

***Mothers' parity and weaning age influence  
the transition from liquid to solid feed in  
female Holstein calves***

**Dissertation to obtain the doctoral degree of Agricultural Sciences  
(Dr. sc. agr.)**

**Faculty of Agricultural Sciences**

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2023

This thesis was accepted as a doctoral thesis (dissertation) in fulfillment of the regulations to acquire the doctoral degree “Doktor der Agrarwissenschaften” by the Faculty of Agricultural Sciences at University of Hohenheim.

Date of the oral examination: June 20<sup>th</sup>, 2023

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## List of Abbreviations

List of Abbreviations excludes abbreviations used in the included manuscripts in Chapter 2.

Alpha-AAA	=	alpha-aminoadipic acid
BCS	=	body condition score
BHB	=	beta-hydroxybutyrate
BW	=	body weight
C0	=	carnitine
C2	=	acetylcarnitine
C3	=	propionylcarnitine
DM	=	dry matter
FLI	=	Friedrich-Loeffler-Institute
IGF-1	=	insulin-like growth factor 1
LSMeans	=	least squares means
LWG	=	live weight gain
MC	=	calves born to multiparous cows
MR	=	milk replacer
NEFA	=	non-esterified fatty acids
ODC	=	ornithine decarboxylase
PC	=	calves born to primiparous cows
PEPCK	=	phosphoenolpyruvate carboxykinase

SE = standard error

SD = standard deviation

TCA = tricarboxylic acid cycle

TMR = total mixed ratio

VFA = volatile fatty acids

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# 1. General Introduction

Natural rearing of calves with a dam differs from current feeding practice in several aspects. The calf received whole milk from its mother. *Ad libitum* access to milk or milk replacer (MR) from an automated feeder led to an intake of 1000 to 2000 g dry matter (DM; equaled 8 – 17 l liquid feed) per day depending on the calf's age (Dennis et al., 2018; Korst et al., 2017; Webb et al., 2014). The daily amount was consumed in 4 – 18 suckling bouts both from the dam and an automated feeder (Korst et al., 2017; Passillé et al., 2011; Passillé and Rushen, 2012; Reinhardt and Reinhardt, 1981; Webb et al., 2014). Holstein bull calves that were allowed to choose their feed preferred MR over solid feed, even when three months old (Webb et al., 2014). The natural weaning process was observed to occur gradually over weeks and varied highly per individual calf (Reinhardt and Reinhardt, 1981; Webb et al., 2014). Therefore, weaning is completed at the age of 7 – 12 months, averaging at 8 – 9 months (Reinhardt and Reinhardt, 1981; Walker, 1962).

In contrast, most calves reared in commercial farms were separated early from their mother and then received restricted, therefore lower than *ad libitum*, amounts of MR, and were weaned much earlier (Khan et al., 2016). These management decisions were mainly based on economic reasons as raising dairy heifers involves high economic investments (Gabler et al., 2000; Tozer and Heinrichs, 2001). With feedstuff accounting for a major part of rearing costs (Gabler et al., 2000), choosing cheaper feedstuff attracts great interest. As MR was higher-priced than solid feedstuff, research focused on reducing MR amount and shortening the liquid feeding period of dairy calves (Khan et al., 2016). Therefore, modern rearing strategies hold potential challenges for growing calves especially regarding nutritional conditions (Weary et al., 2008).

Potential social stressors include separation from the mother or other social companions during group housing. Changing the environment through changing the barn might be stressful for calves as well (Weary et al., 2008). Nutritional challenges can be related to the type and amount

of liquid and solid feedstuff (Khan et al., 2016). Liquid feed for a calf can be milk or MR. Most farmers feed unpasteurized whole marketable milk (89% of Canadian and 28 % of American farms) or MR (50% of Canadian and 70% of American farms) (National Animal Health Monitoring System, 2010; Tautenhahn, 2017; Vasseur et al., 2010). In Germany, the type of liquid feed differs depending on the region. In the north and east of Germany, most farms use MR (60% and 63,1%, respectively), whereas in the south whole milk is the most used liquid feed (51,4%; (Hoedemaker et al., 2020). It is common practice to feed calves restrictively in the USA, Canada and Europe. They receive about 5 - 8 L of milk or MR per day ( Hoedemaker et al., 2020; Spiekers et al., 2009; Tautenhahn, 2017; Urie et al., 2018; Vasseur et al., 2010). There are different technical solutions to offering milk or MR to calves. The most common are buckets, used by most Californian farms, and bottles with teats (Vasseur et al., 2010). In Germany, offering liquid feed differs widely depending on the region. In the south and north of Germany, most calves are fed with a bucket or a bottle with a teat (90,7% and 51% respectively; (Hoedemaker et al., 2020), whereas farms in Eastern Germany mostly use an automatic feeding system ( Hoedemaker et al., 2020; Tautenhahn, 2017). The method of reducing MR or milk during weaning can be challenging for growing calves as well, especially when done abruptly. Therefore, interest has grown in step-down weaning methods (Khani et al., 2017; Valehi et al., 2022), in which the amount of MR until weaning is reduced in several steps, or gradual weaning, in which the amount is reduced gradually (Sweeney et al., 2010). Nutritional challenges might occur *in utero* as well, for example through mothers' parity (Opsomer et al., 2017) or malnourishment (Symonds et al., 2007). All these nutritional experience in early life and associated alterations of development might be persistent through life (Hales et al., 1991; Waterland and Garza, 1999). This phenotypic feature is called *metabolic imprinting* (Waterland and Garza, 1999).

Nutritional challenges during prenatal life through mothers' parity and during postnatal life through weaning age are topics of this thesis. Their potential influences on maturation of calves are discussed in the following subsections.

### **1.1 Maturation of Growing Calves**

Calves undergo remarkable morphological and physiological changes from birth to weaning. They are born as non-ruminant animals that rely on nutrients from milk or MR (Baldwin et al., 2004; Khan et al., 2016). These are digested in the abomasum and the small intestine, similar to the digestion in the stomach and intestine of a monogastric animal (Huber, 1969). Therefore, weaning is characterized by the commissioning of the rumen as the main site of production and absorption of nutrients (Baldwin et al., 2004). At birth, the abomasum yields the same weight as the three forestomachs combined (Becker et al., 1951), whereas the rumen is small and metabolically non-functional (Warner et al., 1956). These proportions, however, change quickly as the calf matures. The abomasum weight relative to live weight remains roughly constant after the age of 3 weeks, whereas the proportionate weight of the reticulorumen increases until the age of 17 weeks (Godfrey, 1961), when it reaches adult proportions of 23 to 36 L/100 kg of ingesta-free body weight (BW; Huber, 1969). Furthermore, the omasum grows relative to BW until about one year of age (Huber, 1969).

To adapt to the changes of nutrient source other organs and metabolic functions need to change as well. Generally, an adequately functioning liver metabolism is important for nutrient utilization (Baldwin et al., 2004). In adult ruminants, most of the dietary carbohydrates are fermented into volatile fatty acids (VFA) in the rumen and less than 10% of the required glucose is directly absorbed from the digestive tract (Aschenbach et al., 2010; Young, 1977). As many organs and cells like brain and erythrocytes have an obligatory requirement for glucose (Aschenbach et al., 2010), gluconeogenesis is an important metabolic pathway for ruminants.

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During weaning, the source of nutrients and energy changes from glucose and milk fat to VFA. Hence, liver metabolism shifts from glycolytic to glucogenic and overall hepatic metabolic activity needs to increase due to weaning (Baldwin et al., 2004). Therefore, the fraction of whole-body oxygen use applied by the liver of ruminants is markedly greater than in pre-ruminant calves. Oxygen use expressed as a percentage of whole body oxygen consumption is 13 – 14 % in pre-ruminant calves, whereas lactating cows and steers use 17 – 31 % (Baldwin et al., 2004).

### **1.1.1 Influence of Mothers' Parity on Maturation**

The average age at first calving was 25 to 29 months in Europe (Froidmont et al., 2013; Hoedemaker et al., 2020; Mohd Nor et al., 2013), although an even younger age (22 – 23 months) was recommended to reduce the costs of heifer rearing (Cole et al., 2013). These young primiparous mothers give birth to calves with lower BW and lower body condition score (BCS; Heinrichs et al., 2005), which kept growing slower than their peers born to multiparous mothers. Body growth during the first 4 months was positively associated with parity of the dam (Place et al., 1998; Simensen and Norheim, 1983). Even at first calving, BW and BCS tended to be lower in heifers born to primiparous mothers (Heinrichs et al., 2005). This might indicate an intrauterine nutritional restriction of these calves compared to calves born to multiparous cows. Young primiparous cows themselves were still growing during pregnancy. Therefore, mother and calf might compete for nutrients to support their growth (Opsomer et al., 2017). Furthermore, the smaller body size of a younger cow might manifest a smaller uterine size, which reduced the offspring's potential growth as well (Snow, 1981). At the same time, multiparous cows were lactating during most of their pregnancy, which likewise might reduce nutrient supply for the calf. Nutrients like proteins and glucose might have been diverted towards the udder instead of to the fetus. This might have been especially distinct for cows with

high milk yields (Opsomer et al., 2017). Furthermore, Cuttance et al. (2022) reported a lower percentage of suckling shortly after birth in calves born to older cows (>7 years) compared to calves born to heifers.

It was demonstrated that maternal nutrient restriction throughout and at specific stages of pregnancy has distinct effects on the embryo, placenta or fetus (Symonds et al., 2007). Late gestation is characterized by enhanced fat deposition and muscle growth of the fetus (Mao et al., 2008), by which BW increases. Nutritional restriction of the mother during late gestation impacts glucose-insulin-homeostasis, birth weight and fetal fat deposition (Symonds et al., 2007). As nutrient requirement of the fetus increases during its intrauterine development, competition between the dairy cow and her offspring might be especially pronounced at the end, potentially leading to long-term impairment of calves' energy metabolism. As discussed before, nutritional experience in early life and associated alterations of development might be persistent throughout an animals' lifetime. As development of the mammary gland begins during intrauterine development, nutritional experiences in fetal life can potentially influence later milk performance (Knight and Sorensen, 2001). This *metabolic imprinting* might be especially deleterious under circumstances of mismatch between the milieu the offspring was prepared for *in utero* and the milieu it is actually born in (Opsomer et al., 2017). The *thrifty phenotype hypothesis* proposes that the associations between poor fetal growth and the subsequent development of metabolic disorders later in life resulted from the effects of poor nutrition in early life, which produced permanent changes in glucose-insulin metabolism and became detrimental under nutritionally adequate circumstances postnatal (Hales et al., 1991; Hales and Barker, 2001). Hence, calves born to primiparous mothers might have developed in a milieu of nutrient restriction and their metabolism has been altered accordingly. Dairy heifers that were born with low weight and then grew fast during the first months of life needed more inseminations to become pregnant (Opsomer et al., 2017). Overall, mothers' parity and related

nutritional supply during pregnancy might influence their calves' development, health and performance later in life.

### **1.1.2 Influence of Weaning Age on Maturation**

The average age of weaning female dairy calves is 6 - 9 weeks in the USA and Canada (National Animal Health Monitoring System, 2010; Urie et al., 2018; Vasseur et al., 2010) and 8 – 11 weeks in Europe (Hoedemaker et al., 2020; Mahendran et al., 2022; Spiekers et al., 2009; Tautenhahn et al., 2020). As discussed before, this was done to reduce the costs of feedstuff. This rearing regimen might lead to stress that could affect health (Brscic et al., 2011; Ferronato et al., 2022; Kim et al., 2011), BW gain (Funaba et al., 1994; Greenwood et al., 1997; Ndumbe et al., 1964), growth (Ndumbe et al., 1964) and animal welfare (Eckert et al., 2015; Weary et al., 2008). It might have contributed to the high rates of calf morbidity and mortality in the dairy industry. Calf mortality rate ranges between 3.5 % to 7.8 % in the USA, Canada and Europe (Gulliksen et al., 2009; Johnson et al., 2017; Hoedemaker et al., 2020; Urie et al., 2018; Tautenhahn, 2017; Santman-Berends et al., 2014; Windeyer et al., 2014), with digestive problems accounting for the majority of pre-weaned heifer morbidity and deaths (Compton et al., 2017; National Animal Health Monitoring System, 2010; Urie et al., 2018).

Interest in delaying weaning has increased in recent scientific literature, as it might affect calves positively (Meale et al., 2015; Passillé et al., 2011). Weaning calves at greater age improved their growth (Passillé et al., 2011). Accordingly, anabolic hormones like insulin-like growth factor 1 (IGF-1), insulin and growth hormone might be influenced by weaning age as well. Contrary, growth hormone concentration was not affected by weaning and was not different whether calves were weaned at 7 or 12 weeks of age (Kato et al., 2004). Weaning caused a significant decrease in plasma IGF-1 and insulin, whether it was performed at the age of 8 weeks or 12 weeks (Breier et al., 1988). Accordingly, calves weaned at 7 or 8 weeks of age had

lower IGF-1 plasma concentrations compared to age-matched calves that still received MR, at least until the age of 12 weeks (Breier et al., 1988; Katoh et al., 2004). Serum IGF-I concentrations reflected the nutritional state of beef cattle (Hayden et al., 1993). It is positively correlated with birth weight (Breier et al., 1988), average daily gain (Blanco et al., 2009) and has been associated with high protein deposition (Blanco et al., 2009; Hayden et al., 1993). Chronically malnourished children had low IGF-1 concentrations and high growth hormone concentrations (Thissen et al., 1994). A similar pattern was observed in energy restricted steers, which had decreased IGF-1 and insulin concentrations and increased growth hormone concentrations (Hayden et al., 1993). An increased BW and IGF-1 concentration during the rearing period until the age of 15 months were associated with a reduced age of first calving (Brickell et al., 2009). As insulin and IGF-1 increased ruminal epithelial cell growth as well (Baldwin, 1999; Sakata et al., 1980), they might be beneficial for ruminal growth in pre-ruminants.

VFA production, especially butyrate, was discussed to be driver and indicator of rumen development (Sutton et al., 1963). Weaning between the age of 4 – 8 weeks increased the total VFA concentrations in the rumen (Anderson et al., 1987; Eckert et al., 2015), which then continued to increase until the age of 24 weeks ( $111.8 \pm 7.5$  mM), indicating a continuous development of rumen function until that age (Hayashi et al., 2006). Furthermore, weaning at 6 weeks of age changed gene expression of molecules that were involved in lipid metabolism, cellular growth and proliferation, cell morphology, molecular transport, and the cell cycle in ruminal epithelia (Connor et al., 2013). The associated functions were free radical scavenging, lipid metabolism and molecular transport (Connor et al., 2013). Changes in transporter gene expression through weaning included 20 different solute carrier including transporters for urea, fatty acids, glucose, copper and amino acids (Connor et al., 2013). Weaning at 6 weeks of age seemed to activate genes that promote ketogenesis and fatty acids beta-oxidation in rumen

epithelial cells (Connor et al., 2013). As all calves in this study were weaned at the same age, these changes in gene expression might have happened regardless of weaning and rather through an ontogenetic regulation. Further, gene expression of enzymes controlling ruminal ketogenesis were expressed independently of intraruminal VFA concentrations and weaning with 7 or 12 weeks of age in lambs (Lane et al., 2002). The mRNA concentrations of the first enzyme in the ketogenic pathway, Acetoacetyl-CoA thiolase, and the rate-limiting enzyme of the ketogenic pathway in non-ruminant liver, 3-hydroxy-3-methylglutaryl-CoA synthase, increased with age independent of diet (Lane et al., 2002). The increase occurred at the age of 42 days in lambs (Lane et al., 2002). Therefore, ruminal development might not only depend on solid feed intake but rather include some ontogenetic regulated features. Further, rumination, microbial fermentation and corresponding ruminal development started before weaning at 8 weeks of age, thus easing transition from liquid to solid feed (Eckert et al., 2015). As discussed before, proportions and functions of reticulorumen increase until the age of 17 – 24 weeks (Godfrey, 1961; Hayashi et al., 2006; Huber, 1969), possibly facilitating weaning at this age. Contrary, early weaning at 4 - 6 weeks of age decreased ruminal pH to an acidotic state (pH 5.0 – 5.7) during the first 2 – 6 weeks after weaning (Anderson et al., 1987; Vazquez-Anon et al., 1993), indicating inefficient rumen buffering function.

Weaning at a greater age might reduce behavioral signs of hunger and distress that commonly occurred during early weaning (Eckert et al., 2015; Passillé et al., 2011; Webb et al., 2014). Two weeks after weaning, calves that were weaned with 6 weeks of age showed higher levels of non-nutritive oral behavior and less time spend with lying compared to calves weaned with 8 weeks of age (Eckert et al., 2015). Therefore, delaying weaning might support animal welfare, which became increasingly important to consumers (Alonso et al., 2020). There was growing willingness to pay for farm animal welfare, especially in German consumers (Lagerkvist and Hess, 2011).

Furthermore, the positive effects of an increased weaning age might continue in later life and improve future performance as well as health. This phenotypic feature called *metabolic imprinting* (Waterland and Garza, 1999) was discussed before. Greater weaning age and associated higher live weight gain (LWG) and higher solid feed intake at weaning was associated with greater milk production (Bach, 2012; Heinrichs and Heinrichs, 2011). Later weaned calves were older when removed from their herd for health or productive reasons (Heinrichs and Heinrichs, 2011). Better health and performance through a greater weaning age might be the effect of a better *resilience*. *Resilience* describes the capacity to cope with environmental and social stressors. A good *resilience* is manifested in a minimal effect or a rapid return to the state before the stressor (Colditz and Hine, 2016). Hypothetically, prolonging the liquid feeding period can be one of many factors influencing the *resilience* of an individual. As culling of milking cows contributed to a major part of the costs of raising dairy heifers (Tozer and Heinrichs, 2001), investing in health and longevity can be an economic advantage. Reducing the milking herd-culling rate by about 1% could reduce the costs of rearing replacement heifers by approximately 1000 – 1500 \$ (Tozer and Heinrichs, 2001). From an economic point of view, the optimum productive lifetime of dairy cows was 7 – 12 years, which is much longer than the average lifespan on farms (Missfeldt et al., 2015). Improved rearing conditions might facilitate the increase of dairy cows' lifespan (Heinrichs and Heinrichs, 2011). Furthermore, milk production has environmentally harmful effects like land usage for feed production or emissions of greenhouse gases (Eide, 2002). A higher milk yield and longer productive life-span might result in fewer animals to produce the needed amount of milk, which led to fewer emissions and land use through these animals (Lichtenberg, 2002). Therefore, outcomes of prolonging liquid feeding might positively affect environmental effects of milk production as well.

## 1.2 Aims and Objectives of the Study

The aim of the current study was to gain a comprehensive understanding of the development of different metabolic features of growing calves and their capacity to cope with the dietary changes associated with weaning. The focus was on identifying the influence of mothers' parity and weaning with 7 or 17 weeks of age. To address these topics, an *in vivo* experiment was designed and carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany. In general, 59 female Holstein calves were examined from birth until 5 months of age. They were weaned either at 7 or 17 weeks of age and calves born to primiparous or multiparous mothers were equally divided between weaning groups. Samples of blood, urine, feces and saliva were taken regularly over the course of the experiment.

Growth performance was evaluated by morphometric measures and weight gain. Metabolic maturity was assessed by indicators such as insulin, leptin, adiponectin, glucose, BHB, NEFA, cholesterol, urea and total protein. Further, a targeted metabolomics approach was used to analyse many metabolites at once including acylcarnitines, amino acids, glycerophospho- and sphingolipids, biogenic amines and monosaccharides (AbsoluteIDQ p180 Kit, Biocrates Life Science AG, Innsbruck, Austria). The development of gastrointestinal tract was assessed by behavioral observations, faecal microbiome analyses and ruminal development markers like rumen sounds per minute and a rumen filling score. Associated acid base homeostasis and nitrogen metabolism was evaluated by measuring urinary nitrogen- containing compounds (creatinine, hippuric acid, uric acid, urea, allantoin) and pH in saliva, faeces, blood and urine. Analyses and results are described and discussed in detail in the three included manuscripts.

## 2. Included Manuscripts

Three manuscripts aroused from the study. The influence of mother's parity and calves' weaning age on growth, protein and energy metabolism and on endocrine regulators like insulin and leptin were addressed in the first manuscript "Weaning Holstein Calves at 17 Weeks of Age Enables Smooth Transition from Liquid to Solid Feed" published in *Animals* (2019; **Manuscript 1**). **Manuscript 2**, entitled "Weaning age influences indicators of rumen function and development in female Holstein calves" published in *BMC Veterinary Research* (2022), addressed behavior, early gastrointestinal development and corresponding systemic metabolic changes in nitrogen metabolism and acid-base homeostasis. Data of fecal microbiome and blood metabolomics analysis, which allow insight in development of gastro-intestinal tract and various metabolic processes, will be published in *animal Microbiome*. This data was combined with growth parameters to gain knowledge in associated metabolic pathways (**Manuscript 3**).

## **2.1 Manuscript 1: Weaning Holstein Calves at 17 Weeks of Age Enables Smooth Transition from Liquid to Solid Feed**

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Published in

Animals (2019), Volume 9, pages 1132 - 1148;

DOI name: 10.3390/ani9121132

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Open access

The original publication is available at <https://doi.org/10.3390/ani9121132>

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**Simple Summary:** Weaning calves from liquid to solid feed can be a stressful event in their life and can affect growth, development and welfare. It is commonly done at the age of 7 to 8 weeks on dairy farms, but weaning at a greater age could potentially reduce the associated stress. Therefore, it might improve growth rates and enable a smooth transition to an adult liver metabolism. To confirm this hypothesis this study evaluated the effect of two different weaning ages (7 vs. 17 weeks of age) on female Holstein calves. Furthermore, the effect of mothers' parity was analyzed (primiparous vs. multiparous). Primiparous cows were often immature and still developing during their first pregnancy. This can lead to negative intrauterine conditions and result in long-term changes in the calf's metabolism. Late-weaned calves consumed high amounts of concentrate feed before weaning despite their high milk replacer intake, indicating the maturation of their rumen. In addition, they experienced a smooth transition to an adult liver metabolism as reflected by steady plasma glucose and cholesterol concentrations. Later weaning corrected the reduced growth of calves born to primiparous cows as well, indicating that those particularly benefitted from late weaning. All benefits were indicated by slower changes of blood metabolites and higher growth rates, which might lead to better health and productivity in their subsequent lifetime.

**Abstract:** Development of calves depends on prenatal and postnatal conditions. Primiparous cows were still maturing during pregnancy, which can lead to negative intrauterine conditions and affect the calf's metabolism. It is hypothesized that weaning calves at higher maturity has positive effects due to reduced metabolic stress. We aimed to evaluate effects of mothers' parity and calves' weaning age on growth performance and blood metabolites. Fifty-nine female Holstein calves ( $38.8 \pm 5.3$  kg birth weight, about 8 days old) were used in a  $2 \times 2$  factorial experiment with factors weaning age (7 vs. 17 weeks) and parity of mother (primiparous vs. multiparous cows). Calves were randomly assigned one of these four groups. Live weight, live weight gain and morphometry increased over time and were greater in calves weaned later.

Metabolic indicators except total protein were interactively affected by time and weaning age. Leptin remained low in early-weaned calves born to primiparous cows, while it increased in the other groups. The results suggest that weaning more mature calves has a positive effect on body growth, and calves born to primiparous cows particularly benefit from this weaning regimen. It also enables a smooth transition from liquid to solid feed, which might reduce the associated stress of weaning.

**Keywords:** weaning age; Holstein calves; growth; milk replacer; metabolism; development

## 1. Introduction

Calves are born as functional monogastric animals that rely on nutrients from milk or milk replacer (MR) [1,2]. Therefore, weaning is a vital event in the young ruminant's life, as it means that lactose and milk fat are no longer available as main sources for energy metabolism. The change from functional monogastric to ruminant not only relies on volatile fatty acids (VFA) production in the rumen to supply energy, but also on well-functioning endocrine and biochemical features such as ruminant-specific insulin homeostasis and hepatic gluconeogenesis. Thus, weaning causes stress [3,4], and could affect animal welfare, growth, development and future performance [5–7]. Calf mortality was high (5%–9%) in the last decades [8,9]. For economic reasons the preweaning period was substantially shortened in dairy cow production systems [10]. In the USA and Canada, the typical weaning age was 6–8 weeks [9,11]. Early weaning was introduced to promote the early intake of concentrate feed and hay, which are cheaper feeds than milk or MR. Therefore, feeding strategies for dairy calves focused on increasing the capacity for solid feed and accelerating the development of the forestomach system. In scientific studies, only a few variables such as beta-hydroxybutyrate in the plasma and rumen epithelium growth are used to indicate this [1,2]. A suggested potential benefit of

early weaning was a faster rumen development [12], whereas the maturity of other parts of the gastrointestinal tract and organs like the liver was not considered. Therefore, little research was done about weaning calves older than 14 weeks [13].

Weaning calves which are more mature was discussed to have many benefits for the growth and development of dairy calves. It has been associated with greater live weight gain (LWG) and improved gastrointestinal development at the time of weaning [5]. For the first 56 days of life, feed efficiency (gain: feed ratio) tended to be greater for calves that were fed milk compared to grain [14]. The utilization of solid feed (corn silage, wheat straw, concentrate) for growth increased until 27 weeks of age [15].

Considering the great impact of weaning on dairy calves, it is crucial to find an optimal age for it. In this study, the optimal age denotes sufficient maturity in all organ and tissue functions, and not only in the ruminal digestion of solid feed. In fact, we define maturity for weaning as the capacity of all organs to fulfill the digestive and metabolic needs for changing to a ruminant status.

In the present study, early weaning was conducted at the age of 7 weeks, as this is a common management decision taken on dairy farms [9,11]. As opposed to that, late weaning was executed at the age of 17 weeks, because the reticulorumen volume of calves reaches adult proportions of 23 to 36 L/100 kg of ingesta-free body weight at 12 to 16 weeks [16].

The prenatal period as well as the early postnatal period are critical stages of development at which metabolic imprinting may occur and have great impact on health and performance in adult life [17]. Opsomer et al. [18] concluded in their review article that the parity of the mother could have a major impact, as older cows are lactating and heifers are still growing during pregnancy. Older cows tended to give birth to larger calves [19]. As birth weight was associated with improved glucose metabolism in humans in adulthood [20], mother's parity can influence

the calf in the long-term. In most studies on calf development, the authors did not consider this parity of the mother as a potential influencing factor of development. Furthermore, few studies examined post-weaning development in female calves for a longer period.

The present study aimed to determine the influence of mother's parity and calves' weaning age on growth, energy and protein metabolism and on endocrine regulators. Growth performance and weight gain was evaluated by morphometric measures, and metabolic maturity was assessed by insulin, leptin and adiponectin as regulators of organ maturation. Since energy and protein metabolism are crucial for growth and development, indicators such as glucose, beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), cholesterol, urea and total protein were measured by spectrophotometric methods.

To assess metabolic imprinting as well as economic aspects, these animals were monitored in an ongoing observational study.

