Institute of Agricultural Sciences
University of Hohenheim
Department of Functional Microbiology of Livestock (460c)
Prof. Dr. Jana Seifert

The intestinal microbiome and metabolome of dairy cows under challenging conditions

Dissertation
submitted in fulfillment of the regulations to acquire the degree
"Doktor der Agrarwissenschaften" (Dr.sc.agr. in Agricultural Sciences)
to the

Faculty of Agricultural Sciences

presented by

Johanna Barbara Tröscher-Mußotter
Weinheim a.d. Bergstraße

2022

This thesis was accepted as a doctoral dissertation in fulfillment of the requirements for the degree "Doctor of Agricultural Sciences" (Dr. sc. agr.) at the Faculty of Agricultural Sciences at the University of Hohenheim.

Dean Faculty of Agricultural Science:	Prof. Dr. Ralf Vögele
Date of the oral examination:	August 30th, 2022
Chair of the committee:	Prof. Dr. Martin Hasselmann
Supervisor:	Prof. Dr. Jana Seifert
Examiner 1:	Jun.-Prof. Dr. Amélia Camarinha Silva
Examiner 2:	Prof. Dr. Helga Sauerwein

Table of contents

List of abbreviations

%	Percentage
°C	Degrees Celsius
2Pprop	2-Phenylpropionate
3Hbut	3-Hydroxybutyrate
4Hbut	4-Hydroxybutyrate
Aca	Acetate
Aco	Acetone
Adi	Adipate
Alla	Allantoin
ANOSIM	One-way analysis of similarity
ap	*Antepartum*
AS	Amino acids
Asp	Aspartate
av.	Average
BCAA	Branched chain amino acids
BCS	Body condition score
BHB	Beta-hydroxybutyrate
BLAST	Basic local alignment search tool
bp	Base pairs
But	Butyrate
BW	Body weight
Ca++	Ionized calcium
Cad	Cadaverine
Car	Carnitine
CAR	L-Carnitine supplemented group
CAZyme	Carbohydrate active enzyme
C-Bifi	Animal cluster dominated by *Bifidobacterium*
C-Clos	Animal cluster dominated by uncl. Clostridiales
CD	Compact disc, data carrier
cHct	Hematocrit
Cho	Choline
Cl	Chloride

CLA	Conjugated linoleic acid
CMR	Commercial milk replacers
CON	Control group
C-Spiro	Animal cluster dominated by uncl. Spirochaetaceae
DFG	German Research Foundation/ Deutsche Forschungsgemeinschaft
DMA	Dimethylamine
DMI	Dry matter intake
DNA	Desoxyribonucleic acid
DUO	Duodenal fluid
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
EtOH	Ethanol
F	Pseudo-F ratio value
FDR	False discovery rate
FEC	Feces
FIA	Flow injection analysis
For	Formate
FRAP	Ferric reducing ability of plasma
g	Times gravity
gBB	Gamma-butyrobetaine
GC-MS	Gas chromatography mass spectrometry
GIT	Gastrointestinal tract
Glu	Glutamate
Gluc	Glucose
Gly	Glycine
Glyol	Glycerol
GPx	Glutathione peroxidase
GR	Absolute granulocytes
GRP	Neutrophile granulocyte percentage
hC	Hours after calving
HCO3	Bicarbonate
HCT	Hematocrit, measured using Celltac
HGB	Hemoglobin
hL	Hours after LPS challenge

HPLC	High performance liquid chromatography
i.s.	*Incertae sedis*
Ibut	Isobutyrate
Imi	Imidazole
Iprop	Isopropanol
Lac	Lactate
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
Leu	Leucine
LogSCC	Logarithmic transformed somatic cell count
LPS	Lipopolysaccharides
LYP	Lymphocytes
Lys	Lysine
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MetOH	Methanol
MitoCow	Mitochondrial functionality in dairy cows
MPV	Mean platelet volumes
mRNA	Messenger ribonucleic acid
MVDISP	Multivariate dispersion
N or n	Number of animals or samples
n.s.	Not significant
NCBI	National Center for Biotechnology Information
NEFA	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
Orn	Ornithine
OTU	Operational taxonomic unit
p	Probability value
Paca	Phenylacetate
Pala	Phenylalanine
PCO	Principal coordinate analysis
pCO2	Partial carbon dioxide pressure
PCR	Polymerase chain reaction
PCT	Thrombocrit

PERMANOVA	Permutational analysis of variance
pH	Potential of hydrogen
Pim	Pimelate
pp	*Postpartum*
ppm	Parts per million
r	Spearman's correlation
R^2	Regression slope
RDP	Ribosomal database project
REI	Residual energy intake
RFI	Residual feed intake
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RUM	Rumen fluid
SARA	Subacute ruminal acidosis
SCFAs	Short chain fatty acids
SEM	Standard error of the mean
Ser	Serine
SIMPER	Similarity percentage analysis
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SOP	Standard operating procedure
spp.	*Species pluralis*
SSC	Somatic cell count
Suc	Succinate
tCO2	Total carbon dioxide
TG	Triglycerides
TMA	Trimethylamine
TMAO	Trimethylamine N-oxid
TML	Trimethyllysine
TpCO2	Partial pressure of carbon dioxide, temperature corrected
Ur	Urea
Vale	Valerate
Vali	Valine

vs.	*Versus*
WBC	White blood cells

List of figures

1 Introduction

Before there were animals there were bacteria! Animals, including humans, and bacteria co-evolved according to respective needs [1]. These complex, sometimes symbiotic relationships took millennia to be formed and modeled the world we know today. Bacteria formed their living habitats with probably endless different niches [1] and so called "old friends" are environmental bacteria [2], that are deeply enrooted to us, our metabolism and our evolution. This led to about 50 % of cells in humans [3] and 20 % of blood metabolites being derived from microbes [1]. In the human body, 20,000 human genes [4] stand against 2 to 20 million microbial genes [5], with the former having approximately 99.1 % bacterial origin [6]. One example would be the mitochondrion, which, via endosymbiosis of an α-Proteobacterium into a Cyanobacterium, is assumed to have built the foundation for extant eukaryotes [7]. This presents the still underestimated role of microbes within humans, and most likely also animals. But why are microbes interested in settling in animals, and what are possible benefits for the animals?

The host provides bacteria continuously with substrate via feed; offers them stable environmental conditions, such as body temperature; and spreads them as vectors. The bacteria in turn provide their host with energy and vitamins, support in digesting food and complex fibers, stimulate and train the immune system, keep up the gut integrity, and support the physiologic and mental health of the host [8-10]. In general, it is suggested that a healthy gut is the foundation of a healthy host [11]. However, this construct is fragile. As soon as hosts turn towards unphysiological habits, the microbiome has to adapt, the former consortium becomes fragile and pathogenic or even formerly commensal bacteria invade the niche [12]. Excessive hygienic behavior, antibiotic use, low or late exposure to beneficial environmental bacteria, host stress, and over- and undernourishment causes this dysbiosis [1, 10]. This is becoming increasingly evident the more medical science digs into the causal chains of diseases. Autoimmune diseases such as irritated bowel disease [9], metabolic syndrome, colorectal cancer, asthma, coeliac disease, and obesity, just to mention a few illnesses, that have emerged in humans in the past decades, are discussed to have their origin in a dysbiosis of the intestinal microbiome [13, 14]. Evidence increases, that diets high in protein, sugar and fat, as well as drugs, pathogens, and toxins may be just a few of the manifold drivers for microbial disbalance and consequently the development of

diseases [13]. This phenomenon is not only seen in humans [15] but also, amongst others, in corals [16], soils [17] and livestock animals [18].

1.1 The modern dairy cow

The cow is one of the most important livestock animals for humans. This is due to the ruminant's ability to convert human indigestible grasses, weeds and other plants as sole feed source into high-quality human edible protein [19]. Hereby, in theory, ruminants do not compete with human edible food sources as monogastric livestock animals do [11, 19]. This makes ruminant products ideal as protein sources, especially in the light of future world food supply strategies. However, for the sake of efficiency and profit, the global cattle and dairy cow livestock animals are largely fed on high-grain and pulses diets in order to increase weight gains and milk yields per unit of time [19]. At the end, this does lead to a direct competition with human diets [19]. Additionally, the demand of ruminant products is increasing dramatically across the past decades [20], demanding an even quicker and therefore high-grain based production regimen. Not only is this discussed critically by society and science in terms of global food supply, also nature is a loser in this scenario. Ruminants produce huge amounts of ammonia (NH_3) and methane (CH_4) [21, 22] via their digestive fermentation processes. This way, they massively contribute to global greenhouse gas emissions [20, 22], and therefore to global warming.

High global milk production in the last decades caused a tremendous decrease in milk price on the global market [23], and entailed a great decline of small dairy farms and their merging into highly cost efficient large scale units with expanding animal numbers per farm. Feed is the most important cost factor in dairy cow husbandry [24, 25]. Hence, milk yield, feed efficiency, and dry matter intake represented the most important breeding targets for decades [26]. The former is improved with increasing amounts of concentrate in the diet, which has a great impact on the longevity and health of the dairy cow [27]. This is due to the high complexity of the ruminant as a host of a broad, complex, and dynamically adapting microbiome which needs to be taken into consideration when feeding animals. Understanding the physiological cascade of digestion of the cow is crucial to understanding the connection between the current struggles in dairy husbandry and its connection to the intestinal microbiome of dairy cows.

1.2 The digestive tract of the dairy cow

Dairy cows as foregut fermenting animals [28] comprise a highly complex and perfectly orchestrated cascade of digestion (Fig. 1.1). Ingested complex plant polysaccharides such as cellulose, starch, pectins and other fibers are degraded by millions of different bacteria, protozoa, archaea, fungi and phages thriving in the intestinal tract. In here, especially bacteria are equipped with a broad set of carbohydrate-active enzymes (CAZymes) such as glycoside hydrolases, polysaccharide lyases, and carbohydrate esterases [29]. They are not only contributing in the breakdown of feedstuff but also, they have been found to greatly influence dairy cow health and milk production [30]. High milk protein, high milk yield cows for example have completely different microbial communities, dominated by *Prevotella* species, compared to low milk protein, low milk yield animals [31]. In parallel, Vitamin B metabolism, branched chain amino acids (BCAA) biosynthesis as well as rumen and blood amino acids are increased in high milk yield and protein animals [31]. This indicates that the bacterial and metabolite profile is strongly associated with the dairy cow's performance and holds true although cows are similarly fed [31].

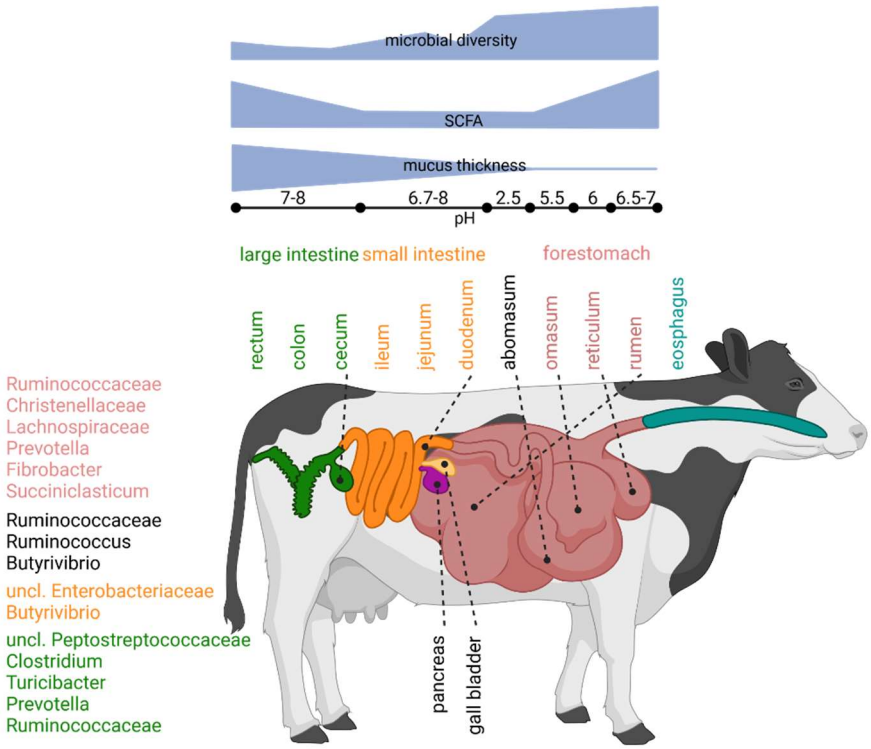


Fig. 1.1 The gastrointestinal tract of the dairy cow and microbial diversities, SCFA production [30], pH [32], and mucus thickness [33] in the respective sections. Dominant bacterial taxa in the respective GIT sections are highlighted in the same color [30, 34]

1.2.1 The upper digestive tract

Microbial digestion begins with the ensalivation of the ingested feed in the mouth at pH 8.0-8.4 [32], as amylase-free saliva is continuously inoculated with ruminal fluid. Enzymes start degrading simple starch molecules, before swallowing of the bolus via the esophagus into the rumen. Now, the bolus is included to the inhomogeneous, but layered mass of previously ingested substrate with a pH between 6 and 7 [30, 32]. Due to smooth muscle contractions of the rumen wall, this is continuously grooved. From ventral to dorsal, fiber size increases and water content decreases [35]. To provide greater surface enlargement and scope for microbial enzymes, cows ruminate larger feed particles from the reticulum, back to the mouth via regurgitation, where they are further mechanically crushed [35, 36]. A dairy cow chews on a bolus around 30 to 70 seconds before it is swallowed [35]. This procedure is repeated after feed ingestion during resting for between 30 seconds to 2 hours per rumination session with up to 20 rumination sessions across the day [35]. The more roughage content is fed, the higher the salivation capacity [37]. Ruminal microbes foster anaerobic conditions and feed fermentation leads to an increased production of organic acids, which lowers the ruminal pH. This is supported by body temperature around 38.6-39.1 °C [38]. Salivation buffers these acids via high contents of sodium bicarbonate [35]. Salivation and fermentation need to be balanced to keep up a healthy ruminal ecosystem. Fermentation results into H_2, methane and CO_2 production and also short chain fatty acids (SCFA), which are the dominant metabolizable energy source for ruminants, making up approximately 50 to 85 % [23] of the total metabolizable energy ingested. SCFA are absorbed via the mucus free rumen epithelium [33] and transported via the portal vein to the liver [28], where they are providing energy and building blocks for, e.g., mid and long-chain fatty acids.

With 10^{12} microbial cells per ml [39], the rumen is assumed to be the most diverse niche within animals [11]. The forestomach is dominated by families Ruminococcaeae, Christenellaceae and Lachnospiraceae as well as the *Prevotella* genus. The latter can make up 19 % of total bacterial reads in rumen digesta samples [30]. Furthermore, microbial genes involved in carbohydrate metabolism are more abundant in the forestomach than in the large intestinal digesta [30]. Pathways of amino sugar and nucleotide sugar metabolism, ribosomes, fructose and mannose metabolism are enriched in the rumen [40].

The smaller the initially ingested feed particle size, the quicker is the passage rate of digesta [41] and the lower is the rumination activity. Well digested, smaller feed particles and rumen liquid enter the omasum, which is responsible for water and electrolyte retention [36]. Thereafter, the abomasum as the monogastric-like stomach of the cow [32, 41] is the place of hydrochloric acid and protease secretion [42] for microbial and dietary protein degradation. Also, it provides a suitable pH of 2 to 3 [32, 39] for pepsin activity [36]. Microbial diversities have been found to be highest up to this section in the total gastrointestinal tract with enriched *Butyrivibrio*, Ruminococcaeae, *Ruminococcus* [30].

1.2.2 The small intestine

Duodenum, jejunum and ileum make up the small intestine, which is primarily the site of animal digestive enzymes secretion and nutrient absorption. The host epithelial gut lining is covered with a layer of mucus, acting as a separator between the digesta and the host epithelial cells [33]. Within the duodenum with about 10^4 microbial cells per ml, the pH of the entering digesta is neutralized by pancreatic bicarbonate (HCO_3^-) [43] to pH 7 until the ileum [30, 39]. Pancreatic enzymes (e.g., α-amylase) further degrade post-ruminal starch and microbial protein [42]. The latter covers ≥50 % of the protein supply [23], whereby methionine, lysine and histidine are the limiting amino acids [44] for milk production. The small intestine is not greatly involved in SCFA production, compared to the rumen and the large intestine [30] and digestion of starch in the small intestine is limited compared to the rumen [45]. Fibrolytic enzymes are not produced by the animal and microbial diversity is much lower than in the forestomach and large intestine [23, 30, 40]. This may be due to a short retention time of digesta [46, 47]. The small intestine is in general dominated by Firmicutes and Proteobacteria [30, 40, 47] and unclassified (uncl.) Enterobacteriaceae are enriched [30].

1.2.3 The large intestine

The large intestine, composing of caecum, colon, and rectum [48] is centrally involved in water absorption [49] and microbial fermentation of protein, secreted mucins, and until so far undigested complex fiber particles. As the large intestinal digesta includes substrate that has been largely acted on in previous intestinal sections, this material is less digestible [48]. Even though not as much as the foregut, the cecum is involved in fermentation and provides the ruminant with 5 to 10 % of the total dietary energy [48].

The large intestinal pH ranges from 7 to 8 [32], whereby the buffer capacity is lower than that in the rumen [48]. Even though the large intestine is not as strongly contributing to nutrient digestion, the hindgut plays an important role in animal performance [48]. Hindgut microbes largely include Peptostreptococcaceae, *Clostridium*, *Turicibacter* [30], *Prevotella,* and Ruminococcaceae [50]. Only 6.5 % of fecal OTUs in cattle were recently found overlapping with rumen derived OTUs, indicating a great transformation from the cranial to the caudal gastrointestinal sections [51].

1.3 Requirements for dairy cow feed

To produce milk, a cow needs a mature mammary gland by giving birth to a calf. After calving, the energy demand increases dramatically due to immediate onset of lactation. Therefore, feed intake and energy density of the diet need to be increased using higher amounts of grain. Additional energy is mobilized from body fat, which is used in the liver via *β*-oxidative processes [52]. This requires the transportation of long-chain fatty acids bound to L-carnitine into the mitochondria. SCFA production increases with increasing feed intake and energy density in the diet, with acetate dominating and mainly contributing to milk fat synthesis. Ruminal butyrate and propionate production largely drive milk yield, while acetate drives milk fat content [53]. This harsh dietary shift from dry to lactation is a very critical event in the dairy cows live [27], apart from calving itself being painful and energy consuming. The adjustments in metabolism from dry to lactation have formerly been described as homeorhetic process [54].

1.4 Energy balance disruption results into microbial disbalance and health problems

The energy demands of a modern dairy cow after calving usually exceeds the physiological capacity of energy ingestion, driving the animal into a negative energy balance (NEBAL) [55]. As compensation for the sake of milk production, cows can mobilize extreme amounts of body fat in form of non-esterified fatty acids (NEFA) during early lactation, which are, as previously mentioned, metabolized in the hepatocytes [55], brain and skeletal muscle tissue [56]. Degradation products are ketone bodies including acetone, acetoacetate and *β*–hydroxybutyrate [55]. However, this metabolic capacity is limited. Surplus long-chain fatty acids can accumulate in the liver (steatosis), leading to fatty liver disease and ketosis, as ketone bodies accumulate in serum. This can cause reduction in feed intake, which accelerates the health threats

since cows at this stage are at high demand of energy. Cows that entered lactation with too high body weights are at higher risk for these diseases [55]. According to the characteristics of L-carnitine described in the former paragraph, increasing the concentration of L-carnitine within the hepatocytes may accelerate the transport of long-chain fatty acids into the mitochondria and hereby reduce the risk for steatosis, and supporting the animal with more energy [52]. Hence, it has been of interest whether feed supplementation of rumen protected L-carnitine can lead to an increased energy usage of feed and body fat deposits, and at the same time reduce the lipid (triglyceride) load of the liver.

Nowadays, the standard procedure in supporting the energy status of the challenged dairy cow is to increase energy concentration per kg of feed in order to meet the demand for high milk yield during lactation [27]. This initiates a cascade of processes. Modern lactating dairy cow diets comprise between 40 to 60 % of concentrate [26]. The much simpler substrate for intestinal microbes, by structure and energy, causes a steep increase of ruminal microbial protein and SCFA production, leading to a lowering of ruminal and even fecal pH [57]. This is due to an accelerated digestive passage rate and higher amounts of not fully digested starch entering the large intestine [57]. The lowered structural fiber intake also lowers rumen motility and the secretion of buffering saliva into the rumen. Together, the more readily fermentable carbohydrates are offered, the higher is the animal at risk to develop production diseases including ruminal acidosis, sub-acute ruminal acidosis [27] and hindgut acidosis [23, 48, 57], laminitis, infertility [58], abomasal displacements, and intestinal leaking [59], which have all become very common in modern dairy cow husbandry. The latter has received increasing attention throughout the last decades and was found to be triggered by a systemic malnourishment of commensal bacteria relying on fiber fermentation [27, 60]. As the diet is the main driver for the formation of the microbial communities, the harsh diet shift is not without drastic consequences for the microbiome [27]. Former microbial communities, especially cellulolytic bacteria, cannot apply their enzymatic tools due to, e.g., acidified conditions [61], and are overgrown by better adapted consortia [27]. This has been found to go along with a reduction in fecal bacterial diversity and richness [57]. Another consequence of high-grain feeding is an increase of lipopolysaccharide endotoxin (LPS) originating from gram-negative bacteria in rumen and fecal material [59, 62]. Low fiber and high concentrate diets decrease the digestive pH, malnourish probiotic bacteria, and reduce the integrity of intestinal epithelium and barrier function

of mucus lining, which are crucial in separating the host from most of its microbiome and digestive agents [62-64]. The blunt epithelial lining can be directly penetrated by pathogens. Resulting perforations increase the risk of intestinal content, including vital bacteria, their metabolites, LPS and digesta, leaking into the serosal site. LPS translocation has not only been observed through the rumen wall but also through the colonic tissue as shown for steers using Ussing chamber experiments [65].

The immune system is highly sensitive to the LPS influx from the intestinal site and reacts with spontaneous fever, latent inflammatory processes [27], lowered feed intake and milk production [66]. This "leaky gut" phenomenon is suggested to be the pivotal element in the cascade of diseases often triggered by inappropriate diets [27].

Most of these diseases appear during the transition period and have their origin in an inadequate and disturbed energy status, which is tried to be compensated by high concentrate feeding. Hence, calving, the immediate feed change [58] and inflammatory processes can be suggested as challenging phases for the modern dairy cow. Cows react very differently towards challenging phases including dietary shifts, calving, poor gastrointestinal health and subsequent metabolic adaptations [27]. The underlying mechanisms to this phenomenon are suggested to be multifactorial but include genetics and microbiome structure [27] and therefore different metabolic potentials.

1.5 Methods to analyze the cow's microbiome

1.5.1 DNA sequencing

Bacteria range in size between 0.2 µm and 750 µm [67] making them difficult to identify and characterize. Cultivation approaches include the first attempts of bacterial studies, whereby medical science played a crucial role in bringing bacterial knowledge to the level that we have today, as various bacteria were causative for diseases throughout history [68]. Continuous improvements in technology and accumulation of knowledge on bacterial behavior towards oxygen, saline and pH gradients dramatically improved the analytical standards. Computer and machine supported science additionally contributed greatly to the vast development of new analytical techniques. This continuously makes bacterial analytics cheaper and quicker, which is why increasing numbers of bacterial niches and habitats are discovered, analyzed and mapped by now.

The difficulty in the characterization and identification of bacteria lies in finding a unique "fingerprint" for each taxon. Even though bacteria comprise a DNA in a circular shape,

much smaller than that of human, sequencing the complete DNA still is very laborious and expensive. Therefore, a smaller fragment is needed. A commonly used nucleotide fragment is the 16S rRNA gene, as it allows for phylogenetic differentiation and is universally found in prokaryotes. The 16S rRNA nucleotide fragment encloses nine hypervariable regions and encodes the small subunit of the ribosomal complex, which is inevitably necessary for protein synthesis, by reading out mRNA. Proteins are the foundation of living beings, which is why their production is ancient and genetically conserved. The gene size (~ 1,500 bp), the presence in all bacteria as well as being functionally constant across time makes this generic fragment useful for taxonomic differentiation [69]. First steps of 16S rRNA fragment analysis encloses sample preparation including weighing and cell lysis using chemical (e.g., detergents), physical (e.g., sonication) and mechanical (e.g., beat-beating) techniques. Cell debris are further separated from nucleic material using precipitation, centrifugation and cleaning applications. DNA extracts are in the following submitted to a two-step polymerase chain reaction (PCR) protocol to amplify the 16S rRNA gene. In the first PCR, flanking reverse and forward primers are used for the hypervariable region of interest such as V3-V4 or V1-V2 together with a barcode. Also, nucleotides (dNTPs), DNA polymerase, and buffer detergents are included to ensure correct amplification. In the second entailed PCR run, multiplexing and index primers are included together with PCR product from the first PCR as template. The index together with the barcode allows for sample-identification and therefore sequencing of multiple samples simultaneously. In case of low initial DNA loads of sample matrices, a preliminary PCR amplification can be used to increase final 16S rRNA gene copy numbers.

Sequencing of the nucleotide fragments in the samples allows for identifying the nucleic code of each read. In a next step the reads are bioinformatically processed. This includes assembly of the raw data, quality controls including PCR primer, adapter, and chimera removal, and filtering of reads with low quality. Today, a threshold of ≥97 % similarity is commonly used to group 16S rRNA fragments into operational taxonomic units (OTUs), clusters of high-similarity bacterial species [70]. Representative OTU reads are thereafter aligned to a database such as SILVA or ribosomal database project (RDP) for taxonomically assignation. At this stage, taxonomical data can be joined with sample metadata information and included to statistical and graphical programs such as R, JMP or PRIMER-E to work on hypothesis ideas.

Even though 16S rRNA amplicon sequencing has emerged to a gold standard method, it lacks information about the vitality of bacteria in a specific niche and quantitative conclusions cannot be drawn. This is particularly detrimental if niches such as intestinal compartments are studied, as digesta flows from the previous to the next compartment, including consortia from the previous segment. Therefore, metagenomics are often combined with other *omics*-technologies such as transcriptomics, proteomics and metabolomic analysis [71] (Fig. 1.2), which give information about which bacteria are actually active and which metabolic pathways are used. There are great hurdles in modern microbiology including the possible underestimation of low abundant bacteria and their contribution to host health and disease. Also, applying different laboratory and data analysis approaches to one sample can lead to particularly different results [72]. Careful comparison and standardization of protocols therefore poses an essential task for future microbial studies.

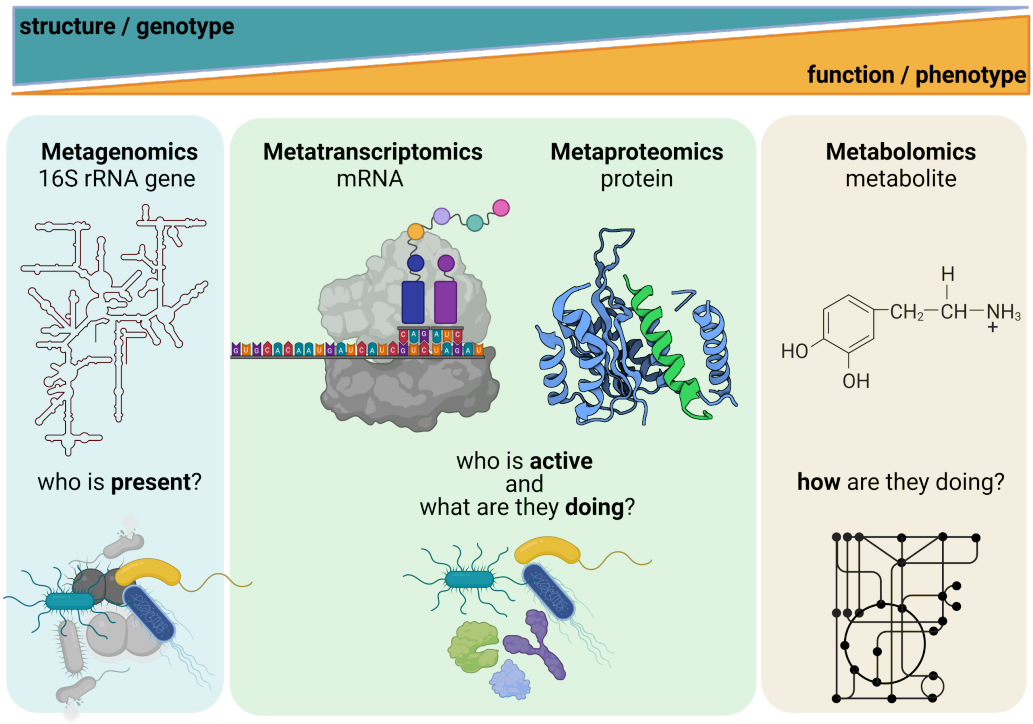


Fig. 1.2 Different options of Omics-technologies for environmental studies on the microbiome target different questions.

1.5.2 Metabolomics

Metabolomics, the entity of metabolites observed in an environment, results from biological and environmental factors and hereby poses the connector between genotypic and phenotypic characteristics [73]. The analysis of metabolites within, e.g., soils, waters, intestines, feces, surfaces or fermenters became a great field of interest and therefore metabolomic technology has emerged and improved in the last decades.

This today allows metabolomic analyses for a great variety of metabolite compounds and sample types.

Bacterial consortia steer their host via signaling molecules such as alcohols, SCFA, proteins (e.g., enzymes) and precursor molecules, which are absorbed from the gut lumen by the host. These bacterial metabolites use different axes to modulate the hosts metabolism, like the gut-brain-axis [74], the gut-lung-axis [75] or the entero-mammary pathway [76]. However, most of these metabolites are not specific to bacterial taxa [77] or even to the host itself and assigning metabolites to one specific origin is not advised. Instead, connecting metabolites with bacterial abundance data and culturing results is of great importance to draw more sound hypotheses [71]. Methods of detecting metabolites are broad. They are not only relevant for microbiome studies but also for, e.g., food technology, plant biotechnology and pharmacology. A diverse spectrum of commercial methods is offered and enables comparability among metabolite-targeted or untargeted studies. Subsequent to sample collection and preparation, metabolite detection techniques include, amongst others, mass spectrometry (MS) and nuclear magnetic resonance (NMR) technology [14].

1.6 Objectives and hypotheses

The ruminant is a complex and fascinating ecosystem, bearing yet undiscovered and little understood relationships between intestinal microbial consortia and the host. More and more it is becoming evident that the intestinal microbiome influences the host via metabolite sensing to various organs such as liver, brain [14], mammary gland, lungs and kidneys. Underlying mechanisms may be best set into focus when contrasting unchallenged phases to challenged phases, visualizing microbial and metabolic shifts at the same time. Hence, the present work aimed to document microbial consortia and metabolites across a particularly long period of time and several challenges, like calving as individual and a lipopolysaccharide (LPS) challenge, induced intravenously, as standardized challenge. Both challenges were more than 100 days apart from each other ensuring positive energy balance during LPS challenge. In parallel, L-carnitine was fed to one half of the herd throughout the complete trial to evaluate its potential of improving the energy metabolism during these challenging phases.

As each gastrointestinal section has individual purposes in the cascade of digestion and different microbial communities, each site may contribute differently to the severity

of host reaction on challenges. Therefore, the first study focused on the upper digestive tract including the rumen and duodenum of multiparous dairy cows, whereby the liquid fraction was studied. The research was set with the hypothesis that the L-carnitine supplementation and/or the two physiological challenges change the microbial and metabolite compositions of the dairy cows ruminal and duodenal fluid. 16S rRNA sequencing was used to obtain the bacterial communities and nuclear magnetic resonance analysis (NMR) was used for the detection of metabolites deriving from host and bacteria. Hereby, up- and down-regulations of bacteria and metabolites after calving and LPS induced inflammatory stimulus, was studied.

The large intestine is suggested to primarily contribute to host health maintenance. Fecal sampling as cheap, quick, and not-invasive technique emerged quickly in animal science to draw connections between host health conditions and the respective fecal microbiome. Yet, it remains quite unclear, what conclusions can be taken from fecal bacterial consortia as well as host and bacterial metabolite profiles especially during challenging phases. Therefore, the second study aimed to uncover microbiome clusters and to close knowledge gaps considering host-metabolite-microbiome interactions. The underlying hypothesis was, that the fecal microbiome and various metabolites shift due to L-carnitine supplementation and along the trial period, especially during the challenges. Also, single taxa may have either beneficial or negative impacts on the fitness of the cow during the challenges. This study was particularly powerful, due to a broad range of different data sets including 16S rRNA sequencing, fecal and blood metabolome parameters as well as health and milk production results across a particularly long period of time.

2 Cow's microbiome from *antepartum* to *postpartum*: a long-term study covering two physiological challenges

Abstract

Little is known about the interplay between the ruminant microbiome and the host during challenging events. This long-term study investigated the ruminal and duodenal microbiome and metabolites during calving as an individual challenge and an LPS induced systemic inflammation as a standardized challenge. Strong inter- and intra-individual microbiome changes were noted during the entire trial period of 168 days and between the 12 sampling time points. Specific progressions for single taxa such as *Bifidobacterium* spp. and *Treponema* spp. were observed. NMR analyses of rumen and duodenum samples identified up to 60 metabolites out of which fatty and amino acids, amines, and urea varied in concentrations triggered by the two challenges. Correlation analyses between these parameters indicated a close connection and dependency of the microbiome with its host at an animal-individual level.

Importance

The interplay between the ruminant and its microbiome is of key importance for health and productivity of the animal. Several physiologic stressors are affecting both organisms during the entire life span and more research has to be done to support future recovering strategies. Our study includes the use of multi-omics for the analyses of rumen and duodenum fluids over a defined productive life span. The present data showed the importance of *Bifidobacterium* around parturition which is mainly studied in calves but not in cows and opens new hypotheses to study the role of bifidobacteria in ruminants.

Keywords: calving, dairy cow, duodenum, L-carnitine, LPS, metabolome, microbiome, rumen, transition

2.1 Introduction

The modern dairy cow is confronted with a multitude of challenges. This, amongst others, includes feed changes, pathogens, heat stress and the transition from dry period to high performance milk production. These might impair the cow's health and hence, productivity as well as product quality. Ketosis, metritis, milk fever, and displaced abomasum might go along with or even originate from a disrupted forestomach and gut microbial community and might appear as a decrease in microbial diversity, richness, and functionality [78, 79]. The gut-brain-axis, the mitochondria-microbiota-intertalk [80] and the entero-mammary pathway [81] are studied intensively, more and more revealing the importance of the microbiome in steering the host metabolism. Nevertheless, since modern animal production requires distinctively different physiological features than those established by evolution over thousands of years, the animal's health is mostly affected negatively, as milk yields continuously increase. Hence, shedding more light on the complex metabolite cross-talk between host and microbes could bear the potential to improve animal welfare and thereby also have crucial economic impacts as medical expenses can be a major burden for animal farmers [82].

The transition from the dry period to lactation is one of the most critical phases in a cow's life. The metabolism focuses on milk production with high energy and protein requirements at limited energy intake, making the cow more susceptible to internal and external challenges and often leading to inflammatory conditions [83]. The requirement of energy increases within hours after calving due to the onset of lactation, which can only be met by enhanced proportions of concentrate feed containing mostly rapidly fermentable carbohydrates. This, in turn, might adversely affect rumination activity and consequently, ruminal and duodenal microbiota. This sudden regimen shift was not anticipated by nature since calving would be during early summer, with ample and steady fresh forage availability [84]. The shift towards intake of rapidly fermentable carbohydrates with insufficient physically effective fiber might trigger a vicious cycle. The subsequent reduction in alkaline saliva production [85] fails to buffer the drop in rumen pH, due to higher ruminal lactate and short-chain fatty acid (SCFA) production, leading to an increase of gram-negative bacteria rich in membrane lipopolysaccharides (LPS) [86, 87]. The shift in dietary composition may also affect the intestinal epithelial lining's permeability, which can represent the gateway for pathogenic organisms and LPS influxes to the blood, causing inflammatory host conditions as the immune system

reacts to the antigens [63, 88]. This impaired health status is often accompanied by secondary symptoms such as fever, lowered feed intake and milk yield, as well as declined rumination activity [88]. The severity of these conditions might vary largely among individuals despite the equal feeding regimen [83] and may derive from varying individual metabolic or energetic capabilities [89]. Cellular energy derives from the mitochondria, whereby their vitality is assumed to play a key role in health and disease of the organism [90]. For mitochondrial energy conversion, L-carnitine plays a crucial role as carrying long-chain fatty acids as acylcarnitines, into the mitochondrial matrix, where they are further included to *β*-oxidation. In previous studies, cows with higher blood serum acylcarnitines had extended productive lifespans compared to those exiting earlier [91]. The supplementation of this metabolite may hence aid the dairy cow through this critical phase.

It is still unclear, which factors contribute to the robustness of individual cows, and how they manage the balance between high productivity at good fitness and therefore contribute to high farm profitability. The response of the microbiome and its possible function towards stimulating the host physiology and metabolism during host challenging situations are also less studied so far. Therefore, scoping into the rumen as the major site of energy conversion and the duodenum as the first site of host nutrient uptake, could enlighten these responses to challenging periods.

This study hypothesizes that modern dairy cows respond to the challenge of calving, the subsequent feed change and a LPS-induced standardized inflammation by dynamic adaptation of ruminal and duodenal bacteriomes. These responses either support or intensify the severity of the reaction towards stress. Additionally, the role of supplemented L-carnitine for the bacterial consortium in the matrices, rumen and duodenal fluid, across a particularly long period of time, is elucidated. This study connects detected metabolites with bacterial abundances and selected host health parameters to pinpoint possible interactions.

2.2 Results

The animal trial covered 168 days and used eight double fistulated dairy cows, which resulted in 149 samples from rumen (RUM) and duodenum fluid (DUO) (Fig. 2.1). From -42 to +14 pH in RUM dropped from an average of 6.9±0.3 (SEM) to 5.8±0.6 (Fig. S2.1 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). Feed adaptation of the bacteriome until +100 and the LPS challenge increased pH in RUM (24hL, pH 6.5±0.4).

The pH values in DUO increased from -42 (2.6±0.5) to shortly after calving (12hC, pH 3.2±0.3) and slowly decreased again until +100 (pH 2.9±0.4). LPS challenge increased the DUO pH (12hL, pH 3.5±0.7) and decreased at 24hL to around 2.7±0.5.

