metatranscriptome, metaproteome, and metabolome analysis (Jiang et al., 2016; AlZahal et al., 2017; Li and Guan, 2017; Wallace et al., 2017) to gain a more

precise understanding of diurnal fermentation patterns and influencing factors. Aspects of the rumen wall physiology (absorption rate and capacity) might also have a substantial influence on the rumen environment (Gäbel et al., 2002; Aschenbach et al., 2011), and these interrelations should be more deeply investigated using in vivo marker-based methods for VFA absorption dynamics (Dijkstra et al., 1993; Resende Júnior et al., 2006) and rumen epithelium gene expression analysis (Penner et al., 2009; Penner et al., 2011; Dieho et al., 2017b). Different studies have also shown a substantial animal genetic component underlying feed efficiency differences (Archer et al., 1999; Arthur et al., 2001; Spurlock et al., 2012), and possible underlying mechanisms, such as differences in nutrient partitioning and metabolic rate (Bauman et al., 1985; Rauw et al., 1998; Drackley, 1999; Nkrumah et al., 2006), require further investigation.

Microbial Diversity and Rumen Fermentation

Concerning species diversity, the data shows a clear association between increased fermentation rate and a decrease in species diversity, illustrated by the clear opposite allocation of the fermentation variables in clusters 3 and 4 (Figure 1). Cluster 3 contains the variables representing the total production of VFA, such as VFA, C2, C3, C4, C5, and iC5 as well as the proportions of C3 and C5, surrounded by only few prokaryotes. These variables are known to increase under an increased fermentation rate, whereas the variables in cluster 4 will concomitantly decrease (pH, C2/C3, C2%; Bergman, 1990; Van Houtert, 1993). As described, cluster 4, together with clusters 5 and 6, contain the largest part of the prokaryotes of the data set, as well as the different species diversity indices. This is in line with various other studies showing a decrease in species diversity whenever the fermentation rate is increased; for example, under the influence of an increased concentrate feeding (Bekele et al., 2010; Kong et al., 2010; McCann et al., 2014a; Dieho et al. 2017a,b; Tapio et al., 2017). In our data set, this is most pronouncedly visible in the plot made for the Shannon index (Figure 5), where a clear negative correlation with the total VFA and C3 concentration stands opposite to a positive correlation with a range of prokaryotes in clusters 4, 5, and 6.

These interrelations are further confirmed by the allocation of the only archaea present in this data set (*Methanobrevibacter*, abbreviated with the letter A) in cluster 5 (in the rectangle A3 at the edge of the circle). Methanobacteria rely on the hydrogen produced by other bacteria from a variety of carbon sources to produce methane by reduction of CO₂ (Valdez-Vazquez and Poggi-Varaldo, 2009; Patra, 2012). Whereas the

Figure 4. Principal component (PC) analysis plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with lactation number (lactation, dark red label, dark gray). Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. Color version available online.

production of acetate is connected to the formation of reducing equivalents, propionate is considered a net sink (Czerkawski, 1986; Russell, 2002). Therefore, under the circumstances of a slow fermentation rate (e.g., due to a low DMI or high-fiber rations) expressed

by a higher pH and increased species diversity, an increase in methane production can also be observed as a consequence of higher acetate proportion (Popova et al., 2011; Patra, 2012; Shabat et al., 2016; Tapio et al., 2017). Interestingly, *Methanobrevibacter* are not

allocated in the center of cluster 4 but at the edge of cluster 5, and are not clearly associated with the other fermentation variables except for pH (Figure 6). One possible explanation could be that the spot sampling of the rumen fluid is only partially representative for the rumen fermentation pattern throughout the day (Hall et al., 2015; Schären et al., 2016b) or that other variables seem to influence the *Methanobrevibacter* occurrence, which have not been covered with our data set. This is supported by the relatively solitary position of the *Methanobrevibacter* in our plot. Different studies have shown that the abundance of methanogens is not necessarily altered due to alterations in the substrate availability for methanogenesis, as well as the fact methanogen abundance is not necessarily linked

to methane production (Hook et al., 2009; Popova et al., 2011; Singh et al., 2013; Schären et al., 2017a) and other complex biochemical interrelations or genetically driven host attributes may be responsible for changes in their abundance (Zhou et al., 2009, 2010; Roehe et al., 2016; Malmuthuge and Guan, 2017). In contrast, others showed a shift in methanogen diversity, but not necessarily total abundance, upon increased fermentation rate (e.g., due to increased concentrate feeding; Liu et al., 2012; Tapio et al., 2017). Different studies have also shown an association between the methanogen abundance and diversity, gene expression, and feed efficiency (Zhou et al., 2009, 2010; Carberry et al., 2014; Roehe et al., 2016; Li and Guan, 2017). These aspects could not be thoroughly investigated in our study due