## **2. Materials and Methods**

In accordance with the German Animal Welfare Act, pertaining to the protection of experimental animals and approved by The Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany, the present trial was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany (file No.: 33.19-42502-04-15/1858). 2.1. Animals, Housing, and Diets

Female German Holstein calves ( $n = 59$ ) were studied from day of birth until day  $149 \pm 2$  (mean  $\pm$  standard deviation (SD)) of life. All calves originated from one established herd of Holstein cows and were born within a seasonal calving period of three months (October–December). They were all vaccinated with inactive *Mannheimia haemolytica* serotype A1 and A6, parainfluenza3 vaccine and bovine respiratory syncytial virus (Bovigrip® RSP plus, MSD

Animal Health, Unterschleißheim, Germany) in weeks 5 and 9 of age, against Trichophyton in weeks 6 and 8 of age with live attenuate vaccine (Bovilis® Ringvac, MSD Animal Health, Unterschleißheim, Germany) and against blue tongue disease (BTV) (Zulvac 8, Zoetis Belgium SA, Louvain-la-Neuve, Belgium) in weeks 11 and 15 of age.

Calves were weighed with an electronic scale directly after birth and received 3 L of colostrum through a nipple bucket within 2 h after birth. The quality of colostrum was evaluated using a colostrum densimeter (Wahl GmbH, Dietmannsried, Germany) and had to be greater than 1035 g/L, otherwise colostrum from another cow from the same herd was used. They were moved 2–3 h after birth into straw-bedded single hutches and were fed twice with three liters of pooled herd milk each day. The pre-experimental feeding period for neonatal calves was done according to standard dairy management practice at the experimental station. In detail, starting at the age of three days, milk replacer (MR) (NOLAC GmbH, Zeven, Germany, Table 1) was mixed with the pooled herd milk, with gradually increasing amounts from 0.3 kg MR powder/d (third day of life) to 0.9 kg MR/d (fifth day of life), while the maximum of 6 L liquid feed with a concentration of 150 g/L MR was available (Table 2). Calves entered the study at a mean age of  $8 \pm 1.9$  days and  $44.5 \pm 5.2$  kg of live weight and moved into straw-bedded stables with MR and concentrate self-feeding systems (Förster-Technik GmbH, Engen, Baden-Württemberg, Germany). Differential feeding and monitoring of feed intake were achieved using a transponder in the calf's ear. They were randomly allocated to either early weaning at 7 weeks of age (early-weaned calves from multiparous cows (earlyMC)/early-weaned calves from primiparous cows (earlyPC)) or late weaning at 17 weeks of age (late-weaned calves from multiparous cows (lateMC)/late-weaned calves from primiparous cows (latePC)) group considering an equal allocation of calves from primiparous cows (PC) and calves from multiparous cows (MC). Our experimental trial started with 0.9 kg MR powder/d, which were available for all calves for the first five experimental days. MR was increased gradually within

the next five days (experimental days 6 to 10) from 0.9 kg MR powder/d to 1.35 kg MR powder/d, and remained at this level until the beginning of the weaning period (early-weaned group = day 28, late-weaned group = day 98). Concentration of MR was continuously at 150 g MR powder/L over the complete experimental time, and a maximum of 9 L liquid feed was available (Table 2). Over the entire trial, all calves received hay and water ad libitum and had access to a maximum of 2 kg concentrate feed per day until weaning. With the start of weaning at experimental day 98, the amount of concentrate feed was reduced to 1 kg/d according to standardized dairy management practice at the experimental station. During weaning, the milk replacer was gradually reduced within 14 days from 1.35 kg/d to 0.3 kg/d. Post-weaning calves were moved to another barn and received hay and a total mixed ration (TMR) consisting of 48% grass, 32% maize silage and 20% concentrate feed.

The ingredients of MR powder and concentrate feed are shown in Table 1. Composition of concentrate feed, roughage, milk replacer and TMR were determined according to the suggestions of the Association of German Agricultural Analysis and Research Centers [21] (Table 3).

**Table 1.** Ingredients of milk replacer (MR) powder and concentrate feed.

<b>Ingredients of MR Powder</b>		<b>Ingredients of Concentrate Feed</b>	
<b>Component</b>	<b>g/kg</b>	<b>Component</b>	<b>g/kg</b>
Skimmed milk powder	320	Soybean meal	300
Sweet whey powder	198	Oat	305
Vegetable fat	140	Barley	180
Whey powder	102	Wheat	170
Whole milk powder	100	Soy bean oil	15
Buttermilk powder	100	Minerals and vitamins *	20
Minerals and vitamins	40	Calcium carbonate	10

\* Ingredients per kg feed: 160 g Ca; 80 g P; 100 g Na; 30 g Mg; 1000 mg Fe; 800 mg Cu; 6000 mg Zn; 50 mg I; 50 mg Se; 30 mg Co; 800,000 IU vitamin A; 80,000 IU vitamin D3; 1000 mg vitamin E.

**Table 2.** Feeding regimen before and during experiment.

Experimental Day	Age in Days	MR Powder (g/L)		Available Volume of Liquid Feed per Day (L)		Available MR Powder per Day (kg)	
		Early Weaned	Late Weaned	Early Weaned	Late Weaned	Early Weaned	Late Weaned
	1–3 *	0	0	6	6	0	0
	3–5	150	150	6	6	Gradually increased from 0.3 to 0.9	Gradually increased from 0.3 to 0.9
1 to 5	6 until start of experiment **	150	150	6	6	0.9	0.9
		150	150	6	6	0.9	0.9
6 to 10		150	150	Gradually increased from 6 to 9	Gradually increased from 6 to 9	Gradually increased from 0.9 to 1.35	Gradually increased from 0.9 to 1.35
11 to 28		150	150	9	9	1.35	1.35
29 to 42		150	150	Gradually decreased from 9 to 2	9	Gradually decreased from 1.35 to 0.3	1.35
42 to 98		0	150	0	9	0	1.35
99 to 112		0	150	0	Gradually decreased from 9 to 2	0	Gradually decreased from 1.35 to 0.3
113 to 140		0	0	0	0	0	0

\*3 L of colostrum within 2 h after birth; pooled herd milk in the first three days of life.

\*\* start of experiment at mean age of  $8 \pm 1.9$  days, ranging from 6–12 days, one animal of earlyMC was 18 days old when entering the experiment.

**Table 3.** Ingredients of concentrate feed, roughage, milk replacer and total mixed ration (TMR).

<b>Feed</b>	<b>DM %</b>	<b>XA g/kg T</b>	<b>XP g/kg T</b>	<b>XL g/kg T</b>	<b>XF g/kg T</b>	<b>NDF g/kg T</b>	<b>ADF g/kg T</b>	<b>Starch g/kg T</b>	<b>Sugar g/kg T</b>
concentrate feed	86.84	63.21	232.31	47.64	68.63	199.09	82.61	363.67	48.32
roughage	86.06	66.61	98.28	22.05	326.74	660.53	365.18		
milk replacer	96.98	79.12	248.56	182.63					
TMR	39.29	43.75	81.22	35.35	217.76	426.36	244.81	301.51	

All ingredients were assessed by Weender analysis. Dry matter (DM), crude ash (XA), crude protein (XP) and crude fat (XL) were analyzed in all feedstuff. Crude fiber (XF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed in the solid feed. In concentrate feed and TMR starch was analyzed and in concentrate feed additionally sugar was analysed.

## 2.2. Morphometry of Calves

Concerning morphometry, the hip height, withers height, back length, body length and heart girth were determined as shown in Table 4 at days 1, 7, 14, 28, 42, 56, 70, 84, 98, 112, 126 and 140 of this trial. Hip and withers height were measured with a folding rule, the other measurements were taken with a measuring tape. Live weight (LW) was recorded on day of birth, and also on days 1, 28, 42, 70, 98, 112 and 140 with an electronic scale. Live weight gain (LWG) in kg per day was calculated from this data by dividing the weight gain between our sample days through the number of days between sampling.

**Table 4.** Morphometry of calves.

<b>Measure</b>	<b>Definition</b>
Withers height	From floor to dorsal process of first thoracic vertebra
Hip height	From floor to sacrum
Back length	From dorsal process of first thoracic vertebra to sacrum
Body length	From shoulder joint to ischium
Heart girth	Behind front leg

Definition of morphometry.

### **2.3. Collection and Analysis of Blood Samples**

Blood samples of each individual animal were taken on experimental days 1, 28, 42, 70, 98, 112 and 140 by jugular venipuncture and collected in serum and ethylenediaminetetraacetic acid (EDTA) plasma tubes (10 mL tubes; Sarstedt, Nuembrecht, Germany). Serum tubes were incubated for 30 min at 30 °C. After centrifugation at 3000× g for 15 min at 15 °C, serum and plasma aliquots were stored at –80 °C for subsequent analyses. Serum leptin concentrations were determined using a competitive enzyme immunoassay according to Sauerwein et al. [22]. Adiponectin concentrations were analyzed in serum with an indirect competitive bovine specific enzyme-linked immunosorbent assay (ELISA) according to Mielenz et al. [23]. Analyses of serum concentrations of beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), cholesterol, urea, total protein and glucose were done by an automatic analyzing system, based on spectrometric measures (Eurolyser, Type VET CCA, Salzburg, Austria). Insulin concentration in plasma was analyzed with a bovine insulin ELISA (Mercodia, Sweden).

### **2.4. Statistical Analysis**

Live weight (LW), live weight gain (LWG), hip and withers height, body length, heart girth,

back length, serum glucose, beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), leptin concentrations and plasma insulin concentrations were presented as least squares means (LSMeans) and standard errors (SEs) which were evaluated by repeated measures using the PROC MIXED procedure in SAS (V 9.4., SAS Institute Inc., Cary, NC, USA), and employing a restricted maximum likelihood model (REML). The model included a fixed factor of time, weaning age, parity of the mother and their interactions while the time was taken into consideration by a “REPEATED” statement. Best fitting covariance structures (compound symmetry, autoregressive and unstructured) was tested and used, based on the Akaike Information Criterion (AICC). Significant effects were further tested with the Tukey–Kramer procedure using the piecewise differentiable (PDIFF) procedure. Visualization and correlations computed as Pearson correlation coefficients were done using GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA). For all statistical tests,  $p < 0.05$  was the level of significance. For visualization, the measurements on serial time points were interpolated linearly.

### **3. Results**

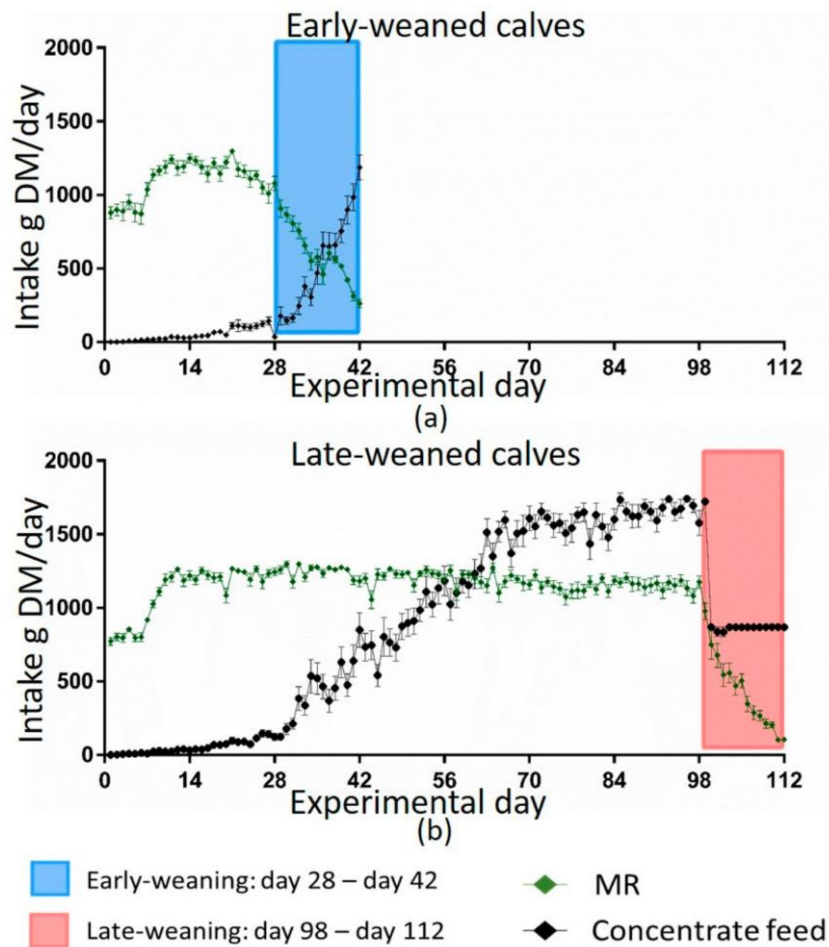
Multiparous cows were  $1592 \pm 805$  days (Mean  $\pm$  SD) old when they gave birth to MC. Their mean lactation number was  $1.875 \pm 0.074$  lactations (Mean  $\pm$  SD). The age of primiparous cows was  $710 \pm 67$  days (Mean  $\pm$  SD) at calving. Birth weight of PC was  $37.9 \pm 4$  kg (Mean  $\pm$  SD) and birth weight of MC was  $39.6 \pm 6$  kg (Mean  $\pm$  SD). 3.1. Feed Intake

There was no difference in feed intake between calves from multiparous (MC) and primiparous cows (PC) in both weaning groups. Therefore, all data from calves of one weaning group were combined for the visualization of feed intake patterns in early and late weaned calves (Figure 1). Both groups had the same MR intake for the first 28 days of trial. Early-weaned calves

consumed 11,288 g MRDM on average during their weaning period (days 28–42) whereas late-weaned calves

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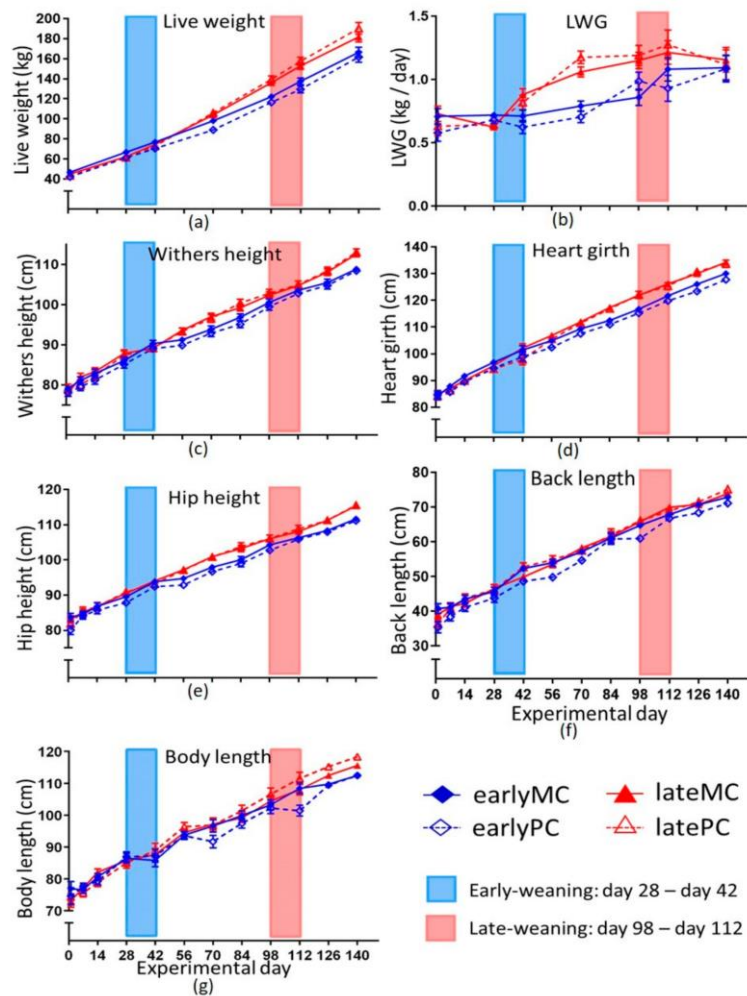


**Figure 1.** Milk replacer (MR) and concentrate feed intake in g dry matter/d for early-weaned calves (a) and late-weaned calves (b). Early-weaned calves ( $n = 24$ ) were weaned gradually between days 28 and 42 of trial. Late-weaned calves ( $n = 28$ ) were weaned gradually between days 98 and 112 of trial. Amount of concentrate feed was limited to 1 kg/d after weaning for early-weaned calves and during weaning period of late-weaned calves. Data shown as means  $\pm$  the standard error of the mean (SEM) evaluated with GraphPad Prism 6. Due to technical problems, not all calves could be included in monitoring feed intake.

### 3.2. Morphometry

Morphometric variables increased over time ( $p < 0.001$ ) and were greater in late-weaned calves. Interactions between time and weaning group were also observed to be highly significant for LW, withers and hip height ( $p < 0.001$ ), heart girth ( $p < 0.01$ ) and body length ( $p < 0.05$ ; Figure

2). LW was greater for all calves in the late-weaned group from day 70 until the end of trial (Figure 2a). On day 140 the mean live weight of early-weaned calves was  $164.1 \text{ kg} \pm 3.65 \text{ kg}$ , whereas that of late-weaned calves was  $186.1 \pm 3.88 \text{ kg}$  ( $p = 0.009$ ). LWG (Figure 2b) was strongly influenced by the weaning age ( $p < 0.001$ ). Additionally, a significant interaction between time and weaning age was found ( $p < 0.001$ ). Late-weaned calves had a significantly higher LWG from day 42 until day 98 of our trial ( $p < 0.05$ ). Withers height differed significantly between late- and early-weaned calves on day 84 ( $p = 0.014$ ) and day 140 ( $p < 0.001$ ). Hip height differed significantly between the weaning groups from day 56 until day 84 ( $p < 0.05$ ) and on day 140 ( $p < 0.001$ ; Figure 2c, d). Late-weaned calves had a greater heart girth from day 84 onwards ( $p < 0.05$ ; Figure 2f). Body length was significantly lower for early-weaned calves at the end of trial on day 126 and day 140 ( $p < 0.01$ ). Back length was the only morphometric variable influenced by parity of the mother as indicated by an interaction between parity and weaning age ( $p = 0.011$ ). EarlyPC had significantly lower back length than earlyMC ( $p < 0.001$ ), whereas it did not differ in the parity groups of late-weaned calves (Figure 2e).



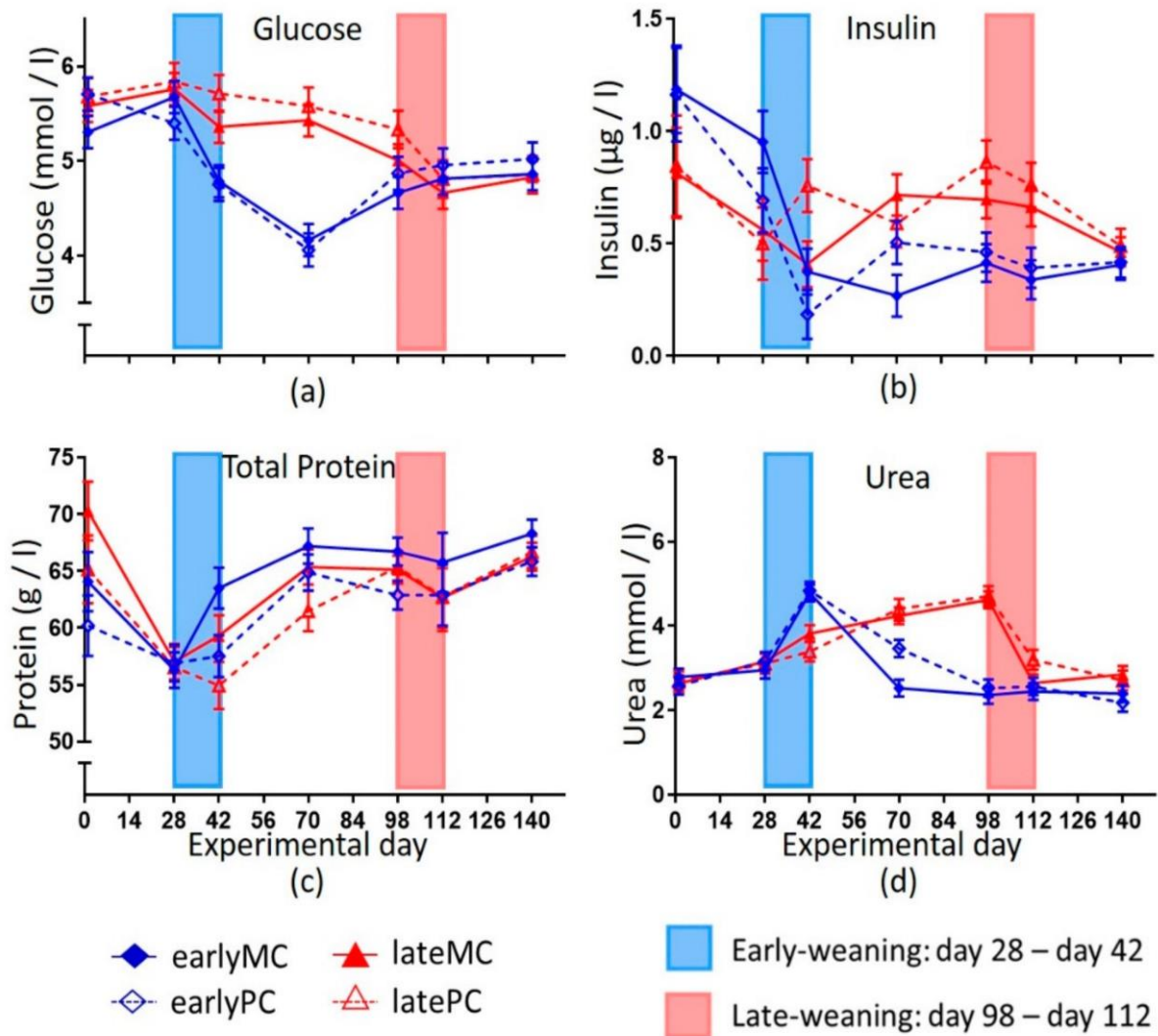
*p*-Values

Parameters	Time (T)	Parity (P)	Weaning Age (W)	T × P	T × W	P × W	T × P × W
Live weight	<0.001	0.396	<0.001	0.685	<0.001	0.099	0.453
LWG	<0.001	0.422	<0.001	0.221	<0.001	0.325	0.556
Withers height	<0.001	0.419	0.005	0.444	<0.001	0.436	0.659
Hip height	<0.001	0.365	0.002	0.233	<0.001	0.364	0.985
Back length	<0.001	0.035	0.008	0.481	0.560	0.011	0.129
Heart girth	<0.001	0.122	0.004	0.899	<0.001	0.537	0.530
Body length	<0.001	0.791	0.023	0.140	0.005	0.116	0.217

**Figure 2.** Morphometry of calves. Shown are live weight (a), live weight gain (LWG) (b), withers (c), heart girth (d), hip height (e) and back length (f). Early-weaned calves were weaned gradually between days 28 and 42 of the trial. Late-weaned calves were weaned gradually between days 98 and 112 of the trial. Data shown as LSmeans ± SEM, early-weaned calves from multiparous cows (earlyMC) *n* = 16, late-weaned calves from multiparous cows (lateMC) *n* = 16, early-weaned calves from primiparous cows (earlyPC) *n* = 15, late-weaned calves from primiparous cows (latePC) *n* = 12.

### 3.3. Blood Parameters

Time had a significant effect on all measured variables in the blood ( $p < 0.001$ ) and there was an interaction of time and weaning age observed for all variables except total protein (Figures 3 and 4). On day 70, which was between the two weaning periods, the two weaning groups differed highly significant in their serum glucose concentration ( $p < 0.001$ , Figure 3a). Blood glucose concentration increased significantly from day 70 to day 112 in the early-weaned calves ( $p = 0.014$ ). Insulin concentration dropped on days 98 and 112 in early-weaned calves and stayed below those insulin concentrations of late-weaned calves during the rest of the trial until day 140 (Figure 3b). Total protein concentration in calves (Figure 3c) was influenced by mother's parity ( $p = 0.021$ ) and was higher in MCs. Urea concentration (Figure 3d) in late-weaned calves increased constantly up to day 98 and started to drop to the initial level during weaning. In early-weaned calves, it increased during weaning and decreased afterwards. Therefore, they reached lower urea concentrations after weaning than late-weaned calves from day 42 until day 98 ( $p < 0.001$ ).

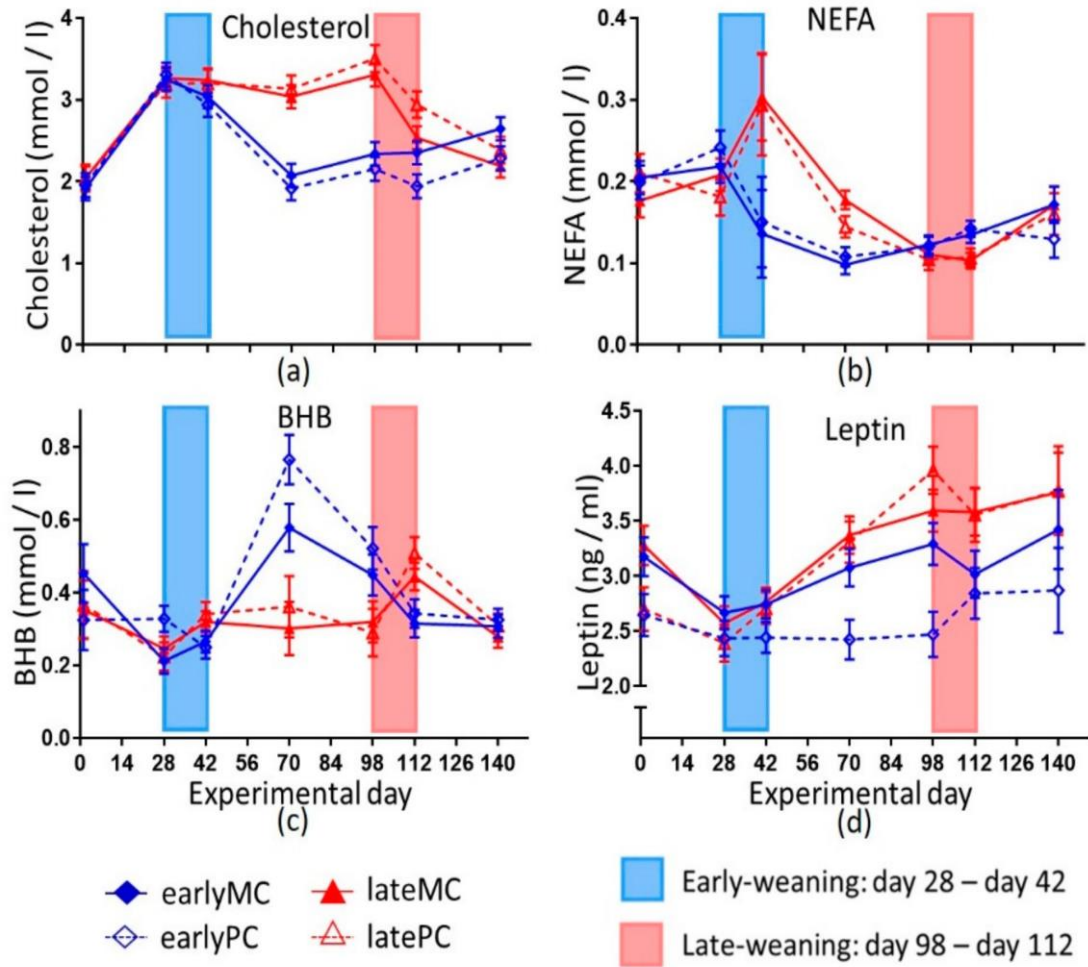


*p*-Values

Parameters	Time (T)	Parity (P)	Weaning Age (W)	T × P	T × W	P × W	T × P × W
insulin	<0.001	0.654	0.089	0.790	<0.001	0.448	0.080
glucose	<0.001	0.224	<0.001	0.538	<0.001	0.620	0.781
total protein	<0.001	<b>0.021</b>	0.779	0.229	0.140	0.597	0.714
urea	<0.001	0.318	<0.001	0.064	<0.001	0.532	0.464

**Figure 3.** Blood concentrations of the glucose (a), insulin (b), total protein (c) and urea (d) of calves. Early-weaned calves were weaned gradually between days 28 and 42 of the trial. Late-weaned calves were weaned gradually between days 98 and 112 of this trial. Data shown as LSmeans ± SEM, early-weaned calves from multiparous cows (earlyMC)  $n = 16$ , late-weaned calves from multiparous cows (lateMC)  $n = 16$ , early-weaned calves from primiparous cows (earlyPC)  $n = 15$ , late-weaned calves from primiparous cows (latePC)  $n = 12$ .