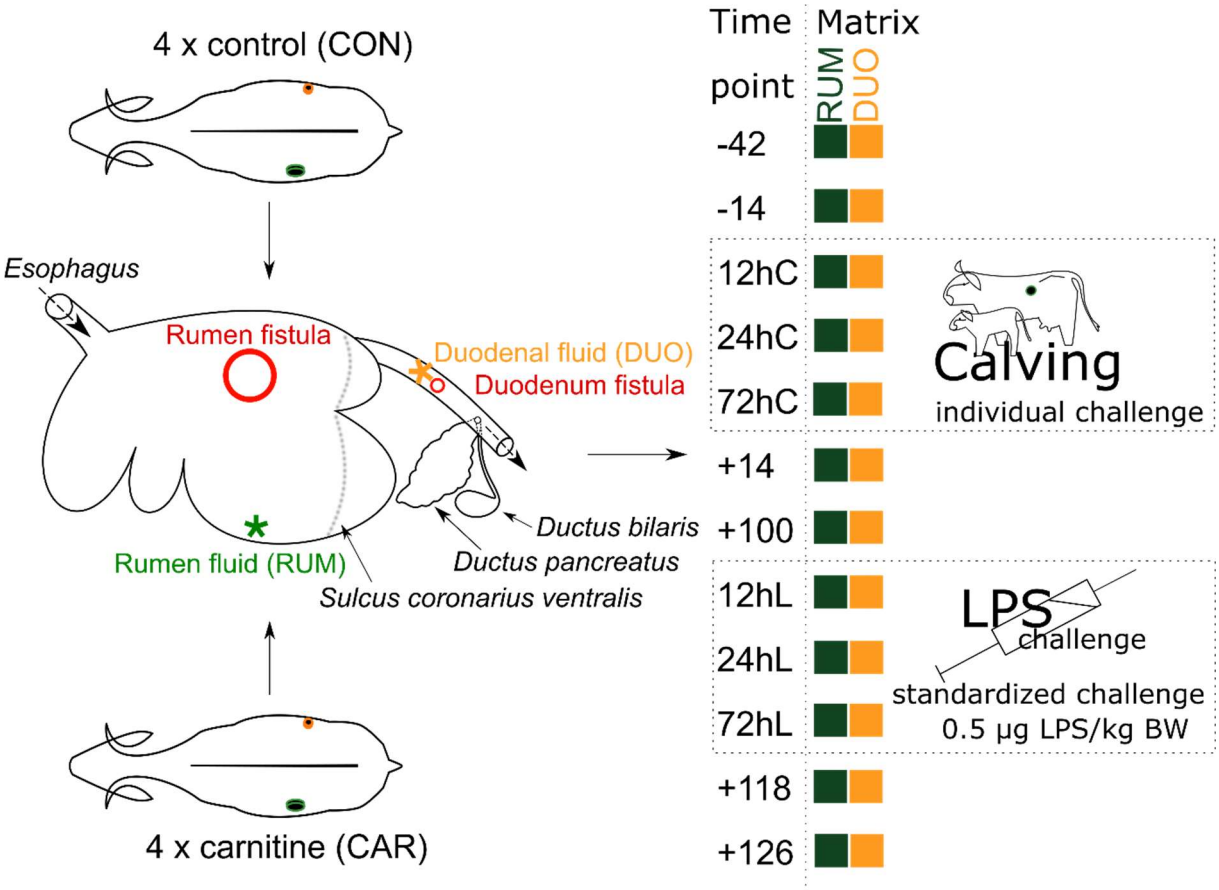


Fig. 2.1 MitoCow - Trial setup. Rumen (RUM) and duodenal (DUO) fluid samples were taken from 4 control and 4 carnitine supplemented animals at 12 different time points. Stars indicate where the samples were taken from, red circles indicate the place of fistulation. Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

2.2.1 Temporal evolvement of the microbiota in rumen and duodenum

OTUs (DissertationCD\Chapter_2\SData\SData_2.1_OTU) were taxonomically assigned to 16 phyla, 65 families and 102 genera. Bacteroidetes (average (av.): RUM= 51%±1.5 (SEM), DUO= 40%±1.4), Firmicutes (RUM= 28%±1.3, DUO= 39%±1.4), Actinobacteria (RUM= 7%±1.1, DUO= 8%±1.1) and Proteobacteria (RUM= 6%±1.0, DUO= 6%±0.7) were the dominant phyla in RUM and DUO samples (DissertationCD\Chapter_2\SData\SData_2.2_Taxa_file).

The two matrices showed similar bacterial community compositions (Fig. S2.2A in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf) throughout the 168 days of sampling, except at -42 and -14, which revealed significant differences between the ruminal and duodenal bacterial compositions (Tab. S2.1 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). At -42 in RUM,

Ruminobacter (OTU104, 1.5 *versus* (*vs*.) 1.2%), *Fibrobacter succinogenes* (OTU285 1.6 *vs.* 0.8%, OTU515 1.2 *vs.* 0.4%), and uncl. Prevotellaceae (OTU630 1.8 *vs*. 0.6%) were higher abundant (all not significant (n.s.)) compared to DUO. In DUO *Succiniclasticum* (OTU38, 2.2 *vs.* 0.9%) and uncl. Gammaproteobacteria (OTU241 0.7 *vs*. 0.5%, OTU310 0.7 *vs*. 0.3%, and OTU403 0.8 *vs*. 0.6%) were higher abundant at -42 (n.s.). At -14, the main contributors to this significant difference were higher fractions of uncl. Bacteroidales (OTU805 1.1 *vs.* 0.2%) and uncl. Gammaproteobacteria (OTU241 0.8 *vs*. 5.6%) (all n.s.) in RUM as well as *Succiniclasticum* (OTU38 2.4 *vs*. 0.3%, $p \leq 0.05$), uncl. Bacteroidetes (OTU739 1.0 *vs.* 0.2%, n.s.) and *Bifidobacterium pseudolongum* (OTU1 0.8 *vs*. 0.2%, n.s.) in DUO. The carnitine supplementation was not affecting bacterial communities in both matrices (Tab. S2.2, Fig. S2.3A,B both in DissertationCD\Chapter_2\Supplementary _Tab_Fig.pdf).

The combined analysis of RUM and DUO samples revealed a separation in two clusters, which were defined by the time points before and after calving (Fig. 2.2, Fig. 2.2B). Comparing RUM at different time points revealed significant differences between -42 (higher abundance of *Ruminobacter* and *F. succinogenes*) to 72hC, 12hL, 72hL, +118, and +126 (all p=0.0003) (DissertationCD\Chapter_2\SData\ SData_2.3_Timepoint_compariosons_RDP). In DUO, -42 (in part higher abundance of *Succiniclasticum* and *Ruminobacter*) was significantly different from +100, 12hL, 24hL, +118, and +126, respectively (all $p \leq 0.0003$).

Alpha-diversity was similar between the matrices and varied throughout the entire trial period on average between 4.0 (+14) to 6.2 (12hC) in RUM and 4.4 (+14) to 5.8 (12hL) in DUO, demonstrating slightly greater diversity fluctuations in RUM (Fig. 2.3, Fig. S2.4 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). Significant negative correlations were observed between α-diversities and *Olsenella*, uncl. Lachnospiraceae and *Roseburia* abundances in RUM and DUO (Tab. S2.3 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf).

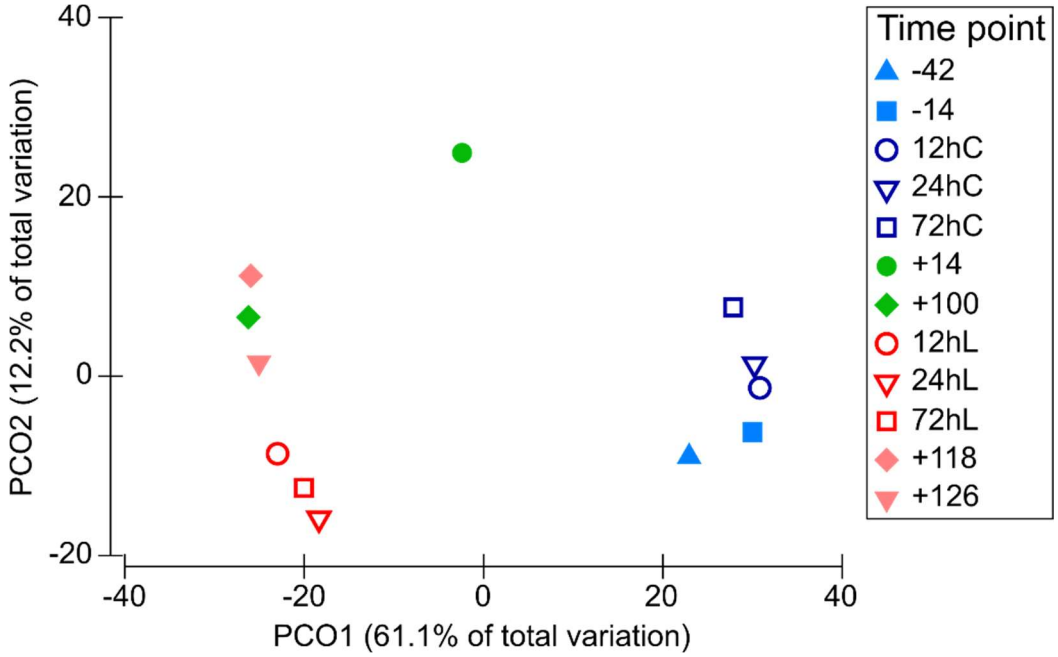


Fig. 2.2 Variation of the bacteriome along the full sampling period. PCO plot of OTU abundances at 12 different time points depicted as centroids of the single samples neglecting matrix definition. Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

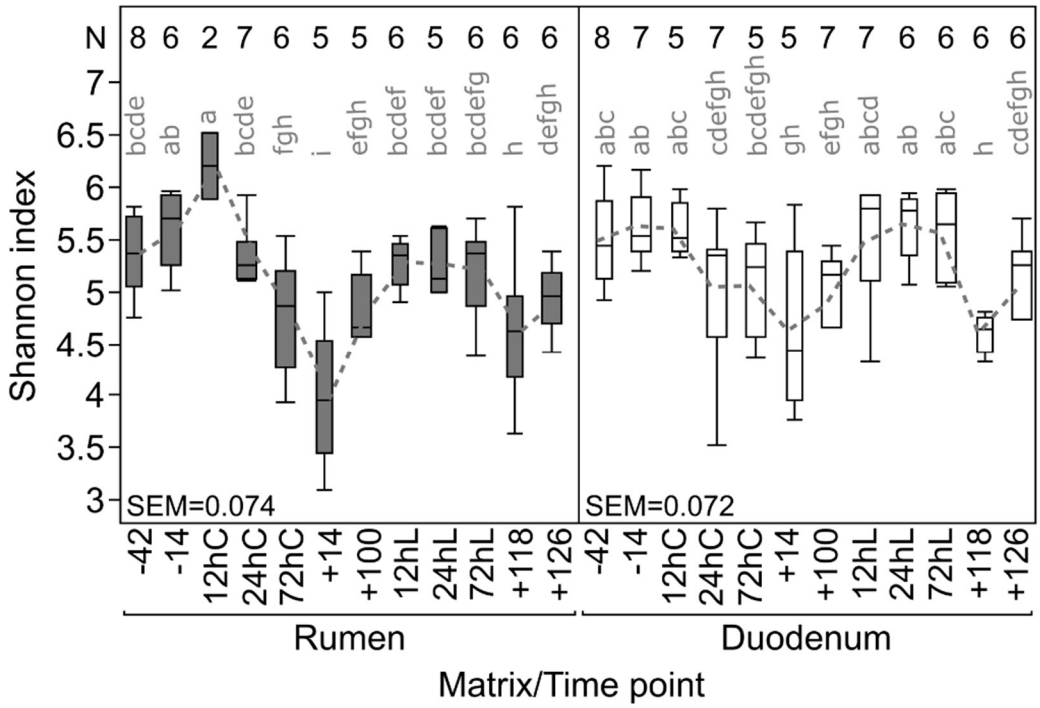


Fig. 2.3 Individual Shannon diversity index throughout the complete trial period for rumen and duodenal fluid samples. Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

2.2.2 Temporal and individual variations of bacterial groups

The overall taxonomic composition showed fluctuations in abundances in both sampling sites along the animal trial caused by calving, changes in feeding regime, and inflammatory challenge (DissertationCD\Chapter_2\SData\SData_2.2_Taxa_file). Bacteroidetes, dominated by species of uncl. Prevotellaceae (RUM= 23.1%, DUO= 16.1%) and *Prevotella* (RUM= 10.1%, DUO= 6.7%), increased gradually after calving, whereas the LPS injection reduced *Prevotella* in both sites. Cow 039 had considerably low *Prevotella* abundances (RUM= 3.0%, DUO= 2.9%) compared to all other animals throughout the complete trial. Actinobacteria showed similar abundance in both matrices throughout the trial phase and was represented by Bifidobacteriaceae (*Bifidobacterium*, *Pseudoscardovia*) and Coriobacteriaceaea (*Olsenella*). *Bifidobacterium* was low at *antepartum* (*ap*) time points (RUM, DUO= 2-3%) and increased in both sites but more gradually in DUO, during hourly samples after calving (Fig. 2.4). At 72hC, this genus peaked in both matrices (RUM= 20%, DUO= 17.7%), which was largely driven by *B. merycicum* and *B. pseudolongum,* whereby species abundances varied greatly among animals. *B. adolescentis* appeared pronounced at this time point in both sites. From here on, total *Bifidobacterium* abundance decreased to +100 (RUM=1.6%, DUO=0.9%) with *B. boum* (OTU183) increasing. During LPS challenge time points, *Bifidobacterium* increased (*B. pseudolongum, B. merycicum*) in RUM and at +118 in DUO (*B. pseudolongum*). However, both peaks were not long-lasting. Amongst both matrices *Bifidobacterium* decreased significantly with lactation number or/and age (Fig. S2.5 in DissertationCD\Chapter_2\Supplementary _Tab_Fig.pdf). *Olsenella* species increased significantly after calving in RUM (+14: 9.2%) and DUO (+14: 8.4%), dominated by an increase of *O. umbonata*, which was replaced by *O. uli* at +100 (Fig. 2.5). At LPS challenge time points, a drop in abundance was observed, which solely recovered in DUO afterwards.

Firmicutes was more abundant in DUO and mainly represented by uncl. Lachnospiraceae, uncl. Clostridiales and uncl. Ruminococcaceae. Each was showing OTU abundances of 6.3% to 7.3% in RUM and 6.7% to 12.9% in DUO (DissertationCD\Chapter_2\SData\SData_2.2_Taxa_file). Unclassified Lachnospiraceae abundance in DUO was negatively (n.s.) affected during the LPS challenge, whereas in RUM, the abundance already dropped at +14. Abundances of uncl. Clostridiales species were higher in DUO (-14: RUM= 8%, DUO= 10%; n.s.). However, after calving and feed change, uncl. Clostridiales abundances dramatically

decreased and ruminal populations remained unaffected between the challenges (RUM= 8.8%, DUO= 5.5%). During the LPS challenge, a significantly higher abundance of uncl. Clostridiales was observed in DUO than in RUM (72hL: RUM= 6%, DUO= 11%; $p \leq 0.05$). Unclassified Ruminococcaceae increased in both matrices evenly from -42 (5-7%) until 12hC (13.3%) and 24hC (12%) in DUO and RUM, respectively, but two days after calving decreased again to 2.6%. In the first hours after the LPS challenge, this species increased up to 8% (RUM) and 6% (DUO).

OTUs belonging to Fibrobacteres were significantly more abundant in DUO than in RUM (p=0.03). *Fibrobacter* spp. were highly abundant at *ap* time points (-42: RUM=4%, DUO=4.3%) and were depleted with higher concentrate levels in the feed ratio (+100: RUM=0.15%, DUO=0.35%). An increase was observed during LPS challenge (24hL: RUM and DUO=1.9%). Cows 245 and 248 exhibited *Fibrobacter* in both matrices, cows 001, 039, 306, and 329 only in DUO.

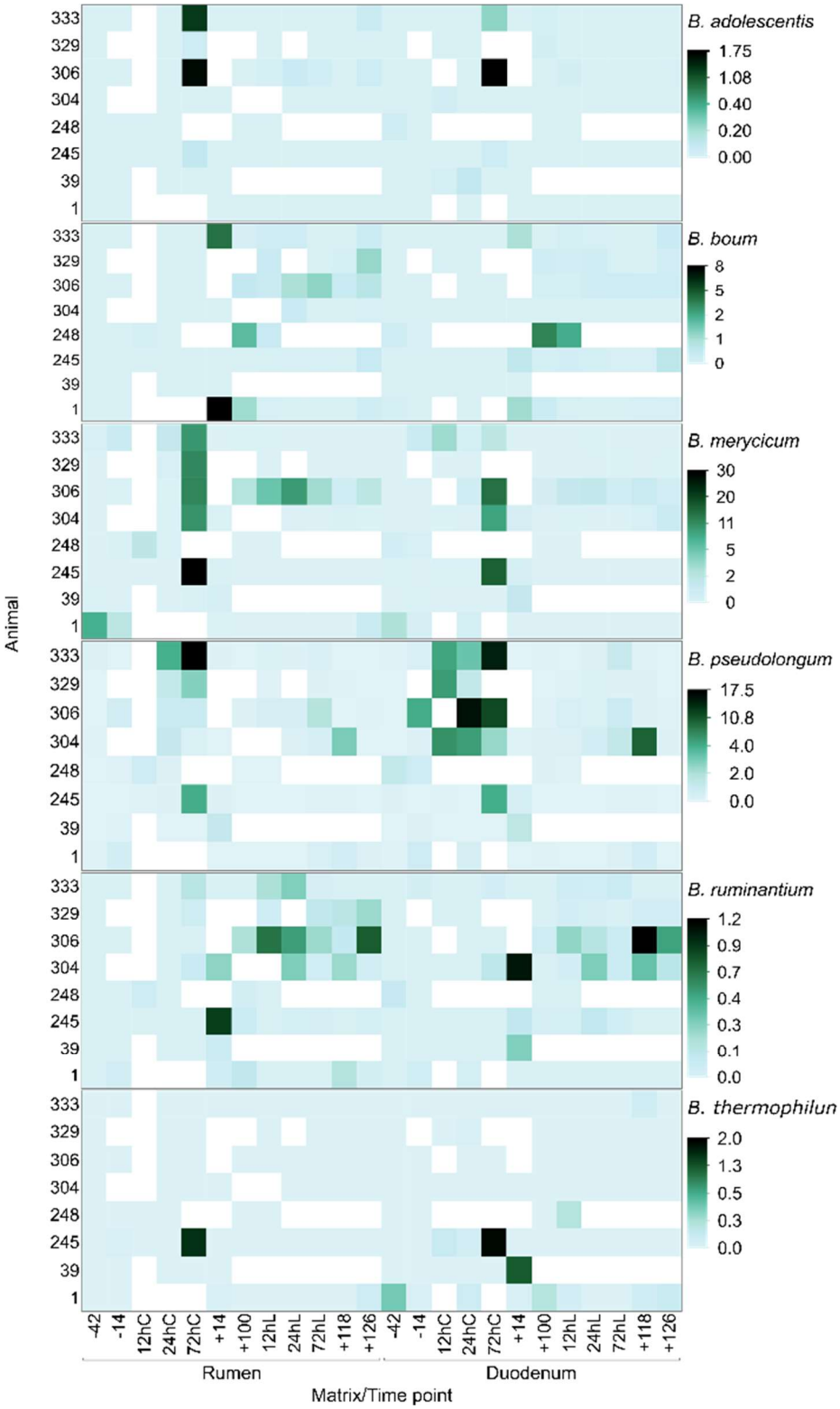


Fig. 2.4 Relative abundance (%) of Bifidobacterium OTUs in rumen and duodenal fluid samples. Legends refer to relative abundance of the complete mean bacteriome in %. Bifidobacterium pseudolongum (OTU1, OTU2); Bifidobacterium merycicum (OTU25, OTU31, OTU4983, OTU60); Bifidobacterium thermophilum (OTU3199, OTU4056); Bifidobacterium adolescentis (OTU1298); Bifidobacterium ruminantium (OTU136); Bifidobacterium boum (OTU183). Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

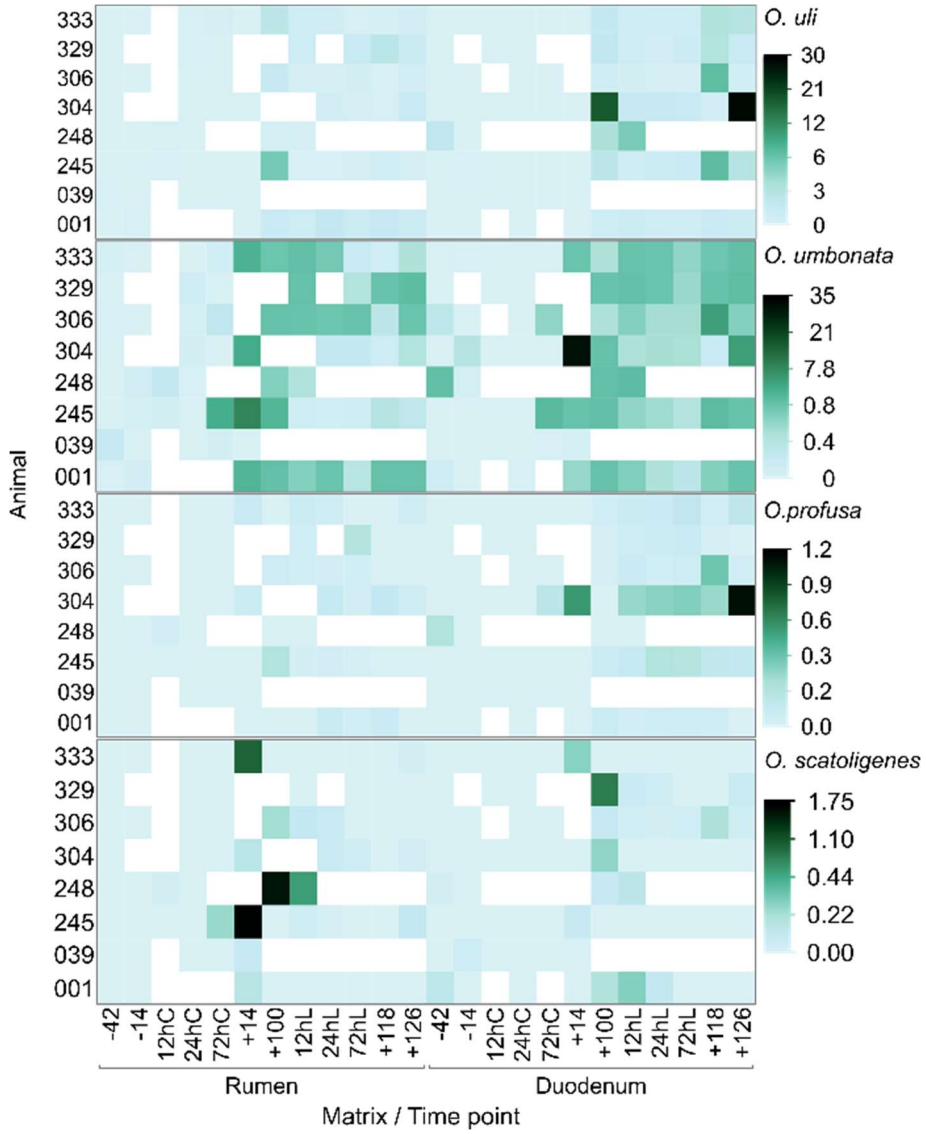


Fig. 2.5 Olsenella species in rumen and duodenal fluid samples across the trial period and all animals. Legends refer to relative abundance of the complete mean bacteriome in %. Olsenella umbonata (OTU6, OTU115, OTU221, OTU1095, OTU6888), Olsenella profusa (OTU227), Olsenella uli (OTU8, OTU2446), Olsenella scatoligenes (OTU321, OTU2511). Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

Proteobacteria was equally abundant in both matrices, especially before calving (9%), with a high genera diversity and evenness of *Ruminobacter*, *Succinimonas*, uncl. Betaproteobacteria and uncl. Gammaproteobacteria (Fig. 2.6), whereas after calving the latter one became the most dominant, summing more than 95% in RUM and 85% in DUO of the total sequenced Proteobacteria. Most *Proteobacteria* genera in rumen disappeared at 72hC (including *Succinimonas, Ruminobacter* and uncl. Gammaproteobacteria), recovering only at 12hL and subsequent time points with a concomitant dominance of uncl. Gammaproteobacteria, which was also observed for duodenal samples. In DUO, a drastic decrease was detected for *Ruminobacter* with a slight recovery at 24hL (Fig. 2.6).

Spirochaetes was low abundant in both matrices (RUM= 0.6%, DUO= 0.8%) and increased from -42 until a few hours after calving (12hC: DUO= 0.8%; 24hC: RUM= 0.7%). Members of the genus *Treponema,* especially the species *Treponema bryantii* (Fig. S2.6 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf), dominated. *Treponema* members after calving/feed change decreased in DUO and even disappeared in RUM at +14. At 12hL, *Treponema* members increase strongly in both matrices.

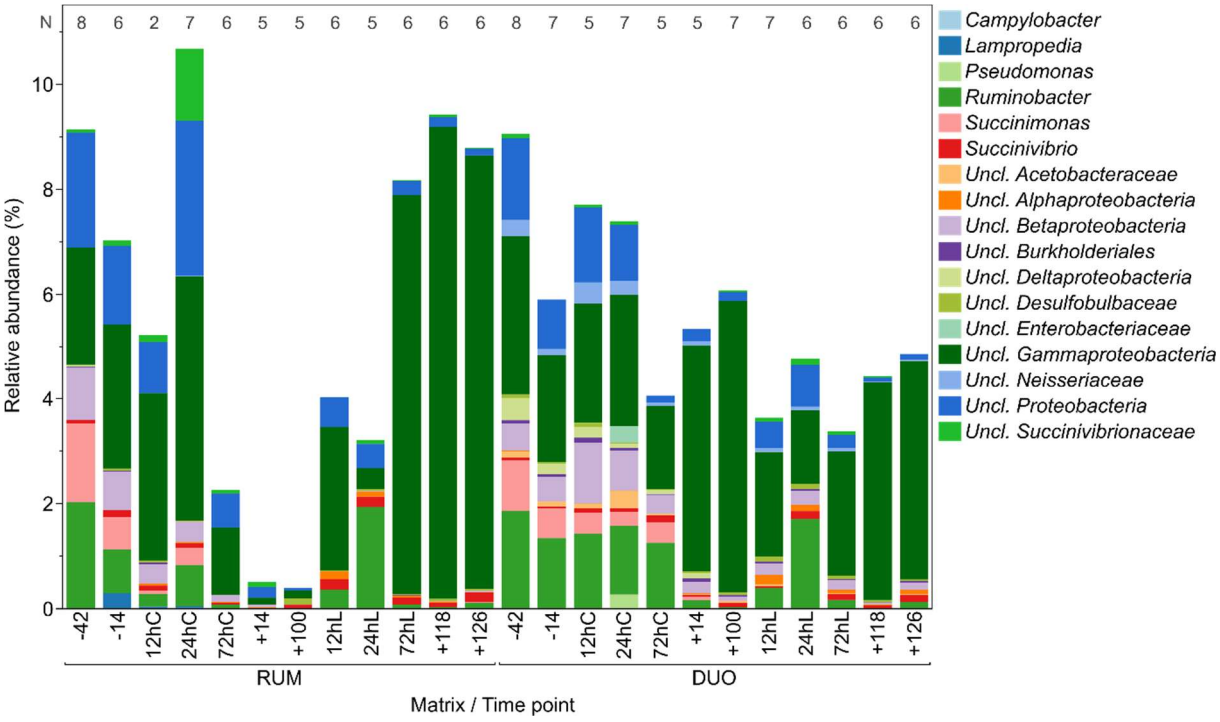


Fig. 2.6 Relative abundance of Proteobacteria genera in rumen (RUM) and duodenal fluid (DUO). N refers to sample number included per time point. Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively.

2.2.3 Variations of rumen and duodenal metabolites

In total, 60 host and bacteria derived metabolites were measured using NMR spectroscopy (DissertationCD\Chapter_2\SData\SData_2.4_NMR_data). Rumen fluids were dominated by acetate (av. 74 mM±1.7 SEM), propionate (av. 27 mM±1.2), and butyrate (av. 12 mM±0.4). The concentration changes of SCFA mirror the changing feeding regimes, such as the increase of propionate with increasing concentrate feeding (Fig. S2.7AB in DissertationCD\Chapter_2\Supplementary _Tab_Fig.pdf). Urea (av. 5.6 mM±1.0), valerate (av. 3.3 mM±0.2), adipate (av. 3.0 mM±0.1), and pimelate (av. 2.6 mM±0.1) were the following dominant metabolites. Isobutyrate (±0.1), lactate (±0.2), isovalerate (±0.03), and glucose (±0.1) varied at around av. 1 mM. Duodenal fluids mainly contained allantoin (av. 3.3 mM±0.4) and

aspartate (av. 2 mM±0.4), followed by glycine (av. 1.8 mM±0.5), dimethylamine (av. 1.6 mM±0.1), glutamate (av. 1.4 mM±0.3), and acetate (both av. 1.4 mM±0.5). Carnitine concentrations were significantly higher in DUO than RUM (p=0.016) and highest in the DUO carnitine (CAR) group (control (CON): av. 0.06 mM, CAR: av. 0.1 mM; Fig. S2.8 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). However, there was no significant difference in the metabolite patterns between CON and CAR in both matrices (Fig. S2.3C,D in DissertationCD\Chapter_2\Supplementary _Tab_Fig.pdf). RUM and DUO samples were significantly grouping apart from each other (p=0.0001, Fig. 7A, 81.1% av. dissimilarity between RUM and DUO) with RUM samples clustering closer together (85.5% av. similarity) compared to DUO samples, which are rather scattered (67.2% av. similarity). Spearman's correlation vectors with r≥0.85 indicated a strong, significant separation along with the 1st component of the PCO, as SCFA (acetate, propionate, and butyrate) were more abundant in RUM samples (Fig. 2.7A). In contrast, allantoin and dimethylamine, were more abundant in the DUO sample cluster. The separation between RUM and DUO was confirmed when depicting the metabolites, however excluding acetate, propionate and butyrate (p=0.0001; Fig. 2.7B). Some RUM samples with a higher concentration of urea and allantoin clustered apart from the main rumen group (Fig. 2.7B). Rumen samples contained significantly higher acetone, valerate, adipate, and isobutyrate concentrations compared to DUO samples (p<0.0001). On the other hand, DUO had significantly higher concentrations of carnitine and serine (p<0.05); phenylalanine, allantoin, glycine, lysine, glutamate, 3-hydroxybutyrate (3Hbut), and cadaverine levels (p≤0.005). In general, amino acids (AA) were considerably elevated in DUO samples throughout the trial. At -42, 12hL, +118, and +126, the concentrations of total AA were two to three times higher as aspartate, proline, and histidine strongly increased, compared to all other time points (Fig. S2.9 in DissertationCD\Chapter_2\ Supplementary_Tab_Fig.pdf). Also, branched-chain amino acids (BCAA), including valine, leucine, and isoleucine were higher in DUO compared to RUM (p=0.022).

Formate concentration was steady along the complete trial in RUM (~0.2 mM), but more than doubled at +14. At -42, DUO formate concentrations were two times higher compared to RUM samples and decreased markedly as soon as animals were fed higher concentrate ratios during and after calving.

According to SIMPER analysis uncovering the metabolites contributing the most to matrix similarity (excluding acetate, propionate, and butyrate) for RUM were adipate,

valerate, urea and pimelate and for DUO, isobutyrate, allantoin, dimethylamine, and aspartate.

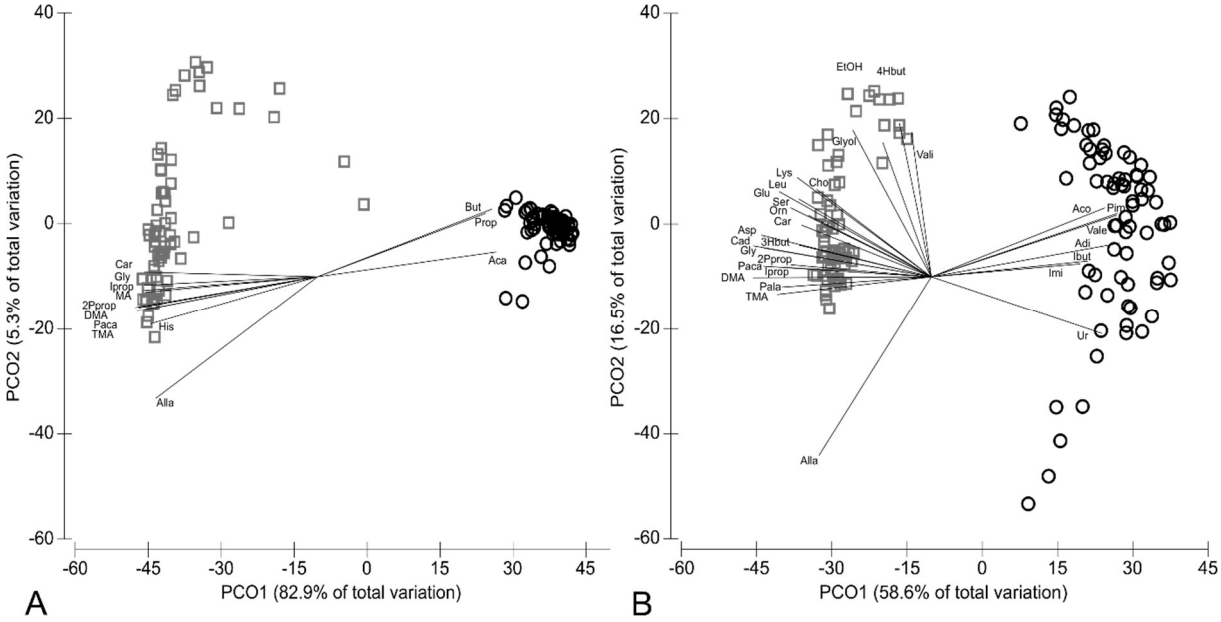


Fig. 2.7 Distribution of metabolites identified by NMR analysis. (A) Metabolites of rumen fluid (RUM, circles) and duodenal fluid (DUO, squares) samples including Spearman's correlation vector (Spearman's r ≥ 0.85). (B) Metabolites of rumen (circles) and duodenal fluid (squares) samples including Spearman's correlation vector (r ≥ 0.65) and excluding acetate, propionate and butyrate. 2Pprop=2-Phenylpropionate, 3Hbut=3-Hydroxybutyrate, 4Hbut=4-Hydroxybutyrate, Aca=Acetate, Aco=Acetone, Adi=Adipate, Alla=Allantoin, Asp=Aspartate, But=Butyrate, Cad=Cadaverine, Car=Carnitine, Cho=Choline, DMA=Dimethylamine, EtOH=Ethanol, Glu=Glutamate, Gly=Glycine, Glyol=Glycerol, Ibut=Isobutyrate, Imi=Imidazole, Iprop=Isopropanol, Leu=Leucine, Lys=Lysine, Orn=Ornithine, Paca=Phenylacetate, Pala=Phenylalanine, Pim=Pimelate, Ser=Serine, TMA=Trimethylamine, Ur=Urea, Vale=Valerate, Vali=Valine.

A possible evolution of the metabolite profiles was checked by clustering RUM and DUO samples separately. No distinguishable metabolic clusters based on time or challenges were observed in both matrices, but variations of metabolite concentrations caused clusters of similarity. RUM samples were divided into three metabolite clusters (Fig. S2.10A in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). Cluster 1 indicated higher valerate concentrations, pimelate, and adipate, whereas fumarate, allantoin, and urea were associated with cluster 2. Cluster 3 grouped ornithine, methanol, glycine, ribose, acetoacetate, 4-hydroxybutyrate, valine, lysine, glutamate, ethanol, maltose, isoleucine, choline, glucose, isoleucine, N-nitrodimethylamine, and carnitine. The metabolite profile of cow 039 was consistent and static over time compared to the other animals. Two clusters could be distinguished for DUO samples (Fig. S2.10B in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). Cluster 1 was particularly driven by higher acetoacetate concentrations, whereas cluster 2 had higher concentrations of allantoin. Cows 245, 248, and 306 at *ap* time points had higher duodenal concentrations of tri- and dimethylamine, isopropanol, and isovalerate. Calving caused a clear separation of the herd, with cow 001, 039, and 329 having

higher acetoacetate concentrations at 24hC in DUO compared to the other animals. Feed change and/or calving (+14 and +100) caused a clear shift of metabolite compositions from *ap* time points. Other time points did not cluster in a clear pattern. Pairwise correlations between metabolites of both matrices can be taken from DissertationCD\Chapter_2\SData\SData_2.5_Pairwise_correlation_all_metabolites.

2.2.4 Correlations between bacteriome and metabolome

Most metabolites in both matrices correlated negatively with high α-diversities, such as in RUM samples valerate (p<0.0001), trimethylamine, and propionate (p<0.0001) as well as pimelate, alanine, and acetone (p<0.0001). In DUO, glycine (p=0.0004), isovalerate (p=0.001), carnitine (p=0.01), and isobutyrate (p=0.006) negatively correlated with high microbial diversities. In rumen, 3-phenylpropionate correlated positively with high α-diversities (p=0.02).

Coriobacteriaceae had particularly significant positive correlations amongst others with RUM valerate, pimelate and 4-hydroxybutyrate (all p<0.0001) as well as with DUO glycine, propionate and isobutyrate concentrations (Tab. S2.4 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf).

Correlating a great number of metabolites and genera and two matrices at twelve different time points brings up a wealth of significant correlating combinations. Counting significant combination repeats between genera and metabolites, such as "SuccinivibrioFormate", across all time points resulted into insights of those combinations appearing the most. The higher the repeat number of the combination across all time points, the more meaningful this combination may be to the host and the ecosystem. Unclassified Succinivibrionaceae was the genus correlating the most commonly with different metabolites across time, counting 15 metabolites (such as proline, aspartate or ribose) with five or more repeats across all time points and both matrices (DissertationCD\Chapter_2\SData\SData_2.6_Repeating_correlations). Members of the genus *SR1 genera incertae sedis* (i.s.) were the second most common across time; however, Bacteroides genus was found to correlate with all 60 metabolites analyzed at least once across the whole trial period. Choline, in turn, was the most common metabolite correlator for bacteria – 86 out of 102 genera correlated with this metabolite, whereby formate was the most frequently repeating correlator across all time points and both matrices, mostly with *Fusobacterium*, *Megasphaera*, *Ruminobacter*, *SR1 genera i.s.*, uncl. Succinivibrionaceae and uncl. Bacteroidia.