Figure 5. Principal component (PC) analysis plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with the rumen microbiome diversity variable Shannon index (Shannon, dark red label, dark gray). Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. Color version available online.

to the abundance threshold of 0.1% in the filtering step during bioinformatics (most archaea or methanogens exhibit a lower abundance and were therefore excluded from our analysis). Considering the importance of understanding the dynamics behind methanogenesis and mitigation strategies, future studies should investigate possible aspects concerning interanimal variation as well as the genetic variation in the methanogenic community more closely (Zhou et al., 2009; Liu et al., 2012; Leahy et al., 2013; McAllister et al., 2015).

Furthermore, for the different VFA concentrations and proportions, no strong correlation pattern can be observed in relation to certain prokaryote groups (Supplemental File S7; <https://doi.org/10.3168/jds.2017-13736>). Molar acetate proportions correlate

positively with serum triglyceride concentrations, most likely due to their involvement in fatty acid metabolism (Bergman, 1990). Molar acetate proportions are also positively associated with serum BHB concentrations, which may point toward an interrelation between slow rumen fermentation rate and an energy deficit (Bergman, 1990). Mainly OTU belonging to the taxon *Firmicutes* (*Butyrivibrio*, *Mogibacterium*, *Ruminococcaceae*, Family XIII *Incertae Sedis*, and *Saccharofermentans*) exhibit a positive correlation with C2 proportion. Molar butyrate proportions exhibit a positive association with several OTU ascribed to *Prevotella*, *Bifidobacterium*, *Ruminococcus*, uncultured *Ruminococcaceae*, *Anaerovibrio*, and *Selenomonas* (Supplemental File S7). Interestingly, none of the members of the taxon *Butyri-*

Figure 6. Principal component (PC) analysis plot from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with the abundance of the archaeal taxon *Methanobrevibacter* (A, label description in Table 1, dark red label, dark gray). Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. Color version available online.

vibrio were positively associated with the propionate proportion (C3%), in contrast to older studies appointing this genus a major role in the ruminal propionate production (Paillard et al., 2007). All these findings are only partially in line with other studies (Wang et al., 2012; Sandri et al., 2014; Dieho et al., 2017a), and the reason for observing only these few interrelations between rumen VFA concentrations and proportions could be that, in our data set, the basal ration and the concentrate proportion was similar across all animals; therefore, (strong) variation among rumen fermentation pattern and rates did most likely not occur. This aspect of variance contribution is discussed in detail in the following section.

Minor Variance Contributing Variables: Metabolism and Immunology

The master plot (Figure 1) illustrates that the main drivers for variation in our data set were milk production (cluster 1), feed efficiency (cluster 2), rumen fermentation (clusters 3 and 4), microbiome diversity (clusters 4, 5, and 6), and their associated prokaryotes. A large part of variables, however, was allocated rather near the center of the plot (cluster 7), indicating that they contribute only little to the variation within the data set. In cluster 7 we find, aside from the mentioned behavioral variables, the largest part of the serum metabolic and immunological variables as well as the rumen LPS concentrations. Therefore, our data set ascribes them little influence or interrelation with the variability of the rumen microbiome and also the other variance driving variables. This is contrary to several other studies showing changes in these variables in connection with alterations in the rumen fermentation and microbiome, such as under the influence of SARA (Gozho et al., 2005; Khafipour et al., 2009; Zebeli et al., 2011). However, it has to be noted that the 36 animals included in our data set were all healthy and in a steady metabolic condition [no (sub)clinical ketosis, and so on], and therefore demonstrated only marginal variation in the health and metabolic variables. Also, the diet fed was not to be expected to elicit any SARA (Zebeli et al., 2008). Thus, we suggest that these well-documented interrelations would only emerge if the animals were metabolically or immunologically challenged.