Cholesterol concentrations increased similarly in all groups from day 1 to day 28. After weaning, it decreased in the early-weaned calves from day 28 to day 70 ( $p < 0.001$ ). Therefore, they showed lower cholesterol concentrations than late-weaned calves until day 112 ( $p < 0.01$ ). From day 70 to day 140 it increased significantly in early-weaned calves ( $p = 0.011$ ; Figure 4a). NEFA concentrations decreased with weaning in early-weaned calves. Therefore, late-weaned calves had higher NEFA concentrations on day 70 ( $p = 0.001$ ; Figure 4b). The serum BHB concentration increased after weaning for the early-weaned calves (day 42–70,  $p < 0.001$ ), whereas it remained low in the late-weaned calves and increased after their weaning period (day 112–140;  $p < 0.001$ ). As a result, a significant difference in serum BHB concentration was observed between the weaning groups on day 70 ( $p = 0.001$ ; Figure 4c). After day 70, BHB concentration decreased until day 140 in early-weaned calves ( $p < 0.001$ ). BHB concentration was negatively correlated with glucose concentration when all treatments and time points were considered collectively ( $p = 0.0001$ ;  $r = -0.1895$ ). Serum leptin concentration showed a significant interaction of weaning age and time ( $p < 0.001$ ), and was also influenced by parity of the mother ( $p < 0.05$ ). Serum leptin concentration increased from day 28 to day 140 in late-weaned calves ( $p = 0.008$ ), whereas no significant increase was found in early-weaned calves (Figure 4d). Calves' plasma leptin concentrations correlated positively with the lactation number of the mother ( $p = 0.0015$ ;  $r = 0.4177$ ; data not shown). All measured blood metabolites except insulin (Figure 3a), total protein (Figure 3c) and NEFA (Figure 4b) were affected by weaning age. Weaning age did not affect Adiponectin concentrations, but there were significant effects of time ( $p < 0.001$ ) and interaction between time and parity ( $p = 0.031$ ; data not shown).



*p*-Values

Parameters	Time (T)	Parity (P)	Weaning Age (W)	T × P	T × W	P × W	T × P × W
cholesterol	<0.001	0.995	<0.001	0.990	<0.001	0.327	0.344
NEFA	<0.001	0.766	0.072	0.737	<0.001	0.735	0.440
BHB	<0.001	0.225	<b>0.026</b>	0.540	<0.001	0.685	0.511
leptin	<0.001	<b>0.025</b>	<0.001	0.448	<0.05	0.100	0.115

**Figure 4.** Indicators for lipid metabolism of calves. Shown are cholesterol (a), non-esterified fatty acids (NEFA) (b), beta-hydroxybutyrate (BHB) (c) and leptin (d). Early-weaned calves were weaned gradually between days 28 and 42 of this trial. Late-weaned calves were weaned gradually between days 98 and 112 of this same trial. Data shown as LSmeans ± SEM, early-weaned calves from multiparous cows (earlyMC)  $n = 16$ , late-weaned calves from multiparous cows (lateMC)  $n = 16$ , early-weaned calves from primiparous cows (earlyPC)  $n = 15$ , late-weaned calves from primiparous cows (latePC)  $n = 12$ .

## **4. Discussion**

This study assessed the impact of two different weaning ages. Furthermore, calves were grouped according to their mother's parity. Precisely, calves born to primiparous and born to multiparous cows were allocated to both weaning groups. To assess the effect of weaning age, a high-quality MR was used, consisting mostly of milk components (Table 2). One liter of MR contained the same amount of protein as whole milk (36.13 g XP/L MR vs. 35 g XP/L whole milk). The amount of MR powder used in literature ranges from 0.383 kg per day to 1.49 kg per day [24], thus the calves in the present study received a high amount of MR, which was quite similar to an ad libitum intake [25]. Voluntary DM intake/day from MR was lower than 1300 g/day in the first 8 weeks of life [26], which was the maximum allowance in the current study. Therefore, the effects of weaning age were assessed under sufficient milk-derived energy and nutrient supply, and not negatively influenced by a low amount and quality of MR.

### **4.1. Feed Intake**

Data from computer-controlled mangers for roughage were not shown as there were technical problems with recognizing the individual calf, precluding data collection. As expected, MR intake was not different in the four groups during the first 28 days of trial when MR allowance was the same for all calves. Afterwards, MR intake followed the regimen of weaning. However, the pattern of voluntary solid feed intake over time varied between the weaning groups. Late-weaned calves consumed concentrate feed—despite consumption of the full amount of MR and even before MR supply was reduced, which led to a greater concentrate feed intake at the beginning of their weaning compared to the early-weaned group. This was in line with previous findings [5,13]. Later weaning (12.7 weeks) resulted in concentrate feed intake before weaning (close to 0.5 kg/d), whereas early-weaned calves (6.7 weeks) did not increase their concentrate

intake before weaning started [13]. Eckert et al. [5] observed the same feed intake pattern for calves that were weaned with 6 or 8 weeks, with calves weaned later consuming more concentrate feed 1 week pre- and post-weaning. Male calves permitted to choose their preferred feed between MR and different solid feed components started to consume concentrate feed at the age of 49 days [26]. Despite the high MR allowance, calves consumed solid feed as observed in the present study. As milk production in the first lactation was associated with a higher intake of grain and forage at weaning [7], voluntary solid feed intake before weaning can be a potential benefit for later life production. Higher starter intake was related to a higher weight gain during weaning [13]. Late-weaned calves probably consumed more energy until their weaning, as they had a higher concentrate feed intake and still consumed MR. This was associated with the higher growth rates of late-weaned calves (Figure 2). This also indicates that even though the late-weaned calves had a high MR supply for 15 weeks, they started to consume solid feed and their rumen probably started to develop and to mature. They even restricted their MR intake during the weaning period voluntarily more than they had to, which might indicate the ability of mature organs to function ruminant-specifically.

It is proposed that solid feed was digested in the rumen as indicated by several rumen development parameters (Schwarzkopf et al., unpublished data). This indicated a development of rumen digestive functions despite high MR intake. Besides gastrointestinal development, however, liquid feeding in addition to voluntary solid feed intake over 17 weeks in the early life of calves might also be of advantage for other body functions and endocrine regulatory processes, as demonstrated in the following sections.

## 4.2. Morphometry of Calves

Weaning calves at a more mature developmental stage (17 weeks of life) resulted in increased LWG and higher LW (Figure 2). This was also demonstrated by several other studies [5,27,28]. The reticulorumen reached its adult proportions at the age of 12 to 16 weeks [16]. Additionally, the utilization of solid feed for body growth increased with age. Berends et al. [15] adjusted the quantity of MR for male calves to achieve the same weight gain across different solid feed levels. This way, they measured an increasing utilization of solid feed until the age of 27 weeks. If this was a sign of maturity and a proper function, it could be an explanation why later-weaned calves had a greater live weight and were able to maintain this at least 4 weeks after weaning. It could mean that the early-weaned young ruminants were physiologically unable to use all the energy provided with solid feed, and their preferred sources of energy remained lactose and milk fat. As promoted by early-weaning, the intake of solid feed instead of MR also resulted in a greater need of energy for ruminal activity. Elevated muscle work in the rumen needs energy. Furthermore, heat production through fermentation in the rumen [29] increased the energetic need for thermoregulation. Thus, the metabolic rate for maintenance might increase by solid feed intake. This could also be an explanation for the lower LWG in early-weaned calves compared to the late-weaned groups. Early-weaned calves were unable to compensate the reduced growth and could not catch up with weight, wither and hip height, body length and heart girth at least until the age of 5 months (Figure 2,  $p < 0.05$ ). Furthermore, back length showed a significant interaction of mother's parity and weaning age ( $p = 0.011$ ), showing shorter back lengths in earlyPC. Reduced back length is a well-known symptom of prenatal imprinting by intrauterine malnutrition in rodents, sheep and humans [30]. Weaning late, however, was advantageous to correct the imprinted change in body proportions, as latePC did not express shorter back lengths.

### 4.3. Blood Metabolites

Collection of blood samples was always in the morning between eight and ten, but it was not controlled for feed intake. Part of the variance of serum insulin and glucose concentration could be a result of different times of feed intake relative to sampling. In milk-fed calves, blood glucose increased 1 h after feeding and then decreased rapidly during the next 2 h [28]. A high capacity for hepatic gluconeogenesis is an essential metabolic feature for a ruminant due to low intestinal glucose availability. Weaning late at 17 weeks of age resulted in a smooth transition of glucose metabolism from lactose to endogenous glucose production by the liver without any signs of dysregulation. In early-weaned calves, weaning led to a strong decrease in blood glucose concentration (Figure 3a), as lactose is the most important source of glucose for young calves. Obviously, early-weaned calves were not able to compensate the lack of dietary glucose (lactose) by hepatic gluconeogenesis. The glucose gap was closed slowly. There was a significant increase in blood glucose concentration from day 70 to day 112 in the early-weaned calves ( $p = 0.014$ ), which indicated that liver gluconeogenetic function matured slowly until 4 months of age. This is in accordance with findings of other authors [27]. Calves that were weaned at the age of 5 weeks had lower blood glucose concentrations than calves that still received MR, even 7 weeks after weaning [28].

Low blood glucose concentrations are detrimental for a developing young animal, since related endocrine status is concomitantly changed. The decrease in glucose concentrations at early-weaning resulted in a decrease in serum insulin concentrations. In more mature calves, the endocrine system was smoothly adapting when weaning was done with only marginal changes in hormone concentrations. The decrease of insulin was steeper for early-weaned calves and resulted in more abrupt changes (Figure 3b). Therefore, weaning stress of calves was attenuated when weaned later, because it has more time to mature and adapt. In accordance, other studies also showed that a lower intake of MR decreased insulin and insulin like growth factor 1 (IGF1)

concentrations in calves [24,31,32]. Therefore, a catabolic status was most likely established in early-weaned calves, as insulin and IGF1 are the strongest anabolic hormones.

Consequently, lipolysis and proteolysis most likely were promoted to gain energy and produce precursors for gluconeogenesis. The catabolic state, however, was not able to increase glucose concentrations for several weeks in early-weaned calves. Both pathways led to an increase in ketone bodies in blood when the oxidative capacity of mitochondria was limited [33]. The negative correlation between glucose and BHB concentrations supported the hypothesis that BHB originated from lipolysis and proteolysis because of glucose shortage. NEFA derived from lipolysis were used in beta-oxidation.

Hence, the blood concentrations decreased in early-weaned calves (Figure 4b). Simultaneously, the blood BHB concentration increased, reflecting a low capacity of hepatic oxidative phosphorylation. Furthermore, an incomplete oxidation of amino acids could also result in a higher BHB concentration. The decrease in cholesterol concentrations might also be linked to higher ketone body production, as the precursor metabolite (3-Hydroxy-3-methyl-glutaryl-CoA) for cholesterol production was used for BHB production [34]. Low insulin concentration led to a lower activation of HMG-CoA-reductase, which is vital for cholesterol biosynthesis [35]. This might also be a reason for lower cholesterol production. There was a significant increase in cholesterol concentration in the early-weaned groups from day 70 to day 140 of this trial ( $p = 0.011$ ), which reflected that the liver was unable to produce as much cholesterol in the young, early-weaned calves on account of later production when they were more mature. A higher energy supply enhanced cholesterol biosynthesis in 16-week-old bull calves [36], which is in line with our findings in late-weaned female calves that received more energy through MR and a higher concentrate feed intake (Figure 1). Moreover, the MR is a nutritional source for cholesterol, which is no longer available after weaning. Therefore, late-weaned calves had a constant supply of cholesterol until day 98 of the trial. The liver of early-weaned calves was

not able to produce enough cholesterol and glucose to compensate the dietary lack through weaning for several weeks. Possibly, these are not the only metabolic pathways that did not mature in early-weaned calves, and other important ones were impaired, as well.

Blood BHB concentration was used as a marker for rumen development as it originated from rumen wall ketogenesis [37]. Many authors observed a rise in blood BHB at weaning and with starter intake [14,38]; while lower concentrations were detected in ad libitum MR fed calves ( $0.14 \pm 0.01$  mmol/L) compared to calves fed with restricted MR ( $0.17 \pm 0.01$  mmol/L) [31]. A similar pattern was observed in this study, as serum BHB concentrations were higher in early-weaned calves compared to late-weaned calves still on MR feeding on day 70 (Figure 4c). But these higher concentrations declined again with age ( $p < 0.001$ ). Thus, their relevance as a marker for rumen development could be questioned. It is likely that to some extent BHB was derived from an incomplete oxidation of nutrients, such as fatty acids and amino acids as described above. Parts of higher NEFA concentration in late-weaned calves on day 70 might also be explained by NEFA content in MR (Figure 4). Other authors had also seen that weaning resulted in lower NEFA concentration compared to calves that received MR [14,39]. Low glucose and high BHB after weaning indicated that capacity of liver functions was less developed in early-weaned calves. The increase in plasma urea concentration during weaning could have resulted from elevated proteolysis as well. After weaning the urea concentration likely decreased because the rumen used it in the ruminohepatic recycling of nitrogen. Another explanation might be a lack of microbial protein and low protein sustenance. Hence, plasma urea concentrations were lower in early-weaned calves than in late-weaned calves that still received MR and thus got enough protein for body protein turn-over.

#### *Influence of Mother's Parity on Early- and Late-weaned Calves*

As discussed before, heifers have to allocate nutrients and energy between their own body needs and the requirements of the fetus. This could create unfavorable conditions such as intrauterine

malnutrition for the unborn calf, which could affect them for their whole life [18]. Besides reduced back length, a lower leptin concentration appeared to be another sign of intrauterine imprinting by malnutrition (Figure 4d). Furthermore, the lactation number of the dams and serum leptin concentrations in their 1-week old calves were positively correlated ( $p = 0.0015$ ;  $r = 0.4177$ ). Leptin plays an important role in the onset of puberty and regulation of the immune system [40,41].

Low leptin concentration in early postnatal life was associated with a leptin resistance in later life in rats [42]. Thus, the lower leptin concentrations in PC could indicate a potential risk factor for a dysregulated energy metabolism and development also in later life [43]. The main effect of leptin is a decrease in feed intake. In general, leptin concentration changes during a long-term negative energy balance in mammals [44]. Hence, the lower serum leptin concentrations in the PC can be hypothesized as a sign of hunger and lack of adipose deposition.

#### **4.4. Implications and Perspectives**

Most of the evaluated blood metabolites did not differ among the groups at the age of 5 months, but changes occurred more abruptly for early-weaned, and slower for later-weaned calves. Hence, early weaning may cause more postnatal metabolic stress. This experience could also lead to metabolic imprinting and affect health and productivity in later life [20]. Kenéz et al. [6] examined a reduced amount of MR supply during the rearing period, which sustainably affected the development and altered the metabolism. These changes could still be seen at first lactation. So, it is possible that, weaning age or mother's parity will affect these animals in later life through metabolic imprinting. The existence and quality of long-term consequences are examined in an ongoing study with the same animals during their first and second lactation. Further research is needed to identify an optimal age to transition from MR to solid feed and an

optimal amount of MR. It is challenging to distinguish the effect of older weaning age from the effect of an overall higher amount of MR that is consumed. Thus, it would be advisable to conduct further research on both factors and their influence on the growth and development of calves. Voluntary MR and solid feed intake are different in individual calves [26,45], and should therefore be considered in further research about optimal weaning age. From this study, a later weaning regimen can be considered as advantageous in early life with potential consequences for later health and metabolic performance. Naturally, calves suckling their dam were weaned at the age of 8–9 months [46,47]. This behavior might have been established through evolution, creating the best possible outcome for calves, and should therefore be considered in re-thinking weaning regimens in dairy calves.

## **5. Conclusions**

Increasing weaning age to 17 weeks enables a smooth transition of physiological functions from the pseudomonogastric status to full ruminant status in dairy calves. However, weaning at 17 weeks of age is not only influenced by milk. The calves ingested up to 2 kg of concentrate feed, despite a high intake of MR. Thus, body maturation was supported by both sources of energy.

**Author Contributions:** Conceptualization and Methodology, K.H., S.D. and J.F.; Investigation, J.F., S.S., A.K., J.K., S.K., U.M.; Formal analysis, J.F.; Visualization, S.S.; Writing—original draft, S.S.; Writing—review & editing, J.F., K.H., S.D., S.S.

**Funding:** This research received no external funding.

**Acknowledgments:** Many thanks to the technical staff of the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany. We also want to thank the workgroup of Sauerwein (Institute of Animal Science, Physiology and Hygiene Unit of the University of Bonn) for leptin and adiponectin analyzes.

**Conflicts of Interest:** The authors declare no conflict of interest.

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### 2.1.1 Correction

The authors wish to make the following correction to their paper [1]. In the Materials and Method Section, Table 3 was incorrect due to some numerical errors. The correct version of the table appears below. The authors apologize for any inconvenience; the change does not affect the scientific results on the animal level.

**Table 3.** Dry matter content and chemical composition of milk replacer, concentrate feed and roughage (hay) fed prior to weaning and total mixed ration (TMR) fed after weaning of the calves.

<b>Feed</b>	<b>DM %</b>	<b>CA g/kg DM</b>	<b>CP g/kg DM</b>	<b>EE g/kg DM</b>	<b>CF g/kg DM</b>	<b>NDF g/kg DM</b>	<b>ADF g/kg DM</b>	<b>Starch g/kg DM</b>	<b>Sugar g/kg DM</b>
<b>Prior to weaning</b>									
milk replacer	97	79	225	184					
concentrate feed	87	63	230	51	63	188	77	371	47
roughage (hay)	88	75	97	18	322	666	379		
<b>After weaning</b>									
TMR	44	73	134	36	212	421	242	182	12

All ingredients were assessed by Weender analysis. Dry matter (DM), crude ash (CA), crude protein (CP) and ether extract (EE) were analyzed in all feedstuff. Crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed in the solid feed. In concentrate feed and TMR starch was analyzed and in concentrate feed additionally sugar was analyzed.

## **2.2 Manuscript 2: Weaning age influences indicators of rumen function and development in female Holstein calves**

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Published in  
BMC Veterinary Research (2022), Volume 18(1), pages 102 – 116;  
DOI name: [10.1186/s12917-022-03163-1](https://doi.org/10.1186/s12917-022-03163-1)

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Open access

The original publication is available at <https://doi.org/10.1186/s12917-022-03163-1>

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## **Abstract**

**Background:** Prenatal and postnatal conditions are crucial for the development of calves.

Primiparous cows are still maturing during pregnancy, thus competing with the nutritional needs of their offspring. Therefore, mature cows might provide a superior intrauterine condition. Furthermore, weaning calves at an older age might affect them positively as well by reducing stress and offering time for various organs and their functions to develop. We aimed to evaluate effects of mothers' parity and calves' weaning age on gastrointestinal development and corresponding acid–base balance. Fifty- nine female German Holstein calves (about 8 days old) were investigated in a  $2 \times 2$  factorial experiment with factors weaning age (7 vs. 17 weeks) and parity of mother (primiparous vs. multiparous). Calves were randomly assigned to one of these four groups. Animal behavior that was observed included resting, chewing and active behavior.

**Results:** Behavioral patterns were interactively affected by time and weaning age. Rumen sounds per 2 min increased in early- weaned calves during their weaning period. In late- weaned calves a consistently increase in rumen sounds was already recorded before their weaning period. Urinary N- containing compounds (creatinine, hippuric acid, uric acid, urea, allantoin) were interactively affected by time and weaning age. Concentrations of all measured compounds except urea increased during early weaning. All except hippuric acid concentration decreased in earlyweaned calves after weaning. In late- weaned calves allantoin and uric acid increased before weaning and did not change during weaning.

**Conclusion:** These results suggest that late- weaned calves developed adequate rumen functions and acid–base balance, whereas early- weaned calves might have suffered from ruminal acidosis and catabolism. Weaning calves at 7 weeks of age might be too early for an adequate rumen development.

**Keywords:** Weaning age, Calves, Rumen function and development, Acid–base balance, Parity

## **Background**

The prenatal and early postnatal periods are critical times of development. Any intervention may cause lifelong changes in metabolism, which is termed metabolic imprinting, and therefore have a great impact on health and performance in later life [1]. The parity of the mother could have a major impact on her calf during the prenatal period [2]. Multiparous cows need to share energy and nutrients between the fetus and the lactating mammary gland, and primiparous cows are still maturing during pregnancy, thereby competing with the needs of their offspring. Both conditions could lead to a nutritional restriction of the calf. Calves are born as non-ruminant animals, which rely mainly on lactose and milk fat as energy supply. At weaning, the dependence on rumen fermentation increases and volatile fatty acids (VFA) become the most important source of energy [3]. This implies great modifications of digestive physiology. Rumen weight and epithelial area increased when liquid feed was replaced by solid feed at early age [4]. Weaning also changed the expression of genes involved in the cell cycle, lipid metabolism, molecular transport, cell morphology and death, cellular growth, and proliferation in the rumen epithelium [5]. Therefore, these indicators of a rapid rumen development appeared to be induced by a forced change from liquid to solid feed. On the contrary, other indicators were not influenced by weaning. In lambs, the expression of ketogenic enzymes in rumen epithelium increased with age, regardless of diet and time of weaning [6]. Therefore, rumen development might at least partly be controlled by an evolutionary blueprint. The growth of different gastrointestinal compartments is usually not finished at weaning. The weight of the reticulorumen and omasum relative to body weight (BW) increased until 17 weeks (wk) of age [7]. The omasum kept growing until 1 year of age [3]. Consequently, early weaning might not be necessary for rumen development. As a hypothesis, gastrointestinal development still proceeds when weaning is delayed. Late weaning may minimise the abrupt changes associated with solid feed intake and related metabolic changes, which occur during early weaning. Parity of the mother might impact their calves' development through potential restriction of resources

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as well. Therefore, the present study aimed to determine the influence of mothers' parity and calves' weaning age on gastrointestinal tract development and associated systemic metabolic changes such as acid base homeostasis and early N metabolism. Transition was assessed by markers of ruminal development, and of corresponding adaptive changes in acid–base balance and gut microbial metabolism.

## **Results**

### **Characterization of feed intake pattern**

All data according to feed intake was previously published in Schwarzkopf et al. [8]. In summary, the intake pattern of milk replacer (MR) and concentrate (C) was similar for both weaning groups until experimental day 28 when weaning was initiated for early-weaned group and there were no significant differences in intake levels (122 – 133 g DM/day). While the late-weaned calves were maintained unchanged at a constant MR level (approximately 1300 g DM/day) and C intake continued to increase until reaching 2 kg on day ~ 70, MR intake for the early-weaned group was gradually reduced until day 42 of the experimental trial. At this time, C intake was similar to the late-weaned group. On experimental days 70 and 98, the early-weaned group was characterized by a TMR feeding pattern while the late-weaned group still consumed significant amount of MR. On experimental day 112, it is assumed that the late-weaned group consumed greater amounts of roughage as MR intake was terminated. At the same time, C intake was reduced to 1 kg/day, so that the general ration type was roughly comparable to that of the early-weaned group. On experimental day 140, all groups received the same ration type.

### **Animal behavior**

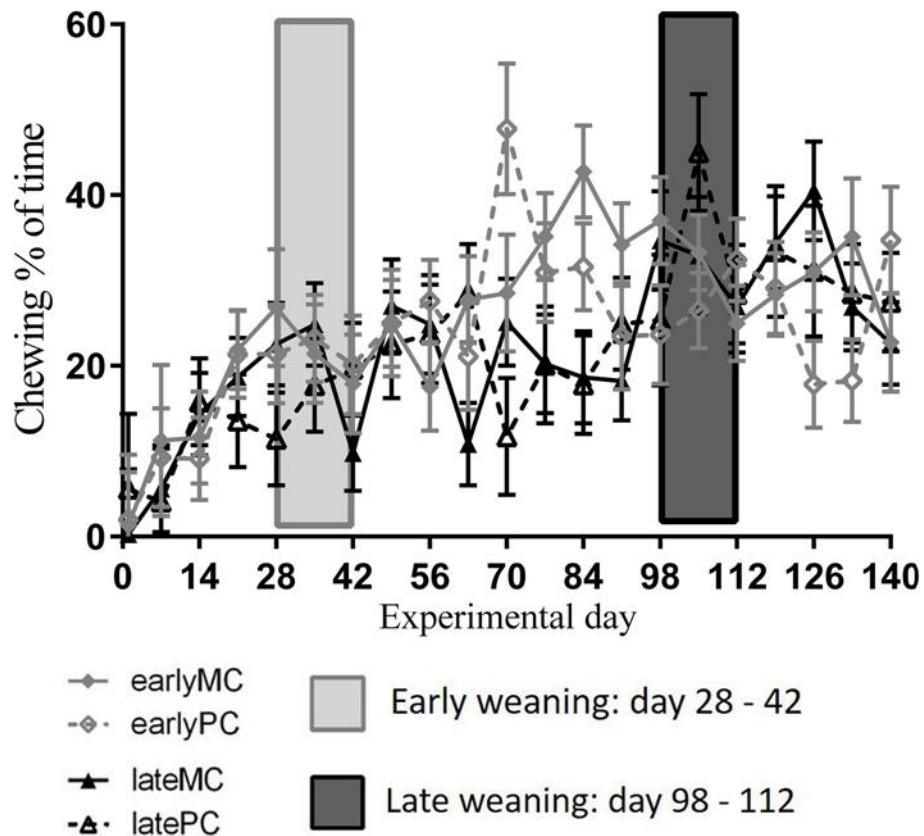
All observed behavior patterns changed over time and were interactively influenced by time and weaning age (Table 2). Percentage of time spent with chewing increased with age (Fig. 1).

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Early-weaned calves increased chewing at d 70 of the trial compared with d 1 ( $p < 0.001$ ). Late-weaned calves increased their time spent with chewing at d 105 of the trial compared to d 1 ( $p = 0.002$ ). Time spent with chewing exceeded 20% after d 21 (earlyMC and earlyPC), 28 (lateMC) or 49 (latePC). Time spent with active behavior increased over time (Fig. 2 A). There was, however, neither a significant difference between the weaning groups on any experimental d nor between the d in each weaning group. Time spent with resting decreased with age in both groups (Fig. 2 B), and the decrease was more pronounced in early-weaned calves. These spent significantly less time resting on d 35 compared to d 1 ( $p = 0.016$ ), whereas late-weaned calves did not decrease the percentage of time spent resting until d 112 ( $p = 0.005$ ).