Together with uncl. Succinivibrionaceae and SR1 genera i.s., *Olsenella* correlated most frequently and positively with the lactate concentration in RUM and DUO.
Significant correlations between L-carnitine and a broad set of bacteria were observed for RUM and DUO uniquely but also shared ones (Tab. S2.5 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf). *Olsenella* was positively and uncl. *Bacteroidales* negatively associated with L-carnitine in both matrices. In RUM *Roseburia* (p<0.0001) and *Syntrophococcus* (p<0.0005) were positively and uncl. Proteobacteria negatively (p<0.03) correlating with L-carnitine. In DUO uncl. Eubacteriaceae (p<0.04) and uncl. Succinivibrionaceae (p<0.004) were positively associated with L-carnitine. Choline, carnitine and trimethylamine are known to be involved in TMAO formation and at various time points correlating significantly with *Olsenella*, *Pseudoscardovia* and uncl. Veillonellaceae abundances (Tab. S2.6 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf).

2.3 Discussion

The present long-time study was conducted to identify the effect of L-carnitine and health challenges on the ruminal and duodenal microbiome at an animal individual level. Throughout 168 days, including calving and an LPS induced inflammatory challenge, samples from eight fistulated dairy cows were collected. In addition to the broad phylogenetic analyses of the bacterial communities, 60 metabolites could be quantified in the RUM and DUO fluids. The animals' observed life span included changes in the feed composition, reflected in changes in the microbiota and metabolite composition. Rumen and duodenal microbial community compositions showed very similar trends throughout the trial. This was also reported in culled lambs [92] but not in samples from culled dairy cows [30]. Thus, these outcomes should be discussed carefully. First, the exact anatomic place of sampling is important, especially as in duodenum the pancreatic duct releases host enzymes, which may have a great impact on the microbial cells. Second, DNA sequencing does not differentiate between live or dead cells and we may describe a fraction of dead ruminal cells in the duodenum. In contrast, the metabolomes of both matrices clearly clustered apart from each other, indicating a different mode of action of the bacterial communities and the host metabolism. This includes a higher abundance of fermentation products in the rumen [31, 93] and protein degradation related metabolites (free amino acids, allantoin, dimethylamine) in duodenal fluid samples. As the duodenal fistula was set before the

pancreatic duct, it is assumed that especially the AA greatly derived from bacteria. Concentration differences between RUM and DUO have to be discussed with care, as water, electrolytes, SCFA, and other metabolites are absorbed in the omasum, influencing duodenal metabolite concentrations. Thus, metabolic routes are hardly absolutely quantifiable.

2.3.1 Rumen and duodenal unique characteristics blur due to high concentrate feeding

The introduction of concentrate to the diet after calving resulted in a drop of pH-values in RUM, due to enhanced formation of SCFA and lactate. This may have caused unfavorable, as more acidic, conditions for fibrolytic rumen bacteria supporting preceding studies [87, 94]. This feed change and/or the calving may have resulted into a displacement of the former microbiome by another, which is better adapted to higher digestive passage rates and lower pH levels. The free ruminal and duodenal AA increased, which may either derived from decayed initial bacteriome members, a higher microbial load due to more energy in the diet compared to *ap* time points and/or an increased rumen wall epithelial abrasion [95]. Only before calving and feed change RUM and DUO communities were significantly different to each other. To the best of our knowledge, this observation was not yet reported in the literature. It is not clear whether this bacterial merging of two physiologically different sites at early lactation was driven by the calving itself or the feed change. Since both events co-occurred simultaneously, subsequent effects cannot be assigned exclusively to one or the other cause. It could be suggested that in the future, the origin of this phenomenon could be investigated using a low-performance breed, not in need of a similarly drastic feed change after calving.

2.3.2 Bacterial taxa correlate with high or low diversities

The α-diversity along the timeline demonstrated that bacterial communities can be different between individuals, even though the feeding regimen and other environmental parameters were similar. The ruminal α-diversities increased towards calving and decreased thereafter, which was already found in other studies [96, 97]. Correlating the abundance of genera with the α-diversity values at same time points might uncover bacteria, which actively promote or benefit from a high or low microbial diversity. This was shown for *Olsenella*, which correlated negatively with high bacterial diversities and has been found to increase during high grain induced subacute ruminal acidosis (SARA) [98] as well as after calving [96]. In the present study, significant

positive correlations were observed in both matrices between the Coriobacteriaceae, propionate, and a great number of free AA. It is known that this family forms a variety of aminopeptidases [99], making AA available for other bacteria or the host. Right after LPS injection, *Olsenella uli* decreased (n.s.) in both matrices, accompanied by reduced level of its fermentation product lactate (Fig. S2.11 in DissertationCD\Chapter_2\ Supplementary_Tab_Fig.pdf) [100].

2.3.3 Uncovering an orchestrated bloom of bifidobacteria after calving

Immediately after calving, *Bifidobacterium*'s abundance increased dramatically in both matrices in five out of six animals sampled and disappeared again at +14. Interestingly, *Bifidobacterium* members decreased strongly with the number of lactation or age and were particularly low in RUM of animals that died in the course of the trial namely 039 and 248. The fecal microbiome analyses of these animals was done previously together with other non-fistulated cows. It could be shown that these animals belonged to a group of animals sorted in microbiome clusters, C-Spiro and C-Clos [101], which were characterized by high average days in illness, body condition scores (BCS), body weights (BW) as well as low milk protein and milk fat yields, fecal *Bifidobacterium* abundances, and residual energy intakes (REI) values around 0 or negative. In contrast, animal 306 was observed with considerably high *Bifidobacterium* abundances, and only had some skin issues, but no infectious diseases or claw infections. The fecal microbiome of this animal could be grouped to the third microbiome cluster (C-Bifi) of the previous study, which was characterized by medium milk yields, positive REI values, lowest BCS and body weights (BW) at high milk protein and milk fat yields [101]. *Bifidobacterium* plays a significant role in the gut's development and resilience as they can produce natural bacteriocins, SCFA, and conjugated linoleic acids (CLA) [102]. *Bifidobacterium* species were detected in fecal samples in cows before calving [103] and the vagina of pregnant cows [104]. The transmission of bifidobacterial strains from the cow to calf was described via vaginal transmission during birth [103] and milk [105], and a colonization of *Bifidobacterium* within the calfs' intestine during the first weeks of life was detected [106]. Our data for the first time showed a significant increase of *Bifidobacterium* spp. in rumen and duodenal fluid samples of cows three days *pp,* which has also been found in the fecal samples of the same animals [101]. The calf was permitted to stay with the cow only until one day *pp*, allowing intense care, licking, and suckling. Hereby, it could be assumed, the cow inoculated herself orally with members of this bacterial genus. It can

be hypothesized that this animal-collective increase of *Bifidobacterium* contributes to the controversially discussed entero-mammary pathway [81], supporting the inoculation of *Bifidobacterium* members via milk. Hence, we suggest that the immediate separation of cow and calf after the newborns colostrum intake may be too early as data suggests a maternal initiation of probiotic stimuli for the calf at three days after calving. Unfortunately, the present study didn't investigate the respective calves. The decreased abundance of *Bifidobacterium* species with aging is known for humans [107] and could be also seen in the present work. Yet, a bigger cohort is needed to verify this decline in dairy cows.

2.3.4 The modern dairy cow's dilemma

Cows in early lactation require high energy, which goes along with low feed intakes [108]. Energy density, therefore, has to be increased in the diet leading to an enhanced SCFA production in the rumen (Fig. S2.7A in DissertationCD\Chapter_2\ Supplementary_Tab_Fig.pdf) [56, 87] and a decrease in rumen pH (via increase of SCFA and lactate) [85]. The high energy demands and low glucose availability cause body fat mobilization and lipid accumulation for mitochondrial *β*-oxidation in the liver. Oxaloacetate is in the meanwhile included into gluconeogenesis and therefore is not available to condense with acetyl-CoAs from *β*-oxidation. Therefore, acetyl-CoAs merge and form ketone bodies including acetoacetate, 3-hydroxybutyrate (3Hbut), and acetone [56, 109]. These ketone bodies can be found in saliva, milk, blood [56] and, according to our study, also in RUM and DUO samples. Together with free glucose, ketones positively correlated amongst each other in RUM and DUO ($r \geq 0.7$) and increased in the rumen when concentrate in the diet was increased (Fig. S2.12 in DissertationCD\Chapter_2\ Supplementary_Tab_Fig.pdf). Ketone bodies serve as an alternative energy source in peripheral tissue [110] if glucose is not available [56]. However, if excessively high, they can have neurotoxic properties [111] and lead to ketosis and fatty liver development [109]. Acetone increased significantly in the rumen fluid during early lactation, where it can be eructed through the esophagus or reduced to isopropanol by microbes [112]. Via the hepatic portal route, isopropanol reaches the liver, where it is oxidized to acetone, which again is transferred in the aforementioned body fluids. This might explain the significant correlation between acetone and isopropanol in the present study. Emissions are promoted via breathing [110] and rumination. But as rumination is reduced with higher concentrate levels in the diet due to the reduction of physical effective fiber [108], an accumulation of acetone in rumen

could be measured (Fig. S2.13 in DissertationCD\Chapter_2\ Supplementary_Tab_Fig.pdf) and this may affect microbial membranes in phospholipid content, fluidity, and stress the former microbes. However, this depends on the general membrane structure and may target individual groups of bacteria more than others [113]. DUO samples had significantly higher 3Hbut concentrations; other ketone bodies were lower than in RUM samples. Intestinal 3Hbut was lately reported as an important driver for intestinal cell differentiation and gut homeostasis [114]. Together, the aforementioned processes might lead to a vicious cycle during the first two weeks of lactation and cause higher incidences of laminitis, ketosis, abomasal displacements, and milk fever [115], which can be confirmed by our findings examining animals with increased health issues, including laminitis, skin erosions and udder injuries.

2.3.5 The "microbial airbag" hypothesis

LPS are natural membrane components of gram-negative bacteria, which are highly abundant in the intestine of ruminants, whereby the rumen contains about 26,000 endotoxin units LPS per ml [86]. An intact gut lining prevents leakage of these molecules into the bloodstream, where they trigger the immune system and hereby cause inflammatory processes. With this aim, an intravenous challenge with a low dose of LPS was done at 111 days (+111) after calving when cows were in positive energy balance.

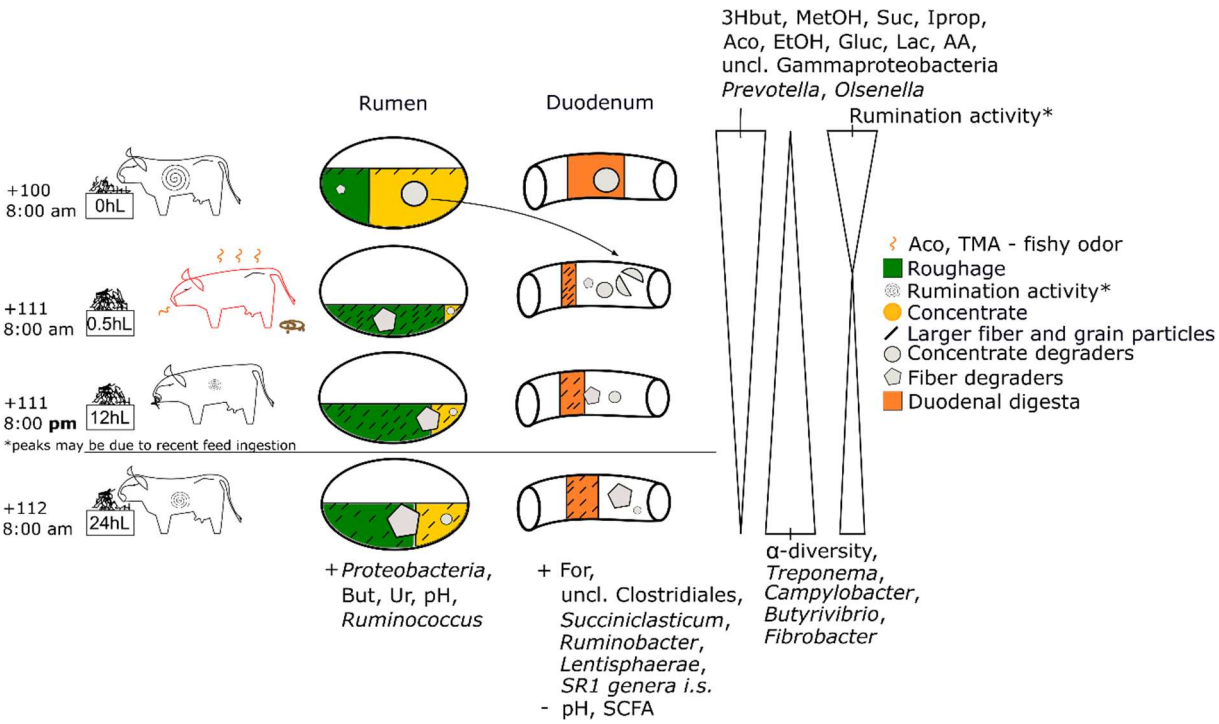


*Fig. 2.8 Microbial and metabolite shifts after LPS injection in rumen and duodenal fluid. Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively. 3Hbut=3-Hydroxybutyrate, Aco=Acetone, But=Butyrate, EtOH=Ethanol, For=Formate, Gluc=Glucose, Iprop=Isopropanol, Lac=Lactate, MetOH=Methanol, Suc=Succinate, TMA=Trimethylamine, Ur=Urea. *Data published in [114].*

Animals were milked and had time for feed intake right before the injection. Immediately after the injection, cows stopped the intake of feed and showed disease symptoms, including fever, diarrhea, muscle twitching, sweating, and a shutdown of rumination and rumen activity for several hours (Fig. 2.8) [116]. It is assumed that simpler carbohydrates in the rumen were quickly degraded by the microbiome, whereas fibers and coarse grain particles were not further mechanically crushed as rumination stopped and gut motility was reduced [116]. This resulted in an increase of fibrolytic bacteria such as uncl. Ruminococcaceae, *Ruminobacter,* duodenal *Treponema*, and *Fibrobacter* as they benefited from the increased retention time and may expressed an evolving, faceted array of bacterial carbohydrate active enzymes. Similar parallels were drawn in steers during fasting [117]. In addition, metabolites such as acetate and butyrate stayed constant or even increased, respectively, during the LPS challenge. In contrast, ruminal glucose, propionate, lactate, ketones, and alcohols significantly declined, which are metabolites related to high milk production and high grain feeding [87] but also to fatty liver disease and ketosis [118]. Interestingly, animals expressed a strong sweet and fishy odor during the first hours after LPS injection, which was probably a result of an accelerated release of acetone and trimethylamine shown to be prominent in RUM and DUO during this time period. It is therefore

suggested that fiber may be an important element in buffering the severity of acute diseases in ruminants. In turn, too small amounts of fiber and therefore material for slow fermentation and energy supply during feed refusal may cause worse outcomes for the animal during a feverish disease.

Fibrobacter succinogenes is the one of the most prominent cellulolytic rumen bacterium and entails increasing microbial diversities due to high cross-feeding activities [119]. Here, it was increased in abundance during the LPS challenge in both matrices. A direct decarboxylation of its primary fermentation product succinate [120] into propionate might have occurred as the latter increased together with its proposed producer such as *Succiniclasticum* [121, 122]. *Treponema* correlated in both matrices with high bacterial diversities and the presence of *Fibrobacter*, matching to previous findings where both genera are associated with high corn levels in the daily ratio of dairy cows [123, 124]. In a different approach, *T. bryantii* was found to be well co-culturable with *F. succinogenes* and *R. albus* on barley straw. Together, they increased SCFA, succinate production and dry matter degradation compared to the pure *Treponema* incubation [125]. This supports the observation in the present work, showing a common increase of these bacteria at stable SCFA production, low rumination activity and possibly higher fiber content in the GIT after LPS injection. The role of *Treponema* members in the ruminant ecosystem needs to be further studied as fluctuations along the trial period are highly related to the patterns of the most important rumen fiber degraders *Ruminococcus* and *Fibrobacter*. Although cellulolytic bacteria are known to be impaired at pH values below 6 [126], *Fibrobacter* members were higher abundant in the acidified duodenal fluid. This can be explained by their fiber attached position, which are, due to less water content after omasum [36], higher in DUO samples. Even though care was taken to use only the liquid fraction of the sample, further DNA extraction steps might have resulted into higher abundances of fiber adhering bacteria in DUO samples.

It is suggested that the aforementioned LPS illness symptoms were mainly affecting the bacterial composition via fasting as described in other studies working with cows in stress and non-intestinal diseases, induced by transportation [127], ketosis [128] or heat [129]. Nevertheless, this bacterial community process was never described as a possible strategy to overcome critical phases. For some taxa, the LPS challenge had a significantly stimulating effect, such as for duodenal uncl. Clostridiales and the rumen Proteobacteria population. Beyond the days of the LPS challenge, ruminal

uncl. Gammaproteobacteria were significantly promoted by this induced inflammation, whereas duodenal Veillonellaceae were significantly decreased. Similar results were observed in human patients with increased stress and irritated bowel disease [130]. Also, increased ruminal abundances of Proteobacteria have been associated with dysbiosis in ruminants [131]. Hence, we assume, that even though health-promoting bacteria, such as *Fibrobacter*, aided the animals in the acute phase of the LPS challenge via fiber fermentation, taxa of potential pathogenic bacteria like uncl. Gammaproteobacteria may have had a benefit from this induced inflammation in the long run.

2.3.6 Fates of L-carnitine, choline and formate in the ruminant ecosystem

In the present study, L-carnitine was supplemented in the feed of four animals to enhance the efficiency in the fatty acid transport into the mitochondria and thus, to likely support the overall physiological capacity of the cows [38]. L-carnitine serves as a nutrient source for bacteria and enhances their robustness in various environments and also can be synthesized by anaerobic bacteria [132]. In the present study, the supplementation did not affect the rumen or duodenal total bacterial community composition nor the metabolome. As a rumen-protected L-carnitine product was applied, significant ruminal concentration differences between CON and CAR animals were not found, and duodenal concentrations were higher compared to rumen fluid samples. High amounts of L-carnitine and choline can increase free trimethylamine (TMA) in the animal, which is further oxidized in the liver to trimethylamine-N-oxide (TMAO) [133]. Genera such as *Olsenella* in both matrices, *Pseudoscardovia,* and uncl. Veillonellaceae in RUM and *Campylobacter* in DUO may be involved in converting L-carnitine and choline to trimethylamine, as they were significantly correlating with the above-mentioned metabolites in the present study. L-carnitine and choline, irrespective of the supplemented group, highly correlate with TMA in RUM but not in DUO (Fig. S2.14 in DissertationCD\Chapter_2\Supplementary_Tab_Fig.pdf), which is why we assume that this conversion rather takes place in the rumen than in the duodenum.

A metabolite with interactions to a large number of genera was choline, which is known to provide an array of health, reproduction and production related functions in the ruminant [134]. For example, it improves lipid transport in the blood and NEFA decrease in the liver. It is mostly ingested via feed in the form of lecithin, which is

hydrolyzed to choline by intestinal mucosal cells and pancreatic enzymes in non-ruminants [135]. Also, choline can be endogenously synthesized and is rapidly degraded in the rumen [136]. However, the present data confirmed that there were significantly (p<0.05) higher choline concentrations found in DUO, either deriving from the microbial activity or a host derived synthesis of choline in between the rumen and duodenum, as no rumen-protected choline was fed [38].

Conclusion

This long-term study showed for the first time how the bacterial communities of cows shift and deal with the challenge of transition into lactation and an LPS injection using a multi-omics approach. Rumen and duodenal fluid bacteriomes were significantly different before calving but thereafter, very similar along a 126-day period. *Bifidobacterium* increased at three days after calving, which was only described for the calf's microbiome before, opening new hypotheses of why this is happening in the cow. The LPS challenge led to a severe illness; nevertheless, data suggests that increasing, health-promoting bacteria cushion the severity of the inflammation, and thereby may act as a microbial "airbag". This is suggested as diversities and rumen lactate concentrations recover to *ap* level, ruminal SCFA concentrations are largely unaffected even though animals stopped feed ingestion during the disease and *Treponema* increased in both matrices. Duodenal fluid samples were, to the best of our knowledge, not yet characterized using NMR analysis, which exposed fundamentally different metabolite patterns than rumen fluid samples. Yet, microbial compositions were highly similar between the matrices underlining the flaws of DNA amplicon sequence data analysis and that microbial similitude does not necessarily imply functional equivalence. Choline was shown to correlate with the broadest set of different genera, whereby formate correlated with different bacteria the most often across all time points and both matrices. *Bacteroides* genus was observed to provide or stimulate the broadest metabolite spectrum, whereby uncl. Succinivibrionaceae was uncovered as the most occurring significant microbial correlator across all time points and both matrices. We hence assume them to act as pivotal elements in the ruminant ecosystem. Further research, perhaps with appropriate cows on pasture all year round and thus on a largely stable diet, even during the transition period, in order to understand the basic biology behind a cow in lactation and its microbiome, is needed.

2.4 Methods

2.4.1 Ethical statement

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Instiut (FLI), in Braunschweig, Germany in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony Office for Consumer Protection and Food Safety, Oldenburg, Germany) (AZ33.19-42502-04-16/2378).

2.4.2 Animal experiment and sampling

This study is part of the cooperative project "Mitochondrial functionality in dairy cows" (MitoCow), funded by the German Research Foundation (DFG) including 59 multiparous German Holstein-Friesian dairy cows [38, 116]. Within this cohort, eight cows were equipped with fistulae in rumen and duodenum. These cows were investigated in the present study. The eight animals were randomly separated into a carnitine (CAR) and control group (CON) (Fig. 2.1). CAR animals received 125 g of Carneon 20 Rumin-Pro (Kaesler Nutrition GmbH, Cuxhaven, Germany), which is equivalent to 25 g of rumen-protected L-carnitine per cow and day included in concentrate feed. This amount is not suggested to result into depression or stimulation of feed intake [38]. CON animals received an equivalent amount of fat (BergaFat F-100 HP, Berg+Schmidt GmbH & Co. KG, Hamburg, Germany) compared to CAR animals, to compensate the Carneon supplementation. During the dry period, silage and hay were offered to the animals with additional access to pasture. Two weeks before initiating the experiment, cows were fed a complete ration for dry cows similar in composition to the control ration fed from day 42 *antepartum* (*ap,* -42). This consisted of 80% roughage and 20% concentrate until calving (day 0) and contained the supplements in the concentrate feed. From the day of calving up to day 14 *postpartum* (*pp*, +14), the amount of concentrate was gradually increased up to a ratio of 50:50. This regimen was continued until the end of the trial. Roughage comprised 70% maize silage and 30% grass silage; water was offered *ad libitum*. Details about the animal experimental setup and feeding are published by for the calving period (days -42 to +110) [38] and the LPS challenge (days +110 to +126) [116]. In short, LPS challenge was conducted at day +111, whereby 0.5 µg/kg BW LPS (E. coli, Serotyp O111:B4, Sigma Aldrich, L2630, St. Louis, Missouri, USA) per cow were injected into the *Vena jugularis externa*. There were no negative controls, all animals were challenged.

Rumen (RUM) and duodenum (DUO) samples were taken regularly at 7 am after milking and at 12 time points between day -42 and day +126, including the sampling time points 12, 24 and 72 hours after calving (12hC, 24hC, 72hC) and an LPS induced inflammatory challenge at day +111 (12hL, 24hL, 72hL) (Fig. 2.1), where the animals are supposed to be out of negative energy balance. RUM samples were collected from the ventral rumen sack using a manual pump, which was thoroughly washed with water between samplings and animals, and fitted with a sieve that filtered larger feed particles. The first spills of pumped rumen fluid were discarded. Duodenal fistulae, placed before the pancreatic and bile duct, were equipped with simple screwing caps. After opening them, the first spill of duodenal fluid was discarded and the further fluid was collected. In total, 76 rumen fluid and 78 duodenal fluid samples with each 30 ml per time point were collected, pH was measured right after sampling (model pH 525; WTW, Weilheim, Germany), and samples were immediately stored at -80°C until further processing. During the trial, animal 039 was euthanized right after time point +14 due to a multifactorial inflammation, and animal 248 died shortly after the samples at time point 12hL were taken after an inflammatory shock. However, samples deriving from these animals have been included in this study.

2.4.3 DNA extraction and Illumina amplicon sequencing

DNA was mechanically extracted from 350 µl sample material following the FastDNA™ Spin Kit (MP Biomedicals, Solon, OH, USA) for soil protocol with minimal changes as described previously [72]. DNA quantity and quality were measured using NanoDrop ONE (Thermo Fisher Scientific, Darmstadt, Germany). The V1-2 region of the 16S rRNA gene was targeted to construct an amplicon Illumina sequencing library using a three-step PCR amplification approach. The first PCR was used to increase the number of amplicons using Takara PrimerStar® HS DNA Polymerase (Takara Bio USA Inc.) and bacterial primers according to Kaewaptee et al. [137] applying a denaturation step for 3 min at 95 °C. This was followed by a 10 x cycling program of denaturation at 98 °C for 10 s, primer annealing at 55 °C for 10 sec and extension at 72 °C for 45 s with a final extension at 72 °C for 2 min. The 2nd and 3rd PCR were conducted to attach barcodes and indexes to the amplicons [137]. PCR amplicons were verified by agarose gel electrophoresis and were normalized using Sequalprep™ Normalization Kit (Thermo Fisher Scientific), following the producers' manual. Samples were pooled per index and purified with MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and the final DNA concentration was measured using Qubit® 2.0

fluorometer (Invitrogen, Waltham, Massachusetts, USA). Samples were sequenced with 250 base pairs (bp) paired-end sequencing chemistry applied on an Illumina MiSeq platform.

2.4.4 Sequencing data analysis

Raw sequence reads obtained from Illumina MiSeq system (Illumina, Inc., San Diego, CA, USA) were analyzed using QIIME2 v 2019 (http://qiime2.org/) [138]. Demultiplexing, quality filtering, and trimming of sequencing reads were done using the default parameters of the pipeline with a maximum sequence length of 360 bp. The resulting dataset went through steps of denoising, dereplication, chimera removal, and merging through DADA2 [139]. Taxonomy assignation was done with a pre-fitted sklearn-based classifier [140], using the SILVA Database (Release 132) (https://www.arb-silva.de/) [141]. A total of 3,270,150 reads were clustered into 19,409 OTUs at 97% identity, further filtered by cutting all OTUs related to chloroplasts (3 OTUs). Additionally, those OTUs with 1 to 10 reads in total across all rumen and duodenal fluid samples and less than or equal to 100 reads per sample were discarded, resulting in 2,658 OTUs and 19,751±1,039 average reads per sample. Six samples with less than 4,000 reads were deleted, resulting in 68 RUM and 75 DUO sequenced samples. The closest representative of each OTU sequence was manually identified using the classifier tool of the Ribosomal Database Project [142, 143] (version 2.11) and the taxonomy levels were screened and adjusted as described by Yarza et al. [144].

2.4.5 Nuclear magnetic resonance (NMR) measurement

A total of 71 RUM and 67 DUO samples were analyzed using the 600 MHz-Bruker AVANCE III HD nuclear magnetic resonance spectroscope as described previously [93]. Sterile filtered and vacuum dried RUM samples were reconstituted using 50 mM sodium phosphate buffer at pH 7, and DUO samples with citrate-phosphate buffer at pH 5, both in deuterium oxide (D_2O, 99.9% Sigma-Aldrich, Germany) containing 5 mM 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (Sigma-Aldrich, Germany). Data were processed using Chenomx NMR Suite 8.2 software [145] (Inc., Edmonton, AB, Canada) to identify and quantify 60 metabolites including, amongst others, fatty, amino and carboxylic acids, amines, and carbohydrates. At a pH of 5, a reliable identification and subsequent quantification of urea and many other compounds is not possible. This

accounts as well to other buffers at a pH of 5 and is not attributed to citrate-phosphate buffer exclusively.

2.4.6 Statistical analysis

PRIMER-E (Plymouth Marine Laboratory, UK) [146] was used for statistical and graphical analysis and the calculation of the Shannon diversity index (α-diversity). The Bray-Curtis coefficient [147] was used to create similarity matrices and principal coordinates analysis (PCO) plots. One-way analysis of similarity (ANOSIM) was used to evaluate similarity between different groups. Other statistical evaluations, such as LSMeans Student's *t*-test and connecting letter reports, multivariate correlation matrices, pairwise correlation (both Pearson product-moment correlation) as well as graphical approaches, were performed using JMP® Pro 15.0.0 software.

2.4.7 Data availability statement

Sequences were submitted to European Nucleotide Archive under the accession number PRJEB41061. NMR data are available from the corresponding author upon request. Supplementary data are provided on the dissertation CD (compact disc) under DissertationCD\Chapter_2\SData. Supplementary tables and figures can be found following DissertationCD\Chapter_2\Supplementary_Tab_Fig.

3 Microbiome clusters disclose physiological variances in dairy cows challenged by calving and lipopolysaccharides

Abstract

Dairy cows respond individually to stressful situations, even under similar feeding and housing conditions. The phenotypic responsiveness might trace back to their microbiome and its interactions with the host. This long-term study investigated the effects of calving, lipopolysaccharide (LPS)-induced inflammation, and L-carnitine supplementation on fecal bacteria and metabolites, dairy cow milk production, health, energy metabolism, and blood metabolites. Fifty-four multiparous Holstein dairy cows were examined over a defined period of life (168 days). The obtained data allowed a holistic analysis combing microbiome data such as 16S rRNA amplicon sequencing and fecal targeted metabolome (188 metabolites) with host parameters. The conducted analyses allowed the definition of three enterotype-like microbiome clusters in dairy cows which could be linked to the community diversity and dynamics over time. The microbiome clusters were discovered treatment-independent, governed by *Bifidobacterium* (C-Bifi), uncl. *Clostridiales* (C-Clos), and uncl. *Spirochaetaceae* (C-Spiro). Animals between the clusters varied significantly in terms of illnesses, body weight, microbiome composition, and milk and blood parameters. C-Bifi animals were healthier and leaner with a less diverse but dynamic microbiome. C-Spiro animals were heavier, but the diversity of the static microbiome was higher. This pioneering study uncovered microbiome clusters in dairy cows, each contributing differently to animal health and productive performance and with a crucial role of *Bifidobacterium*.

Importance

The health of dairy cows has to be carefully considered for a sustainable and efficient animal production. The microbiome of animals plays an important role for the host's nutrient supply and regulation of immune functions. We show that a certain composition of the fecal microbiome, called microbiome clusters, can be linked to animal's health at challenging life events such as calving and inflammation. Cows with a specific set of bacteria have better coped under these stressors as others. This novel information has great potential to implement microbiome clusters as a trait for sustainable breeding strategies.

Keywords: microbiome clusters, dairy cow, metabolome, Bifidobacterium, calving, transition, lipopolysaccharide.

3.1 Introduction

Ever since, dairy cow breeding has focussed on phenotypic ideals with no regard for intestinal bacterial communities, which have evolved within - and the cow itself across evolution. The intestinal symbionts of ruminants are crucial for proper fiber degradation, fermentation, vitamin production, and host immune functions [79, 148]. Even though natural life expectancy can reach up to 20 years, modern dairy cows live between 4.5 - 6 years [149, 150], since production diseases, such as claw lesions and lameness, rumen and hindgut acidosis, ketosis, and reproduction disorders have become more common among herds [27]. These diseases often originate from a non-physiological, high-energy diet necessary for high milk yields, however, it causes perturbation of the established microbiome [27, 79]. This diet malnourishes protective, mucus-stimulating gut bacteria, such as *Bifidobacterium* [63], and promotes potentially pathogenic consortia [151]. Even though it promotes high productivity, this new microbial community is accompanied by gastrointestinal acidosis, followed by epithelial leakiness [152]. The decrease in mucus thickness, soon exposes the underlying bare gut epithelia and increases the risk of infection [27, 63, 152]. Such a "leaky gut" poses open gates for LPS and other immune triggers, reaching the blood stream, and contributing to a latent inflammatory status [27, 86, 151-154]. This cascade of increased intestinal stress makes the host more sensitive to infections or health issues, thereby triggering a downward spiral of the physiological state. The latest studies have associated the aforementioned health issues with an impaired gut microbiome [152, 154], suggesting that the modern dairy industry should focus on maintaining gut health, including its complex ecosystem and integrity, to increase the cows' well-being and performance [152]. Additional stressors, such as calving or infection, can pose the final blow for the animal. In particular, calving, and the subsequent transition period, are the most critical and energy-requiring periods in the dairy cow's life. Energy metabolism in animals is crucial for coping with physiological challenges. However, excessive mobilisation of body fat and enhanced ratios of energy in the diet can cause a metabolic imbalance [155]. Therefore, finding an optimal balance between the animals demands by nature and high performance is a difficult task. Feed supplementation with L-carnitine, a metabolite inevitably necessary for the transport of long-chain fatty acids

into the mitochondria for *β*-oxidation, is suggested to enhance the energetic potential of dairy cows [156]. Cows with an improved energy metabolism could emerge better from stressful phases.

The combined analysis of the fecal microbiome, its metabolites, animal performance variables, and health indicators has the potential to elucidate and understand the cow in its complexity. Hence, the objective of this study was to identify the role of the microbiome in the health of dairy cow during challenging periods. The present work tested physiological and the microbial differences between individuals of the same herd during the calving, transition, and an LPS-induced inflammatory challenge with or without supplementation of rumen-protected L-carnitine (CAR).

3.2 Results

The present study investigated 54 multiparous Holstein dairy cows over 168 days, including calving and an inflammatory challenge induced by LPS injection. Fecal samples were collected at 13 time points to obtain a representative overview of the microbiome changes in the host during two physiological challenges (Fig. 3.1, see DissertationCD\Chapter_3\Data_Sets\Data Set S1-S3). The herd was randomly split into L-carnitine supplemented (CAR) and non-supplemented (CON) cows, defined hereafter as treatment. In general, the time point of sampling had a significant and stronger impact on operational taxonomic unit (OTU)-based community structures (Fig. 3.2A; ANOSIM Global-R=0.298, p=0.0001; PERMANOVA F=6.08, p=0.0001; Fig. S1A,B in DissertationCD\Chapter_3\S_Figures) than the age of the animal (ANOSIM Global-R=-0.001, p=0.504; PERMANOVA F=3.69, p=0.0001) or treatment (ANOSIM Global-R=0.01, p=0.006; PERMANOVA F=2.67, p=0.0001), with drastic changes after calving and feed adjustment (time point +14). Before calving (-14), the microbiomes show the highest within-time similarity (SIMPER average similarity at OTU-level=29.3 %), as greater variations among individual animal communities are seen at all other time points (see DissertationCD\Chapter_3\Data_Sets\Data Set S4_Simper for TimePoint). The highest dissimilarity between time points at OTU-level was observed between 12hC and +42 (86.7 %). The highest genus contribution to time point similarity was observed at 72hC by *Bifidobacterium* (OTU1 and OTU2), accounting for 31.3 % of the total similarity.

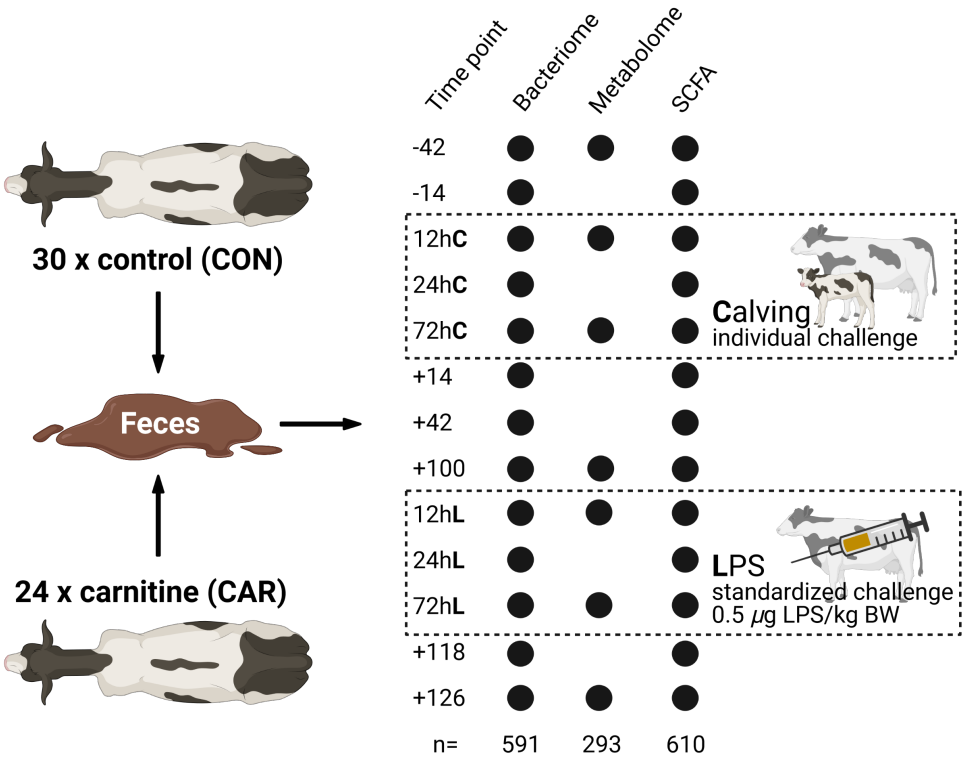


Fig. 3.1 Trial setup of the MitoCow study, including fecal sampling at 13 time points for 16S rRNA sequencing and SCFA measurement as well as at seven time points for Biocrates p180 metabolite analysis. n-Values are the total number of samples measured per analysis. Time points including a "2" or "1" indicate days antepartum or postpartum, respectively, and those including "hC" or "hL" are samples taken at 12, 24 or 72 h after calving or LPS challenge, respectively.

3.2.1 Community dispersion and alpha-diversity cause the formation of distinct microbiome clusters

Changes in nutritional and physiological conditions over a long time period are significant factors to change the microbial composition. But the extend of such dynamic changes may vary at animal-individual level. This was calculated without considering any a priori groups and using the multivariate dispersion (MVDISP) approach. Each animal received a dispersion score depending on its respective samples. This allows for the separation of the herd into a dynamic and static group (Fig. 3.2B) by using the mean of MVDISP=1.000 as the separator. High MVDISP values indicate high heterogeneity between the samples and a more variable bacterial composition, while low values indicate low heterogeneity. Hereby 26 individuals were assigned to dynamic (MVDISP>1.000) and 28 to static (MVDISP≤1.000) over the entire sampling period. In addition, α-diversity calculated as Shannon diversity index matches for the majority of samples with the calculated dispersions, as higher Shannon indexes (≥5.20) are identified for dynamic and lower indexes (<5.19) for static animals (Fig. 3.2C, see DissertationCD\Chapter_3\Data_Sets\Data Set S3_Metadata Experiment).