In this context, it also needs to be discussed whether the role and interrelations we are ascribing in this particular data set are ubiquitous or whether they possibly represent a certain, relatively undisturbed, state. Morgavi et al. (2013) stated that many basic ecological questions concerning the rumen microbiome have not been answered. For example, it is not clear whether numerically dominant rumen microbes also constitute the

key members or if key functions might be carried out by rare members (the so-called “rare biosphere,” with an abundance <0.1%). It might be the case that they only increase their abundance in certain situations (e.g., under the influence of a ration change or toxin degradation) or even carry out key functions despite their low abundance (Morgavi et al., 2013; Shabat et al., 2016). To investigate these subtler interrelations, larger studies across different diets and metabolic states, as well as repeated measurements and the use of different methods that include members of the rare biosphere (e.g., metagenomics analysis or different filtering threshold within an amplicon sequencing method), are required to attain the variance and depth needed (Fouts et al., 2012; Malmuthuge and Guan, 2017).

Taxonomic Classification, Functional Community, and Redundancy

When analyzing the distribution of the OTU of a common taxon on the master plot (Figure 1) it can be observed that some are very closely associated, whereas others are not. This is even more clearly visible when looking at the plots illustrating the Spearman's rank correlations of the different OTU with all the other variables included in the data set (Supplemental File S8B–L; <https://doi.org/10.3168/jds.2017-13736>). As an example, 4 different OTU from the *Prevotella* genus are shown in Figure 7 (abbreviated with the letter E, all OTU of the *Prevotella* genus in Supplemental File S8C; <https://doi.org/10.3168/jds.2017-13736>). The different correlation distribution patterns on the master plot illustrate that the different OTU, even though belonging to a common taxon, have different functional properties. Exemplary for this is the association of only some OTU ascribed to the *Prevotella* species with fat percentage (Supplemental File S2; <https://doi.org/10.3168/jds.2017-13736>), which is in line with the study of Jewell et al. (2015). Bekele et al. (2010) described diet-specific *Prevotella* species, each occupying a distinct metabolic niche, strongly supporting our data. Furthermore, various studies have also shown that the genus *Prevotella* exhibits a broad range of fermentation products (Marounek and Duskova, 1999; Krieg et al., 2009; Emerson and Weimer 2017). Other examples in our data set of taxa containing OTU exhibiting distinct correlation distribution patterns are *Bifidobacterium*, BS11 gut group, RC9 gut group, candidate division TM7, *Christensenellaceae*, Family XIII *Incertae Sedis*, several taxa belonging to the *Lachnospiraceae* (e.g., *Butyrivibrio*), *Ruminococcus*, *Saccharofermentans*, *Erysipelotrichaceae*, *Succinivibrionaceae*, and *Treponema* (Supplemental File S8B–M; <https://doi.org/10.3168/jds.2017-13736>). These findings are in line with studies

Figure 7. Four different principal component (PC) analysis plots from Figure 1, only showing the variables that significantly correlate ($P < 0.05$, Spearman's rank correlation) with a specific OTU ascribed to the *Prevotella* genus [E9 (upper left), E11 (upper right), E30 (lower left), E3 (lower right), label description in Table 1, dark red labels, dark gray]. Positive correlations in green (light gray), negative correlations in orange (light gray with black border). Abbreviations can be found in Materials and Methods. Grouping and labeling of prokaryotes is as in Table 1. More detail available in Supplemental Files (<https://doi.org/10.3168/jds.2017-13736>). Color version available online.

showing that microbes that are phylogenetically close may still exhibit substantially different phenotypes and functions (Schären et al., 2017b), and important varia-

tion in microbial communities lies at a finer resolution than the genus or higher taxonomic levels (Myer et al., 2015). As discussed in Schären et al. (2017b), this

discrepancy between phylogenetic (or genomic) commonality and phenotype can most likely be attributed to differences in gene expression as a result of environmental influences. Different authors have suggested the revision of the polyphasic approach (integration of genotype and phenotype) in prokaryotic taxonomy and repeatedly stated that more effort should go into unravelling functional aspects of the rumen microbiome (Achenbach and Coates, 2000; Kämpfer and Glaeser, 2012; Morgavi et al., 2013; Solden et al., 2016).