### **Rumen fill and sound**

Rumen fill (Fig. 3 A) and sounds (per 2 min, Fig. 3 B) increased significantly over time. A significant interaction between time and weaning age was observed for both variables (Table 2). Interaction between time, weaning group and parity was significant for rumen sounds. Rumen sounds increased during early weaning (d 28 vs. d 42;  $p < 0.001$ ), whereas in late-weaned calves it increased already before weaning (latePC d 1 vs. d 56,  $p = 0.003$ ; lateMC d 1 vs. d 70,  $p < 0.001$ ) and therefore weaning caused no further increase (d 98 vs. d 112,  $p = 0.804$ ). Rumen fill increased from d 1 to d 14 in both weaning groups (early  $p < 0.001$ ; late  $p = 0.003$ ). It was also influenced by parity (Table 2) with PC yielding a greater score. At the end of trial, there were no significant differences between weaning groups regarding rumen fill and sounds. The VFA and ammonia-N concentrations and pH in ruminal fluid at d 140 showed no effects of weaning age, mother's parity and their interaction (Table 1).



**Figure 1.** Percentage of time spent with chewing. Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMeans with SE. Early-weaned calves from multiparous cows (earlyMC), late-weaned calves from multiparous cows (lateMC), early-weaned calves from primiparous cows (earlyPC), late-weaned calves from primiparous cows (latePC).

### pH in saliva, feces, and blood

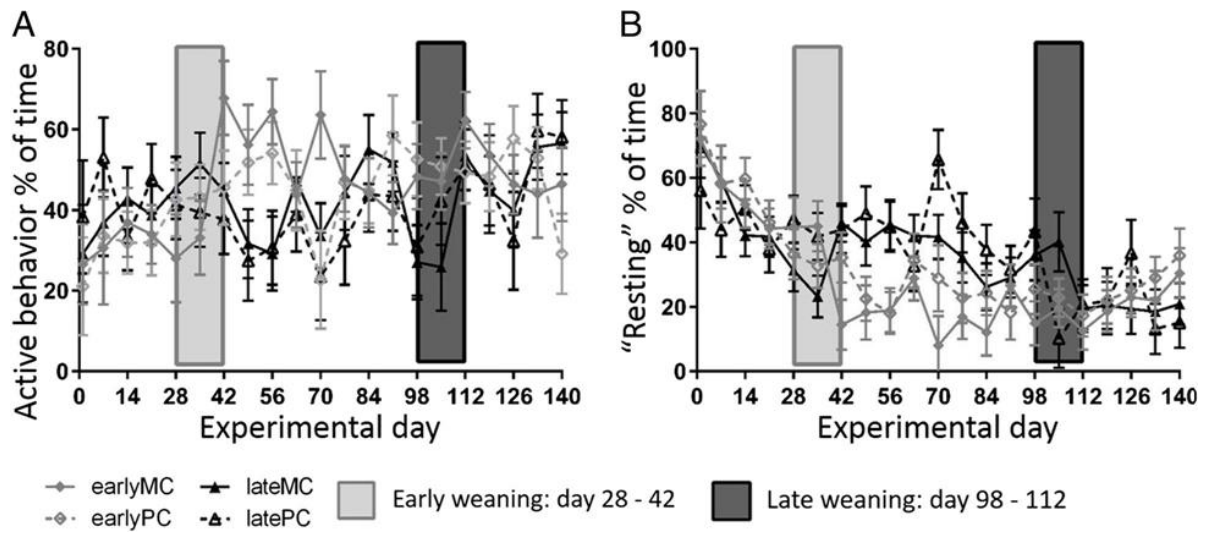
The pH value in saliva increased over time and was not affected by weaning age or mother's parity (Table 2). It increased from d 1 to d 42 ( $p < 0.001$ ) and then from d 42 to d 140 ( $p = 0.011$ ). Interaction between time and weaning age was significant for pH of feces (Table 2). It increased similarly in all groups from experimental d 1 to d 28 ( $p < 0.001$ ). The highest pH values were observed at d 28 of the trial with no significant differences between the groups (earlyMC pH = 7.25; latePC pH = 6.99; lateMC pH = 7.06; earlyPC pH = 7.18). Afterwards, it

decreased over time. The steepest pH decrease was observed during weaning in both weaning groups (early-weaned d 28 vs. d 42,  $p = 0.002$ ; late-weaned d 98 vs. d 112,  $p = 0.018$ ). Hence, it was different between weaning groups on experimental d 98 ( $p = 0.043$ ). However, late-weaned calves had a significant decrease in feces pH before weaning (d 28 vs. d 70,  $p = 0.044$ ). Whole blood pH increased over time (Fig. 4 C) and was also interactively influenced by time and weaning age, but it was not significantly different between groups at any sampling d. It increased from experimental d 42 to d 70 in early-weaned calves ( $p < 0.001$ ).

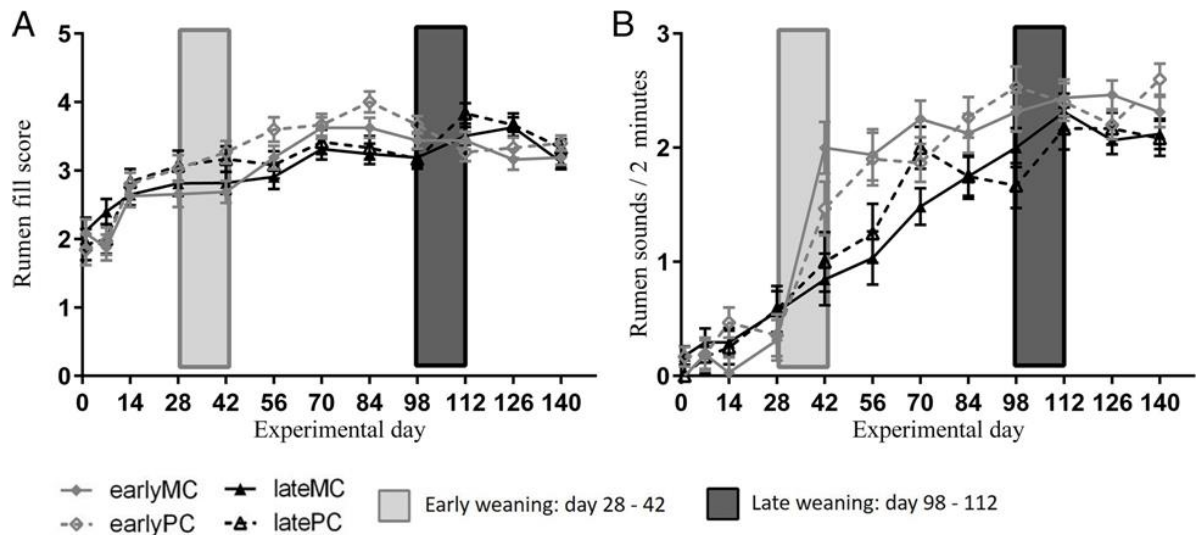
### **Variables in urine**

The pH value in urine was interactively affected by time and weaning age (Table 2). During early weaning, it decreased (d 28 vs. d 42,  $p = 0.010$ ) and then increased within the experimental period from d 42 to d 70 ( $p < 0.001$ ). Afterwards, it remained stable for the rest of the experimental time. For late-weaned calves urinary pH stayed low until d 98 (d 28 vs. d 98,  $p = 0.624$ ) and then increased during weaning (d 98 vs. d 112,  $p = 0.002$ ) without dropping down before.

Specific density of urine was interactively influenced by time and weaning age ( $p < 0.001$ ; data not shown). It increased in early-weaned calves between d 28 and 42 ( $p < 0.001$ ). Therefore, it was higher in early-weaned calves than in late-weaned calves on experimental d 42 ( $p = 0.005$ ). In late-weaned calves, it increased until d 70 ( $p < 0.001$ ) and then remained stable on this level.



**Figure 2.** Percentage of time spent with active behavior (A) and resting (B). Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMMeans with SE. Early-weaned calves from multiparous cows (earlyMC), late-weaned calves from multiparous cows (lateMC), early-weaned calves from primiparous cows (earlyPC), late-weaned calves from primiparous cows (latePC).



**Figure 3.** Rumen fill (A) and sounds (B). Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMeans with SE. Early-weaned calves from multiparous cows (earlyMC)  $n = 16$ , late-weaned calves from multiparous cows (lateMC)  $n = 16$ , early-weaned calves from primiparous cows (earlyPC)  $n = 15$ , late-weaned calves from primiparous cows (latePC)  $n = 12$ .  $P < 0.05$  was set as the level of significance.

Furthermore, specific density did not change through weaning in late-weaned calves ( $p = 0.1$ ). Nitrogen-containing compounds in urine (creatinine, hippuric acid, uric acid, urea and allantoin) were interactively affected by time and weaning age (Table 2).

Concentrations of all compounds except urea increased during weaning in early-weaned calves (d 28 vs. d 42;  $p < 0.001$ ). Urinary allantoin ( $p = 0.004$ ; Fig. 5 A), uric acid ( $p = 0.027$ ; Fig. 5 B) and hippuric acid ( $p = 0.016$ ; Fig. 5 C).

**Table 1.** Effects of weaning age and parity on ammonia, VFA, and pH in rumen fluid evaluated by two-way ANOVA on experimental d 140. Values are presented as Means with SD.

Parameters	Early-weaning		Late-weaning		p-values		
	MC	PC	MC	PC	Weanin g age W	Parit y P	W*P
Ammonia-N (mg/100g)	2.33 ± 1.25	1.91 ± 1.71	2.93 ± 2.07	2.52 ± 1.18	0.144	0.289	1
Acetate (mmol/L)	35.89 ± 6.71	37.99 ± 8.23	36.15 ± 6.78	38.06 ± 5.88	0.971	0.278	0.917
Propionate (mmol/L)	9.44 ± 1.91	10.42 ± 2.18	9.91 ± 2.38	10.65 ± 2.89	0.605	0.170	0.723
Butyrate (mmol/L)	5.13 ± 1.67	5.76 ± 1.67	5.11 ± 1.50	5.52 ± 2.00	0.741	0.234	0.876
Iso-Butyrate (mmol/L)	0.66 ± 0.15	0.74 ± 0.16	0.67 ± 0.15	0.74 ± 0.20	0.948	0.099	0.974
Valerate (mmol/L)	0.70 ± 0.19	0.82 ± 0.21	0.74 ± 0.24	0.72 ± 0.22	0.630	0.337	0.238
Iso-Valerate (mmol/L)	1.10 ± 0.52	1.36 ± 0.55	0.99 ± 0.22	1.15 ± 0.46	0.172	0.078	0.665
Total VFA (mmol/L)	52.92 ± 10.25	57.10 ± 12.12	53.57 ± 10.23	56.84 ± 10.22	0.990	0.189	0.856
Ratio acetate:propionate	3.84 ± 0.47	3.67 ± 0.44	3.74 ± 0.60	3.69 ± 0.56	0.781	0.435	0.643
pH	7.34 ± 0.21	7.32 ± 0.27	7.35 ± 0.21	7.32 ± 0.17	0.857	0.684	0.960

were higher in early-weaned calves on d 42, when they were weaned, compared to late-weaned calves that still received MR. Nitrogen-containing compound concentrations decreased in early-weaned calves after weaning except hippuric acid concentration. The urinary urea concentration decreased from d 42 to d 98 ( $p = 0.004$ ; Fig. 5 D). Urinary creatinine (Fig. 5 E) and uric acid (Fig. 5 B) concentrations decreased from experimental d 42 to d 112 ( $p < 0.01$ ). Urinary allantoin concentration was lower at the end of the experimental period (d 140) compared to the d after weaning (d 42;  $p = 0.019$ ). Urinary hippuric acid concentration increased in early-weaned calves until d 70 (d 28 vs. d 70  $p < 0.001$ ) and did not change significantly thereafter. It also increased in late-weaned calves from d 28 to d 70 ( $p = 0.020$ ), while the steepest increase occurred during weaning ( $p = 0.004$ ; Fig. 5 C). All other N-containing compound concentrations did not change during weaning in late-weaned calves. Urinary creatinine

concentration of late-weaned calves increased over time from experimental d 28 to 112 ( $p < 0.001$ ). The urinary allantoin concentration increased before weaning (d 28 vs. d 70  $p < 0.001$ ). Urea concentration did not change significantly during weaning in both weaning groups and was not different between the groups on any experimental d.

All variables of urine were standardized to the corresponding specific density (data not shown), but no differences to unstandardized data regarding statistical data evaluation were observed. The NABE ( $p < 0.001$ ; Fig. 6 A) and BAR ( $p = 0.005$ ; Fig. 6 B) values in urine were interactively affected by time and weaning age (Table 2). Both increased after weaning in early-weaned calves (d 42 vs. d 70  $p < 0.001$ ). Therefore, they were higher in these than in late-weaned calves on d 70 (NABE  $p = 0.010$ ; BAR  $p = 0.027$ ). NABE values increased in late-weaned calves before weaning (d 42 vs. d 98,  $p = 0.030$ ).

## **Discussion**

Studying weaning at different ages is challenging for research approaches. The regimen of weaning at younger age has different requirements compared to weaning at older age. In this study, for instance, calves at 15 wk of age voluntarily ingest more concentrate than calves at 5 wk of age (8). To provoke an increase in concentrate intake at this early age, MR consumption has to be reduced. As a result, concentrate intake increased to 1 kg/d. In calves at 15 wk of age, the concentrate intake was twice as high compared to the early-weaned calves. Thus, at the beginning of weaning, the concentrate intake was limited to 1 kg/d in the late-weaned group. After weaning, all calves received hay and a TMR. The effects of two different rearing regimens and mother's parity are discussed in the following.

Rumen maturation at early age was assessed by behavioral observations, rumen sounds and scoring of rumen fill. Closely related to rumen maturation, acid base homeostasis and N

metabolism have to adapt to ruminant status. Thus, the pH in blood, urine, feces and saliva was measured to assess changes in systemic acid–basemetabolism going along with rumen maturation. Furthermore, the concentration of several N-containing compounds (creatinine, allantoin, uric acid, hippuric acid and urea) in urine was determined to estimate the onset of microbial activity in the gastrointestinal tract, especially in the rumen, and to assess the development of early N metabolism in growing calves.

Mother’s parity had no effect on any parameter except rumen fill score ( $p = 0.018$ ). This indicated that rearing conditions have a greater impact on rumen development, maturation of acid–base homeostasis and N metabolism than mother’s parity. Rumen fill score was greater in PCs, which might be caused by their smaller body size paired with the same feed intake as MCs.

**Table 2** Effects of time, mother’s parity and weaning age and their interaction on parameters shown in Figs. 1, 2, 3, 4, 5 and 6

***p*-Values**

<b>Parameter</b>	<b>Time (T)</b>	<b>Parity (P)</b>	<b>Weaning age (W)</b>	<b>T x P</b>	<b>T x W</b>	<b>P x W</b>	<b>T x P x W</b>
Chewing	< 0.001	0.440	0.042	0.512	0.016	0.525	0.277
Active behavior	0.037	0.308	0.085	0.750	0.001	0.850	0.803
Resting	< 0.001	0.110	0.004	0.575	< 0.001	0.548	0.442
Rumen fill score	< 0.001	0.018	0.490	0.335	0.001	0.455	0.780
Rumen sounds	< 0.001	0.770	< 0.001	0.965	< 0.001	0.938	0.042
pH saliva	< 0.001	0.638	0.295	0.621	0.674	0.171	0.299
pH feces	< 0.001	0.994	0.155	0.891	0.004	0.047	0.827
pH urine	< 0.001	0.525	0.069	0.652	< 0.001	0.670	0.773
pH blood	< 0.001	0.654	0.206	0.172	0.010	0.290	0.716
Creatinine	< 0.001	0.253	0.628	0.125	< 0.001	0.239	0.544
Allantoin	< 0.001	0.152	0.274	0.126	< 0.001	0.684	0.646
Uric acid	< 0.001	0.057	0.152	0.075	< 0.001	0.431	0.387
Urea	< 0.001	0.815	0.625	0.161	0.005	0.539	0.643
NABE	< 0.001	0.523	0.063	0.411	< 0.001	0.584	0.329
BAR	< 0.001	0.712	0.024	0.528	0.005	0.835	0.312

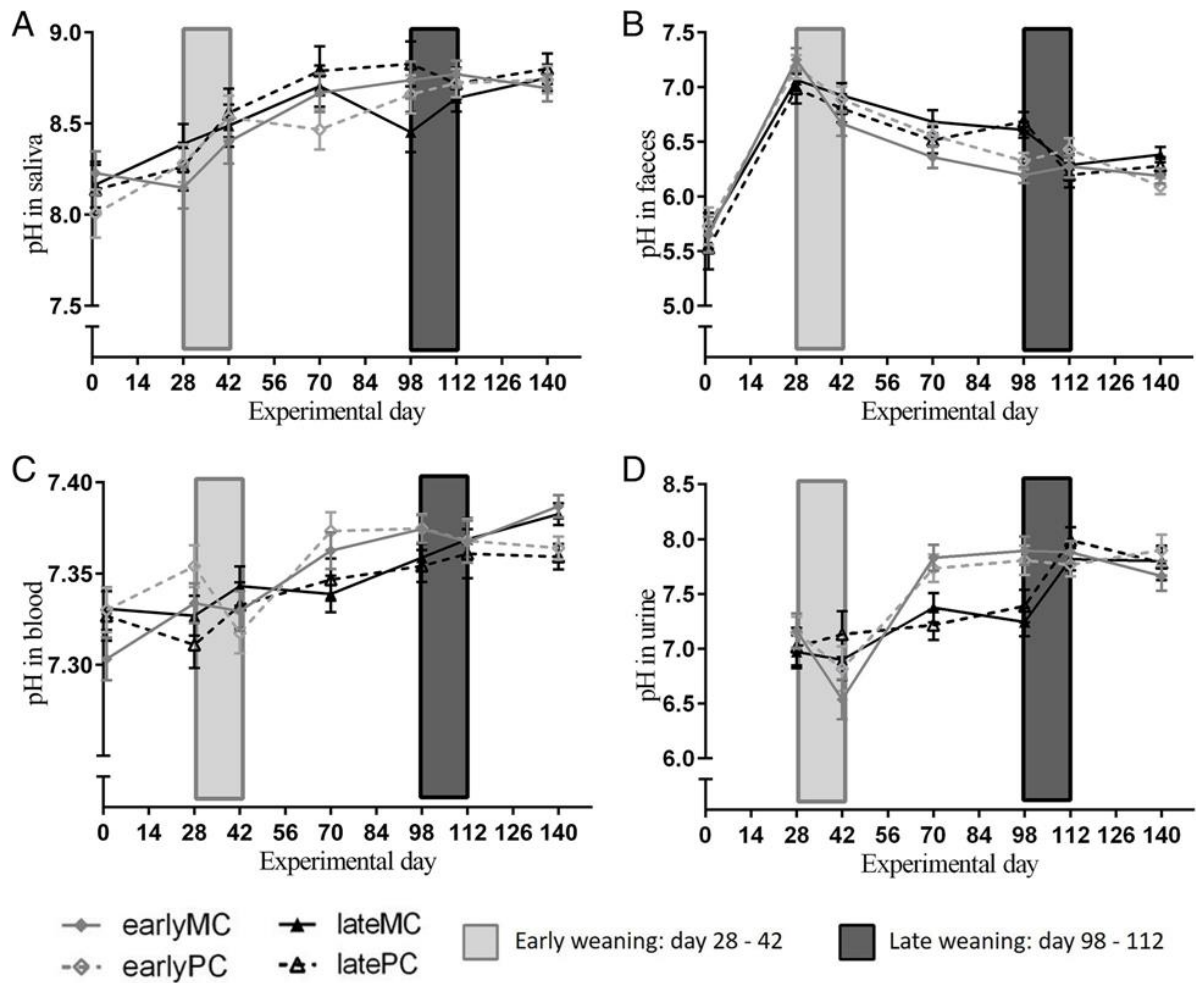
## **Rumen maturation at early age**

Late-weaned calves increased their solid feed intake before weaning [8]. As a consequence, rumen fill score and rumen sound increased before weaning as well. Rumen sounds per 2 min were assessed to get insights into progress of rumen motility, as it is possible to notice the sound of ingesta movements in the rumen with a stethoscope. Biphasic contractions of the reticulum were observed in milk-fed calves at the age of 16 d through ultrasonographic examinations [9]. In the current study, some calves had detectable rumen sounds as early as during the first wk of the trial, that corresponds to an age of ~ 14 days. This indicated that rumen sound and therefore rumen contraction were not only influenced by solid feed intake, but might be determined, at least partly, by an evolutionary blueprint. This might indicate that the nuclear genome was partly determining the age and velocity of maturation of organs and tissues of newborn and young calves with regard to their structure and function. Adverse nutritional interventions, especially during this fragile time window, could deteriorate this process leading to metabolic stress due to immature organs and tissues.

Confirming this assumption, early-weaned calves belatedly increased their time spent chewing from experimental d 70 on, which was 4 wk after weaning. This happened despite the ad libitum supply of hay and TMR, which is as solid feed known to increase rumination. However, late-weaned calves increased chewing during weaning at experimental d 105 already. The immature rumen might have contributed to lower solid feed intake in early-weaned calves. Thus, these calves were undernourished over several wk which was reflected in a lower live weight gain [8]. Concomitantly, early-weaned calves expressed a more active behavior after weaning up to experimental d 70, most likely due to seeking for liquid feed. In late-weaned calves, weaning was not associated with higher activity levels afterwards.

Calves that could freely access different solid feed and MR spent 20% of time with chewing both at the age of 3 and 6 mo [10]. As these calves displayed a low level of non-nutritive oral

behavior, such as tongue playing and oral manipulation of the pen structure or other calves, a chewing level of 20% appeared to be enough to satisfy needs for chewing and rumination. This level was reached by our feeding groups at d 21 (earlyMC and earlyPC), 28 (lateMC) and 49 of the trial (latePC) and remained stable or increased most of the time (Fig. 1). Therefore, weaning at 17 wk of age may not trigger abnormal behaviour. The results of the aforementioned and the current study were similar, although we observed behavior only in a small time frame during the day because chewing was found to have no circadian rhythm in calves [11].



**Figure 4.** PH value of saliva, blood, urine and feces. Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMMeans with SE. Early-weaned calves from multiparous cows (earlyMC) n = 16, late-weaned calves from multiparous cows (lateMC) n = 16, early-weaned calves from primiparous cows (earlyPC) n = 15, late-weaned calves from primiparous cows (latePC) n = 12.

### Maturation of acid–base homeostasis in growing calves

Maturation of the gastrointestinal tract might be associated with maturation of systemic acid–base homeostasis. To assess its development, the pH values in saliva, blood, urine, and feces were measured during early life in all calves. As pH in saliva increased over time, being

unaffected by weaning age or mothers' parity, it seemed to be determined solely by the evolutionary blueprint. The first significant increase in pH compared to experimental d 1 was observed at d 42 ( $p < 0.001$ ) and then again from d 42 to d 140 ( $p = 0.01$ ) in all groups. Therefore, there might be an ontogenetic window for development of ruminant salivary buffer composition during these time points. The salivary gland responsible for secreting large amounts of buffer in ruminants is the Glandula parotis. The main chemical components of Parotis saliva are  $\text{Ca}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , Urea,  $\text{HCO}_3^-$ ,  $\text{HPO}_4^{2-}$ ,  $\text{Cl}^-$  and water. In general, the concentration of these components did not vary much between healthy adult cows and were unaffected by diet. Only urea concentration was affected by diet in adult cows [12]. The saliva buffering capacity and alkaline pH is mainly achieved by  $\text{HCO}_3^-$  and  $\text{HPO}_4^{2-}$  [13]. Therefore, these components are vital for an adequate rumen function. The change in salivary pH indicated that the adequate composition needed time to develop. However, the capacity of salivary glands, especially of the Parotis, to produce adequate volumes of saliva to buffer VFA production in the rumen was not assessed in this study.

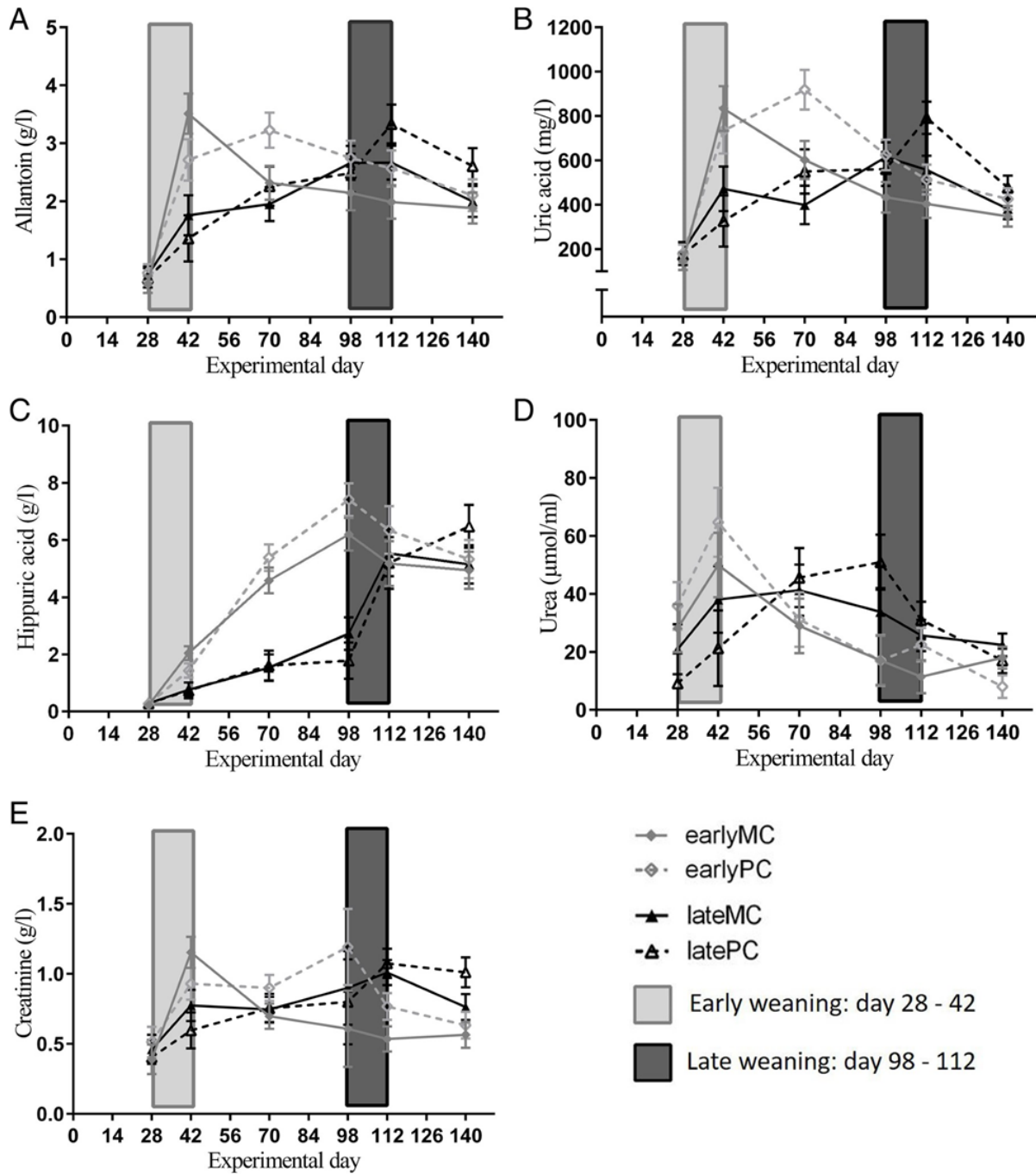
As a consequence of the associated slow rise of salivary buffer capacity, early weaning, particularly before the age of 7 wk, could lead to acidification of rumen fluid. Ingestion of starch-rich concentrate might have increased rumen VFA concentration and thereby, decreased rumen pH value. This possible acidotic condition was most likely not balanced by a sufficient availability of saliva, due to low buffer concentrations and saliva volume, respectively. Additionally, absorption of VFA by the rumen epithelium might not be expressed with a sufficient capacity leading to an accumulation of acids in the rumen at this age. Furthermore, chewing activity, which is strongly influencing saliva secretion rate, is low after weaning in early-weaned calves. Others noticed different rumination patterns attributable to weaning age as well. Calves weaned at the age of 8 wk displayed more rumination before weaning than calves weaned at the age of 6 wk [14]. Therefore, it was hypothesized that early-weaned calves

were at risk for ruminal acidosis, and for higher acid load in plasma as well. Several authors observed an acidic ruminal pH in early-weaned calves [14, 15]. Calves that were weaned at 4 wk of age had a ruminal pH under 5.5 for at least 4 wk after weaning (15). Weaning at 6 wk of age reduced ruminal pH below 5.5 at least for 3 wk as well [14]. As this might be a sign for chronic rumen acidosis at least for adult cows [16], it is possible that these calves suffered from this disease as well. To maintain plasma pH is vital, thus, the lung and the kidneys are important regulators of acid–base homeostasis. While in the lung protons were eliminated as CO<sub>2</sub> and H<sub>2</sub>O, kidneys excrete protons or reabsorb bases to maintain acid–base balance. Physiologically, urinary pH of healthy fully ruminating cattle is neutral to slightly alkaline [17, 18] due to the high amounts of excreted bicarbonate in herbivores. In the condition of metabolic acidosis, the kidney conserved bicarbonate by reabsorption [19]. As a consequence, the urinary pH value declined with decreasing bicarbonate concentration [20]. Consequentially, urinary pH value correlated positively with ruminal pH value [16]. Therefore, urinary pH might reflect the ruminal pH value and the decrease of urinary pH during early-weaning might have indicated rumen acidosis during this time. In early-weaned calves a potentially higher acid load needed more bicarbonate, thus this buffer was reabsorbed in the kidney. Consequently, pH of urine decreased (experimental d 42,  $6.6 \pm 0.1$ ) directly after early-weaning (Fig. 4D). Furthermore, a higher NH<sub>4</sub><sup>+</sup> and phosphate concentration may also be responsible for the lower urinary pH value after early weaning, which might result from still low utilization of N-containing compounds in rumen microbial metabolism. Furthermore, low urinary pH value was associated with greater calcium excretion, probably bound to acid phosphate [21]. As calcium is important for growth and bone development, this could have contributed to the impaired growth of early-weaned calves [8].