3.2.2 Clusters of distinct community composition types are identified and linked to dispersion and diversity

Due to the observed differences in dynamics, the hypothesis was that 54 animals could be grouped into distinct community composition clusters among the whole experimental period. The decision to average the datasets per cow was done based on our intention to identify microbiomes which can be linked to animals with good performance and health rather than to describe microbial changes induced by nutrition. Therefore, the enterotype approach of Arumugam et al. [157] was applied, and three distinct microbiome clusters across the herd and experimental period were revealed (Fig. 3.2D and Fig. 3.3, PERMANOVA F=9.10, *p*=0.0001). Eleven animals were assigned to the "C-Bifi" group (CAR:CON=7:4), revealing *Bifidobacterium*, uncl. Coriobacteriales and uncl. Lachnospiraceae as the dominant genera (SIMPER average similarity at genus level=58.4 %; see DissertationCD\Chapter_3\Data_Sets\Data Set S4_SIMPER for TimePoint). The "C-Clos" group, which comprised of 27 animals (CAR:CON=12:15), was colonized by uncl. *Clostridiales*, uncl. Ruminococcaceae and *Oscillibacter* (SIMPER average similarity at genus level=62.4 %). Sixteen animals were dominated by uncl. Spirochaetaceae, uncl. Bacteroidetes and uncl. Haloplasmatales, and clustered into the "C-Spiro" group (SIMPER average similarity at genus level=65.1 %; CAR:CON=6:10). C-Spiro and C-Bifi genera communities were the most disparate across all time points (ANOSIM Global-R=0.334, *p*=0.0001; SIMPER average dissimilarity at genus level=44.2 %), followed by C-Bifi *vs.* C-Clos (ANOSIM Global-R=0.264, *p*=0.0001; SIMPER average dissimilarity at genus level=44.0 %) and C-Spiro *vs.* C-Clos (ANOSIM Global-R=0.079, *p*=0.0001; SIMPER average dissimilarity at genus level=38.3 %). Combining MVDISP and the microbiome clusters results in 91 % of C-Bifi, 48 % of C-Clos, and 19 % of C-Spiro animals, which are sorted into the dynamic group (Fig. S2 in DissertationCD\Chapter_3\S_Figures). Hence, microbiomes of the C-Bifi animals were largely dynamic and C-Spiro largely static, with C-Clos representing an intermediate cluster. Copy number counts of qPCR-derived total bacteria are only significantly different at +14 with C-Clos, showing a significantly higher count than the other clusters (Fig. S3A in DissertationCD\Chapter_3\S_Figures).

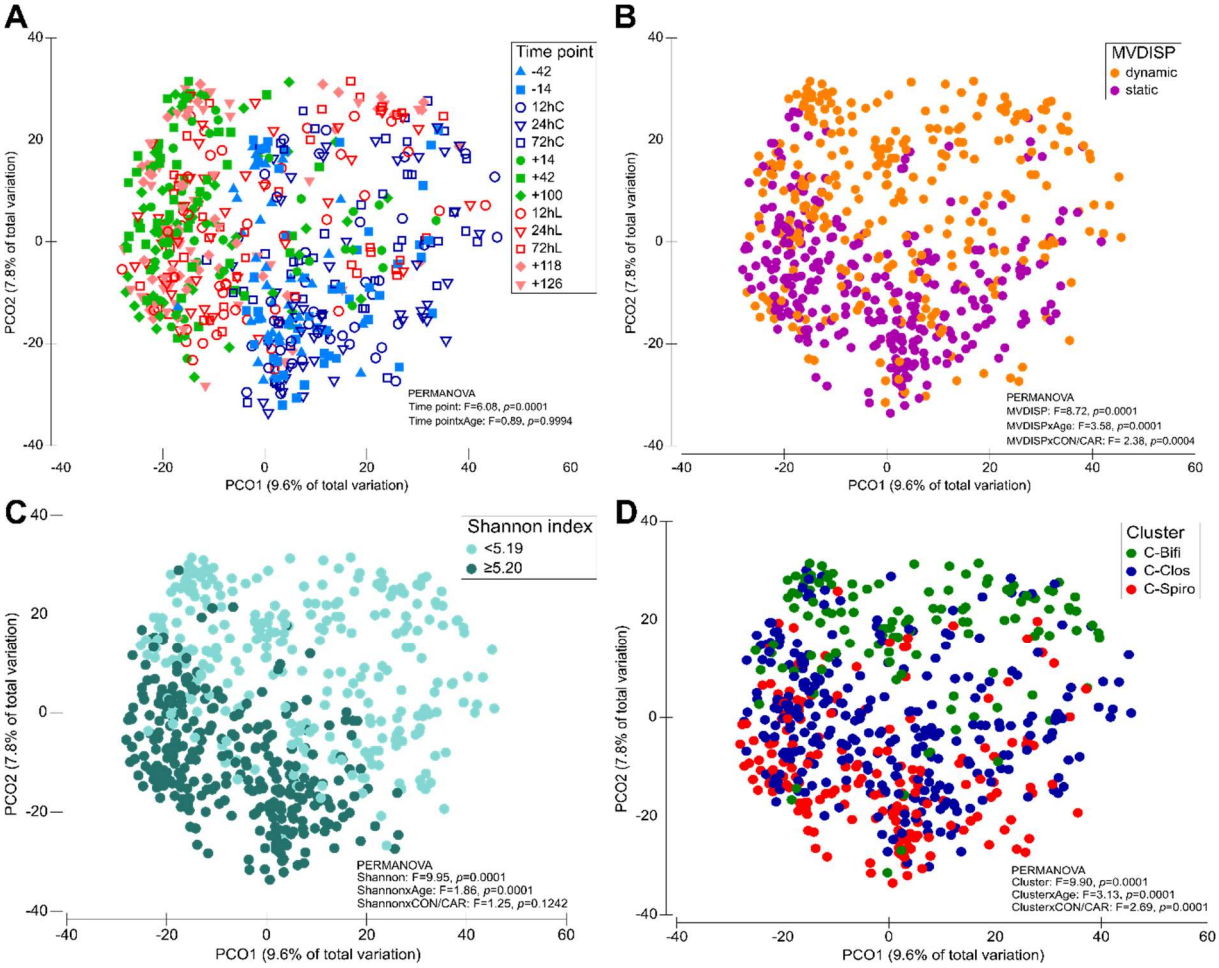


Fig. 3.2 Microbiome dynamics and diversity are associated with defined microbiome clusters. Microbiome analyses included 591 samples of 54 animals with various labels. (A) Time points (time points including a "–" or "1" indicate days antepartum or postpartum, respectively, and time points including "hC" or "hL" are samples taken at 12, 24 or 72 h after calving or LPS challenge, respectively); (B) MVDISP (multivariate dispersion) classification of 26 dynamic and 28 static animals; (C) Shannon diversity indexes separated at the average; (D) animal clusters. PCO data based on Bray-Curtis metrics showed clustering of operational taxonomic units (OTUs).

Diversity across individual time points is significantly higher in C-Spiro, followed by C-Clos and lowest in C-Bifi (Fig. 3.4). A significant decrease in diversity during calving and LPS challenge was observed to different extents. Regression slopes indicated a stronger significant decrease in diversity during calving (-42 to 72hC) in C-Bifi (R^2=0.2, p=0.004) and C-Clos (R^2=0.2, p=0.0001) than C-Spiro (R^2=0.1, p=0.02). The decreasing slope across the LPS challenge (+100 to 72hL) was only significant for C-Bifi (R^2=0.1, p=0.03). ANOSIM analysis reveals significantly stronger effects due to microbiome clusters than due to treatment (see DissertationCD\Chapter_3\Data_Sets\Data Set S4_SIMPER for TimePoint).

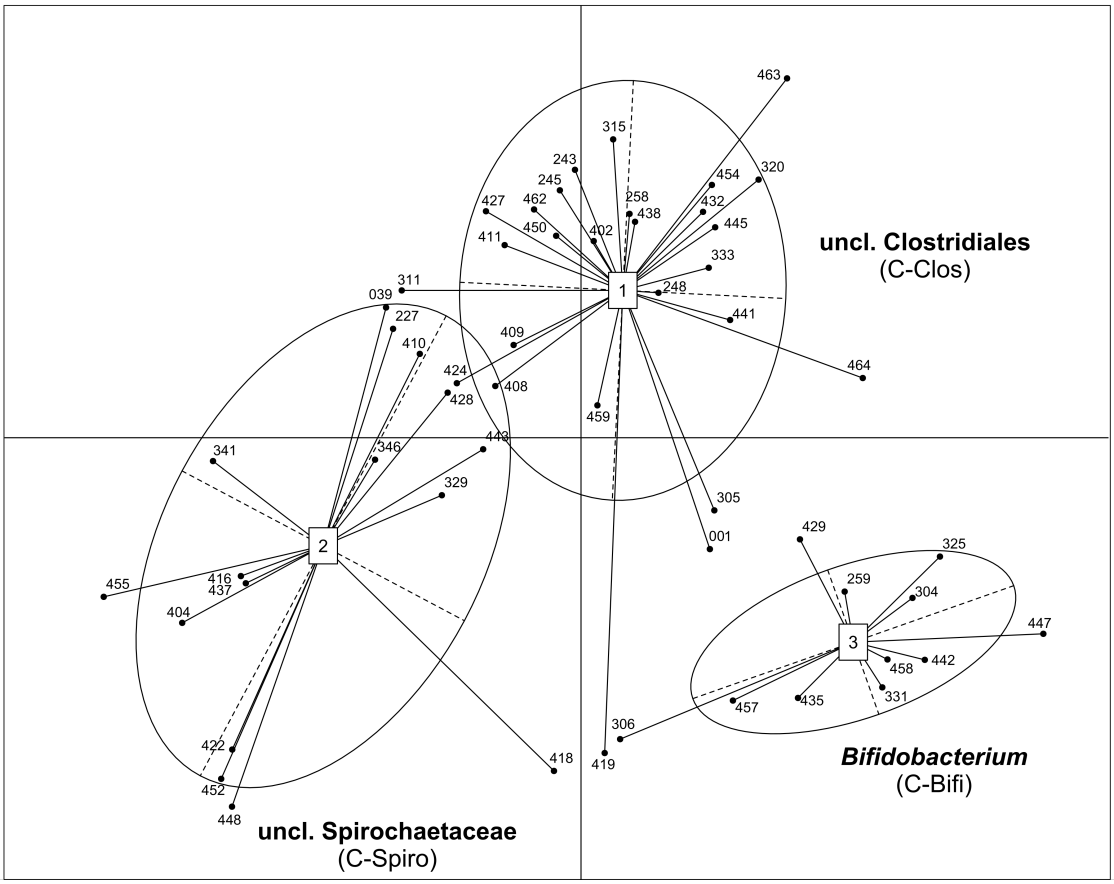


Fig. 3.3 Three clusters of dairy cows within the same herd. Relative abundance genus data were averaged per animal across all 13 time points, resulting in a total of 54 data points included in this R-supported analysis. Labels in the figure indicate the genera with the highest taxon weight of the cluster, as follows: for cluster 1, it was uncl. Clostridiales (C-Clos, n = 27, CAR:CON = 12:15), for cluster 2, it was uncl. Spirochaetaceae (C-Spiro, n = 16, CAR:CON = 6:10), and for cluster 3, it was Bifidobacterium (C-Bifi, n = 11, CAR:CON = 7:4).

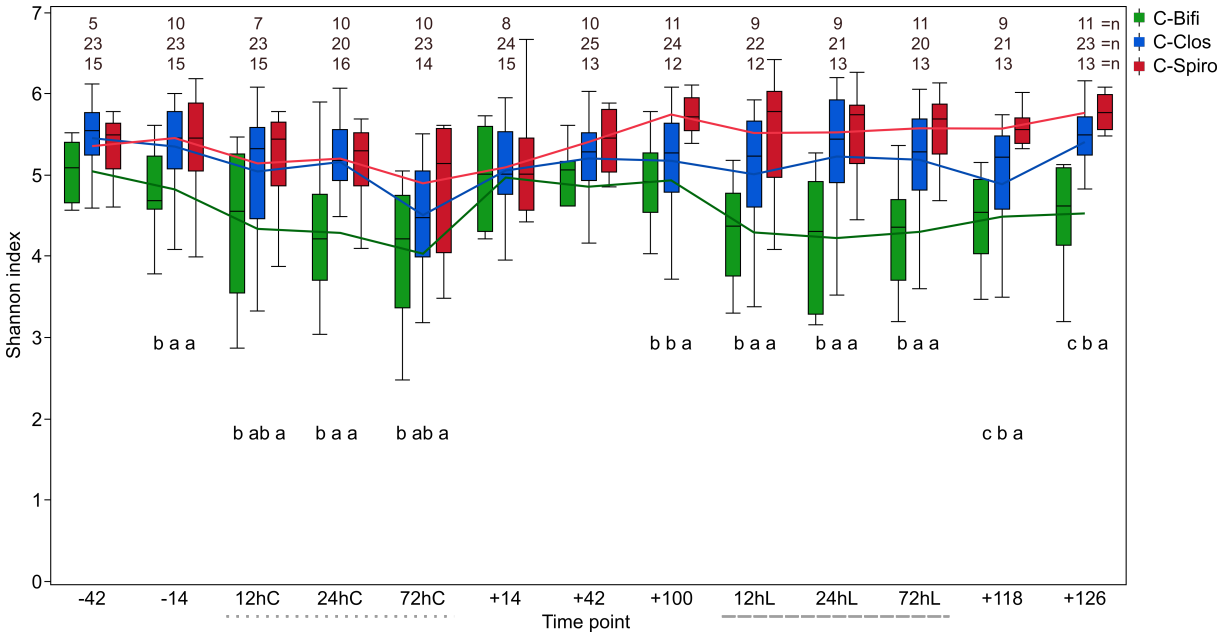


Fig. 3.4 Microbiome clusters with distinct microbial alpha-diversity indices. Shannon diversity indices at the OTU level across 13 time points are given as box plots for each of the three animal clusters. Time points including a "–" or "+" indicate days antepartum or postpartum, respectively, and time points including "hC" or "hL" are samples taken at 12, 24, or 72 h after calving (dotted line) or LPS challenge (dashed line), respectively. Letters below boxes indicate significance by the nonparametric Wilcoxon test (p≤0.05); levels not labeled with the same letter are significantly different. n values are the numbers of cows included per time point and cluster.

3.2.3 Bacterial networks in the microbiome clusters

The main discriminative genus between the microbiome clusters is *Bifidobacterium,* which largely contributes to the total dissimilarity between the three animal clusters (SIMPER average 15.9 %; see DissertationCD\Chapter_3\Data_Sets\Data Set S4_SIMPER for TimePoint). It shows the highest relative abundance in C-Bifi (14.6 %), followed by C-Clos (8.6 %) and C-Spiro (6.9 %) (Fig. S4 and Fig. S5 in DissertationCD\Chapter_3\S_Figures). Copy numbers of *Bifidobacterium* matched Illumina sequencing results in trends (Fig. S6 in DissertationCD\Chapter_3\S_Figures), but are not significantly different between the clusters across different time points (Fig. S3B in DissertationCD\Chapter_3\S_Figures). Members of uncl. Bacteroidales were highest in C-Bifi (28.4 %) than in the other two clusters (C-Clos 23.1 %, C-Spiro 24.2 %), and contributed an average of 13.2 % to dissimilarity among them. Uncl. Lachnospiraceae members were highest in C-Bifi (14.0 %), followed by C-Clos (9.4 %) and C-Spiro (7.9 %), contributing an average of 9.2 % to the dissimilarity. Uncl. *Clostridiales* members were the most abundant in C-Clos animals (15.8 %), followed by C-Spiro (12.7 %) and C-Bifi (9.9 %), contributing an average of 8.2 % to cluster dissimilarity. *Turicibacter* members were lowest in C-Bifi (0.6 %) and C-Spiro (2.9 %) and the highest in C-Clos (4.1 %), contributing an average of 4.6 % to their dissimilarity. Uncl. Ruminococcaceae members contributed an average of 5.4 % to the cluster separation, with higher abundance in C-Clos (9.8 %), followed by C-Spiro (8.0 %) and C-Bifi (5.9 %).

Co-occurrence network analysis on non-parametric Spearman's rank correlation (*r*) offers insights into the main interactions between the dominant cluster genera (*Bifidobacterium*, uncl. Clostridiales and uncl. Spirochaetaceae), and other community members at three levels of interaction (Fig. 3.5). Open circle shapes with overlaying genera indicate 1st level correlations with the respective dominating genus. The inner circle lines indicate genera with 2nd level correlations. From circles outwards directing lines indicate 3rd level correlating genera (solely $|r|\geq 0.5$), which are not directly connected to the respective dominating genus with similar strength. *Bifidobacterium* was negatively associated with 1st level genera in the C-Bifi and C-Clos clusters, but also with uncl. Ruminococcaceae at the 2nd level. Uncl. Marinilabiliaceae was a 1st level member in all clusters, however, it was negatively associated with *Bifidobacterium* in C-Bifi ($r \leq -0.3$) and positively associated with both the other clusters

($r \geq 0.3$). This genus concatenated a wide range of positively correlated genera, such as uncl. Proteobacteria in C-Clos (3rd level), as well as uncl. Peptostreptococcaceae and *Succinivibrio* in C-Spiro (2nd level). Uncl. Spirochaetaceae in C-Spiro showed an enhanced number of positive correlations with genera at the selected threshold compared to the other clusters.

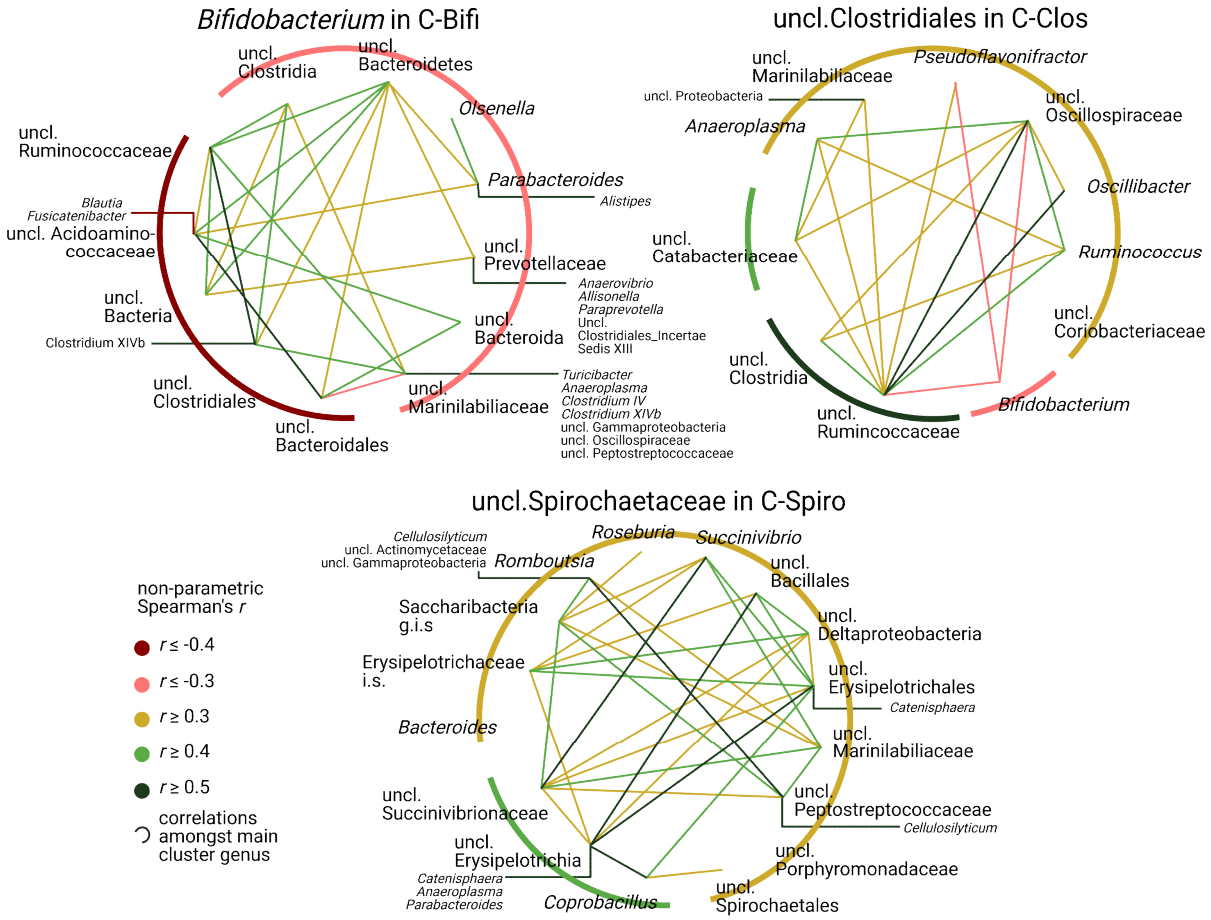


Fig. 3.5 Microbiome clusters are defined by divergent correlations among community members. Circular correlation networks including 591 samples were drawn from nonparametric Spearman's rank analysis (all significant at a P of ≤ 0.006) of microbiome clusters for their respective dominating genera: Bifidobacterium (C-Bifi), uncl. Clostridiales (C-Clos), and uncl. Spirochaetaceae (C-Spiro). Open circle shapes with overlying taxon names indicate first-level correlations with the respective dominating genus. Inner-circle lines indicate genera with second-level correlations. From circles, outwardly directed lines indicate third-level correlating genera (solely $|r| \geq 0.5$), which are not directly connected to the respective dominating genus at a similar strength. For example, Bifidobacterium in C-Bifi individuals is at first rank negatively correlating with Olsenella ($r \leq -0.3$), which in turn was positively correlated at the second level with Parabacteroides ($r \geq 0.4$). Parabacteroides was at the third level positively correlating with Alistipes ($r \geq 0.5$), for which no direct correlation with Bifidobacterium was observed. Correlations were calculated using JMP Pro 15.2.1, and the most important correlations were selected and used to draw the plots.

3.2.4 Fermentation products and microbiome clusters

Short-chain fatty acids (SCFAs) of 610 fecal samples of 54 animals, covering 13 time points, were measured (Fig. S7 in DissertationCD\Chapter_3\S_Figures; Data Set S5_Metabolome in DissertationCD\Chapter_3\Data_Sets). Generally, time had a more significant impact on SCFA (ANOSIM Global-R=0.207, p=0.0001; PERMANOVA

F=21.5, *p*=0.0001) than treatment (ANOSIM Global-R=0.008, p=0.048; PERMANOVA F=5.4, *p*=0.007) or microbiome clusters (ANOSIM Global-R=0.007, *p*=0.265; PERMANOVA F=0.4, *p*=0.83). During early lactation, C-Spiro showed a rapid and enhanced formation of propionate, butyrate, isobutyrate, valerate, and isovalerate. However, during LPS challenge at 12hL, clusters C-Bifi and C-Clos formed higher concentrations of propionate, isobutyrate, valerate, and isovalerate, with a delay at 72hL higher acetate concentrations.

3.2.5 Fecal metabolites and microbiome clusters

A metabolomics approach targeting 188 different metabolites is used to obtain additional information about the host and microbiome function, with respect to time, challenges, and composition (see DissertationCD\Chapter_3\Data_Sets\Data Set S5_Metabolome). A subset of seven time points was chosen to cover the two challenge phases in all animals. Time had a strong impact on metabolite composition (ANOSIM Global-R=0.102, *p*=0.0001; PERMANOVA *F*=11.10, *p*=0.0001) compared to microbiome clusters (ANOSIM Global-R=-0.002, *p*=0.526; PERMANOVA *F*=3.65, *p*=0.0001) and treatment (ANOSIM Global-R=0.016, *p*=0.007; PERMANOVA *F*=3.15, *p*=0.03). The dominant metabolite groups in the fecal samples were amino acids (AA) (>57 %), and hexoses (H1) (>31 %), followed by biogenic amines (>4 %) with alanine (Ala), glutamate (Glu), and glycine (Gly) as major metabolites, which were all highest in C-Bifi. Total fecal metabolite concentration is significantly higher in C-Bifi than in C-Spiro (*p*=0.016), with AA concentrations contributing the most to this difference (Fig. S8 in DissertationCD\Chapter_3\S_Figures, *p*=0.04). *Bifidobacterium* abundance is significantly positively associated with higher levels of histamine (His), Ala, valine (Val), Gly, methionine (Met), leucine (Leu), Glu, isoleucine (Ile), methionine sulfoxide (Met-SO), and symmetric dimethylarginine (SDMA) (Fig. 3.6). Uncl. *Clostridiales* was negatively correlated with PC.aa.C38.6, asymmetric dimethylarginine (ADMA), SDMA, C18:2, His, Ala, Val, Met, and Leu, but positively correlated with lysoPC.a.C14.0. The genus uncl. *Spirochaetaceae* did not show significant correlations with the metabolites. LysoPC.a.C14.0, lysoPC.a.C16.0, lysoPC.a.C17.0, and lysoPC.a.C24.0 were significantly ($p<0.05$) higher in dynamic animals than in static animals. Phosphatidylcholines (PC), irrespective of microbiome clusters, showed a clear gap between time points before 12hC (higher PC.aa.C42.2, Spearman's $r \geq 0.8$) and after 72hC. AA, irrespective of microbiome clusters, revealed a separation between time

points before 12hL and after 72hL (higher His, threonine (Thr), and tryptophan (Trp); all Spearman's $r \geq 0.7$).

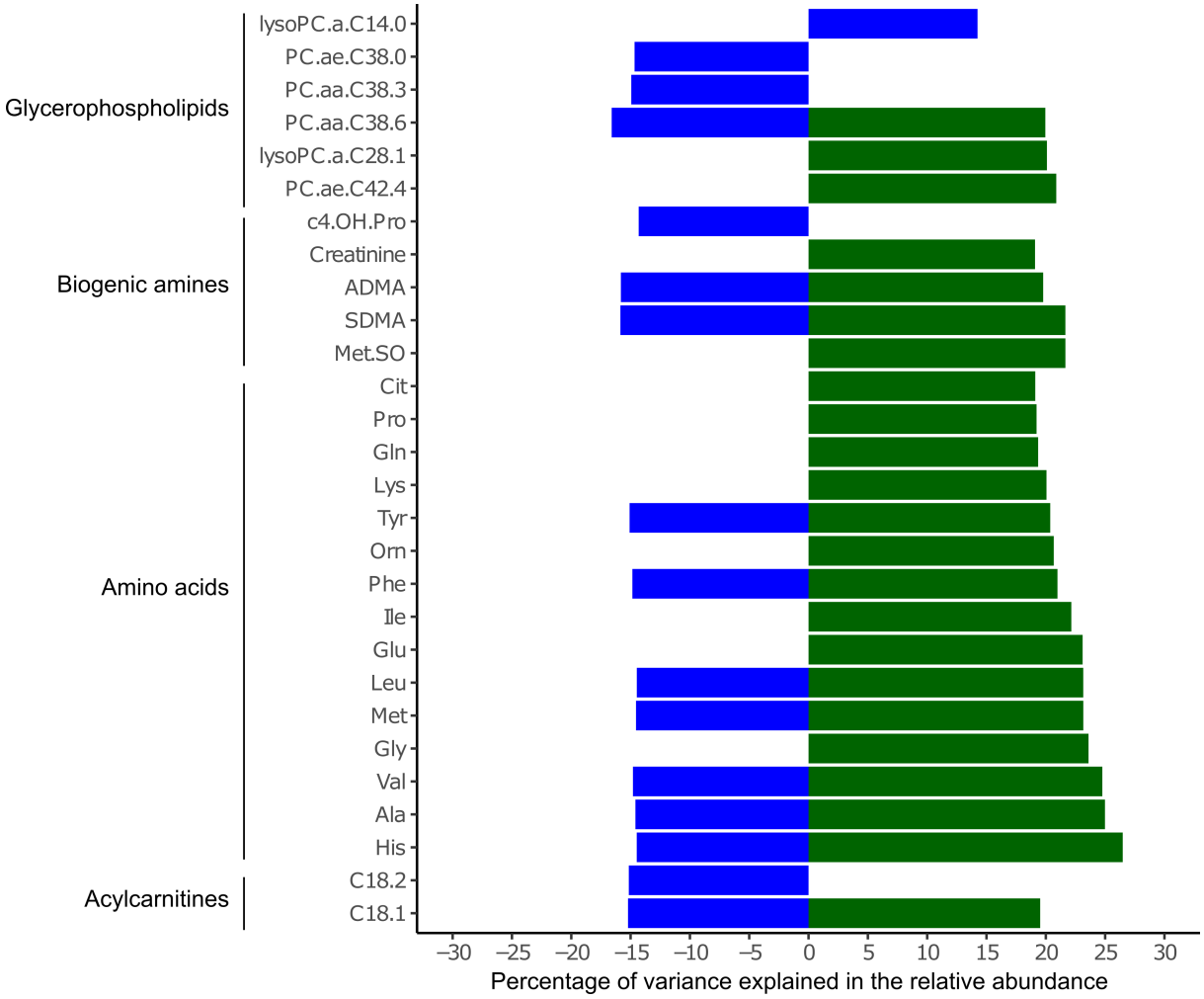


Fig. 3.6 Microbiome clusters are differentially associated with fecal metabolites. Percentages of variance in relative abundance of Bifidobacterium (green) and uncl. Clostridiales (blue) are given for 275 metabolite samples and 47 animals, with significantly associated metabolites (FDR-corrected P = 0.05) derived from the Biocrates p180 panel.

3.2.6 Functional prediction of the microbiome clusters

Rumen-specific functional prediction of the gut microbiome was performed using CowPI precalculated files and PICRUSt. In total, 256 KEGG pathways were predicted among all samples, and 17 AA pathways were manually selected as metabolome analyses, indicating differences in AA profiles. The analysis reveals significant differences primarily in AA-related pathways between the microbiome clusters (Fig. S9 in DissertationCD\Chapter_3\S_Figures). Ala, aspartate (Asp), Glu, cysteine (Cys), Met, and tyrosine (Tyr) metabolism; AA-related enzymes, lysine (Lys), Val, Leu, and Ile biosynthesis are increased in C-Bifi. C-Spiro was higher with amino and nucleotide sugar metabolism and C-Clos with aminobenzoate degradation, D-alanine metabolism, and Val, Leu and Ile degradation.

3.2.7 Microbiome clusters are linked to animal's health records and production parameters

The long-term experimental trial, including calving and inflammatory challenge affected animal health to different extents. Results of daily visual examination of dairy cows are shown in Data Set S6 (see DissertationCD\Chapter_3\Data_Sets\Data Set S6_Health Data) and evaluated with respect to the microbiome clusters and the corresponding animals. Seventy-two percent of C-Bifi animals were ill at least once, struggling with one or more illnesses 6.1 days on average (standard error of mean (SEM): 2.4).

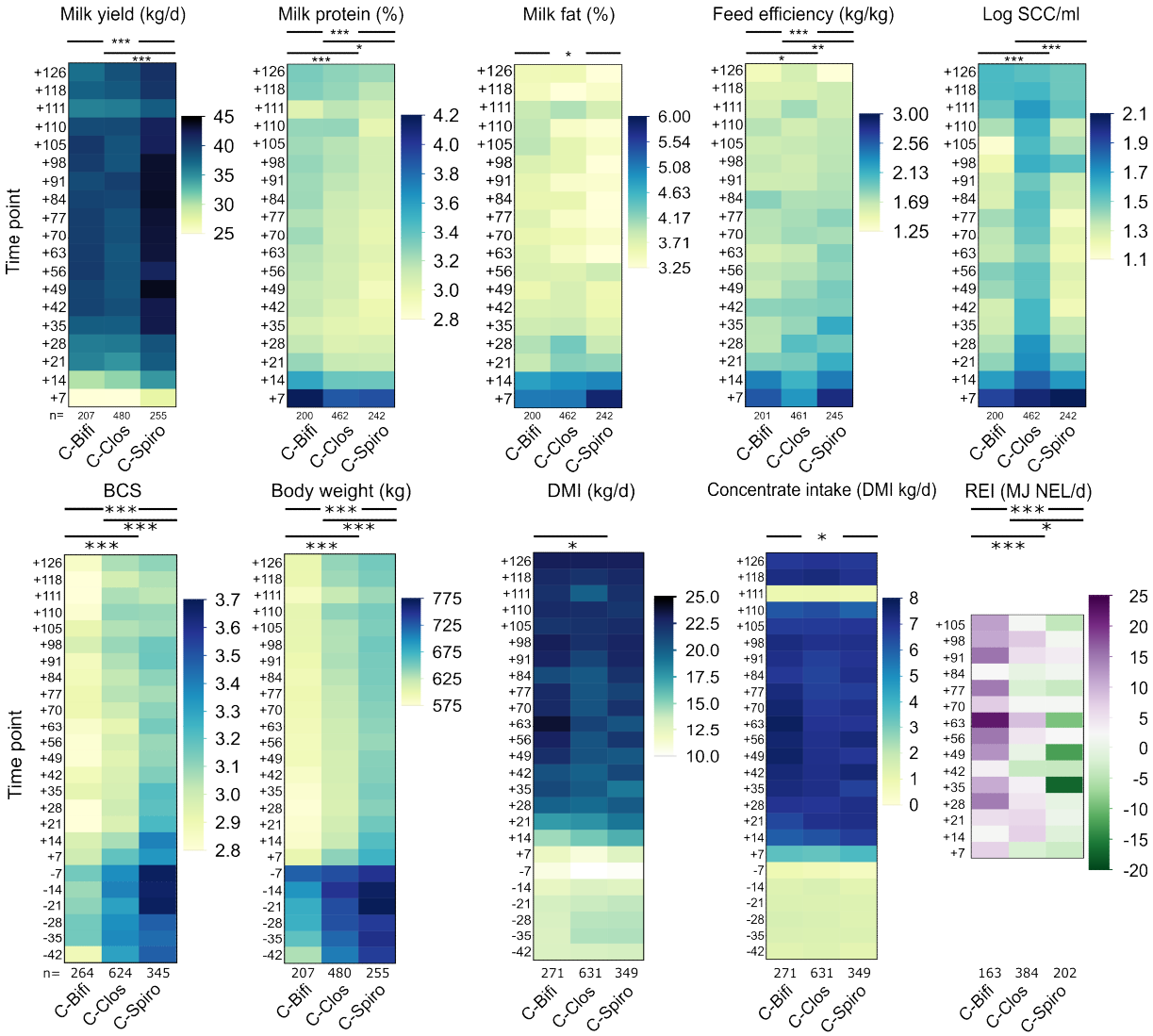


*Fig. 3.7 Microbiome clusters show differences in production and physiological parameters across multiple time points. Significant differences among microbiome clusters C-Bifi (11 animals), C-Clos (27 animals), and C-Spiro (16 animals) are indicated by P values (***, P ≤ 0.0001; **, P ≤ 0.005; *, P ≤ 0.05) derived from the nonparametric Wilcoxon method. n values are the total number of samples averaged per cluster. Time points including a "-" or "+" indicate days antepartum or postpartum, respectively, and time points including "hC" or "hL" are samples taken at 12, 24, or 72 h after calving or LPS challenge, respectively. SSC, somatic cell count; BCS, body condition score; DMI, dry matter intake; REI, residual energy intake.*

Seventy-four percent C-Clos animals were sick 11.4 days on average (SEM: 3.2), compared to 81 % of all C-Spiro animals which were sick 9.8 days on average (SEM: 5.3). Animals belonging to C-Clos experienced a broader spectrum of health issues, followed by C-Spiro and C-Bifi. Milk yields, body condition scores (BCS), body weight (BW), and residual energy intake (REI) are significantly higher ($p \leq 0.0001$) in C-Spiro animals than in C-Bifi animals (Fig. 3.7; see DissertationCD\Chapter_3\Data_Sets\Data Set S7_Production Data). In turn, C-Bifi animals had significantly higher concentrate intakes, REI values, milk protein ($p \leq 0.0001$), and fat ($p \leq 0.05$) concentrations compared to C-Spiro. C-Clos animals had significantly higher milk somatic cell counts (SCC) than both the other clusters, significantly lower dry matter intakes compared to C-Bifi ($p \leq 0.05$), and were mostly intermediate in other parameters. Rectal temperatures were significantly higher at three days *antepartum* for C-Bifi compared to C-Spiro ($p = 0.0293$, see DissertationCD\Chapter_3\Data_Sets\Data Set S6_Health Data).

3.2.8 Influence of microbiome clusters on blood parameters

Blood samples were collected at 37 time points, and 23 time points were included in the present study, covering 62 metabolites and cellular parameters (see DissertationCD\Chapter_3\Data_Sets\Data Set S8_Blood Parameters). The effects of time point and treatment on red blood cell count [158], energy metabolism, and electrolytes [38, 116] have recently been published. Among all the parameters, chloride (Cl) is significantly different ($p < 0.0001$) between each microbiome cluster, with the highest significance in C-Bifi and lowest in C-Spiro (Fig. S10 in DissertationCD\Chapter_3\S_Figures). No significant differences in water intake among the microbiome clusters were observed. In addition, ionized calcium (Ca++) and hematocrit (HCT, measured using Celltac) were significantly different ($p \leq 0.0001$) between each cluster, with the lowest significance for C-Bifi and the highest for C-Spiro. Mean platelet volumes (MPV) were significantly different ($p < 0.0001$) between all three clusters, with C-Clos at the highest and C-Bifi at the lowest level. In contrast, C-Bifi had significantly higher carnitine and gamma-butyrobetaine (gBB) concentration and ferric reducing ability of plasma (FRAP) ($p \leq 0.02$). In addition, this cluster showed a significantly lower mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), glutathione peroxidase (GPx), thrombocrit (PCT), partial pressure of carbon dioxide ($TpCO_2$, temperature corrected), partial carbon dioxide pressure (pCO_2), hemoglobin (HGB), total carbon dioxide (tCO_2), bicarbonate (HCO_3), and non-

esterified fatty acids (NEFA) compared to the other two clusters. For the C-Spiro cluster, blood glucose, lactate, sodium (Na), hematocrit (cHct, measured using blood gas analyser), and superoxide dismutase (SOD) were significantly lower, and trimethyllysine (TML), triglycerides (TG), and beta-hydroxybutyrate (BHB) concentrations were significantly higher than that of the other clusters. Lymphocytes (LYP) were significantly lower; absolute granulocytes (GR), neutrophile granulocyte percentage (GRP), and white blood cells (WBC) were significantly higher in C-Clos animals than in the other two clusters.

3.3 Discussion

The aim of this long-term study was to elucidate the relationship between the dairy cow as a host, its fecal metabolites and microbiome during two challenging periods, and the modulating ability of L-carnitine. Combining these data with blood metabolites and performance data offered insights into different stages of the dairy cow's life, and revealed unknown relationships between the above-mentioned players. L-carnitine supplementation did not affect fecal metabolites or bacterial consortia in the present study. However, CAR animals had higher milk fat and serum triacylglyceride concentrations [38], as well as increased blood platelets and plateletcrit [158] during early lactation. In addition, higher insulin levels and lower NEFA concentrations were observed after LPS injection [116].

