Impact of age and weaning time on the gut microbiome and the potential host-microbe interactions in calves

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LIST OF ABBREVIATIONS

~	Approximately
α	Alpha
ß	Beta
°C	Degree Celsius
μl	Microliter
μM	Micrometre
%	Percentage
16S rRNA gene	16S ribosomal ribonucleic acid gene
5-HT	5-hydroxytryptamine/serotonin
AAs	Amino acids
ABC transporter	ATP-binding cassette transporters
ACs	Acylcarnitines
ad lib	ad libitum
ADG	Average daily gain
ANOSIM	Analysis of similarities
ASVs	Amplicon sequence variants
BAs	Biogenic amines
BHB	Beta-hydroxybutyrate
BLAST	Basic Local Alignment Search Tool
BM	Bulk milk
bp	Base pairs
BS	Buccal swabbing
BSH	Bile salt hydrolase
C	Concentrates
c_	Class
CAZYmes	Carbohydrate-active enzymes
d	Day
DADA2	Divisive Amplicon Denoising Algorithm 2
dIgA	Dimeric immunoglobulin A
DM	Dry matter
DMs	Differential metabolites

DNA	Deoxyribonucleic acid
E	Early
e.g.	For example
EAA	Essential amino acid
earlyC	Early-weaned calves
EC cell	Enterochromaffin cell
ELISA	Enzyme-linked immunoassay
f_	Family
FC	Fold change
FDR	False discovery rate
g	Gram
g_	Genus
GC-MS	Gas chromatography-mass spectrometry
GH	Glycoside hydrolases
GIT	Gastrointestinal tract
GLP-2	Glucagon-like peptide-2
h	Hour
HTR2B	5-hydroxytryptamine receptor 2B
HTR4	5-hydroxytryptamine receptor 4
Ig	Immunoglobulin
IL-2	Interleukin-2
k_	Kingdom
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	Kilogram
L	Liter
L	Late
lateC	Late-weaned calves
LC-MS	Liquid chromatography–mass spectrometry
LW	Live weight
LWG	Live weight gain
m	Month
M cell	Microfold cell
MALTs	Mucosa-associated lymphoid tissues

max	Maximum
MetPA	Metabolic pathway analysis
mg	Milligram
min	Minute
miRNA	MicroRNAs
Ml	Milliliter
mM	Millimolar
MR	Milk replacer
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
n	Number
N.D.	Not defined
NCBI	National Center for Biotechnology Information
NEAA	Non-essential amino acid
NMR	Nuclear magnetic resonance
nt	Nucleotide
o_	Order
OMVs	Outer membrane vesicles
OS	Oral-specific
OTUs	Operational taxonomic units
p	Probability value
p_	Phylum
PAMPs	Pathogen-associated molecular patterns
PCO/PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PCR-SSCP	PCR single strand conformation polymorphism
PE	Paired-end
PERMANOVA	Permutational analysis of variance
pH	Potential of hydrogen
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
pIgR	Polymeric Ig receptor
PLS-DA	Partial least square discriminant analysis

PPs	Peyer's patches
PRRs	Pattern recognition receptors
pWM	Pasteurised waste milk
q2	QIIME 2
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Rumen
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
RS	Rumen-specific
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
S	Supplementary
s	Second
s_	Species
SCFA	Short-chain fatty acids
SEM	Standard error of the mean
sIgA	Secretory immunoglobulin A
SLs	Sphingolipids
SMs	Sphingomyelins
spp.	Species
TLRs	Toll-like receptors
TMR	Total mixed ration
TSS	Total sum normalization
U	Enzyme unit
uncl	Unclassified
v	Version
V1, V2, V3, V4, V5 V6 and V8	Hypervariable regions of prokaryotic 16S rRNA gene
VFA	Volatile fatty acids
VIP	Variable importance in the projection
vs	Versus
WM	Waste milk
x g	Times gravity

CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1 Developmental phases of digestive tract in ruminants

The digestive tract of ruminants is underdeveloped at birth and undergoes developmental phases during the first few weeks of life. The first phase occurs from 0−3 weeks of age and termed as monogastric phase. During this phase, ruminants rely solely on the abomasum for the digestion of feed particle as their rumen, reticulum and omasum are underdeveloped and non-functional (Longenbach and Heinrichs, 1998; Heinrichs and Lesmeister, 2005). The diet of neonatal calves mainly comprises of milk or milk replacer (MR), which bypasses the forestomach system and are digested in the hindgut, where microbial degradation processes synthesize amino acids, volatile fatty acids (VFAs), and vitamins (Guilloteau et al., 2009). The hindgut microbiome is also responsible for the fermentation of amino acids, and carbohydrates that results in the synthesis of branched-chain fatty acids, essential for neonatal calves' development and growth (Song et al., 2018). The second phase occurs from 3−8 weeks of age and termed as transition phase. During this phase, solid feed (starter meal, hay or fresh grass) intake increases and the milk consumption is decreased. The addition of solid feed in the diet activates rumen fermentation activity, that changes drastically with weaning (complete milk removal) and thus alter the gut microbial composition (Meale et al., 2016).

1.2 Pattern of microbial colonization in the digestive tract of ruminants

Ruminants are mainly dependent on their rumen microbiome for the digestion of feed particles and synthesis of metabolic products (Matthews et al., 2019). Although, it is assumed that the ruminants are born with a sterile gastrointestinal tract (GIT) and that the gut microbial colonization starts directly after birth (Figure 1). Maternal contact, environmental exposure, colostrum and feed majorly serve as the initial sources of the gut microbial inoculum during the immediate neonatal period (Mayer et al., 2012; Taschuk and Griebel, 2012; Alipour et al., 2018; Yeoman et al., 2018; Klein-Jöbstl et al., 2019). A vast variety of studies have been conducted in the past to explore the pattern of gut microbial colonization and GIT development in calves. These studies have highlighted several factors that may influence the initial establishment of microbial communities in the neonatal calves GIT including the host genetics and sex (Mayer et al., 2012; Klein-Jöbstl et al., 2014; Fan et al., 2020; Fan et al., 2021), age (Uyeno et al., 2010; Edrington et al., 2012a; Li et al., 2012; Jami et al., 2013; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014; Rey et al., 2014; Guzman et al., 2015; Dill-McFarland et

al., 2017; Dias et al., 2018; Song et al., 2018), diet (Castells et al., 2012; Edrington et al., 2012a; Malmuthuge et al., 2013; Malmuthuge et al., 2015a; Guzman et al., 2016; Kim et al., 2016; Dias et al., 2017; Lin et al., 2018; Dill-McFarland et al., 2019; Maynou et al., 2019; Zhang et al., 2019), rearing environment (O'Hara et al., 2020), weaning time and strategy (Meale et al., 2016; Meale et al., 2017b), usage of antibiotics and feed additives (Langford et al., 2003; Edrington et al., 2012b; Foditsch et al., 2015; Yousif et al., 2018; Kido et al., 2019; Cao et al., 2020; Pereira et al., 2020; Wei et al., 2020).
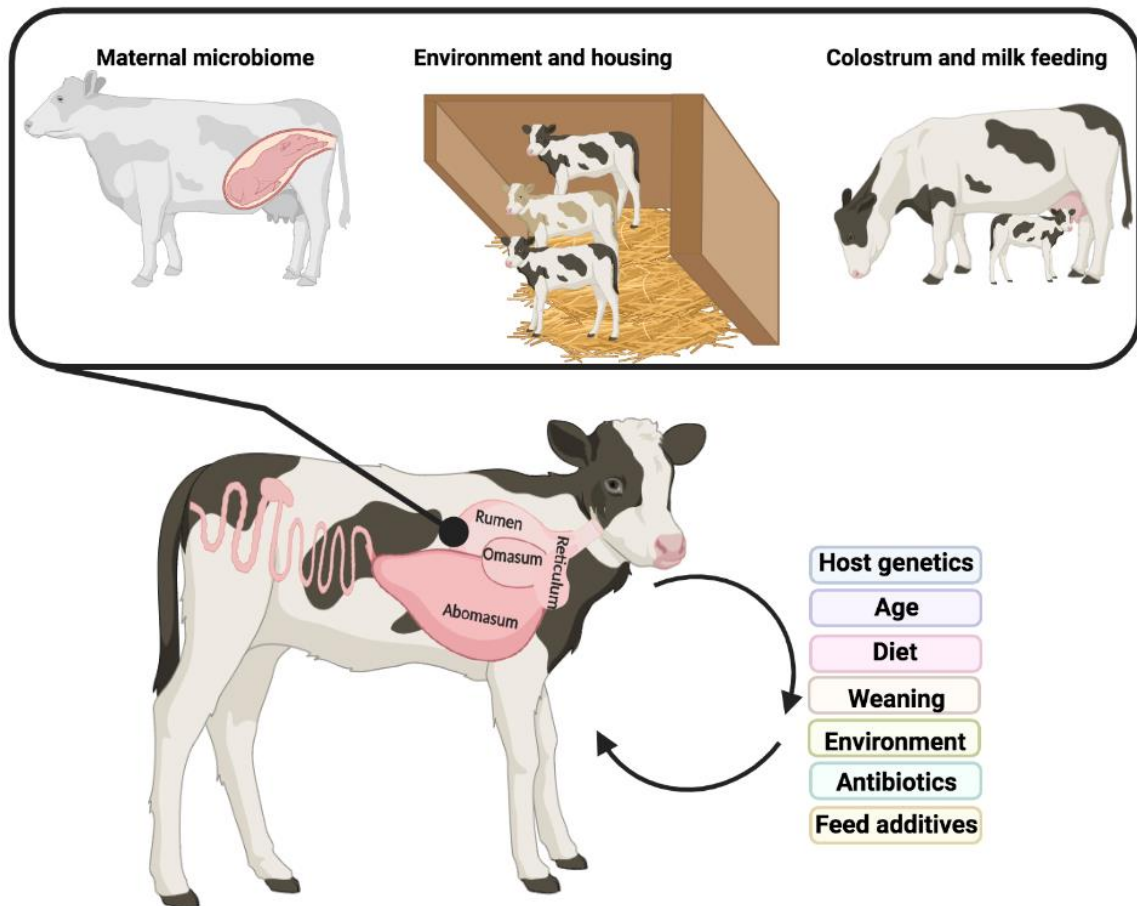
Figure 1 | Factors influencing gut microbial colonization in neonatal calves. Figure created with BioRender.com.

These studies indicated that the gut microbial colonization in neonatal calves follows a sequential pattern with facultative anaerobes (*Enterococcus, Escherichia coli, Lactobacillus,* and *Streptococcus*) as the first colonizers. The strictly anaerobic bacterial community is established within a few days after birth and dominated by *Bifidobacteria* (Fonty et al., 1987; Minato et al., 1992; Jami et al., 2013). The contribution of proteolytic bacteria to the anaerobic microbial community is only 1−2% until 10-weeks but rises up to 10% after 12-weeks of age

(Anderson et al., 1987). *Proteobacteria* and the fibrolytic bacteria can be detected in the rumen of calves as early as 1−2 days after birth. Higher solid feed intake and fiber feeding resulted in high dominance of *Prevotella* (Jami et al., 2013; Rey et al., 2014). Similar to the rumen, the faecal microbiome of dairy calves also changes dynamically within the first 12-weeks, where the age-dependent decrease in dominance of *Bifidobacterium*, *Streptococcus*, and *Enterobacteriaceae*, and an increase in fibrolytic bacterial species (*Fibrobacter* and *Ruminococcus flavefaciens*) were reported (Uyeno et al., 2010).

In addition to the age, the diet fed to the calves during the pre-weaning period directly impact the gut microbial communities as indicated by the high dominance of commensal gut microbes with milk-based diet, while an increased abundance of amylolytic and fibrolytic bacteria with solid-feed intake was observed (Dias et al., 2017; Lin et al., 2018; Song et al., 2018; Dill-McFarland et al., 2019; Zhang et al., 2019; Kumar et al., 2021). Therefore, it can be assumed that both age-dependent gut development and diet serves as the key influencing factors to the establishment of early life microbial communities in calves. In addition, different regions within the GIT differ in their physiology, pH, nutrient profiles and transition rate, the population of immune cells as well the host-microbial interactions (Ward, 2008; Abreu, 2010; Van den Abbeele et al., 2011; Laarman et al., 2012b). Considerable differences also exist between the mucosal epithelium and ingesta (Van den Abbeele et al., 2011). Such differences result in site- and region-specific establishment of microbial communities throughout the GIT of neonatal calves (Malmuthuge et al., 2014; Mao et al., 2015). It has been observed that once the microbial community is established and reached to maturity, the gut microbiome is considered stable thereafter, with the exception of dietary modification, changes in health and physiology of the host (Russell and Rychlik, 2001; Weimer et al., 2010; Henderson et al., 2015).

1.3 Weaning transition and rumen development in calves

Weaning transition is described as the transition period when calves are shifted from liquid diet to the solid feed. This period is critical for rumen development due to the direct influence of dietary composition on rumen microbiome and microbial metabolic products (Petri et al., 2012; Henderson et al., 2015). Feeding calves with starch-based diet during weaning transition increases the production of VFAs and lactic acid, which decreases the rumen pH (Laarman and Oba, 2011). Such decrease in ruminal pH results in higher translocation of free lipopolysaccharides from rumen into the blood that causes immunosuppression and inflammation (Gozho et al., 2005). On the contrary, feeding pre-weaned calves with higher

amount of milk or MR stimulates organ development and health (Geiger et al., 2016; Schäff et al., 2016; Rosenberger et al., 2017), and starter concentrate feeding increases the production of butyrate, which is essential for the development of rumen papillae (Cui et al., 2020). However, feeding ruminants only with starter concentrate increases the chances of parakeratosis and ruminal acidosis, which effects the proper functioning of the GIT, thus influencing animal health and development (Liu et al., 2017; Wang et al., 2017). Therefore, calf starter feeding is usually recommended along with MR during weaning transition as it improves rumen microbial fermentation processes and promotes rumen development, increases the average daily gain (ADG) and decreases the mortality rate in post-weaned calves (Drackley, 2008).

The bovine rumen comprises of diverse commensal microbial communities, dominated by bacteria that account for nearly 95% of the total microbiota, protozoa, methanogenic archaea, fungi and viruses (Choudhury et al., 2015). The rumen microorganisms act interdependently to degrade feed particles and synthesize VFAs, microbial proteins, vitamins and minerals (Mackie, 2002). The rumen microbial community changes drastically during weaning transition (Figure 2), until a mature microbial community is established (Kim et al., 2016; Meale et al., 2016; Dill-McFarland et al., 2017; Li et al., 2020). For example, a delay in the onset of rumen microbial communities was observed with prolonged milk feeding (Lengemann and Allen, 1959), while, an increase in the abundance of rumen carbohydrate-utilising bacteria such as *Megasphaera, Succinivibrio,* and *Sharpea* was observed with the addition of starter in the diet of pre-weaned calves (Dias et al., 2017). However, when dietary supplementation was alfalfa hay, an increased number of fibrolytic bacteria and methanogens was observed within the GIT of calves (Guzman et al., 2016). Solid feed addition also increased the abundance of *Bacteroidetes*, an essential cellulose-degrading bacterium in the rumen (Kim et al., 2016). The rumen fibrolytic microbes ferment the solid feed particles and synthesize VFAs. The VFAs supply energy to the calves for metabolic processes such as growth, immunity and thermoregulation and are also essential for the development of rumen papillae, which further facilitates the absorption of VFAs into the blood (Khan et al., 2011; Rey et al., 2012). Therefore, a higher solid feed intake is necessary for the development of an active rumen as it increases the amount of rumen fibrolytic bacteria and production of VFAs. Subsequently, the surface area of the rumen is increased by expanding its volume and papillation, which contributes to the host's survival success after weaning (Khan et al., 2016). The weaning-related dietary changes switches the major site of feed digestion and absorption from hindgut to the rumen (Liu et al., 2017; Saro et al., 2018; Yang et al., 2018; Kargar and Kanani, 2019).

The buffering ability of rumen and the metabolic processes also increases with the increase in roughage intake (Laarman et al., 2012a; Laarman et al., 2012b; Connor et al., 2013).
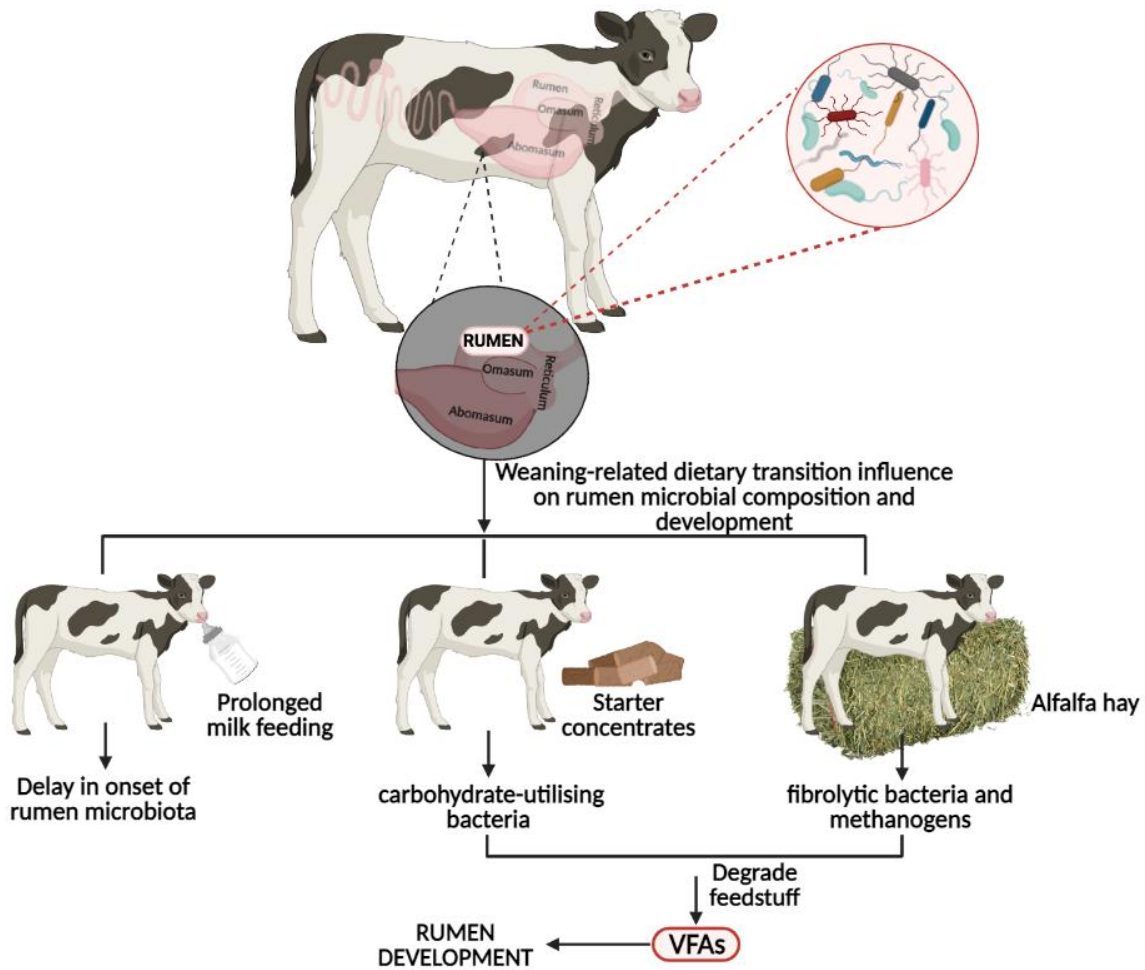
Figure 2 | Influence of weaning-related dietary transitions on rumen development and its associated microbiome. Figure created with BioRender.com.

Dairy farming system are facing the problem of high feed costs that account for nearly 60% of their total production expenses (Ho et al., 2005). Thus, in order to reduce the feed cost, producers are using various approaches to enhance the digestive ability and accelerate rumen development in calves (Klein et al., 1987; Pazoki et al., 2017). Therefore, the dairy industry generally relies on early-weaning concepts by restricting the amount of milk or MR fed to the calves, which stimulate the early consumption of concentrate and thus decreases the weaning age of calves (Hulbert et al., 2011; Dias et al., 2018). The positive impacts of fibrous feed consumption on reticulorumen development, increase in solid feed / concentrate intake and improvement in ADG and performance have been previously reported in calves using barley

straw, grass silages, orchard grass hay or chopped grass hay (Thomas and Hinks, 1982; Khan et al., 2011; Castells et al., 2012). Similar positive impact of alfalfa hay feeding on rumen epithelium development in dairy calves were reported (Cui et al., 2020). However, the usage of fibrous feed is not recommended during the pre-weaning period of calf life due to their limited fibrolytic capacities, as it poses the risk of undigested forage feed accumulation in the rumen and reduces the intake of starter concentrate (Drackley, 2008). In addition, the fermentation of alfalfa hay by rumen microorganisms does not produce adequate amount of VFAs required for optimum development of rumen papillae in dairy calves (Jahani-Moghadam et al., 2015; Mirzaei et al., 2015; Hosseini et al., 2016).

Many studies have focused on the effect of weaning time on ruminant's health and productivity. For example, weaning calves at an early age is beneficial in terms of their improved growth, performance and carcass quality (Myers et al., 1999a; Myers et al., 1999b). However, a premature weaning may result in underdeveloped rumen due to the lack of time for gradual adaptation to solid feed consumption, resulting in increased stress and death rate in calves at weaning than late-weaned calves (Khan et al., 2007). On the contrary, too late weaning increases the feed costs and has a negative impact on the organ development, thus affecting the productive performance of an animal in later stages (Mao et al., 2017). Therefore, the weaning age should be carefully considered by ensuring greater GIT maturation at weaning to lessen such effects (Eckert et al., 2015).

1.4   Role of microorganisms in neonatal calves' growth and health

Calves undergo extreme nutritional stress and metabolic adaptations from birth until the end of weaning period. The gut microbial communities play an essential role in the nutrient digestion and absorption (Karasov et al., 2011; Morgavi et al., 2015), gut and immune system development (Baldwin et al., 2004; Mazmanian et al., 2005; Peterson et al., 2007), maintenance of intestinal homeostasis and maturation of immune system (Yu et al., 2012; Sommer and Bäckhed, 2013; Guzman et al., 2015; Malmuthuge et al., 2015b; Malmuthuge and Guan, 2016, 2017), prevention of pathogenic colonization and diseases (Round and Mazmanian, 2009; Maslowski and Mackay, 2011; Kamada et al., 2013). Certain gut bacteria also serve their host by detoxification of natural feed compounds (Gregg, 1995). In addition, the host-microbe interactions are essential for proper functioning of the host. For example, the interaction between gut mucosa and beneficial bacterial genera (*Bifidobacterium* and *Lactobacillus*) is essential for tight junction formation in the calves GIT during the neonatal period. An alteration in gut microbial composition may result in higher gut permeability and increased infection rate

in neonatal calves (Turner, 2009; Gomez et al., 2019). Changes in the gut microbial community compositions also increases the risk of various pathogenic infections in animals (Hopwood and Hampson, 2003). For example, increase in *Escherichia coli, Salmonella* spp., *Cryptosporidium,* bovine viral diarrhoea virus and *enterovirus* results in neonatal calf diarrhoea (Cho et al., 2013), while, higher abundance of *Faecalibacterium* in the neonatal calf's gut has been linked with lower incidence of diarrhoea (Oikonomou et al., 2013). Similarly, *Bifidobacterium* is part of the healthy gut microbiota and is associated with immunity (Apgar et al., 1993; Picard et al., 2005; Uhde et al., 2008). Furthermore, the early life gut microbial colonization can have long-lasting effects on animal health and productivity (Thompson et al., 2008). The structure and composition of the matured rumen microbiota and its associated functions are determinants of ruminant's production phenotypes such as feed efficiency, methane and nitrogen emissions and health status of the host (Guan et al., 2008; Kittelmann et al., 2014; Shabat et al., 2016; Li and Guan, 2017; Sasson et al., 2017; Paz et al., 2018). Hence, a better understanding of early microbial colonization in neonatal calf's gut and their possible sources could support the progressive development of calf raising management systems including reduced incidences of illness.

Several studies have shown the resilient and host-specific nature of rumen microbes in adult cows, suggesting that once the rumen microbiome is established and reached maturity, it is possibly resistant to long-term perturbations (Jewell et al., 2015; Weimer, 2015; Malmuthuge and Guan, 2017; Weimer et al., 2017). Conversely, this is not the case with young ruminants, which harbour less resistant and heterogenous rumen microbiota within the first few weeks of life as compared to the adult ruminants (Abecia et al., 2014; Yáñez-Ruiz et al., 2015; Abecia et al., 2017; Bu et al., 2020). Therefore, the early life of ruminants represents the promising period for gut microbial modulation in order to achieve the long-term effects in adult animals (Yáñez-Ruiz et al., 2015). Furthermore, with the advance of genetic selection and animal breeding approaches, animals with a preferred gut microbial composition can be selected to obtain higher feed efficiency and thereby reducing methane emissions (Roehe et al., 2016; Zhang et al., 2020).

1.5   Rumen sampling procedures

The development of rumen and its microbiome has been extensively studied in young calves due to its essential role in weaning transitions. Most of the studies used invasive rumen sampling procedures such as rumen cannulation, oral intubation / rumen fistulation, or rumenocentesis to study the development of the rumen (Beharka et al., 1998; Coverdale et al.,

2004; Duffield et al., 2004; Lesmeister and Heinrichs, 2004; Khan et al., 2008) and the rumen-associated microbial communities (Jami et al., 2013; Kim et al., 2016; Meale et al., 2016; Meale et al., 2017b; Lin et al., 2018). However, these invasive rumen sampling methods have both advantages and disadvantages. Rumen cannulation is the most reliable rumen sampling method as it enables more extensive monitoring of rumen parameters as compared to repetitive sampling within the same day or using rumen probes. But fitting a rumen cannula requires invasive surgical procedures, official permission from the ethical commission and animal welfare authorities as well as dedicated animal facilities (Lodge-Ivey et al., 2009; Shen et al., 2012; Terré et al., 2013). Similarly, the collection of rumen samples using stomach tubing or rumen fistula is unpleasant for animals and not preferable when sampling large group of animals is a concern (Tapio et al., 2016). In addition, the rumen samples collected by stomach tubing are often at high risk of contamination with saliva (Shen et al., 2012; Ramos-Morales et al., 2014). Rumenocentesis is another effective rumen sampling method. However, the collection of rumen samples using this procedure requires pricking of abdominal wall with a needle, enabling collection of limited amounts of sample. This procedure is also undesirable for animal health and welfare (Duffield et al., 2004). As discussed previously in other ruminant studies that the choice of rumen sampling procedure can directly impact the study outcomes (Duffield et al., 2004; Terré et al., 2013).

The rumen microbiome is non-static and changes quickly with diet, host physiology and health (Russell and Rychlik, 2001; Weimer et al., 2010; Henderson et al., 2015). In addition, individual animal to animal variations exist under a controlled diet and environmental conditions due to the animal history, health, and the post-feeding sample collection time (Kittelmann et al., 2015). Therefore, a large sample size is required to obtain sufficient statistical power, thus, increasing the demands for non-invasive rumen sampling procedures. The presence of regurgitation activity in ruminants enables them to bring the partly digested ruminal contents back to the mouth (Meale et al., 2017a), thus, the oral cavity of ruminants can serve as a reservoir for some ruminal microbes (Kittelmann et al., 2015; Tapio et al., 2016). If so, the oral samples can serve as non-invasive proxies to study the rumen microbiome, avoiding the need to sacrifice the animals or invasive surgical procedures for rumen sampling.

1.6 Cultivation *vs*. DNA-based methods to study gut microbial communities

Research on ruminants gut microbial communities using culture-based studies was limited by the gut microbial complexity, the inability to culture anaerobic bacterial species, unambiguous quantification of gut microbes and host-microbial interactions (Ziolecki and

Briggs, 1961; Eadie, 1962; Beharka et al., 1998; Rada et al., 2006; Vlková et al., 2006; Collado and Sanz, 2007). However, several cultivation-independent studies were done in the past to study the dynamics of gut ecosystem and pattern of microbial colonization using denaturing gradient gel electrophoresis, sanger sequencing, single strand conformation polymorphism, suppressive subtractive hybridization, and restriction fragment length polymorphism (Fonty et al., 1991; Skillman et al., 2004; McEwan et al., 2005; Fernando et al., 2010; Chen et al., 2011; Michelland et al., 2011; Malmuthuge et al., 2012; Mayer et al., 2012; Malmuthuge et al., 2013). Most of these procedures are limited by low resolution power and high labour cost.

On the contrary, the advance sequencing technologies such as Illumina sequencing and Roche/454 amplicon pyrosequencing enables the characterization of ruminant's gut microbial communities by targeting the hypervariable regions of their 16S rRNA gene (Brulc et al., 2009; Hess et al., 2011; Kittelmann et al., 2013; McAllister et al., 2015). These high-throughput sequencing technologies are widely used nowadays to characterize the abundance and diversity of ruminant's GIT microbial communities and establish the association of gut microbes with specific biological functions of the host. These sequencing methods are highly sensitive, less time consuming, provide the benefits of extraordinary sampling depth, and enable the detection of vast variety of microorganisms without having prior knowledge about their growth conditions and identity (Li et al., 2012; Malmuthuge et al., 2012; Jami et al., 2013; Rey et al., 2014; Kim et al., 2016; Meale et al., 2016; Dias et al., 2017; Dill-McFarland et al., 2017; Meale et al., 2017a; Dias et al., 2018; Lin et al., 2018; Yeoman et al., 2018; Dill-McFarland et al., 2019; Zhang et al., 2019). Additionally, the cost-effectiveness of these sequencing-based methods enables their widespread usage in various scientific fields. However, there is still the need of using proper controls for such kind of studies, so that the effects of environmental contaminants and external sources of microbial DNA such as laboratory reagents and equipment can be excluded (Alipour et al., 2018).

1.7 Targeted *vs.* untargeted metabolomics approaches

Metabolites are synthesized as an intermediate or by-products of several metabolic pathways (Bäßler et al., 2021). The host metabolic profile has significant influence on the host development and physiology especially during early life and an alteration in their concentrations have been linked with metabolic disorders (Monnerie et al., 2020). Generally, the metabolomics studies are accomplished using a variety of analytical platforms such as nuclear magnetic resonance (NMR) spectroscopy, gas chromatography-mass spectrometry (GC-MS), or liquid chromatography–mass spectrometry (LC-MS) (Goodacre et al., 2004).

NMR approach is a powerful method to investigate the metabolic alteration in milk or serum samples (Klein et al., 2012; Palma et al., 2016). On contrary to the NMR, LC-MS and GC-MS techniques are more specific, highly sensitive and can be used to monitor the alteration in the metabolic profiles of blood, milk, and biofluids (Boudonck et al., 2009; Sun et al., 2015). GC-MS technique is limited to small volatile metabolites (Simón-Manso et al., 2013), whereas, LC-MS technique is more commonly used in metabolomic studies to characterize the high molecular weight, non-volatile metabolites with high coverage as compared to the other methods (Siskos et al., 2017). These analytical methods can be combined with a targeted or un-targeted metabolomics approach. Un-targeted approaches enable the detection of several distinct compounds in a single analysis (Chen et al., 2020). However, the lack of standards in such approach increase the probability of false metabolic identifications, which poses hinderance in the interlaboratory comparison of un-targeted metabolomics results (Dunn et al., 2013; Ribbenstedt et al., 2018).

Contrary to the un-targeted approaches, the targeted approaches use standards and promise stability and repeatability due to the correct identification and quantification of metabolites (Ribbenstedt et al., 2018). The most commonly used kits for targeted approach are commercially available from Biocrates Life Science AG and can quantify from 180 metabolic compounds (AbsoluteIDQ® p180 kit) up to 500 compounds (MxP Quant 500 Kit) (Hampel et al., 2019; Kuhring et al., 2020). These kits allow identification of metabolites involved in various pathways including the metabolism of carbohydrate, amino acid, lipid, and energy, oxidation of fatty acids, cell cycle control, insulin resistance, mitochondrial dysfunction, pro-inflammatory signalling and immune regulation (Hampel et al., 2019; Bäßler et al., 2021), thus providing comprehensive assessment of the metabolic impairment mechanisms (Hailemariam et al., 2014a; Hailemariam et al., 2014b). The targeted metabolomic approaches have already been applied on plasma, and serum samples of human and animal origin for identification of affected metabolic pathways in human diabetes, horse insulin dysregulation, and pathobiology of retained placenta (Kühn et al., 2016; Lotta et al., 2016; Dervishi et al., 2018; Kenéz et al., 2018). However, the integration of metabolomics with metagenomics studies can further help us to better understand the associations between diet, age or environmental-derived microbial imbalances in the gut and the host metabolic disturbances, thus, leading to the identifications of predictive disease biomarkers as reported recently (Visconti et al., 2019; Li et al., 2020; Agus et al., 2021).

1.8 Objectives

The period from birth to the complete weaning is critical for the development of digestive tract, metabolic and immune system in calves. The establishment of robust microbial communities and their interactions with the host is essential for gastrointestinal tract development, maturation of host gut, immune system, and health. The current information about the possible factors that may influence gut microbial colonization in pre- and post-weaned calves, the potential host–microbe interactions, and the possible mechanisms of gut microbial modulations are summarized in the first manuscript with the title **"Dynamic progression of the calf's microbiome and its influence on host health"** published in Computational and Structural Biotechnology Journal in 2021.

Oral cavity is a complex habitat, that harbours microorganisms with an essential role in animal health and initial digestion of feed particles. The information about the oral microbiome of ruminants especially young calves is still missing and needed to be explored. Rumen is the major compartment of ruminant's digestive tract, responsible for fermentation of feed stuff and nutrients biosynthesis. Among various factors that influence the development of rumen and its associated microbiome, age, diet and weaning time are crucial. To date, the rumen microbiome studies are limited to the invasive sampling procedures, which are laborious, time-consuming, unpreferable for animal health and impractical for studies targeting large number of animals. Therefore, the next aim of this work was to establish some non-invasive rumen sampling procedure to monitor the age- and weaning-dependent changes in the rumen microbial communities of young calves as summarised in the second manuscript **"Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning"** published in Animal Microbiome Journal in 2021.

Neonatal calves solely rely on their hindgut fermentation due to under-developed rumen, which synthesize metabolites necessary for neonatal growth and development. The hindgut colonization by a stable commensal microbial community is essential to protect calves from invasive pathogens. In addition, the weaning-related dietary transitions switches the major site of feed digestion and absorption from hindgut to the rumen, resulting in a subsequent modification in the hindgut microbiota. Therefore, the third and final aim of this project was to explore changes in the structure of hindgut microbial communities of calves due to age and time of weaning. Changes in blood metabolic profiles have been associated with metabolic disorders in humans and animals. However, the knowledge about the age- and weaning-dependent changes in the plasma metabolic profiles of young calves are still lacking. In order to fill such gap of knowledge the third study was conducted as summarized in the manuscript

"Host metabolome and faecal microbiome shows potential interactions impacted by age and weaning times in calves" submitted to Animal Microbiome Journal on December 6, 2021.

CHAPTER II

FIRST MANUSCRIPT

DYNAMIC PROGRESSION OF THE CALF'S MICROBIOME AND ITS INFLUENCE ON HOST HEALTH

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2. DYNAMIC PROGRESSION OF THE CALF'S MICROBIOME AND ITS INFLUENCE ON HOST HEALTH

2.1 Abstract

The first year of a calf's life is a critical phase as its digestive system and immunity are underdeveloped. A high level of stress caused by separation from mothers, transportation, antibiotic treatments, dietary shifts, and weaning can have long-lasting health effects, which can reduce future production parameters, such as milk yield and reproduction, or even increase the mortality of calves. The early succession of microbes throughout the gastrointestinal tract of neonatal calves follows a sequential pattern of colonisation and is greatly influenced by their physiological state, age, diet, and environmental factors; this leads to the establishment of region- and site-specific microbial communities. This review summarises the current information on the various potential factors that may affect the early life microbial colonisation pattern in the gastrointestinal tract of calves. The possible role of host–microbe interactions in the development and maturation of host gut, immune system, and health are described. Additionally, the possibility of improving the health of calves through gut microbiome modulation and using antimicrobial alternatives is discussed. Finally, the trends, challenges, and limitations of the current research are summarised and prospective directions for future studies are highlighted.

2.2 Introduction

The development of the gastrointestinal tract (GIT) in neonatal humans and animals is a highly dynamic process that is influenced by genetic and environmental factors, nutrition, and the concomitant development of the intestinal microbial communities. This is also true for ruminants, where the first month of life is even more challenging as the rumen is less developed. The rumen is the largest forestomach in ruminants and is highly important for the conversion of ingested feed particles into metabolites that are absorbed and utilised by the host

and the formation of microbial protein sources used by the animals [1]. Young ruminants are functionally monogastric at birth with an underdeveloped forestomach system, including the rumen, reticulum, and omasum. During these first months of life, the abomasum and intestines serve as their major digestion sites [2]. The establishment of a fully mature system requires the development of the reticulo-rumen and the associated microbiomes [3]. The microbial communities in the rumen follow a sequential pattern of colonisation with bacteria as the first colonisers, followed by the methanogenic archaea, anaerobic fungi, and protozoa [4–6]. However, studies using molecular-based techniques showed initial rumen colonisation with facultative anaerobic bacteria (*Enterococcus* and *Streptococcus*) in new-born calves as well as archaea within a few hours after birth [7,8]. A recent study by Malmuthuge et al. (2019) reported on rumen colonisation in neonatal calves with an active bacterial community at birth. The rumen of one-week-old calves were already colonised by active complex-carbohydrate-fermenting bacterial species even in the absence of solid substrates in the diet [9]. These initial gut colonisers utilise the oxygen available in the gut, thus, creating an anaerobic environment favourable for the growth of strict anaerobic gut communities, including *Bifidobacterium* and *Bacteroides* [10,11]. The strict anaerobic bacterial community, including cellulolytic and proteolytic bacteria, together with niche specialists, establish and dominate the gut microbiome within the first two weeks of life [7,12–14].

The establishment of a strict anaerobic bacterial community in the GIT of neonates plays an essential role in mucosal immune system development, and is therefore, a critical phase for the host [15,16]. After the initial gut colonisation, constant exposure of the host GIT to specific microbes is necessary to maintain the host's energy metabolism, health, and mucosal immune system maturation [17,18]. Once the GIT is fully mature and the climax microbial community is established, the intestinal microbiome is considered stable thereafter, except for changes in the host's health, physiological state, and diet [19–21]. However, considerable differences exist in microbial community profiles in different regions of the GIT in ruminants [14]. Similarly, the mucosa-associated microbial communities were found to differ from those occupying the lumen [14,22–25], suggesting a possible role of host–microbe interactions in defining such diverse microbial community structures. In this review, the development of microbial communities across the GIT of calves under the influence of maternal microbiota, age, diet, weaning, and environmental factors (antibiotics and pre/probiotics) (Figure 3 and Table 1), and the possible role of host–microbe interactions in the development of the host's gut, immunity, and health is summarised.
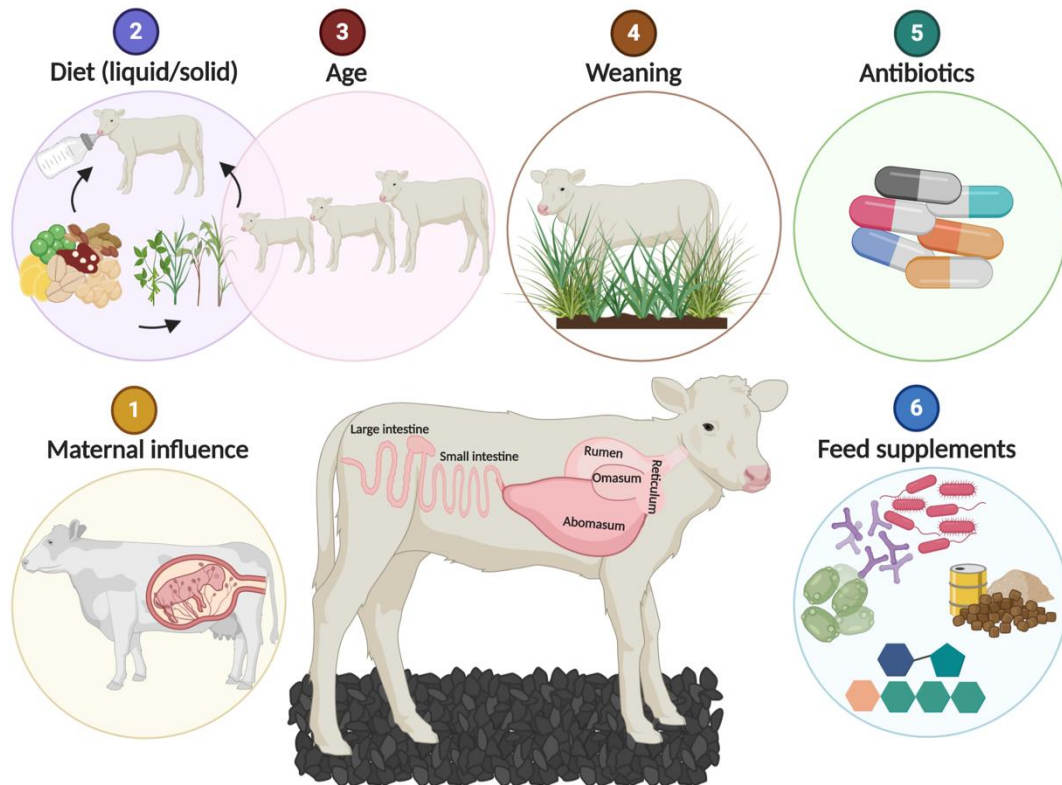
Figure 3 | Factors that influence the initial establishment and development of microbial communities throughout the GIT of neonatal calves. Figure created with BioRender.com.

Table 1 | Overview of major factors that affect the initial colonization of microbial communities throughout the GIT of neonatal calves, host gut and immune system development.