An additional aspect in this context is the functional redundancy of the different members of the rumen microbiome (Weimer 2015). Taxis et al. (2015) showed, by integrating metabolic and metagenomic analyses of a rumen microbiota, that different community metabolic networks may exhibit the same metabolic inputs and outputs but differ in their internal structure. The ability of the different community members to carry out similar metabolic pathways and substitute each other in the different metabolic niches represents a key feature for the plasticity of the rumen microbiome; however, this also represents a major challenge in unravelling the different functional properties of the single members due to large confounding effects. Again, this aspect illustrates that new biotechnical as well as computational methods are needed to capture and integrate the different interrelations, dynamics, and pathways.

Methodological Aspects and Remarks

Concerning the methodological aspects, different studies have shown and discussed how, depending on the sampling technique and time (Li et al., 2009; Kong et al., 2010; Welkie et al., 2010), the DNA extraction method and primer choice (Henderson et al., 2013; Klindworth et al., 2013; Albertsen et al., 2015), the sequencing platform (Goodwin et al., 2016), and the bioinformatical settings used (Majaneva et al., 2015; Malmuthuge and Guan, 2017), the results may vary considerably (Schären et al., 2017a,b). The results presented in ours, as well as in other manuscripts, should therefore be interpreted within this context. The amplicon sequencing analysis applied in our study only covered the largest part of the prokaryotes (due to the primer chosen), but it did not include a thorough differentiation of the protozoa (which were only evaluated using an optical counting method) nor an evaluation of the fungi or virome. The rumen microbiome also is subjected to spatial differences. Several studies have shown that the microbiome associated with feed particles and the rumen epithelium is distinct from the liquid-associated microbiome (which is most frequently sampled for rumen microbiome analysis and was also the focus of the present study), each comprising

scarcely investigated characteristics and functions (Cho et al., 2006; Kim and Yu, 2012; Henderson et al., 2013; Malmuthuge and Guan, 2017; Schären et al., 2017b). Finally, it should be considered that the rumen is only the first part of the gastrointestinal system, and that the microbial community in the lower gastrointestinal tract most certainly plays an important role in nutrient utilization (Myer et al., 2015). Little is known about the relevance of these different aspects in the host-microbiome interaction, and future affordable and powerful sequencing techniques should be used to attain more insight in these interrelations (McAllister et al., 2015; Jiang et al., 2016; Raszek et al., 2016; Malmuthuge and Guan, 2017; Wallace et al., 2017).

Weimer (2015) described a vast amount of studies published during the last decade analyzing interrelations between the gut microbiota and different clinical conditions (especially in humans) and eloquently stated that we “are only occasionally reminded that the ‘conclusions’ of these studies have almost always been based on association, rather than rigorous demonstration of cause and effect.” This also needs to be emphasized when analyzing and discussing the data set of the present study. The principal component together with the correlation analysis have shown that almost all variables in the data set are somehow interrelated; however, the driving force behind certain dynamics is often not clear (e.g., feed efficiency and microbiome; Malmuthuge and Guan, 2017). As highlighted in the different sections of the discussion, larger data sets including more variation (e.g., age, stage of lactation, diet) as well as greater data depth using sophisticated methodologies are needed to further unravel these interrelations (McAllister et al., 2015; Solden et al., 2016; Malmuthuge and Guan, 2017; Wallace et al., 2017).

CONCLUSIONS

Our data set describes several well-known associations between metabolic and production traits in healthy dairy cows, and can therefore be considered a valid basis to investigate interrelations concerning the host-microbiome interaction. We confirmed previously described strong correlations of certain production traits (e.g., feed efficiency and milk production variables) with the rumen microbiome, as well as the association of fermentation rate with microbial diversity. Our hypothesis that the feed intake pattern plays a key role in this context by influencing the ruminal fermentation pattern was not confirmed, and suggests that another undescribed driving force causes the distinct differences in the rumen microbiome between efficient and inefficient animals. Future studies should include more sophisticated methods to describe phenotypical

traits of the host (e.g., rumen physiology and metabolic and genetic aspects) as well as the rumen microbiome (e.g., metagenome, metatranscriptome, metaproteome, and metabolome analysis).

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