As a major novel finding, this study detected three "robust clusters", as previously described in humans and referred to as "enterotypes" [157]. Enterotypes are the strongest separator for microbial community structures in murine models [159], yet have never been reported in dairy cows. The respective microbiome clusters were characterized by different community structures, microbial diversity, fecal SCFA, and metabolite patterns. In addition, the animal's health conditions and performance data were cluster-specific. As previously observed, enterotype distributions varied over time instead of underlying a continuum [160]. Hence, it was decided to refer to them as "microbiome clusters" in the present study.

Finding the "ideal" [161] or "steady state" of the microbiome is a goal in human and livestock animal studies [162, 163] as it is seen as the "healthy" mode. High stability may be beneficial in keeping up inherent functional relationships between the host and its microbiome [164]. However, in the case of strong environmental disturbances, the complete system may be at a higher risk of collapse [164]. The implications of flexible

microbiomes, defined by quick losses and gains of taxa, are receiving more attention. However, the response potential of the host microbiome to environmental changes has not yet been explored [164]. Small sample sizes and the lack of knowledge on physiological fluctuations of microbial communities often do not allow classification into dynamic or static phenotypes. Therefore, the present study offers new insights into the response of static and dynamic dairy cow microbiomes to different challenges. It was observed that animals with a dynamic microbiome had low fecal α-diversities and positive REI values, consistent with the results of previous studies on steers, where high fecal α-diversities correlate with low residual feed intake (RFI) [25]. Animals with a dynamic microbiome, mostly associated with the C-Bifi group, were observed to have higher milk quality (milk LogSCC, protein, and fat), lower body weight, fewer health issues, and lower ketosis risk (low NEFA, BHB, TG; high glucose and carnitine). Therefore, it is suggested that animals with a dynamic microbiome might have a lower risk of ketosis and fatty liver disease. Further research on the flexibility of microbiomes should be conducted to evaluate static or dynamic microbial communities and their implications for the host.

At the start of the lactation period, fiber is considerably replaced by readily fermentable carbohydrates to meet the energy demand of the dairy cow. This increases ruminal SCFA concentrations [27] for energy coverage and subsequent milk production, as well as microbial biomass in feces [165]. Most microbial proteins are absorbed in the small intestine, but also enter the large intestine, where they can be implicated in microbial crosstalk of residential microbes [166]. *Bifidobacterium* and other fiber-degrading bacteria can thrive in the hindgut with remaining microbial proteins and metabolites, complex fibers, and host glycoproteins as nutrient sources. The protein sources were further digested or used for *de novo* synthesis of free AA. In the present study, concentrations of AA in the feces during transition increased with increasing SCFA concentrations. Therefore, high-grain feeding simplified the cascade of digesta degradation, accelerated its passage rate, and confronted post-ruminal sections with a highly degradable substrate [152, 165]. The physiological purpose of gastrointestinal compartments is hereby suspended, and formerly specialized consortia cannot apply their functions [79]. This opens the niche to a more diverse set of invading bacteria, such as facultative anaerobic Proteobacteria [167]. Proteobacteria are involved in inflammaging processes and leaky gut syndrome in mice [168]. In the present study, *Proteobacteria* members were 4.5-fold more abundant in static C-Spiro, than in

dynamic, low α-diversity C-Bifi animals. Interestingly, the latter cluster was found to struggle with fewer health issues compared to C-Clos, which however also included the most individuals. As a result, this may lead to an over-interpretation of the clusters' disease status. Cluster C-Clos covered a much broader range of more cost-intensive health impairments, at highest milk SCC as well as blood MPV, WBC, and granulocytes, which are involved in the immune response during infections and may indicate a disruption of the gut intestinal barrier [154]. C-Clos had higher *Turicibacter* abundances, which were positively associated with high-grain feeding and cecal mucosa damage via IL-6 and IL-12 mRNA expression in goats [169]. This may indicate that enterotypes may be able to group animals according to their inflammatory status [159]. Fecal *Turicibacter* abundances were negatively associated with functional traits such as AA metabolism, biosynthesis of secondary metabolites, enzyme families, and lipid metabolism in a previous dairy cow study [170]. In the present study, high *Turicibacter* abundance was associated with low concentrations of free fecal AA, and a lowered abundance of predicted AA metabolism, which corroborates with previous findings. The respective animal productions were characterized by lower milk protein and milk fat levels. A pronounced increase in *Turicibacter* was observed during the challenging phases for C-Clos and C-Spiro, but was almost absent in C-Bifi animals. The findings of Liu et al. [169] combined with the present results may indicate that C-Clos and C-Spiro might have struggled with intestinal damage. Whether the increase in *Turicibacter* is the cause or consequence of possible inflammation needs to be evaluated.

Clostridiales are involved in AA fermentation in the large intestine, and use ethanol and lactate as substrates to produce CO_2, hydrogen, and SCFA [171], and intestinal CO_2, derived from digestive fermentation, is absorbed from the small intestine [166, 172]. Increased CO_2 levels in the intestine (hypercapnia) promote hypoxia-inducible factor degradation, which plays a major role in intestinal tight junction integrity and mucus stabilization [173, 174]. CO_2 is also produced during hepatic *β*-oxidation and stimulates satiety [175]. Additionally, high blood pCO_2 concentrations have been associated with subacute ruminal acidosis (SARA) [176]. Here, animals with microbiome clusters having a high abundance of uncl. *Clostridiales* (C-Clos>C-Spiro), higher blood pCO_2, and respective OTUs were mostly linked to the degradation of fecal AA, rather than biosynthesis. This is usually accompanied by an increased production of fecal branched-chain fatty acids (isobutyrate and isovalerate) [166], which was not

observed. In addition, saturation signaling via high blood pCO_2 levels in high uncl. Clostridiales animals (C-Clos) are mirrored by significantly lower DMI and concentration intakes, compared to animals with low abundance of uncl. *Clostridiales* (C-Bifi), thereby supporting the findings of previous studies [175]. Together, these results indicate that the microbiome clusters coped differently metabolically and may mirror different health conditions. In addition, high *Turicibacter,* uncl. *Clostridiales*, and uncl. *Ruminococcaeae* abundances may indicate a stiffened and inflexible fecal microbiome with static dispersions and high α-diversity. This was detected in the present study in heavy animals, with impaired energy metabolism (e.g., low blood glucose; high TG, NEFA, BHB), lowered milk quality (LogSCC, fat, protein), and high blood TML concentrations, the precursor of carnitine [177]. C-Bifi was observed with significantly less TML but highest carnitine and gBB concentration and *vice versa* for C-Spiro. Therefore, suggesting that C-Spiro covered carnitine demands via TML upregulation. Both, TML and gBB are known to be involved in cardiovascular diseases [166, 178]. The significantly higher concentration of blood carnitine and gBB in C-Bifi animals might be due to an unbalanced CAR:CON animal ratio (7:4), as significantly higher concentrations were observed previously in the same CAR animals [38].

The fecal microbiome of C-Bifi animals showed an increase in *Bifidobacterium* at 3 days *postpartum*. Other studies on the development of the calf microbiome showed concurrent enrichment with *Bifidobacterium* during the first days after birth[179, 180]. The trigger for this common development is unknown. Increased involvement in AA traits and high milk protein concentrations may indicate that C-Bifi animals possibly provide their calves with higher amounts of prebiotic glycoproteins and possibly even intact probiotic bifidobacteria via the entero-mammary pathway [76, 181]. Milk glycoproteins promote *Bifidobacterium* in the infant's gut, which increases fecal acetate production in human infants [182] and rats [183]. An appropriate acetate increase was confirmed in the present study and during high *Bifidobacterium* abundance in C-Bifi animals. Cows with a microbiome low in *Bifidobacterium,* as well as the conventional premature separation of the calf from the cow, may pose poor initial conditions for the calf and, as data suggests, for the cow at the onset of lactation. To confirm this connection between bifidobacterial strains and *Bifidobacterium*-promoting and/or derived metabolites, a study up to a minimum of seven days *postpartum* should be conducted to examine both, calves and cows. Bifidobacteria are the first settlers in the calf's intestine and produce bacteriocins [184], which may protect against an explosive

or adverse establishment of pathogenic bacteria, which bear the risk of diarrhea, the most common cause of early deaths in calf husbandry [185, 186]. Bifidobacteria may instead allow a controlled settlement of this bare and sensitive niche in new-borns. The same is true for the cow, as bifidobacteria may have protective effects against *Enterobacteriaceae,* obesity during gestation, and ketosis, as observed in women [187]. Largely negative associations of *Bifidobacterium* with other bacteria were found in the C-Bifi and C-Clos animals. This may indicate their ability to modulate the microbial community composition in later stages of the cow's life, possibly via bacteriocin expression. In the present study, animals with lower body weights had higher abundances of *Bifidobacterium*, which has recently been found in human enterotype studies [188]. Furthermore, C-Bifi had negative 3rd level correlations with uncl. Gammaproteobacteria compared to the other two clusters. C-Bifi individuals increased BCS and BW just before calving, to almost the same weight at day -7 as both other clusters. This rather indicates the growth of the fetus in C-Bifi animals, than an increase in body mass, which was different in C-Spiro animals. The latter struggled with higher blood triglycerides (TG) and NEFA levels at low blood glucose levels compared to C-Bifi animals, which suggests a higher fat mobilization, possibly due to negative energy balance [155]. NEFA contribute to milk fat and energy synthesis via *β*-oxidation in the liver [155], brain and skeletal muscle tissue [56]. As milk fat was significantly lower in C-Spiro animals than in C-Bifi animals, NEFA might have largely gone into liver *β*-oxidation, rather than milk fat production. This might have resulted in a higher risk of developing milk fat depression, ketosis, and fatty liver disease in highly productive and "efficient" animals, according to negative REI values [155, 175].

A negative REI has been a breeding target for decades, aiming for highly energy-efficient animals defined by low feed intakes at high milk yields, and therefore high profitability [25, 189], yet at high body mass mobilization. Negative REI values and other breeding targets, such as high milk yields with low SCCs, were found within the static C-Spiro cluster and herein assorted animals would be labeled as "efficient". C-Bifi animals, in turn, would be labeled as "inefficient", due to largely positive REI values [190, 191]. However, C-Bifi individuals recovered faster from LPS injection, indicated by an earlier re-start of SCFA production, quicker fever recovery (not significant), higher blood glucose levels, and higher milk fat and protein levels, at lower BCS, BW, and average days of illness. Hence, the dilemma of modern dairy cows can be addressed by these findings and should be further elucidated by large cohort studies.

Data suggest that grouping dairy cows as "inefficient" and "efficient" according to the REI value, needs to be re-thought, as "inefficient" animals seem to be those with a better fitness. Therefore, breeding positive REI dairy cows may have unbeneficial outcomes for the cow's fitness in the long run.

Previous studies have observed enriched enzymes for protein digestion and AA biosynthesis in the rumen samples of inefficient animals [191]. A similar trend was observed in the C-Bifi animals. However, higher fecal excretion of AA points towards higher environmental pollution potential of C-Bifi animals.

Increased *Bifidobacteriaceae* and largely negative correlation with other families (e.g., *Ruminococcaceae*) have been previously observed in fecal samples of high RFI steers, which together with no significant difference in fecal SCFA concentrations between the groups, is in accordance with the present data [25]. The above-mentioned attributes indicate that higher abundances of *Bifidobacterium* in the large intestine of ruminants might keep the total microbiome more flexible, hence quicker reactions to challenging environmental changes than animals with a more static microbiome.

How is it possible that highly synchronized animals (e.g., parents, diet, reproductive cycle, environment) develop different community structures that have beneficial or harmful health and physiological outcomes? In addition to the supplementation, which did not show strong effects, the trial animals varied by their trial animal history participating in multiple short- and long-term studies, such as milk replacers, antibiotics, or fungal infestation of feed on the cows' physiology. These studies were performed at all life stages of a dairy cow: as calve or mature cow, during gestation, lactation, and rearing. Bifidobacteria and possibly other bacteria are highly sensitive to antibiotics, and long-lasting impacts have been documented, mainly in humans [192, 193]. It is suggested that the identified microbiome clusters may reflect long-term and cumulative effects on manipulated intestinal conditions due to multiple previous trials. As the latest studies discuss host genetic influences on intestinal microbial compositions, it may also be possible that clusters genetically mirror more similar groups. For example, host genetic effects on rumen bacteria have been observed for *Bifidobacterium* and Proteobacteria [194].

The enterotype approach is discussed critically [160]. In the present study, the calculation of the microbiome clusters resulted in a loss of taxonomic information, as an average of all genera per cow was used to obtain a robust cluster formation. Accepting this, the study proves that the power of this analysis lies in its integrative

association with microbiome, metabolite, health, and milk production data and thus has the strength to concatenate blind ends of existing knowledge in dairy cow science.

This long-term study uncovered three distinct microbiome clusters linked to different characteristics of the animal's physical conditions and body parameters. Each microbiome cluster coped differently with a challenging calving period and an LPS-induced inflammatory stimulus. They differed significantly in their bacterial dynamics, composition and diversity indexes, health status, body mass, milk, and blood parameters. The same diet and housing resulted in different community structure outcomes, showing that it is not only the feed itself that matters, but also what the cows' individual microbiome makes out of it. It would be of great interest if cows from regular farms also show microbiome clusters, and if breeding cows with fewer health issues and positive REI values would sooner or later result in herds and animals with higher *Bifidobacterium* abundances.

The study demonstrated that it might not be the stable microbiome animal husbandry should aim for, but rather, dairy cows with a more dynamic microbiome that might be more robust by responding quicker to environmental changes. In the future, the importance of *Bifidobacterium* in lactating dairy cows should be as intensively studied as it is in calves, as this study proved the positive effects of higher fecal abundance of *Bifidobacterium*. Preserving this bifidobacterial community might be a long-term goal, which may yield rich benefits for animal husbandry.

3.4 Materials and methods

3.4.1 Animal experiment and sampling

This study is part of the cooperative project "Mitochondrial functionality in dairy cows" (MitoCow) funded by the German Research Foundation (DFG), including 54 multiparous Holstein Frisian dairy cows ranging between 3 and 7 years of age, and grouped into a control (CON: n=30) and a carnitine supplemented herd (CAR: n=24). Detailed dietary and nutritional composition and study approaches for the calving period [38] and for the LPS challenge [116] have been published previously. In short, 80 % roughage and 20 % of concentrate were fed until calving (day 0) and contained the supplements in the concentrate feed. Until 14 *postpartum* (*pp*, +14), concentrate amounts were gradually increased up to a ratio of 50:50. This regimen was continued until the end of the trial. Roughage comprised 70 % maize silage and 30 % grass silage; water was offered *ad libitum*. Samples were taken regularly at 7 am after milking

at seven time points, between 42 days *antepartum* (*ap,* -42) and 126 days *pp* (Fig. 3.1) as well as at 12, 24 and 72 hours after the calving (hC) and the LPS challenge (hL). This resulted into 13 sampling time points per cow. Calving functioned as an individual, and the LPS challenge at 111 days *postpartum* (*pp,* +111) as a standardized stimulus. At this time point cows are suggested to be out of negative energy balance, which could interfere with the LPS challenge. Before the LPS injection the animals were examined by veterinarians in order to confirm state of health. Each cow received 0.5 µg LPS /kg of body weight, which was applied via *Vena jugularis* to provoke an inflammatory challenge. The cows were head locked at the feeding table during regular sampling and greatly sampled unlocked during the challenges, to reduce stress. Defecation was awaited and the feces collected manually before falling to the ground; using long, disposable gloves and aluminium dishes for temporal storage. The fecal heap was then sampled at three different spots for randomisation reasons using a sterile metal spoon and avoiding the top layer due to excessive oxygen exposure. A total of 626 fecal samples were stored at -80°C, whereby not all samples were included in all analysis. Blood was collected from *Vena jugularis externa* by needle puncture or by indwelling catheters for the frequent sampling during the challenges [116].

3.4.2 Bacterial DNA extraction and amplification for Illumina sequencing

Microbial DNA of 616 fecal samples was extracted using the FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA) following producers' instructions with minimal changes [72]. DNA quantity and quality were measured using NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts, EUA) and subsequently, the DNA extracts were stored at -20°C. The V1-2 region of the 16S rRNA gene was targeted to construct an amplicon Illumina sequencing library amplified using a two-step PCR approach [137]. During the 1st and 2nd PCR, barcodes and indexes were attached to the amplicons using Takara PrimerStar® HS DNA Polymerase (Takara Bio USA Inc.) [137]. PCR amplicons were verified by agarose gel electrophoresis and normalized using Sequalprep™ Normalization Kit (Thermo Fisher Scientific, Waltham, Massachusetts, EUA), following the producers' instructions. Samples were pooled and purified with MinElute PCR Purification Kit (QIAGEN). The final DNA concentration was measured using Qubit® 2.0 fluorometer (Invitrogen) and QuantiFluor® dsDNA System (Promega). Samples were sequenced with 250 base pairs (bp) paired-end sequencing, on an Illumina MiSeq.

3.4.3 Sequencing data analysis and taxonomic assignation

QIIME2 v 2019 (http://qiime2.org) was used to analyze the obtained raw sequences [138]. The default parameters of the pipeline were used to perform quality filtering, trimming, and demultiplexing, resulting in a maximum sequence length of 360 bp. The subsequent dataset was dereplicated, denoised, chimeras removed, and merged through DADA2 [139]. A pre-fitted sklearn-based classifier [140] was used for taxonomy assignation, equipping the SILVA Database (Release 132) (https://www.arb-silva.de/) [141]. Filtration of 11,187,005 reads and 19,409 operational taxonomic units (OTU) (97% identity) was performed by cutting those appearing only once across 616 fecal samples (countif = 1) and that summed up in total 2-10 counts with maximal ≤1,000 reads across all samples (max=2-10). Those OTUs appearing in only 11-20 samples with ≤100 reads were deleted as well. Data filtering resulted in 9,437,285 total reads, grouped into 3,921 OTUs with an average of 15,941 ± 367 reads per sample. The Ribosomal Database Project (RDP) seqmatch tool was used to identify the closest representative of each OTU [142, 143]. Subsequently, the taxonomy levels were assigned following the threshold cut-off values of Yarza, Yilmaz [144]. The blastn tool of the National Center for Biotechnology Information (NCBI) was used to specify the genera uncl. Firmicutes and uncl. Spirochaetaceae for the microbiome cluster analysis.

3.4.4 Microbiome cluster analysis

Sequence data of the samples were clustered based on the mean relative genus abundance of 591 samples representing 54 animals (CON: 30, CAR: 24). The mean was calculated based on all samples per individual (≤13 samples) to detect a global information of the genera contributing to respective microbiome clusters during a defined production lifespan in dairy cows. This resulted in one set of abundance data across all genera per cow (see DissertationCD\Chapter_3\raw_data\R_inputfile_BlastNCBI_fecalsamples_for Enterotype_unclFirmiunclSpiro_54animals). Microbiome clusters among the animals were identified as formerly described by Arumugam, Raes [157] (https://enterotype.embl.de), including the unclassified taxa. Briefly, a Jensen–Shannon divergence matrix was calculated based on the genus-relative abundance using R v 3.6.1 and the "tidyverse" package [195]. After, the partitioning around medoids clustering algorithm was done with "cluster" [196] and the optimal number of clusters, resulting in three clusters, was assessed using the Calinski–Harabasz index

and the Elbow method using "clusterSim" [197] and "factoextra" [198]. Finally, "ade4" was used to perform a principal component analysis of the data and visually explore the clusters [199]. See DissertationCD\Chapter_3\R_script\ Enterotype_script_13tp_NCBInewannotationFirmiSpiro for the code used in R.

3.4.5 Quantitative PCR (qPCR)

Nine time points (-42, 12hC, 24hC, 72hC, +14, +100, 12hL, 72hL and +126) and 11 animals per cluster were randomly selected for qPCR analysis, using the above-mentioned DNA extracts, summing in total 287 samples. Following the principles of Lengowski, Witzig [200], a pooled DNA sample was used as a "sample-derived DNA standard" confirming DNA load using Qubit and Nanodrop. Primer pair products were tested on this pooled DNA standard using a conventional PCR. According to the method described by Lee, Lee [201], a tenfold serial dilution series of each PCR product with six dilutions was used for generating standard curves. For qPCR, two replicates per sample, two negatives, and three replicates of the standard were running on every plate using a CFX Real-Time PCR instrument (BioRad). Quantification of bacterial copy numbers was done using primers 338F 5'-ACTCCTACGGGAGGCAG and 805R 5'-GACTACCAGGGTATCTAATCC with a product length of 468 bp. PCR mix contained 160 nM of each primer, 2.3 mM $MgCl_2$, 3.2 % bovine serum albumin (1 mg/1 ml, BSA), 1x GoTaq qPCR polymerase mix (Promega) and 1 *µ*l of template undiluted DNA. Following conditions were applied to the samples: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C (15 sec), annealing at 50 °C (20 sec) followed by 60 °C for 15 sec (two-step qPCR), and final elongation at 72 °C for 1 min. Thereafter, melting curves were measured at slow heating from 65 °C for 5 sec to 95 °C in 0.5 °C steps. Copy numbers of *Bifidobacterium* were determined using a PCR mixture of 200 nM of each primer (Bifido_5 GATTCTGGCTCAGGATGAACGC, Bifido_3 CTGATAGGACGCGACCCCAT) [202], 1x GoTaq qPCR polymerase mix (Promega), and 1 *µ*l of 1:10 diluted DNA template, resulting into a product length of 236 bp. Cycle conditions were equivalent to total bacteria; however, after denaturation, an annealing step at 60 °C for 1 min was used (one-step qPCR). Total copy numbers per sample were calculated using the standard curves.

3.4.6 Functional prediction

Amplicon data of seven time points (-42, 12hC, 72hC, +100, 12hL, 72hL, +126) chosen in accordance with the fecal metabolite analyses (275 samples and 47 animals) were used to perform a functional prediction of the fecal microbiome using CowPI and PICRUSt in Galaxy as described by Wilkinson, Huws [203].

3.4.7 Short-chain fatty acid measurement

From 610 thawed samples, three aliquots per sample were taken, each weighing 4 g. Samples were homogenised, acidified using sulphuric acid (H_2SO_4), and supplemented with 80 mM of 2-methylvaleric acid in 50 % formic acid as internal standard. The samples were frozen in an Erlenmeyer flask and incubated using a - 30°C ethanol bath under continuous movement. The undissociated fatty acids were distilled with liquid nitrogen under vacuum and 1 ml of distillate sample were used for the determination of acetic (C2), propionic (C3), butyric (C4), isobutyric (C4I), valeric (C5), and isovaleric (C5I) acid. For the analysis of short-chain fatty acids (SCFA) in the fecal samples, gas chromatography (GC Hewlett-Packard 6890; Agilent) connected to a fused silica capillary column (HP-FFAP, 25 m x 0.32 mm, film thickness 0.5 *µ*m HP 7683; Agilent) and a flame ionisation detector (GC-FID) according to Wischer, Boguhn [204] was equipped.

3.4.8 Metabolomic analysis

Targeted measurements of metabolites were performed using 293 fecal samples from a subset of 7 time points (-42, 12hC, 72hC, +100, 12hL, 72hL, +126; samples per time point between 39 and 45 animals). Metabolite extraction was done as suggested by Biocrates Life Science AG using a buffer with a high extraction efficiency for amino acids (AA), biogenic amines, acylcarnitines, and hexoses. The buffer comprised 80 ml ethanol (Supelco, LiChrosolv®) and 320 ml phosphate buffer (20 mM; Sigma, P5244; 0.1 M, pH=7.5 at 25 °C) (v/v)). 200 mg of thawed sample was mixed on ice with 600 *µ*l of Buffer B on a shaker at 200 rpm for 30 min, followed by a centrifugation step for 15 sec at 19,000 x *g*. Thereafter, samples were tip sonicated on ice for 5 min at 100 % amplitude and 0.5 duty cycle (Ultrasonic processor UP50H with MS1 sonotrode, Hielscher, Germany). Cell debris, feed, and other particles were precipitated by centrifugation at 800 x *g* for 10 min and at 2 °C. The supernatant was centrifuged at 19,000 x *g* for 10 min and 2 °C. The clean supernatant was stored at -80 °C until measurements within the next days after processing were done. Fecal samples were

further treated, following the producer's manual for blood plasma samples. Target metabolomics measurements were done using AbsoluteIDQ®p180Kit (Biocrates Life Science AG, Innsbruck, Austria) according to the manufacturer's instructions. Quantified metabolites (188) included amino acids (21), biogenic amines (21), hexoses (1), acylcarnitines (40), glycerophospholipids (90), and sphingomyelins (15). The former two were measured using a liquid chromatography-mass spectrometry (LC-MS/MS). All other metabolites were analysed using a flow injection analysis measurement (FIA)-MS/MS equipping a SCIEX 4000 QTRAP ® (SCIEX, Darmstadt, Germany) or Xevo TQ-S Micro (Waters, Vienna, Austria) machine combined with electrospray ionisation (ESI). The metabolites measurement was described in detail before [205], applying the following adjustments: quantification of the biogenic amines was improved by adding the calibration standard 0.25 to the calibration standard curve. The incubation time with phenylisothiocyanate was extended by 5 min for improved derivatisation of the samples. A nitrogen pressure unit was used to elute the extraction solvent. Afterwards, 50 µl were removed from the filtrate, transferred to a fresh multiwell plate and diluted with 450 µl of 40 % High Performance Liquid Chromatography (HPLC) grade methanol for liquid chromatography mass spectrometry (LC-MS) analysis. For FIA-MS/MS analysis, 10 µl from the filtrate and 490 µl mobile phase solvent were added to a new multiwell plate.

3.4.9 Blood, health and milk production parameters

Heparinized blood samples were analysed immediately after sampling using blood gas analyzer GEM Premier 400 (Werfen, Kirchheim, Germany) [38]. Total blood cell counts were determined in ethylenediaminetetraacetic acid (EDTA) blood samples using an automated hematology analyzer (Celltac-α MEK 6450, Nihon Kohden Corporation, Japan). Blood metabolites (Non-esterified fatty acids, triglycerides, glucose, beta-hydroxybutyrate) were determined in serum samples by using an automatic clinical chemistry analyzer (Eurolyser CCA 180, Eurolyser Diagnostica GmbH, Salzburg, Austria) [158]. Residual energy intake (REI), milk parameters, body weights, and daily visual health examinations by the same veterinarian were recorded and recently published [38, 116]. The average number of sick days per sick cow was calculated, ignoring the quantity of multiple health issues at one day per individual.

3.4.10 Statistical analysis of sequencing data

The total number of reads per sample was standardized by total. Bray-Curtis similarity coefficient [147] was used to calculate and visualize similarity matrices and inter-sample similarity plots (PCO Plots) using PRIMER-E 6 (Plymouth Marine Laboratory, UK) [146]. Alpha-diversity and animal's microbial flexibility along time was evaluated using the Shannon diversity index and multivariate dispersion indices (MVDISP). The average MVDISP across all animals functioned as a separator between "dynamic" (MVDISP>1.000) and "static" (MVDISP≤1.000) individuals (see DissertationCD\Chapter_3\Data_Sets\Data Set S3_Metadata Experiment). Global-R and *p*-values were generated using one-way analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA). PERMANOVA (permutations = 9999) was used to test for significance of time point, age, MVDISP, microbiome cluster and supplementation. The similarity percentages (SIMPER) tool was applied to find the main contributors of differences between groups. Data distribution was analysed using the Shapiro test in JMP Pro 15.2.1 software (SAS Institute, NC, USA). A mixed model analysis was used to evaluated the differences between the predicted functional pathways (CowPI), the animals were included as a random effect nested by the microbiome clusters (275 samples representing 47 animals). Briefly, R (v. 4.0.2) and "tidyverse" [195] were used to transform and arrange the data. Additionally, the linear mixed-effects model was calculated with "ime4" [206], and the differences between each cluster per AA were found using "lmerTest" [207]. Copy numbers of *Bifidobacterium* and total bacteria were obtained by qPCR. Shapiro-test in JMP Pro 15.2.1 software was used to test the normality of distribution of bacteria and metabolites and Wilcoxon/Kruskal-Wallis test was used to test for significance. The same software and herein the Bivariate Fit tool was used to draw regression slopes for the calving period (-42 to 72hC) and the LPS challenge (+100 to 72hL), in order to evaluate how strong each microbiome cluster decreased in α-diversity.

3.4.11 Statistics of metabolomics data

The Biocrates metabolite data were normalized by using the target value of the mean of quality control 2. JMP Pro 15.2.1 software was used to create graphs and to confirm the non-normal distribution of metabolites, including SCFA using the Shapiro test. As JMP does not require deletion of below detection level values (NA), 293 samples and 188 Biocrates derived metabolites were included. Wilcoxon/Kruskal-Wallis test was used for evaluation of significance. PERMANOVA and ANOSIM analysis of the

metabolomics dataset were done using PRIMER-E 6. Working with the Biocrates metabolites in PRIMER-E 6 required a removal of samples which included values below detection level, resulting into 177 fecal metabolites and 275 samples from 47 animals (CAR:CON=22:25).

3.4.12 Correlation analyses

Circular correlation networks on genera across microbiome clusters included 591 samples and were drawn from non-parametric Spearman's *r* multivariate methods, using JMP Pro 15.2.1 software. Only significant ($p \leq 0.006$) and high correlations ($|r| \geq 0.2$) between genera were considered. Third level genera were restricted to $|r| \geq 0.5$. The linear mixed model correlations on genera and fecal metabolites included 275 samples from 47 animals and were corrected by age, L-carnitine supplementation, and time point (see DissertationCD\Chapter_3\R_scripts\LMM_loop for R-script and DissertationCD\Chapter_3\raw_data\Biocrates_normalized_for_R_Metabotype for used raw data). Animals were considered as a random effect and only significant correlating metabolites were included in the figure (False Discovery Rate (FDR) corrected $p < 0.05$). Model was calculated with ime4 [206].

3.4.13 Data and software availability

Finally, the sequences were submitted to the European Nucleotide Archive under the accession number: PRJEB44871. The raw metabolomics datasets are available from the corresponding author on reasonable request. Analyzed data are provided as Data Set S1-S8 on the dissertation CD (DissertationCD\Chapter_3\Data_Sets), so are detailed information on data analyzes and used codes (DissertationCD\Chapter_3\R_scripts).

4 Discussion

The dairy cow's microbial consortia and metabolite compositions throughout the gastrointestinal tract (GIT) during challenging periods of life are rather unexplored. This is due to multiple hurdles, which hinder quick progress in the scientific field of microbiome-metabolite-host intertalk. For example, a great number of intestinal bacteria yet haven't been classified and cultured [208]. Moreover, the ruminant harbours one of the most complex microbial community known in animals and beyond, leading to a great number of bacteria that until now haven't been characterized and assorted on a taxonomic level. Also, cows vary largely between and within herds on their genetic inventory and, or maybe therefore, in their susceptibility towards stressors [27]. In parallel, animal studies see great microbial and metabolic variations between individuals, that can best be buffered by including many individuals into the study. Subsequently, the average metabolite concentrations and microbial abundances across all individuals is focused on, instead of the microbial and metabolite fluctuations of each individual. This offers a certain robustness of the data. The now following difficulty is the interpretation of intertalk between microbial consortia and the host. This takes place via an excessive exchange of a vast bandwidth of metabolites, which are not species or host specific [77, 209]. Understanding who is messenger and who is recipient bears enormous health benefits for modern dairy cow husbandry and therefore economic potential. Also, it could provide strategies for environmental improvements as the cattle and dairy cow husbandry contributes to climate change and competes with the growing human population in terms of diet. Lastly, it is suggested that different microbiomes can fulfill the same functional outcomes within a healthy ecosystem and hence, defining THE healthy microbiome is not straightforward at all [210] and might even be an unresolvable task.

Stressful or challenging periods, as stated above, are phases that can have negative effects on the animal's health, fertility or development [211]. In fact, the modern dairy cow is confronted with a multitude of stressful phases, above all, the recurring transition from dry to lactation as well as microbial infections. Yet, individual cows cope differently with these phases. Whilst parts of the herd manage them well, others develop production diseases and even drop from the herd. Certainly, the different genetic inventories play a great part in this phenomenon, yet also the rumen and gut microbiome might be involved. This is due to its ability of manipulating the hosts

metabolism and immune system [13, 154, 212] and hereby having a direct effect on the production parameters [53, 66]. Hence, analyzing the microbiome and the host-microbiome interplay of metabolites during challenging periods may tell which bacteria contribute to which stages of health or production levels. In the present work, dairy cows were studied during the challenge of calving as an individual stimulus and a challenge of acute illness initiated by intravenously applied lipopolysaccharides constituting a standardized stimulus.

4.1 Changes of microbial communities and metabolites in the rumen and duodenum

Considering intestinal cascades, such as from rumen to duodenum, are of high interest as defoliating bacterial and metabolite evolutions across the GIT of the host take place. The interplay of host, metabolites, and bacteria needs to be evaluated by considering the different environmental conditions and hence their biological purpose within niches of the GIT. The rumen as major site of microbial fermentation and energy conversion is at a high focus of nutritionists [33]. The rumen microbiome has been widely studied, whereas only limited information about duodenal fluid samples is available.

There are different approaches in animal sampling. Slaughtering offers only a single-time point insight into the microbial community, which is a disadvantage compared to rumen tubing or fistulation, a surgically set and permanent application for multiple samplings [213]. The latter offers insights into the core microbiome of an individual [214], which further allows the surveillance of changes due to an induced environmental or dietary stimulus [215].

The digesta of the small intestine is difficult to sample due to limited accessibility, [216] but can be sampled via fistulation or slaughtering. Often, studies fail to state the exact positioning of the duodenal fistula or the place of sampling after slaughtering. Especially at this intestinal section, where the pancreatic duct secretes a variety of different enzymes and digestion molecules and the pH is gradually changing from proximal to distal, it is important to define the exact sampling position to obtain a reproducible sampling procedure.

In the present work, the duodenal fistula was set before the pancreas, what may be the reason why great microbial variations have not been observed between the ruminal and duodenal bacterial consortia across the sampling period, except before calving and feed change. In contrast, in slaughtered cattle, greater community differences have been observed between rumen and duodenal digesta samples [30], whereby the

exact sampling site is not always clearly stated. Yet, as the latter study reported a pH at around 7, it could be assumed that sampling site was located after the pancreatic and bile duct.

Abomasal and pancreatic proteases induce protein hydrolysis and strongly modulate the microbial community by the production of host antimicrobial peptides [42, 217]. Therefore, microbial sampling after the duct might provide a more distinct microbial composition compared to the rumen as for example demonstrated by Mao et al. [30]. Coupling this with metatranscriptomics or - proteomics could additionally enlighten, which bacteria resisted the abomasal, pancreatic, and bile "challenge" and which are actually active at the very start of nutrient absorption by the host.

In sum, even though fistulation at the proximal duodenum gives valuable information about post-ruminal nutrient influx into the small intestine, it could be questioned whether this site is important enough for microbial analysis, as largely covering the ruminal consortium.

4.1.1 The microbial airbag

Which bacteria increase and decrease during calving and a LPS challenge was one question of interest in the present work. Stressful times make the host more susceptible for opportunistic pathogens [218], but it is also known that intestinal microbes can protect the host against them [219].

The present work demonstrates a fecal efflux of amino acids and an increase of fiber-degrading *Fibrobacter* and *Ruminobacter* in rumen and duodenum shortly after the LPS challenge. These have not been dominantly present in the respective periods before. The question arises, if there is a deeper function in this increase. Is it a flush-out effect from previous sections or do they really increase in their functional activity? Even though the latter cannot be answered using 16S rRNA gene sequencing at least the former can be denied, as an increase of these bacteria was also observed in the upmost digestive niche, the rumen.

The LPS challenge was accompanied by a stop of rumination activity and feed ingestion [116] (see chapter 2.3.5). Bacteria depending on continuous feed supply declined, mirrored by the increased amino acids (microbial protein) in feces. It seems that the stop of rumination has a biological purpose, like inducing a clearance or consolidation of the microbial community. Also, a reduced or even stopped rumination offers a longer retention time of fiber in the GIT and consequently more time for the fiber-degrading microbiome, whereby readily fermentable carbohydrates have been

quickly digested. Dietary fibers are commonly known to fulfill a broad spectrum of physiological benefits for the host. Via their fermentation by intestinal bacteria, SCFA are produced, which serve as energy source for the host [23], strengthen the gut epithelium [220], increase the protective mucosal lining [64], and protect against pathogens [221]. Fiber within the GIT is also associated with an improved immune system [212]. Therefore, this "microbial airbag" following a phase of feed refusal and stop of rumination may protect the animal during challenges or times of acute stress against the settlement of unphysiological microbial communities and strengthen the host from the inside. Yet, this is a quite new hypothesis and more studies need to be done to confirm it.

The aforementioned effect may be largely dependent on the quality and composition of the diet, which has been ingested before the trigger. If the animal has largely fed on easily fermentable material before the stop of rumination, it may result in an unphysiological and even unhealthy microbial establishment. This assumption is made as pasture feeding goes along with a lower grade of *E. coli* growth during fasting [222]. Also, high-concentrate diets reduce saliva production that buffers the high fermentation activity, leading to a higher risk for the animal to develop an acidosis. Hence, it can be assumed that larger, fiber-rich feed particles may protect the host better during challenging periods than chopped fibers and concentrates by stimulating fiber-degrading bacteria. It may therefore be beneficial to at least offer effective fiber before and/or during challenging phases – even though this stands in direct conflict with covering the energy demands during lactation.

4.1.2 Bacteria might promote or inhibit microbial diversity

Some bacteria, such as *Fibrobacter* [123], are known to have a great cross-feeding potential and hereby promote a diverse ecosystem. Cross-feeding is a nutritional interaction between community members, in which a microbe synthesizes nutrients for itself but also for another subset of microbes [223]. Other microbes in turn rather compete or even inhibit the growth of other community members and hereby keep the diversity low [223]. If an ecosystem is more diverse, less cross-feeding events are needed to reach a final metabolite and, hence such ecosystems are more energy efficient than less diverse ones [224].

In the present study, *Treponema* was positively associated with high microbial diversities and unlike *Olsenella* decreased with concentrate feeding during early and mid-lactation in the rumen. Interestingly, rumen *Olsenella* populations were associated

with the low-fat marbling and *Treponema* with the high-fat marbling group in Korean beef cattle [225]. As high-fat marbling of skeletal muscle has been associated with an improved immune response [226], future studies could investigate how a more diverse ruminal microbiome may aid in the muscle marbling process and hereby the immune system of the cow.