Sample type	Calf age at the time of sampling[1]	Diet[1,2]	Method[3]	Year	Reference
MATERNAL INFLUENCE					
Faeces	0, 6, 12, 24 and 48h, 3, 7, 14, and 42 days 4 days–20 days	N.D.	DNA, PCR single strand conformation polymorphism (PCR-SSCP) of V4-V5 region	2012	[28]
Faeces	24h and 7 days	Colostrum: 4–6h after birth, followed by pooled cow milk	DNA, qPCR, V3-V4 amplicon sequencing (Illumina)	2018	[29]
Overall GIT	0, 1, 2, 3, 4, 5, 7, 14, and 21 days	Milk replacer (MR) throughout the study	DNA, V3-V4 amplicon sequencing (Illumina)	2018	[25]
Faeces and mouth	Faeces (0.5, 6,12, 24, and 48h); mouth (0.5h)	Colostrum: after 0.5h till the end of trial	DNA, V3, V4, V5 amplicon sequencing (Illumina)	2019	[30]

Sample type	Calf age at the time of sampling[1]	Diet[1,2]	Method[3]	Year	Reference
WEANING					
Rumen and faeces	36 and 54 days	Abrupt weaning: MR until day 48, reduction to 0 within 24h; Gradual weaning: MR slowly reduced from day 36 to day 49; all calves had ad lib. access to water, starter and chopped straw from day 7 to day 54	DNA, V4 amplicon sequencing (Illumina)	2016	[79]
Rumen	N.D., after weaning	Fresh milk: day 1 to day 7; half fresh milk and half MR until day 13; MR and dry feed starter till the end of trial; starter, grass hay and water were available ad lib.	DNA, qPCR	2017	[80]
Rumen and faeces	5, 7, and 9 weeks	Ad libitum access to water, starter, chopped straw and oat straw from birth till the end of trial	DNA, V4 amplicon sequencing (Illumina)	2017	[84]
ANTIBIOTICS					
Rectal swabs and faeces	Newborn, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 weeks	Trial 1: Milk substitute without antibiotics or antibiotic containing fresh milk or fermented milk Trial 2: Standard milk substitute, containing growth promoter or antibiotic containing milk	Culture-based assays	1990	[143]
Rectal swabs	N.D.	Colostrum: within 24h after birth; ad lib. milk with penicillin G and water: until day 37	Culture-based assays	2003	[144]
Faeces	N.D.	Bulk milk (BM) and grain concentrates with or without oxytetracycline: 12 weeks trial	Culture-based assays and PCR for screening of drug resistance genes	2004	[133]
Rectal faecal swabs	0, 2, 4, and 6 weeks	N.D.	Culture-based assays	2005	[145]
Faeces	9 time points during first 6 months	Pasteurized or non-pasteurized waste milk before weaning	Culture-based assays	2012	[146]
Faeces	6, 7, and 12 weeks	Colostrum: within 2–6h after birth; MR without antibiotics or with neomycin sulfate and oxytetracycline hydrochloride antibiotics; all calves ad lib. access to starter grain from day 1; alfalfa hay offered post-weaning	DNA, qPCR, sequencing of target genes	2012	[150]
Faeces	2, 14, 28, and 56 days	Colostrum: within 2–4h after birth; ad lib. hay: from day 1; pasteurized or non-pasteurized (WM and	Culture-based assays	2013	[132]

Sample type	Calf age at the time of sampling[1]	Diet[1,2]	Method[3]	Year	Reference
		BM): from day 3; pelleted calf starter: from day 8 until day 56			
Faeces	12 days	MR: from day 0 with or without bacitracin methylene disalicylate. all calves: ad lib. to concentrate from day 3 until day 56	DNA, V4-V6 amplicon sequencing (454)	2013	[137]
Faeces	3, 5, and 6 weeks	Pasteurized hospital milk throughout the study. Water and calf starter ad lib.	DNA, V1-V2 amplicon sequencing (454)	2015	[138]
Faeces	Newborn, 1, 2, 3, 4, 5, and 6 weeks	Colostrum: within 4h after birth; raw milk without antibiotics or with low concentrations of ampicillin, ceftiofur, penicillin, and oxytetracycline: from day 1 till the end of trial; pelleted calf starter: offered from day 7 until day 42	DNA, V4 amplicon sequencing (Illumina)	2016	[139]
Faecal and nasal swabs	42 days and 1 year	Colostrum: after birth; MR or WM: for 6–12 weeks	Culture-based assays	2017	[147]
Faecal swabs	3, 35, and 56 days	Colostrum: within the 24h after birth; MR without antimicrobials or pasteurized WM with β-lactam residues: until day 49. all calves ad lib. water and textured calf starter: from day 1 to day 56	Culture-based assays and PCR of antimicrobial resistance genes	2017	[148]
Faeces	0, 1, 3, and 6 weeks	Milk without antimicrobials or with low concentrations of ceftiofur, penicillin, ampicillin and oxytetracycline: birth till 6 weeks of age	DNA, and whole genome sequencing (Illumina)	2018	[151]
Faeces, ileum, colon	35 days	Colostrum: within 1h after birth; MR without antibiotics or with low concentrations of antibiotics. all calves ad lib. water and starter feed from day 4 until end of trial	DNA, V3-V4 amplicon sequencing (Illumina)	2018	[140]
Faeces	N.D.	Colostrum: within hours after birth; Pasteurized non-saleable milk: until 56 days of age. Ad lib. water	DNA, whole genome sequencing (Illumina)	2019	[141]
Rumen fluid and tissues	15, 25, and 35 days	Colostrum: within 1h after birth; MR without antibiotics or with low concentrations of penicillin, streptomycin,	DNA, V3-V4 amplicon sequencing (Illumina)	2019	[142]

Sample type	Calf age at the time of sampling[1]	Diet[1,2]	Method[3]	Year	Reference
		tetracycline and ceftiofur. all calves ad lib. starter and water from day 2 until end of trial			
Faeces	N.D.	Colostrum: within 1h after birth; pasteurized non-saleable milk until 56 days of age. ad lib. water	Culture-based assays and PCR	2020	[149]
FEED SUPPLEMENTS **Probiotics**					
Faeces	7–35 days	Trial 1: MR without or with *B. pseudolongum* / *L. acidophilus*: from day 7 to day 42 d; starter: from day 14 to day 56. ad lib. water and dried grass Trial 2: MR without or with *B. thermophilum, E. faecium* and *L. acidophilus*; ad lib. MR without antibiotics and water	Culture-based assays	1995	[154]
Rumen contents and faeces	31–33 days	MR until 6 weeks of age, afterwards a mixture of alfalfa pellets and sweet feed with ad lib. water throughout the trial	Culture-based assays and genomic DNA fingerprinting	1998	[157]
Faeces and blood	1, 3, 5, and 7 weeks	Non-pasteurized colostrum: after birth; acidified non-saleable milk: day 1 - day 56. Ad lib. water and calf starter	DNA, V4 amplicon sequencing (Illumina)	2015	[155]
Blood, and tissue and digesta of jejunum, ileum and colon	Blood (1 and 12h, 1–7 days); Tissue and digesta (1 week)	Colostrum replacer: first 12h; MR: from day 1 to day 7 with or without supplementation of *Saccharomyces cerevisiae boulardii*. Ad lib. water.	Radial immunodiffusion analysis, ELISA, immunohistochemistry, RNA and DNA, RT-qPCR	2020	[159]
Prebiotics					
Rumen fluid and blood	N.D.	Milk and concentrate feed (with or without cellooligosaccharides or kraft pulp supplements): from 4 weeks before weaning till 12- or 16-weeks post-weaning	DNA, qPCR	2019	[161]
Rumen fluid and blood	6.5, 7, 7.5, and 8 months	Ad lib. starter concentrate, chopped oat hay and water: for 1 week; oat hay and concentrate (3:7) with or without astragalus root extract: afterwards	Manual assay for serum; DNA, V3-V4 amplicon sequencing (Illumina)	2020	[162]
Dietary supplements					
Rumen	90 and 160 days	Whole milk: first 30 days; MR and starter concentrate (with or without calcium propionate supplement):	DNA, V4 (bacteria) and V8 (archaea) amplicon	2020	[164]

Sample type	Calf age at the time of sampling[1]	Diet[1,2]	Method[3]	Year	Reference
		day 30 to day 90; starter feed: day 91 till the end of trial; alfalfa hay was only provided at day 91.	sequencing (Illumina)		
Faeces and blood	Faeces (1, 3, 7, and 14 days); Blood (14 days)	Colostrum: within 1h after birth; Raw milk: day 2 to day 4; starter concentrate (with or without zinc supplement): day 4 till the end of trial	ELISA, DNA, V3-V4 amplicon sequencing (Illumina)	2020	[163]
HOST IMMUNE SYSTEM DEVELOPMENT					
Ileum and colon tissues, plasma, adrenal glands	Plasma samples (72h); other (75h)	Colostrum: immediately after birth. three groups: a) colostrum, b) whole milk, c) mixture of 50% colostrum and 50% whole milk: for 72 h	RNA, qRT-PCR and qPCR	2020	[113]
Jejunal mucosa	80 days	Colostrum: immediately after birth; acidified transition milk: first 3 days; MR: day 4 until 8 weeks of age with linear reduced amount during week 9 to 10. Ad lib. water, hay and concentrate from day 10	RNA, Illumina HiSeq sequencing	2018	[115]
Blood, jejunum mucosa	Blood (1, 2, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77 days) Jejunum (day 80)	Colostrum: within 2h after birth; acidified transition milk until day 3; MR: day 4 until day 70. Ad lib. water, hay and concentrate from day 10	RNA, whole transcriptome sequencing	2018	[117]
Rumen, jejunum, ileum, cecum, and colon	3 weeks	Fresh whole milk and calf supplement throughout the trial	DNA, V1-V3 amplicon sequencing (454), qPCR	2014	[14]
Mucosa of rumen, jejunum, ileum, cecum and colon	3 weeks and 6 months	Non-pasteurized whole milk and calf supplement: first 12 weeks; alfalfa hay and oats: for the next 4 months	DNA, fingerprinting, clone libraries, qPCR	2012	[119]
HOST GUT DEVELOPMENT					
Rumen, jejunum and ileum tissues	Newborn, 7, 21, and 42 days	Colostrum: after 30 min. of birth; whole milk: until day 7; ad lib. starter: from day 7 until day 42	DNA and RNA; Illumina RNA-sequencing and qRT-PCR	2014	[122]
Rumen tissue and content	Newborn, 1, 3, and 6 weeks	Colostrum: within the first 3 days; whole milk: day 4 till the end of trial. Ad lib. starter from second week of life	DNA, whole genome sequencing (Illumina), qPCR, RNA, transcriptome (host)	2019	[9]

[1]N.D. = Not defined.

[2]Ad lib. = ad libitum.

[3]Hypervariable regions (V1, V2, V3, V4, V5 V6 and V8) of prokaryotic 16S rRNA.

2.3 Early succession of microbes throughout the GIT of neonatal calves and maternal influence

Birth exposes neonates to the vaginal, skin, and colostrum microbiome of the mother [26,27], which initiates the microbial colonisation of the neonatal GIT. The neonatal microbiome must undergo several modifications prior to weaning (6–12 weeks), and it may take a year for the establishment of a fully functional and stable GIT microbial community [7]. To date, only a few culture-independent studies have examined the effect of maternal sources on the early establishment of microbes in neonatal calves' GIT [25,28–30]. At the genus level, the rectal microbiota of the new-born calves was more similar to the dam's oral microbiota (39%) as compared to the microbiota on the dam's vagina (24%) or faeces (15%), indicating an in-utero transfer route for the inoculation of neonatal gut microbiota [29]. However, the faecal microbiota during the first 48 h of calf life showed a close resemblance to the dam's vaginal microbiota than other maternal sources (faeces or colostrum), indicating the possible transfer of microbes to the neonates via the birth canal [30]. In contrast, Yeoman et al. reported high similarity between the dam's udder skin and calf's GIT microbiota during the first three weeks of life [25]. The inconsistencies among these studies are probably due to differences in sampling sites (calf faeces vs. dam's mouth, vagina, faeces, udder skin, or colostrum), and sampling time. In addition to the influence of maternal interaction/microbiome on the early succession of microbes throughout the neonatal calves' GIT, the facility, farm or location where the calves are born and raised also reported to have a significant impact on the gut microbiota of Holstein dairy cows [31] as well as beef calves [32,33]. Thus, the management practices must be carefully considered because of their unidentified role in shaping gut microbial community structures besides several other factors including genetics, breed, age, diet and study method etc.

2.4 Effect of early feeding regimen and age on the initial establishment and development of microbial communities in the GIT of neonatal calves

Young ruminants are pseudo-monogastric at birth with an underdeveloped reticulo-rumen, relying solely on a milk-based diet [2]. In pre-weaned calves, most of the liquid feed flows straight into the abomasum without entering the rumen; thus, the small and large intestines serve as their major digestion sites. The forestomach system in neonatal calves changes tremendously during the first year of life, with a shift in the activity of intestinal enzymes (lactase and maltase), which facilitates the development of the salivary apparatus, other digestive compartments, and rumination behaviour in calves [34–36]. In addition, rumen

volume increases, and rumen papillary shape and size proliferate, providing better niche environments for the microbial colonisation of the rumen and its subsequent functioning [37]. Concomitant with these morphophysiological adaptations, the changes in microbial composition of pre-weaned calves' GIT are driven by the rearing environment, age, and diet [17,33,36,38,39]. The diet of pre-weaned calves is changed gradually from milk or milk replacer (MR)-based diets to solid feed within the first few weeks of their lives [40]. These dietary shifts seem to have prominent effects on the neonatal calf microbiome. Many studies have explored the effect of liquid/solid diets, including fresh or heated colostrum [41,42], whole milk, waste milk (WM), pasteurised waste milk (pWM) or MR [43–45], starter concentrate [23,46,47] and roughage [48–51], on the initial establishment of bacterial communities in the GIT of neonatal calves.

2.4.1 Colostrum and other liquid feeds

New-born calves are immunodeficient and depend solely on colostrum-associated immunoglobulins [52]. Feeding high-quality colostrum is highly recommended as it can inhibit the growth of pathogens, stimulate the colonisation of the small intestines with beneficial microorganisms [41], increase body weight gain, improve the development and function of the GIT, reduce the risk of diarrhoea [53] and thereby, decrease the mortality rate in calves [54]. However, the lack of proper hygiene practises increases the risk of colostrum contamination with microbes [55]; therefore, adequate heating of colostrum is recommended. Feeding heat-treated colostrum within the first 12 h of life inhibited pathogenic *Escherichia coli* and *Shigella*, and increased the growth of *Bifidobacterium* [41,42]. The increase in *Bifidobacterium* was also observed in 51-hour-old dairy calves using a similar treatment [56].

After colostrum feeding, the nutrient composition of the subsequent feeding again defines the microbiome composition. In general, the rumen bacterial community of one- to three-day-old colostrum-fed calves was dominated by *Proteobacteria* [7,57], but as the calves aged and started to consume MR and starter concentrate-based diet, *Proteobacteria* was slowly replaced by *Bacteroidetes* in the rumen [7,12,57]. Similar to the rumen, *Proteobacteria* dominated the faecal microbiota of 24–48-hour-old calves, showing a depletion and a subsequent increase in *Firmicutes* within the first seven days of a calf's life without any diet change [29,30]. Similarly, *Firmicutes* was the dominant phylum in the faecal microbiota of one- to seven-week-old calves [13]. Yeoman et al. also reported higher abundance of *Firmicutes* in the colon and faeces, while *Bacteroidetes* was more abundant in the rumen, reticulum, omasum, and abomasum within the first three weeks of a calf's life [25].

Shifting the diet of pre-weaned calves (7–28 days) from colostrum to whole milk increased the abundance of typical milk- utilising bacteria (*Lactobacillus, Parabacteroides*, and *Bacteroides*) in their rumen [47]. Feeding milk to two-week-old calves also increased the abundance of *Ruminococcus flavefaciens*, a fibrolytic bacterium in the rumen [46]. Similarly, a recent study by Malmuthuge et al. reported the colonisation of a whole milk-fed one-week-old calf's rumen with active *R. flavefaciens*, whose density increased with increasing age, suggesting the possible use of milk as a substrate for *R. flavefaciens* [9]. Feeding a milk-based diet also had prominent effects on the lower gut microbiota of pre-weaned calves as indicated by the high levels of the *Bacteroides–Prevotella* group and *Faecalibacterium* in the faecal samples of MR-fed one-week-old calves [58]. Similar levels were also reported in the colon samples of three-week-old whole milk-fed calves [14], indicating that faecal samples represent the microbiome of the large intestine in an adequate manner [14]. Similarly, Alipour et al. also observed a high dominance of *Faecalibacterium* and *Bacteroides* in the faecal samples of seven-day-old milk-fed calves [29].

The cost benefits of WM over whole milk and MR [59,60] and the increased use of on-farm pasteurisers have facilitated the use of waste milk in calf feeding programmes. Feeding WM modified the rumen bacterial community composition by decreasing *Prevotella* 7 and increasing *Butyrivibrio* 2, the *Rikenellaceae* RC9 gut group, and *Prevotellaceae* UCG-003 in two-month-old calves [45]. The opposite was true when WM feeding was prolonged during the first six months, and higher abundance of *Prevotella* 7 and *Succinvibrionaceae* UCG-001 and lower abundance of *Prevotellaceae* UCG-003, *Rikenellaceae* RC9 gut group, *Selenomonas* 1, and others were observed [45]. Pasteurisation inactivates the vegetative bacterial cells, reduces the risk of disease transmission and mortality and improves the growth rate of calves [61]. A relatively high abundance of *Prevotella* and low abundance of *Streptococcus* and *Histophilus* were observed in the nasal microbiota of pWM-fed 42-day-old calves [44]. In addition, feeding pWM increased faecal bacterial diversity from two weeks to six months of age; a higher prevalence of faecal *Bacteroidetes* and lower prevalence of *Firmicutes*, and no *Salmonella* were detected in young pWM-fed calves [43]. An opposite, but non-significant, ratio of *Firmicutes* and *Bacteroidetes* was also found in pWM-fed calves as compared to that in MR-fed calves [44]. The effects of MR-compositions on faecal microbial communities were studied recently, and it was found that the faecal microbiota of seven-day-old calves fed with MR enriched with conjugated milk oligosaccharides had higher relative abundance of *Faecalibacterium prausnitzii* and *Bifidobacterium* species than did those consuming MR with high free milk oligosaccharides [62]. *F. prausnitzii* is a beneficial bacterium for neonatal calves

due to its positive correlation with body weight gain and reduced diarrhoea [13]. Furthermore, Yak calves reared in isolation on a standard MR-, starter concentrate-, and hay-based diet were found to have better organ development, growth rate, immune function, and higher abundance of non-fibrous carbohydrate-utilising bacterial genera [63] than the maternally nursed and grazed calves that had a higher abundance of fibrous carbohydrate-utilising bacterial genera [63,64]. Thus, the early feeding regimen shapes the microbiome structure in pre-weaned calves by providing different substrates for growth and establishment of various ecological niches. In addition, drinking water offered to the calves immediately after birth seems to have a prominent impact on gut microbial composition, as indicated by the increased abundances of *Faecalibacterium* and *Bacteroides* at two weeks and *Faecalibacterium* and *Bifidobacterium* in the six-week-old calves [65]. In addition, calves consuming drinking water from birth had higher body weight, digestibility of fibre, and feed efficiency than the calves that started to receive drinking water from 17 days of age [66].

2.4.2 Consumption of solid feed reshapes the gut microbiota in pre-weaned calves

The solid feed intake begins around two to three weeks of life, which initiates the critical transition process leading to the establishment of a fully functional rumen. It is usually characterised by a constant or gradual supply of concentrate and ad-libitum hay in addition to milk feeding. Thus, the effects of solid feed intake should be considered as complex responses to enhanced starch-rich and moderate fibrous feed ingredients together. Generally, an increased abundance of amylolytic and fibrolytic bacteria, such as *Succinovibrionaceae*, *Fibrobacteraceae*, and *Prevotellaceae*, in the rumen microbiome has been described in almost all studies of this feeding period [7,46,48–50,57,67–70]. *Prevotellaceae* is the predominant family in the rumen fluid and has a broad genetic capacity to use a variety of soluble sugars, starch, protein, and peptides [71–73]. The enzymes involved include carbohydrate-degrading enzymes (CAZYmes), such as glycoside hydrolases (GH2, GH3, GH42, and GH92), which are detectable in pre-ruminant rumen samples [12]. The activity of amylase and xylanase has already been shown in two-day-old calves, even in the absence of complex dietary carbohydrates [74]. Thus, the presence of glycoside hydrolase activity together with the production of short-chain fatty acids (SCFA) reveals that the metabolically active rumen microbiome is established soon after birth in neonatal calves, even in the absence of solid feed. SCFA are important for rumen tissue metabolism, rumen papillae, and epithelium development [9,40] and they are absorbed into the bloodstream through the papillae and provide energy for calf metabolism and growth [40]. Depending on the solid feed source, changes in the pH and

SCFA amount and composition are observed. Forage feeding improves the ruminal environment by increasing rumen liquid pH [40,48], reducing the chances of subacute ruminal acidosis, and modifying the structure of the rumen microbiome, leading to the establishment of a fully functional rumen during weaning [49,75]. Furthermore, the particle size as well as the physical form of diet seems to influence the morphophysiological and microbial development of the rumen [76,77]. Feeding a ground diet to calves reduces the growth of their rumen papillae, lowers the pH of their rumen liquid, reduces the number of cellulose-degrading bacteria, and increases the number of amylose degraders [76]. This finding strongly indicates the potential role of effective fibre feeding for the modification of the rumen environment as well as the associated microbial community composition.

The establishment of an archaeal community in the GIT of calf is important for the required hydrogen balance during bacterial fermentation. The dietary modifications also seemed to have obvious effects, and a higher abundance of *Methanosphaera* and lower abundance of *Methanobrevibacter* were observed in the rumen of pre-weaned calves fed a milk plus starter concentrate-based diet as compared to the milk-fed calves [47]. Starter concentrate feeding also increased the dominance of *Methanomicrobiales mobile* in the abomasum, caecum, and faeces and *Methanobrevibacter* in the caecum and faeces of 20-day-old calves, as well as decreasing the abundance of *Methanococcales votae* [46]. Additionally, a decrease in the rumen bacterial diversity, and an increase in the rumen archaeal diversity as well as fungal richness were observed with silage supplementation [51].

2.5 Effect of weaning age and management on microbial colonisation of the GIT in calves

Among the most important factors influencing further animal development in general, and the forestomach system in particular, are the date (age) and strategy of weaning. The abrupt weaning of calves from a milk-based diet to the consumption of solid feed decreases their solid feed intake and average daily gain [40,78]. However, no effect of the weaning strategy (abrupt vs. gradual) was observed on the establishment of rumen and faecal microbial community composition [79], suggesting that the progressive development of the microbial community into a mature state occurs with age [12]. The date of weaning is an important factor in the development of the rumen. Weaning calves, at eight weeks of age, increased their average daily gain [80], and improved carcass quality, feedlot growth, and performance [81,82]. Rumen enzyme activity was also improved [80], probably due to a greater concentrate intake [83], indicating that the consumption of solid feed triggers the development of the adult-like rumen

bacterial community. However, calves weaned six weeks after birth abruptly shifted the ß-diversity of their rumen and faecal microbiomes compared to the calves weaned eight weeks after birth [84]. This sudden change in the microbial community structure of early weaned calves reflects pre-mature rumen development, paralleled by their reduced growth rate [85], whereas gradual rumen development, [84] improved feed intake, and growth rates were observed when calves were weaned at eight weeks of age [85]. Thus, a balanced weaning management and an appropriate weaning age are important to minimise the side effects.

Rumen fermentation activity begins with the addition of solid feed in the diet and concomitantly alters the microbial composition of a calf's GIT. An increase in the abundance of *Firmicutes* and *Proteobacteria* and a decrease in the abundance of *Bacteroidetes* were observed in the rumen microbial community from pre- to post-weaned state [79]. *Bacteroidetes* dominated the rumen microbiota of 42-day-old [12] and two-month-old pre-weaned calves [7]. A similar weaning-related decrease in the abundance of *Bacteroidetes* and a subsequent increase in *Firmicutes* were observed, regardless of the calf's age at weaning [84]. This suggests that the rumen of pre-weaned calves contains the same dominant phyla, including *Bacteroidetes, Firmicutes,* and *Proteobacteria*, as found in the rumen of mature post-weaned calves, although the abundance of these phyla varies depending on the developmental stage [86]. At the genus level, *Prevotella* dominated the rumen microbial community of both pre- and post-weaned calves and showed no changes in the abundance regardless of weaning age or strategy [79,84]. Similarly, high dominance of *Prevotella* in the mature rumen of two-month- to two-year-old cattle has previously been reported [7,12] Nevertheless, the genus level composition of MR-fed pre-weaned calves' rumen showed a higher relative abundance of *Bacteroides* and *Succinivibrio* than did that of post-weaned calves fed a high-starch diet [79]. In contrast to this depletion, the abundance of *Sharpea* increased by weaning, making it the second dominant genus in the rumen of post-weaned calves [79]. The increase in starter and forage intake from pre- to post- weaned period [79,87] was positively correlated with the calf's body weight and the abundance of *Sharpea* [79]. However, the abundances of *Shuttleworthia* and *Dialister* increased drastically in early weaned calves across weaning, while no differences were observed in late-weaned calves before and after weaning [84]. *Dialister* spp. are capable of degrading starch [88], and the increased abundance of *Dialister* in early weaned calves was probably due to increased consumption of starter concentrate across weaning [84]. In addition, early weaned calves had higher number of *Fibrobacter succinogenes* and *Ruminococcus albus*, with a lower number of *Butyrivibrio fibrisolvens*, than did late-weaned calves [80]. *Ruminococcus* abundance was positively correlated with solid feed intake and body weight

gain in calves [79], likely reflecting the cellulolytic capabilities of *Ruminococcus* species, which are found in the mature rumen [7,89]. Therefore, it can be speculated that as soon as the calf started to consume the solid feed, the bacterial community resembling the mature rumen is established.

Contrary to the bacterial community of the rumen, the faecal bacterial community of pre-weaned calves showed a high dominance of *Firmicutes* being replaced by *Bacteroidetes* in post-weaned calves [79]. At the genus level, the abundance of faecal *Bacteroides* decreased due to weaning, but it remained the predominant genus in both the pre- and post-weaned state [79]. Furthermore, an increase in the abundance of *Prevotella* was observed due to weaning [79]. However, the abundances of the major faecal bacteria remained unaffected by weaning [84]. Nevertheless, an increased abundance of *Ruminococcus* and a decreased abundance of *Blautia* were observed in post-weaned calf faeces [79,84], likely reflecting a shift from intestinal to ruminal fermentation in post-weaned calves.

Rumen carbohydrate metabolism showed an age-dependent increase between 5 and 9 weeks, regardless of weaning. Conversely, a decline in faecal carbohydrate metabolism was observed from the pre- to post-weaned state [84]. Additionally, a decrease in rumen bacterial diversity and evenness and an increase in faecal bacterial diversity, richness, and evenness were observed in post-weaned calves [79]. This was probably due to the higher solid feed intake in the post-weaning period, resulting in a greater amount of substrates reaching the lower intestine [79]. Thus, the higher substrate availability and lower pH variability of the hindgut favoured higher bacterial diversity in the lower digestive tract of ruminants.

2.6 Distinct bacterial communities are associated with the mucosal epithelium and luminal digesta of the GIT of calves

The bacterial composition in the GIT of animals and humans varies among the gut regions, with considerable differences between the microbes associated with the epithelial mucosa and those occupying the luminal digesta. This is also true for calves [14,22–25] and adult ruminants [90]. The mucosa-associated microbial community in calves is found to have higher individual variation, diversity, richness, and a lower microbial load than the microbiota in digesta samples [17,22–24,90]. These differences are caused by variations in host physiological state and immunity, interactions between the symbiotic bacteria and host epithelium, pH, oxygen gradient, nutrient profile, and dietary transition rates [91,92]. Each of these factors defines the microbial colonisation potential of each site, thus resulting in site- and region-specific microbial community establishment.

Digesta-associated gut communities within the first 21 days of a calf's life, except for the colon, showed a high dominance of *Firmicutes* [14,25], whereas a higher abundance of *Bacteroidetes* was observed in the mucosa-associated communities, except jejunal tissues, suggesting that the early life mucosal environment favours the colonisation by *Bacteroidetes* than *Firmicutes* [14]. *Proteobacteria* were also more abundant in the mucosa than the digesta samples [14,25], suggesting that the mucosa-associated *Proteobacteria* spp. might play an essential role in scavenging blood oxygen and ruminal ammonia oxidation [14]. This would promote an anaerobic environment for the colonisation and fermentative activities of rumen microorganisms [14]. Such compositional changes in the mucosa or digesta-associated communities were more prominent at the genus level, where *Bacteroides* dominated the digesta-associated communities in the reticulum, rumen, omasum, abomasum, caecum, and colon [14,25]. In contrast, the mucosa-associated bacterial communities of the rumen, ileum, caecum, and colon were dominated by *Prevotella* [14]. Moreover, the abundance of *Escherichia* exceeded *Bacteroides* in the mucosal samples of the omasum, abomasum, ileum, colon, and faeces [25]. Similar to this study, the hindgut microbiota of one-week-old calves showed a high dominance of mucosa-associated *Escherichia-Shigella* groups, indicating greater disease susceptibility in young calves [24]. The digesta-associated community of the duodenum was dominated by *Lactobacillus*, while *Pseudomonas* dominated in the mucosa [25]. Furthermore, the mucosa-associated communities of the jejunum showed high abundances of *Prevotella, Pseudomonas, Acinetobacter, Rikenellaceae* RC9 group, and *Delftia* [25]. The high dominance of aerobic/facultative anaerobic bacteria (*Pseudomonas, Acinetobacter, Delftia,* and *Escherichia*) in several mucosal samples suggests that these bacteria prefer gastrointestinal epithelium for growth due to higher availability of oxygen concentration [93]. In contrast, the jejunal digesta-associated communities were dominated by *Sharpea, Butyrivibrio, Ruminococcus, Lactobacillus* [14], *Streptococcus,* and *Escherichia* [25]. *Sharpea* spp. are capable of fermenting a vast variety of sugars [94]. Their high dominance in jejunal digesta of three-week-old calves is indicative of their important role in the fermentation of milk during early calf life [14].

The mucosa-associated bacterial community composition was also affected by calves' age and was significantly correlated with SCFA concentrations, indicating that the host physiology as well as diet play a role in shaping mucosal microbial communities [24]. The abundance of mucosa-associated *Escherichia-Shigella* was negatively correlated with acetate concentration and the inhibition of *E. coli* growth was observed due to high concentration of acetate [95]. SCFA can also influence the turnover of intestinal epithelial cells [96], indicating

a possible interaction between mucosa-associated microbial communities and digesta-associated microbial metabolites [24].

2.7 Influence of host genetics on gut microbial colonisation and systemic immunity in neonatal calves

In recent years, many studies have evaluated the influence of host genetics on gut microbiota in cattle [97–101] and the possible association of heritable gut microbes with nutrition and gut health in calves [102], methane emissions and feed efficiency in beef and dairy cattle [98,101]. Majority of these studies used animals belonging to different populations with variable genetic distance, age and diet, thus, masking the real influence of host genetics on gut microbiota. However, a recent study by Fan and colleagues reported genetic influences on gut microbiota based on 228 calves with linearly varying breed (Angus to Brahman), raised under controlled diet and environmental conditions [102]. The three-month-old pre-weaned calves with higher Brahman proportion harboured more butyrate-producing and fibre-digesting bacteria, carbohydrate metabolism genes, less opportunistic pathogenic bacteria and mucin-degraders, lower level of primary antibody (plasma IgG1) and less weight gain than higher Angus proportion calves that harboured bacterial taxa rapidly involved in amino acids and lipids metabolism [102]. This indicates that the host genetics not only shapes the early life gut microbiota composition but can also have strong impact on systemic immunity, which is further associated with health and growth of an animal. However, the studies addressing the role of host genetic influence on neonatal calves' microbiota are still very scare and needed to be explore further.

2.8 Gut microbiota and the host immune system development

Gut microbial communities are essential for the development of the mucosal epithelium and immune system of the host [18]. The mucosal epithelial cells line the upper respiratory tract, GIT, and uterus and are the primary responders to the microorganisms [103]. The mucosal immune system contains various physical and chemical barriers as well as pattern recognition receptors (PRRs), which enable the mucosal epithelium to coexist with its resident symbiotic microorganisms and provides protection against invading pathogens [104–106]. Notably, these signalling cascades are essential for maintaining the intestinal homoeostasis, integrity, antimicrobial peptide expression, and modulation of the mucosal barrier functions and immune responses [91,107,108]. The immune response at the mucosal surface is generally initiated by mucosa-associated lymphoid tissues (MALTs) [38,103]. In ruminants, the initiation of MALT

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development occurs in utero when the microbial communities are not yet established [109]. These in utero MALTs are capable of initiating specific immune responses through secretory IgA production [110]. However, IgA+ and IgG+ cells appear in Peyer's patches (PPs) only after birth due to the absence of in utero infections [109]. The complete development of germinal centres of PPs requires exposure to the gut microbiome [18]. In the absence of gut microbial exposure, the ileal PPs of new-born lambs showed pre-mature lymphoid follicle involution; however, when the gut microbiome was restored at four weeks, the involution was reversed [111]. This finding demonstrates that the gut microbiome provides signals for the production of a vast variety of pre-immune B cells (Figure 4A).
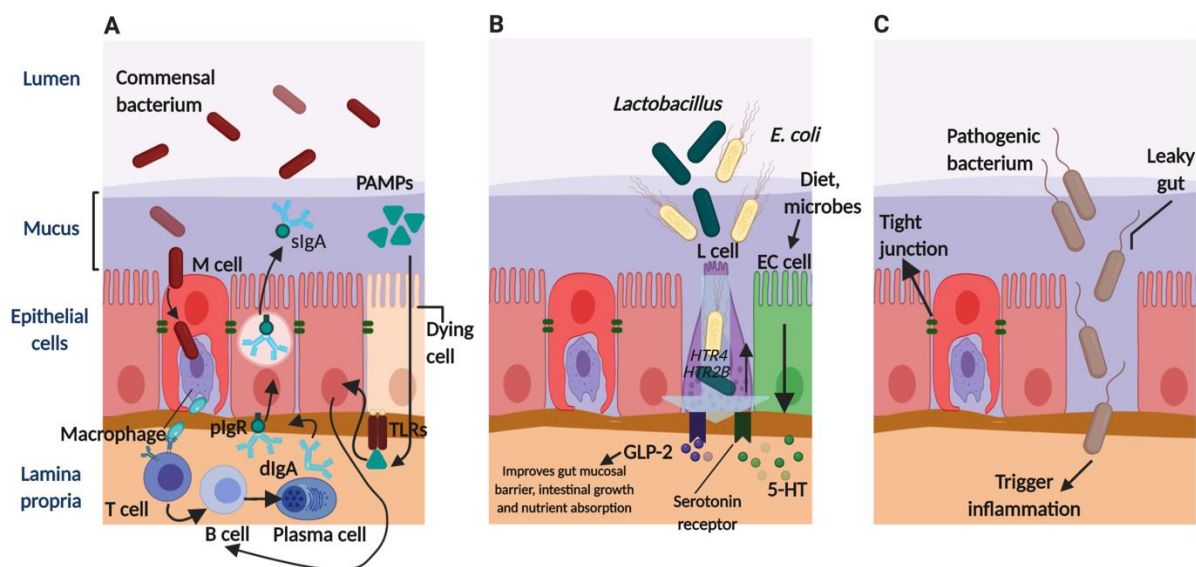
Figure 4 | Mucosa-associated lymphoid tissues (MALTs) dependent activation of immune responses in mucosal surface of calves. A) Microfold (M) cell transport microbial antigens from the luminal surface to the underlying MALT cells, where they stimulate specific T- and B- lymphocytes, resulting in the production of dIgA by B-cells, which are translocated as sIgA to the apical epithelial surface. PAMPs can alter the expression of TLRs and activate host immunity. B) Upregulation of HTR4 and HTR2B genes expression by mucosa-associated bacteria. These gene codes for the serotonin receptors that regulate GLP-2 secretion by enteroendocrine L cells via interaction of 5-HT with serotonin receptors. C) Breakdown of tight junctions, transport of pathogens and activation of inflammatory responses. Abbreviations: PAMPs, pathogen-associated molecular patterns; dIgA, dimeric immunoglobulin A; sIgA, secretory immunoglobulin A; pIgR, polymeric Ig receptor; TLRs, toll-like receptors; EC cell, enterochromaffin cell; 5-HT, 5-hydroxytryptamine/serotonin; HTR4, 5-hydroxytryptamine

receptor 4; HTR2B, 5-hydroxytryptamine receptor 2B; GLP-2, glucagon-like peptide-2. Figure created with BioRender.com.

In addition to the gut microbiome, diet (colostrum, intensive feeding of milk or MR), and environment (toxins) were also found to have a strong influence on the mucosal immune system development in neonatal calves [112]. Extended colostrum feeding during early life resulted in higher abundances of active mucosa-associated *Lactobacillus* and *E. coli* and upregulated the expressions of serotonin and adrenergic receptors genes in the calf's intestines (Figure 4B) [113]. These receptors are involved in the regulation of glucagon-like peptide-2 secretion by enteroendocrine L cells, which decreases the apoptosis of epithelial cells, reduces the motility and permeability of the gut, and increases mesenteric blood flow, intestinal growth, and nutrient absorption [114]. A positive correlation was observed between the abundances of *Lactobacillus* and *E. coli* and serotonin receptor gene expression in the colon, suggesting that the early feeding regimen may affect the host–microbe interactions, and thus play a critical role in host immune system development in new-born calves [113]. Likewise, the intensive feeding of milk or MR during the pre-weaning period stimulated the expression of long noncoding RNAs with a potential role in the synthesis of tight junction proteins in the jejunal mucosa of calves [115]. The tight junctions are protective mucosal barriers whose breakdown results in leaky gut syndrome (Figure 4C) [103,116]. In addition, it was shown that an ample supply of nutrients is essential for maturation of the intestinal immune system [117], suggesting that the pre-weaning period is critical for the development and maturation of the mucosal immune system in calves [39].

The host identifies commensal microorganisms using PRRs such as toll-like receptors (TLRs) [107]. Mucosa-attached bacteria can also alter the expression of TLRs [118] and cause PRR-dependent activation of the host immunity [14]. In contrast, pathogen-dependent activation of TLR signalling generally activates inflammatory responses [107]. Furthermore, an age-dependent decrease in mucosal TLR gene expression [119] and an increase in T lymphocytes such as CD3+, CD4+, and CD8+ cells in the mucosa of the jejunum and ileum of calves were observed [120]. Such changes may cause a decrease in the innate immunity and an increase in the adaptive immunity with age. This age-dependent downregulation of the innate immunity protects the host from harmful inflammatory responses [121]. It has been suggested that TLRs act as a primary mechanism of innate immunity in neonatal calves. They are substituted by antimicrobial-peptide-dependent innate immune mechanisms over time and protect the animal from unnecessary inflammatory responses [119]. Additionally, a potential

link between age-dependent alteration in mucosal immune mechanisms and the gut microbial communities was shown by the negative correlation between TLRs (TLR2, TLR6, and TLR9) in the mucosa of the rumen, jejunum, and caecum and the mucosa-attached bacterial population [119]. Moreover, the host–microbe interactions play a crucial role in the regulation of GIT development, as demonstrated by bovine transcriptome analyses [122].

A positive correlation was observed between the gene copy numbers of *Lactobacillus* or *Bifidobacterium* spp. and microRNAs (miRNA) expression levels. These miRNAs act as promoters of GIT development and include miR-15/16 (immune cells development), miR-29 (maturation of dendritic cells), and miR-196 (lymphoid tissue development) [122]. Likewise, the microbial-driven transcriptional regulation of developing rumen in calves via miRNAs was suggested recently [9]. They identified three miRNA-mRNA pairs involved in the development of rumen ''miR-25 and fatty acid-binding protein 7, miR-29a and platelet-derived growth factor a polypeptide, and miR-30 and integrin-linked kinase" [9].

2.9   Role of the microbiota in gut health and treatment strategies

The previous sections have summarised the current knowledge about the essential co-evolution of GIT in ruminants and the colonising microbiome. Disturbances result in an imbalanced symbiosis, leading to gut microbial dysbiosis which can induce several enteric disorders [123]. The pre-weaning period is critical due to the high susceptibility of neonatal calves to a vast variety of bacterial and viral infections, which cause diarrhoea (the major cause of death in neonatal calves) [124]. A decreased incidence of diarrhoea was correlated with a higher abundance of *Faecalibacterium* in faecal samples of one-week-old calves and in the large intestine of three-week-old calves [13,14,58]. *F. prausnitzii* promotes anti-inflammatory responses, maintains intestinal homoeostasis [125] and produces butyrate in the large intestine [13]. A high abundance of this species during the pre-weaning period may provide health benefits to the neonates by decreasing their susceptibility to enteric infections. More recently, the idea of a microbiota transplantation to stabilise the gut microbiome was applied in ruminants by transferring the rumen microbiome of adult animals orally to young calves. Although the overall microbiome structure was not affected, the incidence of calf diarrhoea decreased [126].

2.9.1 Early life antimicrobial treatments and emergence of resistant bacterial strains in the calf gut

The dairy industry relies on the use of antimicrobials to cure various diseases, resulting in the production of milk with residual concentrations of antimicrobials [127,128]. In addition to the presence of antimicrobial residues in the milk, it may contain a high number of pathogens and somatic cells [129]. Thus, the milk from antimicrobial-treated cows is generally used by the dairy industry as a feed for dairy calves [59,60]. Antimicrobials are also fed directly to the calves as medicated MR to increase their growth rate and prevent diseases [123,130]. Nevertheless, this direct or indirect exposure of neonatal calves to antimicrobials modifies their intestinal microbial community structure, resulting in the emergence of resistant bacterial strains as well as the transfer of resistance genes to other bacteria [131,132]. There is increasing evidence of the presence of highly resistant enteric microbes in young animals compared to adults [133–135], probably due to high faecal–oral transmissions and higher antimicrobial usage in young animals [136]. Several studies have reported the effects of antimicrobial usage on the gut microbial composition [137–142], the development of antimicrobial-resistant bacterial strains [132,143–149], genes involved in antimicrobial resistance [133,148,150], and antimicrobial-dependent changes in the functional profile of gut microbiota [151].

Feeding calves with WM containing residual antibiotics (oxytetracycline, ceftiofur, ampicillin, and penicillin) resulted in lower abundances of faecal *Clostridium* and *Streptococcus* in pre-weaned calves [139]. Similarly, when calves were fed with medicated MR containing tetracycline, ceftiofur, penicillin, and streptomycin, reduced abundance of *E. coli* in the ileum [140] and *Prevotella* in the rumen [142] was observed. However, feeding calves with MR supplemented with only ceftiofur reduced the abundance of *Comamonas* in the ileum [140]. Decreased abundance of beneficial bacteria (*Faecalibacterium, Roseburia, Prevotella,* and *Eubacterium*) and increased abundance of pathogenic bacteria (*Shigella, Escherichia,* and *Enterococcus*) in calf faeces were observed using the antibiotic bacitracin methylene disalicylate antibiotics [137]. Enrofloxacin treatment decreased the abundance of *Bacteroides* and increased the abundance of *Blautia, Desulfovibrio,* and *Coprococcus* in calf faeces [141]. As the concentration of residual antibiotics in the WM increases, a higher number of antibiotic-resistant bacterial strains emerge in the gut [144]. A higher prevalence of antimicrobial-resistant faecal *E. coli* phenotypes and the increased detection of ß-lactamase resistance genes in these populations was observed in WM-fed calves than in bulk milk or MR-fed calves [132,147,148]. Feeding drug residues containing milk to the pre-weaned calves also resulted in lower abundance of genes involved in regulation and cell signalling, stress response

and nitrogen metabolism [151]. In addition, the direct treatment of calves with antibiotics may also result in the emergence of antibiotic-resistant bacterial strains [149]. However, other studies have reported that the occurrence of multi-drug resistant bacterial strains is not dependent on recent antimicrobial usage but rather on other environmental variables, age, and diet [145–147]. A decreased prevalence of multi-drug resistant faecal *E. coli* with increasing age of calves indicated that the underdeveloped digestive system of neonatal calves serves as an excellent niche for the growth of resistant microbes due to limited competition for resources [146]. However, Thames et al. reported an age-dependent increase in tetracycline resistance genes in calf faeces [150]. These studies suggest that the direct and indirect exposure of the gut of neonatal calves to the antimicrobials modifies the composition and functional profile of the microbiome and the development of antibiotic resistance is mainly influenced by host-specific factors.