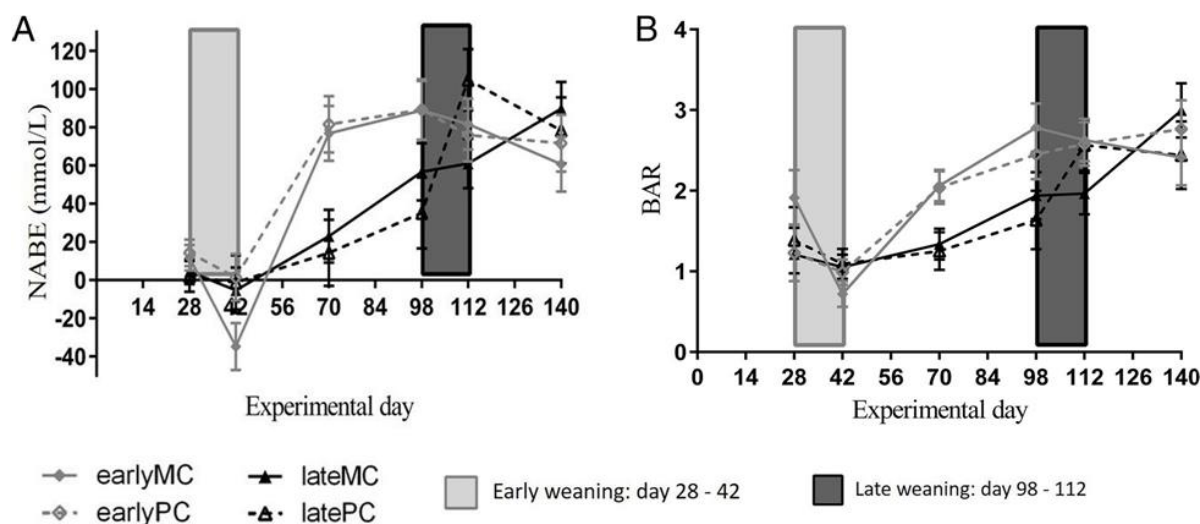
In adult cows, values of NABE and BAR in urine were used to diagnose a mild metabolic acidotic burden [22]. In calves this might not be advisable, as both variables are low in milk-

fed calves (Fig. 6). The values of NABE and BAR increased over time, which reflected the transient change in dietary composition. In early-weaned calves, the increase was steep after weaning from experimental d 42 until d 70, but not during their weaning. The metabolic adaptation to an adult status seemed to occur after weaning was already done. On the other hand, the increase of NABE values in late-weaned calves was not as steep but occurred before weaning. They might have experienced a smoother transition to adult status.

The pH value in feces decreased during weaning (Fig. 4 B). Lohakare et al. [23] measured a pH of 7 in feces of calves at 40—42 d of age. Confirming, in the current study calves were  $37 \pm 2$  d of age at experimental d 28 and had a faecal pH around 7 as well. Lohakare et al. [23] observed a decrease of faecal pH through weaning as well, but this increased again to a pH of 6.8 – 7.0 at 42 d after weaning. This increase was not detected in the current study, in which faecal pH continued to decline. Adult cattle had a faecal pH from 7 – 8 [24, 25], which, in this study, was not reached by any group until the end of the trial. This may be a result of high intake of starch through concentrate and TMR which could not be digested in rumen and therefore was transported into the large intestine. In the hindgut, it could be fermented, but emerging VFA could not be fully absorbed, subsequently leading to a decrease in pH value. The decrease in faecal pH through increased energy intake and carbohydrate infusion has been tested in steers [25], as well as a strong correlation between faecal starch content and pH [24]. Calves had a higher percentage of faecal starch after weaning, especially when weaned early at the age of 6 wk [14]. Hence, the low fecal pH could be a sign for inadequate digestion in the rumen, especially of starch. As the concentration of starch was common for calve and heifer feed (concentrate 371 g/kg dry matter (DM); TMR 182 g/kg DM) the rumen might not have been developed sufficiently to digest a typical adult cow diet even at the age of 5 months.



**Figure 5.** Concentrations of nitrogen-containing compounds in urine. Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMeans with SE. Early-weaned calves from multiparous cows (earlyMC) n = 16, late-weaned calves from multiparous cows (lateMC) n = 16, early-weaned calves from primiparous cows (earlyPC) n = 15, late-weaned calves from heifers (lateHC) n = 12



**Figure 6.** Values of net acid base excretion (NABE) and base-acid ratio (BAR) in urine. Early-weaned calves were weaned gradually between experimental d 28 and 42 of the trial. Late-weaned calves were weaned gradually between experimental d 98 and 112 of trial. Data shown as LSMMeans with SE. Early-weaned calves from multiparous cows (earlyMC) n = 16, late-weaned calves from multiparous cows (lateMC) n = 16, early-weaned calves from primiparous cows (earlyPC) n = 15, late-weaned calves from heifers (lateHC) n = 12.

### Maturation of N metabolism in growing calves

Nitrogen-containing metabolites in blood and urine indicate changes in the diet, endogenous protein metabolism, and in developing microbial metabolism in the rumen. Dietary effects on N-containing metabolites were most likely associated with changes in microbial metabolism. Urea-N was measured in plasma [8] and urine, while non-urea-N was measured only in urine. Creatinine is the only N-containing metabolite in urine which is derived solely from muscle protein break-down, thus of endogenous origin [26]. Early weaning resulted in a sudden increase in urinary creatinine and urea, which indicated a catabolic status. Due to the change of highly digestible milk protein and energy to less digestible solid feed protein and energy, calves failed to adapt to weaning at early age. A fasting status was most likely established at the period of early weaning. Concomitantly, this was confirmed by a strong increase in plasma urea and

betahydroxybutyrate and a low insulin concentration at early weaning [8] Furthermore, the quick rise in excretion of allantoin and uric acid, end products of metabolism of endogenous nucleic acids, might also be attributable to a catabolic state and/or a more stimulated microbial activity compared to late-weaned calves at this time. While the latter group showed a continuous increase of these compounds the calves weaned at experimental day 42 and changed to a TMR onwards, failed to further increase allantoin and uric acid concentration in urine. This might indicate a relative deficiency of energy available for adequate ruminal microbial protein synthesis due to a lower supply of easily fermentable substrates. Later, urinary urea concentrations decreased steadily in early-weaned calves, most likely due to recycling into the developing rumen to serve as N source for the microbial growth. Due to the prolonged high availability of milk protein and energy in late-weaned calves, urea concentrations in blood [8] and in urine were stable on a high level until weaning. At weaning, urea decreased by recycling into the rumen to compensate a lower dietary crude protein (CP) supply.

The purines from rumen microbes were metabolized and the end products hypoxanthine, xanthine, uric acid and allantoin were excreted in the urine [27]. Xanthine and hypoxanthine were only found in small amounts in cows' urine, whereas uric acid and allantoin were the abundant purine derivatives [28]. This is due to the fact that, xanthine and hypoxanthine are converted to uric acid, which is further converted to allantoin [27]. Therefore, urinary concentrations of allantoin and its precursor, uric acid, were used as a predictor for microbial CP production in the rumen [29]. Both increased in earlyweaned calves during weaning, but as they decreased again until the end of trial, their importance as a marker for rumen microbial activity can be contested. This has already been refuted for cows in different stages of lactation [29]. As stated above, the steep initial increase in allantoin and uric acid might be provoked by endogenous catabolic processes and only to a small extent by the increase in microbial activity at early weaning. Lateweaned calves expressed steady increases in urinary allantoin and uric

acid concentrations, which slightly peaked around weaning. Therefore, it can be assessed that provision of liquid feed together with voluntary solid feed intake promoted a slow but effective rumen development and microbial activity. However, for the full functioning of rumen, the capacity for fibre digestion must be developed. For that, hippuric acid is a well-known marker [30]. It is formed in the liver from benzoic acid and glycine. Benzoic acid is synthesised by microbial metabolism of plant-derived phenolic cinnamic acids in the rumen [31]. Therefore, urinary hippuric acid concentration was strongly linked to diet composition in adult cows [32, 33], which seemed to be the same in calves as urinary hippuric acid concentration increased after weaning in both groups. Early-weaned calves, however, did not reach mature hippuric acid concentrations before experimental d 70, which could indicate an insufficient fibre digestion in the rumen, although they were on solid feed from d 42 on. Concomitantly, time spent chewing was increased only after experimental d 70, supporting the assumption that fibre digestion was not developed after early weaning and fibre intake was low, respectively. The potential restriction was reflected in a lower live weight gain and growth rates after early-weaning [8].

In addition, urinary N is a source of N<sub>2</sub>O emission [26], which affects the environment and can influence surface water, biodiversity and climate change in a negative way [34]. Therefore, attempts should be pursued in optimal rearing strategies to lower the excretion of urinary N-containing compounds.

### **Rumen maturity at the age of 5 months**

All variables linked to rumination, such as chewing behavior, rumen fill and sound, increased over time in both groups, despite the high MR allowance. These variables as well as VFA concentration and pH of ruminal fluid were not significantly different between the weaning

groups at the end of the trial (d 140), which indicated that both groups reached a concordant status of rumen development. Therefore, rumen and its functions appeared to be equally developed at the age of 5 months, which corresponds to experimental d 140. Late-weaned calves showed no sign of impaired rumen development at that age despite of the higher weaning age. Ad libitum intake of MR in the first 5 wk of life did not retard rumen development [35]. High MR allowance and higher weaning age did not avert the transition to a functional ruminant status. Additionally, this transition was smoother in late-weaned calves [8]. On the contrary, in early-weaned calves these metabolic changes occurred relatively abrupt, which might have put a lot more strain on organs and tissues. In mammals, development of organs and tissues and their physiological functions in adulthood can be affected by early nutrition [36, 37]. As mentioned before, metabolic imprinting might occur through nutritional experiences in early life and can have a great impact on later health and performance [1]. Therefore, these abrupt changes in nutrition and consequent metabolic adaptations might lead to future impairment of health and performance. Increased MR intake by calves resulted in greater relative kidney weights [38, 39]. Hence, kidney development seemed to be affected by early-life nutrition. The secretion of protons and the rapid change of urinary composition during earlyweaning might strain the calves' kidney massively. This might also result in coping mechanisms that imprint these vital organs and alter their functions later in life. Although there was no difference in the measured variables at the age of 5 months, early-weaning might have caused a metabolic imprinting which might result in negative outcomes later in life. This might be true for calves born to primiparous mothers as well [40], even though we did not observe a considerable effect of parity on measured parameters. To assess the metabolic imprinting, these animals were further monitored in an ongoing observational study.

## **Conclusion**

Weaning calves at 7 wk of age appears to be too early for an adequate rumen development according to the evolutionary blueprint. This became apparent in an inefficient rumen function and a not fully developed metabolic adaption in early-weaned calves before experimental d 70, although they were fully weaned with 42 d, corresponded to an age of 7 wk. This indicated that calves needed more time to mature. They reached functional ruminant status at about 11 wk of age, which might be a more appropriate age for weaning. None of the response variables of behavior, rumen development, and urinary excretion of N-containing compounds were significantly different at the end of the trial. This indicated an equal functional status of the rumen despite the prolonged MR feeding in late-weaned calves. However, long-lasting effects of a metabolic imprinting for later life in terms of health and performance are possible for these future dairy cows and need to be studied.

## **Material and methods**

In accordance with the German Animal Welfare Act and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany, the present trial was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany (file No.: 33.19–42,502-04–15/1858).

### **Animals, housing, and diets**

Detailed information about the study design were described elsewhere [8]. In short, female German Holstein calves (n = 59) were studied from the age of (mean  $\pm$  standard deviation)  $8 \pm 2$  days (d) until d  $149 \pm 2$  of life. All calves originated from one established herd of German Holstein cows and were born within a seasonal calving period of 3 months (October -

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December). After the feeding trial, the animals remained in the institute's own herd. They received 3 L of colostrum through a nipple bucket within 2 h after birth. Consistent quality of colostrum was evaluated using a colostrum densimeter (Wahl GmbH, Dietmannsried, Germany) and had to be defined as good at a value greater than 1035 g/L. In the pre-experimental feeding period, milk replacer (MR; NOLAC GmbH, Zeven, Germany) was mixed to the pooled herd milk, starting at the age of 3 d, with gradually increasing amounts from 0.30 kg MR powder/d (d 3 after birth) to 0.90 kg MR/d (d 5 after birth), when the maximum of 6 L liquid feed with a concentration of 150 g/L MR powder was available. Calves entered the study at an average live weight of  $44.5 \pm 5.2$  kg at experimental d 1 and were moved into straw-bedded stables with provision of MR and concentrate through self-feeding systems (Förster-Technik GmbH, Engen, Germany). They were randomly allocated to either early weaning at 7 wk of age (early-weaned calves from multiparous cows (earlyMC, n = 16) and primiparous cows (earlyPC, n = 15)) or late weaning at 17 wk of age (late-weaned calves from multiparous cows (lateMC, n = 16) and primiparous cows (latePC, n = 12)) group assuring an equal allocation of calves from primiparous cows (PC, n = 17) and calves from multiparous cows (MC, n = 32). Early weaning was conducted at the age of 7 weeks, as this is a common management decision take on dairy farms, while late weaning was executed at the age of 17 weeks, because the reticulorumen volume approximately reaches adult proportions [3]. The trial started with 0.90 kg MR powder/d, which were available for all calves for the first 5 experimental d. Then MR was increased gradually within the next 5 d to 1.35 kg MR powder/d (9 L liquid feed) and remained at this level until the beginning of the weaning period (early-weaned group = experimental d 28; lateweaned group = experimental d 98). Over the entire trial, all calves received hay and water for ad libitum consumption. They had access to a maximum of 2 kg concentrate feed per d, which was reduced with start of late weaning (experimental d 98) to 1 kg/d. During weaning, the MR was reduced step-down within 14 d from 1.35 kg/d to 0.30 kg/d. Post-weaning calves were moved to another barn and received hay

and a total mixed ration (TMR) consisting of 48% grass, 32% maize silage and 20% concentrate for ad libitum consumption.

### **Behavioral assessments and measures of rumen variables**

Calf behavior was recorded using instantaneous scan sampling ([41] at 10 min intervals during 2 h (h) once a wk between 8:00 and 12:00 h. Observation was done in a quiet atmosphere and the observer entered the barn 10 min before beginning to let calves accommodate themselves to the situation. As calves were raised on the farm, all typical activities and sounds outside the barn were accepted as characteristics for their d-to-d life and did not influence observation. Lateral movement of the mandibular in a lying position with a raised head was defined as chewing. Regurgitated boli were not included in the definition as they were difficult to observe. Therefore, chewing included both non-ruminating and ruminating activities. Lying on the ground with bent legs without movement of the mandibular was defined as resting. All other behaviors were registered as active behavior. To assess the percentage of each behavior during a given observational period, the number of observations of that defined behavior was divided by the total of 12 observations and multiplied with 100. For analysis, a given sampling d was combined as that  $d \pm 3$  d.

Rumen motility was assessed by counting the number of rumen sounds within 2 min with a stethoscope in the Fossa paralumbalis. Rumen fill was scored through palpation of the Fossa paralumbalis using a scale from 1 to 5, which was modified based on Zaaijer and Noordhuizen [42] (score 1 = empty, rectangular; score 2 = empty, triangular; score 3 = a little sunken, soft; score 4 = scarce blending between Fossa paralumbalis and Arcus costalis, firm; score 5 = Arcus costalis hardly visible, Fossa paralumbalis convex, firm). Scoring and measuring of rumen sounds were done on experimental d 1, 7, 14, 28, 42, 56, 70, 84, 98, 112, 126 and 140.

## **Collection and analysis of blood, urine, saliva, rumen fluid and fecal samples**

All samples were collected between 8 AM and 1 PM. Feces and urine were collected after spontaneous release or manual stimulation and filled in clean and sealable tubes. The pH value in feces and urine was measured 1 to 4 h after collection with a pH meter (pH 7119, WTW, Weilheim, Germany). For collection of saliva, two sterile cotton wool swabs were offered each animal on a clamp for chewing. Afterwards they were put into a commercially available salivette (Sarstedt, Nümbrecht, Germany) and cooled on ice. Salivettes were centrifuged at  $2000 \times g$  for 3 min and the pH value was measured with a pH meter (pH 7119, WTW, Weilheim, Germany). Urine was stored at  $-20\text{ }^{\circ}\text{C}$  before analysis of net acid base excretion (NABE), urea, allantoin, creatinine, hippuric acid and uric acid were done. NABE and the base–acid ratio (BAR) were determined by the titrimetric method as described by Kutas [43]. Following dilution and filtration of urine samples, the purine derivatives (allantoin, uric acid, hippuric acid) and creatinine were conducted by reverse-phase HPLC (LC-20A prominence, Shimadzu Europe GmbH, Duisburg, Germany) according to the method of Shingfield and Offer [44]. Urinary urea was analyzed with a colorimetric assay kit (Biovision, Milpitas, CA). Specific density of urine was estimated with a handheld refractometer (RPI, Optech srl, Turin, Italy). The pH values in whole blood were measured by an automated blood gas analyser (GEM4000, Werfen GmbH, Munich, Germany). On experimental d 140 ruminal fluid was collected using a rubber tube modified to Geishauser [45]. The equipment consisted of an oro-ruminal probe with flexible tube and a manual suction pump. The probe was inserted orally into the ventral sac of the rumen and a volume of approximately 100 ml of rumen fluid was collected after discarded the first part of the sample to avoid contamination by saliva. Ruminal pH was immediately determined using a pH meter (pH 7119, WTW, Weilheim, Germany).

Ruminal fluid was centrifuged (Heraeus Varifuge®,  $2400 \times g$ , 5 min), acidified with a mixture of phosphoric acid/formic acid (5%) and stored at  $-20\text{ }^{\circ}\text{C}$  before analysis of VFA (acetate,

propionate, butyrate, iso-butyrate, valerate, iso-valerate) using a gas chromatograph (Clarus 689, PerkinElmer LAS GmbH, Rodgau, Germany) equipped with a flame ionisation detector as described by Geissler et al. [46]. Ruminal ammonia-N was determined directly after centrifugation using stream distillation according to DIN38406, E5-2 [47].

### **Statistical Analysis**

The pH value in feces and saliva was measured in two samples per animal and point in time, and the mean value was used for further analyses. NABE and BAR of the urine were calculated as: NABE = base excess – (acid excess + ammonium ions) BAR = sum of bases/sum of acids.

All variables, except for variables in ruminal fluid, were evaluated as repeated measures using PROC MIXED procedure in SAS (V 9.4., SAS Institute Inc., Cary, NC). The model included fixed factors of time, weaning age, parity of the mother and their interactions. Results were presented as least squares means (LSMeans) with standard errors (SE). All pair-wise differences of LSMean were tested with the Tukey–Kramer procedure. Variables in ruminal fluid were evaluated by two-way ANOVA with weaning age, mother's parity and their interaction as independent variables using SAS (V 9.4., SAS Institute Inc., Cary, NC). For all statistical tests,  $p < 0.05$  was the level of significance. Visualization was done using GraphPad Prism 6.0 (GraphPad software, San Diego, USA), whereby measurements on serial time points were interpolated linearly.

### **Abbreviations**

ADF: Acid detergent fibre; BAR: Base–acid ratio; BW: Body weight; CF: Crude fiber; CP: Crude protein; d: Day; DM: Dry matter; earlyMC: Early- weaned calves from multiparous cows; earlyPC: Early- weaned calves from primiparous cows; EE: Ether extract; g: Gram; FLI:

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Friedrich- Loeffler Institut; h: Hour; HPLC: High performance liquid chromatography; kg: Kilogram; L: Litre; lateMC: Lateweaned calves from multiparous cows; latePC: Late- weaned calves from primiparous cows; LAVES: Lower Saxony State Office for Consumer Protection and Food Safety; LSMMeans: Least squares means; mg: Milligrams; mmol: Millimoles; MC: Multiparous cows; Mo: Months; MR: Milk replacer; N: Nitrogen; NABE: Net acid base excretion; NDF: Neutral detergent fibre; P: Parity; PC: Primiparous cows; SE: Standard errors; SD: Standard deviation; T: Time; TMR: Total mixed ration; VFA: Volatile fatty acids; W: Weaning age; wk: Week.

### **Acknowledgements**

The authors would like to gratefully acknowledge the technical staff of the experimental station of the Institute of Animal Nutrition, Friedrich- Loeffler- Institute (FLI), Braunschweig, Germany.

### **Author contributions**

Conceptualization: SD, KH, JF; Investigation: SS, JF, AK; Data Curation: SS; Methodology: SS, LS, LH, SK, K- HS; Writing original draft preparation: SS; Review and editing: AK, LH, SK, K- HS, KH, SD, JF; Supervision: KH, JF, SD; Project administration: KH, SD. All authors read and approved the final manuscript.

### **Funding**

Open Access funding enabled and organized by Projekt DEAL.

### **Availability of data and materials**

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

### **Declarations**

#### **Ethics approval and consent to participate**

All animals were obtained from the experimental station of the Institute of Animal Nutrition, Friedrich- Loeffler Institut, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (file number: 33.19–42502- 04–15/1858).

#### **Consent for publication**

Not applicable.

#### **Competing interests**

The authors declare no conflict of interest.

Received: 9 July 2021 Accepted: 25 Januarz 2022

Published online: 17 March 2022

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## **2.3 Manuscript 3: Host metabolome and faecal microbiome shows potential interactions impacted by age and weaning times in calves**

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Submitted to Animal Microbiome.

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## **Abstract**

**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.

**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.

**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,

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suggesting a less abrupt but more-persistent effect of dietary changes on host metabolome compared to the microbiome.

**Keywords:** Calves, Faeces, Age, Weaning time, Microbiome, Plasma metabolites

## **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–

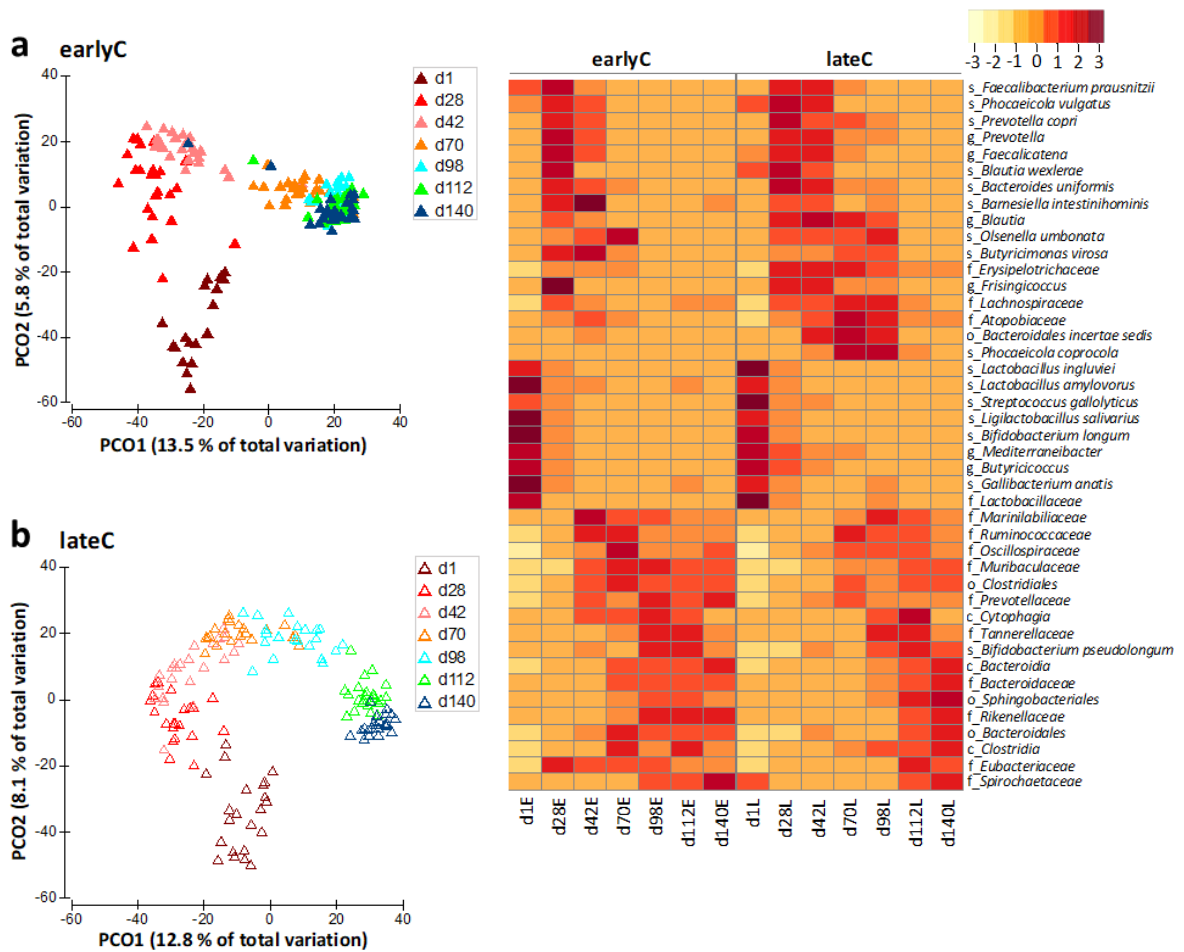
microbe's metabolic interactions and possible identifications of predictive biomarkers for diseases [11, 12]. With the advanced metabolomic analysis tools, it is now possible to detect several classes of metabolites such as amino acids (AAs), biogenic amines (BAs), acylcarnitines (ACs), and sphingomyelins (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.