The present study shows that rumen and duodenal fluid become more similar on microbiome level after concentrate feeding is increased and/or the onset of lactation. It may be assumed that the decrease in complex fibers decreases the bandwidth of intermediate metabolites and, therefore, the microbial diversity. This phenomenon has been already observed [27]. Additionally, it is known that this loss of dietary complexity causes an increased passage rate, in turn causing an increased influx of yet undigested starch into post-ruminal sections. As starch is usually greatly degraded in the rumen, starch degrading bacteria will increase also in post-ruminal sections. This could lead to an increasing similitude of microbial consortia amongst subsequent sections (Fig. 4.1). This may go along with a loss of niche characteristic metabolic processes and possibly cause intestinal and metabolic diseases. Yet, this needs to be evaluated in future with bigger cohorts.

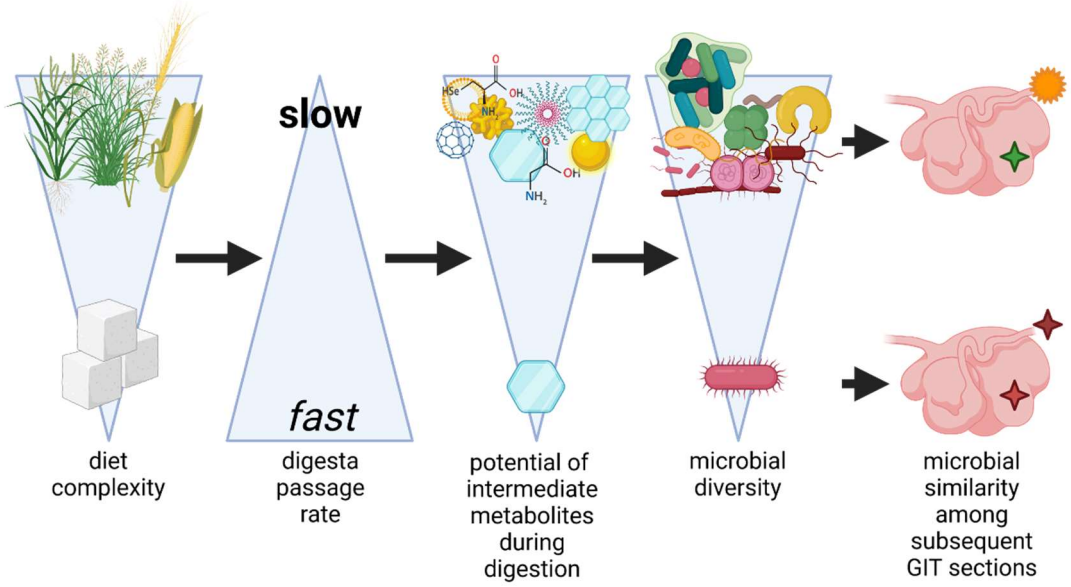


Fig. 4.1 Possible consequence on microbial differentiation among subsequent gastrointestinal sections with different feeding regimen including more or less fiber (diet complexity) on digesta passage rate, diversity of metabolite intermediates and microbial diversity.

4.2 Microbial and metabolite evolutions in fecal matter

Fecal sampling became gold standard in microbiome studies as it is non-invasive, easy, and cheap. Yet, fecal samples are basically a pooled digesta sample from all preceding intestinal compartments and probably rather mirror the microbial communities and metabolomic structures of lower intestinal compartments.

In the present study a great number of fecal samples was collected, which were 16S rRNA sequenced for accessing the bacterial patterns and measured for SCFA, amino acids, acylcarnitines, and other metabolite groups. Together with health and production parameters, this work could cluster three distinct animal groups, demonstrating that fecal samples are sufficient to picture these groups, if enough samples have been collected.

4.2.1 Disclosed microbiome clusters revealed metabolic groups

The observation of three different microbiome clusters that went along with distinct production and health status emphasizes that the hosts microbiome is strongly associated with the hosts physiological capacity. Future enterotype studies should clarify, which factors contribute the most to cluster formation such as genetics, prenatal experiences, diets, antibiotic treatments, mitochondrial capacities and others. Of interest is also whether single nucleotide polymorphism (SNP) genotyping and mitochondrial haplotypes would result in a similar clustering pattern as observed in the present study. If so, the approach here would provide a simple procedure to predict animal groups. The greatest challenge is the interpretation of clusters, as health and disease have multiple faces mirrored by the microbiome. It has to be underlined that the collection of metabolites and production data included in the present work, contributed massively to the observation of microbiome clusters. Yet, it demanded enormous sampling efforts, which by far would exceed the time capacity demanded from herd managers with daily business becoming more and more bureaucratic. Also, the here observed microbiome clusters may not be identical as in other herds, as environments and diets greatly shape the intestinal consortia. Hence, it is of emerging importance to find metabolite-bacteria markers and thresholds in order to understand to which each cluster might respond to, including their metabolic, performance and health implications. This, however, is a highly complex task as metabolites are produced and used by a great range of different taxa from different microbial clades.

Most studies test animals with phenotypic characteristics such as milk production [31] or diseases (e.g. ketosis, abomasal displacement, mastitis, claw infections) and

compare them to a control group to further analyse differences in microbial communities. The present work, however, chose the opposite direction. The herd was grouped according to microbiome community similarities, which uncovered phenotypic similarities (REI, body weight, milk yield etc.). Whether there are stronger grouping effects than those tested needs to be further analyzed using the manifold data collected in the MitoCow study.

There is a broad discussion about the robustness of enterotypes [227]. In the pioneering study of Arumugam et al. [157] a significant robustness of enterotypes was stated, whereby another study suggested enterotypes may not be as robust as blood types but stable along adulthood and largely formed via diet [10]. Knights et al. (2014) claims enterotypes to be continuous and varying within an individual [160]. In the present work, cluster assignments of individuals highly depended on data included to the analysis (e.g. number of time points). However, some bacterial taxa kept their dominance within the respective clusters. It is therefore not yet clear how robust animal clusters or enterotypes really are. Hence, it is suggested to further analyze the characteristics of enterotypes using long term studies on large sample sets to assess how easily individuals switch between clusters.

Human studies using enterotyping approaches used individuals from different countries [157] or the same country but varying environments [228]. The latter study found a strong influence on enterotypes by "long-term dietary habits". The present work included animals, that were environmentally (e.g. housing and diet) and genetically more standardized than the mentioned human studies and still microbiome clusters were observed. This indicates that there must be other influences for the observed animal clusters. For instance, it may be of interest to what extend enterotypes are coupled to the genetics of the host [229].

Keystone taxa [230], "old friends" [231], and enterotypes [157] are all hypotheses pointing to a similar direction. Yet, the former two are unified by the suggestion that a specific set or even single taxa are crucial for the animals or the niches wellbeing and flourishment. If the contact to those taxa is lost, the host or niche loses crucial metabolic potentials or resilience. Enterotypes are uncoupled from a qualitative assessment and therefore it is not clear if there are "better" or "worse" clusters. Also, enterotypes rather mirror actual community compositions instead of selecting single genera or even species. Yet, it is strongly assumed that the inclusion of single taxa

into an ecosystem does not necessarily lead to the unfoldment of the complete potential of the bacterium as it depends on specific consortium members for cross-feeding and signaling actions. Also, the "artificial" inclusion of bacteria does not necessarily lead to a long-term settlement of the specific taxon, indicating that bacterial consortia at the end not only depend on host diet but also strongly underlie host-genetics and the predominance of the enterotype consortium. The present work may contribute puzzle pieces in this field.

Bifidobacterium, uncl. Clostridiales, and uncl. Spirochaetaceae were the leading genera in the observed animal clusters which all belong to different phyla namely Actinobacteria, Firmicutes, and Spirochaetes. As bacteria of different phyla have fundamentally different metabolic characteristics, it might be suggested that these are expressed via the animals different metabolic and productive potentials.

Even though the present study observed multiple parameters that showed very interesting differences between the enterotypes, fecal pH was not measured. This, however, might have answered the question whether there were animals with unphysiologically low or high pH values in the large intestine and if they also grouped according to the microbiome clusters. This is assumed as a previous study on breast fed infants observed strong negative associations between Bifidobacteriaceae and fecal pH values [232], possibly because this family includes (e.g., *B. lactis*) [182] or supports (e.g., *B. breve*) [183] acetate and lactate forming genera. Clostridiaceae and Peptostreptococcaceae in turn were positively correlated with alkaline pH values [232]. All three taxa in this work were highly abundant in C-Bifi, C-Clos and C-Spiro clusters. Therefore, it is suggested to regularly include fecal pH measurements in upcoming studies, as it might be a particularly interesting biomarker for an unbalanced gut environment.

4.2.2 Dynamic and static microbiomes may function as health parameters

It is generally accepted that an alteration of the gut microbiome is associated with disease [210]. The rumen ecosystem was described as "stable and at the same time dynamic" [209]. Other studies tried to assess the time the rumen microbiome needs to stabilize after a nutritional change [162]. The present work uncovered that the fecal microbiomes of animals belonging to the same herd and environment show differences in their "reactivity" across time.

Significantly different microbiomes between babies and adults have already been found in humans [107] this is also true for the cow. The calf's microbiome is completely different to that of a lactating dairy cow [233], what may largely be due to fundamentally different feed sources [234]. Dynamic adaptation of the microbiome has been demonstrated also in shorter periods of time, in feeding [235], and environmental [236] studies. Yet, it is quite new, that herd individuals exposed to the same environment group according to their responsiveness towards triggers. The question arises whether there is a qualitative difference between these groups, obtaining a static or dynamic microbiome. Is it beneficial to react quick or rather slow to environmental triggers? In fact, those animals showing dynamic microbiomes were those showing higher production and better health parameters and hereby challenge current knowledge as those animals were also those with a low fecal Shannon diversity. The present work proposes that there might be biological consequences or causations according to the reactivity of the microbiome, that might be worth further evaluation.

The decision for using the Multivariate Index of Dispersion (MVDISP) approach arose after identifying animals that did not vary too much in their microbiome and that even died during the course of the trial. However, this approach has not been widely used in dairy cow studies until now. This might also be due to the fact that studies with perspective on the individual animal are not very common and animal numbers may not be sufficient enough to observe these herd variations. Yet, this procedure was very easy, as it is embedded in the PRIMER-E package and could in future be used as a routine investigation for larger cohorts and interest in individual animal reactions. One disadvantage in this procedure is that the chronological sequence of samples is not considered but rather the dispersion of all samples of one individual. Yet, it was ground-breaking in indicating the microbiome clusters, which might not have been found without this mathematical analysis.

4.2.3 Breeding for efficiency implies breeding towards a specific microbiome

The residual energy intake (REI) is the estimated difference between the actual and predicted energy intake that is required for lactation and maintenance of an animal [237]. The population average resides at 0, energy efficient animals have negative, and inefficient ones have positive REI values. The residual feed intake (RFI) follows generally spoken the same principal, but is rather used in fattening animals. Selecting for animals with negative REI may soon result into a population of animals that foster

highly energy efficient rumen microbial consortia [191]. These produce less methane and have better energy and carbon channeling [191]. Yet, there are not many studies on how this selection influences the fecal microbiome and also health parameters. The present work suggests that there is a strong redundancy of REI groups and microbiome clusters that were calculated independently from each other. This may demonstrate that genetics and microbial communities are inherited hand in hand to a yet unknown degree.

4.3 A holistic view on the dairy cow's gastrointestinal tract

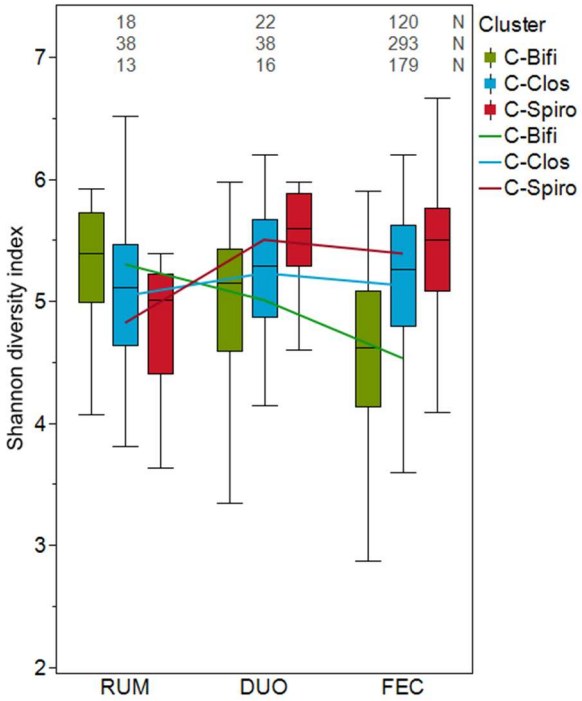
Sampling the upper gastrointestinal tract of the ruminant poses a much greater difficulty than collecting fecal samples. Rumen and duodenal fistulation, as well as rumen tubing via mouth are applied techniques, but in case of the fistula highly expensive and elaborate. However, as this offers collecting samples from the same animal across multiple time points, it bears much more potential in understanding the dynamics of the ruminant ecosystem. Often, animals are sampled after culling, which is quite cheap. This, however, has flaws as the microbiome might be distorted through pre-culling stress or post-culling movements (e.g., hanging) of the carcass and therefore causing unphysiological movements of the body fluids. Therefore, results from culled and live animals can differ and should be compared with care. Studies analyzing all three matrices are very rare, therefore the present work might contribute to the knowledge of dairy cow physiology and microbiology.

4.3.1 Rethinking the Shannon diversity index

Rumen, duodenum, and large intestine vary greatly in their physiological purpose and therefore harbour different microbiomes [238] – but also different microbial diversities? The Shannon index as a measure of diversity of an ecosystem [239] is a commonly used value in microbiome studies as the scale and implications are easy to understand and the calculation adaptable for any ecosystem of interest.

The present work shows that robust animals with good milk quality, medium milk yield, high body weights, and positive REI values are those with continuously decreasing microbial diversities (Fig. 4.2) from rumen fluid to feces, similar to the findings of Mao et al. [30] (Fig. 1.1). The C-Spiro animals, with increasing diversities from caudal to rectal matrices, had negative REI values, high body weights, low milk fat and protein at highest milk yields. Welch et al. [25] compared high and low-RFI steers and observed no difference in diversity between rumen samples, but, similar to our findings,

higher fecal diversities in low- compared to high-RFI animals. How this unusual slope of microbial diversity (compare with Mao et al. [30]) may contribute to, or result in the C-Spiro phenotype could be a subject of future studies. As the present work indicates that the group showing the highest bacterial diversity in feces is not necessarily the healthier one, the Shannon diversity needs to be discussed more carefully. If possible, it may also be valuable to look at the diversity slope along the GIT rather than on the single matrix diversity.



Level	- Level	Std Err Dif	*p*-Value
RUMC-Spiro	DUOC-Clos	4.77659	0.0351*
RUMC-Spiro	FECC-Clos	25.07803	0.0280*
RUMC-Spiro	RUMC-Bifi	3.30898	0.0182*
DUOC-Spiro	DUOC-Bifi	3.65115	0.0166*
FECC-Clos	DUOC-Spiro	22.93791	0.0124*
FECC-Spiro	DUOC-Bifi	13.14138	0.0083*
RUMC-Clos	DUOC-Spiro	4.68849	0.0062*
FECC-Bifi	DUOC-Bifi	9.54031	0.0027*
RUMC-Spiro	DUOC-Spiro	3.17934	0.0009*
RUMC-Clos	FECC-Spiro	11.21443	0.0005*
RUMC-Spiro	FECC-Spiro	15.96206	0.0002*
RUMC-Clos	FECC-Bifi	8.51688	0.0001*
FECC-Spiro	FECC-Clos	12.93960	<.0001*
RUMC-Bifi	FECC-Bifi	10.10573	<.0001*
FECC-Bifi	DUOC-Spiro	10.48712	<.0001*
FECC-Bifi	DUOC-Clos	8.51689	<.0001*
FECC-Clos	FECC-Bifi	12.93702	<.0001*
FECC-Spiro	FECC-Bifi	10.20054	<.0001*

Fig. 4.2 Shannon diversity indexes of rumen (RUM, C-Bifi in green N=18; C-Clos in blue N=38; C-Spiro in red N=13), duodenum (DUO, C-Bifi N=22; C-Clos N=38; C-Spiro N=16) and fecal samples (FEC, C-Bifi N=120; C-Clos N=293; C-Spiro N=179). The table indicates Wilcoxon test at p≤0.05. Asterisk (*) indicates significance.

Sampling of dead *vs.* alive animals, working with different extraction kits, sequencing regions, and/or OTU clustering approaches can cause differences in Shannon values even if using the same sample matrix. For example, Shannon diversities of digesta from culled animals were found to be highest in the rumen and decreased dramatically in the further sections, whereby duodenal and rectal samples were similar in their diversity [30]. This is in contrast to the present results, as all three sections had similarly high Shannon diversity indexes, rather mirroring the diversities of the mucosal samples [30].

In the present work, the amplitudes throughout the study period were greatest for the rumen and lowest for the fecal samples (compare lines of the mean in Fig. 4.3), indicating a stronger sensitivity of the ruminal community towards the studied

challenges compared to the fecal microbiome. Therefore, the challenges, which have been selected, were able to disturb previous microbial communities (Fig. 4.3), as in former studies no significant day-to-day variations of Shannon diversities in rumen and fecal samples of unchallenged lactating dairy cows were observed [170].

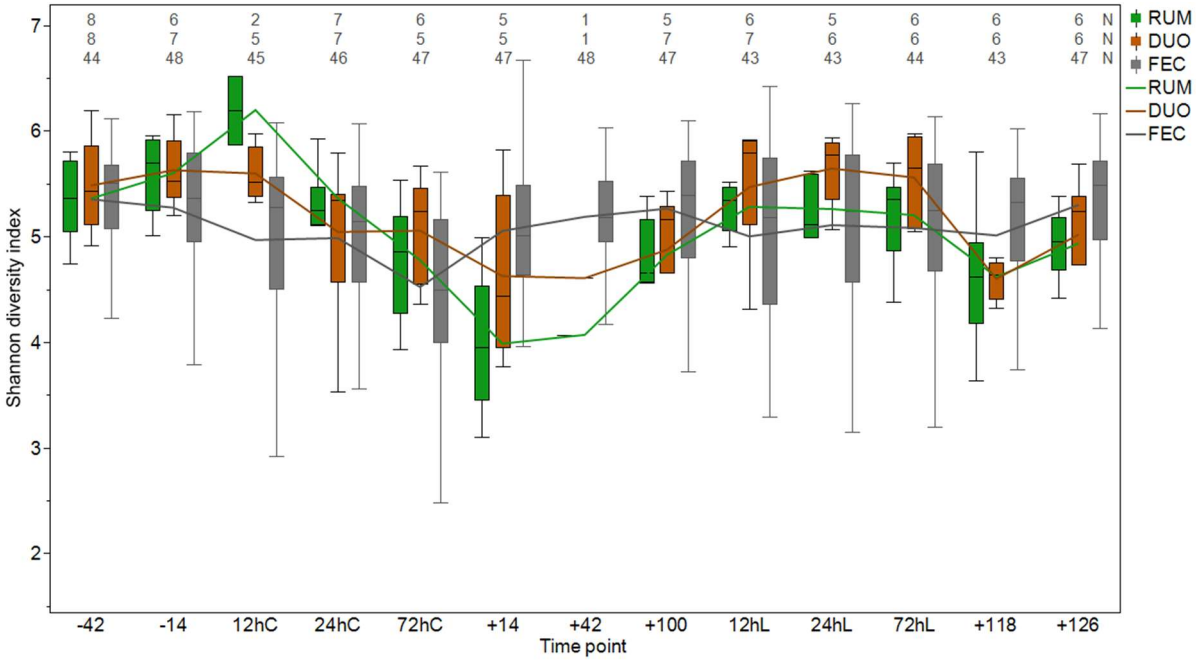


Fig. 4.3 Shannon diversity index along time for rumen fluid (RUM), duodenal fluid (DUO) and feces (FEC). Time points including a "–" or "+" indicate days antepartum or postpartum and time points including "hC" or "hL" are samples taken at 12, 24 or 72 hours after calving or LPS challenge, respectively. Lines connecting time points indicate Shannon index means along the trial period for each matrix (green=rumen fluid, orange=duodenal fluid, gray=feces).

4.3.2 Dominating genera of microbiome cluster in all three matrices

The analyses of the sequencing data showed three bacterial taxa which seem to be key players for the microbiome along GIT, namely *Bifidobacterium*, uncl. Clostridiales, and uncl. Spirochaetaceae. As these genera were detected in the feces, it would be of interest how high their abundances were in the upper digestive tract. Since the sample number is very low, this can only be discussed with some caution.

Bifidobacterium in rumen, duodenum and feces were significantly higher in C-Bifi animals than in the other two clusters (Fig. 4.4). This may indicate that the abundance of this genus in the upper GIT may also be predicted by looking at the fecal abundance. Interestingly, the abundance along the GIT increased in high-RFI steers stronger than in low-RFI steers [25], matching the present results.

For uncl. Clostridiales, the picture is a different one. The highest abundance of this genus was observed in rumen fluid samples of C-Clos animals but in duodenal samples for C-Bifi animals. In bovines fed forage instead of concentrate based diets

[240], uncl. Clostridiales was found having the highest heritability among rumen microbes in beef cattle [229]. This may be the reason why this cluster included the most animals. Also, uncl. Clostridiales are proposed to be involved in ruminal biohydrogenation, as associated with the production of C18:0 [241]. Hence, as low ruminal uncl. Clostridiales prevents excessive hydrogenation and hereby increases concentration of unsaturated fatty acids available for milk fat synthesis, this may argue for C-Bifi producing higher milk fat concentrations than both other microbiome clusters. The role of uncl. Clostridiales members in dairy cows need to be further studied in future works.

Uncl. Spirochaetaceae are often associated with digital dermatitis and other diseases in cattle and were highest in the feces C-Spiro cluster. Unidentified Spirochaetaceae have even been associated with an increased susceptibility of dairy cows to develop subacute ruminal acidosis (SARA) in a recent rumen fluid study [242]. Together with the results gained from the present work, it can be suggested that there might be health implications depending on which cluster a cow is in.

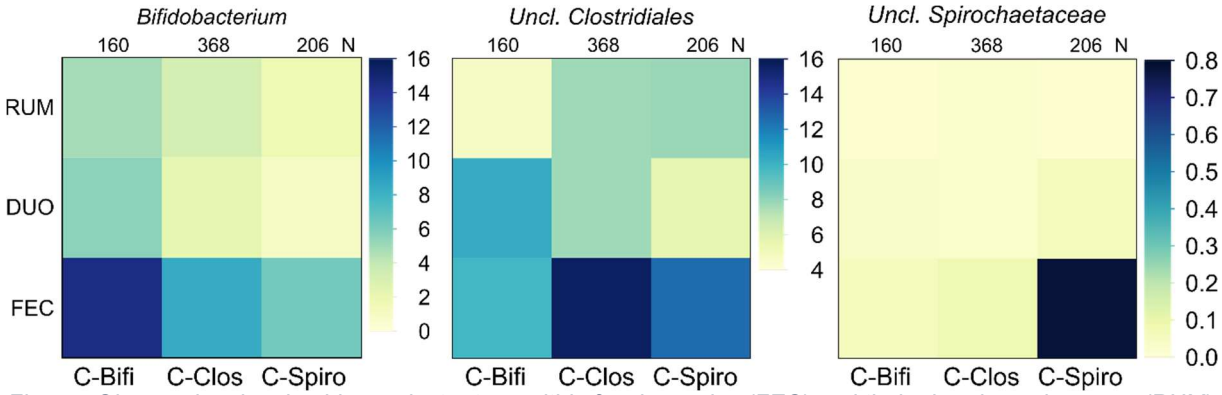


Fig. 4.4 Observed main microbiome cluster taxa within fecal samples (FEC) and their abundance in rumen (RUM) and duodenal fluid (DUO) samples in the animal clusters C-Bifi, C-Clos and C-Spiro. N refers to sample number included per microbiome cluster.

4.3.3 Bifidobacteria in the multiparous dairy cow

Bifidobacteria are gram-positive, non-sporeforming, non-motile bacteria [243] and the first settlers of the calf's gut [186]. Specific, complex oligosaccharides in the milk of humans and bovines are fermented as main substrates for *Bifidobacterium* species [244], their abundance being strongly dependent on diet and age [245]. As milk is the only significant food source for mammals in the first weeks of life, and Bifidobacteria are dominating the newborns GIT, it is suggested that non-milk-oligosaccharide degraders cannot grow under this bifidobacterial dominance and environment [244]. Calves receiving commercial milk replacers (CMR) without complex oligosaccharides

showed lower abundances of this bacterial genus. CMR often contain particularly higher amounts of minerals and lactose, and their application in calves diet are brought together with an increased permeability of the calves GIT [246]. Therefore, CMR might, applied too early, promote a much broader set of gut microbes in the calf's intestines with yet unclear implications. Many studies on *Bifidobacterium* refer to their stimulation via milk [247] and their abundance in the calf's gut [248], but less in the GIT of the adult cow. In feces of cows *B. pseudolongum* is the most dominant *Bifidobacterium* followed by *B. thermophilum* [243]. Bifidobacteria stabilize the colonic epithelial integrity and mucus growth in rodent models [63, 183], produce health promoting metabolites such as CLA in the rumen [249] and hereby have probiotic properties. Also, they are suggested to play a great role in aging, gut homeostatic, and health of humans [102]. According to the present work, *Bifidobacterium* species are potent in suppressing a broad range of bacteria, hereby modeling the microbiome in feces of dairy cows probably via their expression of bacteriocins [245].

Even though the abundance of *Bifidobacterium* is highly dependent on the diet, a stronger dependence on the feed efficiency status of sheep was observed [250]. This would explain why cows receiving the same diet but showing different residual energy efficiencies have different microbiomes and hence *Bifidobacterium* abundances.

The present work demonstrates a steep increase of this genus at three days after calving in rumen, duodenum, and feces, irrespectively of the microbiome cluster. This indicates a deeper programmed mechanism after calving, which as of yet has not been observed in dairy cows. The deeper purpose behind this mechanism and whether there are benefits for the cow would be of great interest. Also, it remains unclear how the cow inoculates herself with this taxon. The study strongly suggests a closer investigation of the importance of this axis, especially how it is affecting the cow's health and whether an extended calf-mother period would support this process.

4.3.4 Proteobacteria as microbial marker for dysbiosis

As stated before, one major aim was to sample a number of animals before and after stressful phases in order to evaluate if these entail some signs of microbial shift or even dysbiosis in all individuals or animal groups. The term "dysbiosis", however, has become a dictum in science. It is unclear how a normal and abnormal microbiome is defined [251], especially for a highly specialized animal such as the dairy cow. Most studies referring to dysbiosis claim it to be a stage of imbalance, change, or the increase or decrease of specific bacterial taxa (e.g., increase of Proteobacteria or

decrease of Firmicutes) [251]. Especially in the light of the here observed microbiome clusters, it seems to be even more difficult to discover and describe a dysbiotic microbiome. Is each of the microbiome cluster having a dysbiotic stage or is the C-Spiro group the dysbiotic stage of the C-Bifi cluster?

Microbiome studies are until now not able to clearly separate between healthy and unhealthy, eubiotic and dysbiotic microbiomes. Future studies need to evaluate if these classifications are even possible, when considering the huge mass of different bacterial species, each contributing to host health and disease by very minute actions. Increased abundances of intestinal Proteobacteria have been reviewed in humans with metabolic disorders, intestinal inflammations, and cancer [252]. Also, they have been associated with increased concentrate feeding, going along with increasing antimicrobial resistance genes in cattle [131]. Significantly high abundances of this phylum were observed in the present thesis for C-Spiro, followed by C-Clos (Fig. 4.5A,C). This was mainly driven by significantly higher *Ruminobacter*, uncl. Neisseriaceae, uncl. Burkholderiales ($p \leq 0.01$), uncl. Betaproteobacteria, uncl. Proteobacteria and *Succinivibrio* (Wilcoxon test $p \leq 0.0001$) abundances in both mentioned clusters compared to C-Bifi, and elevated abundances of uncl. Gammaproteobacterium in C-Clos animals (Fig. 4.5C). Interestingly, the number of fecal Proteobacteria was double as high in static animals as in dynamic ones (Fig. 4.5B), particularly due to significantly higher uncl. Proteobacteria, uncl. Deltaproteobacteria, and uncl. Succinivibrionaceae abundances (Wilcoxon test $p \leq 0.0001$, Fig. 4.5D) in static animals. As in parallel the C-Spiro animals stood out with more average days in disease, this may underline the hypothesis that high Proteobacteria in the gut may pose a "diagnostic signature" of disease and dysbiosis [252].

Animals assigned to C-Clos microbiome cluster had higher abundances of Proteobacteria in the upper GIT compared to the other two clusters, particularly after the LPS challenge (Fig. 4.6). This may support that C-Clos animals were more prone to dysbiosis induced by the inflammatory challenge than both other clusters. It remains unclear which role Proteobacteria play during stress and dysbiosis – yet we see cluster specific movements matching to physiological patterns (health, production data, body weight etc.) and hope that the present data offers material and motivation for future work in this field.

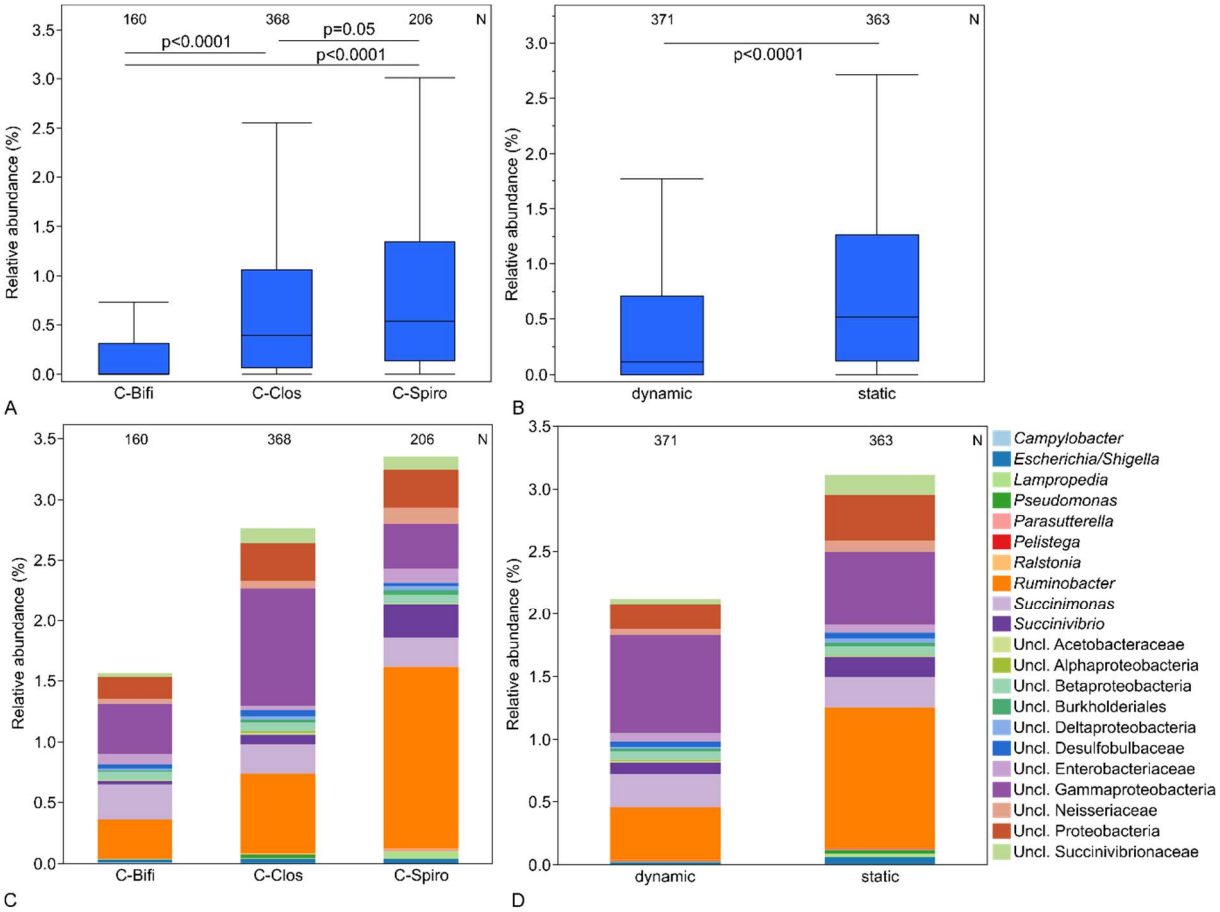


Fig. 4.5 Average relative abundance of Proteobacteria in total of rumen fluid, duodenal fluid and fecal samples on phylum level for (A) the animal clusters and (B) the dynamic and static group and on genus level for (C) the animal clusters and (D) the dynamic and static group. N refers to sample number included per group. N-Values refer to total samples per group. P-values are based on non-parametric Wilcoxon test.

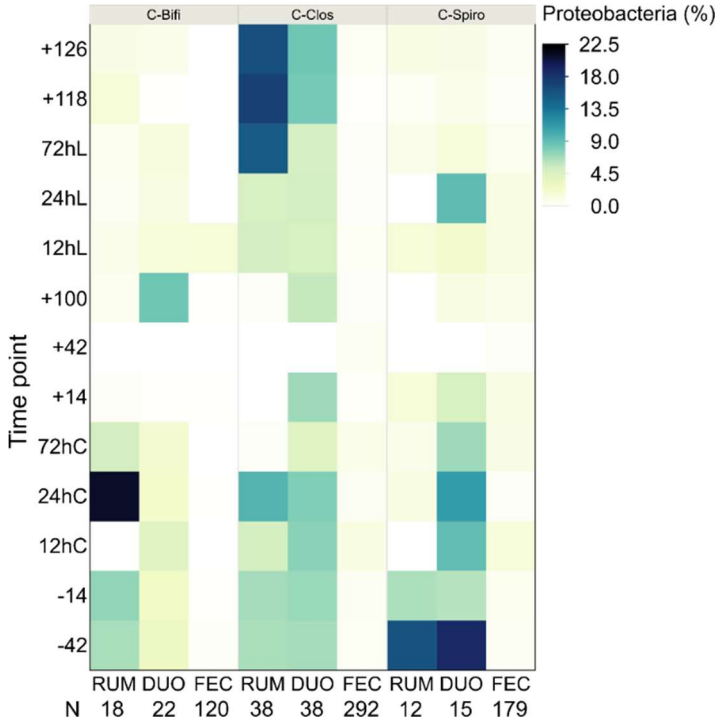


Fig. 4.6 Average relative abundance across all time points of Proteobacteria in rumen (RUM), duodenum (DUO) and feces (FEC) among the three observed microbiome clusters. N-Values refer to total samples per group.

4.3.5 Metabolite variations throughout the GIT

Metabolites along the GIT disclosed several commonly known fluctuations throughout GIT sections and the productive life of the cow. For example, acetate, propionate, and butyrate, increased in rumen, duodenum, and feces after calving and diet change. However, similar to previous studies [57], community differences between, e.g., microbiome clusters did not lead to great variations in SCFA profiles. Glucose and amino acids increased in rumen, duodenal, and fecal samples with the introduction of more concentrate in the diet due to increased influx of starch and hence fuel for microbial protein synthesis [41]. LPS challenges resulted in reduced feed intakes and therefore in lower SCFA concentrations in all three matrices, as well as reduced serum glucose levels.

Acylcarnitine, an acyl-CoA group bound to carnitine, can be transported into the inner side of the mitochondrion where the acyl-CoA is used for oxidative catabolism [253]. Recent murine studies have observed a colonic uptake of acylcarnitines and their oxidation in mitochondria [253]. The increase of acylcarnitines has been found to be associated with a diseased state [253]. As we see an increase shortly after calving and during LPS challenge, increasing acylcarnitine concentrations may in general be an indicator for stressful periods and that this is a mechanism from the guts side to supply the animal with energy. The stress response of the animal clusters was different in

intensity, with C-Bifi showing much higher concentrations of fecal acylcarnitines during the LPS challenge, whereby C-Clos had the highest during calving. Therefore, C-Clos may be at higher risk to develop reactive oxygen species after calving compared to the other two clusters. During the LPS challenge, all animals were out of negative energy balance, the rumination activity and feed intake were both very low. High concentrations of acylcarnitines may therefore be a welcomed energy source for the immunologically compromised animal. Whether Bifidobacterium or the microbiome along with the C-Bifi cluster enables a quicker and stronger acylcarnitine supply needs to be analyzed in the future.

C-Bifi animals had particularly higher amino acid concentrations than C-Clos and C-Spiro, not only in feces but also in duodenal fluid samples. Therefore, as protein concentrations are higher in milk of C-Bifi animals, it could be due to improved amino acid uptake in the duodenum. As the rumen fluid amino acid concentrations have remained similar across the clusters, a microbial "wash-out" effect can be dismissed.

Formate was found to be the metabolite with the highest number of significant correlations among rumen and duodenal fluid samples and significantly higher in C-Spiro compared to C-Bifi animals. The antimicrobial and proteolysis inhibitory effect of formate has already been described [254], underlining low rumen diversity and low duodenal protein concentration of C-Spiro. Anaerobic fermentation of polysaccharides results in the formation of pyruvate and thereafter formate. The latter is quickly metabolized to methane [255], which leads to the assumption, that this animal cluster might have produced more methane than the others and hereby having greater energy losses. Yet, this cannot be confirmed so far.

Significantly higher carnitine concentrations were observed in C-Bifi blood and feces samples with significantly lower blood NEFA concentrations. This antagonistic correlation was already observed in goats [256]. Paired with the improved milk quality (fat and protein), this may again be an indicator for C-Bifi having an improved metabolic status compared to the other two animal clusters.

Significantly higher glycine concentrations have been observed in plasma samples of positive-REI Charolais Bulls [257] and duodenal fluid samples of positive-REI C-Bifi animals (compared to C-Clos). Bifidobacteria are able to resist bile salts via bile-salt hydrolases and thereafter de-conjugation of bile salts [258], resulting into free glycine. As cows were sampled using a T-shaped fistula at the proximal duodenum [259], before the pancreatic and bile duct, bile salts shouldn't be present here. Yet, this

phenomenon could be explained by occurring back-flows of boluses, as cannulas can greatly impact the natural peristalsis of digesta flow [260]. Assuming that these effects may be identical among all cannulated animals, it can be assumed that fat digestion differed between negative and positive REI animals. Future work could focus on this connection.

4.4 Limitations of the study and future improvements

The MitoCow project is a large-scaled study including a particularly diverse consortium of animal scientists. Studies of this size bear great potentials to gain a deeper understanding of metabolic cycles, actions, and reactions in the respective animal. However, planning and execution of the study demand great team work and accuracy and bear massive implementation difficulties. The MitoCow project seeks its peers in the field of dairy cow science as highly multi-leveled, complex, and offering a wealth of data. Nevertheless, some features of the study could be considered for improvements. One significant limitation of the project is the lack of control animals running in parallel, as each animal served as its own control. It would have been of interest how unchallenged and unsupplemented animals would have gone thought the trial. For example, milk somatic cell counts after the LPS challenge remained high in all animal clusters until the end of the trial. Whether this was a lasting LPS effect or unrelated from the challenge needs to be investigated in future. Also, the animals' diet needed to be changed simultaneously with the event of calving. Subsequent microbial and metabolite shifts can therefore not be assigned to one or the other trigger. Future studies may include animals on pasture all year through to evaluate the "bare" effect of calving on the microbiome. This also could be conducted with special focus on the bifidobacterial axis between calf and cow.