2.9.2 Improvement of calf gut health by feed supplements

The use of antimicrobials to support calves' health and to prevent or treat certain diseases can be avoided by using direct-fed microbes, prebiotics, and probiotics. This has been widely practised in order to improve gut health and productivity of livestock [152,153]. Supplementation of new-born calves with *Lactobacillus* and *Bifidobacterium* within the first seven days of life decreased diarrhoea and increased feed conversion ratio and weight gain [154]. Similarly, supplementation with *F. prausnitzii* in the first week of calf life decreased the calf death rate and diarrhoea [155]. Administration of *Lactobacillus* spp. to young calves also increased their serum IgG levels, suggesting a potential role of the host–microbe interactions in modulating calf health [156]. Apart from influencing host health, microbial manipulations also affect the gut microbial community structure. Feeding pre-weaned calves with probiotic strains decreased their intestinal colonisation with pathogenic *E. coli* [157]. Similarly, a decrease in faecal *E. coli* load was observed using direct-fed microbes [158]. Supplementation of the diet of neonatal calves with *Saccharomyces cerevisiae boulardii* immediately after birth increased the abundance of beneficial bacteria (*F. prausnitzii* and *Lactobacillus*) in the intestinal microbiota, as well as increasing the concentrations of endogenous secretory IgA, thus enhancing immunity and intestinal homoeostasis of calf GIT [159]. Feeding heated colostrum soon after birth benefited young calves with increased colonisation with *Bifidobacterium* and decreased colonisation with *E. coli* in the small intestine, suggesting the potential role of colostrum as a natural prebiotic associated with reduced risk of diarrhoea [41,53]. Prebiotics supplementation immediately after birth was found to have more prominent

effects than supplementation at a later stage. Higher abundances of *Bifidobacterium* and *Lactobacillus* were detected in the colon of two-week-old than in four-week-old calves fed with galactooligosaccharides [160]. Supplementation of grazing calf diet with cellooligosaccharides decreased the proportions of archaea at weaning and *Fibrobacter* within the first four weeks post-weaning. In contrast, an increase in *Fibrobacter* was detected using kraft pulp as prebiotics at four weeks post-weaning [161]. Addition of astragalus root extract in the diet of early weaned calves at a dose of 2% dry matter intake, increased the body weight, average daily gain, serum concentrations of interleukin-2 (IL-2), IgG, superoxide dismutase, and the abundance of fibrolytic bacteria [162]. Increasing the dose of astragalus root extract to 5% and 8% dry matter intake fortified these effects [162]. Supplementation of calf diet with zinc oxide (104 mg/d) effectively reduced the incidence of diarrhoea from days 1–3, increased the abundance of beneficial *Faecalibacterium* and *Lactobacillus* within the first seven days of life and improved the immunity by increasing the concentrations of serum immunoglobulins (IgM and IgG) [163]. However, when zinc methionine (457 mg/d) was supplemented, a prolonged reduction in diarrhoea was observed from days 1–14, and increased abundances of *Faecalibacterium* and *Collinsella* (day 7), and *Ruminococcus* (2 weeks) were detected [163]. These results suggest the essential role of zinc in the treatment of neonatal calf diarrhoea. In addition, calcium propionate supplementation increased the body weight and decreased the relative abundance of *Bacteroidetes* in both pre- and post-weaning groups, but increased *Proteobacteria* (*Succinivibrionaceae*) and *Methanobrevibacter* only the post-weaning group [164]. These studies suggest that microbial manipulations are easier to perform during early life, and these effects may persist longer when manipulations are performed in early life of animal.

2.10   Summary and outlook

Understanding the pattern of microbial succession throughout the GIT of pre-weaned calves is essential as it influences the development and maturation of the host gut, immune system, and health. The microbial colonisation of the GIT of neonatal calves begins during the birthing process or even in utero, but the microbial community structure changes rapidly within the first few weeks of life and is strongly affected by the genetic background, rearing environment, early life antibiotic treatments, age and feeding conditions. The majority of the studies reported the early life microbial succession patterns using DNA-based methods without any information about the viability, genetic potential (metagenomics), or even gene or protein expression (metatranscriptomics and metaproteomics) of the detected microbial communities.

Thus, there is still a lot to understand the underlying mechanisms of the possible interactions between the gut microbial communities and their mammalian host. In addition, the results obtained by various DNA-based studies are limited by different sample types and locations, extraction methods, gene regions being sequenced, sequencing methods, sequence depth, and the pipeline used for the analysis. In addition to the region-specific establishment of microbial communities along the GIT of calves, the microbiota associated with the epithelial mucosa was clearly different from those occupying the luminal digesta and had a potential role in host immune system development [113]. Thus, to better understand the host– microbe interactions, a thorough knowledge of microbial segregation between mucosal epithelium and luminal digesta throughout the pre-weaning period is of utmost importance. In the future, genome-wide association studies should be conducted to track the possible associations between host single nucleotide polymorphisms and the abundances of commensal bacterial taxa. Furthermore, more emphasis should be placed on the microbial dysbiosis caused by in-feed antimicrobials and the possibility of using the gut microbiome, prebiotics, and probiotics as antimicrobial substitutes. In addition to the control of neonatal calf diseases using antimicrobial alternatives, one can also predict the onset of diseases based on early life gut microbiota composition, and the predictive modelling approach was recently suggested by Ma et al. [165]. The combination of collecting big data with machine learning algorithms can support the establishment of prediction tools for output targets or disease outbreaks, and helps to design preventive treatment strategies (Figure 5). We conclude by mentioning that future studies must focus on the ecologic as well as metabolic activity of the detected microbiome based on advanced machine learning and prediction modelling approaches.
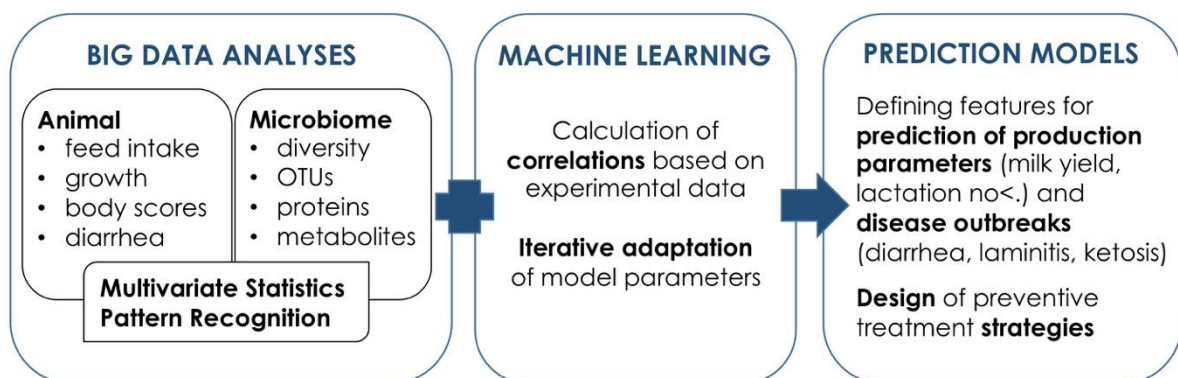
Figure 5 | Combination of big data repositories with machine learning algorithms to create prediction tools for sustainable animal productions strategies.

2.12   References

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CHAPTER III

SECOND MANUSCRIPT

EVOLUTION OF RUMEN AND ORAL MICROBIOTA IN CALVES IS INFLUENCED BY AGE AND TIME OF WEANING

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3. EVOLUTION OF RUMEN AND ORAL MICROBIOTA IN CALVES IS INFLUENCED BY AGE AND TIME OF WEANING

3.1 Abstract

3.1.1 Background

The rumen bacterial communities are changing dynamically throughout the first year of calf's life including the weaning period as a critical event. Rumen microbiome analysis is often limited to invasive rumen sampling procedures but the oral cavity of ruminants is expected to harbour rumen microbes due to regurgitation activity. The present study used buccal swab samples to define the rumen core microbiome and characterize the shifts in rumen and oral microbial communities occurring as result of calf's age as well as time of weaning.

3.1.2 Results

Buccal swab samples of 59 calves were collected along the first 140 days of life and compared to stomach tubing sample of the rumen at day 140. Animals were randomly divided into two weaning groups. Microbiota of saliva and rumen content was analysed by 16S rRNA gene amplicon sequencing. Our study showed that most rumen-specific bacterial taxa were equally observed in rumen samples as well as in the buccal swabs, though relative abundance varied. The occurrence of rumen-specific OTUs in buccal swab samples increased approximately 1.7 times from day 70 to day 140, indicating the gradual development of rumen as calf aged. The rumen-specific bacterial taxa diversity increased, and inter-animal variations decreased with age. Early weaning (7 weeks of age) rapidly increased the rumen microbial diversity from pre- to post-weaned state. Rumen microbiota of early-weaned calves seemed to have a suppressed growth of starch- and carbohydrate-utilizing bacteria and increased fibre degraders. Whereas, in late-weaned calves (17 weeks of age) no impact of dietary modifications on rumen microbiota composition was observed after weaning. Oral-specific bacterial community composition was significantly affected by calf's age and time of weaning.

3.1.3 Conclusions

The present study showed the significant impact of calf's age and weaning on the establishment of rumen- and oral-specific bacterial communities utilizing buccal swab samples. The results emphasize the possibility of using buccal swab samples as a replacement of complex stomach tube method for large-scale predictive studies on ruminants. For in-depth

rumen microbiome studies, the time of sampling should be carefully considered using an active phase of regurgitation.

3.2 Background

Dairy calves have an immature gastrointestinal tract (GIT) at birth, with a non-functional rumen [1]. The rumen proportions are relatively smaller than in adult cows and lack some major functional components (i.e., rumen wall villi), which are essential for nutrient absorption [1]. During the first 3 weeks of life, milk is the major component of diet, which is directly carried by an oesophageal groove into the abomasum without entering the rumen [1]. Therefore, the rumen contribution to nutrient digestion, absorption and generation of energy-rich substrates are marginal in young calves than in more advanced developmental stages.

The pre-weaning stage is a crucial period for the development of GIT and immune system in calves. The consumption of solid food as "starter feed" begins around second to third week of life. The highly palatable starter feed rich in rapidly fermentable carbohydrates triggers the growth and establishment of rumen microbiota, especially starch-degrading bacteria. The increase in fermentation products and microbial biomass result in structural and physiological modifications of rumen characteristics [2, 3], with subsequent establishment of a fully functional rumen and adult-like microbiota near weaning [4].

Several negative impacts of stressful weaning transition on animal feed intake and growth have previously been reported [5]. Weaning calves at 6 weeks of age rapidly shifted their rumen and faecal microbiome beta-diversity [6] and reduced their growth rate during weaning as compared to the late-weaned calves [7]. On the contrary, weaning calves at 8 weeks of age gradually shifted the beta-diversity, indicating a gradual increase in starter concentrate consumption and a progressive rumen development as compared to the early-weaned calves [6]. The premature weaning can increase the death rate in calves and delayed weaning could lead to increase feed cost and growth retardation of animal's digestive organs, thereby, affecting productive performance of animals during maturity [8]. However, these effects can be minimized by careful consideration of weaning age of an animal, to ensure better intestinal and ruminal maturation before weaning [7].

The ruminal microbiome is non-static and changes continuously with diet [9, 10], host breed [11], age [12], as well as sampling time and location [13]. Moreover, inter-animal variations in rumen microbial community composition on a defined diet could be observed due to animal history, body condition and post-feeding sampling time, thereby, a larger sample size is needed to obtain ample statistical power. The majority of rumen sampling procedures in

practice are invasive procedures such as rumenocentesis [14], oral intubation and rumen cannulation [15], which are not only unpleasant for the health and welfare of an animal but are also impractical for large scale animal sampling campaigns. Thus, the identification of more efficient, non-invasive, extensive rumen sampling procedure is needed for rumen microbiome studies.

Ruminants possess regurgitation activity that enables them to bring ruminal contents back to the mouth for chewing partially digested plant material [1]. Therefore, it is highly expected to obtain good representation of particle- and liquid-associated microbiota of the rumen utilising the buccal swab samples [16]. The concept of buccal fluid sampling as replacement of invasive rumen sampling procedures has already been tested in sheep fed on four different diet [16], and cows fed on grass silage-based diets with or without lipid supplementation [17]. However, for practical implication of the proof of concept, large scale non-invasive animal studies are needed. In the present study, buccal swab samples were collected using sterile cotton wool swabs at five different time points from 59 female Holstein calves weaned at 7 or 17 weeks of age. Bacterial communities of buccal swab samples were compared with rumen samples collected by stomach tubing from same animals at the end of experiment. The shifts in rumen and oral microbial communities occurring as a result of calf's age as well as the time of weaning were also characterized.

3.3   Results

3.3.1   Characterization of feed intake pattern in relation to saliva sampling scheme

The microbial composition of buccal swabs is related to feed intake pattern, such as proportions of milk replacer (MR), concentrate feed (C) and roughage intake. The pattern of MR and C intake was similar for both weaning groups until day 28 when weaning was initiated for early-weaned (earlyC) group and there were no significant differences in intake amounts (122–133 g DM/day). While the late-weaned (lateC) group was maintained at a constant MR level of approximately 1300 g DM/day and continued to increase C intake until 2 kg was reached at day ~ 70, the MR intake of the earlyC group was gradually reduced until day 42. At this time, the C intake level of earlyC was similar to the lateC group. From day 42 onward, the earlyC group was fed a TMR. However, the intake level could not be recorded due to technical reasons. Saliva samples were taken at days 42, 70, 98, 112 and 140. Due to some technical and health-related issues, there was no access to all animals of the herd at each time point. In addition, some of the calves' samples had to be removed during bioinformatic analysis due to poor sequence quality and low read counts. Thus, the final number of buccal swab (BS) samples

used for the data analyses were: 11 (day 42), 26 (day 70), 51 (day 98), 51 (day 112), and 47 (day 140). Forty-seven samples were available from rumen (R) at day 140.

3.3.2 Comparison of rumen and salivary bacterial communities of different age group calves

Amplicon sequencing of BS and R samples revealed $17,716 \pm 1590$ mean read counts per sample for stomach tubing and $21,014 \pm 2014$ for BS samples and a total of 4906 unique bacterial operational taxonomic units (OTUs) were obtained. Bacterial communities in samples collected by stomach tubing clustered separately from those in samples collected via buccal swabs (Figure 6a). Analysis of similarity revealed significant differences between sampling methods (stomach tubing vs. buccal swabbing; $p < 0.001$; R = 0.38; ANOSIM) as well as the calves age groups ($p < 0.001$; R = 0.37; ANOSIM). This was due to high relative abundances of potential oral bacterial taxa in the BS samples. Exclusion of OTUs related to oral bacteria was done by using a previously described mathematical filtering approach [16]. According to this approach, all the bacterial genus-level taxa with maximum relative abundance of $\geq 1\%$ (arbitrary cut-off) in BS samples as compared to any sample collected by stomach tubing were classified as "true" oral bacteria. This resulted in an oral-specific (OS) dataset of 1190 OTUs corresponding to 141 genera as potential oral bacteria. The rumen-specific (RS) dataset included 3479 OTUs, where 29 genus-level taxa were grouped as potential rumen bacteria. The OS-taxa excluded with the mathematical filtering approach made up 36.0, 66.2, 65.0, 53.2 and 57.1% of the total bacterial communities of the day 42, 70, 98, 112 and 140 BS samples, respectively. In addition, the bacterial taxa with maximum relative abundance of $< 1\%$ in all of the BS and R samples (237 OTUs corresponding to 104 genus-level taxa) were classified as rare taxa (Additional file 2: Table S8.1 and S8.2). The rare taxon accounted for a maximum of 0.01–0.97% contribution to the total bacterial community across all samples, thus considered not important for the study and eliminated from further analysis.

Following normalization of the 29 potential RS genus-level taxa to account for a total of 100% in each sample, analysis of beta-diversity revealed better clustering of samples by calves age groups ($p < 0.001$; R = 0.31; ANOSIM) rather than by the sampling method used ($p = 0.032$; R = 0.07; ANOSIM) (Figure 6b).
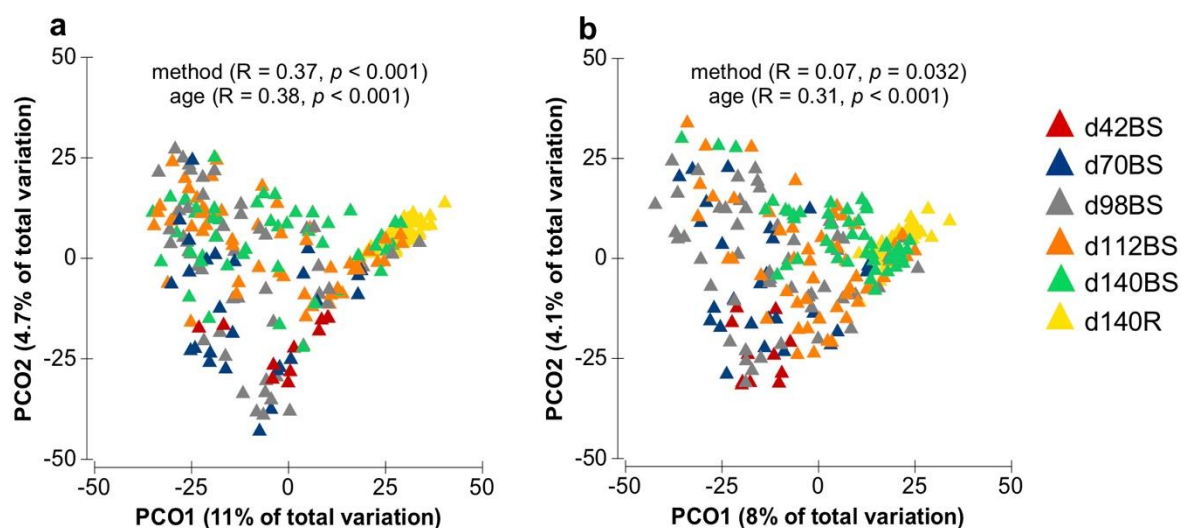
Figure 6 | Principal coordinates analysis plots depicting the distribution of bacterial communities in 233 samples collected via two different sampling methods (buccal swabbing (BS) and stomach tubing (R)) from different age group calves, without exclusion (a) or after exclusion (b) of potential oral taxa by mathematical filtering approach. Each point represents one sample. Different age groups are indicated by different coloured triangles.

3.3.3 Effect of calves age on rumen-specific microbiota

The effect of calves age on RS microbiota was analysed without taking into consideration the time of weaning. There was a significant effect of calves age ($p < 0.01$) on RS bacterial diversity, as indicated by a significant gradual increase in alpha-diversity from 3.91 (day 42BS) to 4.27, 4.36 and 4.50 at days 98, 112 and 140 BS samples, respectively (Additional file 1: Figure S1). In addition, the inter-animal variations decreased with calves age as indicated by a lower spread of Bray-Curtis values in older animals (Figure 6b). An increased within-group similarity from 9.6% at day 70 to 18.9% at day 140 (Additional file 1: Figure S7), as well as higher number of shared RS-OTUs with animal age (Additional file 1: Figure S2) were observed. However, exception was observed for day 42 BS samples, which showed a higher within group similarity (15.0%) compared to the BS samples of all other time points. This was probably due to the low sample number ($n = 8$) compared to the other time points ($n = 24$–48) as well as the influence of feed intake. At this time point, milk replacer (MR) intake in earlyC animals was low, whereas the C intake level of earlyC was similar to the lateC group. Thus, the overall feed composition was similar consisting predominantly of MR and C. However, at days 70 and 98, the two weaning groups clearly had different dietary

conditions. While, at day 112 and 140, both weaning groups started to receive the comparable dietary ration, thus, resulting in increased within-group similarity in older animals.

Calves age significantly modified the RS bacterial community composition as indicated by a decrease in relative abundance of phylum *Actinobacteria* ($p < 0.001$) and an increase in *Bacteroidetes* ($p < 0.001$), *candidatus Saccharibacteria* ($p < 0.001$), *Fibrobacteres* ($p < 0.019$), *Proteobacteria* ($p < 0.015$), and *SR1* ($p < 0.001$) with age of calves (Additional file 1: Figure S3a, Additional file 2: Table S1). At genus-level, a continuous significant decrease in relative abundances of genera *Olsenella*, unclassified *Prevotellaceae*, unclassified *Lachnospiraceae*, and a subsequent significant increase in unclassified *Bacteroidetes*, unclassified *Bacteroidales*, unclassified *Saccharibacteria genera incertae sedis*, *Fibrobacter*, *Ruminobacter*, and unclassified *SR1 genera incertae sedis* from days 42–140 was observed (Additional file 1: Figure S3b, Additional file 2: Table S1).

In addition to the calves age, sampling method also significantly affected the RS bacterial community composition as indicated by high relative abundances of genera: unclassified *Saccharibacteria genera incertae sedis*, unclassified *Clostridiales*, unclassified *Ruminococcaceae*, unclassified *SR1 genera incertae sedis*, and lower relative abundance of unclassified *Prevotellaceae* in rumen samples as compared to the BS (Additional file 1: Figure S3b, Additional file 2: Table S1).

The developing calves' rumen core microbiome was defined at the described circumstances of housing and feeding conditions based on BS samples collected from 70 to 140-day-old calves (irrespective of weaning time), as the microbiota of 6–12-week-old calves resembled more closely to the adult-like microbiota rather than early developmental stages [18]. A total of 3425 unique RS-OTUs were defined in this time period showing varying numbers at the single time points. The occurrence of RS OTUs in BS samples increased with age of calves from 726 OTUs (day 70) to 1243 OTUs (day 140), indicating the gradual development of rumen and its microbiome. Out of this, 614 RS-OTUs were defined as "core bacterial OTUs" commonly found in day 70BS, day 98BS, day 112BS, day 140BS and day 140R samples (Additional file 1: Figure S2). These core OTUs were taxonomically associated to 8 bacterial phyla and 27 genus-level taxa, with 331 OTUs assigned to *Bacteroidetes*, 196 OTUs to *Firmicutes*, 19 OTUs to *Actinobacteria*, 14 OTUs to *candidatus Saccharibacteria*, 10 OTUs to *Proteobacteria*, 9 OTUs to *Fibrobacteres*, 7 OTUs to SR1, 2 OTUs to *Tenericutes* and 26 OTUs were assigned to an unknown bacterial phylum (Additional file 2: Table S2).

In scatter plots, the relative abundances of 29 RS bacterial genus-level taxa from BS samples of five age group calves (days 42, 70, 98, 112 and 140) were compared individually

with day 140 rumen samples and the strength of similarity among sample types was assessed based on overall Spearman correlation coefficient (Figure 7). BS samples collected from 140-day-old calves were most similar (R = 0.73), while the ones collected from other age group calves were less similar (R = 0.63–0.69) with the rumen bacterial composition. In addition, an overall high correspondence was observed between all BS samples regardless of calves age, with R-value ranging between 0.68–0.78. The extent to which the BS samples collected at day 140 reflected the rumen microbial community composition at the same time point was assessed using a Mantel test. The BS Bray-Curtis dissimilarity matrix had a significant relationship with the rumen Bray-Curtis dissimilarity matrix (Mantel statistic R = 0.28, $p < 0.001$) meaning that samples which became more dissimilar in terms of RS microbial community composition in BS samples also became more dissimilar in terms of microbial community composition in R (Additional file 2: Table S9). The fitness of the BS-RS approach in reflecting the rumen microbiome composition was further elucidated based on Spearman correlation coefficient between the OTUs relative abundance along the d140R samples with its abundance over the RS portion of the d140BS samples yielding an average R-value of 0.21 (Additional file 1: Figure S8).
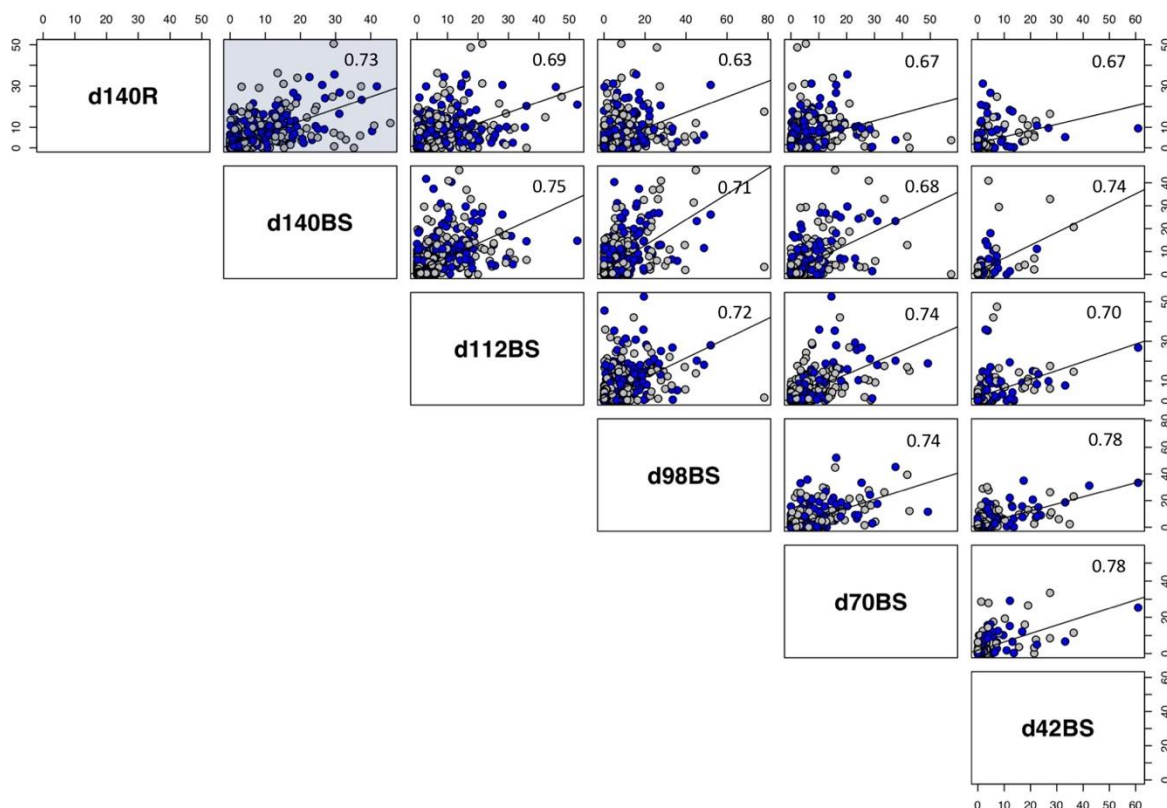
Figure 7 | Scatter plots for analysis of differences in the relative abundances of each RS bacterial taxon, among sample types. The circles (o) are representing RS bacterial taxon in R

and BS samples. The same animals were compared among sample types: a total of 36 animals (day 140R vs. day 140BS), 36 animals (day 140R vs. day 112BS), 35 animals (day 140R vs. day 98BS), 19 animals (day 140R vs. day 70BS) and 6 animals (day 140R vs. day 42BS). Spearman correlation coefficients (R-values) are indicated. Correlation in terms of microbial taxa abundance between d140R vs. d140BS is illustrated in the upper left corner highlighted with a light blue background. Grey dots represent RS samples and blue dots represent BS samples.

3.3.4 Effect of weaning time on rumen-specific microbiota in calves

In addition to the calves age, the RS bacterial communities in BS samples were also affected by the time of weaning, as indicated by separate clustering of RS bacterial communities of earlyC and lateC groups specifically during days 70 and 98 (Figure 8). Analysis of similarity (ANOSIM) revealed significant differences between weaning groups at days 70, 98 and 112 (Additional file 2: Table S3).
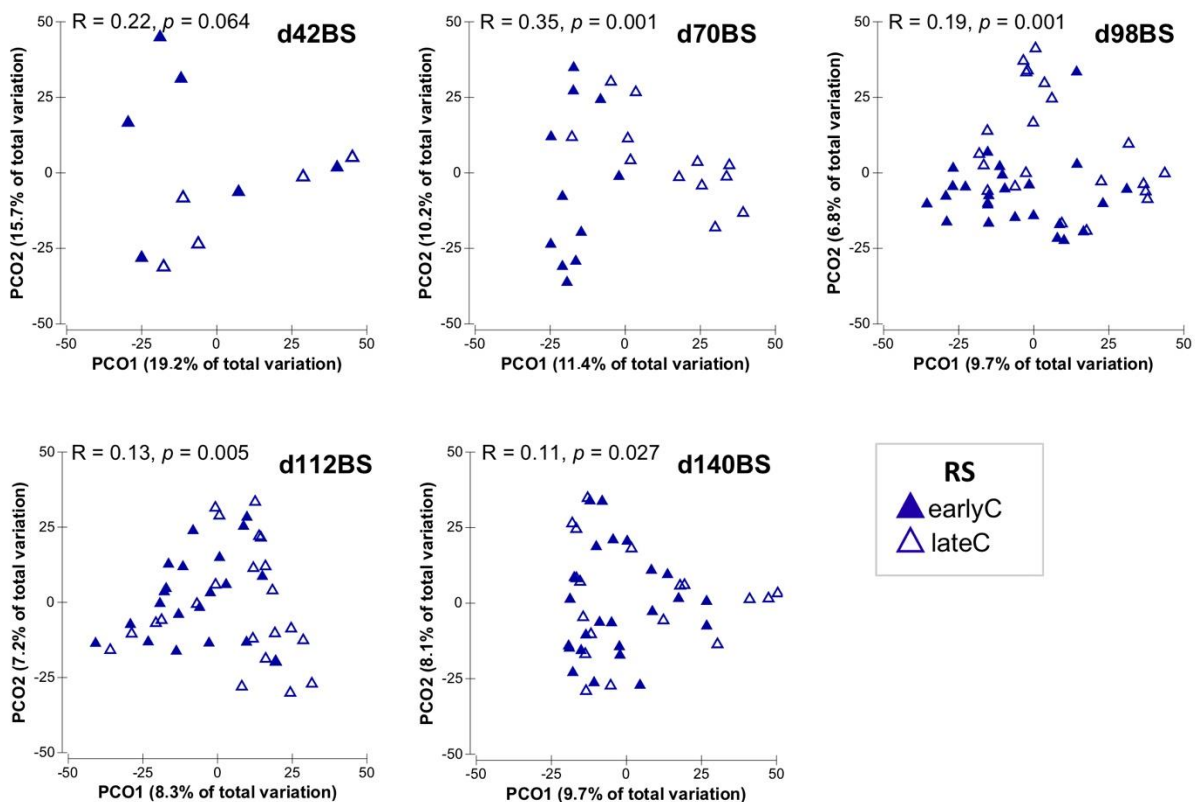
Figure 8 | Principal coordinates analysis plots depicting RS bacterial communities in BS samples of earlyC and lateC groups. Each triangle represents one sample.

Significant differences were also observed in bacterial diversity (Shannon index; $p < 0.001$). EarlyC had higher taxa diversity with a rapid increase from pre- to post-weaning period compared to the lateC group. However, the lateC group only showed a gradual increase in taxa diversity with calves age without any prominent impact of weaning (Figure 9).
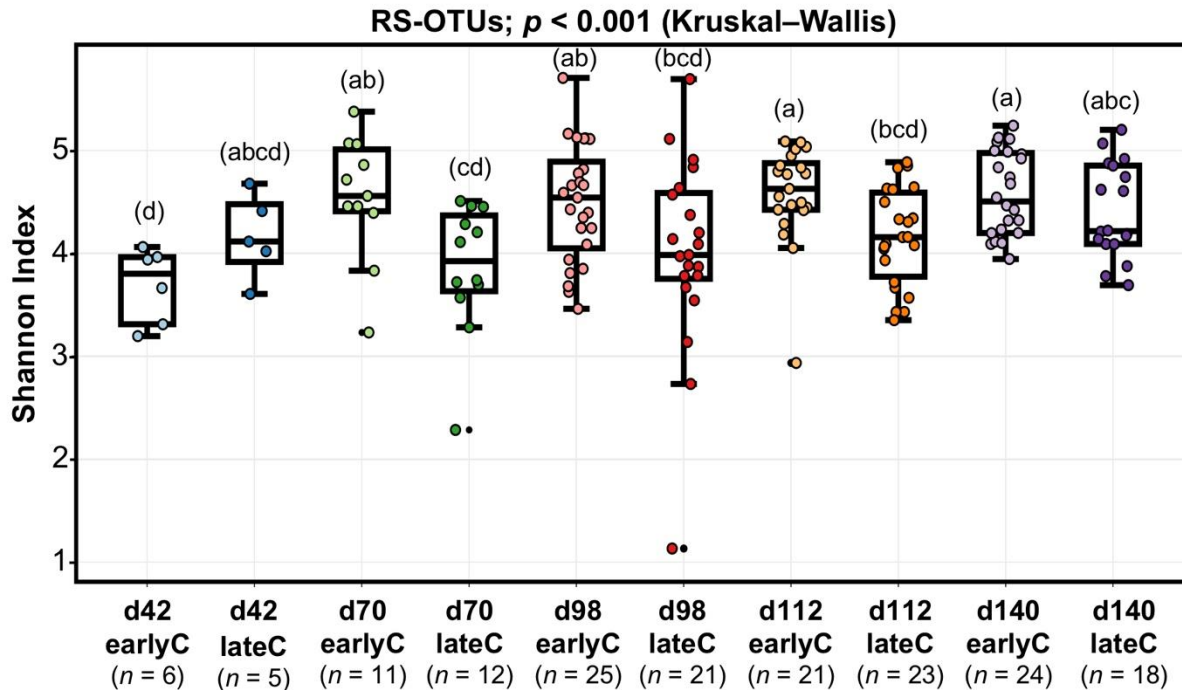
Figure 9 | Shannon index of RS bacterial communities in BS samples of different weaning groups of calves. Different weaning periods within each age group are shown by different colours.

Comparing the RS bacterial community composition of earlyC group with the same day-old lateC group, no significant effects of weaning time were observed at the phylum-level ($p > 0.05$) (Additional file 2: Table S4). Nevertheless, at the genus-level, earlyC group showed significant higher relative abundance of genus unclassified *Clostridia* ($p = 0.002$) at day 70 as compared to the same day-old lateC group. In contrast, lateC group showed a significant higher relative abundance of genus *Olsenella* ($p < 0.001$) and lower relative abundances of unclassified *Bacteroidetes* and *Butyrivibrio* at day 98 as compared to the same day-old earlyC group. No significant differences were observed between RS bacterial communities of weaning groups at days 42, 112 and 140 (Figure 10, Additional file 2: Table S4).
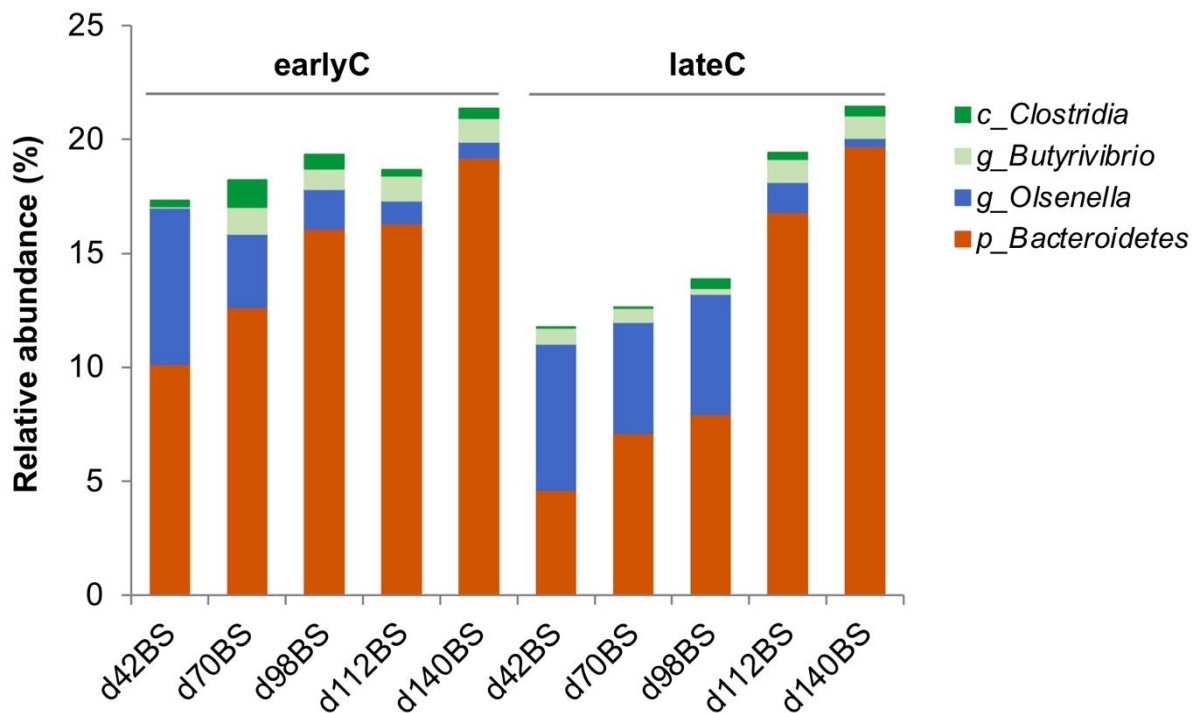
Figure 10 | Average relative abundances of RS bacterial genus-level taxa in BS samples of different weaning groups of calves. Each bar represents an average value for animals at each age group-weaning period combinations: day 42BS (6 & 5 animals), day 70BS (11 & 12 animals), day 98BS (25 & 21 animals), day 112BS (21 & 23 animals) and day 140BS (24 & 18 animals) for earlyC and lateC groups respectively. Only taxa that showed significant differences ($p \leq 0.05$) between the two weaning groups in a given sampling day are indicated.

3.3.5 Effects of calves age and time of weaning on oral-specific microbiota

The effects of calves age and time of weaning on OS microbiota were analysed separately. Following normalization of the 141 potential OS genus-level taxa to account for a total of 100% in each sample, analysis of beta-diversity revealed minor age-related clustering of the OS bacterial communities of calves ($p = 0.001$; R = 0.11; ANOSIM) (Additional file 1: Figure S4A). At phylum-level, a significant decrease in relative abundance of *Fusobacteria* ($p = 0.014$) and a subsequent significant increase in *Proteobacteria* ($p = 0.048$) with age of calves was observed (Additional file 1: Figure S4b, Additional file 2: Table S5). At genus-level, an age-dependent decrease in relative abundances of unclassified *Flavobacteriaceae*, unclassified *Porphyromonadaceae, Corynebacterium, Acidaminococcus, Roseburia, Anaerostipes, Bacteroides, Actinomyces, Selenomonas, Blautia,* and *Ruminococcus* 2 were observed. Some of these genera (*Bacteroides, Actinomyces, Selenomonas, Blautia,* and *Ruminococcus* 2) were

highly abundant at day 42 as compared to the BS samples of other time points and it was probably not related to calf's age or weaning rather an effect of low sample number at this specific time point. In addition, few OS genera also showed increased abundances with age such as *Acinetobacter, Burkholderia, Rhizobium, Arthrobacter, Aerococcus, Variovorax* and *Clavibacter* (Additional file 1: Figure S5, Additional file 2: Table S5).

Weaning affected the OS microbiome mainly at days 70 and 98 of calf's life, as indicated by separate clustering of OS bacterial communities of earlyC and lateC groups during respective days (Figure 11, Additional file 2: Table S3). Both calves age and time of weaning were found to have no significant impact on OS bacterial diversity (Shannon index; $p > 0.05$, Additional file 1: Figure S6).
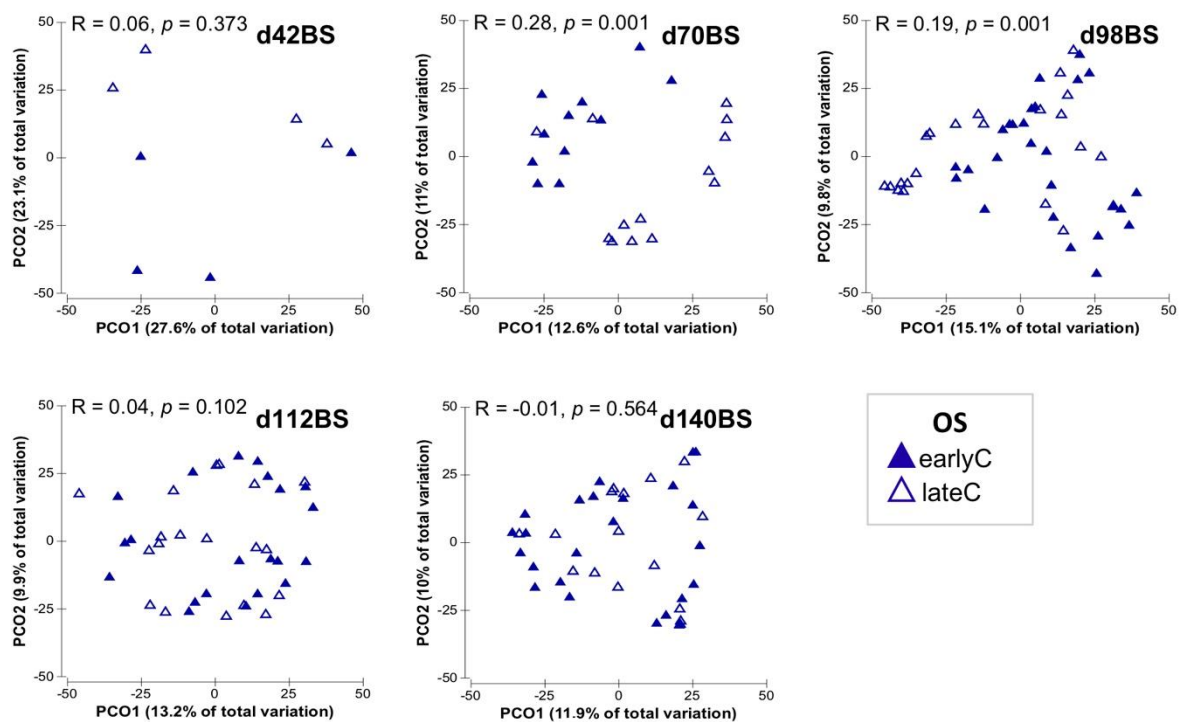
Figure 11 | Principal coordinates analysis plots of OS bacterial communities in BS samples of earlyC and lateC groups. Each triangle represents one sample.

Comparing the OS bacterial community composition of the two weaning groups, earlyC group showed a significant decrease in relative abundance of phylum *Fusobacteria* at day 98 as compared to same day-old lateC group and the relative compositions of other OS-phyla remained unaffected by the time of weaning (Additional file 2: Table S6). Nevertheless, weaning time clearly influenced the OS bacterial community composition at the genus-level,

where the earlyC group had significantly higher abundances of genera *Sphingobacterium* (days 42–70), *Kurthia* (day 70), and lower abundances of genera *Dialister* (day 42), *Acidaminococcus* (day 70), unclassified *Lactobacillales* (day 98), unclassified *Porphyromonadaceae* and unclassified *Leptotrichiaceae* (days 70–98), and unclassified *Streptococcaceae* (days 42–98) as compared to the same day-old lateC group. No significant differences were observed between OS bacterial communities of weaning groups at days 112 and 140 (Figure 12, Additional file 2: Table S6).
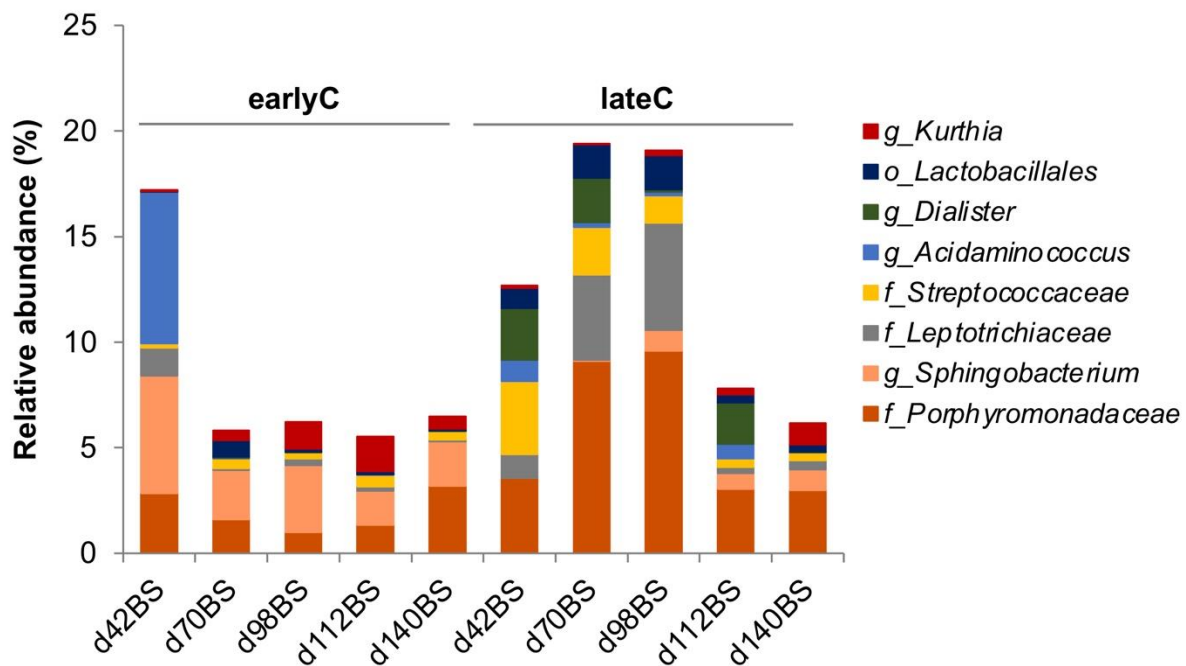
Figure 12 | Average relative abundances of OS bacterial genus-level taxa in BS samples of different weaning groups of calves. Each bar represents an average value for animals at each age group-weaning period combinations: day 42BS (4 & 4 animals), day 70BS (11 & 13 animals), day 98BS (27 & 21 animals), day 112BS (21 & 20 animals) and day 140BS (24 & 16 animals) for earlyC and lateC groups respectively. Only taxa that showed significant differences ($p \leq 0.05$) between the two weaning groups in a given sampling day are indicated.