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## Results

### 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 (Fig. 1A, 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: Fig. S1A). 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: Fig. S1B, Additional file 2: Table S1).



**Figure 1.** Age-dependent changes in the faecal bacterial communities of earlyC and lateC calves. (a,b) Bacterial compositional profiles of different age group earlyC and lateC 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.) *Butyricoccus*, uncl. *Lactobacillaceae*, and uncl. *Mediterraneibacter*, showing significant

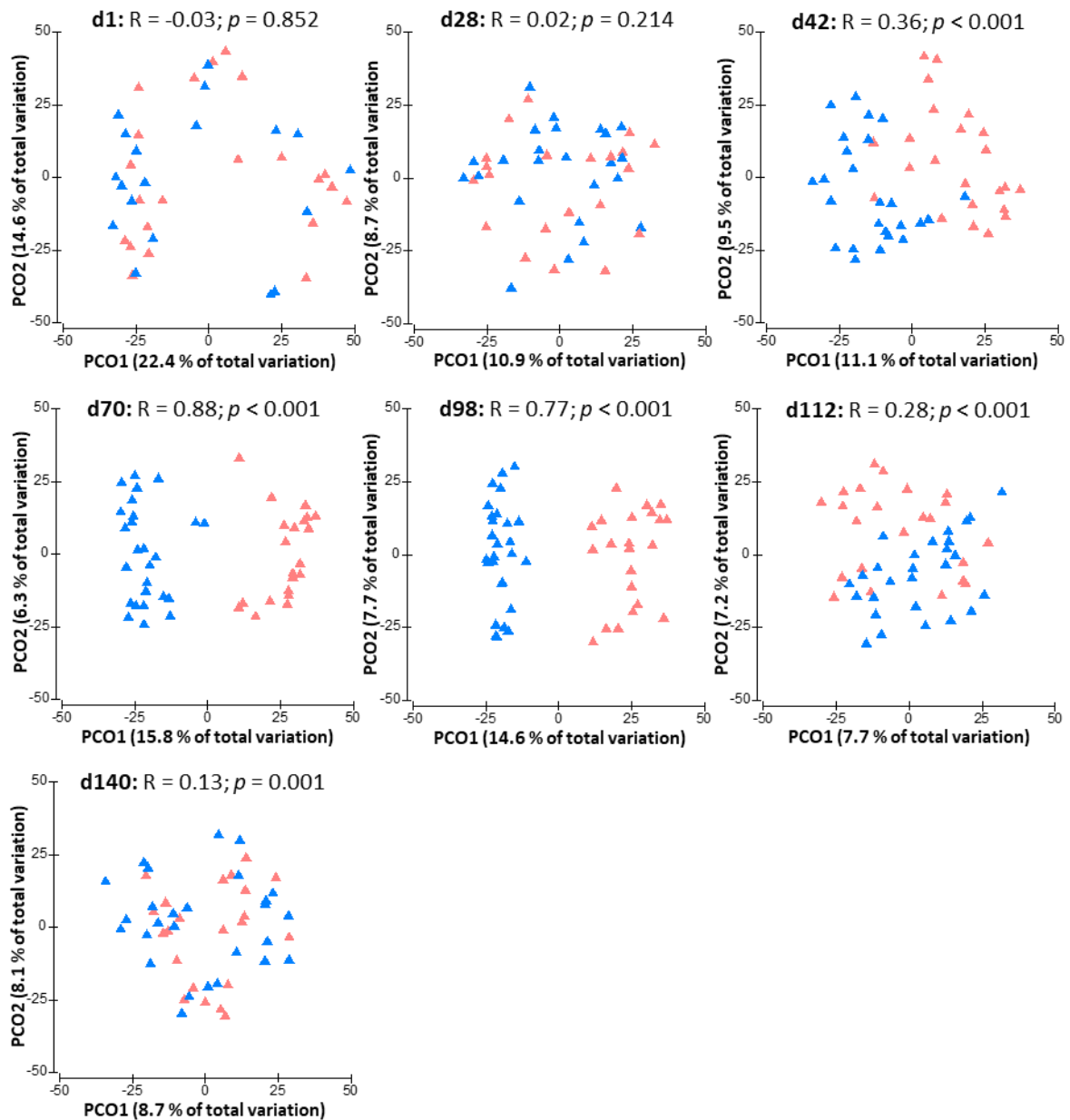
decrease in abundance with age (d1–d140) in both weaning groups. In addition, *Bacteroides uniformis*, *Barnesiella intestinihominis*, *Blautia wexlerae*, *Faecalibacterium prausnitzii*, *Phocaeicola vulgatus*, *Prevotella copri*, uncl. *Faecalicatena*, and uncl. *Prevotella* were significantly more 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 (Fig. 1C, Additional file 2: Table S1).

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

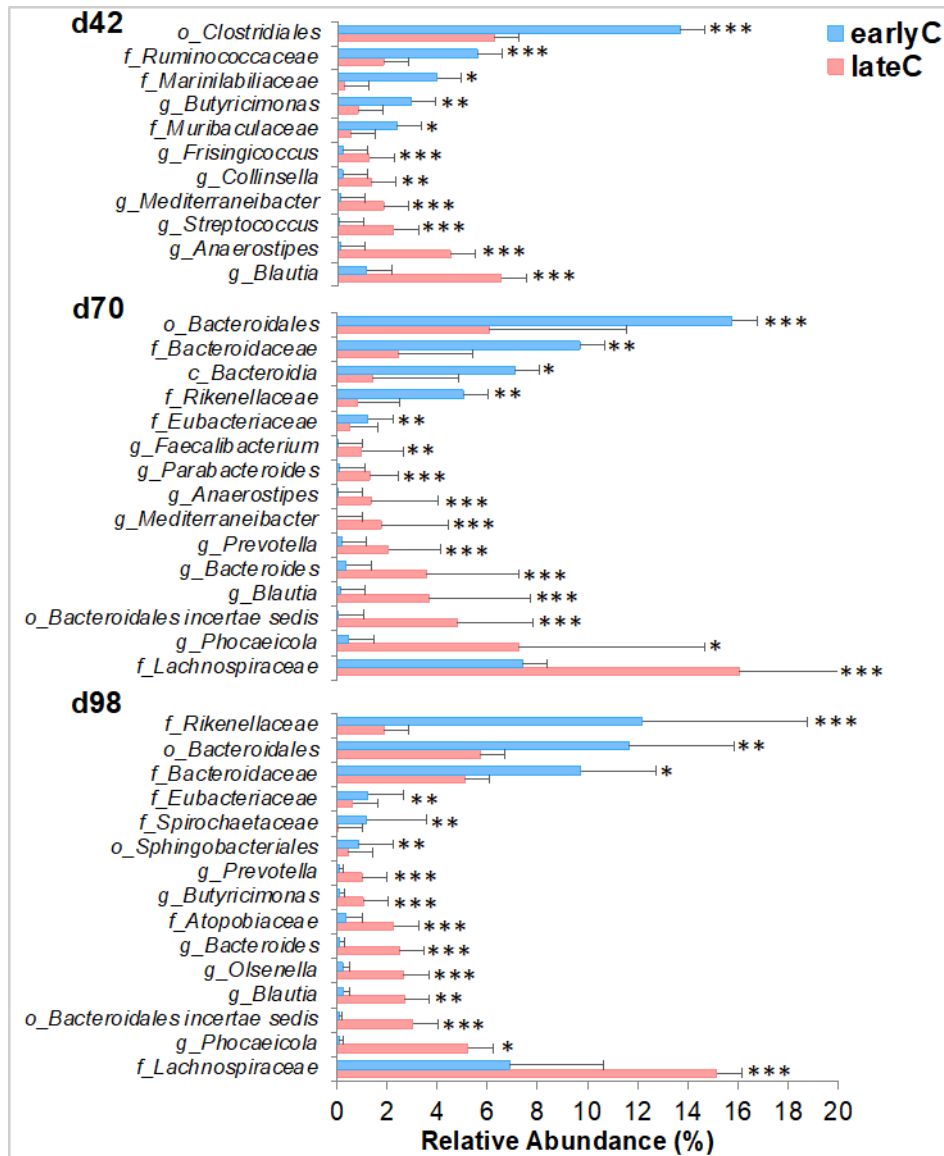
In addition to the age-related maturation, the time point of weaning also significantly influenced the faecal bacterial compositional profiles as indicated by the separate clustering of weaning groups during days 42–112. In contrast, no significant difference was detected before or after this period (Fig. 2). Both weaning groups had distinct bacterial taxonomic compositions during days 42–98 (Fig. 3, Additional file 2: Table S1). Early-weaning at seven weeks triggered an increase in the relative abundance of Bacteroidetes and a decrease of Firmicutes (Additional file 1: Fig. S1B) 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* (Fig. 3, Additional file 2: Table S1). 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*,

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Frasingicoccus, 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 2.** 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.



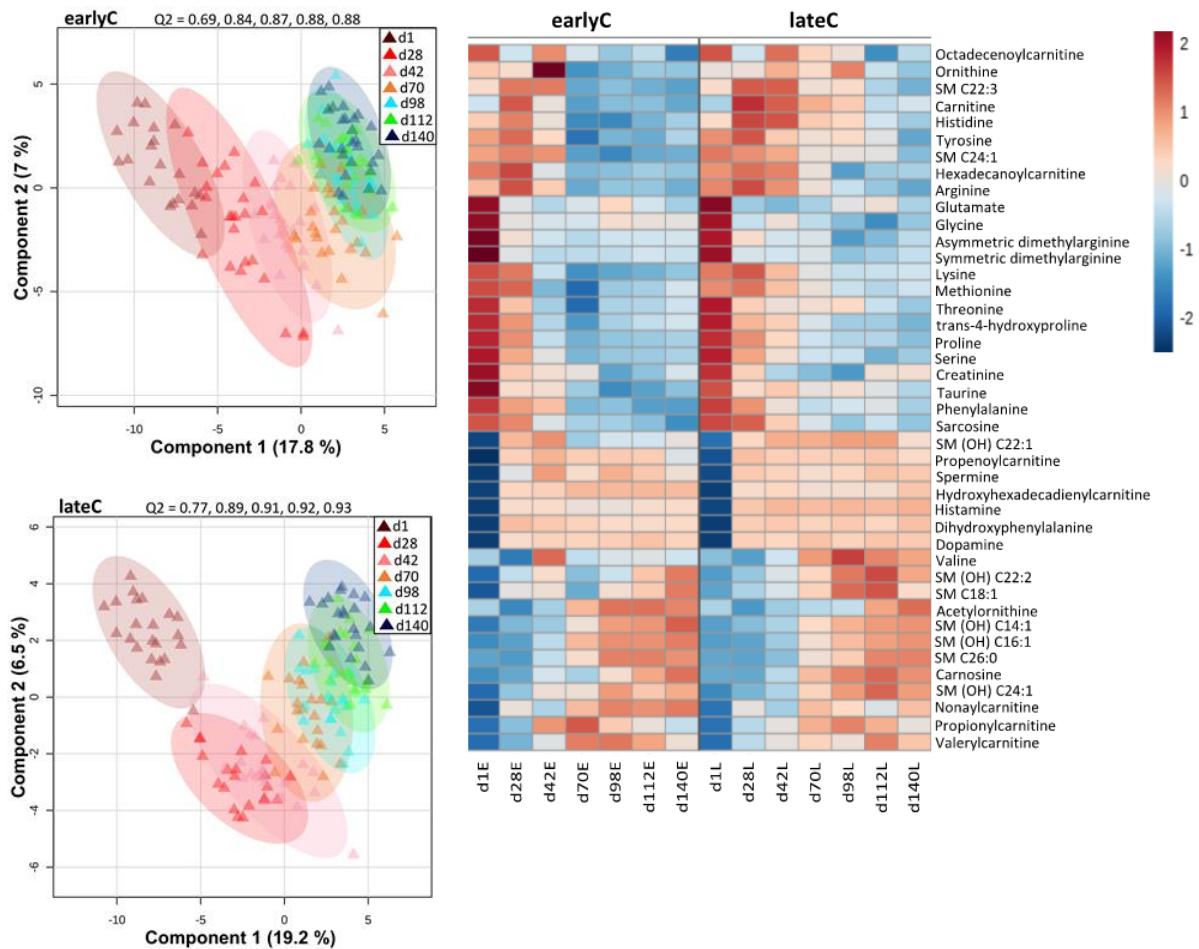
**Figure 3.** Changes in faecal bacterial communities of calves due to weaning event. Significantly different bacterial genera with relative abundance ( $\geq 1\%$ ) and  $p \leq 0.05$  (Kruskal-Wallis test) among same-age old weaning groups are shown. Each bar represents an average value for animals: 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: Fig. S2). 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.

### **Plasma metabolome and the impact of calves' age**

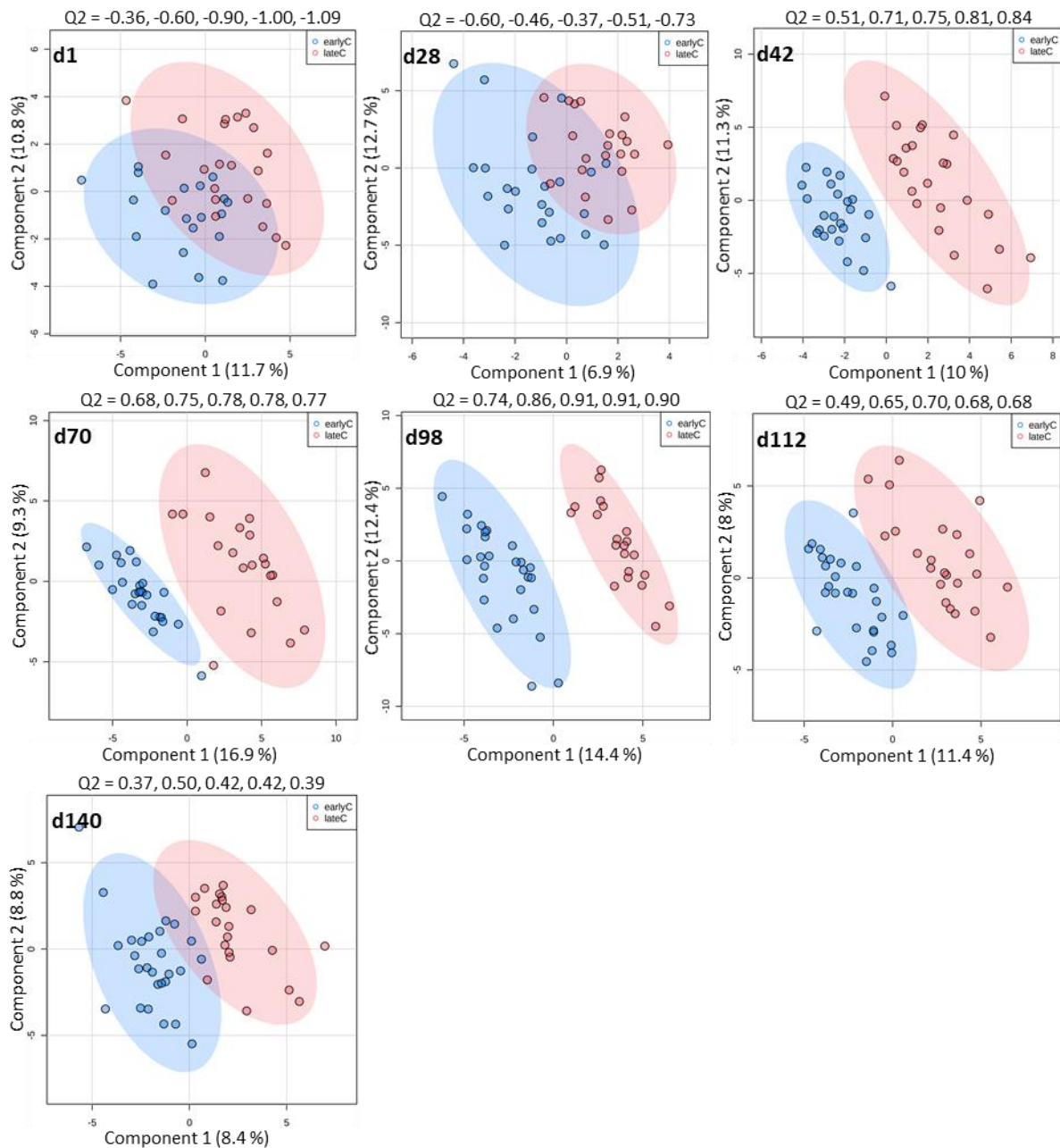
The differences between the plasma metabolic profiles of calves from different age groups were shown by a supervised partial least square discriminant analysis (PLS-DA), that resulted in clear age-dependent clustering for both weaning groups (Fig. 4A, B). Metabolites showing significant difference due 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) (Fig. 4C, Additional file 2: Table S2).



**Figure 4.** Age-dependent changes in plasma metabolites concentrations of earlyC and lateC calves. (a, b) Metabolic profiles of different age group earlyC and lateC plasma samples of calves 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.

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 (Fig. 4C). 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 (Fig. 4C).

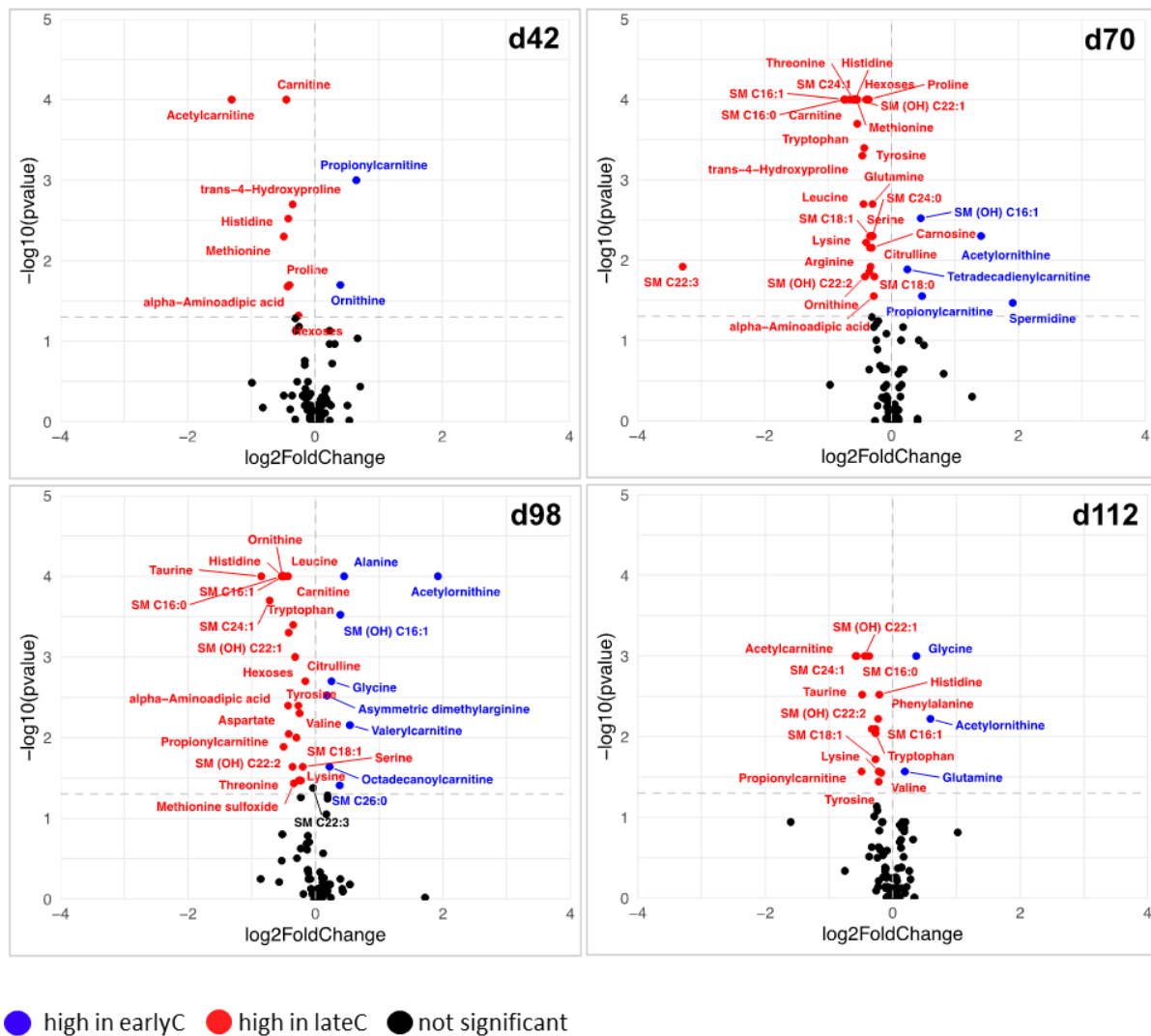


**Figure 5.** Partial least squares-discriminate analysis for identification of metabolic differences among weaning groups. Each circle indicates one sample and ellipse indicating the 95 % confidence region. The quality of the models was assessed using Q2 as performance measure and tenfold cross-validation method. The Q2 values for the first 5 components are shown.

## 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 (Fig. 5). 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 log<sub>2</sub> 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 (Fig. 6). 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-amino adipic 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; Fig. 6). The ratio between kynurenine/tryptophan was lower at day 70 and 98 (Additional file 1: Fig. S3) 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: Fig. S4, pathway impact  $\geq 0.1$ , FDR  $< 0.01$ ).



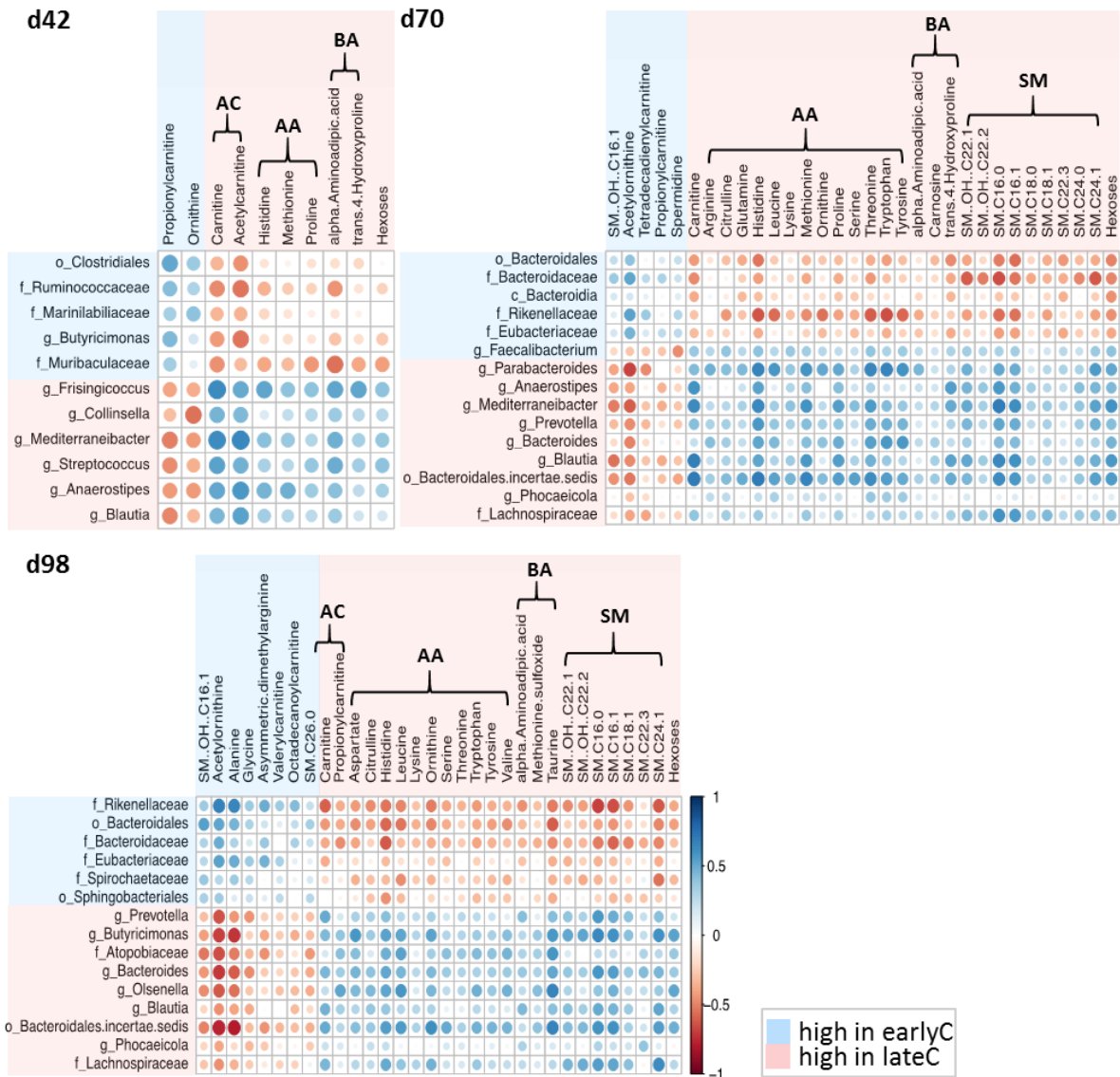
**Figure 6.** Volcano plots of the weaning-dependent changes in the plasma metabolic profiles of weaning groups. The identification of significantly altered metabolites due to weaning event within each age group was based on a VIP  $> 1$ , FDR  $< 0.05$  (t-test) and log<sub>2</sub> FC  $> 0.1$  or  $< -0.1$ . Each circle indicates one metabolite.

## **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 (Fig. 7). 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* (Fig. 7).

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-amino adipic 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 Butyrivibrio with acetylcarnitine, uncl. Spirochaetaceae with SM C24:1 ( $R < -0.50$ ,  $p < 0.05$ ).



**Figure 7.** 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 (AC, AA, BA and SM) indicates following metabolites classes: acylcarnitines, amino acids, biogenic amines, and sphingomyelins respectively.

## **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 S3) 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 acetylnithine 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 acetylnithine (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 acetylmethionine (d98).

## **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 composition 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.

## 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.

## Methods

### Animals and experimental procedures

The experiment was performed using 59 female German Holstein calves, raised under controlled environmental conditions from birth until  $149 \pm 2$  days of life. The experimental design was the same as described previously [22]. Briefly, the experimental period started when calves were  $8 \pm 1.9$  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 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].