A major difficulty in animal or human studies is the individual effect. Age, gender [261], live style and diet [262] have been found to strongly impact the metabolism and gut microbiome. For the present study, animals of different ages, lactation numbers, and experimental trial history were used. As the former two did not correlate with the microbiome clusters, it could be suggested that the latter might have an impact on this formation. The clusters might mirror long lasting impacts of previous trials, e.g., on the effect of fungal toxins, antibiotics, and weaning approaches. A comparative study with focus on the microbiome clusters here observed, contrasted to previous trial animal

groupings would be an easily feasible and highly interesting approach to assess the long-lasting effect of dietary supplements, toxins or husbandry strategies.

Working on large sample sets is a great challenge in terms of data handling but can provide much more space for interpretation, as the individual variation becomes a smaller factor. This was the case when working with the fecal samples. The dataset of the rumen and duodenal fluid samples was much smaller, making statistical analysis difficult to perform. Also, time and budget restrictions demanded restriction to subsets of samples, leading to gaps in sample data, making integration of data very difficult. As the present work suggests that the microbiome clusters deriving from fecal information, seem to be consistent with the microbiomes of the upper GIT, it would have been of particular interest to have more samples from these upper sections from more animals. Working on these two different sample sets, upper GIT and feces, truly emphasized how precious large datasets are.

The present work focused on the bacteriome of the trial animals. However, the GIT is also settled by different other microbes such as protozoa, fungi, and archaea. Whilst protozoa degrade protein and carbohydrates [263], archaea channel the excess of hydrogen into methane, a major contributor to climate change [21]. Their analysis was also planned for the present work, yet the design of accurate primers poses a challenge. The mycobiome, the entity of fungi, is also playing a great role in the ruminant ecosystem in degrading complex plant cell walls [264]. In the light of modern dairy cow diets containing great amounts of concentrate, it is plausible that recent studies observed significant decreases of the fungal community in dairy cows [265]. It could be suggested that the host-bacterial community hereby loses fungal key players for cross-feeding activities [266].
In general, it is of significant interest how these microbes interact with each other and the host, and how they behave during transition and stressful periods. The analyses of the whole microbiome should be considered in future experiments and is becoming increasingly realistic as suitable primers and analyses tools improve continuously.

5 Summary

The modern dairy cow is confronted with a multitude of stressors throughout live. Especially calving, transition, and microbial infections are strong challenges that can have long-lasting impacts on the cow's health and performance. Yet, individuals can differ in their response towards these challenges, raising the question which characteristics in the dairy cow contribute to a more or less robust animal. Apart from genetics, the gut microbiome and the entailed metabolome is assumed to play an important role in buffering or promoting host stress. This is also due to the fact that the gut microbiome is strongly involved in the hosts energy metabolism and immune system. As dairy cows often show performance impairments during high energy demanding periods, it could be suggested that improving energy metabolism in these specific phases might reduce the negative phenotypic outcomes. This was tested using dietary L-carnitine, a metabolite inevitably necessary for energy metabolism. However, no supplement effects on the intestinal microbiome or metabolome have been found in the present work. Supplementation was continued throughout the complete trial. Calving functioned as an individual stimulus, and an intra-venous LPS injection induced a standardized inflammatory challenge, as a specific amount of LPS per kg of bodyweight was applied per cow. Supplemented animals were compared to a control group. In total, the animals were studied across 168 days and sampled extensively at several sites. The focus of this thesis was to analyze the bacterial consortia and metabolites of both, host and bacteria, in rumen, duodenum, and feces throughout the given period. This was to elucidate the metabolic reactions and bacterial shifts during the mentioned challenging periods and their response to the L-carnitine supplementation.

First, the ruminal and duodenal fluid microbiome of eight double cannulated animals during the two respective challenges was analysed. Before calving and feed change, rumen and duodenal fluid bacterial consortia were significantly different, thereafter very alike. Strong microbial community shifts were observed throughout the complete trial irrespectively of the matrix. Both matrices varied in their metabolite patterns indicating functional variation among sites. Also, a strong increase of *Bifidobacterium* at three days after calving was observed in almost all animals pointing towards a strong biological purpose. This needs to be investigated in upcoming studies. The study could show increasing ketogenic activities in the animals after calving and proposes a

possible protective host-microbial interaction, against a ruminal collapse induced by LPS challenge, here described as "microbial airbag".

The second part included fecal samples of the same animals, which were analyzed for their bacterial consortia and targeted metabolites. Different dynamics and diversities of microbial communities amongst the individuals were observed, according to which animals could be grouped into three microbiome clusters. These showed in part fundamentally different metabolic, health, and performance parameters, indicating strong host-microbiome-metabolite interactions. The study demonstrated that microbiome clustering may contribute to identifying different metabo- and production types. Again, the study observed a strong increase of *Bifidobacterium* at three days after calving and even during the LPS challenge supporting the findings of the former study. This strengthens the hypothesis that also for the cow *Bifidobacterium* may have protective effects, as this genus is largely involved in health promoting activities.

The power of this project lies in the massive sampling of different body sites in dairy cows across a very long period of time and finally, merging of the collected data. This, however, requires high computational efforts as numerous time points, matrices, animals, measurements, treatments, feeding regimen, and challenges resulted into a large bandwidth of parameters and metadata. Yet, it bears the potential to better elucidate and understand actions and reactions of the host, its microbiome and metabolism, as well as organ-axes in dairy cows and thereby gaining a more holistic picture of these complex animals.

The aim of analyzing the host, its microbiome and metabolome throughout challenging periods resulted into the following main findings. Time, calving, and feed change remarkably change the microbial communities and to a lesser extent the metabolomes in all three matrices. Rumen and proximal duodenal fluid samples significantly differ in their metabolomes but not in their microbiome. In all matrices, an increase of *Bifidobacterium* is seen within three days after calving, which has to be further researched. Across the herd, three distinct microbiome clusters are found, which significantly differ in their production and health parameters.

6 Zusammenfassung

Die moderne Milchkuh steht während ihres Lebens einer Vielzahl an Herausforderungen gegenüber. Insbesondere Kalbungen, die Transitphasen, sowie mikrobielle Infektionen sind einschneidende Herausforderungen, welche lebenslange Folgen für die Gesundheit und die Leistung haben können. Trotzdem können Individuen in ihrer Stressantwort stark variieren, weshalb sich die Frage aufdrängt, welche Charakteristika die einzelne Milchkuh zu einem mehr oder weniger resilienten Tier machen. Neben der Genetik wird vermutet, dass das Darmmikrobiom und das daraus resultierende Metabolom eine wichtige Rolle bei der Pufferung oder Förderung von Wirtsstress spielen. Dies liegt auch daran, dass das Darmmikrobiom wesentlich den Energiestoffwechsel und das Immunsystem des Wirts beeinflusst. Da Milchkühe in Phasen hohen Energiebedarfs häufig Leistungseinbußen zeigen, könnten, den Energiestoffwechsel unterstützende Maßnahmen die negativen phänotypischen Ergebnisse abmildern. Daher untersuchte die vorliegende Arbeit den Effekt einer L- Carnitin Supplementierung auf den Energiestoffwechsel. Dieser Metabolit ist wichtig für den Abbau langkettiger Fettsäuren, die vor allem bei der Mobilisierung von Fettreserven nach der Kalbung entstehen. In dieser Arbeit wurden jedoch keine Auswirkungen der Supplementierung auf das Darmmikrobiom oder das Metabolom gefunden.

Die hier beschriebene Studie umfasste die Transitphase, das Kalben als individuellen Stimulus und eine intravenöse, durch LPS induzierte entzündliche Noxe. Letzteres stellte einen standardisierten Stressor dar, da jeder Kuh eine spezifische LPS-Menge pro kg Körpergewicht appliziert wurde. Supplementierte Tiere wurden mit einer Kontrollgruppe verglichen und 168 Tage lang ausgiebig beprobt. Der Fokus dieser Dissertation lag auf der Analyse der bakteriellen Gemeinschaften und Metaboliten des Wirtes und der Bakterien innerhalb des Pansens, Duodenums und Kotes. Hierbei sollten die Stoffwechselreaktionen und bakteriellen Verschiebungen während der erwähnten herausfordernden Perioden und die Reaktion auf die L-Carnitin-Supplementierung aufgedeckt werden.

Zuerst wurde das Mikrobiom der Pansen- und Duodenaldigesta von acht doppelt fistulierten Tieren, während der Noxen analysiert. Vor dem Futterwechsel und der Kalbung waren die Bakterienkonsortien des Pansens und Duodenums signifikant unterschiedlich, danach sehr ähnlich. Während des gesamten Versuchs wurden, unabhängig von der Matrix, starke Veränderungen des Mikrobioms beobachtet. Beide

Matrizen variierten in ihren Metabolitenmustern, was auf die unterschiedlichen Funktionen der Sektionen zurückzuführen ist. Zudem, wurde bei fast allen Tieren ein klarer Anstieg von *Bifidobacterium* drei Tage nach dem Kalben beobachtet, was auf einen starken biologischen Zweck hindeutet, der in zukünftigen Studien untersucht werden muss. Eine zunehmende ketogene Aktivität der Tiere nach dem Kalben konnte gezeigt werden. Der "mikrobielle Airbag" beschreibt einen möglichen Mechanismus zwischen dem Wirt und seinem Mikrobiom, welcher den Wirt während der LPS challenge vor einem Pansenkollaps schützt.

Der zweite Teil der Arbeit befasste sich mit den Kotproben derselben Tiere, die auf ihr Mikrobiom und einer Auswahl an Metaboliten analysiert wurden. Es zeigten sich unterschiedliche Dynamiken und Diversitäten mikrobieller Gemeinschaften zwischen den Individuen, welche sich hierdurch in drei verschiedene Mikrobiom Gruppen einteilen ließen. Diese Gruppen zeigten zum Teil grundlegend unterschiedliche Stoffwechsel-, Gesundheits- und Leistungsparameter, was auf starke Wechselwirkungen zwischen Wirt, Mikrobiom und Metabolom hindeutet. Es konnte gezeigt werden, dass Enterotypisierungen dazu beitragen können, verschiedene Metabo- und Produktionstypen zu identifizieren. Auch hier konnte ein starker Anstieg von *Bifidobacterium* drei Tage nach dem Kalben und sogar während der LPS challenge gezeigt werden, was die Ergebnisse aus der vorausgegangenen Studie mit Pansen und Duodenalsaft unterstützt. Da diese Gattung in hohem Maße an gesundheitsfördernden Aktivitäten beteiligt ist, erhärtet sich die Hypothese, dass *Bifidobacterium* auch für das Muttertier schützende Wirkungen haben könnte.

Die Besonderheit dieses Projekts liegt in den umfassenden Beprobungen verschiedener Matrizen der Milchkühe über einen sehr langen Zeitraum hinweg und schließlich die Zusammenführung der gesammelten Daten. Dies erfordert ein hohes Maß an Datenmanagement, da zahlreiche Zeitpunkte, Matrizen, Tiere, Messungen, Behandlungen, Fütterungsregime und Noxen zu einer großen Bandbreite an Parametern und Metadaten führt. Es birgt jedoch das Potenzial, Aktionen und Reaktionen des Wirts, seines Mikrobioms und Stoffwechsels, sowie der Organachsen bei Milchkühen besser zu verstehen und so ein ganzheitlicheres Bild dieser komplexen Tiere zu gewinnen.

Die Untersuchung der Milchkuh, sowie deren Mikrobiom und Metabolom in kritischen Zeiträumen führte zu den folgenden Hauptergebnissen. Die Zeit, das Kalben und der Futterwechsel haben das Mikrobiom und in geringerem Maße die

Zusammensetzungen der Metaboliten aller untersuchter Matrizes verändert. Proben des Pansens und des proximalen Duodenums unterschieden sich signifikant in ihren metabolischen Zusammensetzungen, jedoch nicht in ihren mikrobiellen Gemeinschaften. In allen Matrizen konnte drei Tage nach der Kalbung ein Anstieg von *Bifidobacterium* festgestellt werden, den es weiter zu beleuchten gilt. Über die Herde hinweg konnten drei Individuengruppen erfasst werden, welche sich hinsichtlich ihres Mikrobioms sowie in ihren Leistungs- und Gesundheitsparametern signifikant unterschieden.

References

1. Rook, G., et al., *Evolution, human-microbe interactions, and life history plasticity.* The Lancet, 2017. **390**(10093): p. 521-530. DOI: 10.1016/S0140-6736(17)30566-4.
2. Mills, J.G., et al., *Urban habitat restoration provides a human health benefit through microbiome rewilding: the microbiome rewilding hypothesis.* Restoration Ecology, 2017. **25**(6): p. 866-872. DOI: 10.1111/rec.12610.
3. Sender, R., S. Fuchs, and R. Milo, *Are we really vastly outnumbered? Revisiting the ratio of bacterial to host cells in humans.* Cell, 2016. **164**(3): p. 337-340. DOI: 10.1016/j.cell.2016.01.013.
4. IHGSC, I.H.G.S.C., *Finishing the euchromatic sequence of the human genome.* Nature, 2004. **431**(7011): p. 931-945. DOI: 10.1038/nature03001.
5. Knight, R., et al., *The microbiome and human biology.* Annual Review of Genomics and Human Genetics, 2017. **18**: p. 65-86. DOI: 10.1146/annurev-genom-083115-022438.
6. Qin, J., et al., *A human gut microbial gene catalogue established by metagenomic sequencing.* Nature, 2010. **464**(7285): p. 59-65. DOI: 10.1038/nature08821.
7. Keeling, P.J. and J.D. Palmer, *Horizontal gene transfer in eukaryotic evolution.* Nature Reviews Genetics, 2008. **9**(8): p. 605-18. DOI: 10.1038/nrg2386.
8. Shreiner, A.B., J.Y. Kao, and V.B. Young, *The gut microbiome in health and in disease.* Current Opinion in Gastroenterology, 2015. **31**(1): p. 69-75. DOI: 10.1097/MOG.0000000000000139.
9. Yamamoto, E.A. and T.N. Jørgensen, *Relationships between vitamin D, gut microbiome, and systemic autoimmunity.* Frontiers in Immunology, 2020. **10**: p. 3141. DOI: 10.3389/fimmu.2019.03141.
10. Rinninella, E., et al., *What is the healthy gut microbiota composition? A changing ecosystem across age, environment, diet, and diseases.* Microorganisms, 2019. **7**(1): p. 14. DOI: 10.3390/microorganisms7010014.
11. Belanche, A., et al., *Gut microbiome modulation in ruminants: enhancing advantages and minimizing drawbacks.* Frontiers in Microbiology, 2021. **11**. DOI: 10.3389/fmicb.2020.622002.
12. Tlaskalová-Hogenová, H., et al., *Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases.* Immunology Letters, 2004. **93**(2-3): p. 97-108. DOI: 10.1016/j.imlet.2004.02.005.
13. Carding, S., et al., *Dysbiosis of the gut microbiota in disease.* Microbial Ecology in Health and Disease, 2015. **26**(1): p. 26191. DOI: 10.3402/mehd.v26.26191.
14. Xie, G., et al., *Metabolomics approaches for characterizing metabolic interactions between host and its commensal microbes.* Electrophoresis, 2013. **34**(19): p. 2787-2798. DOI: 10.1002/elps.201300017.
15. Malard, F., et al., *Introduction to host microbiome symbiosis in health and disease.* Mucosal Immunology, 2020: p. 1-8. DOI: 10.1038/s41385-020-00365-4.
16. Corinaldesi, C., et al., *Multiple impacts of microplastics can threaten marine habitat-forming species.* Communications Biology, 2021. **4**(1): p. 1-13. DOI: 10.1038/s42003-021-01961-1.
17. Geisen, S., D.H. Wall, and W.H. van der Putten, *Challenges and opportunities for soil biodiversity in the anthropocene.* Current Biology, 2019. **29**(19): p. R1036-R1044. DOI: 10.1016/j.cub.2019.08.007.

18. Yuan, L., et al., *Microbiota in viral infection and disease in humans and farm animals.* Progress in Molecular Biology and Translational Science, 2020. **171**: p. 15. DOI: 10.1016/bs.pmbts.2020.04.005.
19. Ertl, P., et al., *The net contribution of dairy production to human food supply: the case of austrian dairy farms.* Agricultural Systems, 2015. **137**: p. 119-125. DOI: 10.1016/j.agsy.2015.04.004.
20. Du, Y., et al., *A global strategy to mitigate the environmental impact of China's ruminant consumption boom.* Nature Communications, 2018. **9**(1): p. 1-11. DOI: 10.1038/s41467-018-06381-0.
21. Eger, M., et al., *Application of MootralTM reduces methane production by altering the archaea community in the rumen simulation technique.* Frontiers in Microbiology, 2018. **9**: p. 2094. DOI: 10.3389/fmicb.2018.02094.
22. Rong, L., et al., *Effect of climate parameters on air exchange rate and ammonia and methane emissions from a hybrid ventilated dairy cow building.* Energy and Buildings, 2014. **82**: p. 632-643. DOI: 10.1016/j.enbuild.2014.07.089.
23. Harmon, D. and K. Swanson, *Nutritional regulation of intestinal starch and protein assimilation in ruminants.* Animal, 2020. **14**(S1): p. 17-28. DOI: 10.1017/S1751731119003136.
24. VandeHaar, M.J., et al., *Harnessing the genetics of the modern dairy cow to continue improvements in feed efficiency.* Journal of Dairy Science, 2016. **99**(6): p. 4941-4954. DOI: 10.3168/jds.2015-10352.
25. Welch, C.B., et al., *The impact of feed efficiency selection on the ruminal, cecal, and fecal microbiomes of Angus steers from a commercial feedlot.* Journal of Animal Science, 2020. **98**(7): p. skaa230. DOI: 10.1093/jas/skaa230.
26. Eastridge, M., *Major advances in applied dairy cattle nutrition.* Journal of Dairy Science, 2006. **89**(4): p. 1311-1323. DOI: 10.3168/jds.S0022-0302(06)72199-3.
27. Plaizier, J., et al., *Enhancing gastrointestinal health in dairy cows.* Animal, 2018. **12**(s2): p. 399-418. DOI: 10.1017/S1751731118001921.
28. Godoy-Vitorino, F., et al., *Comparative analyses of foregut and hindgut bacterial communities in hoatzins and cows.* The ISME Journal, 2012. **6**(3): p. 531-541. DOI: 10.1038/ismej.2011.131.
29. Gharechahi, J., et al., *Metagenomic analysis reveals a dynamic microbiome with diversified adaptive functions to utilize high lignocellulosic forages in the cattle rumen.* The ISME Journal, 2021. **15**(4): p. 1108-1120. DOI: 10.1038/s41396-020-00837-2.
30. Mao, S., et al., *Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function.* Scientific Reports, 2015. **5**(1): p. 1-14. DOI: 10.1038/srep16116.
31. Xue, M.-Y., et al., *Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance.* Microbiome, 2020. **8**(1): p. 1-19. DOI: 10.1186/s40168-020-00819-8
32. Vandamme, T.F. and K. Ellis, *Issues and challenges in developing ruminal drug delivery systems.* Advanced Drug Delivery Reviews, 2004. **56**(10): p. 1415-1436. DOI: 10.1016/j.addr.2004.02.011.
33. Steele, M.A., G.B. Penner, and F. Chaucheyras-Durand, *Development and physiology of the rumen and the lower gut: targets for improving gut health.* Journal of Dairy Science, 2016. **99**(6): p. 4955-4966. DOI: 10.3168/jds.2015-10351.

34. Holman, D.B. and K.E. Gzyl, *A meta-analysis of the bovine gastrointestinal tract microbiota.* FEMS Microbiology Ecology, 2019. **95**(6). DOI: 10.1093/femsec/fiz072.
35. Beauchemin, K., *Invited review: current perspectives on eating and rumination activity in dairy cows.* Journal of Dairy Science, 2018. **101**(6): p. 4762-4784. DOI: 10.3168/jds.2017-13706.
36. Hofmann, R.R., *Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system.* Oecologia, 1989. **78**(4): p. 443-457. DOI: 10.1007/BF00378733.
37. Maekawa, M., K. Beauchemin, and D. Christensen, *Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows.* Journal of Dairy Science, 2002. **85**(5): p. 1165-1175. DOI: 10.3168/jds.S0022-0302(02)74179-9.
38. Meyer, J., et al., *Effects of a dietary l-carnitine supplementation on performance, energy metabolism and recovery from calving in dairy cows.* Animals, 2020. **10**(2): p. 342. DOI: 10.3390/ani10020342.
39. Frey, J., et al., *Comparative studies of microbial populations in the rumen, duodenum, ileum and faeces of lactating dairy cows.* Journal of Applied Microbiology, 2010. **108**(6): p. 1982-1993. DOI: 10.1111/j.1365-2672.2009.04602.x.
40. Zhu, Y., et al., *Comparative study of the bacterial communities throughout the gastrointestinal tract in two beef cattle breeds.* Applied Microbiology and Biotechnology, 2021. **105**(1): p. 313-325. DOI: 10.1007/s00253-020-11019-7.
41. Webster, J., *Understanding the dairy cow*. 3 ed. 2020: John Wiley & Sons.
42. Sissons, J.W., *Digestive enzymes of cattle.* Journal of the Science of Food and Agriculture, 1981. **32**(2): p. 105-114. DOI: 10.1002/jsfa.2740320202.
43. Loeffler, K. and G. Gäbel, *Anatomie und Physiologie der Haustiere*. Vol. 13. 2015: UTB.
44. Schwab, C.G. and G.A. Broderick, *A 100-year review: protein and amino acid nutrition in dairy cows.* Journal of Dairy Science, 2017. **100**(12): p. 10094-10112. DOI: 10.3168/jds.2017-13320.
45. Brake, D. and K. Swanson, *Ruminant nutrition symposium: effects of postruminal flows of protein and amino acids on small intestinal starch digestion in beef cattle.* Journal of Animal Science, 2018. **96**(2): p. 739-750. DOI: 10.1093/jas/skx058.
46. Van den Abbeele, P., et al., *The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept.* FEMS Microbiology Reviews, 2011. **35**(4): p. 681-704. DOI: 10.1111/j.1574-6976.2011.00270.x.
47. Liu, J., et al., *Diet-induced changes in bacterial communities in the jejunum and their associations with bile acids in angus beef cattle.* Animal Microbiome, 2020. **2**(1): p. 33. DOI: 10.1186/s42523-020-00051-7.
48. Gressley, T., M. Hall, and L. Armentano, *Ruminant nutrition symposium: productivity, digestion, and health responses to hindgut acidosis in ruminants.* Journal of Animal Science, 2011. **89**(4): p. 1120-1130. DOI: 10.2527/jas.2010-3460.
49. Hecker, J. and W. Grovum, *Rates of passage of digesta and water absorption along the large intestines of sheep, cows and pigs.* Australian Journal of Biological Sciences, 1975. **28**(2): p. 161-168. DOI: 10.1071/BI9750161.
50. Myer, P., et al., *Analysis of the gut bacterial communities in beef cattle and their association with feed intake, growth, and efficiency.* Journal of Animal Science, 2017. **95**(7): p. 3215-3224. DOI: 10.2527/jas.2016.1059

51. Kim, M. and J.E. Wells, *A meta-analysis of bacterial diversity in the feces of cattle.* Current Microbiology, 2016. **72**(2): p. 145-151. DOI: 10.1007/s00284-015-0931-6.
52. Carlson, D., et al., *Dietary l-carnitine affects periparturient nutrient metabolism and lactation in multiparous cows.* Journal of Dairy Science, 2007. **90**(7): p. 3422-3441. DOI: 10.3168/jds.2006-811.
53. Seymour, W., D. Campbell, and Z. Johnson, *Relationships between rumen volatile fatty acid concentrations and milk production in dairy cows: a literature study.* Animal Feed Science and Technology, 2005. **119**(1-2): p. 155-169. DOI: 10.1016/j.anifeedsci.2004.10.001.
54. Bauman, D.E. and W.B. Currie, *Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis.* Journal of Dairy Science, 1980. **63**(9): p. 1514-1529. DOI: 10.3168/jds.S0022-0302(80)83111-0.
55. Herdt, T.H., *Overview of ketosis in cattle.* MERCK Veterinary Manual, 2019. **12**.
56. Laffel, L., *Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes.* Diabetes Metabolism Research and Reviews, 1999. **15**(6): p. 412-426. DOI: 10.1002/(sici)1520-7560(199911/12)15:6<412::aid-dmrr72>3.0.co;2-8
57. Neubauer, V., et al., *Starch-rich diet induced rumen acidosis and hindgut dysbiosis in dairy cows of different lactations.* Animals, 2020. **10**(10): p. 1727. DOI: 10.3390/ani10101727.
58. Roche, J., et al., *Fertility and the transition dairy cow.* Reproduction, Fertility and Development, 2018. **30**(1): p. 85-100. DOI: 10.1071/RD17412.
59. Li, S., et al., *Effects of subacute ruminal acidosis challenges on fermentation and endotoxins in the rumen and hindgut of dairy cows.* Journal of Dairy Science, 2012. **95**(1): p. 294-303. DOI: 10.3168/jds.2011-4447.
60. Kvidera, S., et al., *Intentionally induced intestinal barrier dysfunction causes inflammation, affects metabolism, and reduces productivity in lactating Holstein cows.* Journal of Dairy Science, 2017. **100**(5): p. 4113-4127. DOI: 10.3168/jds.2016-12349.
61. Russell, J.B. and D.B. Wilson, *Why are ruminal cellulolytic bacteria unable to digest cellulose at low pH?* Journal of Dairy Science, 1996. **79**(8): p. 1503-1509. DOI: 10.3168/jds.S0022-0302(96)76510-4.
62. Plaizier, J., et al., *Subacute ruminal acidosis (SARA), endotoxins and health consequences.* Animal Feed Science and Technology, 2012. **172**(1-2): p. 9-21. DOI: 10.1016/j.anifeedsci.2011.12.004.
63. Schroeder, B.O., et al., *Bifidobacteria or fiber protects against diet-induced microbiota-mediated colonic mucus deterioration.* Cell Host and Microbe, 2018. **23**(1): p. 27-40. e7. DOI: 10.1016/j.chom.2017.11.004.
64. Desai, M.S., et al., *A dietary fiber-deprived gut microbiota degrades the colonic mucus barrier and enhances pathogen susceptibility.* Cell, 2016. **167**(5): p. 1339-1353. e21. DOI: 10.1016/j.cell.2016.10.043.
65. Emmanuel, D., et al., *Acidosis and lipopolysaccharide from Escherichia coli B:055 cause hyperpermeability of rumen and colon tissues.* Journal of Dairy Science, 2007. **90**(12): p. 5552-5557. DOI: 10.3168/jds.2007-0257.
66. Zebeli, Q., et al., *Intermittent parenteral administration of endotoxin triggers metabolic and immunological alterations typically associated with displaced abomasum and retained placenta in periparturient dairy cows.* Journal of Dairy Science, 2011. **94**(10): p. 4968-4983. DOI: 10.3168/jds.2011-4194.

67. Schulz, H.N. and B.B. Jørgensen, *Big bacteria.* Annual Review of Microbiology, 2001. **55**(1): p. 105-137. DOI: 10.1146/annurev.micro.55.1.105.
68. Mendelsohn, J.A., *'Like all that lives': biology, medicine and bacteria in the age of Pasteur and Koch.* History and Philosophy of the Life Sciences, 2002: p. 3-36. DOI: 10.1080/03919710210001714293.
69. Patel, J.B., *16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory.* Molecular Diagnosis, 2001. **6**(4): p. 313-321. DOI: 10.1007/BF03262067.
70. Edgar, R.C., *Updating the 97% identity threshold for 16S ribosomal RNA OTUs.* Bioinformatics, 2018. **34**(14): p. 2371-2375. DOI: 10.1093/bioinformatics/bty113.
71. Deusch, S., et al., *News in livestock research—use of omics-technologies to study the microbiota in the gastrointestinal tract of farm animals.* Computational and Structural Biotechnology Journal, 2015. **13**: p. 55-63. DOI: 10.1016/j.csbj.2014.12.005.
72. Burbach, K., et al., *Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions.* MicrobiologyOpen, 2016. **5**(1): p. 70-82. DOI: 10.1002/mbo3.312.
73. Schrimpe-Rutledge, A.C., et al., *Untargeted metabolomics strategies—challenges and emerging directions.* Journal of the American Society for Mass Spectrometry, 2016. **27**(12): p. 1897-1905. DOI: 10.1007/s13361-016-1469-y.
74. Cryan, J.F., et al., *The microbiota-gut-brain axis.* Physiological Reviews, 2019. DOI: 10.1152/physrev.00018.2018.
75. Dang, A.T. and B.J. Marsland, *Microbes, metabolites, and the gut–lung axis.* Mucosal Immunology, 2019. **12**(4): p. 843-850. DOI: 10.1038/s41385-019-0160-6.
76. Young, W., et al., *Transfer of intestinal bacterial components to mammary secretions in the cow.* PeerJ, 2015. **3**: p. e888. DOI: 10.7717/peerj.888.
77. Seifert, J., et al., *Bioinformatic progress and applications in metaproteogenomics for bridging the gap between genomic sequences and metabolic functions in microbial communities.* Proteomics, 2013. **13**(18-19): p. 2786-2804. DOI: 10.1002/pmic.201200566.
78. Pitta, D.W., et al., *Temporal dynamics in the ruminal microbiome of dairy cows during the transition period.* Journal of Animal Science, 2014. **92**(9): p. 4014-22. DOI: 10.2527/jas.2014-7621.
79. Khafipour, E., et al., *Effects of grain feeding on microbiota in the digestive tract of cattle.* Animal Frontiers, 2016. **6**(2): p. 13-19. DOI: 10.2527/af.2016-0018.
80. Saint-Georges-Chaumet, Y. and M. Edeas, *Microbiota–mitochondria inter-talk: consequence for microbiota–host interaction.* Pathogens and Disease, 2015. **74**(1). DOI: 10.1093/femspd/ftv096.
81. Rainard, P., *Mammary microbiota of dairy ruminants: fact or fiction?* Veterinary Research, 2017. **48**(1): p. 25. DOI: 10.1186/s13567-017-0429-2.
82. Schlau, N., L. Guan, and M. Oba, *The relationship between rumen acidosis resistance and expression of genes involved in regulation of intracellular pH and butyrate metabolism of ruminal epithelial cells in steers.* Journal of Dairy Science, 2012. **95**(10): p. 5866-75. DOI: 10.3168/jds.2011-5167.
83. Bradford, B., et al., *Invited review: Inflammation during the transition to lactation: New adventures with an old flame.* Journal of Dairy Science, 2015. **98**(10): p. 6631-6650. DOI: 10.3168/jds.2015-9683.

84. Krasiński, Z. and J. Raczyński, *Bisoniana XXV. The reproduction biology of European bison living in reserves and in freedom.* Acta Theriologica, 1967. **12**(29): p. 407-444.
85. Yang, W. and K. Beauchemin, *Effects of physically effective fiber on chewing activity and ruminal pH of dairy cows fed diets based on barley silage.* Journal of Dairy Science, 2006. **89**(1): p. 217-228. DOI: 10.3168/jds.S0022-0302(06)72086-0
86. Khafipour, E., D. Krause, and J. Plaizier, *A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation.* Journal of Dairy Science, 2009. **92**(3): p. 1060-1070. DOI: 10.3168/jds.2008-1389.
87. Saleem, F., et al., *A metabolomics approach to uncover the effects of grain diets on rumen health in dairy cows.* Journal of Dairy Science, 2012. **95**(11): p. 6606-6623. DOI: 10.3168/jds.2012-5403.
88. Baumgard, L.H. and R.P. Rhoads Jr, *Effects of heat stress on postabsorptive metabolism and energetics.* Annual Review of Animal Biosciences, 2013. **1**(1): p. 311-337. DOI: 10.1146/annurev-animal-031412-103644.
89. Jami, E., et al., *Exploring the bovine rumen bacterial community from birth to adulthood.* The ISME Journal, 2013. **7**(6): p. 1069-79. DOI: 10.1038/ismej.2013.2.
90. Duchen, M.R., *Roles of mitochondria in health and disease.* Diabetes, 2004. **53**(suppl 1): p. S96-S102. DOI: 10.2337/diabetes.53.2007.s96.
91. Huber, K., et al., *Metabotypes with properly functioning mitochondria and anti-inflammation predict extended productive life span in dairy cows.* Scientific Reports, 2016. **6**(1): p. 1-11. DOI: 10.1038/srep24642
92. Perea, K., et al., *Feed efficiency phenotypes in lambs involve changes in ruminal, colonic, and small-intestine-located microbiota.* Journal of Animal Science, 2017. **95**(6): p. 2585-92. DOI: 10.2527/jas.2016.1222
93. Deusch, S., et al., *A structural and functional elucidation of the rumen microbiome influenced by various diets and microenvironments.* Frontiers in Microbiology, 2017. **8**: p. 1605. DOI: 10.3389/fmicb.2017.01605.
94. Plaizier, J.C., et al., *Changes in microbiota in rumen digesta and feces due to a grain-based subacute ruminal acidosis (SARA) challenge.* Microbial Ecology, 2017. **74**(2): p. 485-95. DOI: 10.1007/s00248-017-0940-z.
95. Hollmann, M., et al., *Downregulation of cellular protective factors of rumen epithelium in goats fed high energy diet.* PLoS One, 2013. **8**(12). DOI: 10.1371/journal.pone.0081602
96. Bach, A., et al., *Changes in the rumen and colon microbiota and effects of live yeast dietary supplementation during the transition from the dry period to lactation of dairy cows.* Journal of Dairy Science, 2019. **102**(7): p. 6180-6198. DOI: 10.3168/jds.2018-16105
97. Lima, F.S., et al., *Prepartum and postpartum rumen fluid microbiomes: characterization and correlation with production traits in dairy cows.* Applied and Environmental Microbiology, 2015. **81**(4): p. 1327-37. DOI: 10.1128/aem.03138-14.
98. Kim, Y.-H., et al., *Changes in ruminal and reticular pH and bacterial communities in Holstein cattle fed a high-grain diet.* BMC Veterinary Research, 2018. **14**(1): p. 310. DOI: 10.1186/s12917-018-1637-3.
99. Clavel, T., P. Lepage, and C. Charrier, *The family Coriobacteriaceae*, in *The Prokaryotes: Actinobacteria*, E. Rosenberg, et al., Editors. 2014, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 201-38.

100. Dewhirst, F.E., et al., *Characterization of novel human oral isolates and cloned 16S rDNA sequences that fall in the family Coriobacteriaceae: description of Olsenella gen. nov., reclassification of Lactobacillus uli as Olsenella uli comb. nov. and description of Olsenella profusa sp. nov.* International Journal of Systematic and Evolutionary Microbiology, 2001. **51**(5): p. 1797-1804. DOI: 10.1099/00207713-51-5-1797.
101. Tröscher-Mußotter, J., et al., *Microbiome clusters disclose physiologic variances in dairy cows challenged by calving and lipopolysaccharides.* mSystems, 2021. **5**(5): p. e00856-21. DOI: 10.1128/mSystems.00856-21.
102. Arboleya, S., et al., *Gut bifidobacteria populations in human health and aging.* Frontiers in Microbiology, 2016. **7**: p. 1204. DOI: 10.3389/fmicb.2016.01204
103. Makino, H., et al., *Mother-to-infant transmission of intestinal bifidobacterial strains has an impact on the early development of vaginally delivered infant's microbiota.* PLoS One, 2013. **8**(11): p. e78331. DOI: 10.1371/journal.pone.0078331.
104. Laguardia-Nascimento, M., et al., *Vaginal microbiome characterization of Nellore cattle using metagenomic analysis.* PLoS One, 2015. **10**(11): p. e0143294. DOI: 10.1371/journal.pone.0143294.
105. Addis, M., et al., *The bovine milk microbiota: insights and perspectives from-omics studies.* Molecular BioSystems, 2016. **12**(8): p. 2359-2372. DOI: 10.1039/C6MB00217J.
106. Alipour, M., et al., *The composition of the perinatal intestinal microbiota in cattle.* Scientific Reports, 2018. **8**(1): p. 10437. DOI: 10.1038/s41598-018-28733-y.
107. Yatsunenko, T., et al., *Human gut microbiome viewed across age and geography.* Nature, 2012. **486**(7402): p. 222-227. DOI: 10.1038/nature11053.
108. Brandstetter, V., et al., *Chewing and drinking activity during transition period and lactation in dairy cows fed partial mixed rations.* Animals, 2019. **9**(12): p. 1088. DOI: 10.3390/ani9121088.
109. Kuhla, B., C. Metges, and H. Hammon, *Endogenous and dietary lipids influencing feed intake and energy metabolism of periparturient dairy cows.* Domestic Animal Endocrinology, 2016. **56**: p. S2-S10. DOI: 10.1016/j.domaniend.2015.12.002.
110. Ruzsanyi, V. and M.P. Kalapos, *Breath acetone as a potential marker in clinical practice.* Journal of Breath Research, 2017. **11**(2): p. 024002. DOI: 10.1088/1752-7163/aa66d3.
111. Fedorovich, S.V., P.P. Voronina, and T.V. Waseem, *Ketogenic diet versus ketoacidosis: what determines the influence of ketone bodies on neurons?* Neural Regeneration Research, 2018. **13**(12): p. 2060-2063. DOI: 10.4103/1673-5374.241442.
112. Bruss, M. and M. Lopez, *Mixed ruminal microbes of cattle produce isopropanol in the presence of acetone but not 3-D-hydroxybutyrate.* Journal of Dairy Science, 2000. **83**(11): p. 2580-2584. DOI: 10.3168/jds.S0022-0302(00)75151-4.
113. Dyrda, G., et al., *The effect of organic solvents on selected microorganisms and model liposome membrane.* Molecular Biology Reports, 2019. **46**(3): p. 3225-3232. DOI: 10.1007/s11033-019-04782-y.
114. Wang, Q., et al., *Ketogenesis contributes to intestinal cell differentiation.* Cell Death and Differentiation, 2017. **24**(3): p. 458-468. DOI: 10.1038/cdd.2016.142.
115. Goff, J. and R. Horst, *Physiological changes at parturition and their relationship to metabolic disorders.* Journal of Dairy Science, 1997. **80**(7): p. 1260-1268. DOI: 10.3168/jds.S0022-0302(97)76055-7.