3.4   Discussion

The progressive development of rumen to mature state occurs with age and the transition phase at weaning [6]. The start of solid food intake initiates ruminal fermentation processes which greatly modifies the rumen microbial community composition. In the present study, we defined the rumen core microbiome using BS samples and then characterized the

shifts in rumen and oral microbial communities occurring as result of calf's age as well as the time of weaning. Obtaining rumen samples via stomach tubing is usually a laborious and technically challenging procedure [19]. It is a stressful event for the animals and can have negative impacts on animal health. Therefore, rumen fluid samples from the young calves (days 42, 70, 98 and 112) were not collected in our study. On the contrary, collection of BS samples from oral cavity of an animal is a less time consuming, non-invasive method and can possibly be used as a replacement of complex stomach tube method to study rumen microbiota [16, 17]. Many previous studies characterized the rumen microbial communities of pre-ruminant calves using sacrificed animals, with the limitation of long-term monitoring of single animal [18, 20,21,22]. This can be circumvented with the use of BS, as it replaces the need to sacrifice the animals and enables the monitoring of animals across their entire life span, without having any harmful impact on animal's health due to repeated non-invasive sampling procedures. In the present study, the major RS bacterial taxa observed in the stomach tube samples were also detected in the BS samples, though relative abundances varied. Exceptions were *Anaeroplasma, Fibrobacter, Ruminobacter*, unclassified *candidatus Saccharibacteria* and unclassified *Elusimicrobiales* that were absent in the BS samples of 42-day-old calves, which is in agreement with other studies that reported these bacterial taxa to be very low abundant or totally absent in the rumen of 2 months old calves [12, 23]. Moreover, the occurrence of RS-OTUs in BS samples increased approximately 1.7 times from day 70 to day 140 independent of the time of weaning, indicating the gradual development of rumen as calf aged. In general, the BS samples of 140-day-old calves showed high overall correspondence and similar bacterial taxa diversity to the stomach tubing samples collected at the same day. Besides the valid representation of rumen microbiota, OS bacterial taxa were also identified. As the time passes between the regurgitation activity and sampling, the amount of typical oral bacteria increases in the buccal swabs [16]. Therefore, the dataset was filtered to remove these oral bacterial taxa using a mathematical filtering approach [16], that resulted in a clear clustering of bacterial communities by calf's age or time of weaning rather than by sampling method used.

In the present study, hay (ad libitum) and concentrate feed (max. 2 kg/day) were available for the calves throughout the experimental period [24], thus it could be suggested that rumen fermentation processes have already started prior to weaning. Calf's age affected the diversity of RS microbiome as shown by an increase in taxa diversity and a decrease in inter-animal variation with age. Our results are in agreement with a recent study on age-dependent

shifts in gut communities of dairy cows, showing lower beta-diversity and a higher alpha-diversity with age [18].

Weaning also affected the bacterial diversity in the rumen. Early weaning (7 weeks of age) rapidly increased the microbial diversity from pre- to post-weaned state (days 42–70) proven by a similar diversity in more mature age (days 98–140). Such an abrupt shift in microbial diversity of earlyC group reflects the sudden alteration in the source of nutrients for calves, paralleled by an overall reduced growth of earlyC compared to the lateC group [24]. A Spearman correlation analysis also showed strong positive correlations between bacterial alpha-diversity and body length ($R = 0.58$; $p = 0.048$) only during day 70 in lateC group (Additional file 2: Table S7). Conversely, lateC group (17 weeks of age) showed a gradual increase in microbial diversity with age rather than weaning, indicating age-dependent gradual increase in intake of concentrate [24], and perhaps progressive development of rumen as compared to the earlyC group. Overall, lateC group showed lower microbial diversity than earlyC from day 70 to day 112 and this was perhaps due to consumption of high amount of concentrate feed (starch) in lateC group prior to weaning. A reduction in rumen bacterial diversity was previously observed with dietary starch addition in Holstein cows [25] and was suggested to be linked to an improved feed efficiency in dairy cows [26].

Changes in diversity were correlated to phylogenetic modifications. The relative abundance of phylum *Actinobacteria* decreased, while *Bacteroidetes, candidatus Saccharibacteria, Fibrobacteres, Proteobacteria,* and *SR1* increased with age of calves. However, the time of weaning did not modify the rumen bacterial composition at the phylum-level. The genus-level composition showed that the dominant genera belonging to *Actinobacteria* namely *Olsenella* was affected by both calves age as well as time of weaning, as indicated by a significant decrease in its relative abundance with age and lower relative abundance in earlyC group as compared to the lateC group. *Actinobacteria* were described to be dominant in newborn calves exclusively fed with colostrum and showed an age-dependent decrease and compositional change in older animals [12]. This verified their role as early colonizers of neonate's gut and their importance for the conversion of milk components. In addition, they are related to the consumption of starch to produce lactic acid [27] and *Olsenella* ferments carbohydrates to produce lactic, formic and acetic acid [28]. A decrease in *Olsenella* abundance with dietary forage inclusion was recently reported [29]. Thus, it can be speculated that the decrease in *Olsenella* abundance with age and after day 70 in the earlyC group was probably due to weaning related dietary modifications as milk replacer was substituted by a total mixed ration (TMR) including 48% grass silage.

Bacteroidetes showed an increase in abundance with age and after weaning events in the present study. The main reason was the change in diet and the corresponding availability of plant polysaccharides which could be used by *Prevotella* spp. and other members of the *Bacteroidetes* phylum, inventing a huge number of carbohydrate active enzymes [9]. Changes within the *Firmicutes* phylum were especially observed in the increased abundance of unclassified *Clostridia* after weaning (day 70 earlyC). The high abundance of unclassified *Clostridia* in post-weaned microbiota of earlyC group was diet-dependent, as high abundances of *Clostridia* have previously been reported using diets containing forages and mixed forages [30]. In addition, an age-dependent increase in the abundance of *Fibrobacteres* and it corresponding *Fibrobacter* genus was observed. *Fibrobacteres* are major degraders of cellulose in the rumen [31] and their abundance in rumen decreases with increasing dietary concentrate proportions [32]. Thus, the increased abundance of *Fibrobacteres* in the rumen of mature calves in our study seemed to be reasonable due to the presence of hay and a total mixed ration in their diet.

The calves age as well as the time of weaning also affected the OS microbiota at both phylum- and genus-level. A recent study reported that the oral microbiota of neonatal calves matured quickly and contained similar microbial composition to the adult cow oral microbiota by four-weeks of age [33]. In the present study, the oral samples were collected at seven-weeks of age, thus, it is presumable that the oral microbiota was matured and the major changes observed in OS microbiota composition were mainly caused by weaning related dietary shifts. Weaning influenced the OS microbiota mainly at days 70 and 98 of the calf's life, where the earlyC group showed significant higher abundances of genera *Kurthia* and *Sphingobacterium*, and lower abundances of *Dialister, Acidaminococcus*, and unclassified *Lactobacillales* as compared to the same day-old lateC group. *Kurthia* occupied the normal intestinal microbiota of high-roughage fed cattle [34]. *Dialister* was frequently isolated from the oral cavity, with some species as causative agents of periodontitis [35]. This genus was positively correlated with starch degradation [36] and the decreased abundance of this genus in earlyC group after weaning was due to weaning related dietary shifts. *Lactobacilli* are common members in GIT of human and animals, in mouth and female genital tract and exert certain beneficial effect on host health such as reduced diarrhoea and increased weight gain in neonatal calves, provide protection against pathogenic bacteria, promote gut health and reduce gastrointestinal inflammatory responses [37]. *Lactobacilli* concentration was high in milk consuming calves [2] and negatively affected by weaning [38]. The higher abundance of *Lactobacilli* observed in 98-day-old lateC group in our study was probably due to higher availability of rapidly

fermentable substrates (e.g., starch and lactose) in their diet compared to the earlyC group, receiving a total mixed ration.

The present data showed an age-dependent decrease in potential pathogenic bacteria such as *Corynebacterium*, unclassified *Flavobacteriaceae*, and unclassified *Porphyromonadaceae*. *Corynebacterium* colonizing the skin and membranes in animal and humans [39], including several disease-causing species such as *C. bovis*, a causative agent of bovine mastitis [40]. *Flavobacteriaceae* family members were found in human oral cavity, dog mouth and other habitats [41, 42]. Certain *Flavobacteriaceae* genera (*Flavobacterium* and *Bergeyella*) can cause dental caries [43]. Similarly, the weaning related dietary shifts also benefited the post-weaned microbiota of earlyC group in terms of decreased abundances of potential pathogenic bacteria such as *Porphyromonadaceae* and *Leptotrichiaceae*. Species belonging to *Porphyromonadaceae* are ubiquitously present in oral cavities and GIT of animals and humans with some causing infections [44]. Genera belonging to *Leptotrichiaceae* such as *Leptotrichia* are commonly found in human oral cavity and are causative agents of dental plague [45]. In addition, no significant differences were observed between RS as well as OS bacterial communities of weaning groups at days 112 and 140, indicating greater ruminal maturation and enhanced feed adaptability of calves' microbiota at 17 weeks as compared to 7 weeks of age.

3.5   Conclusion

Our study showed the significant impact of calves age and time of weaning on the establishment of RS and OS bacterial communities using BS samples. This sampling strategy eliminated the need of animal slaughtering or invasive rumen sampling and enabled sample collection from a large number of animals over a longer time span. The results of our study are emphasizing the possibility of using BS samples in large-scale predictive studies on ruminants, where direct access to the ruminal contents is not an option. However, the BS dataset should be carefully evaluated, when analysing the abundances of RS microbiome. The oral health of an animal and the gap between the regurgitation activity and sampling can increase the amount of typical oral or pathogenic bacteria in the buccal swabs decreasing the predictive power of buccal swabbing procedure. In addition, sampling via buccal swabs might serve as a potential tool for the establishment of a fast-screening methodology to monitor the weaning status of calves. Prospective lab-on-chip techniques using probes specific for OS and RS taxa could be developed to provide an easy-to-use diagnostic tool for the farmers and to avoid illegal calf trading. This study identified 614 "core RS bacterial OTUs" corresponding to 27 genus-level

taxa that were ubiquitously observed in BS samples of 70–140-day-old calves. The obtained dataset might serve as starting point to define potential biomarker OTUs in future predictive studies on ruminants. In addition, our study exposed the beneficial effects of late weaning in terms of relatively stable rumen and oral microbial community composition, quick adaptability of microbiota to dietary changes and better growth performance in lateC group.

3.6   Methods

3.6.1   Animals management and diets

The experimental design was previously described by Schwarzkopf et al. [24]. Briefly, 59 female German Holstein calves born to an established herd in a seasonal calving period (October to December) were monitored from birth until 149 ± 2 days of life. Calves were fed initially after birth with 3 L of colostrum using nipple bucket. Within 2–3 h after birth, calves were shifted into straw-bedded single hutches and were fed twice a day with 2 L of pooled herd milk. During the pre-experimental period (starting from 3 days of age), pooled herd milk was mixed with milk replacer (MR; NOLAC GmbH, Zeven, Germany). The milk replacer was first dissolved in temperature-adjusted water and then mixed with pooled herd milk. The amount of MR was increased gradually from 0.3 kg/d (day 3) to 0.9 kg/d (day 5), with a maximum amount of liquid feed available at a concentration of 150 g/L MR. The experimental period started by shifting calves at an average age $(8 \pm 1.9 \, \text{days})$ and live weight $(44.5 \pm 5.2 \, \text{kg})$ into two separate, straw-bedded free barns within one housing facility and animals were kept randomized in the groups until weaning. Both compartments of the barn were under the same climatic conditions and equipped with MR and concentrate self-feeding stations (Förster-Technik GmbH, Engen, Germany). Each calf was equipped with an ear transponder for automatic recording of the daily individual intake of MR and concentrate. During the first 5 days of experimental period, both groups received 0.9 kg MR powder/d. MR amount was gradually increased from 0.9 kg/d (day 6) to 1.35 kg/d (day 10) and remained at constant level until start of weaning. Over the entire experimental period, the maximum amount of liquid feed was available at a concentration of 150 g/L MR, water was fed ad libitum and a maximum amount of 2 kg concentrate feed/d was available until weaning. At the time of weaning, calves were moved to another straw-bedded barn in groups of different sizes. EarlyC group was weaned at 7 weeks of age (days 28–42) and lateC group at 17 weeks of age (days 98–112). During weaning, the amount of milk replacer was reduced in a 14 days step-down approach from 1.35 kg/d to 0.3 kg/d. Concentrate amount was reduced from 2 kg/d to 1 kg/d during weaning at day 98 for lateC animals. Reducing the concentrate in lateC group was intended to

reduce the risk of acidification of the rumen during weaning and to stimulate roughage intake and consequently rumen development at the same time. After weaning all calves were housed irrespective of their weaning group affiliation together into two compartments within one straw-bedded barn under same housing conditions and were fed ad libitum with hay and a total mixed ration comprising of grass silage (48%), maize silage (32%) and concentrate feed (20%). The individual concentrate intake until weaning was previously published by Schwarzkopf et al. [24].

3.6.2 Sampling procedures

3.6.2.1 Buccal swabbing using sterile cotton wool swabs

On experimental days, buccal swabs were taken in the morning between 8.00 and 11.00 am. Since the calves had no fixed feeding times, sampling was independent of the feeding time. Two sterile cotton wool swabs were placed on a clamp in the mouth of each calve for at least 30 s, at day 42, 70, 98, 112, and 140 of the experimental periods. Cotton wool swabs were immediately inserted into salivette (Sarstedt, Nümbrecht, Germany) and cooled on ice. The salivettes were centrifuged at 2000 g for 3 min and the swabs were frozen individually in plastic bags at − 80 °C. The bacterial cells were eluted from the BS samples by mixing them with 4 mL PBS buffer, incubated in fridge for 1 h, followed by 30 s sonication using ultrasonication bath. The liquid was squeezed from the swabs with sterile forceps. The extracted sample was centrifuged at 2500 g for 10 min, supernatant was transferred into clean tubes and centrifuged again at 19,000 g for 10 min. Half of the supernatant was discarded, and pellet was resuspended in the remaining supernatant. After 15 s sonication step, the liquid was directly added into Lysing Matrix E tubes for DNA extraction.

3.6.2.2 Stomach tubing

Rumen samples were collected from each calve in the morning between 8:00 and 10:00 am. at the end of the experimental trial on day 140 using an oral stomach tube modified according to Geishauser (1993) [46]. The instrument consisted of an oro-ruminal probe, a flexible tube and a manual suction pump. The probe was inserted orally into the ventral sac of the rumen and approximately 100 ml ruminal fluid sample was collected, while 200 ml of the fluid obtained at the beginning of sampling was discarded to avoid salvia contaminations. Rumen fluid samples were immediately frozen at − 80 °C until further analysis.

3.6.3 DNA extraction

DNA from the rumen fluid and BS cell suspension was extracted using FastDNA™ SPIN Kit for Soil (MP Biomedical, Solon, OH, USA) with slight modifications in the manufacturer protocol as described previously [47]. The DNA extraction method included a bead-beating procedure to ensure effective cell lysis as proposed by [19]. The concentration and quality of DNA extracts was checked with NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.6.4 PCR amplification and Illumina amplicon sequencing

The V1-V2 region of bacterial 16S rRNA gene was amplified with PCR and Illumina library was prepared as described previously [48]. A barcode (6-nt) and a linker (2-nt) sequence was added in the forward primer. Both primers were additionally linked to the overhang adapter sequences to make amplicons compatible with the Illumina MiSeq sequencing. The PCR mixture was the same as described by [9]. Thermocycling conditions for PCR involved a 3 min initial denaturation step at 95 °C, followed by 20 cycles including 10 s of denaturation at 98 °C, 10 s of annealing at 59 °C, 45 s of extension at 72 °C and 2 min final extension step at 72 °C. PCR product (1 μl) was used in second PCR (15 cycles) step under same thermocycling conditions with reverse primer that contained additional sequence with integration of Illumina index primers and Illumina multiplexing sequence [48]. Amplicons were quality controlled by gel electrophoresis, purified and normalized with SequalPrep Normalization Kit (Invitrogen Inc., Carlsbad, CA, USA) and sequenced utilizing paired-end (2×250 bp) sequencing chemistry on an Illumina MiSeq platform. Sequences were submitted to European Nucleotide Archive under the accession number PRJEB41435.

3.6.5 Bioinformatic analysis

The bioinformatic analysis of Illumina amplicon sequencing datasets covering V1-V2 region of 16S rRNA gene was done using QIIME 2 (2019.10) [49]. The paired-end (PE) Illumina raw sequences (2×250 bp) were imported in QIIME 2 using MultiplexedPairedEndBarcodeInSequence semantic type. The PE sequences were demultiplexed using cutadapt (v2.6) within QIIME 2 with q2-cutadapt plugin and demux-paired command, increasing the default error tolerance to 0.2. The residual artificial sequences such as barcodes, forward primer (22 bp) and reverse primer (19 bp) were trimmed by implementing cutadapt (v2.6) in QIIME 2 with q2-cutadapt plugin and trim-paired command [50]. The quality filtration step and joining of PE reads was done by implementing DADA2

pipeline in QIIME 2 with q2-dada2 plugin and denoise-paired command [51]. The trimmed PE sequences were quality filtered by retaining high quality bases (average quality score above 30) and PE reads were joined at a mean length of 313 ± 6 bp, chimeric sequences, non-overlapping regions and singletons were discarded and FeatureTable [Frequency] and FeatureData [Sequence] QIIME 2 artifacts were generated. The PE sequences from each sequencing run were processed separately throughout the analysis resulting in FeatureTable [Frequency] and FeatureData [Sequence] QIIME 2 artifacts per sequencing run after DADA2 step. The filtered FeatureTable [Frequency] artifacts were merged with qiime feature-table merge command and FeatureData [Sequence] artifacts with qiime feature-table merge-seqs command resulting in a total of 6,141,120 reads, with $23,262 \pm 1758$ reads (mean $\pm$ SEM) per sample. Taxonomic classification was performed with q2-feature-classifier plugin and classify-sklearn method using sklearn-based taxonomy classifier (pre-trained on SILVA reference database for 16S rRNA (release_132), under a default confidence of 0.7 [52, 53]. Sequences assigned to cyanobacteria and chloroplast as well as non-bacterial and unassigned sequences from FeatureData [Sequence] and FeatureTable [Frequency] artifacts were removed using q2-taxa plugin in QIIME 2 and a taxonomy-based filtering step using qiime taxa filter-seqs and qiime taxa filter-table commands. All low reads samples (< 5000 reads) were removed from FeatureTable [Frequency] and FeatureData [Sequence] artifacts with qiime feature-table filter-samples and qiime feature-table filter-seqs commands. A biom feature table (FeatureTable [Frequency]-with-taxonomy annotations) was produced with biom add-metadata command in QIIME 2 that was later converted into txt format with biom convert command. The feature table was filtered again by following strict criteria to remove the low abundance OTUs ($\leq 0.2\%$ of total reads per sample), thus, resulting in a total of 4,741,355 reads, with mean read counts for stomach tubing samples $17,716 \pm 1590$ and for buccal swab samples $21,014 \pm 2014$ (mean $\pm$ SEM) per sample and a total of 4906 unique bacterial OTUs. All unique bacterial OTUs were taxonomically reassigned using RDP database [54] and naïve Bayesian RDP classifier [55]. The output taxonomy table was filtered according to [56] with a defined confidence threshold cut-off value for each taxonomic level such as: genus (94.5%), family (86.5%), order (82.0%), class (78.5%) and phylum (75.0%) and the taxonomic assignments were omitted if they fall below the following sequence identity thresholds.

3.6.6 Statistical analysis

Prior to any statistical analysis, the OTU count data was standardized with total sum normalization (TSS) method by dividing the OTU read counts by total reads in each sample.

For alpha-diversity analysis, samples were rarefied to the lowest read counts in the dataset (a read depth of 1008; RS-dataset) and (a read depth of 1157; OS-dataset). Principal coordinates analysis (PCO/PCoA) was performed on standardized OTU abundance data, using Bray-Curtis dissimilarity matrix in Primer-e (PRIMER 6.1.16 and PERMANOVA+ 1.0.6 [57]; to visualize the samples clustering within specific group and between groups (sampling method, calf age and weaning). Bray-Curtis dissimilarity is a coefficient with value bound between 0 and 1, where 0 represents high similarity between two samples (share all species, with same abundance) and 1 represents no similarity between the two samples (share no species) [58]. Analysis of similarities (ANOSIM) test was performed in Calypso v8.84 [59] to confirm statistically significant differences between groups. ANOSIM statistic R corresponds to the mean rank differences between and within groups, with value between -1 and $+1$, where 0 represents complete random grouping [60]. To access how well the RS microbiota in BS samples represented that in rumen, scatter plots were generated using "pairs" function in R v4.0.3 [61]. A linear regression line to the scatter plots was added using R "abline" function. Pairwise taxonomy comparisons among sample types (rumen vs. buccal swabbing) were performed by calculating Spearman correlation coefficients using rcorr() function from "Hmisc" package in R. In order to further elucidate the extent to which the rumen composition similarities between the animals' rumen samples in day 140 were reflected by their oral microbiome, a Bray-Curtis dissimilarity matrix was calculated for day 140 rumen samples and another Bray-Curtis dissimilarity matrix for day 140 RS portion of the BS samples and the two dissimilarity matrices were examined for correlation using Mantel test in R. The same animals were selected for both matrices (36 animals for day 140R and the same 36 animals for day 140BS) and both OTU abundance datasets were normalized separately to account for a total of 100% relative abundance in each sample before calculation of Bray-Curtis dissimilarity matrices. For OS-dataset, the standardized relative abundance table of bacterial genus-level taxa was scaled by row to generate heat maps using "gplots" package in R based on Spearman correlation and hierarchal average linkage clustering method. The microbial composition at phylum- and genus-level as well as alpha-diversity index was compared between groups using Kruskal–Wallis test and Dunn's post hoc test in R. For Dunn's statistical test, Benjamini–Hochberg algorithm [62] was used for p-value adjustment into false discovery rate (FDR). The FDR-adjusted p-values were considered significant at a probability of $p < 0.05$. The correlation matrix between bacterial alpha-diversity indices of earlyC and lateC groups and their body growth parameters (hip height, withers height, back length, body length, heart girth and live weight gain) was built based on the Spearman correlation coefficients.

3.7   Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42523-021-00095-3

Additional file 1:

Figure S1 | Shannon index of RS bacterial communities in R and BS samples of different age group calves.

Figure S2 | Overlap of RS-OTUs covering V1-V2 region of bacterial 16S rRNA.

Figure S3 | Average relative abundances of RS bacterial phylum- and genus-level taxa in rumen and buccal swab samples of different age group calves.

Figure S4 | Principal coordinates analysis plot of bacterial communities in 186 BS samples of different age group calves, after exclusion of potential RS taxa by mathematical filtering approach.

Figure S5 | Average relative abundances of OS bacterial genus-level taxa in BS samples of different age group calves.

Figure S6 | Shannon index of OS bacterial communities in BS samples of different weaning groups of calves.

Figure S7 | Bar-plot depicting within group similarity (mean and standard deviations) along the time.

Figure S8 | The Spearman correlation coefficients (R-values) between OTU's relative abundance along the d140R samples with its abundance over the RS portion of the d140BS samples.

Additional file 2:

Table S1 | Average relative abundance of RS bacterial taxa in samples of different age group calves.

Table S2 | Core RS bacterial OTUs in R and BS samples of different age group calves.

Table S3 | ANOSIM analysis between weaning groups.

Table S4 | Average relative abundances of RS bacterial taxa in BS samples of different weaning groups.

Table S5 | Average relative abundance of OS bacterial taxa in BS samples of different age group calves.

Table S6 | Average relative abundances of OS bacterial taxa in BS samples of different weaning groups.

Table S7 | Correlation matrix between bacterial alpha-diversity indices of earlyC and lateC groups and their body growth parameters.

Table S8.1 | Rare taxa OTUs in BS and R samples.

Table S8.2 | Mean, median and standard deviations of rare genus-level taxa in BS and R samples.

Table S9 | Mantel test statistics showing correlation between Bray-Curtis dissimilarity matrices of d140R samples and day 140 RS portion of the BS samples.

3.8   Acknowledgements

3.9   Authors' contributions

Conceptualization: KH, JF, SD, JS; Project administration and Funding acquisition: KH, JF, SD and JS; Supervision: KH, JF, SD, ACS and JS; Writing original draft: NA and JS; Methodology: NA, SS, AK, JTM, ACS and JS; Formal analysis and software: NA, ACS; Investigation and Visualization: NA, ACS and JS; Review and editing: all authors. The author(s) read and approved the final manuscript.

3.10   Funding

3.11   Availability of data and materials

Sequences were submitted to European Nucleotide Archive under the accession number PRJEB41435.

3.12   Ethics approval and consent to participate

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (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, Germany, file No.: 33.19–42502–04-15/1858).

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CHAPTER IV

THIRD MANUSCRIPT

HOST METABOLOME AND FAECAL MICROBIOME SHOWS POTENTIAL INTERACTIONS IMPACTED BY AGE AND WEANING TIMES IN CALVES

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4. HOST METABOLOME AND FAECAL MICROBIOME SHOWS POTENTIAL INTERACTIONS IMPACTED BY AGE AND WEANING TIMES IN CALVES

4.1 Abstract

4.1.1 Background

Calves undergo nutritional, metabolic, and behavioural changes from birth to the entire weaning period. An appropriate selection of weaning age is essential to reduce the negative effects caused by weaning-related dietary transitions. This study monitored the faecal microbiome and plasma metabolome of 59 female Holstein calves during different developmental stages and weaning times (early *vs.* late) and identified the potential associations of the measured parameters over an experimental period of 140 days.

4.1.2 Results

A progressive development of the microbiome and metabolome was observed with significant differences according to the weaning groups (weaned at 7 or 17 weeks of age). Faecal samples of young calves were dominated by bifidobacterial and lactobacilli species, while their respective plasma samples showed high concentrations of amino acids (AAs) and biogenic amines (BAs). However, as the calves matured, the abundances of potential fiber-degrading bacteria and the plasma concentrations of sphingomyelins (SMs), few BAs and acylcarnitines (ACs) were increased. Early-weaning at seven weeks significantly restructured the microbiome towards potential fiber-degrading bacteria and decreased plasma concentrations of most of the AAs and SMs, few BAs and ACs compared to the late-weaning event. Strong associations between faecal microbes, plasma metabolites and calf growth parameters were observed during days 42–98, where the abundances of *Bacteroides*, *Parabacteroides*, and *Blautia* were positively correlated with the plasma concentrations of AAs, BAs and SMs as well as the live weight gain or average daily gain in calves.

4.1.3 Conclusions

The present study reported that weaning at 17 weeks of age was beneficial due to higher growth rate of late-weaned calves during days 42–98 and a quick adaptability of microbiota to weaning-related dietary changes during day 112, suggesting an age-dependent maturation of the gastrointestinal tract. However, the respective plasma samples of late-weaned calves

contained several metabolites with differential concentrations to the early-weaned group, suggesting a less abrupt but more-persistent effect of dietary changes on host metabolome compared to the microbiome.

4.2   Background

The commercial calf rearing facilities are continuously challenged by cost reduction without affecting animal health and performance. Even a slight reduction in the weaning age can significantly reduce the feed cost. However, weaning age should be carefully considered as calves undergo extreme nutritional, metabolic, and behavioural changes from birth to the entire weaning period [1]. Feeding minimal plane of nutrition before weaning could result in long-term detrimental effects on calf's growth and metabolic health [2]. The composition of the gut microbiome is unstable during the first three months of a calf's life due to the change in physiological state, age, diet, weaning, and other environmental factors [3]. Besides other factors, pre-weaning calf diet contributes most strongly to the establishment of gut microbial communities and mucosal immune system [4]. The activity of gut microbes in turn benefit the host through digestion of complex dietary substrates, maturation of host immune system, intestinal epithelium development, maintenance of gut integrity and protection against pathogens [5-8]. The gut microorganisms produce a wide variety of metabolites either through direct fermentation of dietary substrates or through utilization of endogenous compounds produced by other gut microbes and the host [9]. These microbial metabolites are absorbed by the intestinal epithelium, enter the bloodstream to provide energy and nutrition to the host, regulate target organs and thus, alter the host's metabolic state [10].

Most recent studies have highlighted the importance of integrating data from the microbiome and metabolome instead of solely microbial taxonomic profiling to better understand the host–microbe's metabolic interactions and possible identifications of predictive biomarkers for diseases [11, 12]. With the advanced metabolomic analysis tools, it is now possible to detect several classes of metabolites such as amino acids (AAs), biogenic amines (BAs), acylcarnitines (ACs), and sphingomyelins (SMs) in a broad spectrum of matrixes such as blood or digestive material. These metabolites can provide a broader image of metabolic shifts and enable us to understand the underlying mechanisms caused by gut microbial dysbiosis [13]. Given the role of AAs in protein synthesis, energy generation and metabolic pathways regulation [14], plasma AAs quantification can provide an insight into the nutritional status, health and disease pathogenesis [15]. Similarly, high levels of BAs during rumen acidosis are regarded as a biomarker for bacterial dysbiosis [16], due to their important role in

immunological, muscular, cardiovascular and neurological functions, as well as anti-inflammatory and anti-oxidative reactions [17]. Acylcarnitines were suggested as lipid mobilization biomarkers [18] and their high concentrations in plasma have been linked with both the healthy and diseased status of the host [19]. Sphingolipids are bioactive molecules, involved in several cellular and pathological processes including proliferation, cell division and differentiation, cell death, and pro-inflammatory responses [20]. Thus, it can be speculated that stress-related gut microbial dysbiosis can strongly impact the levels of metabolites [12]. To our knowledge, the association of gut microbiota with the plasma concentrations of AAs, BAs, ACs and SMs in pre- and post-weaned calves has not been examined so far. Although this evaluation should be done with care as a strong influence of host genetics on serum metabolites was described before [21], a more recent study found that 47% of the microbe-associated blood metabolites to be nonheritable [11]. This suggests the important role of gut microorganisms on the systemic metabolism, which is independent of the host's genome. Here, we explored the changes in the calf's faecal microbiome and plasma metabolome due to the developmental stage and the early and late-weaning event, inherently associated with qualitative and quantitative aspects of nutrient intake pattern.

4.3   Results

4.3.1   Age-dependent changes in the compositional profile of calves' faecal microbiome

The differences between the faecal bacterial community structure associated with age, weaning and parity of the mother were identified using Permutational Analysis of Variance (PERMANOVA) that showed a significant impact of age ($p < 0.001$), weaning time ($p < 0.001$), parity ($p = 0.007$) and the interaction between age and weaning time ($p < 0.001$) but parity was non-significant within the respective age and weaning groups. A clustering of bacterial communities based on amplicon sequence variants (ASVs) was observed by calves age in both weaning groups (Figure 13A, B), which was further confirmed with the analysis of similarity test (ANOSIM) that showed significant differences between age groups (ANOSIM; $p < 0.001$; R = 0.65 and 0.75; earlyC and lateC, respectively). Both weaning groups showed a significant increase in faecal bacterial alpha-diversity with age ($p < 0.001$) as indicated by the lowest Shannon index values of 2.68 and 2.98 (d1) to the highest values of 4.94 and 4.95 (d140) in earlyC and lateC groups, respectively (Additional file 1: Figure S9A). However, no significant impact of weaning time on diversity index was observed. With respect to the faecal bacterial taxonomic composition, a significant age-dependent decrease in the relative abundances of

Firmicutes and *Actinobacteria*, while an increase in *Bacteroidetes*, *Spirochaetes* and *Elusimicrobia* was observed (Additional file 1: Figure S9B, Additional file 2: Table S10).
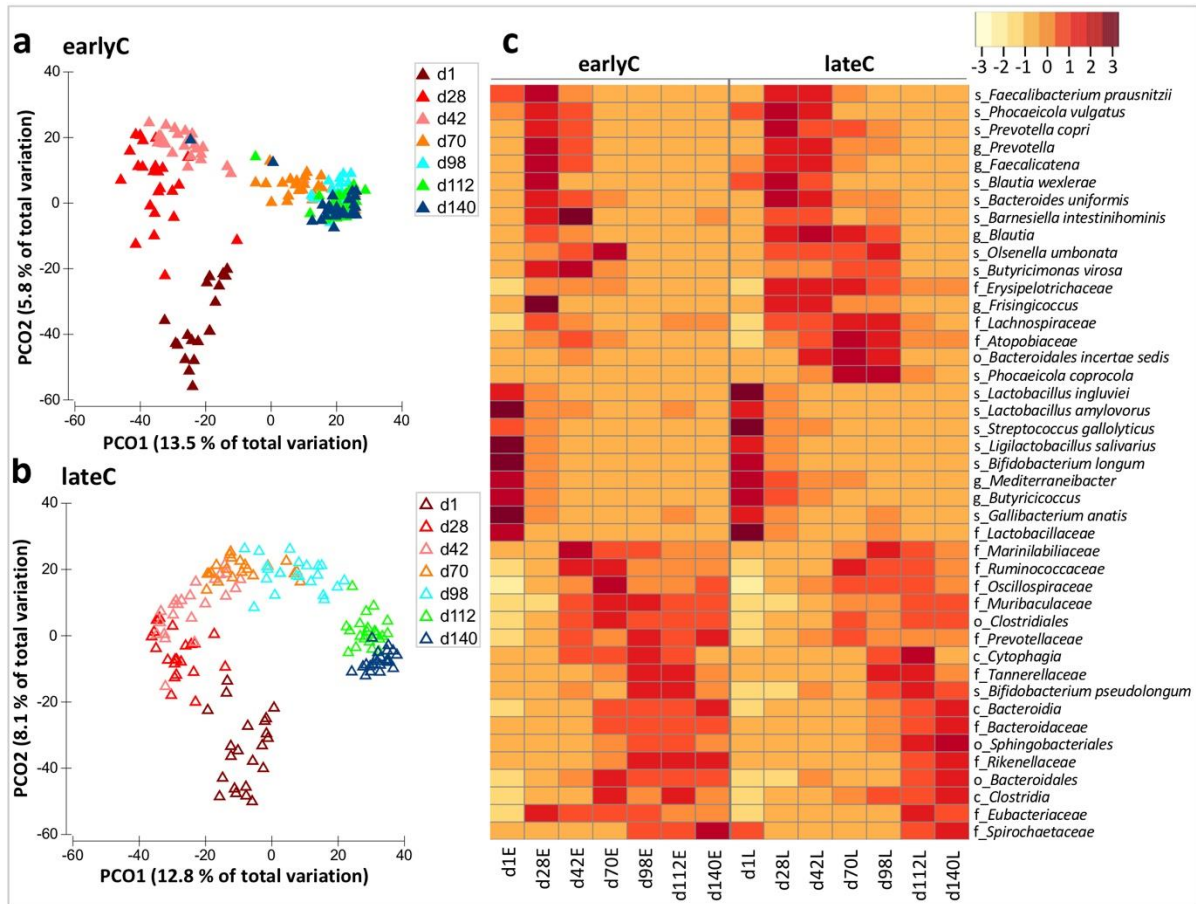
Figure 13 | Age-dependent changes in the faecal bacterial communities of earlyC and lateC calves. (a, b) Bacterial compositional profiles of different age group earlyC ($n = 176$) and lateC ($n = 154$) faecal samples based on ASVs visualized using principal-coordinate analysis plots. Each triangle indicates one sample. (c) Significantly different bacterial species ($p < 0.05$; Kruskal-Wallis test). Each day represents an average value for animals: d1 (20 & 22), d28 (24 & 21), d42 (25 & 23), d70 (26 & 21), d98 (27 & 22), d112 (27 & 23), and d140 (27 & 22) animals for earlyC and lateC groups, respectively.

At species-level, the earliest time point (d1) had significantly higher abundances of *Bifidobacterium longum*, *Gallibacterium anatis*, *Lactobacillus amylovorus, Lactobacillus ingluviei*, *Ligilactobacillus salivarius*, *Streptococcus gallolyticus,* unclassified (uncl.) *Butyricicoccus,* uncl. *Lactobacillaceae,* and uncl. *Mediterraneibacter,* showing significant decrease in abundance with age (d1−d140) in both weaning groups. In addition, *Bacteroides uniformis, Barnesiella intestinihominis*, *Blautia wexlerae*, *Faecalibacterium prausnitzii*,

Phocaeicola vulgatus, Prevotella copri, uncl. *Faecalicatena*, and uncl. *Prevotella* were significantly more abundant during days 28−42 and less abundant during later time points. On the contrary, *Bifidobacterium pseudolongum*, uncl. *Bacteroidia*, uncl. *Bacteroidales*, uncl. *Bacteroidaceae*, uncl. *Clostridia*, uncl. *Clostridiales*, uncl. *Eubacteriaceae*, uncl. *Muribaculaceae*, uncl. *Oscillospiraceae*, uncl. *Prevotellaceae,* uncl. *Ruminococcaceae,* uncl. *Rikenellaceae,* uncl. *Sphingobacteriales,* and uncl. *Tannerellaceae* were less abundant during early time points and showed a significant increase with age (Figure 13C, Additional file 2: Table S10).

4.3.2 Weaning-dependent modifications in the faecal bacterial composition and their predicted function in calves

In addition to the age-related maturation, the time point of weaning also significantly influenced the faecal bacterial compositional profiles as indicated by the separate clustering of weaning groups during days 42−112. In contrast, no significant difference was detected before or after this period (Figure 14).

Both weaning groups had distinct bacterial taxonomic compositions during days 42−98 (Figure 15, Additional file 2: Table S10). Early-weaning at seven weeks triggered an increase in the relative abundance of *Bacteroidetes* and a decrease of *Firmicutes* (Additional file 1: Figure S9B) during days 42−98 ($p < 0.05$). At genus-level, earlyC calves had significantly higher abundances of *Butyricimonas* and certain unclassified members of *Bacteroidetes*, *Firmicutes*, as well as *Spirochaetes* (Figure 15, Additional file 2: Table S10).

Early-weaning also significantly decreased the abundances of potential lactose- and starch-degraders as well as potential butyrate-producing bacteria including *Faecalibacterium*, *Blautia, Prevotella, Bacteroides, Parabacteroides, Butyricimonas, Olsenella*, *Anaerostipes, Streptococcus, Frisingicoccus, Phocaeicola, Mediterraneibacter,* uncl. *Atopobiaceae,* uncl. *Bacteroidales incertae sedis,* and uncl. *Lachnospiraceae.* In addition, the abundance of potential pathogenic bacteria, such as *Collinsella,* was reduced due to the weaning event in the earlyC group (Figure 15, Additional file 2: Table S10).
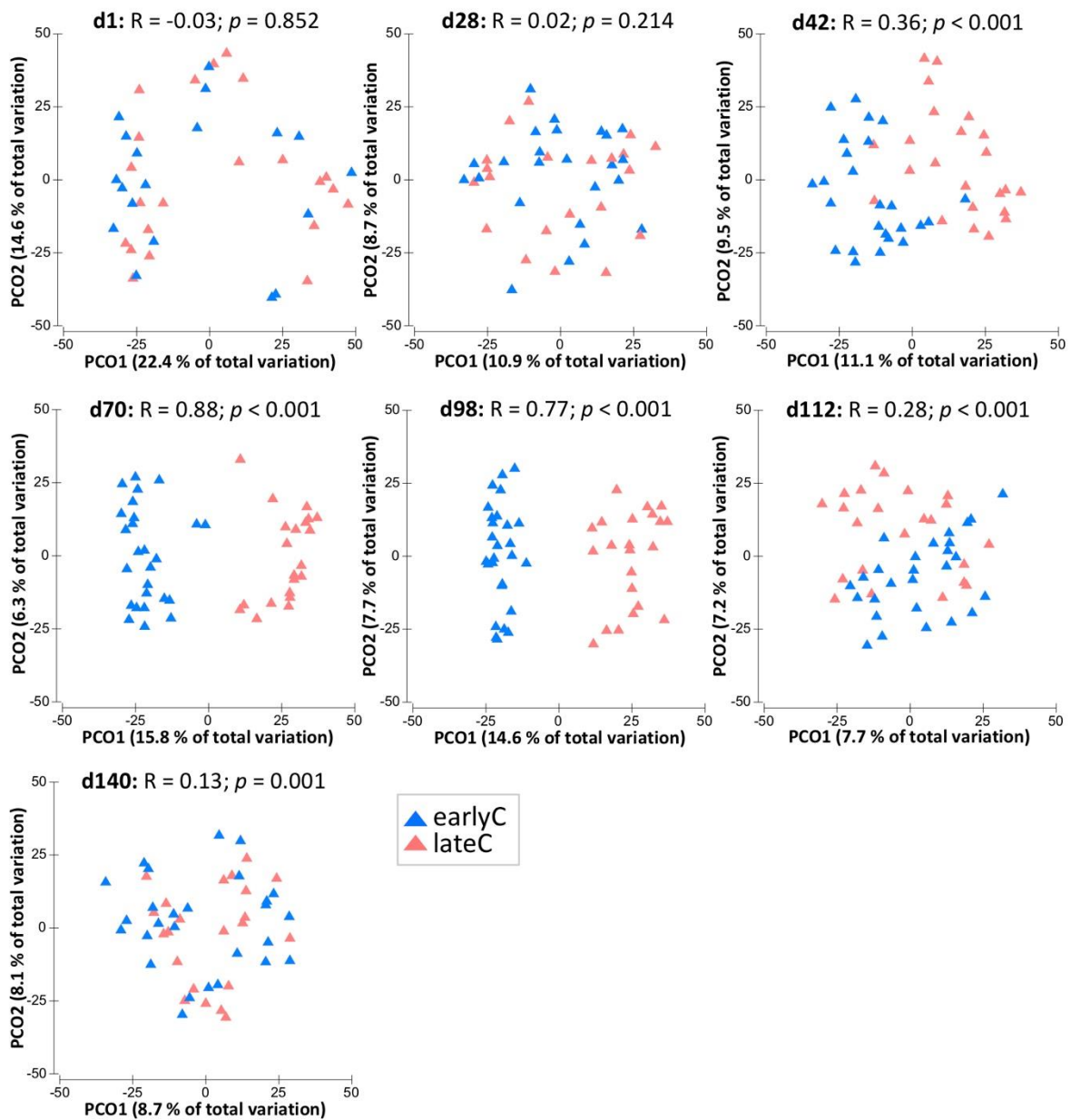
Figure 14 | Principal-coordinate analysis plots showing changes in bacterial compositional profiles of faecal samples due to weaning event. Each triangle indicates one sample. The significant differences between same-age-old weaning groups, separated based on PCO analysis, were confirmed using analysis of similarities test (ANOSIM), with R- and p-values indicated.
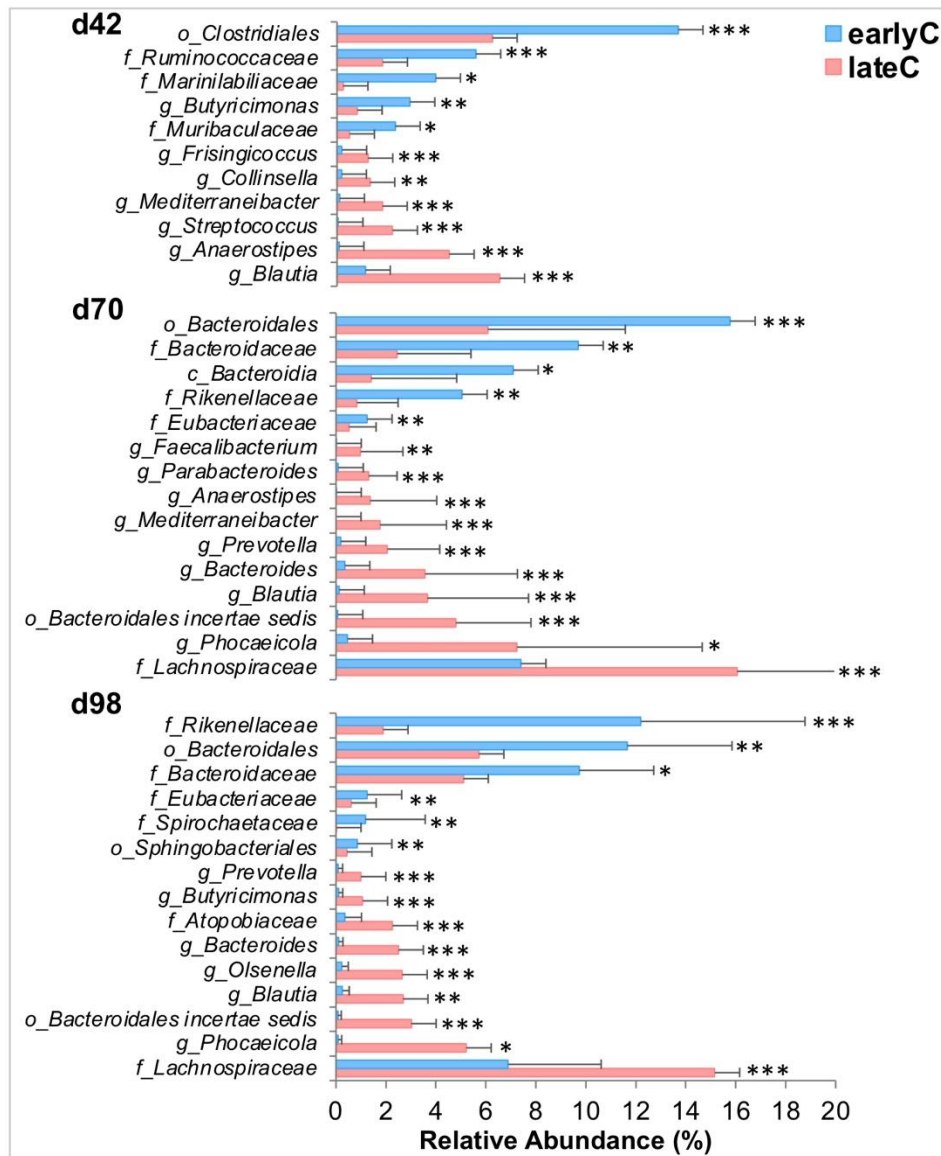
Figure 15 | Changes in faecal bacterial communities of calves due to weaning event. Significantly different bacterial genera with relative abundance (≥ 1%) and $p ≤ 0.05$ (Kruskal-Wallis test) among same-age old weaning groups are shown. Each bar represents an average value for animals: d42 (25 & 23), d70 (26 & 21), d98 (27 & 22) animals for earlyC and lateC groups, respectively.