## **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, Nürnberg, 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.

## **Faecal bacterial community profiling**

The genomic DNA was isolated from the faecal samples (250 mg) using the FastDNATM SPIN Kit for Feces (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 × g for 15 min. The DNA concentration and quality were accessed using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

## **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 ± 1,183 reads (mean ± 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  $\leq 0.2\%$  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  $24,495 \pm 777$  reads (mean  $\pm$  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 the 16S 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.

### **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 ( $\mu\text{mol/l}$ ). 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].

## **Statistical analysis**

### **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.

### **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 Q<sup>2</sup> as performance measure and tenfold cross-validation method. Q<sup>2</sup> indicates the predictive ability of the model, with high Q<sup>2</sup> means good prediction and negative Q<sup>2</sup> 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.

## **Declarations**

## **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).

## **Consent for publication**

Not applicable.

### **Availability of data and material**

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

### **Competing interests**

The authors declare no conflicts in this study.

### **Funding**

N.A.

### **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.

### **Acknowledgements**

We would like to thank the animal farm in Braunschweig for their tremendous support during the animal experiment and samplings. We thank Beate Mezger for her support with DNA extractions. We thank A. Kenez for his initial guidance of the metabolomic analysis. The authors acknowledge support by the High Performance and Cloud Computing Group at the Zentrum für Datenverarbeitung of the University of Tübingen, the state of Baden-Württemberg

through bwHPC and the German Research Foundation (DFG) through grant no INST 37/935-1 FUGG.

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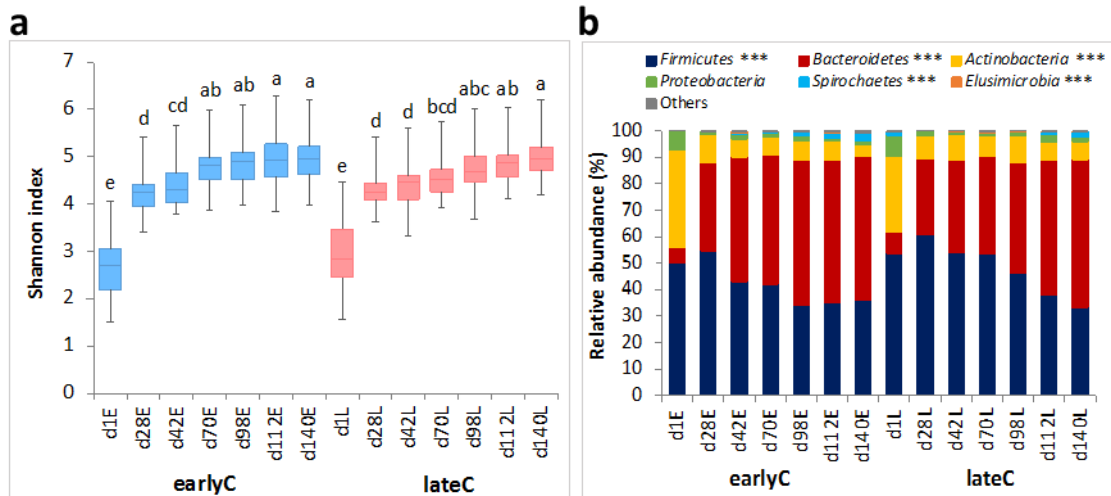
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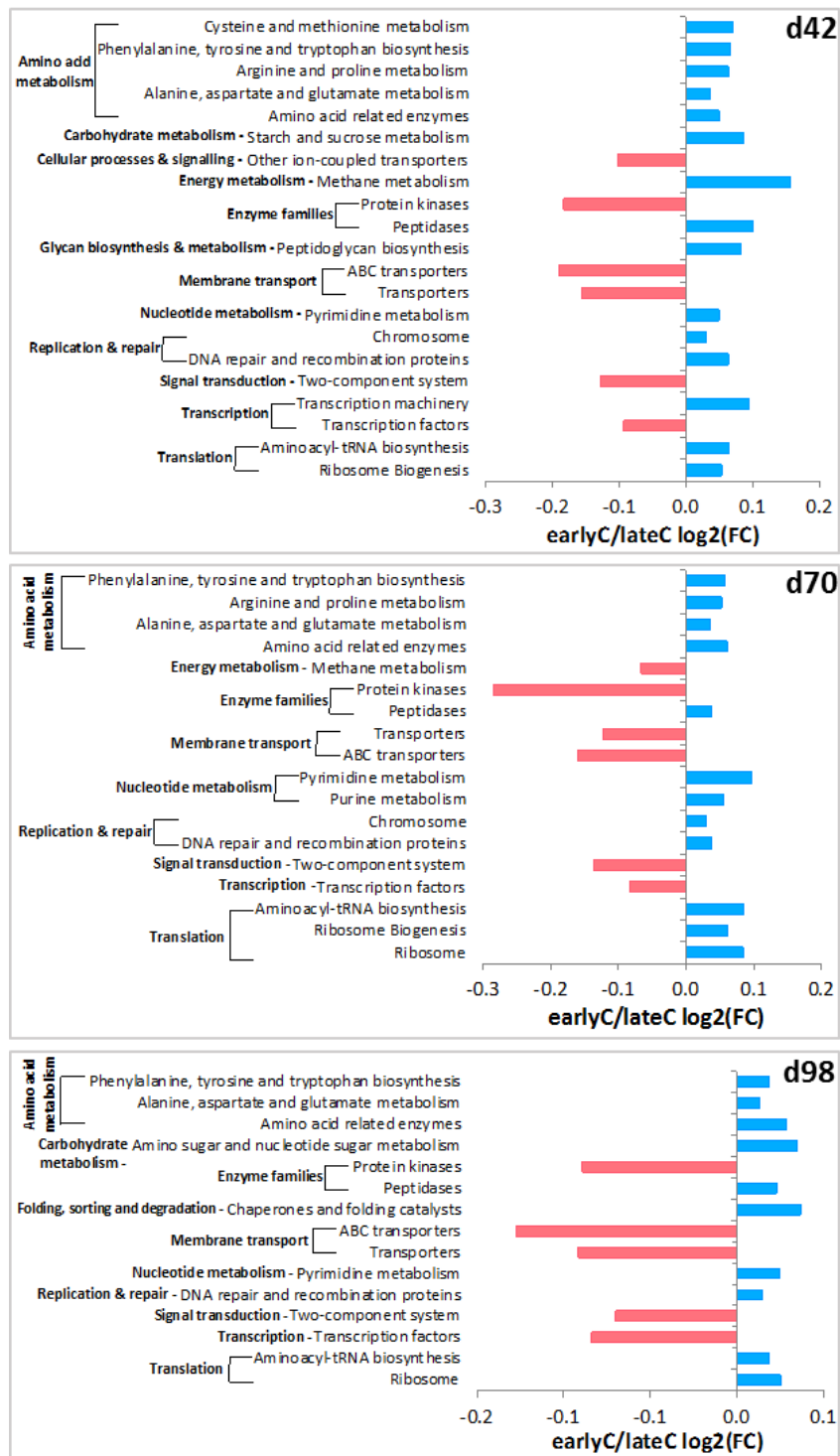
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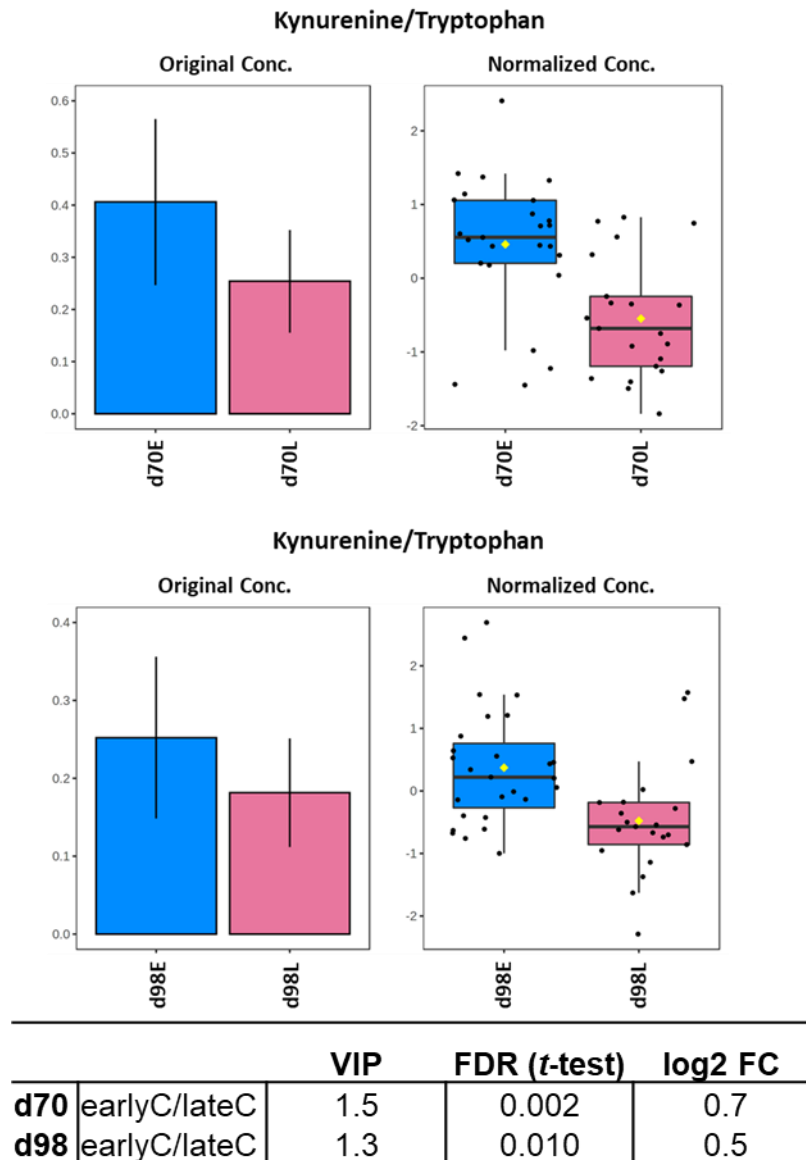
## Additional Files



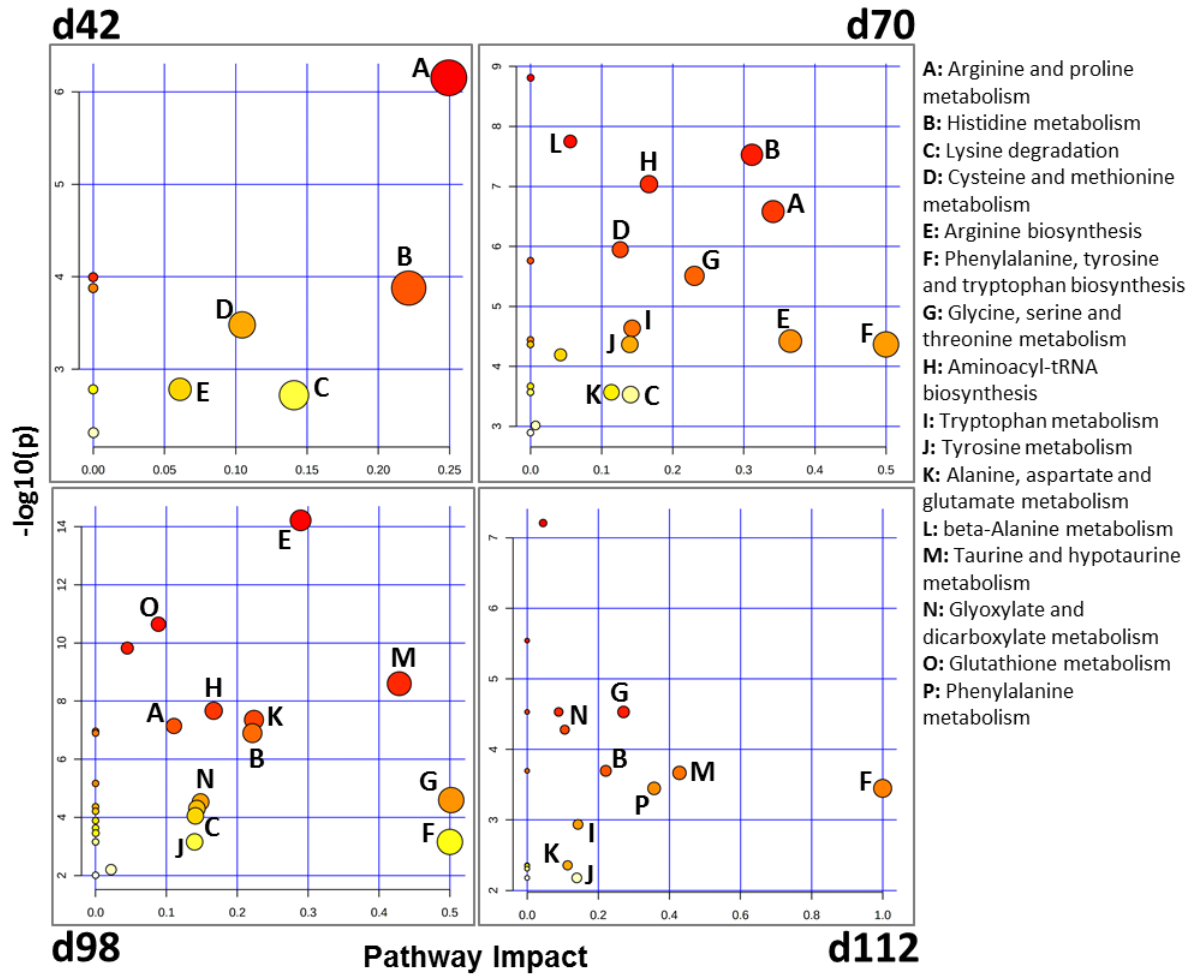
**Figure S1.** Age- and weaning-dependent changes in the faecal bacterial compositional profile of calves. (a) Changes among alpha-diversity index. abcdeGroups that share superscript letters are not significantly different ( $p > 0.05$ ; Dunn's post-hoc test). Standard deviations are indicated by error bars. (b) Significantly different bacterial phyla. \*\*\*Phyla with  $p < 0.001$  (age x weaning effect; Kruskal-Wallis test) are shown.



**Figure S2.** Microbial functional predictions using KEGG pathways and the CowPI database. EarlyC/lateC log<sub>2</sub>(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 (> 1.0 %) in at least 50 % of the animals and FDR adjusted  $p < 0.05$  (Kruskal-Wallis test) are shown.



**Figure S3.** Calculation of kynurenine/tryptophan ratio at d70 and d98 for early weaned calves (E) and late weaned calves (L).



**Figure S4.** Metabolic pathway analysis based on significantly differential plasma metabolites between weaning groups. Circle size indicates pathway impact and colours (yellow to red) show different levels of significance.

**Table S1.** Average relative abundances of faecal bacterial communities in early- and late-weaned calves.

<sup>1</sup> Taxon	<sup>2</sup> earlyC							<sup>3</sup> lateC							<sup>4</sup> p-value
	d1E	d28E	d42E	d70E	d98E	d112E	d140E	d1L	d28L	d42L	d70L	d98L	d112L	d140L	
<b>Phyla</b>															
<i>Firmicutes</i>	50.05	54.51	43.08	42.01	33.93	35.33	35.96	53.66	60.77	54.05	53.60	46.37	37.82	33.23	< 0.001
<i>Bacteroidetes</i>	6.05	33.33	46.87	48.77	55.07	53.84	54.30	8.02	28.59	34.86	36.92	41.57	51.13	55.89	< 0.001
<i>Actinobacteria</i>	36.80	10.64	6.90	7.04	7.40	6.86	4.62	28.58	8.66	9.66	7.83	10.12	6.59	6.75	< 0.001
<i>Proteobacteria</i>	7.08	1.35	1.89	1.47	1.63	1.29	1.47	7.98	1.95	1.29	0.66	1.68	2.86	1.62	0.194
<i>Spirochaetes</i>	0.00	0.03	0.45	0.29	1.33	1.82	2.69	1.28	0.01	0.03	0.04	0.06	1.18	2.14	< 0.001
<b>Genera</b>															
<i>o_Bacteroidales</i>	0.06	4.30	7.55	15.75	11.66	14.39	14.17	0.06	1.77	6.83	6.07	5.72	12.70	15.60	< 0.001
<i>o_Bacteroidales incertae sedis</i>	0.00	0.17	0.80	0.07	0.07	0.09	0.27	0.01	0.00	3.05	4.79	3.01	0.11	0.33	< 0.001
<i>g_Blautia</i>	0.18	6.43	1.14	0.13	0.25	0.23	0.27	2.40	7.96	6.54	3.65	2.68	0.31	0.19	< 0.001
<i>g_Prevotella</i>	0.72	4.72	2.34	0.19	0.08	0.13	0.11	0.20	4.90	3.75	2.05	1.00	0.08	0.02	< 0.001
<i>g_Mediterraneibacter</i>	4.32	2.09	0.10	0.00	0.04	0.06	0.04	5.23	4.13	1.82	1.76	0.66	0.00	0.00	< 0.001
<i>g_Anaerostipes</i>	0.64	3.76	0.08	0.01	0.09	0.11	0.06	0.82	3.93	4.51	1.36	0.35	0.01	0.01	< 0.001
<i>g_Parabacteroides</i>	0.16	2.63	3.60	0.09	0.03	0.06	0.30	0.28	2.16	1.79	1.30	0.75	0.16	0.01	< 0.001
<i>g_Faecalicatena</i>	0.19	2.06	0.81	0.01	0.01	0.05	0.12	0.30	1.46	1.51	0.72	0.24	0.00	0.12	< 0.001
<i>g_Streptococcus</i>	2.34	0.62	0.04	0.01	0.11	0.00	0.01	5.87	1.69	2.23	0.64	0.14	0.01	0.01	< 0.001
<i>g_Frisingicoccus</i>	0.01	2.27	0.19	0.15	0.11	0.11	0.09	0.01	1.32	1.24	0.61	0.39	0.19	0.09	< 0.001
<i>g_Collinsella</i>	0.03	2.88	0.18	0.00	0.03	0.02	0.02	0.75	2.13	1.32	0.53	0.35	0.00	0.00	< 0.001
<i>o_Sphingobacteriales</i>	0.00	0.12	0.10	0.65	0.84	0.77	0.63	0.00	0.00	0.01	0.00	0.44	1.29	1.60	< 0.001
<i>f_Lachnospiraceae</i>	1.75	11.15	10.54	7.39	6.89	7.62	8.02	1.78	12.91	12.53	16.03	15.16	7.70	7.14	< 0.001
<i>g_Bacteroides</i>	2.80	8.80	7.27	0.35	0.10	0.17	0.68	3.56	8.85	6.08	3.56	2.50	0.25	0.02	< 0.001
<i>f_Bacteroidaceae</i>	0.10	0.19	2.08	9.68	9.74	9.37	9.60	0.01	0.05	0.52	2.44	5.10	9.74	11.93	< 0.001
<i>f_Rikenellaceae</i>	0.04	0.04	0.28	5.04	12.20	12.27	11.49	0.02	0.02	0.07	0.83	1.88	7.05	11.52	< 0.001
<i>g_Faecalibacterium</i>	1.29	3.78	0.61	0.01	0.01	0.00	0.02	0.34	2.36	2.49	0.98	0.12	0.01	0.00	< 0.001
<i>f_Eubacteriaceae</i>	0.00	1.60	1.31	1.24	1.25	0.94	1.07	0.01	0.48	0.61	0.52	0.61	1.58	1.13	< 0.001
<i>c_Cytophagia</i>	0.00	0.02	0.65	0.70	1.11	0.72	0.06	0.05	0.00	0.02	0.14	0.79	1.55	0.24	< 0.001
<i>c_Bacteroidia</i>	0.02	0.46	2.55	7.08	7.62	7.05	8.18	0.02	0.31	0.86	1.40	4.11	7.62	8.84	< 0.001
<i>g_Phocaecicola</i>	1.90	5.79	4.66	0.46	0.07	0.10	0.33	3.59	7.71	7.26	7.23	5.21	0.50	0.06	< 0.001
<i>f_Atopobiaceae</i>	0.71	0.76	1.80	0.87	0.35	0.32	0.36	0.02	0.93	1.53	2.84	2.26	0.89	1.00	< 0.001
<i>f_Muribaculaceae</i>	0.01	0.22	2.34	3.16	3.24	2.77	2.89	0.00	0.01	0.50	1.55	2.07	2.83	2.25	< 0.001
<i>g_Alistipes</i>	0.01	1.16	1.12	0.33	0.08	0.05	0.06	0.06	0.44	0.91	0.47	0.31	0.16	0.01	< 0.001
<i>o_Clostridiales</i>	0.23	4.47	13.71	15.70	13.44	13.70	12.77	0.48	4.70	6.24	12.18	10.75	14.21	11.64	< 0.001
<i>f_Spirochaetaceae</i>	0.00	0.00	0.16	0.21	1.17	1.44	2.51	1.10	0.00	0.01	0.00	0.00	1.11	2.07	< 0.001
<i>g_Barnesiella</i>	0.02	1.15	2.05	0.02	0.00	0.02	0.44	0.02	0.87	0.89	0.07	0.49	0.03	0.01	< 0.001
<i>g_Gemmiger</i>	0.11	2.00	0.03	0.00	0.01	0.02	0.00	0.03	1.02	0.35	0.02	0.06	0.00	0.00	< 0.001
<i>g_Butyricicoccus</i>	2.82	0.54	0.01	0.00	0.01	0.01	0.01	3.17	1.38	0.54	0.03	0.08	0.00	0.00	< 0.001
<i>g_Butyricimonas</i>	0.02	2.02	2.93	0.47	0.10	0.14	0.09	0.05	0.42	0.81	1.48	1.06	0.28	0.02	< 0.001
<i>f_Marinilabiliaceae</i>	0.02	0.18	3.96	1.72	1.96	0.95	0.85	0.04	0.01	0.24	1.26	2.94	2.13	0.86	< 0.001
<i>f_Ruminococcaceae</i>	0.55	2.23	5.58	5.77	3.64	3.54	3.42	0.52	1.81	1.83	5.38	4.14	4.63	3.40	< 0.001
<i>g_Olsenella</i>	0.05	1.61	1.66	3.26	0.23	0.47	0.29	0.04	2.06	2.21	2.62	2.65	0.33	0.17	< 0.001

<i>f_Prevotellaceae</i>	0.13	0.41	1.51	1.04	1.55	1.30	1.87	0.02	0.39	0.73	1.28	0.88	0.80	1.02	< 0.001
<i>g_Clostridium</i>	1.88	0.48	0.07	0.78	0.31	0.23	0.64	1.83	1.25	0.17	0.39	0.22	0.31	0.16	< 0.001
<i>c_Clostridia</i>	0.02	0.94	0.88	2.21	1.37	2.09	1.28	0.01	0.71	0.76	1.22	1.68	1.60	2.21	< 0.001
<i>o_Erysipelotrichales</i>	0.00	0.48	0.31	0.10	0.11	0.21	0.23	0.02	0.58	2.06	0.35	0.23	0.16	0.18	< 0.001
<i>f_Lactobacillaceae</i>	6.38	0.05	0.01	0.06	0.01	0.00	0.01	8.11	0.14	0.01	0.00	0.12	0.02	0.01	< 0.001
<i>f_Oscillospiraceae</i>	0.00	1.37	1.84	3.46	2.07	1.76	2.17	0.05	1.11	1.91	2.16	2.10	2.56	1.80	< 0.001
<i>f_Tannerellaceae</i>	0.01	0.05	0.15	1.17	3.84	2.88	1.09	0.00	0.00	0.05	0.49	3.03	3.34	1.45	< 0.001
<i>f_Erysipelotrichaceae</i>	0.00	0.82	0.70	0.72	0.25	0.29	0.28	0.03	1.27	1.43	1.20	1.17	0.50	0.72	< 0.001
<i>g_Ligilactobacillus</i>	7.82	1.53	0.02	0.01	0.03	0.08	0.01	4.23	1.15	0.07	0.01	0.05	0.00	0.00	< 0.001
<i>g_Bifidobacterium</i>	34.59	4.48	3.11	2.47	6.47	5.57	3.71	26.98	2.96	4.06	1.73	4.72	5.19	5.22	< 0.001
<i>g_Gallibacterium</i>	4.56	0.05	0.00	0.00	0.01	0.10	0.01	2.25	0.22	0.02	0.00	0.05	0.00	0.01	< 0.001
<i>g_Enterococcus</i>	2.22	0.07	0.00	0.00	0.00	0.00	0.00	1.22	0.13	0.01	0.00	0.01	0.00	0.02	< 0.001
<i>g_Lactobacillus</i>	8.24	0.64	0.10	0.04	0.01	0.13	0.00	4.92	0.25	0.02	0.01	0.07	0.01	0.00	< 0.001
<i>g_Citrobacter</i>	0.97	0.18	0.03	0.02	0.11	0.07	0.01	1.33	0.34	0.02	0.00	0.00	0.01	0.01	< 0.001
<i>g_Limosilactobacillus</i>	5.28	0.69	0.02	0.02	0.05	0.00	0.01	8.75	0.24	0.04	0.01	0.03	0.00	0.01	< 0.001
<i>g_Schaalia</i>	1.39	0.01	0.00	0.00	0.01	0.00	0.00	0.69	0.01	0.00	0.00	0.00	0.00	0.00	< 0.001
<i>g_Turicibacter</i>	0.00	0.00	0.01	0.13	0.21	0.25	0.58	0.00	0.00	0.03	1.04	0.85	0.59	0.40	< 0.001