116. Meyer, J., et al., *Dietary l-carnitine supplementation modifies the lipopolysaccharide-induced acute phase reaction in dairy cows.* Animals, 2021. **11**(1). DOI: 10.3390/ani11010136.
117. Kim, J.N., et al., *Effects of short-term fasting on in vivo rumen microbiota and in vitro rumen fermentation characteristics.* Asian-Australasian Journal of Animal Sciences, 2019. **32**(6): p. 776. DOI: 10.5713/ajas.18.0489
118. Mahapatra, R. and A. Sahoo, *Ketosis and fatty liver in dairy animals: preventive nutritional approaches.* Clinical Nutrition of Livestock and Pets, 2006. **23**(3): p. 45-53.
119. Kobayashi, Y., T. Shinkai, and S. Koike, *Ecological and physiological characterization shows that Fibrobacter succinogenes is important in rumen fiber digestion - review.* Folia Microbiologica, 2008. **53**(3): p. 195-200. DOI: 10.1007/s12223-008-0024-z.
120. Guiavarch, E., et al., *Application of a data reconciliation method to the stoichiometric analysis of Fibrobacter succinogenes growth.* Applied Biochemistry and Biotechnology, 2008. **151**(2-3): p. 201-210. DOI: 10.1007/s12010-008-8172-9
121. Rainey, F.A., *Succiniclasticum*, in *Bergey's Manual of Systematics of Archaea and Bacteria*, W.B. Whitman, Editor. 2015, John Wiley & Sons, Inc., in association with Bergey's Manual Trust.
122. Scheifinger, C.C. and M.J. Wolin, *Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium.* Applied and Environmental Microbiology, 1973. **26**(5): p. 789-795. DOI: 10.1128/am.26.5.789-795.1973.
123. Xie, X., et al., *Persistence of cellulolytic bacteria Fibrobacter and Treponema after short-term corn stover-based dietary intervention reveals the potential to improve rumen fibrolytic function.* Frontiers in Microbiology, 2018. **9**: p. 1363. DOI: 10.3389/fmicb.2018.01363
124. Sun, H.-Z., et al., *Multi-omics reveals functional genomic and metabolic mechanisms of milk production and quality in dairy cows.* Bioinformatics, 2020. **36**(8): p. 2530-37. DOI: 10.1093/bioinformatics/btz951.
125. Kudo, H., K.-J. Cheng, and J. Costerton, *Interactions between Treponema bryantii and cellulolytic bacteria in the in vitro degradation of straw cellulose.* Canadian Journal of Microbiology, 1987. **33**(3): p. 244-248. DOI: 10.1139/m87-041.
126. Lynd, L.R., et al., *Microbial cellulose utilization: fundamentals and biotechnology.* Microbiology and Molecular Biology Reviews, 2002. **66**(3): p. 506-577. DOI: 10.1128/MMBR.66.3.506-577.2002.
127. Deng, L., et al., *Ground transport stress affects bacteria in the rumen of beef cattle: A real-time PCR analysis.* Animal Science Journal, 2017. **88**(5): p. 790-7. DOI: 10.1111/asj.12615.
128. Wang, X., et al., *Correlation between composition of the bacterial community and concentration of volatile fatty acids in the rumen during the transition period and ketosis in dairy cows.* Applied and Environmental Microbiology, 2012. **78**(7): p. 2386-92. DOI: 10.1128/AEM.07545-11
129. Zhao, S., et al., *Effect of heat stress on bacterial composition and metabolism in the rumen of lactating dairy cows.* Animals, 2019. **9**(11): p. 925. DOI: 10.3390/ani9110925.
130. Humbel, F., et al., *Association of alterations in intestinal microbiota with impaired psychological function in patients with inflammatory bowel diseases in*

remission. Clinical Gastroenterology and Hepatology, 2019. **18**(9): p. 2019-29.e11. DOI: 10.1016/j.cgh.2019.09.022.
131. Auffret, M.D., et al., *The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle.* Microbiome, 2017. **5**(1): p. 159. DOI: 10.1186/s40168-017-0378-z.
132. Meadows, J.A. and M.J. Wargo, *Carnitine in bacterial physiology and metabolism.* Microbiology, 2015. **161**(Pt 6): p. 1161. DOI: 10.1099/mic.0.000080.
133. Roncal, C., et al., *Trimethylamine-N-Oxide (TMAO) Predicts Cardiovascular Mortality in Peripheral Artery Disease.* Scientific Reports, 2019. **9**(1): p. 1-8. DOI: 10.1038/s41598-019-52082-z.
134. Jayaprakash, G., et al., *Rumen-protected choline: A significance effect on dairy cattle nutrition.* Veterinary World, 2016. **9**(8): p. 837. DOI: 10.14202/vetworld.2016.837-841.
135. Pour, H.A., et al., *Effect of choline on performance quality of non-ruminant.* Entomology and Applied Science Letters, 2014. **1**(1): p. 14-18.
136. Pinotti, L., et al., *The role of micronutrients in high-yielding dairy ruminants: choline and vitamin E.* Ankara Üniv Vet Fak Derg, 2020. **67**: p. 209-14. DOI: 10.33988/auvfd.695432.
137. Kaewtapee, C., et al., *Effect of Bacillus subtilis and Bacillus licheniformis supplementation in diets with low-and high-protein content on ileal crude protein and amino acid digestibility and intestinal microbiota composition of growing pigs.* Journal of Animal Science and Biotechnology, 2017. **8**(1): p. 1-15. DOI: 10.1186/s40104-017-0168-2.
138. Bolyen, E., et al., *Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2.* Nature Biotechnology, 2019. **37**(8): p. 852-857. DOI: 10.1038/s41587-019-0209-9.
139. Callahan, B.J., et al., *DADA2: high-resolution sample inference from Illumina amplicon data.* Nature Methods, 2016. **13**(7): p. 581-583. DOI: 10.1038/nmeth.3869.
140. Pedregosa, F., et al., *Scikit-learn: machine learning in Python.* Journal of Machine Learning Research, 2011. **12**: p. 2825-2830.
141. Quast, C., et al., *The SILVA ribosomal RNA gene database project: improved data processing and web-based tools.* Nucleic Acids Research, 2012. **41**(D1): p. D590-D596. DOI: 10.1093/nar/gks1219.
142. Cole, J.R., et al., *Ribosomal database project: data and tools for high throughput rRNA analysis.* Nucleic Acids Research, 2014. **42**(D1): p. D633-D642. DOI: 10.1093/nar/gkt1244.
143. Wang, Q., et al., *Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy.* Applied and Environmental Microbiology, 2007. **73**(16): p. 5261-5267. DOI: 10.1128/AEM.00062-07.
144. Yarza, P., et al., *Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences.* Nature Reviews Microbiology, 2014. **12**(9): p. 635-645. DOI: 10.1038/nrmicro3330.
145. Weljie, A.M., et al., *Targeted profiling: quantitative analysis of ^{1}H NMR metabolomics data.* Analytical Chemistry, 2006. **78**(13): p. 4430-42. DOI: 10.1021/ac060209g.
146. Clarke, K.R., et al., *Change in marine communities: an approach to statistical analysis and interpretation*. 3rd Edition ed. 2014, Plymouth, UK: Primer-E Ltd.

147. Bray, J.R. and J.T. Curtis, *An ordination of the upland forest communities of southern Wisconsin.* Ecological Monographs, 1957. **27**(4): p. 325-349. DOI: 10.2307/1942268.
148. Weiss, W.P., *A 100-year review: from ascorbic acid to zinc—mineral and vitamin nutrition of dairy cows.* Journal of Dairy Science, 2017. **100**(12): p. 10045-10060. DOI: 10.3168/jds.2017-12935.
149. De Vries, A. and M. Marcondes, *Overview of factors affecting productive lifespan of dairy cows.* Animal, 2020. **14**(S1): p. 155-164. DOI: 10.1017/S1751731119003264.
150. Bach, A., *Associations between several aspects of heifer development and dairy cow survivability to second lactation.* Journal of Dairy Science, 2011. **94**(2): p. 1052-1057. DOI: 10.3168/jds.2010-3633.
151. Plaizier, J., et al., *Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences.* The Veterinary Journal, 2008. **176**(1): p. 21-31. DOI: 10.1016/j.tvjl.2007.12.016.
152. Sanz-Fernandez, M.V., et al., *Targeting the hindgut to improve health and performance in cattle.* Animals, 2020. **10**(10): p. 1817. DOI: 10.3390/ani10101817.
153. Boyle, E.C. and B.B. Finlay, *Leaky guts and lipid rafts.* Trends in Microbiology, 2005. **13**(12): p. 560-563. DOI: 10.1016/j.tim.2005.10.003.
154. Eckel, E.F. and B.N. Ametaj, *Bacterial endotoxins and their role in periparturient diseases of dairy cows: mucosal vaccine perspectives.* Dairy, 2020. **1**(1): p. 61-90. DOI: 10.3390/dairy1010006.
155. van Knegsel, A.T., et al., *Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle.* Reproduction Nutrition Development, 2005. **45**(6): p. 665-688. DOI: 10.1051/rnd:2005059
156. LaCount, D., J. Drackley, and D. Weigel, *Responses of dairy cows during early lactation to ruminal or abomasal administration of l-carnitine.* Journal of Dairy Science, 1995. **78**(8): p. 1824-1836. DOI: 10.3168/jds.S0022-0302(95)76807-2.
157. Arumugam, M., et al., *Enterotypes of the human gut microbiome.* Nature, 2011. **473**(7346): p. 174-180. DOI: 10.1038/nature09944.
158. Kononov, S.U., et al., *Effects of dietary l-carnitine supplementation on platelets and erythrogram of dairy cows with special emphasis on parturition.* Dairy, 2021. **2**(1): p. 1-13. DOI: 10.3390/dairy2010001.
159. Hildebrand, F., et al., *Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice.* Genome Biology, 2013. **14**(1): p. R4. DOI: 10.1186/gb-2013-14-1-r4.
160. Knights, D., et al., *Rethinking "Enterotypes".* Cell Host and Microbe, 2014. **16**(4): p. 433-437. DOI: 10.1016/j.chom.2014.09.013.
161. Lozupone, C.A., et al., *Diversity, stability and resilience of the human gut microbiota.* Nature, 2012. **489**(7415): p. 220-230. DOI: 10.1038/nature11550.
162. Clemmons, B.A., et al., *Temporal stability of the ruminal bacterial communities in beef steers.* Scientific Reports, 2019. **9**(1): p. 9522. DOI: 10.1038/s41598-019-45995-2.
163. Coyte, K.Z., J. Schluter, and K.R. Foster, *The ecology of the microbiome: networks, competition, and stability.* Science, 2015. **350**(6261): p. 663-666. DOI: 10.1126/science.aad2602.

164. Voolstra, C.R. and M. Ziegler, *Adapting with microbial help: microbiome flexibility facilitates rapid responses to environmental change.* BioEssays, 2020. **42**(7): p. 2000004. DOI: 10.1002/bies.202000004.
165. Van Vliet, P., et al., *Effects of cow diet on the microbial community and organic matter and nitrogen content of feces.* Journal of Dairy Science, 2007. **90**(11): p. 5146-5158. DOI: 10.3168/jds.2007-0065.
166. Krautkramer, K.A., J. Fan, and F. Bäckhed, *Gut microbial metabolites as multi-kingdom intermediates.* Nature Reviews Microbiology, 2020: p. 1-18. DOI: 10.1038/s41579-020-0438-4.
167. Litvak, Y., M.X. Byndloss, and A.J. Bäumler, *Colonocyte metabolism shapes the gut microbiota.* Science, 2018. **362**(6418). DOI: 10.1126/science.aat9076.
168. Fransen, F., et al., *Aged gut microbiota contributes to systemical inflammaging after transfer to germ-free mice.* Frontiers in Immunology, 2017. **8**: p. 1385. DOI: 10.3389/fimmu.2017.01385.
169. Liu, J., et al., *High-grain feeding alters caecal bacterial microbiota composition and fermentation and results in caecal mucosal injury in goats.* British Journal of Nutrition, 2014. **112**(3): p. 416-427. DOI: 10.1017/S0007114514000993.
170. Huang, S., et al., *The day-to-day stability of the ruminal and fecal microbiota in lactating dairy cows.* MicrobiologyOpen, 2020. **9**(5): p. e990. DOI: 10.1002/mbo3.990.
171. Oliphant, K. and E. Allen-Vercoe, *Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health.* Microbiome, 2019. **7**(1): p. 1-15. DOI: 10.1186/s40168-019-0704-8.
172. Kalantar-Zadeh, K., et al., *Intestinal gases: influence on gut disorders and the role of dietary manipulations.* Nature Reviews Gastroenterology and Hepatology, 2019. **16**(12): p. 733-747. DOI: 10.1038/s41575-019-0193-z.
173. Singhal, R. and Y.M. Shah, *Oxygen battle in the gut: hypoxia and hypoxia-inducible factors in metabolic and inflammatory responses in the intestine.* Journal of Biological Chemistry, 2020. **295**(30): p. 10493-10505. DOI: 10.1074/jbc.REV120.011188.
174. Weber, G.J., D.M. van Sambeek, and S.C. Pearce, *Hypoxia and hypercapnia diminish intestinal barrier integrity in a human enteroid model system.* The Federation of American Societies for Experimental Biology Journal, 2020. **34**(S1): p. 1-1. DOI: 10.1096/fasebj.2020.34.s1.05850.
175. Allen, M.S. and P. Piantoni, *Metabolic control of feed intake: implications for metabolic disease of fresh cows.* Veterinary Clinics of North America: Food Animal Practice, 2013. **29**(2): p. 279-297. DOI: 10.1016/j.cvfa.2013.04.001.
176. Gianesella, M., et al., *Subacute ruminal acidosis and evaluation of blood gas analysis in dairy cow.* Veterinary Medicine International, 2010. **2010**. DOI: 10.4061/2010/392371.
177. Maas, M.N., et al., *Trimethyllysine: from carnitine biosynthesis to epigenetics.* International Journal of Molecular Sciences, 2020. **21**(24): p. 9451. DOI: 10.3390/ijms21249451.
178. Skagen, K., et al., *The carnitine-butyrobetaine-trimethylamine-N-oxide pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis.* Atherosclerosis, 2016. **247**: p. 64-69. DOI: 10.1016/j.atherosclerosis.2016.01.033.
179. Oikonomou, G., et al., *Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth.* PLoS One, 2013. **8**(4): p. e63157. DOI: 10.1371/journal.pone.0063157.

180. Amin, N., et al., *Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning.* Animal Microbiome, 2021. **3**(1): p. 31. DOI: 10.1186/s42523-021-00095-3.
181. Kordy, K., et al., *Contributions to human breast milk microbiome and enteromammary transfer of Bifidobacterium breve.* PLoS One, 2020. **15**(1): p. e0219633. DOI: 10.1371/journal.pone.0219633.
182. Mohan, R., et al., *Effects of Bifidobacterium lactis Bb12 supplementation on body weight, fecal pH, acetate, lactate, calprotectin, and IgA in preterm infants.* Pediatric Research, 2008. **64**(4): p. 418-422. DOI: 10.1203/PDR.0b013e318181b7fa.
183. Robles-Vera, I., et al., *Probiotic Bifidobacterium breve prevents DOCA-salt hypertension.* The FASEB Journal, 2020. **34**(10): p. 13626-13640. DOI: 10.1096/fj.202001532R.
184. Korzhenkov, A.A., et al., *A dataset of four probiotic Bifidobacterium strains genome assemblies.* Data in Brief, 2021. **34**: p. 106710. DOI: 10.1016/j.dib.2020.106710.
185. Kim, H.S., et al., *Longitudinal evaluation of fecal microbiota transplantation for ameliorating calf diarrhea and improving growth performance.* Nature Communications, 2021. **12**(1): p. 1-16. DOI: 10.1038/s41467-020-20389-5.
186. Malmuthuge, N., et al., *Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves.* Journal of Dairy Science, 2015. **98**(11): p. 8044-8053. DOI: 10.3168/jds.2015-9607.
187. Santacruz, A., et al., *Gut microbiota composition is associated with body weight, weight gain and biochemical parameters in pregnant women.* British Journal of Nutrition, 2010. **104**(1): p. 83-92. DOI: 10.1017/S0007114510000176.
188. Christensen, L., et al., *Microbial enterotypes in personalized nutrition and obesity management.* The American Journal of Clinical Nutrition, 2018. **108**(4): p. 645-651. DOI: 10.1093/ajcn/nqy175.
189. Fischer, A., R. Delagarde, and P. Faverdin, *Identification of biological traits associated with differences in residual energy intake among lactating Holstein cows.* Journal of Dairy Science, 2018. **101**(5): p. 4193-4211. DOI: 10.3168/jds.2017-12636.
190. Patil, R.D., et al., *Poor feed efficiency in sheep is associated with several structural abnormalities in the community metabolic network of their ruminal microbes.* Journal of Animal Science, 2018. **96**(6): p. 2113-2124. DOI: 10.1093/jas/sky096.
191. Shabat, S.K.B., et al., *Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants.* The ISME Journal, 2016. **10**(12): p. 2958-2972. DOI: 10.1038/ismej.2016.62.
192. Uzan-Yulzari, A., et al., *Neonatal antibiotic exposure impairs child growth during the first six years of life by perturbing intestinal microbial colonization.* Nature Communications, 2021. **12**(1): p. 1-12. DOI: 10.1038/s41467-020-20495-4.
193. Korpela, K., et al., *Antibiotics in early life associate with specific gut microbiota signatures in a prospective longitudinal infant cohort.* Pediatric Research, 2020. **88**: p. 438-443. DOI: 10.1038/s41390-020-0761-5.
194. Gonzalez-Recio, O., et al., *Signs of host genetic regulation in the microbiome composition in 2 dairy breeds: Holstein and Brown Swiss.* Journal of Dairy Science, 2018. **101**(3): p. 2285-2292. DOI: 10.3168/jds.2017-13179.
195. Wickham, H., et al., *Welcome to the Tidyverse.* Journal of Open Source Software, 2019. **4**(43): p. 1686. DOI: 10.21105/joss.01686.

196. Walesiak, M. and A. Dudek, *The choice of variable normalization method in cluster analysis*, in *Education Excellence and Innovation Management: A 2025 Vision to Sustain Economic Development during Global Challenges*, K. Soliman, Editor. 2020. p. 325-340.
197. Maechler, M., et al. *Package 'cluster'*. 2014; Available from: https://mran.microsoft.com/snapshot/2014-12-11/web/packages/cluster/cluster.pdf.
198. Kassambara, A. and F. Mundt. *Package 'factoextra': extract and visualize the results of multivariate data analyses*. 2017 [cited 76; Available from: https://rpkgs.datanovia.com/factoextra/index.html.
199. Thioulouse, J., et al., *Multivariate analysis of ecological data with ade4*. 2018, New York, NY: Springer. Xi, 329.
200. Lengowski, M.B., et al., *Effects of corn silage and grass silage in ruminant rations on diurnal changes of microbial populations in the rumen of dairy cows.* Anaerobe, 2016. **42**: p. 6-16. DOI: 10.1016/j.anaerobe.2016.07.004.
201. Lee, C., et al., *Real-time PCR determination of rRNA gene copy number: absolute and relative quantification assays with Escherichia coli.* Applied Microbiology and Biotechnology, 2008. **78**(2): p. 371-376. DOI: 10.1007/s00253-007-1300-6.
202. Monteagudo-Mera, A., et al., *High purity galacto-oligosaccharides (GOS) enhance specific Bifidobacterium species and their metabolic activity in the mouse gut microbiome.* Beneficial Microbes, 2016. **7**(2): p. 247. DOI: 10.3920/BM2015.0114.
203. Wilkinson, T.J., et al., *CowPI: a rumen microbiome focussed version of the PICRUSt functional inference software.* Frontiers in Microbiology, 2018. **9**: p. 1095. DOI: 10.3389/fmicb.2018.01095.
204. Wischer, G., et al., *Effect of monensin on in vitro fermentation of silages and microbial protein synthesis.* Archives of Animal Nutrition, 2013. **67**(3): p. 219-234. DOI: 10.1080/1745039X.2013.793050.
205. Kenéz, Á., et al., *A metabolomics approach to characterize phenotypes of metabolic transition from late pregnancy to early lactation in dairy cows.* Metabolomics, 2016. **12**(11): p. 1-11. DOI: 10.1007/s11306-016-1112-8.
206. Bates, D., et al., *Fitting linear mixed-effects models using lme4.* arXiv preprint arXiv:1406.5823, 2014.
207. Kuznetsova, A., P.B. Brockhoff, and R.H. Christensen, *lmerTest package: tests in linear mixed effects models.* Journal of Statistical Software, 2017. **82**(1): p. 1-26. DOI: 10.18637/jss.v082.i13.
208. Bellali, S., et al., *Running after ghosts: are dead bacteria the dark matter of the human gut microbiota?* Gut Microbes, 2021. **13**(1): p. 1-12. DOI: 10.1080/19490976.2021.1897208.
209. Kamra, D.N., *Rumen microbial ecosystem.* Current Science, 2005: p. 124-135.
210. McBurney, M.I., et al., *Establishing what constitutes a healthy human gut microbiome: state of the science, regulatory considerations, and future directions.* The Journal of Nutrition, 2019. **149**(11): p. 1882-1895. DOI: 10.1093/jn/nxz154.
211. Moberg, G.P., *Biological response to stress: key to assessment of animal well-being?*, in *Animal Stress*, G.P. Moberg, Editor. 1985, Springer New York: New York, NY. p. 27-49.
212. Shang, Q., et al., *Source of fiber influences growth, immune responses, gut barrier function and microbiota in weaned piglets fed antibiotic-free diets.* Animal Nutrition, 2021. **7**(2): p. 315-325. DOI: 10.1016/j.aninu.2020.12.008.

213. Castillo, C. and J. Hernández, *Ruminal fistulation and cannulation: a necessary procedure for the advancement of biotechnological research in ruminants.* Animals, 2021. **11**(7): p. 1870. DOI: 10.3390/ani11071870
214. Hamady, M. and R. Knight, *Microbial community profiling for human microbiome projects: tools, techniques, and challenges.* Genome Research, 2009. **19**(7): p. 1141-1152. DOI: 10.1101/gr.085464.108
215. Shade, A. and J. Handelsman, *Beyond the Venn diagram: the hunt for a core microbiome.* Environmental Microbiology, 2012. **14**(1): p. 4-12. DOI: 10.1111/j.1462-2920.2011.02585.x.
216. Harmoinen, J.A., et al., *Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function.* Comparative Medicine, 2001. **51**(3): p. 252-256.
217. Ahuja, M., et al., *Orai1-mediated antimicrobial secretion from pancreatic acini shapes the gut microbiome and regulates gut innate immunity.* Cell Metabolism, 2017. **25**(3): p. 635-646. DOI: 10.1016/j.cmet.2017.02.007.
218. Dowd, S.E., T.R. Callaway, and J. Morrow-Tesch, *Handling may cause increased shedding of Escherichia coli and total coliforms in pigs.* Foodborne Pathogens and Disease, 2007. **4**(1): p. 99-102. DOI: 10.1089/fpd.2006.53.
219. Abt, M.C. and E.G. Pamer, *Commensal bacteria mediated defenses against pathogens.* Current Opinion in Immunology, 2014. **29**: p. 16-22. DOI: 10.1016/j.coi.2014.03.003.
220. Beukema, M., M.M. Faas, and P. de Vos, *The effects of different dietary fiber pectin structures on the gastrointestinal immune barrier: impact via gut microbiota and direct effects on immune cells.* Experimental and Molecular Medicine, 2020. **52**(9): p. 1364-1376. DOI: 10.1038/s12276-020-0449-2.
221. Van Deun, K., et al., *Butyrate protects Caco-2 cells from Campylobacter jejuni invasion and translocation.* British Journal of Nutrition, 2008. **100**(3): p. 480-484. DOI: 10.1017/S0007114508921693.
222. Gregory, N., et al., *Effect of preslaughter feeding system on weight loss, gut bacteria, and the physico-chemical properties of digesta in cattle.* New Zealand Journal of Agricultural Research, 2000. **43**(3): p. 351-361. DOI: 10.1080/00288233.2000.9513434.
223. D'Souza, G., et al., *Ecology and evolution of metabolic cross-feeding interactions in bacteria.* Natural Product Reports, 2018. **35**(5): p. 455-488. DOI: 10.1039/c8np00009c.
224. Larsen, O.F.A. and E. Claassen, *The mechanistic link between health and gut microbiota diversity.* Scientific Reports, 2018. **8**(1): p. 2183. DOI: 10.1038/s41598-018-20141-6.
225. Kim, M., et al., *Association between rumen microbiota and marbling score in korean native beef cattle.* Animals, 2020. **10**(4): p. 712. DOI: 10.3390/ani10040712.
226. Roudbari, Z., et al., *Identification of biological pathways contributing to marbling in skeletal muscle to improve beef cattle breeding.* Frontiers in Genetics, 2020. **10**: p. 1370. DOI: 10.3389/fgene.2019.01370.
227. Cheng, M. and K. Ning, *Stereotypes about enterotype: the old and new ideas.* Genomics, Proteomics and Bioinformatics, 2019. **17**(1): p. 4-12. DOI: 10.1016/j.gpb.2018.02.004.
228. Liang, C., et al., *Diversity and enterotype in gut bacterial community of adults in Taiwan.* BMC Genomics, 2017. **18**(1): p. 932. DOI: 10.1186/s12864-016-3261-6.

229. Li, F., et al., *Host genetics influence the rumen microbiota and heritable rumen microbial features associate with feed efficiency in cattle.* Microbiome, 2019. **7**(1): p. 1-17. DOI: 10.1186/s40168-019-0699-1.
230. Gibbons, S.M., *Keystone taxa indispensable for microbiome recovery.* Nature Microbiology, 2020. **5**(9): p. 1067-1068. DOI: 10.1038/s41564-020-0783-0.
231. Rook, G.A., *99th Dahlem conference on infection, inflammation and chronic inflammatory disorders: Darwinian medicine and the 'hygiene' or 'old friends' hypothesis.* Clinical and Experimental Immunology, 2010. **160**(1): p. 70-79. DOI: 10.1111/j.1365-2249.2010.04133.x.
232. Henrick, B.M., et al., *Elevated fecal pH indicates a profound change in the breastfed infant gut microbiome due to reduction of Bifidobacterium over the past century.* mSphere, 2018. **3**(2): p. e00041-18. DOI: 10.1128/mSphere.00041-18.
233. Klein-Jöbstl, D., et al., *Microbiota of newborn calves and their mothers reveals possible transfer routes for newborn calves' gastrointestinal microbiota.* PLoS One, 2019. **14**(8): p. e0220554. DOI: 10.1371/journal.pone.0220554.
234. Wang, L., et al., *Exploring the goat rumen microbiome from seven days to two years.* PLoS One, 2016. **11**(5): p. e0154354. DOI: 10.1371/journal.pone.0154354.
235. Fernando, S.C., et al., *Rumen microbial population dynamics during adaptation to a high-grain diet.* Applied and Environmental Microbiology, 2010. **76**(22): p. 7482-7490. DOI: 10.1128/AEM.00388-10.
236. Kim, D.-H., et al., *Differential dynamics of the ruminal microbiome of jersey cows in a heat stress environment.* Animals, 2020. **10**(7): p. 1127. DOI: 10.3390/ani10071127.
237. Zamani, P., *Efficiency of lactation*, in *Milk Production - an up-to-date overview of animal nutrition, management and health*. 2012, IntechOpen. p. 139-150.
238. Martinez-Guryn, K., V. Leone, and E.B. Chang, *Regional diversity of the gastrointestinal microbiome.* Cell Host and Microbe, 2019. **26**(3): p. 314-324. DOI: 10.1016/j.chom.2019.08.011.
239. Hill, T.C., et al., *Using ecological diversity measures with bacterial communities.* FEMS Microbiology Ecology, 2003. **43**(1): p. 1-11. DOI: 10.1111/j.1574-6941.2003.tb01040.x.
240. Henderson, G., et al., *Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range.* Scientific Reports, 2015. **5**(1): p. 1-15. DOI: 10.1038/srep14567.
241. Huws, S.A., et al., *As yet uncultured bacteria phylogenetically classified as Prevotella, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal biohydrogenation.* Environmental Microbiology, 2011. **13**(6): p. 1500-1512. DOI: 10.1111/j.1462-2920.2011.02452.x.
242. Hu, X., et al., *The effect of rumen microbiota in the susceptibility of subacute ruminal acidosis in dairy cows.* Research Square, 2021. DOI: 10.21203/rs.3.rs-250349/v1.
243. Gavini, F., et al., *Bifidobacterium species isolated from animal feces and from beef and pork meat.* Journal of Food Protection, 2006. **69**(4): p. 871-877. DOI: 10.4315/0362-028X-69.4.871.
244. Lawson, M.A.E., et al., *Breast milk-derived human milk oligosaccharides promote Bifidobacterium interactions within a single ecosystem.* The ISME Journal, 2020. **14**(2): p. 635-648. DOI: 10.1038/s41396-019-0553-2.

245. Darvishi, N., N.A. Fard, and M. Sadrnia, *Genomic and proteomic comparisons of bacteriocins in probiotic species Lactobacillus and Bifidobacterium and inhibitory ability of Escherichia coli MG 1655.* Biotechnology Reports, 2021. **31**: p. e00654. DOI: 10.1016/j.btre.2021.e00654.
246. Wilms, J., H. Berends, and J. Martín-Tereso, *Hypertonic milk replacers increase gastrointestinal permeability in healthy dairy calves.* Journal of Dairy Science, 2019. **102**(2): p. 1237-1246. DOI: 10.3168/jds.2018-15265.
247. Petschow, B.W. and R.D. Talbott, *Response of Bifidobacterium species to growth promoters in human and cow milk.* Pediatric Research, 1991. **29**(2): p. 208-213. DOI: 10.1203/00006450-199102000-00021.
248. Badman, J., et al., *The effect of milk replacer composition on the intestinal microbiota of pre-ruminant dairy calves.* Frontiers in Veterinary Science, 2019. **6**: p. 371. DOI: 10.3389/fvets.2019.00371.
249. Jaglan, N., et al., *Isolation, characterization and conjugated linoleic acid production potential of bifidobacterial isolates from ruminal fluid samples of Murrah buffaloes.* Anaerobe, 2019. **56**: p. 40-45. DOI: 10.1016/j.anaerobe.2019.02.001.
250. Ellison, M., et al., *Diet and feed efficiency status affect rumen microbial profiles of sheep.* Small Ruminant Research, 2017. **156**: p. 12-19. DOI: 10.1016/j.smallrumres.2017.08.009.
251. Hooks, K.B. and M.A. O'Malley, *Dysbiosis and its discontents.* mBio, 2017. **8**(5). DOI: 10.1128/mBio.01492-17.
252. Shin, N.-R., T.W. Whon, and J.-W. Bae, *Proteobacteria: microbial signature of dysbiosis in gut microbiota.* Trends in Biotechnology, 2015. **33**(9): p. 496-503. DOI: 10.1016/j.tibtech.2015.06.011.
253. Smith, S.A., et al., *Mitochondrial dysfunction in inflammatory bowel disease alters intestinal epithelial metabolism of hepatic acylcarnitines.* The Journal of Clinical Investigation, 2021. **131**(1). DOI: 10.1172/JCI133371.
254. Kara, K., et al., *Effect of dietary formic acid on the in vitro ruminal fermentation parameters of barley-based concentrated mix feed of beef cattle.* Journal of Applied Animal Research, 2018. **46**(1): p. 178-183. DOI: 10.1080/09712119.2017.1284073.
255. Asanuma, N., M. Iwamoto, and T. Hino, *The production of formate, a substrate for methanogenesis, from compounds related with the glyoxylate cycle by mixed ruminal microbes.* Nihon Chikusan Gakkaiho, 1999. **70**(2): p. 67-73. DOI: 10.2508/chikusan.70.67.
256. Kaçar, C., et al., *The effects of l-carnitine administration on energy metabolism in pregnant Halep (Damascus) goats.* Turkish Journal of Veterinary and Animal Sciences, 2010. **34**(2): p. 163-171. DOI: 10.3906/vet-0805-11.
257. Meale, S.J., et al., *Exploration of biological markers of feed efficiency in young bulls.* Journal of Agricultural and Food Chemistry, 2017. **65**(45): p. 9817-9827. DOI: 10.1021/acs.jafc.7b03503.
258. Ruiz, L., A. Margolles, and B. Sánchez, *Bile resistance mechanisms in Lactobacillus and Bifidobacterium.* Frontiers in Microbiology, 2013. **4**: p. 396. DOI: 10.3389/fmicb.2013.00396.
259. Von Soosten, D., et al., *Excretion pathways and ruminal disappearance of glyphosate and its degradation product aminomethylphosphonic acid in dairy cows.* Journal of Dairy Science, 2016. **99**(7): p. 5318-5324. DOI: 10.3168/jds.2015-10585.
260. Wenham, G. and R. Wyburn, *A radiological investigation of the effects of cannulation on intestinal motility and digesta flow in sheep.* The Journal of

Agricultural Science, 1980. **95**(3): p. 539-546. DOI: 10.1017/S0021859600087943.
261. Fushuku, S. and K. Fukuda, *Gender difference in the composition of fecal flora in laboratory mice, as detected by denaturing gradient gel electrophoresis (DGGE).* Experimental Animals, 2008. **57**(5): p. 489-493. DOI: 10.1538/expanim.57.489.
262. Jha, A.R., et al., *Gut microbiome transition across a lifestyle gradient in Himalaya.* PLoS Biology, 2018. **16**(11): p. e2005396. DOI: 10.1371/journal.pbio.2005396.
263. Williams, A.G. and G.S. Coleman, *The rumen microbial ecosystem: the rumen protozoa*. 1997: Springer, Dordrecht.
264. Solomon, K.V., et al., *Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes.* Science, 2016. **351**(6278): p. 1192-1195. DOI: 10.1126/science.aad1431.
265. Xue, F., et al., *Metagenome sequencing to analyze the impacts of thiamine supplementation on ruminal fungi in dairy cows fed high-concentrate diets.* Applied Microbiology and Biotechnology Express, 2018. **8**(1): p. 1-12. DOI: 10.1186/s13568-018-0680-6.
266. Pérez, J.C., *The interplay between gut bacteria and the yeast Candida albicans.* Gut Microbes, 2021. **13**(1): p. 1979877. DOI: 10.1080/19490976.2021.1979877.

Supplementary material

The supplementary material can be found in electronic form only on CD ("DissertationCD") and is structured as follows:

Chapter_2	SData	• *SData_2.1_OTU.xlsx*
		• *SData_2.2_Taxa_file.xlsx*
		• *SData_2.3_Timepoint_comparisons_RDP.xlsx*
		• *SData_2.4_NMR_data.xlsx*
		• *SData_2.5_Pairwise_correlation_all_metabolites.xlsx*
		• *SData_2.6_Repeating_correlations.xlsx*
	Supplementary_Tab_Fig.pdf	
Chapter_3	Data_Sets	• *Data Set S1_OTU.xlsx*
		• *Data Set S2_Taxa.xlsx*
		• *Data Set S3_Metadata Experiment.xlsx*
		• *Data Set S4_SIMPER for TimePoint.xlsx*
		• *Data Set S5_Metabolome.xlsx*
		• *Data Set S6_Health Data.xlsx*
		• *Data Set S7_Production Data.xlsx*
		• *Data Set S8_Blood Parameters.xlsx*
	raw_data	• *Biocrates_normalized_for_R_Metabotype.csv*
		• *R_inputfile_BlastNCBI_fecalsamples_for Enterotype_unclFirmiunclSpiro_54animals.txt*
	R_scripts	• *Enterotype_script_13tp_NCBInewannotationFirmiSpiro.R*
		• *LMM_loop.R*
	S_Figures.pdf	
Affidavit1		
Affidavit2		
Curriculum_vitae		
Dissertation		

Acknowledgements

Writing a doctoral thesis has been the most challenging thing that I have accomplished so far. It would have not been possible without the aid, encouragement and motivation of quite a list of people, which I am very thankful for.

The block module, where I got to know Prof. Dr. Jana Seifert has been both fascinating and scary, as in my former study program bacteria were only sparsely discussed. I am happy and grateful she accepted me as a master student and later as doctoral candidate. She always had new ideas which forced me to push my intellectual boundaries and go beyond my comfort zones and always had an open door, when it was needed. Thanks for believing in me and for giving me a platform to grow.
Also, I would like to thank Jun.-Prof. Dr. Amélia Camarinha Silva for her continuous support, feedback and advice. Both female scientists were excellent teachers and role models, for women and mothers in science.

In the past years, not only did I learn about bacteria in dairy cows but also about the beauty of an international working group. The conversations about science and a good sip of nonsense were the best and a good stress relief during challenging times in lab and office. There were many ups and downs, devastations and beautiful lucky moments. For this unforgettable time, I would like to thank all the fellows that I met along way of my PhD, especially, Angélica, Andrej, Daniel, Simon, Katharina and Bruno!
Throughout the - in part stressful - lab work, the good souls of the lab, Beate, Petra, Tanja and Helga, have always been a help and often calmed me down, when things got sticky, tricky or were just not going through the filter! Thanks for everything!

In the toughest time during the work, the animal trial in Brunswick, Sandra Grindler and me shared a flat. We have been labeled by the Brunswick institute folks as “das doppelte Lottchen” as we ALWAYS sticked together. That was our winning formula! Thanks for these many lovely nights and days in Brunswick. 1/2 Hohenheim out – now it's your turn!

For contracts, keys, holidays, payroll accountings, orders and all other administrative tasks I had to face, I could always count on the support of Birgit and Ingrid. Even for

ordering delicious free-ranged angus beef! Thanks for your, literally, open doors and ears, throughout all these years!

Hohenheim has not only been a wonderful chapter in my life, it also is the place where my parents have met. As their child, Hohenheim somehow is where my story begins. Their passion for the beauty of nature has been successfully reseeded in the next generation. Thanks Papa for reading so many pieces of my work and for always having an ear. It is always fun yarning ideas about how we can embrace the power of nature. With this work, I have proved to myself, that I have inherited a bit of the persistence of my mother. She's not a quitter! That makes her a very tough opponent for political colleagues and weeds in the garden. But also it makes her the most solid person I know.

Special thanks to Dr. Angelica Hernandez, Dr. Maximilian Mußotter, Dr. Arnulf Tröscher and Dr. Simon Champ for taking time to proof read the final thesis. I know it can be difficult to read other peoples works.

To all the grandparents of Thea, but in particular to Dorothea Mußotter and Susanne Tröscher, thank you for taking care of Thea in the last months. This gave me time to focus on the final work and I cannot and don't want to imagine how I would stand without your precious help!

Throughout the years as a PhD candidate, there has been this one person, that always pushed and supported me. Not only was he my solid rock in turbulent waters, but also often a pain in the neck when it came to keeping my work professional, accurate and - objective. Max, thanks for walking aside me!