CowPI-based predictive analysis showed a significant enrichment of function with particular involvement in the metabolism of amino acid, carbohydrate, energy and nucleotide, and glycan biosynthesis in the earlyC group (days 42–98) (Additional file 1: Figure S10). In contrast, a significant reduction in some of the general metabolic functions with essential role

in microbial survival such as protein kinases, ABC transporters, two-component system, transcription factors, and other ion-coupled transporters were also predicted in the earlyC group corresponding to the weaning event.

4.3.3 Plasma metabolome and the impact of calves' age

The differences between the plasma metabolic profiles of calves from different age groups were shown by a supervised partial least square discriminant analysis (PLS-DA), that resulted in clear age-dependent clustering for both weaning groups (Figure 16A, B). Metabolites showing significant difference due to the age of the calves were selected based on the variable importance in the projection (VIP) threshold > 1 and a false discovery rate (FDR) < 0.001 (ANOVA) (Figure 16C, Additional file 2: Table S11). The plasma concentrations of most of the metabolites including AAs, BAs, ACs, and SMs were affected by both calves age and the time of weaning (Figure 16C).
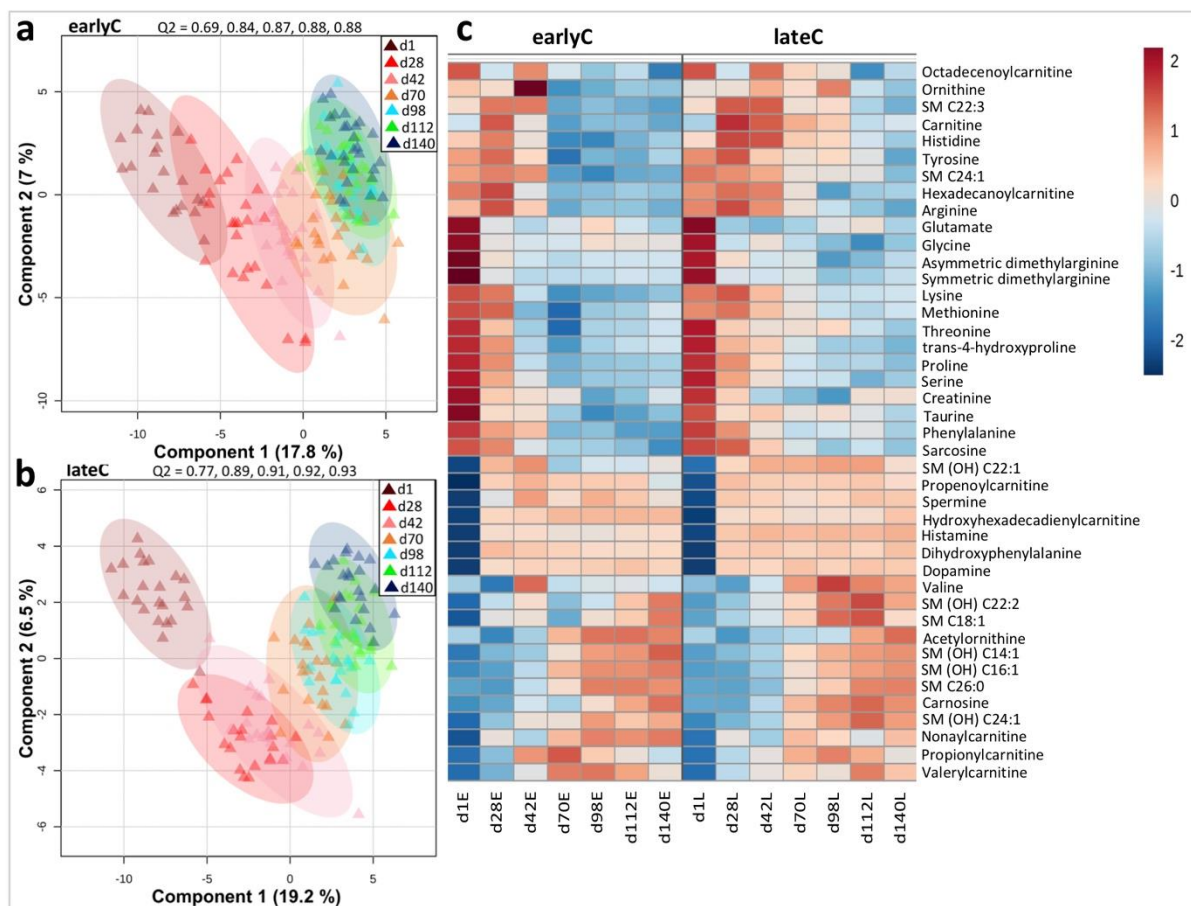
Figure 16 | Age-dependent changes in plasma metabolites concentrations of earlyC and lateC calves. (a, b) Metabolic profiles of different age group earlyC ($n = 174$) and lateC ($n = 153$)

plasma samples visualized using PLS-DA score plots. Each shape indicates one sample coloured according to the age group with ellipse indicating the 95% confidence region. (c) Heatmap of the significantly altered metabolites due to calves age (VIP > 1, FDR < 0.001, ANOVA). Each day represents an average concentration of metabolites for animals: d1 (20 & 22), d28 (24 & 21), d42 (24 & 23), d70 (25 & 21), d98 (27 & 21), d112 (27 & 23), and d140 (27 & 22) animals for earlyC and lateC groups, respectively.

In both weaning groups, a significant age-dependent decrease in the concentrations of AAs (arginine, lysine, methionine, phenylalanine, threonine, proline, serine, tyrosine, glutamate, glycine, and histidine), BAs (taurine, trans-4-hydroxyproline, creatinine, sarcosine, asymmetric dimethylarginine, and symmetric dimethylarginine), AC (carnitine) and SM (SM C24:1) was observed. However, as the calves aged and became more mature (days 70−140), the plasma concentrations of BAs (carnosine, acetylornithine, dopamine, spermine, histamine, and dihydroxyphenylalanine), ACs (hydroxyhexadecadienylcarnitine, and valerylcarnitine), and most of the sphingomyelins (SM (OH) C14:1, SM (OH) C16:1, SM (OH) C22:1, SM (OH) C22:2, SM (OH) C24:1, SM C18:1, SM C26:0) were increased (Figure 16C).

4.3.4 Weaning-dependent modifications in the plasma metabolome of calves

Similar to the weaning-related shifts in the faecal microbial profiles, the supervised PLS-DA showed clear separation among metabolic profiles of earlyC and lateC calves during days 42−112 (Figure 17). The identification of metabolites altered due to the weaning event within each age group was based on a VIP > 1, FDR < 0.05 (*t*-test) and log2 FC > 0.1 or < -0.1. Mother's parity showed no significant influence on DMs within each weaning group (earlyC PC *vs.* earlyC MC and lateC PC *vs.* lateC MC). A total of 10, 32, 32, and 18 significantly differential metabolites (DMs) were identified between earlyC and lateC groups at days 42, 70, 98, and 112, respectively. During days 42–112, the relative concentrations of 2, 5, 8 and 3 metabolites were significantly higher in the plasma of earlyC calves, and the relative concentrations of 8, 27, 24, and 15 metabolites were significantly higher in the plasma of lateC calves (Figure 18).
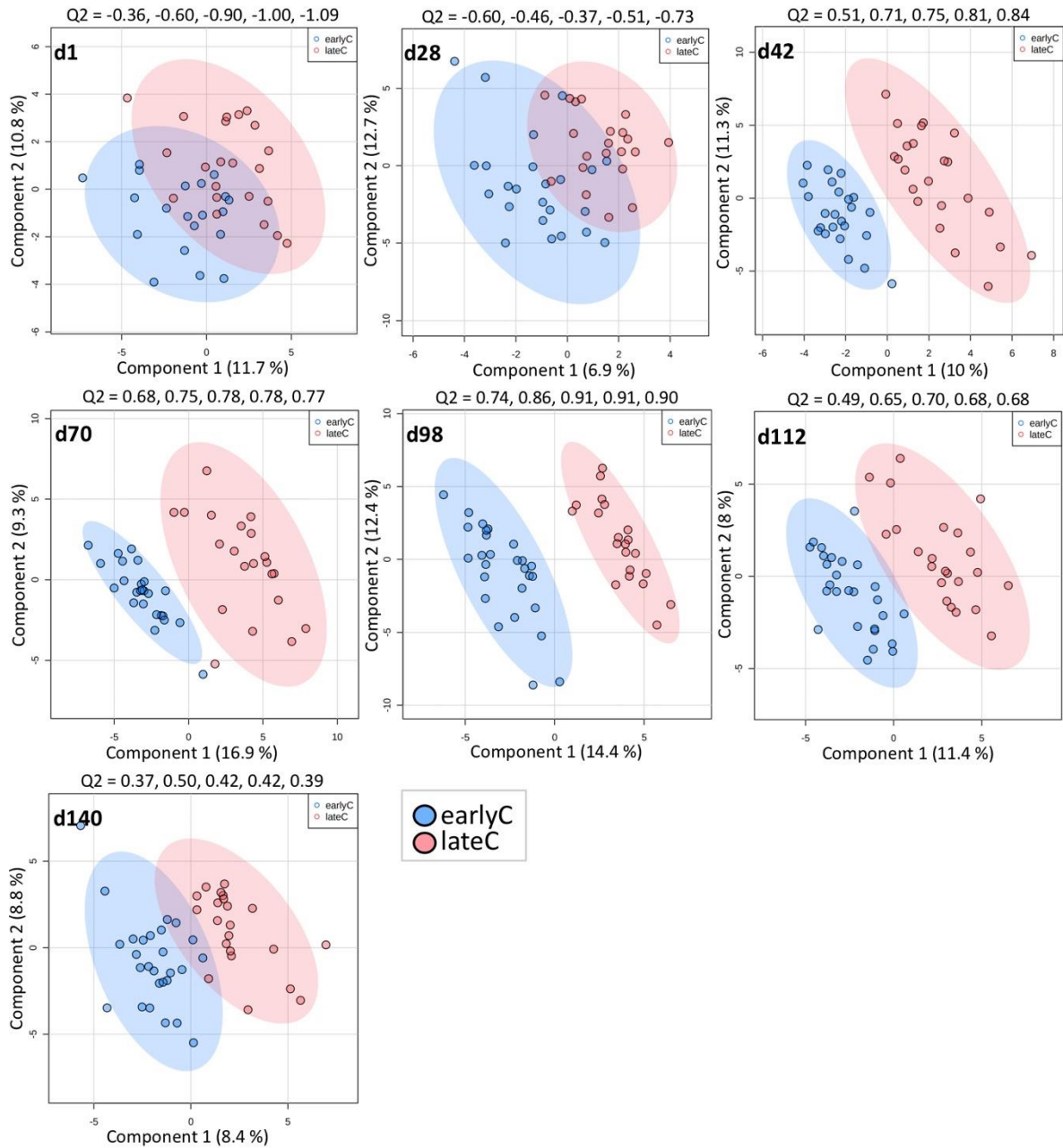
Figure 17 | Partial least squares-discriminate analysis for identification of metabolic differences among weaning groups. Each circle indicates one sample and ellipse indicating the 95% confidence region. The quality of the models was assessed using Q2 as performance measure and tenfold cross-validation method. The Q2 values for the first 5 components are shown.
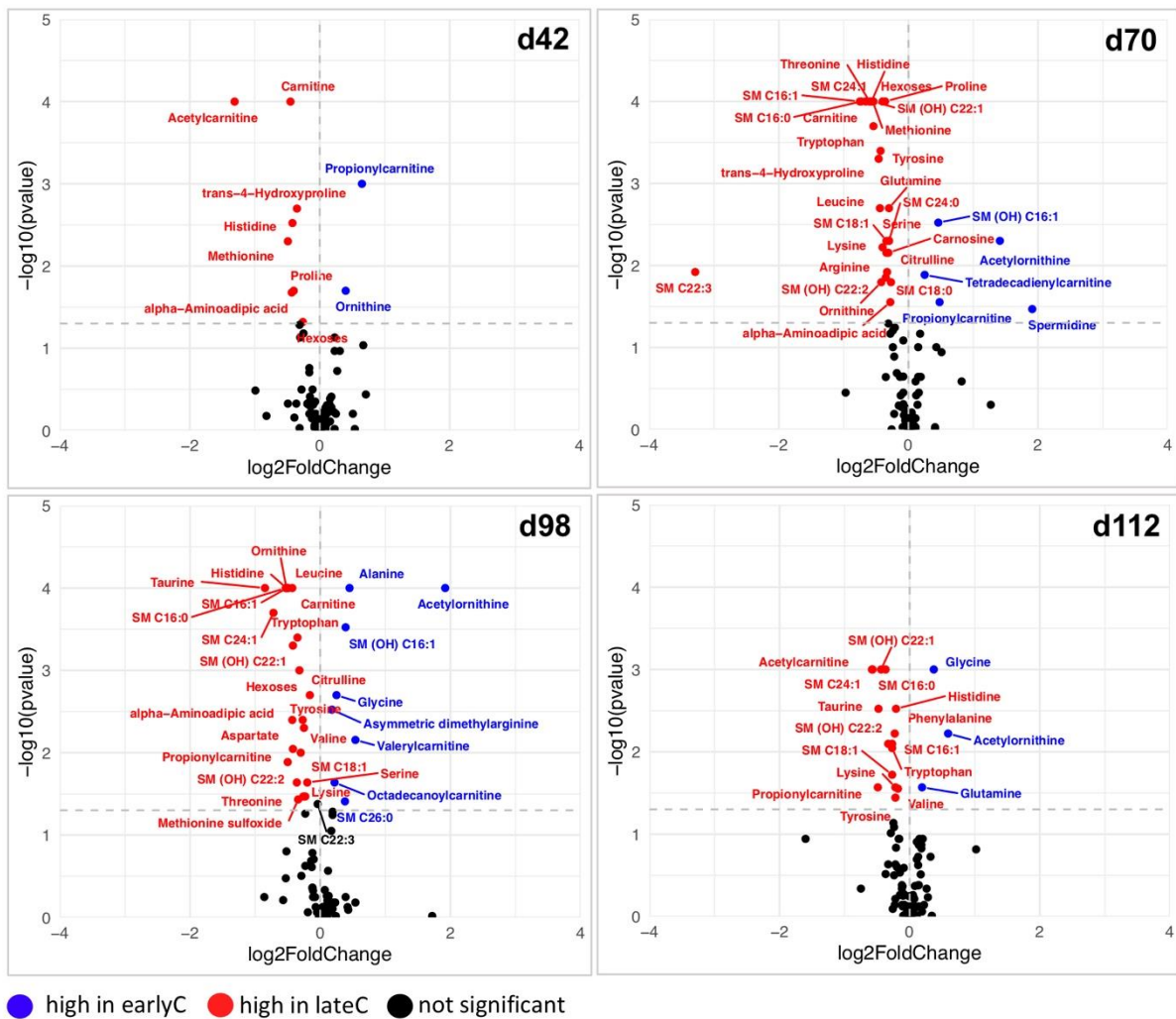
Figure 18 | Volcano plots of the weaning-dependent changes in the plasma metabolic profiles of weaning groups. The identification of significantly altered metabolites due to weaning event within each age group was based on a VIP > 1, FDR < 0.05 (*t*-test) and log2 FC > 0.1 or < -0.1. Each circle indicates one metabolite.

In general, earlyC calves had significantly lower concentrations of most of the essential amino acids (EAAs; arginine, histidine, leucine, lysine, methionine, phenylalanine, valine, threonine, tryptophan), and non-essential amino acids (NEAAs; aspartate, glutamine, proline, serine, tyrosine, citrulline, and ornithine), BAs (taurine, trans-4-hydroxyproline alpha-aminoadipic acid, carnosine, and methionine sulfoxide), ACs (carnitine, acetylcarnitine, and propionylcarnitine), and SMs (SM (OH) C22:1, SM (OH) C22:2, SM C16:0, SM C16:1, SM C18:0, SM C18:1, SM C22:3, SM C24:0, SM C24:1) as compared to the same-day-old lateC group (days 42–112; Figure 18). The ratio between kynurenine/tryptophan was lower at day 70

and 98 (Additional file 1: Figure S11) in the lateC group. Similar to the microbiome dataset, no significant differences between metabolic profiles of weaning groups were observed during days 1–28, but the plasma samples of 112 days old earlyC and lateC calves showed a large number of DMs. A metabolic pathway analysis (MetPA) was done using DMs identified between the weaning groups. The enrichment of 5 (d42), 12 (d70), 13 (d98), and 9 (d112) pathways mainly related to AAs metabolism was shown to be significantly different between the weaning groups (Additional file 1: Figure S12, pathway impact $\geq$ 0.1, FDR < 0.01).

4.3.5 Associations between differential faecal microbial genera and plasma metabolites of weaning groups

To identify the weaning-dependent shifts in the potential host-microbe metabolic interactions, Spearman's rank correlations were calculated between the differentially abundant faecal microbial genera and plasma metabolites of weaning groups, separately for each time point (Figure 19). The potential lactose- and starch-degrading bacterial genera that were reduced by the early-weaning events during days 42–98 were strongly positively correlated (R > 0.50, p < 0.05) with the plasma concentrations of AAs, BAs and SMs. Aspartate was positively correlated with *Butyricimonas,* histidine with *Frisingicoccus, Blautia, Bacteroides, Prevotella, Mediterraneibacter, Anaerostipes, Parabacteroides, Butyricimonas* and *Olsenella,* methionine and proline with *Blautia, Mediterraneibacter* and *Parabacteroides,* leucine and ornithine with *Parabacteroides* and *Butyricimonas,* threonine, tryptophan and tyrosine with *Bacteroides* and *Parabacteroides,* leucine with *Olsenella,* and threonine *with Mediterraneibacter.* Similar positive correlations were observed between the plasma concentrations of BAs such as alpha-aminoadipic acid with *Frisingicoccus,* taurine with *Bacteroides, Butyricimonas* and *Olsenella*, trans-4-hydroxyproline with *Frisingicoccus, Blautia, Mediterraneibacter* and *Anaerostipes.* The plasma SMs concentrations were positively correlated with bacterial abundances; SM (OH) C22:1 with *Blautia, Mediterraneibacter* and *Butyricimonas*, SM (OH) C22:2 with *Butyricimonas,* SM C24:1 with *Mediterraneibacter, Parabacteroides, Blautia* and *Butyricimonas,* SM C16:0 with *Blautia, Prevotella, Mediterraneibacter, Anaerostipes, Parabacteroides, Bacteroides* and *Butyricimonas,* and SM C16:1 with *Blautia, Mediterraneibacter, Bacteroides* and *Butyricimonas* (Figure 19).
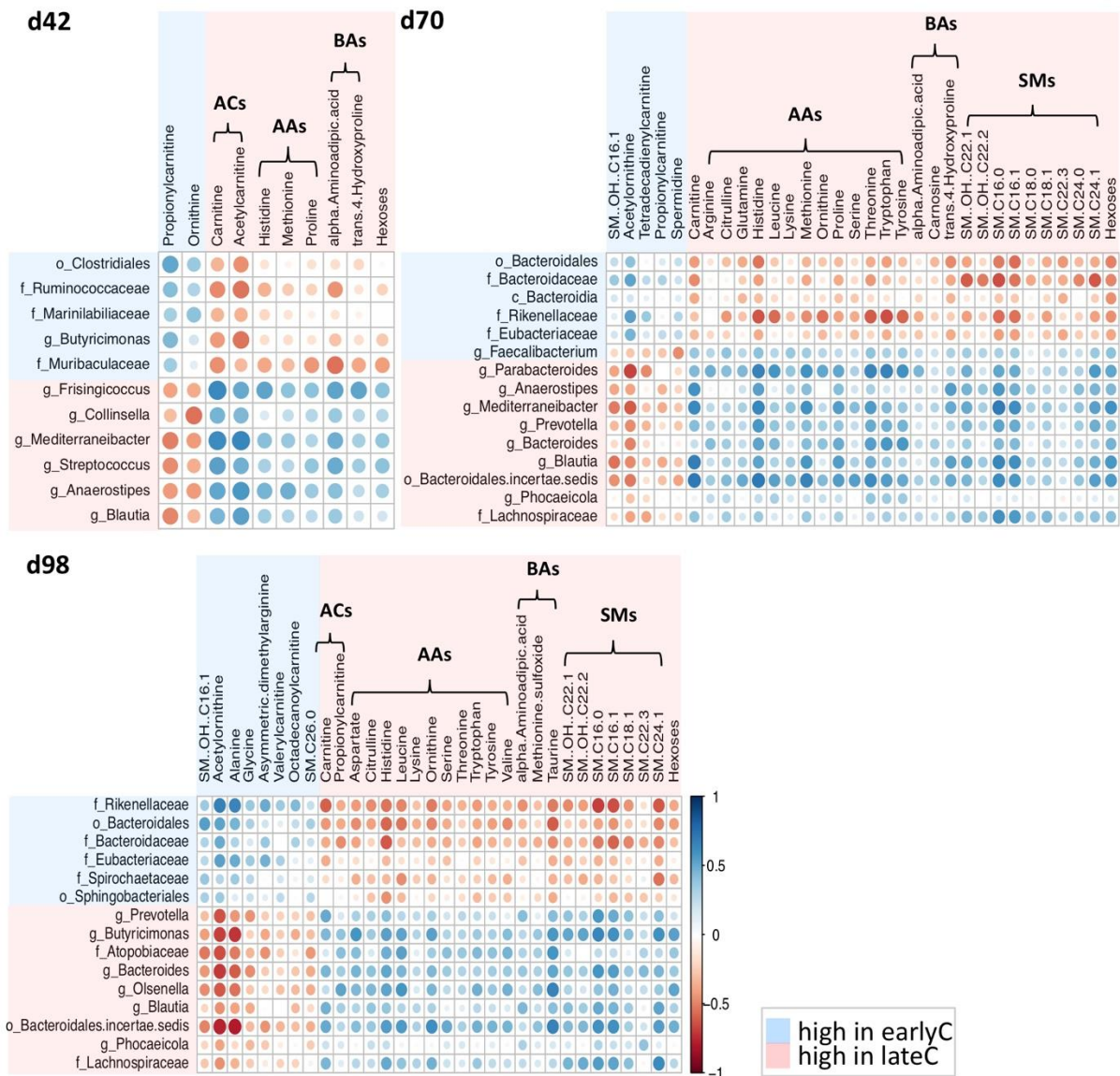
Figure 19 | Heatmaps showing the Spearman's rank correlations between differentially abundant faecal microbial genera and plasma metabolites of weaning groups. Colours indicates the correlation between microbiome and metabolome (blue: significant positive, red: significant negative, and white: non-significant). Only Spearman correlation coefficients with $p < 0.05$ are shown. Abbreviations (ACs, AAs, BAs and SMs) indicates following metabolites classes: acylcarnitines, amino acids, biogenic amines, and sphingomyelins respectively.

In addition, the genera that were significantly higher in abundance in the earlyC group during days 42–98 were also strongly positively correlated to the following plasma metabolites: uncl. *Rikenellaceae* with alanine, uncl. *Clostridiales* with propionylcarnitine, uncl. *Bacteroidales, Bacteroidaceae, Rikenellaceae* and *Eubacteriaceae* with acetylornithine, and

uncl. *Bacteroidales* with SM (OH) C16:1 (R > 0.50, *p* < 0.05). Furthermore, few strong negative correlations also existed between uncl. *Bacteroidales* with histidine, leucine, taurine, SM C16:0, and SM C16:1, uncl. *Bacteroidaceae* with SMs (SM (OH) C22:1, SM (OH) C22:2, SM C16:0, SM C16:1, and SM C24:1) as well as with AA (histidine), uncl. *Muribaculaceae* with alpha-aminoadipic acid, uncl. *Rikenellaceae* with carnitine, AAs (histidine, leucine, ornithine, threonine, tryptophan, tyrosine), taurine, and SMs (SM C16:0, SM C16:1, and SM C24:1), uncl. *Ruminococcaceae* and *Butyricimonas* with acetylcarnitine, uncl. *Spirochaetaceae* with SM C24:1 (R < -0.50, *p* < 0.05).

4.3.6 Associations between morphometric variables of calves, differential faecal microbial genera and plasma metabolites of weaning groups

Live weight (LW), live weight gain (LWG) or average daily gain (ADG), and morphometric variables such as withers height, hip height, back length, heart girth and body length increased with age (*p* < 0.001) and were higher in the lateC group [22]. The data were checked for strong positive (R > 0.50) or strong negative correlations (R < -0.50; *p* < 0.05, Additional file 2: Table S12) with microbiome and metabolome data. LWG or ADG was significantly higher for lateC group from days 42–98, showing strong positive correlations with the abundances of *Mediterraneibacter, Parabacteroides, Prevotella, Blautia,* uncl. *Bacteroidales incertae sedis*, uncl. *Lachnospiraceae* (d70), and *Olsenella* (d98), as well as the plasma concentrations of threonine, tryptophan, tyrosine, histidine, methionine, proline, carnitine, hexoses, SM C16:0, and SM C16:1 (d70), while strong negative correlation with uncl. *Rikenellaceae* abundance and acetylornithine concentration (d70) were observed. LateC group had significantly higher LW from days 70–140, which was strongly positively correlated with uncl. *Bacteroidales incertae sedis* (d70 and d98), uncl. *Atopobiaceae* (d98), and plasma concentrations of methionine, serine, trans-4-hydroxyproline, and carnitine (d70), tryptophan, tyrosine, valine, leucine, ornithine, taurine, hexoses, SM C24:1, SM C16:0, SM C16:1 (d98), and threonine (d70 and d98), and negatively correlated with uncl. *Rikenellaceae* (d98), spermidine (d70) and acetylornithine (d98). Hip height was significantly different between the weaning groups only during days 70 and 140, and positively correlated with the abundances of *Blautia, Mediterraneibacter, Prevotella* and uncl. *Bacteroidales incertae sedis*, and plasma concentrations of lysine, threonine, histidine, methionine, serine and carnitine, while negatively correlated with spermidine and SM (OH) C16:1 (d70). Heart girth was greater for lateC group from days 98 onwards and had a strong positive association with the abundance of unclassified *Bacteroidales incertae sedis*, plasma concentration of tryptophan, valine, leucine,

ornithine, SM C24:1, SM C16:0 and SM C16:1, and strong negative association with uncl. *Rikenellaceae* abundance and acetylornithine (d98).

4.4 Discussion

This study examined the age- and weaning-dependent changes in the calves' faecal microbiome, plasma metabolome and explained the potential host-microbe associations. We showed an age-dependent increase in the faecal bacterial alpha-diversity as reported in other studies [23, 24], which might have assisted GIT development and liquid to solid diet transition post-weaning [25]. At species-level, the faecal bacterial community of young calves was dominated by potential lactose- and starch-degrading bacteria, which was replaced by potential fiber-degrading bacteria with age. A similar age-related decrease in the abundances of *Bifidobacterium*, *Lactobacillus,* and *Faecalibacterium* [25, 26], and an increase in fiber-degrading *Ruminococcus* was recently reported [25]. Bifidobacteria can utilize carbohydrates freely available in the pre-weaned calf GIT [27] and are usually isolated from faecal samples of new born calves, and young ruminants [27-29]. Similarly, *F. prausnitzii* was found in faecal samples of 3–4-week-old calves, showing an age-dependent decrease in abundance as observed in our study [30]. The high abundance of *F. prausnitzii* has also been linked with increased weight gain and lower incidence of diarrhoea in dairy heifers and Holstein calves during the pre-weaning period [23, 31]. We also reported an age-dependent decrease in certain potential pathogenic bacteria such as *Streptococcus gallolyticus,* found in newborn calves with purulent lesions and meningitis [32], and *Gallibacterium anatis,* isolated from cattle with respiratory diseases [33], indicating an age-dependent maturation of the immune system in calves.

In addition to the age, the time at which animals were weaned (7 or 17 weeks of age) had an important role in shaping their gut microbial communities. The major differences between the bacterial compositions of weaning groups were observed during days 42–98. The earlyC group was characterized by a significantly higher abundance of phylum *Bacteroidetes* and potential fiber-degrading bacteria. In contrast, the lateC group was dominated by *Firmicutes* and potential lactose- and starch-degraders. The differential bacterial composition of weaning groups during days 42–98 was due to their different feed intake pattern as described previously [22]. During day 42, lateC group had higher milk replacer (MR) intake, while earlyC had higher roughage and concentrate (C) intake. However, during days 70–98, earlyC group was characterized by a total mixed ration (TMR) feeding pattern, while, the lateC group still consumed substantial amounts of MR and C. Castro and colleagues suggested that the increase in MR intake may result in higher lactose flux in the hindgut, serving as a prebiotic and a

growth substrate for certain beneficial microorganisms [34]. In accordance with this study, the faecal microbiota of lateC calves (days 42–98) had high dominance of *Bacteroides, Prevotella, Faecalibacterium*, *Butyricimonas, Blautia*, and *Olsenella*. Few other studies have reported an increased dominance of *Bacteroides, Prevotella, Faecalibacterium* and *Blautia* in MR-fed pre-weaned calves' faeces [35]. Likewise, a positive association between MR intake and faecal *Blautia* abundance in pre-weaned calves [36] and a negative association between dietary forage inclusion and faecal *Bacteroides, Olsenella* abundances have been reported [37, 38]. The high abundance of *Bacteroides*, *Faecalibacterium,* and *Butyricimonas* has also been linked with lower disease susceptibility in calves [39, 40]. Thus, it can be speculated that the decrease in the abundances of major lactic-acid producing bacteria with age and after day 42 in the earlyC group was due to their increased fiber ingestion and the decreased milk consumption, resulting in limited nutrient availability for the growth of potential lactose- and starch-degrading microorganisms. In addition to the beneficial microorganism, we also observed a significantly higher abundance of pathogenic bacterial genus *Collinsella* in 42-day-old lateC calves' faeces. This bacterial genus reduces the expression of tight junctions and increases intestinal permeability, resulting in gut leakage and pro-inflammatory dysbiosis [41, 42]. Their abundance was linked with host dietary intake, such as higher abundance in MR-fed calves' faeces [43] and lower abundance with fiber-rich diet [44]. Thus, the low abundance of *Collinsella* in 42-day-old earlyC group in our study was probably due to the introduction of roughages in their post-weaning diet. Moreover, no significant differences in the bacterial composition of the weaning groups were observed during later time points (days 112−140) indicating a rapid adaptation of the lateC microbiome to the weaning-related dietary changes without causing dysbiosis.

Besides the differences described above, the plasma metabolic profiles of calves also showed age- and weaning-dependent modifications. The plasma samples of young calves (days 1–28) had high concentrations of most of the AAs, but their concentrations declined with age and after weaning event in the earlyC group. The plasma AAs concentrations are dependent on many factors such as synthesis and breakdown of proteins, and it is known that highly digestible milk protein levels lead to an improved AAs absorption which results in higher blood levels [45]. A high plasma concentration of EAAs and NEAAs was observed after MR-feeding in Holstein bull calves [46]. Similarly, feeding a high amount of milk during the pre-weaning period increased the levels of plasma arginine and lysine in Holstein heifer calves [47], suggesting that the liquid diet could provide specific metabolites that can be transported into the bloodstream through GIT [48]. In ruminants, depending on the stage of development,

digestion and fermentation takes place in different sections of the GIT. Neonatal ruminants mainly rely on their hindgut for digestion of feed and metabolites synthesis [49], this restricts the absorption of certain metabolites as the absorption capacity in the colon is limited. With the development of the rumen, the major microbial activity is located in the forestomach and the microbial metabolites are absorbed through the epithelium of the rumen or the lower GIT and supply energy to the host [50, 51]. Therefore, a lower level of plasma metabolites at the early life of a ruminant is true to the fact of the limited absorption capacities in the hindgut and have to be considered for the interpretation.

Not only the plasma AAs concentrations were affected, but we also observed distinct profiles of BAs at different developmental stages. The early-weaning event lowered the concentrations of certain BAs (taurine, trans-4-hydroxyproline, alpha-aminoadipic acid, carnosine, and methionine sulfoxide) as well as plasma ACs (carnitine, acetylcarnitine, and propionylcarnitine) compared to the late-weaning event. The difference in plasma BAs and ACs concentrations of weaning groups was probably due to their different dietary composition as the carbohydrates rich diet may result in higher levels of BAs [52]. A high concentration of serum taurine was observed in high-grain fed dairy cows [53]. A decreased level of plasma acylcarnitines was observed after feeding calves with a limited amount of MR in another study [2]. Similar to the AAs, BAs and ACs, the plasma concentrations of most of the SMs were also lower in the earlyC compared to the lateC group. The functional aspects of the changed sphingomyelin profile in calves are still unclear, however, lower concentrations of blood SMs (SM OH C14:1 and SM OH C16:1) were linked with metabolic stress in periparturient cows [54]. It may be assumed that the lower level of plasma SMs in earlyC calves was probably due to the stressful weaning event as the animals were not fully matured and sudden dietary changes might have resulted in quick transitioning from a non-ruminant to a pre-ruminant. Contrary to the microbiome dataset that had no significant differences between samples of 112-day-old early- and late-weaned calves, the plasma revealed several metabolites with differential concentrations, suggesting that the weaning related-dietary changes had less abrupt but more-persistent impact on host metabolism compared to the microbiome.

The associations between the faecal microbial genera, plasma metabolites and calf growth parameters were assessed during the weaning event to track the weaning-dependent modifications in the potential host-microbe metabolic interactions. LWG or ADG was higher in the lateC group during days 42–98 and correlated with the faecal abundances of *Parabacteroides, Blautia*, *Mediterraneibacter, Olsenella, Prevotella,* and the plasma concentrations of histidine, threonine, tryptophan, tyrosine, methionine, proline, carnitine,

hexoses, SM C16:0, and SM C16:1. High abundances of *Blautia* and *P. copri* were observed in steers with high ADG [55] and a positive correlation between *Blautia*, *Prevotella* abundances and ADG was recently reported [36, 56], indicating the importance of these bacterial group for ruminants. The early-weaning event not only decreased the LWG or ADG, but the plasma concentrations of most of the AAs, BAs and SMs as well as the abundances of several potential lactose- and starch-degrading bacteria were reduced. Plasma AAs are essential for health and an alteration in their concentrations may result in immune responses and inflammation. Proline possesses antioxidant properties and protects against reactive oxygen species [57]. Leucine involvement in tissues and cells protein synthesis was previously reported in pigs and mice [58, 59]. Tryptophan and its degradation product kynurenine are used as indicators for low-grade chronic inflammation in humans [60]. Here, lateC animals had lower ratio at d70 and d98, which is indicates a possible increased inflammatory status of the earlyC animals during this time period and matches to previous findings reporting a lower kynurenine/tryptophan in healthy dairy cows [19]. The lower plasma levels of arginine, glutamine, methionine, histidine have been linked with the increased incidence of diarrhoea in calves [61]. Our study reported that the weaning event affected the predicted AAs metabolic pathways, specifically during days 42–98. At the same time, higher plasma concentrations of histidine, threonine, tryptophan, and tyrosine were measured in the lateC group. These AAs were positively correlated with the abundances of *Bacteroides* and *Parabacteroides*. Similar trends were observed with methionine, proline, and histidine concentrations that were positively correlated with *Blautia* abundance, while the concentrations of leucine, ornithine, methionine, and proline, were positively associated with the *Parabacteroides*. *Bacteroides* members are essential for AAs metabolism in the large intestine [62]. Similarly, *Parabacteroides*, which was assigned to the *Bacteroides* genus prior to reclassification in 2006 [63], also produces a wide range of AAs such as alanine, glutamate, histidine, isoleucine, lysine, methionine, phenylalanine, proline, and valine [64]. A recent study also reported the significant correlation of *Bacteroides* and *Blautia* abundances with the faecal metabolites involved in AAs metabolism (proline, and leucine) [65]. *Butyricimonas* abundance was relatively higher in the lateC group at day 98 and it was positively correlated with plasma aspartate concentration. Similar positive association between *Butyricimonas* and N-acetylaspartate was reported in young pigs [66]. The lower plasma AA levels and their associations with diet-related diminished abundance of AAs producing bacteria in the earlyC group is understandable. However, the identification of the causal relationships of the observed

correlations are challenging as the plasma AA concentrations are not only determined by diet but also strongly by liver and muscle metabolisms which are yet to be explored.

In addition to the AAs, the plasma concentration of taurine and faecal *Bacteroides* abundance was significantly higher in the lateC group during day 98 and were positively correlated with each other. Similar to our study, a high dominance of *Bacteroides* in MR-fed pre-weaned calves' faeces [35] and its negative association with dietary forage inclusion have previously been reported [38]. Taurine can be derived directly from the diet, absorbed through the epithelial cells and transported to the blood [17]. A significant increase in serum taurine concentration was reported with high-grain feeding in dairy cows [53]. However, endogenous synthesis of taurine from methionine and cysteine majorly takes place in liver and tissues [67]. Taurine is released into the gut as conjugated bile salts [68], where it is deconjugated by bacterial bile salt hydrolases (BSH) [69], expressed by several member of *Bacteroides* (*B. vulgatus* and *B. uniformis*) [70]. This process increases the concentrations of bile salts and taurine in the lower digestive tract [71], which can further be absorbed from the distal ileum and transported to the blood as reported in recent human study [17]. Taurine plays an essential role in regulation of gut micro-ecology through inhibition of potential pathogenic bacteria, reduction of lipopolysaccharides concentrations and acceleration of SCFA synthesis [72]. The association of plasma taurine concentration with liver functionality has previously been reported in cows [73]. This confirms our previous findings reporting lower liver cholesterol production to compensate weaning-related dietary lack in earlyC group as compared to the lateC group [22]. Thus, it can be speculated that the weaning-dependent addition of dietary roughages might have resulted in lower availability of dietary taurine, reduced abundances of bile salt hydrolysing bacterial genera and the resultant lower absorption of taurine from the gut due to insufficient BSH activity in the 98-day-old earlyC group.

In addition, the plasma concentrations of several SMs (SM (OH) C22:1, SM (OH) C22:2, SM C24:1, SM C16:0, SM C16:1) were significantly higher in the lateC group and positively associated with the abundances of *Bacteroides*, *Parabacteroides, Prevotella, Anaerostipes, Blautia, Butyricimonas,* and *Mediterraneibacter* during days 70–98. *Bacteroides, Parabacteroides,* and *Prevotella* are sphingolipids (SLs)-producing bacterial genera [74]. *Bacteroides* are among the few bacteria that can synthesize SLs and utilize them to survive in the stressful intestinal environment [75]. The *Bacteroides* members produce SLs-rich outer membrane vesicles (OMVs) [76], which are described to penetrate the intestinal mucosa and exert immune-related effects on the host [77]. Some recent studies reported the possible processing of *Bacteroides*-SLs via mammalian SL pathways [78] and the utilization

of bacteria-derived SLs during food deprivation periods [74]. Thus, the higher abundance of SLs-producing bacteria in the lateC group might be one of the contributing factors towards their higher plasma SMs concentrations. Hence, a change in the composition of faecal microbiome and plasma metabolic profiles over the course of development, the higher abundances of several beneficial bacterial genera in lateC group and their positive association with AAs, BAs and SMs concentrations suggesting that the gut microbial colonization might play a certain role in this phenomenon.

4.5   Conclusion

Our study showed that the progressive development of faecal microbiome and plasma metabolome in calves depends on their developmental stage and the time of weaning. A high dominance of potential lactose- and starch-degrading bacteria and a high concentration of the plasma AAs and BAs were observed in young calves, but as the calves aged, the abundances of unclassified members of potential fiber-degrading bacteria and the plasma concentrations of SMs and few BAs and ACs were increased. Higher consumption of roughages at day 42 in the earlyC group declines the abundances of potential lactose- and starch-degraders, and the plasma concentrations of most of the AAs and SMs, few BAs and ACs. This weaning-dependent modification in the microbiome composition and plasma metabolic profiles of calves were significantly correlated. On the contrary, the faecal microbial communities of lateC group showed quick adaptability to the weaning-dependent dietary changes, indicating an established microbial consortium compared to the earlyC group. Nevertheless, the plasma samples of lateC group at day 112 showed several metabolites with differential concentrations to the earlyC group, suggesting that the weaning-dependent dietary changes had a less abrupt but more-persistent impact on host metabolome compared to the microbiome. Altogether, the integration of faecal microbiome and plasma metabolome provided us initial insight into the host–microbe's interactions in calves during weaning. However, the plasma metabolic profiles are not only dependent on diet and microbiome, but are also linked to liver and muscle metabolism, as well as the host genetics. Therefore, further studies are needed, where the associations between gut microbiome, gut metabolome, blood metabolome, liver and muscle metabolism must be explored to better understand the role of the microbiome in host metabolism and possible identifications of predictive biomarkers for diseases.

4.6   Methods

4.6.1   Animals and experimental procedures

The experiment was performed using 59 female German Holstein calves, raised under controlled environmental conditions from birth until 149 ± 2 days of life. The experimental design was the same as described previously [22]. Briefly, the experimental period started when calves were 8 ± 1.9 days old. Calves were randomly allocated into two weaning groups, weaned at 7 weeks (experimental days 28–42, earlyC) and 17 weeks of age (experimental days 98–112, lateC). Both weaning groups comprised of equal number of calves born from primiparous cows (PC) and multiparous cows (MC), with similar pattern of MR and C intake until day 28 of the trial. A step-down weaning approach was followed by gradually reducing MR amount (1.35 kg/d–0.3 kg/d) over a period of 14 days. In the earlyC group, MR amount was reduced from day 28 until day 42. However, the lateC group consumed a constant level of MR (~ 1300 g DM/d) until day 98 followed by a gradual reduction in MR amount until day 112. All calves received a maximum of 2 kg/day concentrate feed (C) and ad libitum hay over the entire experimental period. The consumption of C started in both weaning groups at around day 21 of the trial. Intake of C increased in earlyC during their weaning period (days 28–42). However, lateC group continued to increase their C intake until day 63 and then consumed a constant level of C (1500–1700 g DM/d) until weaning. When weaning started for lateC group at day 98, C amount was reduced to 1 kg/d to lower the risk of rumen acidification and increase roughage intake. EarlyC group started to consume roughage from day 42, however, the lateC group increased their roughage intake when the MR supply was reduced at day 98. The post-weaning calves' diet was comprised of hay and a total mixed ration (TMR) containing grass (48%), maize silage (32%), and C (20%). Ingredients and chemical composition of the diets were shown in a companion paper [22].

4.6.2 Sample collection and preparation

On experimental days 1, 28, 42, 70, 98, 112 and 140 blood and faecal samples were taken from each calf. Blood samples were obtained from *Vena jugularis externa* by needle puncture and collected into tubes (10 ml tubes, Sarstedt, Nürnbrecht, Germany) containing ethylenediaminetetraacetic acid (EDTA). After centrifugation (15 min, 3000 x g, Varifuge 3.0, Heraeus, Hanau, Germany), aliquots of plasma samples were stored at -80°C until analysis. Faecal samples were taken directly from the calves' rectum and collected in sample pans. Homogeneous samples were then transferred in sample cups and stored at -80°C until the microbiome was analysed. Some of the calves' samples were discarded due to technical issues as well as during bioinformatic and statistical analysis, thus, resulting in a total of 330 samples over 7 timepoints.

4.6.3 Faecal bacterial community profiling

The genomic DNA was isolated from the faecal samples (250 mg) using the FastDNA^{TM} SPIN Kit for Soil (MP Biomedical, Solon, OH, USA) according to the manufacturer's protocol with minor modifications. For an effective lysis of cells, a bead-beating procedure was performed for 40 sec at a speed of 6 m/sec using FastPrep®-24 instrument (MP Biomedical), followed by centrifugation at $14,000 \times g$ for 15 min. The DNA concentration and quality were accessed using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

4.6.4 Illumina amplicon sequencing and bioinformatic analysis

PCR amplification of the faecal DNA extracts targeting V1-V2 region of bacterial 16S rRNA gene, and Illumina amplicon sequencing was done as described previously by [79]. Briefly, 20 µl PCR mixture was prepared by adding primers (0.2 µM), dNTP mixture (2.5 mM), PrimeSTAR HS DNA polymerase (2.5 U) and 1 µl DNA template. Forward primers comprised of a linker (2-nt) and a barcode (6-nt) sequence. Additionally, an overhang adapter sequences compatible to the Illumina platform were added to both primers. The PCR conditions comprised of an initial denaturation step for 3 min at 95°C, followed by 20 cycles involving denaturation for 10 s at 98°C, annealing for 10 s at 59°C, extension for 45 s at 72°C and 72°C final extension step for 2 min. The resultant PCR product (1 µl) was used in the second PCR step that was performed under similar conditions and comprised of 15 cycles with reverse primer containing additional sequence for integration of Illumina multiplexing sequence and index primers. The PCR products were quality controlled, purified, normalized and sequenced using paired-end (250 bp) Illumina MiSeq sequencing platform.

Bioinformatic analysis of sequencing dataset was performed using QIIME 2 (2019.10) workflow [80]. Briefly, cutadapt (v2.6) was employed within the QIIME 2 for demultiplexing of paired-end (PE) reads according to the barcode sequence of each sample, followed by the trimming of barcodes and primers. The demultiplexed sequences were then quality filtered to remove bases with quality score less than 30, followed by joining of PE reads (mean length 315 ± 14 bp) and removal of non-overlapping regions, chimeras and singletons, thus, resulted in amplicon sequence variant (ASVs) table after DADA2 step. Fourteen faecal samples with < 5,000 reads were discarded from the feature table, resulting in a total of 10,221,260 reads for 339 faecal samples with $30,151 \pm 1,183$ reads (mean $\pm$ SEM) per sample. The negative control samples had an average of 125 reads per sample and therefore were not included in further

analysis. For taxonomic assignments to ASVs, three different reference databases for 16S rRNA gene were employed i.e., the initial classification was performed using pre-trained naïve Bayesian classifier trained on SILVA 132 clustered at 99% similarity. After initial taxonomic classification, an additional filtration step was employed where the unassigned ASVs and those assigned to chloroplast, cyanobacteria, and non-bacterial taxon were removed, the least abundant features (ASV) with ≤ 0.2% contribution to the total reads per sample were discarded and again the low reads samples (< 5,000 reads) were removed, thus, resulting in a total of 8,083,449 reads for 330 faecal samples with 24,495 ± 777 reads (mean ± SEM) per sample and a total of 4,229 unique bacterial ASVs. For taxonomic reassignments of the unique bacterial ASVs, RDP database [81] was used as a reference with naïve Bayesian RDP classifier [82]. The RDP-based taxonomic assignments were then compared with NCBI non-redundant nucleotide database using BLAST [83]. The BLAST results table was filtered with a defined sequence identity threshold for each taxonomic level [84], resulting in removal of taxonomic assignments that fall below the defined threshold; 97.0% (species), 94.5% (genus), 86.5% (family), 82.0% (order), 78.5% (class) and 75.0% (phylum).

For prediction of microbial functional profiles, CowPI was used [85], which is an improved version of PICRUSt, with 16S rDNA inference for rumen [86]. The functional prediction was based on the16S rRNA gene sequence reads of the differential microbial genera due to the weaning event. Only those level-3 KEGG pathways were used for the downstream analysis that had relative abundance > 1% in at least 50% of the animals within each age group.

4.6.5 Plasma metabolome analysis

The targeted metabolomic measurements in plasma samples were performed using AbsoluteIDQ p180 kit (Biocrates Life Science AG, Austria) according to the manufacturer's standard protocol to identify 188 metabolites belonging to 5 compound classes: acylcarnitine, proteinogenic and modified amino acids, glycerophospho- and sphingolipids and hexose. All metabolites were evaluated in absolute concentrations (µmol/l). The assay based on phenylisothiocyanate derivatization in the presence of internal standards followed by FIA-MS/MS (acylcarnitine, hexose, glycerophospho- and sphingolipids) and LC-MS/MS (amino acids, biogenic amines). The experimental measurement technique is described in detail by patent US 8,265,877 B2 [87].

4.6.6 Statistical analysis
4.6.6.1 Microbiome data

The microbiome dataset standardization was performed with the total sum normalization method, where ASVs read counts were divided by the total number of read in a sample. Alpha-diversity analysis was performed in Calypso v8.84 [88] by rarefying samples to a read depth of 4,702 (lowest read counts). Permutational Analysis of Variance (PERMANOVA) at feature level (ASV) was used to identify the differences between the faecal bacterial community structure between groups. The clustering of samples within/between groups (age, weaning time) was visualized using principal-coordinates analysis (PCO) plots in Primer-e (PRIMER 6.1.16 and PERMANOVA+ 1.0.6 [89], that was based on standardized ASV count data and Bray-Curtis as dissimilarity matrix. The significant differences between groups, separated based on PCO analysis, were confirmed using analysis of similarities (ANOSIM) test. Age and weaning-dependent changes in the bacterial diversity and taxonomic composition were tested for statistical significance based on Kruskal–Wallis test in R (https://www.r-project.org; [90]). For multiple comparisons, Dunn's post-hoc test was used with Benjamini–Hochberg algorithm as *p*-value adjustment method and the FDR adjusted $p < 0.05$ was considered significant [91]. The bacterial species-level taxa that were significantly affected by calves age were visualized using heatmap. Heatmap was generated based on hierarchal clustering method using R "gplots" package. The relative abundance table was scaled by row and pairwise distances between species were calculated based on Spearman correlation. These distances were then used to create a dendrogram using average linkage method. Weaning-dependent changes in the predicted metabolic pathways were tested for statistical significance based on Kruskal–Wallis test in R.

4.6.6.2 Metabolome data

Based on targeted metabolomics, a total of 180 metabolic compounds were identified in the plasma samples of calves including free carnitine (1), acylcarnitines (39), amino acids (21), biogenic amines (21), sphingolipids (15), sum of hexoses (1), phosphatidylcholines (76) and lysophosphatidylcholines (14). The latter two metabolite groups were removed from the subsequent analysis as functional aspects of them in calves' gut are not yet understood. The multivariate and statistical analysis of plasma metabolome data was performed in MetaboAnalyst 5.0 [92]. The data containing the absolute concentrations of 98 compounds was normalized before analysis through log-transformation, mean centering and unit variance scaling method. The maximum separation between groups (age, weaning time, parity of the mother) was explained based on supervised partial least squares-discriminant analysis (PLS-DA). The quality of the PLS-DA models was assessed using Q2 as performance measure and

tenfold cross-validation method. Q2 indicates the predictive ability of the model, with high Q2 means good prediction and negative Q2 means overfitting of the model [93]. The dataset containing normalized concentrations of 98 identified metabolic compounds was analysed by one-way ANOVA for age effect and Tukey's HSD test as post-hoc analysis method. *P*-values were adjusted using false discovery rate (FDR) correction and FDR-adjusted $p < 0.05$ was considered statistically significant. To demonstrate the metabolites that were significantly affected by age in calves (VIP > 1, FDR-adjusted $p < 0.05$, ANOVA), a heatmap was generated. For heatmap, the normalized concentration table was scaled by row, pairwise distances between metabolic compounds were calculated based on Euclidean distance measure and ward clustering algorithm. The differential metabolites (DMs) due to the weaning event at each timepoint were selected based on the variable importance in the projection (VIP > 1.0, FDR-adjusted $p < 0.05$ (*t*-test) and earlyC/lateC fold change (FC) > 1.0). The volcano plots with DMs at each timepoint were generated using "ggplot2" package in R. Metabolic pathway analysis (MetPA) was performed based on DMs using *Bos taurus* library as reference [94]. The significantly altered pathways due to the weaning event were selected based on the pathway impact value > 0.1 and FDR-adjusted $p < 0.01$, obtained from pathway enrichment analysis. The associations between bacterial genera, plasma metabolites and morphometric variables of calves were calculated based on Spearman's rank correlation using cor() function in R and the correlation matrix was visualized using corrplot() function. The correlations with $p < 0.05$ were considered significant.

4.7 Supplementary information

Additional file 1:
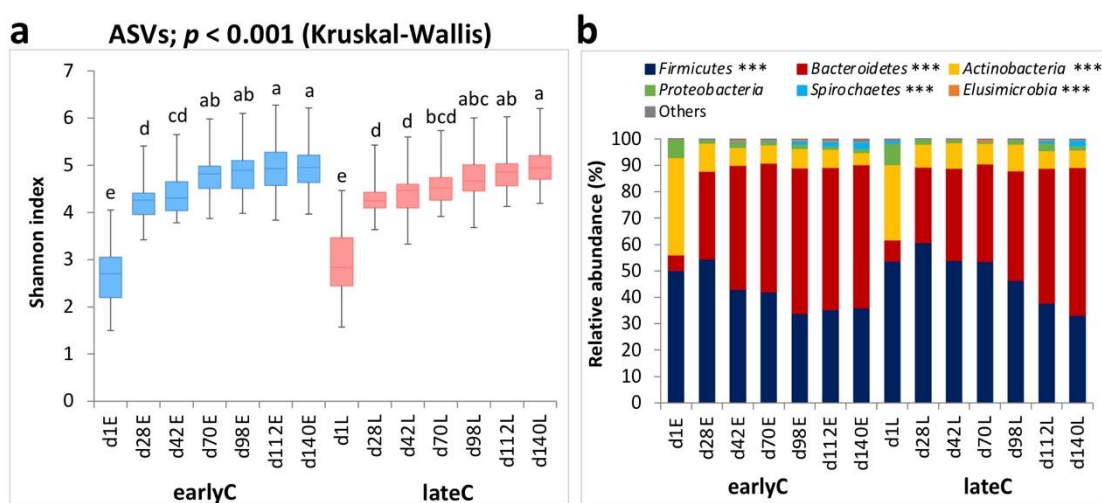
Figure S9 | Age- and weaning-dependent changes in the faecal bacterial compositional profiles of calves. (a) Changes among alpha-diversity index. [abcde] Groups that share superscript letters are not significantly different ($p > 0.05$; Dunn's post-hoc test). Standard deviations are indicated by error bars. (b) Significantly different bacterial phyla. ***Phyla with $p < 0.001$ (age x weaning effect; Kruskal-Wallis test) are shown.
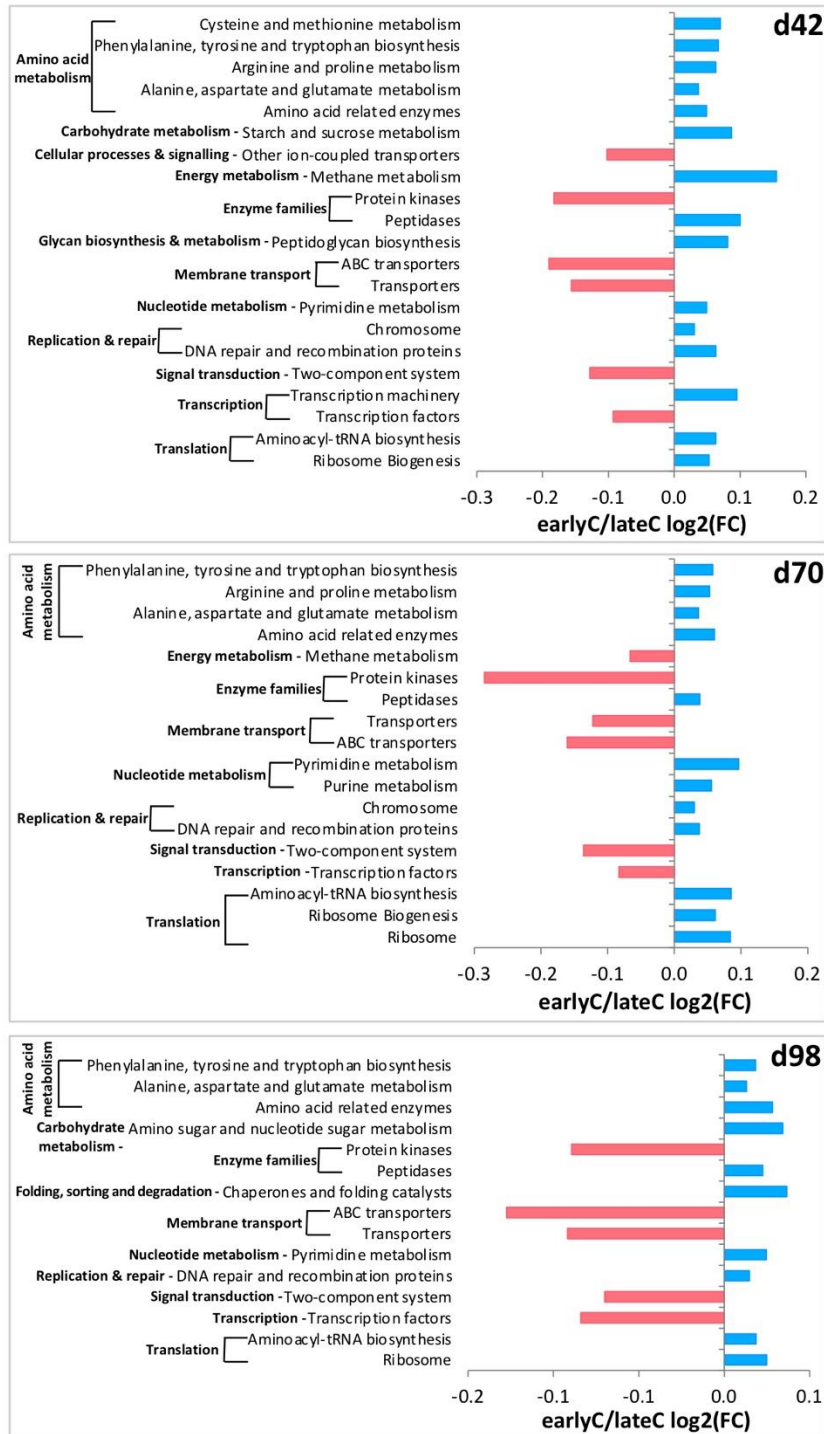
Figure S10 | Microbial functional predictions using KEGG pathways and the CowPI database. EarlyC/lateC log2(FC) shows differences in level-3 KEGG microbial pathways between d42, d70 and d98 earlyC (blue) and lateC (red) calves. Only metabolic pathways with relative abundance (> 1%) in at least 50% of the animals and FDR adjusted $p < 0.05$ (Kruskal-Wallis test) are shown.
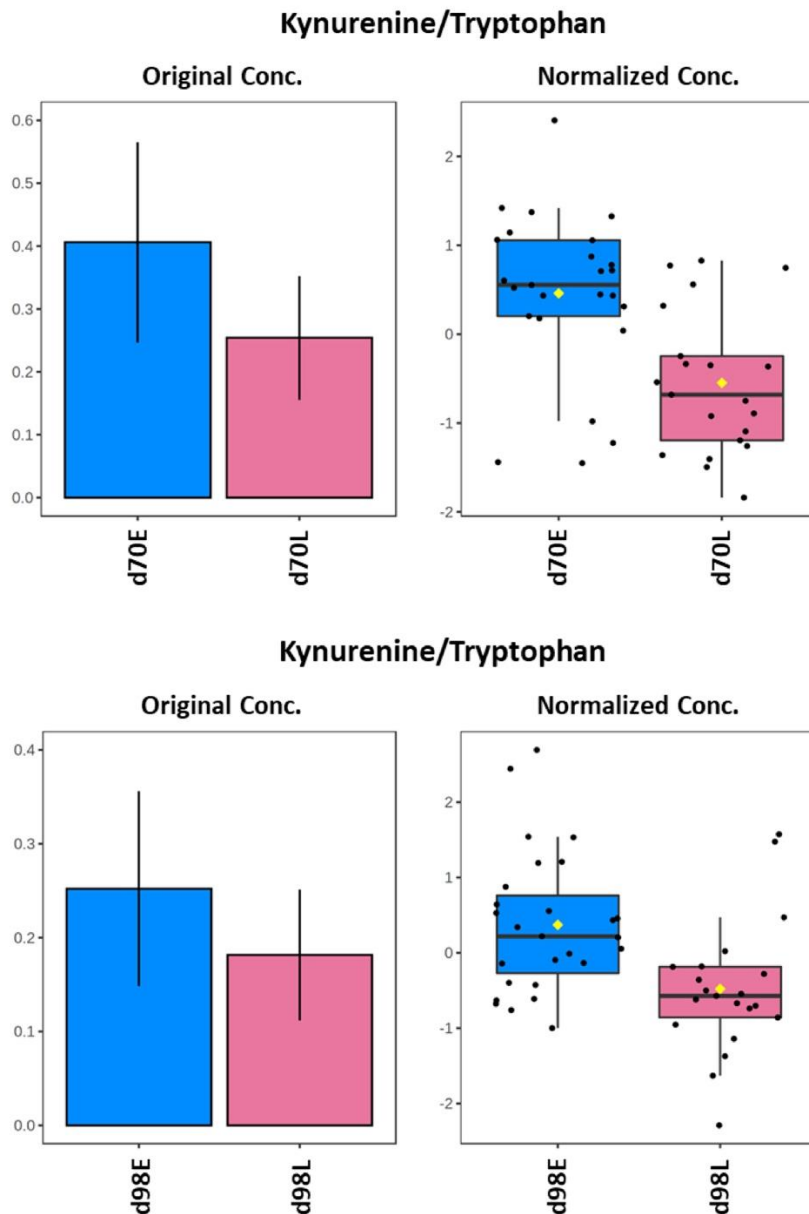
		VIP	FDR (*t*-test)	log2 FC
d70	earlyC/lateC	1.5	0.002	0.7
d98	earlyC/lateC	1.3	0.010	0.5

Figure S11 | Calculation of kynurenine/tryptophan ration at d70 and d98 for early weaned calves (E) and late weaned calves (L).
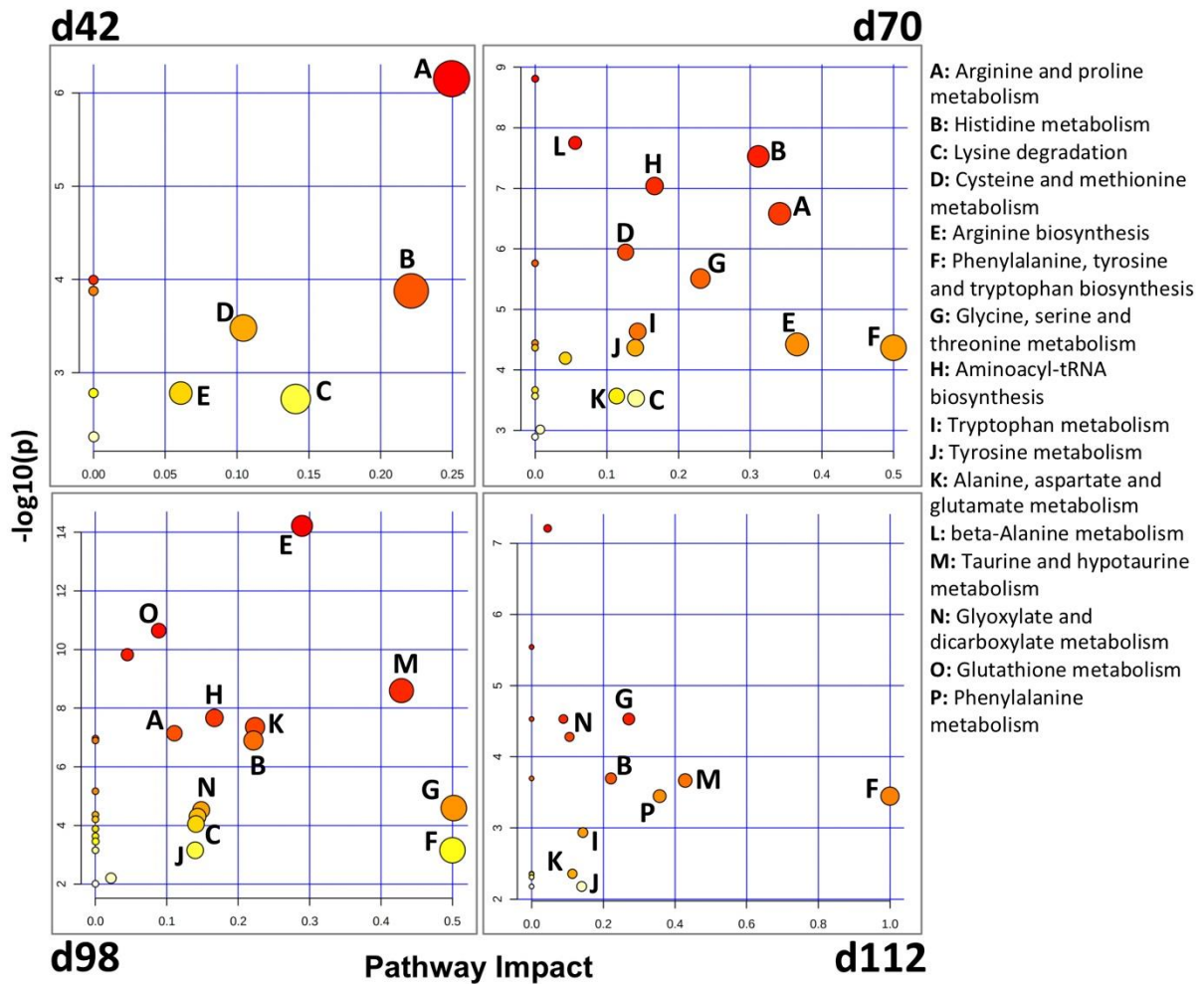
Figure S12 | Metabolic pathway analysis based on significantly different plasma metabolites of weaning groups. Circle size indicates pathway impact and colours (yellow to red) show different levels of significance.

Additional file 2:

Table S10 | Average relative abundances of faecal bacterial communities in early- and late-weaned calves.

	[2]earlyC							[3]lateC						
							Experimental day							
[1]**Taxon**	**1E**	**28E**	**42E**	**70E**	**98E**	**112E**	**140E**	**1L**	**28L**	**42L**	**70L**	**98L**	**112L**	**140L**	[4]***p*-value**
Phyla														

[1]Taxon	[2]earlyC							[3]lateC							[4]p-value
	Experimental day														
	1E	28E	42E	70E	98E	112E	140E	1L	28L	42L	70L	98L	112L	140L	
Firmicutes	50.1	54.5	43.1	42.0	33.9	35.3	36.0	53.7	60.8	54.0	53.6	46.4	37.8	33.2	< 0.001
Bacteroidetes	6.0	33.3	46.9	48.8	55.1	53.8	54.3	8.0	28.6	34.9	36.9	41.6	51.1	55.9	< 0.001
Actinobacteria	36.8	10.6	6.9	7.0	7.4	6.9	4.6	28.6	8.7	9.7	7.8	10.1	6.6	6.8	< 0.001
Proteobacteria	7.1	1.3	1.9	1.5	1.6	1.3	1.5	8.0	1.9	1.3	0.7	1.7	2.9	1.6	0.194
Spirochaetes	0.0	0.0	0.4	0.3	1.3	1.8	2.7	1.3	0.0	0.0	0.0	0.1	1.2	2.1	< 0.001
Genera															
o_Bacteroidales	0.1	4.3	7.6	15.8	11.7	14.4	14.2	0.1	1.8	6.8	6.1	5.7	12.7	15.6	< 0.001
o_Bacteroidales incertae sedis	0.0	0.2	0.8	0.1	0.1	0.1	0.3	0.0	0.0	3.1	4.8	3.0	0.1	0.3	< 0.001
g_Blautia	0.2	6.4	1.1	0.1	0.3	0.2	0.3	2.4	8.0	6.5	3.7	2.7	0.3	0.2	< 0.001
g_Prevotella	0.7	4.7	2.3	0.2	0.1	0.1	0.1	0.2	4.9	3.8	2.1	1.0	0.1	0.0	< 0.001
g_Mediterraneibacter	4.3	2.1	0.1	0.0	0.0	0.1	0.0	5.2	4.1	1.8	1.8	0.7	0.0	0.0	< 0.001
g_Anaerostipes	0.6	3.8	0.1	0.0	0.1	0.1	0.1	0.8	3.9	4.5	1.4	0.4	0.0	0.0	< 0.001
g_Parabacteroides	0.2	2.6	3.6	0.1	0.0	0.1	0.3	0.3	2.2	1.8	1.3	0.8	0.2	0.0	< 0.001
g_Faecalicatena	0.2	2.1	0.8	0.0	0.0	0.1	0.1	0.3	1.5	1.5	0.7	0.2	0.0	0.1	< 0.001
g_Streptococcus	2.3	0.6	0.0	0.0	0.1	0.0	0.0	5.9	1.7	2.2	0.6	0.1	0.0	0.0	< 0.001
g_Frisingicoccus	0.0	2.3	0.2	0.2	0.1	0.1	0.1	0.0	1.3	1.2	0.6	0.4	0.2	0.1	< 0.001
g_Collinsella	0.0	2.9	0.2	0.0	0.0	0.0	0.0	0.8	2.1	1.3	0.5	0.4	0.0	0.0	< 0.001
o_Sphingobacteriales	0.0	0.1	0.1	0.7	0.8	0.8	0.6	0.0	0.0	0.0	0.0	0.4	1.3	1.6	< 0.001
f_Lachnospiraceae	1.8	11.2	10.5	7.4	6.9	7.6	8.0	1.8	12.9	12.5	16.0	15.2	7.7	7.1	< 0.001
g_Bacteroides	2.8	8.8	7.3	0.4	0.1	0.2	0.7	3.6	8.9	6.1	3.6	2.5	0.3	0.0	< 0.001
f_Bacteroidaceae	0.1	0.2	2.1	9.7	9.7	9.4	9.6	0.0	0.1	0.5	2.4	5.1	9.7	11.9	< 0.001
f_Rikenellaceae	0.0	0.0	0.3	5.0	12.2	12.3	11.5	0.0	0.0	0.1	0.8	1.9	7.1	11.5	< 0.001
g_Faecalibacterium	1.3	3.8	0.6	0.0	0.0	0.0	0.0	0.3	2.4	2.5	1.0	0.1	0.0	0.0	< 0.001
f_Eubacteriaceae	0.0	1.6	1.3	1.2	1.3	0.9	1.1	0.0	0.5	0.6	0.5	0.6	1.6	1.1	< 0.001
c_Cytophagia	0.0	0.0	0.7	0.7	1.1	0.7	0.1	0.1	0.0	0.0	0.1	0.8	1.6	0.2	< 0.001
c_Bacteroidia	0.0	0.5	2.6	7.1	7.6	7.1	8.2	0.0	0.3	0.9	1.4	4.1	7.6	8.8	< 0.001
g_Phocaeicola	1.9	5.8	4.7	0.5	0.1	0.1	0.3	3.6	7.7	7.3	7.2	5.2	0.5	0.1	< 0.001
f_Atopobiaceae	0.7	0.8	1.8	0.9	0.4	0.3	0.4	0.0	0.9	1.5	2.8	2.3	0.9	1.0	< 0.001
f_Muribaculaceae	0.0	0.2	2.3	3.2	3.2	2.8	2.9	0.0	0.0	0.5	1.6	2.1	2.8	2.3	< 0.001
g_Alistipes	0.0	1.2	1.1	0.3	0.1	0.1	0.1	0.1	0.4	0.9	0.5	0.3	0.2	0.0	< 0.001
o_Clostridiales	0.2	4.5	13.7	15.7	13.4	13.7	12.8	0.5	4.7	6.2	12.2	10.8	14.2	11.6	< 0.001
f_Spirochaetaceae	0.0	0.0	0.2	0.2	1.2	1.4	2.5	1.1	0.0	0.0	0.0	0.0	1.1	2.1	< 0.001
g_Barnesiella	0.0	1.2	2.1	0.0	0.0	0.0	0.4	0.0	0.9	0.9	0.1	0.5	0.0	0.0	< 0.001
g_Gemmiger	0.1	2.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.4	0.0	0.1	0.0	0.0	< 0.001
g_Butyricicoccus	2.8	0.5	0.0	0.0	0.0	0.0	0.0	3.2	1.4	0.5	0.0	0.1	0.0	0.0	< 0.001
g_Butyricimonas	0.0	2.0	2.9	0.5	0.1	0.1	0.1	0.1	0.4	0.8	1.5	1.1	0.3	0.0	< 0.001
f_Marinilabiliaceae	0.0	0.2	4.0	1.7	2.0	1.0	0.9	0.0	0.0	0.2	1.3	2.9	2.1	0.9	< 0.001
f_Ruminococcaceae	0.6	2.2	5.6	5.8	3.6	3.5	3.4	0.5	1.8	1.8	5.4	4.1	4.6	3.4	< 0.001
g_Olsenella	0.1	1.6	1.7	3.3	0.2	0.5	0.3	0.0	2.1	2.2	2.6	2.7	0.3	0.2	< 0.001
f_Prevotellaceae	0.1	0.4	1.5	1.0	1.6	1.3	1.9	0.0	0.4	0.7	1.3	0.9	0.8	1.0	< 0.001
g_Clostridium	1.9	0.5	0.1	0.8	0.3	0.2	0.6	1.8	1.3	0.2	0.4	0.2	0.3	0.2	< 0.001
c_Clostridia	0.0	0.9	0.9	2.2	1.4	2.1	1.3	0.0	0.7	0.8	1.2	1.7	1.6	2.2	< 0.001
o_Erysipelotrichales	0.0	0.5	0.3	0.1	0.1	0.2	0.2	0.0	0.6	2.1	0.4	0.2	0.2	0.2	< 0.001
f_Lactobacillaceae	6.4	0.1	0.0	0.1	0.0	0.0	0.0	8.1	0.1	0.0	0.0	0.1	0.0	0.0	< 0.001
f_Oscillospiraceae	0.0	1.4	1.8	3.5	2.1	1.8	2.2	0.0	1.1	1.9	2.2	2.1	2.6	1.8	< 0.001
f_Tannerellaceae	0.0	0.1	0.2	1.2	3.8	2.9	1.1	0.0	0.0	0.1	0.5	3.0	3.3	1.4	< 0.001
f_Erysipelotrichaceae	0.0	0.8	0.7	0.7	0.2	0.3	0.3	0.0	1.3	1.4	1.2	1.2	0.5	0.7	< 0.001
g_Ligilactobacillus	7.8	1.5	0.0	0.0	0.0	0.0	0.1	4.2	1.2	0.1	0.0	0.1	0.0	0.0	< 0.001
g_Bifidobacterium	34.6	4.5	3.1	2.5	6.5	5.6	3.7	27.0	3.0	4.1	1.7	4.7	5.2	5.2	< 0.001
g_Gallibacterium	4.6	0.0	0.0	0.0	0.0	0.1	0.0	2.3	0.2	0.0	0.0	0.1	0.0	0.0	< 0.001
g_Enterococcus	2.2	0.1	0.0	0.0	0.0	0.0	0.0	1.2	0.1	0.0	0.0	0.0	0.0	0.0	< 0.001
g_Lactobacillus	8.2	0.6	0.1	0.0	0.0	0.1	0.0	4.9	0.2	0.0	0.0	0.1	0.0	0.0	< 0.001
g_Citrobacter	1.0	0.2	0.0	0.0	0.1	0.1	0.0	1.3	0.3	0.0	0.0	0.0	0.0	0.0	< 0.001
g_Limosilactobacillus	5.3	0.7	0.0	0.0	0.0	0.0	0.0	8.7	0.2	0.0	0.0	0.0	0.0	0.0	< 0.001
g_Schaalia	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	< 0.001
g_Turicibacter	0.0	0.0	0.0	0.1	0.2	0.3	0.6	0.0	0.0	0.0	1.0	0.9	0.6	0.4	< 0.001
Species															
s_Prevotella copri	0.1	1.3	0.7	0.0	0.0	0.1	0.0	0.1	1.9	1.1	0.8	0.3	0.0	0.0	< 0.001
g_Prevotella	0.5	3.4	1.5	0.1	0.0	0.1	0.1	0.1	3.0	2.6	1.3	0.7	0.1	0.0	< 0.001
s_Barnesiella intestinihominis	0.0	1.2	2.0	0.0	0.0	0.0	0.4	0.0	0.9	0.9	0.1	0.5	0.0	0.0	< 0.001
f_Prevotellaceae	0.1	0.4	1.5	1.0	1.6	1.3	1.9	0.0	0.4	0.7	1.3	0.9	0.8	1.0	< 0.001
s_Faecalibacterium prausnitzii	1.2	3.5	0.5	0.0	0.0	0.0	0.0	0.2	2.1	2.1	0.9	0.1	0.0	0.0	< 0.001
g_Faecalicatena	0.2	2.1	0.8	0.0	0.0	0.1	0.1	0.3	1.5	1.5	0.7	0.2	0.0	0.1	< 0.001
c_Clostridia	0.0	0.9	0.9	2.2	1.4	2.1	1.3	0.0	0.7	0.8	1.2	1.7	1.6	2.2	< 0.001
s_Bacteroides uniformis	0.0	1.0	0.9	0.3	0.0	0.0	0.1	0.2	1.4	1.2	0.4	0.4	0.0	0.0	< 0.001
s_Blautia wexlerae	0.0	3.4	0.1	0.0	0.0	0.0	0.0	1.6	4.0	1.3	0.1	0.1	0.0	0.0	< 0.001
f_Lactobacillaceae	6.4	0.1	0.0	0.1	0.0	0.0	0.0	8.1	0.1	0.0	0.0	0.1	0.0	0.0	< 0.001

[1]Taxon	[2]earlyC							[3]lateC							[4]*p*-value
	Experimental day														
	1E	28E	42E	70E	98E	112E	140E	1L	28L	42L	70L	98L	112L	140L	
s_Phocaeicola vulgatus	1.9	4.5	4.0	0.1	0.1	0.1	0.3	3.6	6.3	5.0	0.8	0.6	0.0	0.0	< 0.001
s_Gallibacterium anatis	4.5	0.0	0.0	0.0	0.0	0.1	0.0	2.3	0.2	0.0	0.0	0.1	0.0	0.0	< 0.001
f_Oscillospiraceae	0.0	1.4	1.8	3.5	2.1	1.8	2.2	0.0	1.1	1.9	2.2	2.1	2.6	1.8	< 0.001
f_Tannerellaceae	0.0	0.1	0.2	1.2	3.8	2.9	1.1	0.0	0.0	0.1	0.5	3.0	3.3	1.4	< 0.001
s_Ligilactobacillus salivarius	7.8	1.5	0.0	0.0	0.0	0.1	0.0	4.2	1.2	0.1	0.0	0.1	0.0	0.0	< 0.001
s_Bifidobacterium pseudolongum	1.3	1.6	2.9	2.0	5.5	4.7	2.9	0.5	0.9	3.3	1.1	3.8	5.0	4.4	< 0.001
g_Butyricicoccus	2.8	0.5	0.0	0.0	0.0	0.0	0.0	3.1	1.3	0.5	0.0	0.1	0.0	0.0	< 0.001
s_Bifidobacterium longum	28.9	2.7	0.1	0.1	0.3	0.2	0.0	20.9	1.8	0.5	0.2	0.3	0.0	0.0	< 0.001
s_Lactobacillus amylovorus	6.6	0.1	0.1	0.0	0.0	0.1	0.0	4.3	0.1	0.0	0.0	0.1	0.0	0.0	< 0.001
s_Lactobacillus ingluviei	3.6	0.0	0.0	0.0	0.0	0.0	0.0	5.5	0.0	0.0	0.0	0.0	0.0	0.0	< 0.001
g_Mediterraneibacter	2.4	0.3	0.0	0.0	0.0	0.0	0.0	2.2	1.0	0.6	0.7	0.2	0.0	0.0	< 0.001
s_Streptococcus gallolyticus	1.6	0.2	0.0	0.0	0.0	0.0	0.0	4.7	0.3	0.1	0.0	0.0	0.0	0.0	< 0.001
f_Rikenellaceae	0.0	0.0	0.3	5.0	12.2	12.3	11.5	0.0	0.0	0.1	0.8	1.9	7.0	11.5	< 0.001
f_Eubacteriaceae	0.0	1.6	1.3	1.2	1.2	0.9	1.1	0.0	0.5	0.6	0.5	0.6	1.6	1.1	< 0.001
f_Ruminococcaceae	0.6	2.2	5.6	5.8	3.6	3.5	3.4	0.5	1.8	1.8	5.4	4.1	4.6	3.4	< 0.001
o_Bacteroidales	0.1	4.3	7.5	15.7	11.7	14.4	14.2	0.1	1.8	6.8	6.1	5.7	12.7	15.6	< 0.001
f_Bacteroidaceae	0.1	0.2	2.1	9.7	9.7	9.4	9.6	0.0	0.0	0.5	2.4	5.1	9.7	11.9	< 0.001
o_Clostridiales	0.2	4.5	13.7	15.7	13.4	13.7	12.8	0.5	4.7	6.2	12.2	10.7	14.2	11.6	< 0.001
c_Bacteroidia	0.0	0.5	2.6	7.1	7.6	7.0	8.2	0.0	0.3	0.9	1.4	4.1	7.6	8.8	< 0.001
o_Sphingobacteriales	0.0	0.1	0.1	0.6	0.8	0.8	0.6	0.0	0.0	0.0	0.0	0.4	1.3	1.6	< 0.001
f_Muribaculaceae	0.0	0.2	2.3	3.2	3.2	2.8	2.9	0.0	0.0	0.5	1.6	2.1	2.8	2.2	< 0.001
s_Olsenella umbonata	0.0	0.7	0.9	1.8	0.2	0.3	0.2	0.0	1.2	1.1	1.3	1.5	0.2	0.1	< 0.001
o_Bacteroidales incertae sedis	0.0	0.2	0.8	0.1	0.1	0.1	0.3	0.0	0.0	3.0	4.8	3.0	0.1	0.3	< 0.001
f_Lachnospiraceae	1.8	11.1	10.5	7.4	6.9	7.6	8.0	1.8	12.9	12.5	16.0	15.2	7.7	7.1	< 0.001
f_Erysipelotrichaceae	0.0	0.8	0.7	0.7	0.2	0.3	0.3	0.0	1.3	1.4	1.2	1.2	0.5	0.7	< 0.001
g_Blautia	0.1	2.1	1.0	0.1	0.2	0.2	0.2	0.5	3.1	4.4	3.3	2.3	0.3	0.2	< 0.001
f_Atopobiaceae	0.7	0.8	1.8	0.9	0.4	0.3	0.4	0.0	0.9	1.5	2.8	2.3	0.9	1.0	< 0.001
f_Spirochaetaceae	0.0	0.0	0.2	0.2	1.2	1.4	2.5	1.1	0.0	0.0	0.0	0.0	1.1	2.1	< 0.001
f_Marinilabiliaceae	0.0	0.2	4.0	1.7	2.0	0.9	0.8	0.0	0.0	0.2	1.3	2.9	2.1	0.9	< 0.001
g_Frisingicoccus	0.0	2.3	0.2	0.1	0.1	0.1	0.1	0.0	1.3	1.2	0.6	0.4	0.2	0.1	< 0.001
c_Cytophagia	0.0	0.0	0.7	0.7	1.1	0.7	0.1	0.1	0.0	0.0	0.1	0.8	1.6	0.2	< 0.001
s_Butyricimonas virosa	0.0	1.6	2.2	0.3	0.1	0.1	0.1	0.0	0.3	0.7	1.1	0.9	0.2	0.0	< 0.001
s_Phocaeicola coprocola	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	3.0	2.5	0.3	0.0	< 0.001

[1]Taxon	[5]Dunn's test
Phyla	
Firmicutes	42E-42L; 70E-70L; 98E-98L; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-98E; 42E-112E; 70E-98E; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
Bacteroidetes	42E-42L; 70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
Actinobacteria	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L
Spirochaetes	98E-98L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-98E; 28E-112E; 28E-140E; 1L-112L; 1L-140L; 28L-140L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
Genera	
o_Bacteroidales	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
o_Bacteroidales incertae sedis	70E-70L; 98E-98L; 1E-140E; 1L-70L; 1L-98L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-140L; 42L-70L; 42L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Blautia	42E-42L; 70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-98E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; ; ; ; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Prevotella	70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 28L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L

[1]Taxon	[5]Dunn's test
g_Mediterraneibacter	42E-42L; 70E-70L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Anaerostipes	42E-42L; 70E-70L; 1E-28E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L
g_Parabacteroides	70E-70L; 98E-98L; 112E-112L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L; 112L-140L
g_Faecalicatena	70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-112L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Streptococcus	42E-42L; 70E-70L; 98E-98L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Frisingicoccus	42E-42L; 70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
g_Collinsella	42E-42L; 70E-70L; 1E-28E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
o_Sphingobacteriales	70E-70L; 98E-98L; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Lachnospiraceae	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Bacteroides	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Bacteroidaceae	70E-70L; 98E-98L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Rikenellaceae	70E-70L; 98E-98L; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Faecalibacterium	70E-70L; 1E-28E; 1E-70E; 1E-112E; ; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L
f_Eubacteriaceae	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
c_Cytophagia	42E-42L; 70E-70L; 112E-112L; 1E-42E; 1E-70E; 1E-98E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 42E-140E; 70E-140E; 112E-98E; 98E-140E; 112E-140E; 1L-70L; 1L-98L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 98L-140L; 112L-140L
c_Bacteroidia	70E-70L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-112E; 42E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; ; 28L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
g_Phocaeicola	70E-70L; 98E-98L; 112E-112L; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 28L-140L; 42L-140L; 70L-140L
f_Atopobiaceae	98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Muribaculaceae	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-98E; 42E-112E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
g_Alistipes	98E-98L; 112E-112L; 1E-28E; 1E-42E; 1E-70E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L; 112L-140L
o_Clostridiales	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
f_Spirochaetaceae	98E-98L; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-112E; 42E-140E; 1L-140L; 28L-140L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Barnesiella	98E-98L; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-42L; 1L-98L; 42L-140L; 98L-140L
g_Gemmiger	42E-42L; 1E-28E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
g_Butyricicoccus	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
g_Butyricimonas	28E-28L; 42E-42L; 98E-98L; 112E-112L; 1E-28E; 1E-42E; 1E-70E; 28E-42E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-42L; 28L-70L; 28L-98L; 42L-140L; 70L-140L; 112L-98L; 98L-140L; 112L-140L
f_Marinilabiliaceae	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-112L

¹Taxon	⁵Dunn's test
f_Ruminococcaceae	42E-42L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; ; 70E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
g_Olsenella	98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; ; 28E-42E; 28E-140E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Prevotellaceae	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 1L-112L; 1L-140L
g_Clostridium	1E-42E; 28E-140E; 42E-140E; 70E-140E; 98E-140E; 1L-42L
c_Clostridia	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-112E; 42E-70E; 42E-112E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-140L
o_Erysipelotrichales	1E-28E; 1E-42E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 42L-98L; 42L-112L; 42L-140L
f_Lactobacillaceae	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-112L; 28L-140L
f_Oscillospiraceae	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; ; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L
f_Tannerellaceae	1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L
f_Erysipelotrichaceae	1E-28E; 1E-42E; 1E-70E; 1E-112E; 1L-28L; 1L-42L; 1L-70L; 1L-98L
g_Ligilactobacillus	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Bifidobacterium	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L;
g_Gallibacterium	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Enterococcus	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Lactobacillus	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Citrobacter	1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Limosilactobacillus	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Schaalia	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; ; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L;
g_Turicibacter	1E-112E; 1E-140E; 28E-112E; 28E-140E; 42E-140E; 1L-98L; 28L-98L
Species	
s_Prevotella copri	70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
g_Prevotella	98E-98L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-140L; 98L-140L
s_Barnesiella intestinihominis	98E-98L; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-42L; 1L-98L; 42L-140L; 98L-140L
f_Prevotellaceae	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 1L-112L; 1L-140L
s_Faecalibacterium prausnitzii	70E-70L; 1E-28E; 1E-112E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L
g_Faecalicatena	70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-112L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
c_Clostridia	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 28E-70E; 28E-112E; 42E-70E; ; 42E-112E; ; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-140L
s_Bacteroides uniformis	98E-98L; 1E-28E; 1E-42E; 28E-98E; 28E-112E; 28E-140E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-140L; 98L-140L
s_Blautia wexlerae	42E-42L; 1E-28E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-112L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L; 112L-98L
f_Lactobacillaceae	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-112L; 28L-140L
s_Phocaeicola vulgatus	28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L
s_Gallibacterium anatis	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-112L; 28L-140L
f_Oscillospiraceae	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-140E; ; ; ; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L
f_Tannerellaceae	1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L
s_Ligilactobacillus salivarius	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
s_Bifidobacterium pseudolongum	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-98E; 28E-112E; 1L-42L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 70L-112L
g_Butyricicoccus	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L

[1]Taxon	[5]Dunn's test
s_Bifidobacterium longum	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 112L-98L
s_Lactobacillus amylovorus	1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-112L; 28L-140L
s_Lactobacillus ingluviei	1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-112L; 28L-140L
g_Mediterraneibacter	42E-42L; 70E-70L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 70L-140L
s_Streptococcus gallolyticus	1E-112E; 28E-112E; ; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
f_Rikenellaceae	70E-70L; 98E-98L; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Eubacteriaceae	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Ruminococcaceae	42E-42L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 70E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
o_Bacteroidales	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Bacteroidaceae	70E-70L; 98E-98L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
o_Clostridiales	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
c_Bacteroidia	70E-70L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-112E; 42E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
o_Sphingobacteriales	70E-70L; 98E-98L; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Muribaculaceae	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-98E; 42E-112E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-70L; 42L-98L; 42L-112L; 42L-140L
s_Olsenella umbonata	98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 28E-42E; 28E-70E; 28E-140E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
o_Bacteroidales incertae sedis	70E-70L; 98E-98L; 1E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 28L-140L; 42L-70L; 42L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Lachnospiraceae	70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Erysipelotrichaceae	1E-28E; 1E-42E; 1E-70E; 1E-112E; 1L-28L; 1L-42L; 1L-70L; 1L-98L
g_Blautia	42E-42L; 70E-70L; 98E-98L; 1E-28E; 1E-42E; 1E-98E; 1E-112E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 28L-112L; 28L-140L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Atopobiaceae	98E-98L; 1E-28E; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-42L; 28L-70L; 28L-98L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Spirochaetaceae	98E-98L; 1E-98E; 1E-112E; 1E-140E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-112E; 42E-140E; 1L-140L; 28L-140L; 42L-140L; 70L-112L; 70L-140L; 112L-98L; 98L-140L
f_Marinilabiliaceae	42E-42L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 1E-140E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 1L-98L; 1L-112L; 1L-140L; 28L-98L; 28L-112L; 28L-140L; 42L-112L
g_Frisingicoccus	42E-42L; 70E-70L; 98E-98L; 1E-28E; 1E-42E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 28E-140E; 42E-140E; 1L-28L; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-112L; 70L-140L; 98L-140L
c_Cytophagia	42E-42L; 70E-70L; 112E-112L; 1E-42E; 1E-70E; 1E-98E; 1E-112E; 28E-42E; 28E-70E; 28E-98E; 28E-112E; 42E-140E; 70E-140E; 112E-98E; 98E-140E; 112E-140E; 1L-70L; 1L-98L; 1L-112L; 1L-140L; 28L-70L; 28L-98L; 28L-112L; 28L-140L; 42L-98L; 42L-112L; 42L-140L; 70L-98L; 70L-112L; 98L-140L; 112L-140L
s_Butyricimonas virosa	28E-28L; 42E-42L; 98E-98L; 112E-112L; 1E-28E; 1E-42E; 1E-70E; 28E-42E; 28E-98E; 28E-112E; 28E-140E; 42E-70E; 42E-98E; 42E-112E; 42E-140E; 70E-98E; 70E-112E; 70E-140E; 1L-42L; 1L-70L; 1L-98L; 1L-112L; 28L-42L; 28L-70L; 28L-98L; 42L-140L; 70L-140L; 112L-98L; 98L-140L; 112L-140L
s_Phocaeicola coprocola	70E-70L; 98E-98L; 112E-112L; 1L-70L; 1L-98L; 1L-112L; 28L-70L; 28L-98L; 28L-112L; 42L-98L; 42L-112L; 98L-140L; 112L-140L

[1]Phyla and their corresponding genera and species are indicated and only those taxa with maximum relative abundance > 1% in at least 1 group are shown

[2]earlyC group weaned at 7 weeks of age (experimental days 28–42)

[3]lateC group weaned at 17 weeks of age (experimental days 98–112)

[4]P-values were obtained using Kruskal-Wallis test and $p \leq 0.05$ indicates bacterial taxa that were significantly different between the samples due to age x weaning effect

[5]Pairwise comparisons were done using Dunn's test and $p \leq 0.05$ was considered significant.

Table S11 | Average relative concentrations (µmol/L) of plasma metabolites in early- and late-weaned calves.

[1]Metabolites list	[2]earlyC							[3]lateC							[4]p-value
	1E	28E	42E	70E	98E	112E	140E	1L	28L	42L	70L	98L	112L	140L	
Carnitine	10.2	14.8	10.8	8.4	8.8	8.8	8.4	9.5	15.9	14.8	12.6	11.9	9.8	10.4	< 0.001
Propionylcarnitine	0.2	0.2	0.6	0.8	0.5	0.4	0.3	0.2	0.3	0.4	0.6	0.7	0.6	0.4	< 0.001
Propenoylcarnitine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	< 0.001
Valerylcarnitine	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.1	0.2	0.2	< 0.001
Nonaylcarnitine	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	< 0.001
Hexadecanoyl carnitine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	< 0.001
Hydroxyhexadeca dienylcarnitine	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	< 0.001
Octadecenoyl carnitine	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	< 0.001
Arginine	203	238	192	144	151	151	145	223	238	216	182	166	153	142	< 0.001
Glutamate	154	104	92.3	98.7	108.1	97.1	89.8	157	89.1	85.2	90.9	101	104.1	91.3	< 0.001
Glycine	627	441	424	422	448	445	438	612	413	442	408	377	344	386	< 0.001
Histidine	101	117	91.4	66.5	66.8	72.9	78.5	98.5	124	122	98.6	95.5	84.5	77.5	< 0.001
Lysine	283	267	185	151	156	159	168	261	276	229	200	183	184	188	< 0.001
Methionine	50.1	48.6	29.2	24.4	30.6	31.2	33.8	44.1	46.5	41.0	35.8	33.2	31.7	33.1	< 0.001
Ornithine	98.1	93.2	135	72.2	74.7	78.6	78.8	92.5	90.5	102	96.5	107	85.1	78.4	< 0.001
Phenylalanine	106	93.8	89.2	71.5	72.8	68.9	68.6	104	94.7	84.1	77.6	79.3	80.8	75.0	< 0.001
Proline	200	152	91.1	73.1	78.7	81.0	83.9	194	149	120	94.0	87.0	86.4	76.6	< 0.001
Serine	202	143	108	81.4	85.7	87.5	90.8	196	143	116	100	98.4	79.2	88.3	< 0.001
Threonine	156	125	90.9	71.3	93.3	95.1	99.8	164	119	110	108	112	98.5	90.0	< 0.001
Tyrosine	86.9	93.4	77.6	56.5	63.6	62.7	67.4	89.2	94.0	82.0	75.9	77.0	73.1	61.6	< 0.001
Valine	276	248	333	278	286	286	282	271	256	284	321	342	323	314	< 0.001
Acetylornithine	1.1	0.6	1.5	4.7	6.6	6.8	7.2	1.4	0.6	1.0	1.8	1.7	4.6	7.0	< 0.001
Asymmetric dimethylarginine	2.1	1.6	1.5	1.5	1.5	1.5	1.5	2.0	1.6	1.5	1.5	1.3	1.4	1.5	< 0.001
Carnosine	9.8	10.0	11.6	10.8	12.5	13.9	15.0	10.1	10.0	11.1	13.4	14.6	15.2	14.7	< 0.001
Creatinine	81.7	67.3	65.9	63.7	55.8	57.9	62.2	78.0	66.7	60.6	57.6	54.4	65.0	64.6	< 0.001
Dihydroxyphenyl-alanine	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	< 0.001
Dopamine	0.0	2.0	2.4	2.7	2.8	2.9	2.5	0.0	2.3	2.1	3.0	2.5	3.1	2.8	< 0.001
Histamine	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.3	0.3	0.3	0.3	0.3	0.3	< 0.001
Sarcosine	7.6	6.5	2.6	7.5	6.8	3.9	1.1	8.1	7.6	5.4	3.3	2.3	1.9	1.3	< 0.001
Symmetric dimethylarginine	1.1	0.7	0.6	0.6	0.6	0.6	0.6	1.0	0.7	0.6	0.7	0.6	0.6	0.6	< 0.001
Spermine	0.0	0.8	1.6	1.1	1.5	1.8	0.9	0.0	1.5	1.1	0.9	1.1	1.4	1.3	< 0.001
trans-4-hydroxyproline	88.7	73.1	47.5	38.4	46.3	49.4	51.2	91.8	65.4	60.7	53.0	44.9	45.3	42.0	< 0.001
Taurine	116	50.9	51.6	37.6	26.7	30.8	34.6	84.5	50.3	57.4	45.7	48.2	43.0	38.0	< 0.001
SM (OH) C14:1	2.9	3.6	3.9	5.1	6.6	6.7	7.7	3.4	3.6	4.1	5.4	6.2	6.7	6.8	< 0.001
SM (OH) C16:1	1.1	1.3	2.3	4.6	5.8	5.8	6.5	1.3	1.4	1.9	3.4	4.5	5.5	5.8	< 0.001
SM (OH) C22:1	4.6	9.8	10.8	6.9	7.8	7.7	8.2	5.2	8.9	10.0	10.0	10.4	10.4	8.6	< 0.001
SM (OH) C22:2	2.4	3.3	3.7	3.0	3.5	3.9	4.4	2.8	3.0	3.4	3.8	4.5	4.9	4.2	< 0.001
SM (OH) C24:1	0.5	0.6	0.8	0.8	0.9	0.9	0.9	0.5	0.6	0.7	0.8	1.0	1.1	0.9	< 0.001
SM C18:1	2.8	4.0	4.1	3.3	4.1	4.3	4.8	3.3	3.6	3.8	4.2	5.0	5.1	4.2	< 0.001
SM C22:3	0.1	0.2	0.2	0.0	0.1	0.1	0.0	0.0	0.3	0.2	0.1	0.1	0.1	0.0	< 0.001
SM C24:1	12.8	14.2	13.4	6.2	5.9	6.4	6.7	14.3	13.5	12.3	9.8	9.7	9.5	7.0	< 0.001
SM C26:0	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	< 0.001

[1]Metabolites list	[5]Tukey's HSD
Carnitine	28E-112E; 28L-112E; 42E-112E; 42L-112E; 70L-112E; 98L-112E; 28E-112L; 28L-112L; 42L-112L; 70L-112L; 140L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70L-140E; 98L-140E; 28E-140L; 28L-140L; 42L-140L; 70E-140L; 70L-140L; 28E-1E; 28L-1E; 42L-1E; 70E-1E; 70L-1E; 28E-1L; 28L-1L; 42L-1L; 70L-1L; 98L-1L; 42E-28E; 70E-28E; 98E-28E; 98L-28L; 42E-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 42L-42E; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 98L-42L; 70L-70E; 98L-70E; 98E-70L; 98L-98E
Propionylcarnitine	1E-112E; 1L-112E; 28E-112E; 42E-112E; 70E-112E; 98L-112E; 140E-112L; 140L-112L; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42L-112L; 70E-112L; 1E-140E; 1L-140E; 42E-140E; 70E-140E; 70L-140E; 98E-140E; 98L-140E; 1E-140L; 1L-140L; 28E-140L; 42E-140L; 70E-140L; 98L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 42L-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 42L-42E; 70E-42E; 70L-42L; 98L-42L; 70L-70E; 98E-70E
Propenoylcarnitine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Valerylcarnitine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 70L-28E; 98E-28E; 70E-28E; 98E-28L; 70E-42E; 98E-42E; 98E-42L
Nonaylcarnitine	
	1E-112E; 1L-112E; 1E-140E; 1L-140E; 70E-1E; 98E-1E; 98E-1L
Hexadecanoylcarnitine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42L-140E; 1E-140L; 28E-140L; 28L-140L; 42L-140L; 70E-1E; 98E-1E; 98L-1E; 70E-1L; 98E-1L; 98L-1L; 42E-28E; 70E-28E; 98E-28E; 98L-28E; 70E-28L; 98E-28L; 98L-28L; 70E-42L; 98E-42L; 98L-42L
Hydroxyhexadecadienylcarnitine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28L-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Octadecenoylcarnitine	1L-112E; 1E-112L; 1L-112L; 42E-112L; 42L-112L; 1E-140E; 1L-140E; 42E-140E; 42L-140E; 70L-140E; 1L-140L; 98E-1E; 98E-1L; 98E-42E; 98E-42L
Arginine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70L-140L; 70E-1E; 98E-1E; 70E-1L; 98E-1L; 98L-1L; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 98E-42E; 98L-42L; 70L-70E
Glutamate	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 98E-42L
Glycine	112L-112E; 1E-112E; 1L-112E; 140E-112L; 1E-112L; 1L-112L; 28E-112L; 42E-112L; 42L-112L; 70E-112L; 98E-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 98L-98E
Histidine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 70L-112E; 98L-112E; 28E-112L; 28L-112L; 42L-112L; 70E-112L; 98E-112L; 28E-140E; 28L-140E; 42L-140E; 28E-140L; 28L-140L; 42L-140L; 70E-1E; 98E-1E; 28L-1L; 42L-1L; 70E-1L; 98E-1L; 70E-28E; 98E-28E; 42E-28L; 70E-28L; 98E-28L; 98L-28L; 42L-42E; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E; 98E-70L; 98L-98E
Lysine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 98E-42E; 70L-70E
Methionine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 70E-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 70E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 70E-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 70E-140L; 42E-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70E-1L; 98E-1L; 98L-1L; 42E-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 70E-28L; 98E-28L; 98L-28L; 42L-42E; 70E-42L; 98E-42L; 70L-70E; 98E-70E; 98L-70E
Ornithine	42E-112E; 98L-112E; 42E-112L; 42E-140E; 42L-140E; 98L-140E; 42E-140L; 98L-140L; 42E-1E; 70E-1E; 42E-1L; 42E-28E; 70E-28E; 42E-28L; 42L-42E; 70E-42E; 70L-42E; 98E-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E; 98L-98E
Phenylalanine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 70L-28E; 98E-28E; 70E-28L; 70L-28L; 98E-28L; 70E-42E; 98E-42E
Proline	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-140L; 70L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 42L-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 42L-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 42E-42E; 70E-42E; 70E-42L; 70L-42L; 98E-42L; 98L-42L; 70L-70E
Serine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70L-112L; 98L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 42L-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 42L-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E

[1]Metabolites list	[5]Tukey's HSD
Threonine	1E-112E; 1L-112E; 70E-112E; 1E-112L; 1L-112L; 70E-112L; 1E-140E; 1L-140E; 70E-140E; 1E-140L; 1L-140L; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 70E-28L; 70E-42E; 70L-70E; 98E-70E; 98L-70E
Tyrosine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 70E-112L; 1L-140E; 28E-140E; 28L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-140L; 70E-1E; 98E-1E; 70E-1L; 98E-1L; 70E-28E; 98E-28E; 70E-28L; 98E-28L; 70E-42E; 70E-42L; 70L-70E; 98L-70E
Valine	1L-112E; 28E-112L; 28L-112L; 98L-140E; 28E-140L; 28L-140L; 42E-1E; 98L-1E; 42E-1L; 98L-1L; 42E-28E; 70L-28E; 98L-28E; 42E-28L; 70L-28L; 98L-28L; 98L-42L; 98L-70E
Acetylornithine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70L-112E; 98L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70L-112L; 98L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70L-140E; 98L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70L-140L; 98L-140L; 70E-1E; 98E-1E; 70E-1L; 98E-1L; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 70E-28L; 98E-28L; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E; 98E-70E; 98L-98E
Asymmetric dimethylarginine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 98E-28L; 98L-28L
Carnosine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 70E-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70E-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-140L; 70E-140L; 70L-1E; 98E-1E; 98L-1E; 70L-1L; 98L-1L; 70L-28E; 98L-28E; 70L-28L; 98L-28L; 98L-42L; 98L-70E
Creatinine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 98E-28E; 98L-28E; 98E-28L; 98L-28L
Dihydroxyphenylalanine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Dopamine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Histamine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Sarcosine	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 98E-28E; 98L-28E; 42E-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L
Symmetric dimethylarginine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
Spermine	1E-112E; 1L-112E; 1E-112L; 1L-112L; 1E-140E; 1L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L
trans-4-hydroxyproline	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 70E-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 70E-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-140L; 70L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 42L-42E; 70E-42L; 70E-42L; 98E-42L; 98L-42L; 70L-70E
Taurine	112L-112E; 1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70E-112E; 98L-112E; 1E-112L; 1L-112L; 98E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 98L-140E; 1E-140L; 1L-140L; 42L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 98E-28E; 98E-28L; 98E-42E; 70E-42L; 98E-42L; 98E-70E; 98L-98E
SM (OH) C14:1	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70E-112E; 70L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70E-140E; 70L-140E; 98L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70E-140L; 70L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 70L-42E; 98E-42E; 98L-42E; 70E-42L; 70L-42L; 98E-42L; 98L-42L; 98E-70E
SM (OH) C16:1	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70E-112E; 70L-112E; 98L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70E-140E; 70L-140E; 98L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70L-140L; 98L-140L; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 42L-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 42E-28L; 42L-28L; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 70L-42E; 98E-42E; 98L-42E; 70E-42L; 70L-42L; 98E-42L; 98L-42L; 70L-70E; 98E-70E; 98L-70E; 98L-70L; 98L-98E
SM (OH) C22:1	112L-112E; 1E-112E; 1L-112E; 28E-112E; 42E-112E; 42L-112E; 70L-112E; 98L-112E; 140E-112L; 1E-112L; 1L-112L; 70E-112L; 98E-112L; 1E-140E; 1L-140E; 42E-140E; 98L-140E; 1E-140L; 1L-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 28E-1L; 28L-1L; 42E-1L; 42L-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 98E-28E; 70E-28L; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E; 98E-70E; 98L-98E
SM (OH) C22:2	1E-112E; 1L-112E; 28L-112E; 70E-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 98E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 70E-140E; 98E-140E;

[1]Metabolites list	[5]Tukey's HSD
	1E-140L; 1L-140L; 28L-140L; 70E-140L; 28E-1E; 42E-1E; 42L-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70L-1L; 98L-1L; 98L-28E; 98L-28L; 98L-42L; 98L-70E; 98L-98E
SM (OH) C24:1	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42L-140L; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70L-28E; 98E-28E; 98L-28E; 70L-28L; 98E-28L; 98L-28L; 98E-42L; 98L-42L
SM C18:1	1E-112E; 1L-112E; 70E-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 98E-112L; 1E-140E; 1L-140E; 28L-140E; 42L-140E; 70E-140E; 1E-140L; 1L-140L; 70E-140L; 28E-1E; 28L-1E; 42E-1E; 42L-1E; 70L-1E; 98E-1E; 98L-1E; 70L-1L; 98E-1L; 98L-1L; 98L-28E; 98L-28L; 98L-42L; 70L-70E; 98L-70E
SM C22:3	28E-112E; 28L-112E; 42E-112E; 42L-112E; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70L-140E; 98L-140E; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70E-1E; 70L-1E; 70E-28E; 98E-28E; 70E-28L; 98E-28L; 70E-42E; 98E-42E; 70E-42L; 98E-42L; 98L-70E
SM C24:1	112L-112E; 1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70L-112E; 98L-112E; 140E-112L; 140L-112L; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 98E-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 70L-140E; 98L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70L-140L; 98L-140L; 70E-1E; 98E-1E; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 70E-42E; 70L-42E; 98E-42E; 98L-42E; 70E-42L; 98E-42L; 70L-70E; 98L-70E; 98E-70L; 98L-98E
SM C26:0	1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70E-112E; 70L-112E; 1E-112L; 1L-112L; 28E-112L; 28L-112L; 42E-112L; 42L-112L; 70E-112L; 70L-112L; 1E-140E; 1L-140E; 28E-140E; 28L-140E; 42E-140E; 42L-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70E-140L; 70L-140L; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 70E-1L; 70L-1L; 98E-1L; 98L-1L; 42E-28E; 70E-28E; 70L-28E; 98E-28E; 98L-28E; 70E-28L; 70L-28L; 98E-28L; 98L-28L; 98E-42E; 70E-42L; 70L-42L; 98E-42L; 98L-42L; 98E-70E; 98E-70L

[1]Metabolites with VIP > 1, FDR < 0.001, ANOVA are shown

[2]earlyC group weaned at 7 weeks of age (experimental days 28–42)

[3]lateC group weaned at 17 weeks of age (experimental days 98–112)

[4]P-values were obtained using one-way Analysis of Variance (ANOVA) and FDR-adjusted p ≤ 0.05 indicates plasma metabolites that were significantly different between the samples due to the age effect

[5]Pairwise comparisons were done using Tukey's HSD test

Table S12 | Spearman's rank correlations between morphometric variables of calves, differential faecal microbial genera and plasma metabolites of weaning groups.

		d70			
		LWG or ADG	LW (kg)	Wither height (cm)	Hip height (cm)
Faecal bacterial genera	f_Rikenellaceae	-0.52	---	---	---
	g_Parabacteroides	0.68	---	---	---
	g_Mediterraneibacter	0.59	---	0.55	0.63
	g_Prevotella	0.54	---	---	0.51
	g_Blautia	0.60	---	0.50	0.51
	o_Bacteroidales incertae sedis	0.60	0.55	0.61	0.68
	f_Lachnospiraceae	0.53	---	---	---
plasma metabolites	Histidine	0.59	---	---	0.53
	Lysine	---	---	0.53	0.52
	Methionine	0.61	0.58	0.54	0.56
	Proline	0.53	---	---	---
	Serine	---	0.59	0.54	0.61
	Threonine	0.55	0.50	---	0.54
	Tryptophan	0.56	---	---	---
	Tyrosine	0.53	---	---	---
	Acetylornithine	-0.52	---	---	---
	trans-4-hydroxyproline	---	0.51	---	---

	LWG or ADG	LW (kg)	Heart girth (cm)	Hip height (cm)	Body length (cm)
Spermidine	---	-0.61	-0.54	-0.57	
Carnitine	0.54	0.51	0.54	0.58	
SM C16:0	0.57	---	---	---	
SM C16:1	0.60	---	---	---	
SM (OH) C16:1	---	---	-0.51	-0.55	
Hexoses	0.58	---	---	---	

d98					
	LWG or ADG	LW (kg)	Heart girth (cm)	Hip height (cm)	Body length (cm)
Faecal bacterial genera					
f_*Rikenellaceae*	---	-0.59	-0.54	---	---
f_*Atopobiaceae*	---	0.53	---	---	---
g_*Olsenella*	0.55	---	---	---	---
o_*Bacteroidales incertae sedis*	---	0.59	0.51	---	---
plasma metabolites					
Leucine	---	0.64	0.61	---	0.55
Lysine	---	---	---	---	0.53
Ornithine	---	0.59	0.61	---	0.53
Threonine	---	0.51	---	---	0.51
Tryptophan	---	0.58	0.52	---	---
Tyrosine	---	0.56	---	---	0.50
Valine	---	0.54	0.51	---	---
Taurine	---	0.64	---	---	---
Acetylornithine	---	-0.53	-0.51	---	---
Asymmetric dimethylarginine	---	---	---	-0.53	---
SM C16:0	---	0.57	0.50	---	---
SM C16:1	---	0.56	0.52	---	---
SM C24:1	---	0.57	0.57	---	0.51
Hexoses	---	0.54	---	---	---

Live weight gain (LWG), average daily gain (ADG), live weight (LW)

blue to red colour scale - strong positive to strong negative correlations

Spearman's rank correlations with R > 0.50 or < -0.50 and $p < 0.05$ are shown

4.8 Acknowledgements

4.9 Author Contributions

Conceptualization: KH, JF, SD, JS; Project administration and Funding acquisition: KH, JF, SD and JS; Supervision: KH, JF, SD, ACS and JS; Writing original draft: NA, SS and JS; Methodology: NA, SS, JTM, ACS and JS; Formal analysis and software: NA, SS, ACS; Investigation and Visualization: NA, ACS and JS; Review and editing: all authors.

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N.A.

4.11 Availability of data and material

Sequences were submitted to European Nucleotide Archive under the accession number PRJEB48866.

4.12 Ethics approval and consent to participate

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (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, Germany, file No.: 33.19-42502-04-15/1858).

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CHAPTER V

DISCUSSION

5. DISCUSSION

5.1 Buccal swabbing (BS) technique as an alternative for invasive rumen sampling procedures

Rumen is the largest compartment of ruminant's forestomach system, responsible for major feed digestion and metabolites synthesis through the activity of diverse microbial communities (Mackie, 2002). The establishment of a fully-functional rumen and its associated forage- and concentrate-degrading microorganisms is essential for smooth weaning transitions. The present animal experiment monitored the dynamic progression of oral, rumen and faecal bacterial communities of 59 female Holstein calves with repeated and long-term sampling (over 140 days). All the rumen sampling procedures are highly invasive and are not preferable when frequent and prolonged monitoring of single animals are required, thus, highlighting the need of some non-invasive rumen sampling procedures. Based on the regurgitation activity of ruminant, which enables them to bring the partially digested rumen contents back to the mouth and the results of some previous studies (Kittelmann et al., 2015; Tapio et al., 2016), it was hypothesized that the oral samples might serve as non-invasive alternative to predict rumen microbiota (Chapter 3). The proof of concept was done by comparing the rumen bacterial communities of 140-day-old calves with the bacterial communities of their buccal swabs collected at days 42, 70, 98, 112 and 140 of the experimental trial. A large number of operational taxonomic units (OTUs) were shared between the two sampling methods used (Figure 20).
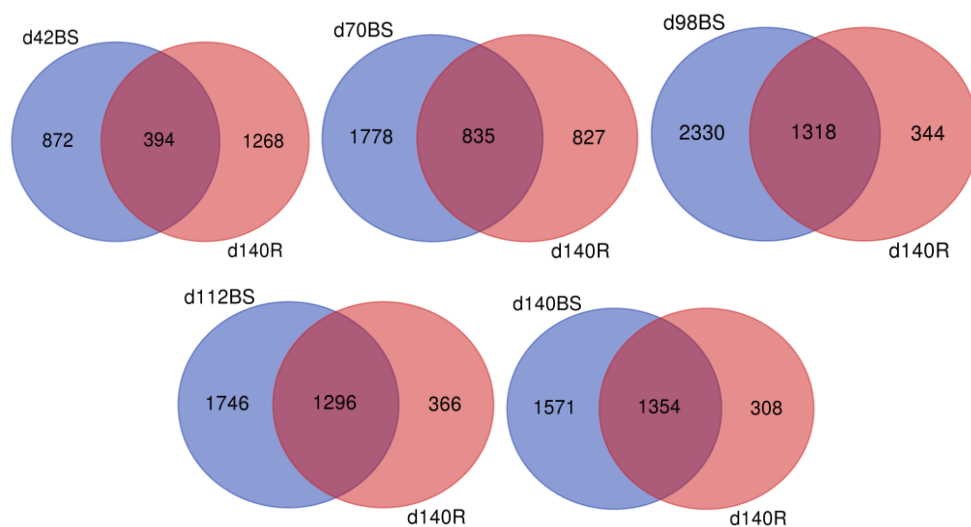
Figure 20 | Overlap of OTUs covering V1-V2 region of bacterial 16S rRNA. A total of 4906

OTUs identified in the rumen fluid (R) and buccal swab (BS) samples collected from 42, 70, 98, 112 and 140-day-old calves were included in the Venn diagram.

These shared OTUs were taxonomically assigned to various genus-level taxa, commonly found in the rumen and buccal swabs, though relative abundance varied (Table 2).

Table 2 | Average relative abundances of bacterial taxa shared between R and BS samples of different age groups of calves. Taxonomic designations were based on RDP database.

| | | Average relative abundance (%) | | | | | |
| | | d42BS | d70BS | d98BS | d112BS | d140BS | d140R |
Taxa	OTUs	($n = 11$)	($n = 26$)	($n = 51$)	($n = 51$)	($n = 47$)	($n = 47$)
o_*Clostridiales*	523	4.4	2.4	2.8	4.2	4.3	13.4
f_*Lachnospiraceae*	456	9.2	4.1	3.3	5.1	2.7	8.6
o_*Bacteroidales*	439	5.8	4.1	4.6	7.1	5.0	6.2
p_*Bacteroidetes*	372	5.5	3.9	4.7	8.2	9.3	23.6
k_*Bacteria*	307	1.8	1.6	1.4	1.2	1.3	5.7
f_*Ruminococcaceae*	297	0.7	0.9	1.0	1.1	1.0	6.5
f_*Prevotellaceae*	295	20.1	6.1	5.6	7.5	6.6	8.0
p_*Firmicutes*	214	2.2	0.9	0.7	1.0	0.6	2.2
g_*Prevotella*	125	5.5	2.6	4.4	4.1	4.0	6.7
p_*Proteobacteria*	65	0.0	0.2	0.3	0.7	0.4	3.2
c_*Clostridia*	63	0.1	0.1	0.2	0.1	0.1	0.6
c_*Bacteroidia*	56	0.1	0.2	0.2	0.4	0.3	0.7
c_*Gammaproteobacteria*	50	0.3	0.6	0.5	0.4	0.3	0.2
c_*Saccharibacteria genera incertae sedis*	33	0.0	0.3	0.2	0.2	0.4	1.9
g_*Olsenella*	25	4.4	1.1	0.9	0.3	0.3	0.6
f_*Erysipelotrichaceae*	24	0.6	0.5	0.4	0.1	0.1	0.5
g_*Bifidobacterium*	23	1.0	1.3	1.3	1.0	0.7	2.8
g_*Fibrobacter*	20	0.0	0.1	0.3	0.4	0.8	0.7
c_*SR1 genera incertae sedis*	19	0.0	0.4	0.3	0.7	0.7	2.8
g_*Butyrivibrio*	18	0.2	0.4	0.3	0.5	0.6	1.3
g_*Ruminococcus*	14	0.0	0.0	0.0	0.0	0.1	0.3
c_*Alphaproteobacteria*	14	0.0	0.0	0.1	0.1	0.1	0.3
g_*Succiniclasticum*	12	0.9	0.6	0.4	1.1	1.0	1.4
g_*Pseudobutyrivibrio*	4	0.0	0.1	0.1	0.2	0.1	0.5
g_*Ruminobacter*	2	0.0	0.1	0.1	0.0	1.2	0.3

In conclusion of the study conducted in chapter 3, it has been confirmed that the BS technique has a potential to be used as a proxy for predicting rumen microbial communities of large animal cohorts over a longer time span without the need of stomach tubing or slaughtering. In contrast to the present evaluation, the work by Kittelmann and colleagues was based on samples collected from 24 sheep and only a single collection time (Kittelmann et al., 2015). Similarly, the study of Tapio and colleagues, was also limited by the number of animals being used (5 cows) and the sampling time (Tapio et al., 2016). The rumen microbiome is unstable and is affected by the host breed, age, diet, as well as sampling location, post-feeding sampling time, and other environmental factors (Li et al., 2009; Petri et al., 2012; Jami et al., 2013; Paz et al., 2016; Deusch et al., 2017). In addition to these factors, animal-to-animal variations in microbial communities exists naturally even under controlled diet and environmental conditions probably due to their body conditions, health status, and animal history, thus limiting the statistical power of a study, especially when minor differences between treated groups are expected. Therefore, large number of samples are needed in animal studies. In accordance, this study collected BS samples from 59 female Holstein calves at 5 different timepoints over a period of 140 days, thus, enabling us to monitor the initial establishment of oral and rumen microbial communities from 42 days of calf's life until the end of weaning period.

5.2   A core rumen microbiota of developing calves based on BS samples

Besides the establishment of BS technique as a proxy for predicting rumen microbial communities (chapter 3), this study also defined for the first time the rumen core microbiome of developing calves based on BS samples (Amin et al., 2021). By a highly strict definition, only those OTUs that were ubiquitously observed during each timepoint (days 70, 98, 112 and 140) were counted to a core rumen microbiome, resulting in a total of 614 core bacterial OTUs that were taxonomically assigned to 8 phyla and 27 genera, including *Prevotella* (57 OTUs), *Butyrivibrio* (11 OTUs), *Bifidobacterium* (8 OTUs), *Succiniclasticum* (7 OTUs), and unclassified members of *Bacteroidetes* (96 OTUs), *Prevotellaceae* (91 OTUs), *Bacteroidales* (78 OTUs), *Clostridiales* (68 OTUs), *Lachnospiraceae* (64 OTUs), *Ruminococcaceae* (19 OTUs), *Saccharibacteria genera incertae sedis* (14 OTUs), *Firmicutes* (13 OTUs), and *SR1 genera incertae sedis* (7 OTUs) were the "dominant" rumen bacteria among all samples (Amin et al., 2021). These rumen-specific (RS) taxa have also been reported in other calves-based studies (Li et al., 2012; Jami et al., 2013; Rey et al., 2014; Kim et al., 2016; Meale et al., 2016; Dias et al., 2017; Dill-McFarland et al., 2017; Meale et al., 2017a; Dias et al., 2018; Lin et al.,

2018; Zhang et al., 2019). Based on the results of rumen core microbiome study by Henderson and colleagues using 32 species of ruminant from 35 countries, *Prevotella*, *Butyrivibrio*, unclassified members of *Bacteroidales, Lachnospiraceae*, and *Ruminococcaceae* were reported to be the "dominant" core bacteria in the rumen. Similarly, in a recent study to access the stability of rumen microbial communities in single lactation dairy cows revealed 176 bacterial OTUs to be stable in bovine rumen. These stable bacterial OTUs were taxonomically associated to *Prevotella* (36 OTUs), unclassified members of *Prevotellaceae* (27 OTUs), *Bacteroidales* (25 OTUs), and *Lachnospiraceae* (15 OTUs) (Zhu et al., 2021).

Taking a closer look at the predominant core *Prevotella* genus in the present study and its association with core and stable rumen microbiome in other studies (Henderson et al., 2015; Zhu et al., 2021)*,* the importance of these bacteria in the rumen of developing calves can be emphasized. Therefore, the 57 OTUs that were previously assigned to the *Prevotella* genus based on RDP database (Cole et al., 2014) were compared with NCBI non-redundant nucleotide database using BLAST (Bazinet et al., 2018), and the resulting BLAST table was filtered at a sequence identity threshold of 97.0% for species-level identifications. A total of 14 OTUs were classified as *Prevotella ruminicola*, 2 OTUs as *Prevotella bryantii B14*, 1 OTU as *Prevotella albensis*, and 1 OTU as *Prevotella brevis* (Table 3).

Table 3| Average relative abundances of core *Prevotella* species in R and BS samples of different age groups of calves.

Identified taxa	OTUs	Average Relative Abundance (%)				
		d70BS	d98BS	d112BS	d140BS	d140R
g_*Prevotella*	39	3.33	5.51	4.28	4.06	3.88
s_*Prevotella ruminicola*	14	1.19	2.10	2.05	2.88	1.58
s_*Prevotella bryantii B14*	2	0.12	0.19	0.14	0.34	0.33
s_*Prevotella albensis*	1	0.01	0.05	0.07	0.02	0.12
s_*Prevotella brevis*	1	0.01	0.03	0.01	0.01	0.00

Similar to the present study, Malmuthuge and colleagues also observed an increase in the active *P. ruminicola* density with the age of calves (Malmuthuge et al., 2019). *Prevotella* members are the most common rumen microbes (Stevenson and Weimer, 2007; Henderson et al., 2015), showing a vast variety of functions from fibrolytic, cellulolytic, and amylolytic activities to ruminal protein and peptide degradation (Avgustin et al., 1994; Avgustin et al., 1997; Bekele et al., 2010). *Prevotella ruminicola* and *Prevotella bryantii* play an essential role

in volatile fatty acids biosynthesis (Osborne and Dehority, 1989; Chiquette et al., 2008) and an increase in the concentration of milk fat (Chiquette et al., 2008). In addition to the degradation of ingested feed particles, the positive associations of *Prevotella* with average daily gains in calves (Lourenco et al., 2019), and ruminal microbial metabolites involved in carbohydrate and amino acid metabolism have recently been reported (Xue et al., 2020). This functional flexibility of *Prevotella* demands further investigation to understand the multi-faceted role of this genus members in the developing rumen of young calves, especially during weaning transitions.

Additionally, the high contribution of unclassified members of *Prevotellaceae*, *Lachnospiraceae*, and *Bacteroidales* towards the core rumen microbiome in the present study and other studies (Henderson et al., 2015; Zhu et al., 2021), and their associations with the dairy cows gross feed efficiency (Jewell et al., 2015), suggesting their essential and conserved functionality to their ruminant host.

5.3 Implication of BS technique for long-term monitoring of animal rumen microbiome

In order to access whether the BS approach mentioned in chapter 3 can be used to predict the rumen microbial communities of older animals. The bacterial communities of 5-month-old BS samples were compared with the bacterial communities of the rumen samples collected by stomach tubing from the same animals at 5, 7, 10, and 18 months of age (Figure 21).
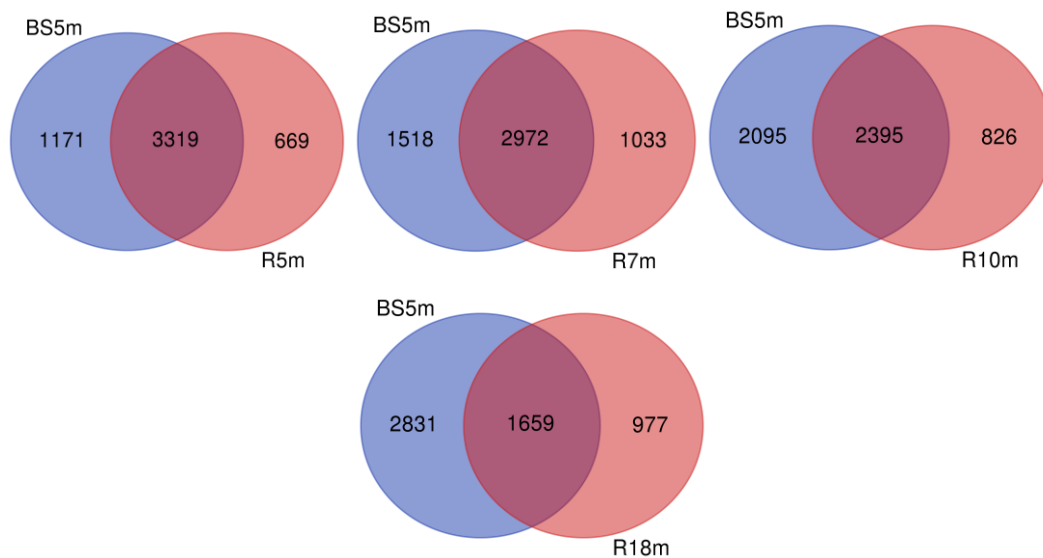
Figure 21 | Overlap of ASVs covering V1-V2 region of bacterial 16S rRNA. A total of 5908

ASVs identified in the BS and R samples collected from 5, 7, 10 and 18-month-old calves were included in the Venn diagram.

The 5-month-old calves BS samples shared large number of amplicon sequence variants (ASVs) with the rumen (R) samples of 5–18-month-old calves. These shared ASVs were taxonomically assigned to various genus-level taxa commonly found in BS and all R samples (5–18-months), though relative abundance varied (Table 4).

Table 4 | Average relative abundances of bacterial taxa shared between BS and R samples of 5–18-month-old calves. Taxonomic designations were based on SILVA database (release 138.1). The genus-level taxa with average relative abundance of > 1% in at least 1 age group are shown.

Taxa	ASVs	Average relative abundance (%)				
		BS5m $(n = 59)$	R5m $(n = 51)$	R7m $(n = 54)$	R10m $(n = 51)$	R18m $(n = 56)$
g_*Prevotella*	650	10.0	14.8	12.8	17.8	23.4
g_*Rikenellaceae RC9 gut group*	307	3.3	7.6	8.8	10.1	9.9
g_*Christensenellaceae R-7 group*	300	1.2	7.1	6.6	9.0	3.4
f_*F082*	239	2.5	9.7	8.2	2.9	5.9
o_*Clostridia UCG-014*	205	0.5	4.4	1.9	2.7	1.7
g_*Treponema*	165	0.3	1.4	3.3	0.5	1.7
g_*Ruminococcus*	160	0.4	3.8	5.5	4.4	1.0
g_*Prevotellaceae UCG-003*	112	0.6	0.7	1.3	1.3	1.6
g_*Candidatus Saccharimonas*	110	0.3	2.2	1.2	2.2	1.1
o_*RF39*	109	0.1	1.6	0.5	0.3	0.5
g_*NK4A214 group*	107	0.4	2.4	2.5	5.0	1.3
g_*Prevotellaceae UCG-001*	93	0.5	1.5	2.3	0.8	2.6
f_*Muribaculaceae*	93	1.0	1.6	1.2	0.6	0.6
f_*Lachnospiraceae*	91	0.5	2.9	0.7	0.7	0.9
g_*Lachnospiraceae NK3A20 group*	60	0.6	1.5	2.3	3.6	1.0
g_*Acetitomaculum*	58	0.1	0.8	1.0	0.8	0.4
g_*Lachnospiraceae AC2044 group*	57	0.3	0.6	0.5	0.7	1.1
g_*Butyrivibrio*	55	0.3	1.5	0.4	1.2	1.2
g_*Saccharofermentans*	53	0.3	1.1	0.6	1.1	1.3
c_*Clostridia*	48	0.2	1.0	1.6	2.2	1.8
g_*Fibrobacter*	46	0.7	0.6	0.8	1.4	2.5

Taxa	ASVs	Average relative abundance (%)				
		BS5m (*n* = 59)	R5m (*n* = 51)	R7m (*n* = 54)	R10m (*n* = 51)	R18m (*n* = 56)
f_*[Eubacterium] coprostanoligenes group*	41	0.1	0.4	0.8	1.8	2.3
f_*UCG-011*	38	0.1	0.9	0.9	1.4	1.9
g_*UCG-004*	38	0.1	0.5	0.3	0.2	1.0
g_*Anaeroplasma*	36	0.0	0.4	0.1	0.1	1.0
g_*Acinetobacter*	34	2.4	0.0	0.0	0.1	0.0
g_*Corynebacterium*	27	4.0	0.0	0.1	0.0	0.0
g_*Sphingobacterium*	25	1.1	0.1	0.0	0.0	0.0
o_*Absconditabacteriales (SR1)*	24	0.3	2.3	2.1	1.8	1.9
g_*Pseudobutyrivibrio*	22	0.1	0.7	0.6	1.3	1.3
g_*Staphylococcus*	20	1.7	0.2	0.0	0.0	0.3
f_*Bacteroidales BS11 gut group*	20	0.1	1.4	0.5	1.3	1.6
g_*Succiniclasticum*	13	0.6	1.1	1.4	2.5	2.3
g_*Bifidobacterium*	13	0.7	2.4	1.1	0.5	0.0
g_*Succinivibrionaceae UCG-002*	13	0.1	0.3	0.9	1.4	1.7
g_*Porphyromonas*	11	1.1	0.0	0.0	0.0	0.0
g_*Burkholderia-Caballeronia-Paraburkholderia*	8	0.4	0.0	2.5	0.0	0.0
g_*Succinivibrionaceae UCG-001*	5	0.1	1.3	0.6	0.0	0.0
g_*Ralstonia*	3	5.5	0.0	0.3	0.0	0.0
g_*Cutibacterium*	2	0.0	0.0	1.0	1.0	0.1

Prevotella was the most dominant genus (650 ASVs) in both sampling methods (BS *vs.* R) as well as during each developmental stage. Since, *Prevotella* members occupies a large portion of the bovine rumen total bacterial populations (60%) (Stevenson and Weimer, 2007) and are the most common rumen microbes irrespective of the diet (Henderson et al., 2015). Therefore, this age-dependent increase in the abundance of *Prevotella* in the present study is understandable.

5.4 Potential drawbacks of BS technique

Although, the BS samples used in the present study shared large number of ASVs / OTUs with the R samples, though relative abundances of taxa assigned to these ASVs / OTUs varied between the sampling methods. Therefore, the suitability of BS technique as a substitute for invasive rumen sampling procedures must be carefully evaluated. As discussed in chapter 3, the oral hygiene of each individual animal as well as the post-feeding sampling time is

important to increase the predictive power of the BS procedure. That means that larger the gap between regurgitation activity and buccal swabbing, the higher the chances of obtaining oral bacteria in the BS approach (Amin et al., 2021). In conclusion, the study conducted in chapter 3 added significant information to the previous observations (Kittelmann et al., 2015; Tapio et al., 2016) and broadened the concept of applying the BS approach on a large number of animals and various sampling time points. However, there is still the need of establishing reference database of ruminants-associated oral bacteria, as the mathematical approach used in the present study and by Kittelmann and colleagues (Kittelmann et al., 2015) requires rumen samples as a reference for bioinformatic filtration of potential rumen bacteria from the BS datasets, thus, defeating the purpose of BS approach. Such reference database is not available at this stage, therefore, BS approach may be powerful tool for large-scale predictive ruminant studies, where direct ruminal access is not possible.

5.5   Development of stable microbial communities with age

The neonatal calves' gastrointestinal tract (GIT) relies on hindgut for digestion due to poorly-developed forestomach system (Davis and Drackley, 1998). The development and maturation of the reticulo-rumen and the associated microorganisms is influenced by several factors as discussed in chapter 2, including the maternal influence, host genetics, age, diet, physiological state, weaning, usage of antibiotics and feed supplements as well as other environmental factors, thus, leading to region- and site-specific establishment of microorganisms (Amin and Seifert, 2021). The GIT microbial communities in turn interacts with their host and are involved in host mucosal epithelium development and immune system maturation (Sommer and Bäckhed, 2013).

Up-to-date, many studies explored the age-dependent development of the GIT communities in calves (Uyeno et al., 2010; Edrington et al., 2012b; Li et al., 2012; Malmuthuge et al., 2012; Mayer et al., 2012; Jami et al., 2013; Oikonomou et al., 2013; Klein-Jöbstl et al., 2014; Rey et al., 2014; Dill-McFarland et al., 2017; Dias et al., 2018; Koringa et al., 2019; Hang et al., 2020; Kim et al., 2021), however, most of these studies were limited by the number of animals being sampled and the sampling procedures used i.e., need of animal slaughtering or invasive sampling procedures. In contrast, the current studies (chapter 3 and 4) monitored the age-dependent progression of the oral, rumen and faecal bacterial communities of 59 female Holstein calves using non-invasive sampling procedures (Figure 22).

p_*Actinobacteria*
g_*Olsenella*
f_*Prevotellaceae*
f_*Lachnospiraceae*

p_*Bacteroidetes*
p_*Fibrobacteres*
p_*Proteobacteria*
p_*candidatus Saccharibacteria*
p_*SR1*
c_*Saccharibacteria genera incertae sedis*
c_*SR1 genera incertae sedis*
o_*Bacteroidales*
g_*Fibrobacter*
g_*Ruminobacter*

Rumen

Buccal cavity

p_*Firmicutes*
p_*Actinobacteria*
f_*Lactobacillaceae*
g_*Butyricicoccus*
g_*Faecalicatena*
g_*Prevotella*
g_*Mediterraneibacter*
s_*Bifidobacterium longum*
s_*Gallibacterium anatis*
s_*Lactobacillus amylovorus*
s_*Lactobacillus ingluviei*
s_*Ligilactobacillus salivarius*
s_*Streptococcus gallolyticus*
s_*Bacteroides uniformis*
s_*Barnesiella intestinihominis*
s_*Blautia wexlerae*
s_*Faecalibacterium prausnitzii*
s_*Phocaeicola vulgatus*
s_*Prevotella copri*

p_*Fusobacteria*
f_*Flavobacteriaceae*
f_*Porphyromonadaceae*
g_*Corynebacterium*
g_*Acidaminococcus*
g_*Roseburia*
g_*Anaerostipes*
g_*Bacteroides*
g_*Actinomyces*
g_*Selenomonas*
g_*Blautia*
g_*Ruminococcus 2*

Faeces

p_*Bacteroidetes*
p_*Spirochaetes*
p_*Elusimicrobia*
c_*Bacteroidia*
c_*Clostridia*
o_*Bacteroidales*
o_*Clostridiales*
o_*Sphingobacteriales*
f_*Bacteroidaceae*
f_*Eubacteriaceae*
f_*Muribaculaceae*
f_*Oscillospiraceae*
f_*Prevotellaceae*
f_*Ruminococcaceae*
f_*Rikenellaceae*
f_*Tannerellaceae*
s_*Bifidobacterium pseudolongum*

p_*Proteobacteria*
g_*Acinetobacter*
g_*Burkholderia*
g_*Rhizobium*
g_*Arthrobacter*
g_*Aerococcus*
g_*Variovorax*
g_*Clavibacter*

Figure 22 | Summary of the age-dependent decrease (red) or increase (black) in the abundances of gut bacterial communities of the female German Holstein calves from 8-days to 5-month of age. Figure created with BioRender.com.

5.5.1 Colonization of microbes in the buccal cavity of calves

Mouth is a complex habitat, harbouring microorganisms on the soft mucosal tissues and hard teeth surfaces. Oral microbiome not only plays an essential role in initiation of digestion but are also important for maintenance of oral and systemic health of the host (Deo and Deshmukh, 2019). However, there is still lack of knowledge about the ruminants-associated oral bacteria especially in young calves. Besides an age-dependent increase in the abundance

of *Burkholderia*, previously associated with the oral cavity of healthy cattle (Borsanelli et al., 2018), the present data reported a continuous age-dependent decrease in the abundances of certain pathogens including *Actinomyces*, *Corynebacterium*, unclassified members of *Flavobacteriaceae* and *Porphyromonadaceae*. *Actinomyces* species are widely distributed from oral cavity to the intestine. The changes in *Actinomyces* composition and its interaction with other microbial species can cause numerous diseases of alimentary tract i.e., periodontal disease (Li et al., 2018). *Corynebacterium* are colonizer of membranes and skin in both humans and animals (Tsuzukibashi et al., 2015), with several infectious species including *Corynebacterium bovis,* responsible for bovine mastitis (Gonçalves et al., 2016). The bacteria belonging to *Flavobacteriaceae* have previously been isolated from the oral cavity of humans and animals (Brenner et al., 1989; Vandamme et al., 1996) and genera such as *Flavobacterium* and *Bergeyella* are causative agents of dental caries (Jiang et al., 2013). Similarly, species belonging to *Porphyromonadaceae* can be ubiquitously found in the oral cavities and intestinal tract of humans and animals, with some species causing infections (Sakamoto, 2014). Thus, the decrease in the abundance of certain pathogenic oral bacterial genera with age might indicate the age-dependent maturation of the host immune system.

5.5.2   Establishment of rumen bacterial communities in calves

Rumen is the largest compartment of ruminant's forestomach system, which is mainly responsible for the digestion of feed particles. The GIT in new born calves has smaller proportions of rumen than in adult cattle, and lack rumen wall villi, responsible for nutrient absorption (Meale et al., 2017a). The first year of calf's life is critical for the development and maturation of their forestomach system and immunity. The microbial colonization of neonatal calves GIT begins right after birth with the concentration of microbes in rumen fluid as 10^9 cells/ml, and the strict anaerobic bacteria becoming dominant a day after birth (Fonty et al., 1989). Not only the rumen microbial communities change with the age of calves, an increase in rumen volume, papillae size and shape are observed, which provides better environment for rumen microbial colonisation and functioning (Li et al., 2011). Thus, the development of rumen in neonatal calves is linked to age and diet of the host, the rumen microbial community establishment and synthesis of the microbial metabolic products. The results of the present study in chapter 3 showed that the ruminal bacterial communities of 7-weeks to 5-month-old calves (experimental days 42–140) were dominated by phylum *Bacteroidetes* (54.77%), *Firmicutes* (28.82%), *Actinobacteria* (8.71%) and *Proteobacteria* (4.71%), with median

relative abundance value shown in brackets. As discussed in chapter 3, the relative abundance of phylum *Actinobacteria* and its dominant genus *Olsenella* decreased with age. *Actinobacteria* are generally associated with newborn calf's rumen (Jami et al., 2013; Rey et al., 2014), showing a decrease in abundance and compositional changes with age of animal (Jami et al., 2013), suggesting their essential role in digestion of milk component of neonate's gut.

In contrast to the phylum *Actinobacteria*, the present study also reported an increase in the abundances of phylum *Bacteroidetes, Fibrobacteres,* and *Proteobacteria* and the corresponding genera unclassified *Bacteroidales, Fibrobacter,* and *Ruminobacter* from 7 weeks to 5-months of age. Similarly, few other studies have also reported a high dominance of phylum *Bacteroidetes* in the rumen of 3-day-old (Rey et al., 2014), 42-day-old (Li et al., 2012) and two-month-old pre-weaned calves (Jami et al., 2013). This confirms that the phylum-level composition of pre-weaned calf's rumen is the same as the rumen of post-weaned calves, though the abundances of dominant phyla varies based on their developmental stage (Jami and Mizrahi, 2012). Phylum *Fibrobacteres* comprises of major cellulose-degrading bacteria in the rumen (Ransom-Jones et al., 2012). These fiber-utilizing bacteria are reported to be low abundant in the rumen of milk-replacer fed pre-weaned calves (Li et al., 2012), but as the proportions of concentrate feed decreases in the diet their abundance increases (Lourenco et al., 2020). Thus, the age-dependent increase in the abundance of rumen *Fibrobacteres* and other fiber-utilizing bacteria were in accordance with the dietary addition of hay and a total mixed ration with age.

5.5.3 Progression of calves' faecal bacterial communities from 1 week to 5 months of age

Neonatal calves are considered functionally as non-ruminants (Baldwin et al., 2004), fed with a milk-based diet, which is mainly digested in their hindgut (Guilloteau et al., 2009). The bacterial colonization of calves GIT begins right after birth or even during the birthing process. Bacteria can be found in neonatal calves' faeces as soon as after 12 hours of birth (Klein-Jöbstl et al., 2014), but the microbial composition is dynamically changing during the pre-weaning period (Uyeno et al., 2010; Edrington et al., 2012a; Oikonomou et al., 2013), until the establishment of a complex and dense microbial ecosystem with age (Uyeno et al., 2010). The GIT microbial communities are essential for digestion of feed particles, absorption of nutrients, GIT development and protection against pathogens that causes diarrhoea and other infectious diseases in calves (O'Hara and Shanahan, 2006; Morgavi et al., 2015). Therefore, it is essential to understand the development of healthy intestinal microbiota during the pre-

weaning period to reduce the mortality rate of calves caused by diarrhoea and other infectious intestinal diseases.

The results of the study in chapter 4 showed the establishment of dense microbial communities in the faeces of 8-day-old (experimental day 1) pooled herd milk and milk replacer fed Holstein calves, dominated by phyla *Firmicutes and Actinobacteria* and lactose- and starch-degrading bacterial species including *Bifidobacterium longum, Lactobacillus amylovorus, Lactobacillus ingluviei, Ligilactobacillus salivarius,* unclassified members of *Butyricicoccus, Lactobacillaceae,* and *Mediterraneibacter*. Bifidobacteria and Lactobacilli are carbohydrate-utilizing bacteria commonly observed in the GIT of pre-weaned calves (Kelly et al., 2016). A high abundance of *Lactobacillus* was reported in calves' faeces within the first three weeks of life (Klein-Jöbstl et al., 2014), showing a decrease in prevalence with age (Oikonomou et al., 2013; Klein-Jöbstl et al., 2014; Dill-McFarland et al., 2017; Virgínio Júnior and Bittar, 2021) as well as with the reduction of milk feeding, and weaning event (Vlková et al., 2006; Uyeno et al., 2010; Oikonomou et al., 2013). There is an increasing evidence about the health benefits of Bifidobacteria and Lactobacilli species such as *Lactobacillus ingluviei*, previously related with weight gain (Uyeno et al., 2010; Million et al., 2012). These bacterial genera when used as probiotics supplement can protect host GIT against infectious diseases including diarrhoea (Servin, 2004; Timmerman et al., 2005). Thus, it can be suggested that the high prevalence of these carbohydrate-utilizing bacteria in the young calves' faeces was due to milk feeding as the addition of hay and TMR resulted in a decreased abundance over time as suggested by (Vlková et al., 2008). The present study also reported a high abundance of certain opportunistic pathogenic bacteria in the faeces of 8-day-old calves such as *Streptococcus gallolyticus*, associated with meningitis and purulent lesions in neonatal calves (Aydın et al., 2019) and *Gallibacterium anatis*, involved in cattle respiratory diseases (Van Driessche et al., 2020). However, the abundances of these pathogenic bacteria decreased as the calves aged. Thus, it can be assumed that the colonization of neonatal gut with beneficial bacterial genera and the decreased abundances of pathogenic bacteria in the gut of older calves indicates a balanced health status of the intestine.

Similar to the microbial communities of other GIT compartments, the faecal bacterial composition of dairy calves also undergoes dynamic changes during the first 5 months of life (Amin et al., under review in Animal Microbiome Journal). These changes include the appearance of new species such as *Bacteroides uniformis, Barnesiella intestinihominis*, *Blautia wexlerae*, *Faecalibacterium prausnitzii, Phocaeicola vulgatus, Prevotella copri,* and unclassified *Faecalicatena* during 5–7-weeks of age (experimental days 28–42), and

unclassified members of fiber-degrading bacteria such as *Bacteroidia*, *Bacteroidales*, *Bacteroidaceae*, *Clostridia*, *Clostridiales*, *Eubacteriaceae*, *Muribaculaceae*, *Oscillospiraceae*, *Prevotellaceae*, *Ruminococcaceae, Rikenellaceae, Sphingobacteriales,* and *Tannerellaceae* at 5-months of age, suggesting that both diet and the age-dependent gut development may drive changes in the bacterial community composition during early life. High abundances of *F. prausnitzii* were reported in calves' faeces within the first 4 weeks of life with a decrease over time (Klein-Jöbstl et al., 2014; Kim et al., 2021). The increase in intensity of *Bacteroides* spp. were observed after 7-days of life (Mayer et al., 2012), showing highest prevalence within the first 3-weeks (Klein-Jöbstl et al., 2014) and even after the fourth week of calf life (Oikonomou et al., 2013). Similar high dominance of *Faecalibacterium*, *Bacteroides, Blautia*, and *Prevotella* in the faeces of milk replacer-fed pre-weaned calves (Uyeno et al., 2010) and the positive associations of faecal *F. prausnitzii and Bacteroides* abundances with lower incidence of diarrhoea and weight gain in pre-weaned calves have been reported (Oikonomou et al., 2013; Foditsch et al., 2015; Hennessy et al., 2021). In addition, *B. uniformis, B. wexlerae, P. copri* and *Barnesiella* are essential for host gut functionality and health due to their role in maintenance of host intestinal immune homeostasis (Brooke et al., 2019; Benítez-Páez et al., 2020) and protection against GIT inflammatory diseases (Mazmanian et al., 2005; Weiss et al., 2014). Thus, it is possible to assume that the age-dependent decrease in major lactic-acid producing bacteria in the present study was due to the decreased milk consumption and increased fiber ingestion, that resulted in lower availability of nutrients for the lactose- and starch-degrading bacterial species with age of calves.

5.6   Impact of early *vs.* late weaning on the development of microbiome and metabolome in calves

The ruminant's forestomach system becomes functional within the first few months of life through processes initiated by intake of solid-feed and activity of rumen microorganisms (Warner et al., 1956). In order to meet the demand of milk and meat consumption and reduce the early-life feed costs, dairy calves raised in commercial farms are usually fed with restricted amounts of liquid diet (milk, or milk replacer) in order to stimulate the intake of starter concentrate and thus, accelerating rumen development (Dias et al., 2018). However, the transition from liquid-diet to solid-feed is critical for GIT development and its associated microorganisms, thus, affecting the growth and production performances of calves during the post-weaning stage (Dias et al., 2018). As discussed in chapter 2 (Amin and Seifert, 2021), the age of animal during weaning clearly influences the development of their forestomach system.

Weaning calves at an early age (6 weeks) may result in pre-mature development of rumen and reduced growth rate and feed intake as compared to the late-weaning at 8 weeks of age (Eckert et al., 2015; Meale et al., 2017b). Thus, the selection of an appropriate weaning age is essential to minimise the side effects. The present study also monitored the impact of early- *vs.* late-weaning (earlyC *vs.* lateC; 7 *vs.* 17 weeks of age) on the oral, rumen and faecal microbial communities by sampling calves at 7 different time points over a period of 140 days (Chapter 3 and 4) (Figure 23).
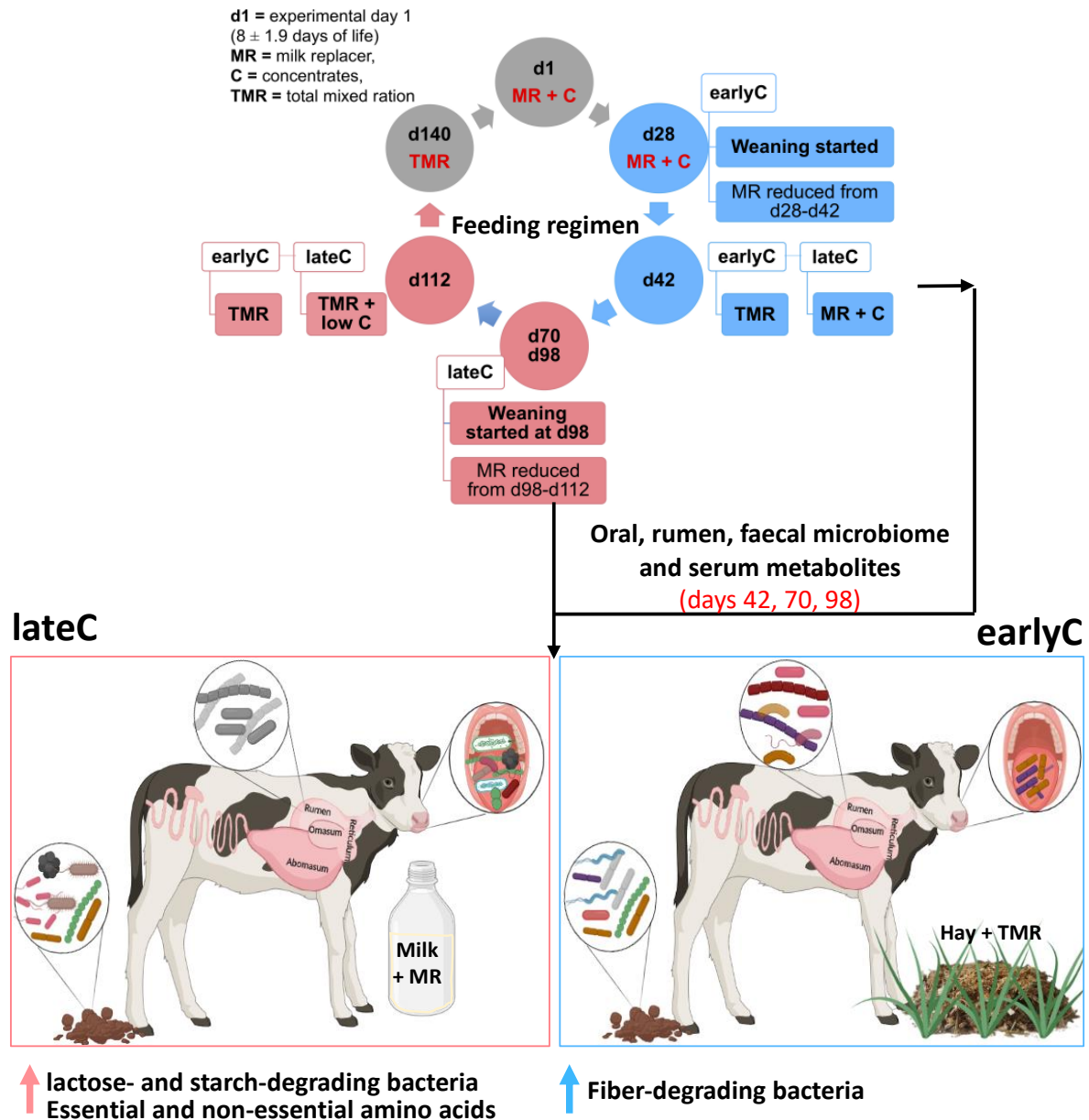

Figure 23 | Summary of the feeding regimen during the experimental trial and weaning-dependent modification in the gut bacterial communities and plasma metabolites of the earlyC and lateC calves during experimental days 42−98. Figure created with BioRender.com.

Calves were gradually weaned in a 14-days stepdown approach during experimental days 28−42 for earlyC and days 98−112 for lateC group, followed by the ad libitum addition of hay and total mixed ration comprising of grass silage (48%), maize silage (32%), and concentrate feed (20%) (Amin et al., 2021). A clear impact of weaning time on the oral and faecal microbiome was observed during experimental days 42−98 (7−15 weeks of age), and the rumen microbiome during days 70−98 (11−15 weeks of age) (Figure 23). As discussed in chapter 3 and 4, the early introduction of roughages in the diet of pre-weaned calves at 7 weeks of age resulted in higher abundances of bacterial genera such as *Kurthia*, which was reported in the intestine of high-roughage-fed cattle (Maki and Picard, 1965), and *Butyrivibrio*, which belongs to the class *Clostridia*, involved in fiber- and protein-degradation and butyrate production in the rumen (Krause et al., 2003; John Wallace et al., 2006). An increase in the abundance of *Butyrivibrio* with higher fiber intake (Mrázek et al., 2006; Chuang et al., 2020) and their positive association with papillae length in the rumen have also been reported (Yang et al., 2018). The current study also reported an increase in the abundances of several unclassified members of phylum *Bacteroidetes* (class *Bacteroidia*), *Firmicutes* (class *Clostridia*, *Sphingobacteria*), and *Spirochaetes* (class *Spirochaetia*) in the post-weaned rumen and faecal microbiota of earlyC group and it was probably diet-dependent, as high dominance of rumen *Bacteroidia* and *Clostridia* was observed in Moxotó goat that were grazing on plant fibers (Cunha et al., 2011). Similarly, Petri and colleagues also reported high abundance of *Clostridia* using forages- and mixed forages-based diets (Petri et al., 2013), thus, suggesting the essential role of these bacteria in the degradation of plant fibers (Cunha et al., 2011). In addition to plant fiber degradation, the positive association of faecal *Clostridia* abundance with butyrate production have also been recently reported (Guo et al., 2020), which is the primary source of energy for the neonatal gut epithelial cells (Pryde et al., 2002). Thus, the observed high abundances of fiber-degrading bacteria in the post-weaned microbiota of earlyC group in current study was due to the addition of roughages in their diet post-weaning.

In addition to the increase in fiber-degrading bacteria, early weaning of calves at 7 weeks of age also negatively impacted the abundances of potential lactose- and starch-degrading bacteria including *Dialister,* a frequent oral isolate (Ghayoumi et al., 2002), positively associated with starch digestion (Wang et al., 2016), unclassified *Lactobacillales,* that showed high concentration in milk consuming calves (Alipour et al., 2018) and negatively impacted by weaning (Salvetti and O'Toole, 2017). Similar to the oral microbes, rumen and faecal microbiome of earlyC group also showed similar trends, with decreased abundances of *Olsenella*, a carbohydrate fermenting bacterial genera (Kraatz et al., 2011), whose abundance

is negatively impacted by dietary forage inclusion (Kim et al., 2016). In addition, earlyC calves in this study also showed lower abundances of several faecal bacterial genera with carbohydrate-utilizing abilities such as *Blautia, Butyricimonas, Streptococcus* and *Mediterraneibacter* (Li et al., 2015; Lau et al., 2018; Togo et al., 2018; Adeyemi et al., 2020), lactate-utilizing *Anaerostipes* genus (Duncan et al., 2004) as well as *Bacteroides, Prevotella,* and *Faecalibacterium* genera that were reported to be abundant in the faeces of MR-fed calves (Uyeno et al., 2010; Castro et al., 2016; Meale et al., 2016; Meale et al., 2017b; Maynou et al., 2019; Kumar et al., 2021), and their abundances are negatively impacted by weaning i.e., a high fiber / forage diet resulted in lower abundances of faecal *Bacteroides*, *Blautia,* and *Faecalibacterium* (Kim et al., 2014; Meale et al., 2016). Thus, it can be assumed that the weaning-dependent reduction in major lactic-acid producing bacteria of the earlyC group was linked to their reduced milk consumption and higher fiber digestion post-weaning.

As discussed in chapter 4, weaning time not only impacted the GIT microbiome but also the plasma metabolic profiles of calves mainly involved in amino acid (AAs) metabolism during days 42–112, where the low plasma concentrations of majority of the essential EAAs (methionine, arginine, valine, leucine, histidine, phenylalanine, threonine, lysine, tryptophan), and non-essential NEAAs (glutamine, serine, citrulline, aspartate, tyrosine, proline, and ornithine) were reported in the earlyC as compared to the lateC group. The concentration of AAs in plasma is net result of several factors such as the breakdown and synthesis of protein, absorption of AAs from GIT, as well as protein obtained from the diet (milk proteins) (Maeda et al., 2012). Diet serves as the major influencing factor on the plasma AAs concentrations in calves than in mature cows (Ghaffari et al., 2017) as indicated by a stable AAs concentration in the plasma of dairy cows throughout the day (Halfpenny et al., 1969) and a high plasma concentration of EAAs in calves after milk feeding (Blum and Hammon, 1999). Similar increase in the concentration of both essential and non-essential AAs in the plasma of MR-fed Holstein bull calves (Ghaffari et al., 2017) and a high plasma level of lysine and arginine in milk-fed Holstein heifer calves were reported (Leal et al., 2021). Thus, a significant decrease in the concentrations of most of the AAs in the plasma samples of earlyC group in the present study was probably connected with the stressful weaning related dietary transition, confirming that the pre-weaning liquid diet can serve as a source of various metabolites to their host that are transported through GIT into their bloodstream (Qi et al., 2018). The plasma EAAs are critical for protein synthesis, higher concentrations resulted in higher protein synthesis (Bohé et al., 2003) and an alteration in their concentration have also been associated with disruption of metabolic processes, inflammation and immune responses i.e., lower plasma concentration

of valine, leucine, and isoleucine resulted in reduction of liver metabolic processes and lower methionine concentrations was associated with reduced development of enterocytes in weaned piglets (Dobrowolski and S´liwa, 2008). Similarly, lower concentration of plasma methionine, arginine, glutamine, and histidine resulted in higher occurrence of diarrhoea in calves (Tsukano and Suzuki, 2019). Thus, the weaning-dependent alteration in the quantity of dietary protein, and nutrients in the present study might have resulted in considerable changes in the AAs metabolism of the earlyC group. In addition to the AAs, the concentrations of several other plasma metabolites such as biogenic amines, acylcarnitines and sphingomyelins were also negatively impacted by the early-weaning event. This weaning-dependent modification in the plasma metabolic profiles and the exact functionality of these metabolites in young calves are yet to be explored in future.

5.7   Conclusions and Perspective

The present study summarised previously published data on calves' microbiome to date and highlighted several intrinsic and extrinsic factors that act synergistically resulting in the establishment of site- and region-specific gut microbial communities. The potential role of microbes in the gut development, health and productive performance of the host and the possibility of gut microbial modulations though dietary interventions are discussed.

Ruminants are mainly dependent on their rumen microbiome for feed digestion, nutrient synthesis and absorption. The non-static nature of rumen microbiome demands repetitive and frequent sampling from large animal cohorts, which is restricted by invasive sampling protocols. The current study showed the successful implication of buccal swabbing technique as an alternative to the invasive stomach tubing method for predicting the rumen microbial communities in young calves. However, the lack of ruminants-specific microbial reference databases (especially oral-specific microbiome) currently poses major hinderance on the applicability of BS approach, which must be explored in future studies.

This study monitored large number of animals over a period of 140 days and with frequent sampling confirmed that both age and weaning time has significant influence on the establishment of oral, rumen, faecal bacterial communities and plasma metabolic profile in young calves. The benefits of late-weaning in terms of better growth performance, stable gut microbiota with quick feed adaptability than early-weaning event is discussed. However, besides age, diet and weaning, there are several other factors that can influence the gut microbial composition of calves, such as host genetics and sex (Fan et al., 2020; Fan et al., 2021), as well as other environmental factors such as antibiotics and pre- or probiotics (Amin

and Seifert, 2021). Host genetics seems to play an important role not only in shaping gut microbiota but also the development of systemic immunity in pre-weaned calves, which is further related to the growth and health of an animal (Fan et al., 2020). A more recent study by Fan and colleagues, showed that the breed composition can have strong influence on gut microbial communities throughout life (Fan et al., 2021). Similar to the gut microbiome, a strong effect of host genetics on blood metabolites have also been reported (Long et al., 2017). However, the studies addressing the role of host genetic on neonatal calves' microbiota during the challenging life events such as weaning and exposure to pathogens are still very scare and are limited by the difficulty of controlling genetic distance, population variation, diet, age, and environmental conditions. Therefore, further studies are needed in future, to explore the integrated role of host genetics in shaping gut microbial communities and metabolic profiles of calves during the stressful weaning event for better understanding the host-microbe metabolic interactions and possible identifications of prognostic biomarkers for neonatal diseases.

CHAPTER VI

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6. BIBLIOGRAPHY

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CHAPTER VII

SUMMARY

7. SUMMARY

The period from birth until the end of weaning is critical for calves as they undergo extreme stress caused by maternal separation, transportation, and weaning related dietary shifts, that can cause long-lasting effects on animal behaviour, health as well as future production parameters. Monitoring the development of microbial ecosystem throughout the gastrointestinal tract of calves and host-microbe interactions during the challenging life periods such as perinatal and weaning is essential for sustainable ruminant production. The present thesis provided new insight on the suitability of buccal swabs as an alternative to complex stomach tubing method for predictive analysis of rumen microbial communities. The changes in oral, rumen and faecal microbial community structure of female German Holstein calves from 8-days to 5-months of age as well as during early- and late-weaning event were identified. The oral microbiota plays a crucial role in animal health. A high dominance of oral pathogens was observed during the first 11-weeks of calves' life. Similar to the oral microbiota, faeces of 8-day-old calves also showed high abundances of certain opportunistic pathogenic bacteria. Both oral and faecal pathogens showed a decrease in abundance with age and after weaning event in the earlyC group, indicating the age and weaning-dependent maturation of the host immune system. The establishment of dense microbial communities in the faeces of 8-day-old (experimental day 1) pooled herd milk and milk replacer fed Holstein calves was shown and it was dominated by phyla *Firmicutes and Actinobacteria* and potential lactose- and starch-degrading bacterial species, but as the calves aged and became more mature (5-months of age), their rumen and faecal bacterial communities were dominated by potential fibre-utilizing bacterial genera. The weaning related dietary transitions are critical for calves as their gastrointestinal tract undergoes several modifications, enabling them to digest plant-based diet during the postweaning period. Thus, it was proposed that the age at which animals should be weaned must be carefully considered as it clearly impacted the gastrointestinal tract microbial communities and plasma metabolic profiles of calves in the present study. Early introduction of roughages in the diet of 7-week-old calves increased the abundances of plant fiber degrading bacteria and decreased the abundances of potential lactose- and starch-degrading bacteria in the buccal cavity, rumen and faeces, indicating the weaning-related increase in fiber ingestion and the decrease in milk consumption of the early-weaned group. However, when roughages were introduced in the diet of late-weaned calves at 17-weeks of age, no significant

modifications in the structure of gastrointestinal tract microbial communities were observed. Similar to the microbiome, plasma metabolic profiles of early-weaned calves during days 42–112, showed lower concentrations of most of the amino acids, few biogenic amines, and sphingomyelins as compared to the late-weaned calves, suggesting that the liquid diet could provide certain metabolites that can be transported into the bloodstream through gastrointestinal tract. Similarly, the weaning-dependent changes in the quantity of dietary protein, fat and carbohydrates resulted in substantial changes in amino acid metabolism of the early-weaned group. The early-weaning event not only impacted the host microbiome and metabolome but also the host-microbe metabolic interactions as the abundances of potential lactose- and starch degrading bacteria and plasma concentrations of amino acid, biogenic amines and sphingomyelins were strongly positively correlated, both were negatively impacted by the early-weaning event. Thus, it can be concluded that late-weaning was beneficial as it allowed better adaptability of microbes to weaning-related dietary shifts, perhaps due to the greater maturation of their gastrointestinal tract with age as compared to the early-weaning group.

CHAPTER VIII

ZUSAMMENFASSUNG

8.   ZUSAMMENFASSUNG

Der Zeitraum von der Geburt bis zum Ende des Absetzens ist für Kälber von entscheidender Bedeutung, da sie durch die Trennung von der Mutter, den Transport und die mit der Entwöhnung verbundene Umstellung der Ernährung extremem Stress ausgesetzt sind, der langfristige Auswirkungen auf das Verhalten und die Gesundheit der Tiere sowie auf künftige Produktionsparameter haben kann. Die Überwachung der Entwicklung des mikrobiellen Ökosystems im gesamten Gastrointestinaltrakt von Kälbern und der Interaktionen zwischen Wirt und Mikroben während der schwierigen Lebensphasen wie der Perinatalperiode und dem Absetzen ist für eine nachhaltige Wiederkäuerproduktion unerlässlich. Die vorliegende Arbeit lieferte neue Erkenntnisse über die Eignung von Wangenabstrichen als Alternative zur komplexen Pansenfistelmethode für die prädiktive Analyse der mikrobiellen Pansengemeinschaften. Es wurden die Veränderungen in der Zusammensetzung der mikrobiellen Gemeinschaften im Maul, im Pansen und im Kot von weiblichen deutschen Holstein-Kälbern im Alter von 8 Tagen bis 5 Monaten sowie während des frühen und späten Absetzens ermittelt. Die orale Mikrobiota spielt eine entscheidende Rolle für die Tiergesundheit. Eine hohe Dominanz oraler Pathogene wurde in den ersten 11 Lebenswochen der Kälber beobachtet. Ähnlich wie die orale Mikrobiota wies auch der Kot von 8 Tage alten Kälbern hohe Abundanzen bestimmter opportunistisch-pathogener Bakterien auf. Sowohl die oralen als auch die fäkalen Pathogene nahmen mit dem Alter und nach dem Absetzen in der earlyC-Gruppe ab, was auf die alters- und absetzungsabhängige Entwicklung des Wirtsimmunsystems hinweist. Es wurde gezeigt, dass sich im Kot von 8 Tage alten (Versuchstag 1) mit gepoolter Herdenmilch und Milchaustauschern gefütterten Holstein-Kälbern dichte mikrobielle Gemeinschaften bildeten, die von den Phyla *Firmicutes* und *Actinobacteria* sowie potenziell laktose- und stärkeabbauenden Bakterienarten dominiert wurden. Die mit dem Absetzen verbundenen Ernährungsumstellungen sind für Kälber von entscheidender Bedeutung, da ihr Magen-Darm-Trakt mehreren Veränderungen unterliegt, die es ihnen ermöglichen, pflanzliche Nahrung in der Zeit nach dem Absetzen zu verdauen. Daher wurde vorgeschlagen, dass das Alter, in dem die Tiere entwöhnt werden sollten, sorgfältig überlegt werden muss, da es sich in der vorliegenden Studie eindeutig auf die mikrobiellen Gemeinschaften des Magen-Darm-Trakts und die Stoffwechselprofile des Plasmas von Kälbern auswirkte. Die frühe Einführung von Raufutter in das Futter von 7 Wochen alten Kälbern erhöhte die Häufigkeit von Pflanzenfasern abbauenden Bakterien und verringerte die

Häufigkeit von potenziell laktose-und stärkeabbauenden Bakterien in der Maulhöhle, im Pansen und in den Fäkalien, was auf die absetzungsbedingte Zunahme der Faseraufnahme und den Rückgang des Milchkonsums der früh abgesetzten Gruppe hinweist. Als im Alter von 17 Wochen Raufutter in die Ernährung der spät abgesetzten Kälber eingeführt wurde, wurden keine signifikanten Veränderungen in der Struktur der mikrobiellen Gemeinschaften des Magen-Darm-Trakts beobachtet. Ähnlich wie das Mikrobiom wiesen auch die Stoffwechselprofile des Plasmas von früh abgesetzten Kälbern in den Tagen 42-112 niedrigere Konzentrationen der meisten Aminosäuren, weniger biogener Amine und Sphingomyeline auf als bei den spät abgesetzten Kälbern, was darauf hindeutet, dass die Flüssignahrung bestimmte Stoffwechselprodukte liefern könnte, die über den Magen-Darm-Trakt in den Blutkreislauf gelangen können. In ähnlicher Weise führten die vom Absetzen abhängigen Veränderungen in der Menge des Nahrungsproteins, des Fetts und der Kohlenhydrate zu erheblichen Veränderungen im Aminosäurestoffwechsel der früh abgesetzten Gruppe. Das frühe Absetzen wirkte sich nicht nur auf das Mikrobiom und das Metabolom des Wirts aus, sondern auch auf die metabolischen Interaktionen zwischen Wirt und Mikroben, da die Häufigkeit potenzieller laktose- und stärkeabbauender Bakterien und die Plasmakonzentrationen von Aminosäuren, biogenen Aminen und Sphingomyelinen stark positiv korreliert waren wobei beide durch das frühe Absetzen negativ beeinflusst waren. Daraus lässt sich schließen, dass die späte Entwöhnung vorteilhaft war, da sie eine bessere Anpassungsfähigkeit der Mikroben an entwöhnungsbedingte Ernährungsumstellungen ermöglichte, möglicherweise aufgrund der umfangreicheren Reifung des Magen-Darm-Trakts mit zunehmendem Alter im Vergleich zur früh entwöhnten Gruppe.

CHAPTER IX

APPENDIX

CURRICULUM VITAE

Personal Data

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Work Experience

PhD student and scientific assistant at the department of Functional Microbiology of Livestock, Institute of Animal Science, University of Hohenheim, Stuttgart, Germany (October 2019 – December 2021)

Scientific research assistant at the department of Agronomy, Animals, Food, Natural Resources and Environment (DAFNAE), University of Padova, Italy (October 2017 – September 2019)

Education

Doctoral student at the Faculty of Agricultural Science (October 2019 – December 2021)
Department of Functional Microbiology of Livestock, Institute of Animal Science, University of Hohenheim, Stuttgart, Germany
Degree Dissertation:
"*Impact of age and weaning time on the gut microbiome and the potential host-microbe interactions in calves*"

M.Sc. in Microbiology and Biochemistry (October 2014 – July 2016)
University of Göttingen, Germany
Degree Dissertation:
"*Bioremediation potential of heavy metal resistant bacterial communities in copper mine waste*"

BS Hons in Zoology (October 2010 – July 2014)
University of the Punjab, Lahore, Pakistan
Degree Dissertation:
"*Cultivation of Bacillus subtilis-A4, a fish growth escalating probiotic in sugarcane bagasse*"

Awards

EXPERTS III scholarship for master's degree (October 2014 – July 2016)
Gold medal for highest grades in bachelor's degree (December 2015)
Bronze medal in IGEM final competition, held in Boston, US (September 2015)
Scholarship for excellent academic performance in bachelor's degree (October 2012 – July 2014)
Scholarship for excellent academic performance in higher secondary education (August 2008 – July 2010)

Stuttgart, 7th of December 2021

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic
Impact of age and weaning time on the gut microbiome and the
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potential host-microbe interactions in calves
………………………………………………………………………………....

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, 7th of December 2021
_____   _____

Place, Date   Signature

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