### Species

<i>s_Prevotella copri</i>	0.15	1.27	0.71	0.04	0.03	0.07	0.02	0.10	1.92	1.13	0.79	0.30	0.02	0.00	< 0.001
<i>g_Prevotella</i>	0.52	3.45	1.53	0.14	0.03	0.06	0.09	0.10	2.98	2.61	1.26	0.70	0.06	0.02	< 0.001
<i>s_Barnesiella</i>															
<i>intestinihominis</i>	0.02	1.15	2.05	0.02	0.00	0.02	0.44	0.02	0.87	0.89	0.07	0.49	0.03	0.01	< 0.001
<i>f_Prevotellaceae</i>	0.13	0.41	1.51	1.04	1.55	1.30	1.87	0.02	0.39	0.73	1.28	0.88	0.80	1.02	< 0.001
<i>s_Faecalibacterium</i>															
<i>prausnitzii</i>	1.17	3.54	0.51	0.01	0.01	0.00	0.02	0.23	2.12	2.14	0.86	0.10	0.01	0.00	< 0.001
<i>g_Faecalicatena</i>	0.16	2.06	0.81	0.01	0.01	0.05	0.12	0.30	1.46	1.51	0.72	0.24	0.00	0.12	< 0.001
<i>c_Clostridia</i>	0.02	0.94	0.88	2.21	1.37	2.09	1.28	0.01	0.71	0.76	1.22	1.68	1.60	2.21	< 0.001
<i>s_Bacteroides</i>															
<i>uniformis</i>	0.02	0.99	0.92	0.29	0.01	0.02	0.11	0.15	1.40	1.25	0.44	0.39	0.03	0.01	< 0.001
<i>s_Blautia wexlerae</i>	0.03	3.37	0.07	0.00	0.02	0.03	0.01	1.57	3.97	1.25	0.09	0.08	0.00	0.00	< 0.001
<i>f_Lactobacillaceae</i>	6.38	0.05	0.01	0.06	0.01	0.00	0.01	8.11	0.14	0.01	0.00	0.12	0.02	0.01	< 0.001
<i>s_Phocaeicola</i>															
<i>vulgatus</i>	1.89	4.50	3.97	0.12	0.06	0.09	0.27	3.55	6.30	5.03	0.83	0.58	0.02	0.02	< 0.001
<i>s_Gallibacterium</i>															
<i>anatis</i>	4.54	0.05	0.00	0.00	0.01	0.10	0.01	2.25	0.22	0.02	0.00	0.05	0.00	0.01	< 0.001
<i>f_Oscillospiraceae</i>	0.00	1.37	1.84	3.46	2.07	1.76	2.17	0.05	1.11	1.91	2.16	2.10	2.56	1.80	< 0.001
<i>f_Tannerellaceae</i>	0.01	0.05	0.15	1.17	3.84	2.88	1.09	0.00	0.00	0.05	0.49	3.03	3.34	1.45	< 0.001
<i>s_Ligilactobacillus</i>															
<i>salivarius</i>	7.81	1.53	0.02	0.01	0.03	0.08	0.01	4.19	1.15	0.07	0.01	0.05	0.00	0.00	< 0.001
<i>s_Bifidobacterium</i>															
<i>pseudolongum</i>	1.29	1.57	2.86	1.98	5.54	4.75	2.89	0.51	0.92	3.34	1.14	3.84	4.98	4.37	< 0.001
<i>g_Butyricoccus</i>	2.80	0.52	0.01	0.00	0.01	0.01	0.01	3.12	1.33	0.53	0.03	0.08	0.00	0.00	< 0.001
<i>s_Bifidobacterium</i>															
<i>longum</i>	28.90	2.73	0.06	0.13	0.32	0.19	0.02	20.88	1.76	0.49	0.16	0.30	0.02	0.02	< 0.001
<i>s_Lactobacillus</i>															
<i>amyovorvus</i>	6.61	0.13	0.10	0.04	0.01	0.09	0.00	4.26	0.08	0.00	0.00	0.06	0.00	0.00	< 0.001
<i>s_Lactobacillus</i>															
<i>ingluviei</i>	3.56	0.05	0.02	0.01	0.04	0.00	0.01	5.54	0.05	0.00	0.01	0.02	0.00	0.00	< 0.001
<i>g_Mediterraneibacter</i>	2.41	0.32	0.01	0.00	0.02	0.03	0.02	2.17	0.98	0.62	0.73	0.20	0.00	0.00	< 0.001
<i>s_Streptococcus</i>															
<i>gallyticus</i>	1.59	0.22	0.02	0.01	0.01	0.00	0.01	4.70	0.30	0.09	0.00	0.00	0.00	0.00	< 0.001
<i>f_Rikenellaceae</i>	0.04	0.04	0.28	5.04	12.20	12.27	11.49	0.02	0.02	0.07	0.83	1.88	7.05	11.52	< 0.001
<i>f_Eubacteriaceae</i>	0.00	1.60	1.31	1.24	1.25	0.94	1.07	0.01	0.48	0.61	0.52	0.61	1.58	1.13	< 0.001

<i>f_Ruminococcaceae</i>	0.55	2.23	5.58	5.77	3.64	3.54	3.42	0.52	1.81	1.83	5.38	4.14	4.63	3.40	< 0.001
<i>o_Bacteroidales</i>	0.06	4.30	7.55	15.75	11.66	14.39	14.17	0.06	1.77	6.83	6.07	5.72	12.70	15.60	< 0.001
<i>f_Bacteroidaceae</i>	0.10	0.19	2.08	9.68	9.74	9.37	9.60	0.01	0.05	0.52	2.44	5.10	9.74	11.93	< 0.001
<i>o_Clostridiales</i>	0.23	4.47	13.71	15.70	13.44	13.70	12.77	0.48	4.70	6.24	12.18	10.75	14.21	11.64	< 0.001
<i>c_Bacteroidia</i>	0.02	0.46	2.55	7.08	7.62	7.05	8.18	0.02	0.31	0.86	1.40	4.11	7.62	8.84	< 0.001
<i>o_Sphingobacteriales</i>	0.00	0.12	0.10	0.65	0.84	0.77	0.63	0.00	0.00	0.01	0.00	0.44	1.29	1.60	< 0.001
<i>f_Muribaculaceae</i>	0.01	0.22	2.34	3.16	3.24	2.77	2.89	0.00	0.01	0.50	1.55	2.07	2.83	2.25	< 0.001
<i>s_Olsenella</i>															
<i>umbonata</i>	0.02	0.69	0.94	1.81	0.18	0.33	0.22	0.02	1.16	1.11	1.32	1.49	0.22	0.10	< 0.001
<i>o_Bacteroidales</i>															
<i>incertae sedis</i>	0.00	0.17	0.80	0.07	0.07	0.09	0.27	0.01	0.00	3.05	4.79	3.01	0.11	0.33	< 0.001
<i>f_Lachnospiraceae</i>	1.75	11.15	10.54	7.39	6.89	7.62	8.02	1.78	12.91	12.53	16.03	15.16	7.70	7.14	< 0.001
<i>f_Erysipelotrichaceae</i>	0.00	0.82	0.70	0.72	0.25	0.29	0.28	0.03	1.27	1.43	1.20	1.17	0.50	0.72	< 0.001
<i>g_Blautia</i>	0.15	2.08	0.99	0.13	0.23	0.18	0.22	0.53	3.10	4.42	3.31	2.26	0.31	0.19	< 0.001
<i>f_Atopobiaceae</i>	0.71	0.76	1.80	0.87	0.35	0.32	0.36	0.02	0.93	1.53	2.84	2.26	0.89	1.00	< 0.001
<i>f_Spirochaetaceae</i>	0.00	0.00	0.16	0.21	1.17	1.44	2.51	1.10	0.00	0.01	0.00	0.00	1.11	2.07	< 0.001
<i>f_Marinilabiliaceae</i>	0.02	0.18	3.96	1.72	1.96	0.95	0.85	0.04	0.01	0.24	1.26	2.94	2.13	0.86	< 0.001
<i>g_Frisingicoccus</i>	0.01	2.27	0.19	0.15	0.11	0.11	0.09	0.01	1.32	1.24	0.61	0.39	0.19	0.09	< 0.001
<i>c_Cytophagia</i>	0.00	0.02	0.65	0.70	1.11	0.72	0.06	0.05	0.00	0.02	0.14	0.79	1.55	0.24	< 0.001
<i>s_Butyricimonas</i>															
<i>virosa</i>	0.02	1.62	2.21	0.33	0.07	0.11	0.07	0.03	0.31	0.65	1.14	0.86	0.23	0.01	< 0.001
<i>s_Phocaeicola</i>															
<i>coprocola</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.24	2.96	2.54	0.31	0.02	< 0.001

<sup>5</sup>earlyC vs. lateC Dunn's test

<sup>1</sup> Taxon	d1	d28	d42	d70	d98	d112	d140
<b>Phyla</b>							
<i>Firmicutes</i>	0.552	0.390	0.042	0.023	0.002	0.433	0.547
<i>Bacteroidetes</i>	0.857	0.622	0.032	0.033	0.006	0.551	0.797
<i>Actinobacteria</i>	0.885	0.805	0.223	0.619	0.074	0.860	0.498
<i>Proteobacteria</i>	0.854	0.823	0.651	0.676	0.807	0.650	0.981
<i>Spirochaetes</i>	0.536	0.993	0.067	0.081	0.030	0.691	1.000
<b>Genera</b>							
<i>o_Bacteroidales</i>	0.913	0.302	0.634	0.000	0.006	0.679	0.598
<i>o_Bacteroidales incertae sedis</i>	0.666	0.718	0.579	0.000	0.000	0.929	0.315
<i>g_Blautia</i>	0.287	0.997	0.000	0.000	0.003	0.834	0.797
<i>g_Prevotella</i>	0.552	0.813	0.180	0.000	0.001	0.635	0.986
<i>g_Mediterraneibacter</i>	0.751	0.435	0.000	0.000	0.060	0.884	0.697
<i>g_Anaerostipes</i>	0.759	0.570	0.000	0.000	0.417	0.955	0.480
<i>g_Parabacteroides</i>	0.558	0.474	0.256	0.000	0.000	0.044	0.630
<i>g_Faecalicatena</i>	0.713	0.814	0.213	0.000	0.003	0.536	0.514
<i>g_Streptococcus</i>	0.817	0.251	0.000	0.000	0.006	0.819	0.910
<i>g_Frisingicoccus</i>	0.943	0.529	0.000	0.000	0.015	0.212	0.801
<i>g_Collinsella</i>	0.295	0.965	0.007	0.000	0.187	0.979	1.000
<i>o_Sphingobacteriales</i>	0.828	1.000	0.827	0.000	0.005	0.950	0.086
<i>f_Lachnospiraceae</i>	0.948	0.723	0.142	0.001	0.000	0.940	0.567
<i>g_Bacteroides</i>	0.874	0.933	0.914	0.001	0.000	0.156	0.777
<i>f_Bacteroidaceae</i>	0.883	0.909	0.318	0.002	0.023	0.880	0.455
<i>f_Rikenellaceae</i>	0.898	0.950	0.583	0.003	0.000	0.219	0.889
<i>g_Faecalibacterium</i>	0.529	0.892	0.584	0.006	0.379	0.449	0.637
<i>f_Eubacteriaceae</i>	0.977	0.475	0.661	0.007	0.004	0.304	0.928
<i>c_Cytophagia</i>	0.738	0.226	0.000	0.014	0.771	0.041	0.182
<i>c_Bacteroidia</i>	0.901	0.811	0.242	0.020	0.230	0.939	0.624
<i>g_Phocaeicola</i>	0.685	0.916	0.964	0.038	0.023	0.021	0.551
<i>f_Atopobiaceae</i>	0.847	0.550	0.826	0.099	0.001	0.664	0.828
<i>f_Muribaculaceae</i>	0.969	0.750	0.011	0.107	0.494	0.973	0.977
<i>g_Alistipes</i>	0.760	0.280	0.577	0.120	0.002	0.022	0.576
<i>o_Clostridiales</i>	0.994	0.936	0.000	0.146	0.209	0.923	0.798
<i>f_Spirochaetaceae</i>	0.502	0.592	0.890	0.161	0.009	0.501	0.945
<i>g_Barnesiella</i>	0.918	0.365	0.093	0.197	0.003	0.281	0.988
<i>g_Gemmiger</i>	0.369	1.000	0.000	0.197	0.843	0.883	0.825
<i>g_Butyricococcus</i>	0.887	0.357	0.001	0.203	0.266	0.903	0.672
<i>g_Butyricimonas</i>	0.930	0.001	0.003	0.272	0.000	0.009	0.979
<i>f_Marinilabiliaceae</i>	0.867	0.757	0.012	0.286	0.798	0.361	0.857
<i>f_Ruminococcaceae</i>	0.797	0.541	0.000	0.366	0.927	0.511	0.908
<i>g_Olsenella</i>	0.831	0.110	0.943	0.776	0.000	0.765	0.975
<i>f_Prevotellaceae</i>	0.892	0.895	0.092	0.392	0.271	0.389	0.691
<i>g_Clostridium</i>	0.627	0.087	0.637	0.100	0.111	0.425	0.242
<i>c_Clostridia</i>	0.909	0.409	0.876	0.148	0.683	0.585	0.064
<i>o_Erysipelotrichales</i>	0.773	0.784	0.085	0.145	0.621	0.674	0.980

<i>f_Lactobacillaceae</i>	0.994	0.890	0.750	0.758	0.789	0.743	0.957
<i>f_Oscillospiraceae</i>	0.815	0.822	0.978	0.241	0.785	0.773	0.704
<i>f_Tannerellaceae</i>	0.969	0.817	0.837	0.184	0.058	0.793	0.771
<i>f_Erysipelotrichaceae</i>	0.507	0.751	0.764	0.799	0.106	0.795	0.797
<i>g_Ligilactobacillus</i>	0.794	0.904	0.373	0.882	0.882	0.802	0.878
<i>g_Bifidobacterium</i>	0.983	0.867	0.388	0.590	0.914	0.846	0.863
<i>g_Gallibacterium</i>	0.741	0.485	0.855	0.867	0.967	0.860	0.969
<i>g_Enterococcus</i>	0.792	0.869	0.278	0.997	0.529	0.883	1.000
<i>g_Lactobacillus</i>	0.795	0.800	0.922	0.743	0.642	0.916	0.874
<i>g_Citrobacter</i>	0.586	0.318	0.982	0.992	0.938	0.947	0.908
<i>g_Limosilactobacillus</i>	0.930	0.575	0.623	0.972	0.481	0.952	0.927
<i>g_Schaalia</i>	0.950	0.966	0.768	1.000	1.000	1.000	1.000
<i>g_Turicibacter</i>	0.791	0.859	0.653	0.233	0.058	0.936	0.853

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**Species**

<i>s_Prevotella copri</i>	0.847	0.510	0.100	0.000	0.000	0.989	0.630
<i>g_Prevotella</i>	0.253	0.981	0.386	0.058	0.030	0.254	0.998
<i>s_Barnesiella</i>							
<i>intestinihominis</i>	0.918	0.365	0.093	0.197	0.003	0.281	0.988
<i>f_Prevotellaceae</i>	0.892	0.895	0.092	0.392	0.271	0.389	0.691
<i>s_Faecalibacterium</i>							
<i>prausnitzii</i>	0.532	0.872	0.594	0.006	0.359	0.445	0.620
<i>g_Faecalicatena</i>	0.656	0.813	0.213	0.000	0.003	0.536	0.512
<i>c_Clostridia</i>	0.909	0.409	0.876	0.148	0.683	0.585	0.064
<i>s_Bacteroides</i>							
<i>uniformis</i>	0.321	0.965	0.947	0.954	0.012	0.619	0.955
<i>s_Blautia wexlerae</i>	0.251	0.832	0.000	0.275	0.349	0.653	0.878
<i>f_Lactobacillaceae</i>	0.994	0.890	0.750	0.758	0.789	0.743	0.957
<i>s_Phocaecicola vulgatus</i>	0.849	0.900	0.845	0.418	0.152	0.749	0.888
<i>s_Gallibacterium</i>							
<i>anatis</i>	0.729	0.689	0.841	0.864	0.970	0.754	0.970
<i>f_Oscillospiraceae</i>	0.815	0.822	0.978	0.241	0.785	0.773	0.704
<i>f_Tannerellaceae</i>	0.969	0.817	0.837	0.184	0.058	0.793	0.771
<i>s_Ligilactobacillus</i>							
<i>salivarius</i>	0.793	0.904	0.373	0.882	0.882	0.802	0.878
<i>s_Bifidobacterium</i>							
<i>pseudolongum</i>	0.910	0.957	0.454	0.162	0.448	0.850	0.828
<i>g_Butyricococcus</i>	0.888	0.368	0.001	0.203	0.262	0.903	0.672
<i>s_Bifidobacterium</i>							
<i>longum</i>	0.884	0.634	0.600	0.886	0.260	0.905	0.950
<i>s_Lactobacillus</i>							
<i>amylovorus</i>	0.748	0.569	0.604	0.540	0.782	0.956	0.966
<i>s_Lactobacillus</i>							
<i>ingluviei</i>	0.944	0.493	0.694	0.938	0.288	0.958	0.972
<i>g_Mediterraneibacter</i>	0.883	0.065	0.000	0.000	0.073	0.968	0.659
<i>s_Streptococcus</i>							
<i>gallolyticus</i>	0.070	0.844	0.085	0.374	0.820	1.000	0.760
<i>f_Rikenellaceae</i>	0.898	0.950	0.583	0.003	0.000	0.219	0.889
<i>f_Eubacteriaceae</i>	0.977	0.475	0.661	0.007	0.004	0.304	0.928
<i>f_Ruminococcaceae</i>	0.797	0.541	0.000	0.366	0.927	0.511	0.908
<i>o_Bacteroidales</i>	0.913	0.302	0.634	0.000	0.006	0.679	0.598
<i>f_Bacteroidaceae</i>	0.883	0.909	0.318	0.002	0.023	0.880	0.455
<i>o_Clostridiales</i>	0.994	0.936	0.000	0.146	0.209	0.923	0.798
<i>c_Bacteroidia</i>	0.901	0.811	0.242	0.020	0.230	0.939	0.624

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<i>o_Sphingobacteriales</i>	0.828	1.000	0.827	0.000	0.005	0.950	0.086
<i>f_Muribaculaceae</i>	0.969	0.750	0.011	0.107	0.494	0.973	0.977
<i>s_Olsenella umbonata</i>	0.968	0.064	0.888	0.944	0.001	0.875	0.890
<i>o_Bacteroidales</i>							
<i>incertae sedis</i>	0.666	0.718	0.579	0.000	0.000	0.929	0.315
<i>f_Lachnospiraceae</i>	0.948	0.723	0.142	0.001	0.000	0.940	0.567
<i>f_Erysipelotrichaceae</i>	0.507	0.751	0.764	0.799	0.106	0.795	0.797
<i>g_Blautia</i>	0.429	0.616	0.000	0.000	0.004	0.720	0.739
<i>f_Atopobiaceae</i>	0.847	0.550	0.826	0.099	0.001	0.664	0.828
<i>f_Spirochaetaceae</i>	0.502	0.592	0.890	0.161	0.009	0.501	0.945
<i>f_Marinilabiliaceae</i>	0.867	0.757	0.012	0.286	0.798	0.361	0.857
<i>g_Frisingicoccus</i>	0.943	0.529	0.000	0.000	0.015	0.212	0.801
<i>c_Cytophagia</i>	0.738	0.226	0.000	0.014	0.771	0.041	0.182
<i>s_Butyricimonas virosa</i>	0.937	0.000	0.003	0.172	0.000	0.008	0.929
<i>s_Phocaeicola</i>							
<i>coprocola</i>	0.883	0.901	0.893	0.019	0.000	0.000	0.423

<sup>5</sup>earlyC vs. earlyC Dunn's test

<sup>1</sup> Taxon	d1E - d28E	d1E - d42E	d1E - d70E	d1E - d98E	d1E - d112E	d1E - d140E	d28E - d42E	d28E - d70E	d28E - d98E	d28E - d112E	d28E - d140E
<b>Phyla</b>											
<i>Firmicutes</i>	0.432	0.208	0.114	0.000	0.001	0.001	0.021	0.009	0.000	0.000	0.000
<i>Bacteroidetes</i>	0.005	0.000	0.000	0.000	0.000	0.000	0.021	0.006	0.000	0.000	0.000
<i>Actinobacteria</i>	0.002	0.000	0.000	0.000	0.000	0.000	0.222	0.201	0.078	0.101	0.004
<i>Proteobacteria</i>	0.814	0.962	0.941	0.982	0.920	0.937	0.830	0.949	0.827	0.941	0.810
<i>Spirochaetes</i>	0.328	0.009	0.013	0.002	0.002	0.001	0.058	0.127	0.019	0.017	0.019
<b>Genera</b>											
<i>o_Bacteroidales</i>	0.015	0.000	0.000	0.000	0.000	0.000	0.116	0.000	0.000	0.000	0.000
<i>o_Bacteroidales incertae sedis</i>	0.716	0.120	0.378	0.076	0.098	0.020	0.247	0.661	0.151	0.200	0.057
<i>g_Blautia</i>	0.000	0.002	0.432	0.035	0.093	0.129	0.000	0.000	0.000	0.000	0.000
<i>g_Prevotella</i>	0.000	0.000	0.844	0.989	0.535	0.550	0.102	0.000	0.000	0.000	0.000
<i>g_Mediterraneibacter</i>	0.257	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>g_Anaerostipes</i>	0.002	0.876	0.394	0.888	0.465	0.833	0.000	0.000	0.000	0.000	0.000
<i>g_Parabacteroides</i>	0.000	0.000	0.978	0.328	0.276	0.270	0.604	0.000	0.000	0.000	0.000
<i>g_Faecalicatena</i>	0.000	0.000	0.185	0.216	0.411	0.811	0.166	0.000	0.000	0.000	0.000
<i>g_Streptococcus</i>	0.248	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>g_Frisingicoccus</i>	0.000	0.002	0.087	0.117	0.062	0.248	0.000	0.000	0.000	0.000	0.000
<i>g_Collinsella</i>	0.000	0.193	0.336	0.964	0.282	0.331	0.000	0.000	0.000	0.000	0.000
<i>o_Sphingobacteriales</i>	0.996	0.718	0.000	0.000	0.000	0.000	0.699	0.000	0.000	0.000	0.000
<i>f_Lachnospiraceae</i>	0.000	0.000	0.000	0.002	0.001	0.000	0.509	0.043	0.010	0.034	0.120
<i>g_Bacteroides</i>	0.001	0.002	0.154	0.016	0.003	0.005	0.772	0.000	0.000	0.000	0.000
<i>f_Bacteroidaceae</i>	0.894	0.028	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000
<i>f_Rikenellaceae</i>	0.797	0.276	0.000	0.000	0.000	0.000	0.131	0.000	0.000	0.000	0.000
<i>g_Faecalibacterium</i>	0.049	0.347	0.048	0.158	0.029	0.162	0.373	0.000	0.000	0.000	0.000
<i>f_Eubacteriaceae</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.385	0.204	0.062	0.118	0.176
<i>c_Cytophagia</i>	0.493	0.000	0.000	0.000	0.001	0.244	0.001	0.000	0.000	0.012	0.659
<i>c_Bacteroidia</i>	0.056	0.001	0.000	0.000	0.000	0.000	0.156	0.008	0.001	0.000	0.000
<i>g_Phocaeicola</i>	0.189	0.141	0.622	0.313	0.100	0.197	0.955	0.029	0.006	0.002	0.003
<i>f_Atopobiaceae</i>	0.001	0.000	0.000	0.001	0.000	0.000	0.095	0.163	0.975	0.831	0.844
<i>f_Muribaculaceae</i>	0.805	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>g_Alistipes</i>	0.000	0.000	0.000	0.175	0.577	0.779	0.443	0.045	0.000	0.000	0.000
<i>o_Clostridiales</i>	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>f_Spirochaetaceae</i>	0.876	0.459	0.104	0.011	0.004	0.004	0.273	0.044	0.004	0.002	0.004
<i>g_Barnesiella</i>	0.075	0.000	0.957	0.799	0.697	0.915	0.093	0.040	0.017	0.011	0.025
<i>g_Gemmiger</i>	0.000	0.240	0.816	1.000	0.975	1.000	0.000	0.000	0.000	0.000	0.000
<i>g_Butyricoccus</i>	0.980	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
<i>g_Butyricimonas</i>	0.000	0.000	0.000	0.312	0.607	0.977	0.020	0.493	0.000	0.000	0.000
<i>f_Marinilabiliaceae</i>	0.689	0.002	0.001	0.002	0.003	0.002	0.005	0.004	0.006	0.011	0.008
<i>f_Ruminococcaceae</i>	0.009	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.021	0.053	0.116
<i>g_Olsenella</i>	0.001	0.000	0.000	0.041	0.011	0.173	0.022	0.067	0.195	0.448	0.045
<i>f_Prevotellaceae</i>	0.372	0.003	0.037	0.003	0.003	0.003	0.047	0.243	0.056	0.067	0.068
<i>g_Clostridium</i>	0.073	0.047	0.063	0.084	0.208	0.863	0.870	0.954	0.871	0.593	0.031
<i>c_Clostridia</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.771	0.030	0.264	0.028	0.551

<i>o_Erysipelotrichales</i>	0.006	0.000	0.053	0.024	0.001	0.004	0.559	0.565	0.661	0.658	0.994
<i>f_Lactobacillaceae</i>	0.007	0.000	0.000	0.000	0.000	0.000	0.024	0.024	0.110	0.008	0.016
<i>f_Oscillospiraceae</i>	0.004	0.000	0.000	0.000	0.000	0.000	0.365	0.004	0.044	0.141	0.037
<i>f_Tannerellaceae</i>	0.737	0.678	0.013	0.000	0.000	0.000	0.387	0.002	0.000	0.000	0.000
<i>f_Erysipelotrichaceae</i>	0.005	0.001	0.002	0.098	0.015	0.067	0.687	0.874	0.349	0.720	0.417
<i>g_Ligilactobacillus</i>	0.064	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.013	0.000	0.000
<i>g_Bifidobacterium</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.592	0.825	0.825	0.689
<i>g_Gallibacterium</i>	0.018	0.000	0.000	0.000	0.000	0.000	0.015	0.016	0.202	0.070	0.118
<i>g_Enterococcus</i>	0.021	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>g_Lactobacillus</i>	0.870	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>g_Citrobacter</i>	0.271	0.086	0.013	0.019	0.038	0.029	0.854	0.327	0.498	0.688	0.636
<i>g_Limosilactobacillus</i>	0.040	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.012	0.000	0.000
<i>g_Schaalia</i>	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.976	1.000	0.993	1.000
<i>g_Turicibacter</i>	0.950	0.673	0.520	0.326	0.044	0.024	0.593	0.438	0.272	0.031	0.015

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**Species**


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<i>s_Prevotella copri</i>	0.000	0.001	0.569	0.630	0.513	0.378	0.131	0.000	0.000	0.000	0.000
<i>g_Prevotella</i>	0.000	0.009	0.690	0.858	0.644	0.871	0.132	0.000	0.000	0.000	0.000
<i>s_Barnesiella</i>											
<i>intestinihominis</i>	0.075	0.000	0.957	0.799	0.697	0.915	0.093	0.040	0.017	0.011	0.025
<i>f_Prevotellaceae</i>	0.372	0.003	0.037	0.003	0.003	0.003	0.047	0.243	0.056	0.067	0.068
<i>s_Faecalibacterium</i>											
<i>prausnitzii</i>	0.028	0.240	0.076	0.214	0.028	0.169	0.357	0.000	0.000	0.000	0.000
<i>g_Faecalicatena</i>	0.000	0.000	0.209	0.247	0.457	0.863	0.167	0.000	0.000	0.000	0.000
<i>c_Clostridia</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.771	0.030	0.264	0.028	0.551
<i>s_Bacteroides uniformis</i>	0.003	0.005	0.119	0.840	0.776	0.874	0.947	0.180	0.001	0.001	0.001
<i>s_Blautia wexlerae</i>	0.000	0.838	0.663	0.877	0.879	0.882	0.000	0.000	0.000	0.000	0.000
<i>f_Lactobacillaceae</i>	0.007	0.000	0.000	0.000	0.000	0.000	0.024	0.024	0.110	0.008	0.016
<i>s_Phocaeicola vulgatus</i>	0.191	0.200	0.328	0.167	0.054	0.127	0.964	0.010	0.002	0.001	0.002
<i>s_Gallibacterium anatis</i>	0.019	0.000	0.000	0.000	0.000	0.000	0.015	0.016	0.193	0.069	0.113
<i>f_Oscillospiraceae</i>	0.004	0.000	0.000	0.000	0.000	0.000	0.365	0.004	0.044	0.141	0.037
<i>f_Tannerellaceae</i>	0.737	0.678	0.013	0.000	0.000	0.000	0.387	0.002	0.000	0.000	0.000
<i>s_Ligilactobacillus</i>											
<i>salivarius</i>	0.064	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.013	0.000	0.000
<i>s_Bifidobacterium</i>											
<i>pseudolongum</i>	0.200	0.037	0.007	0.000	0.000	0.004	0.450	0.203	0.006	0.013	0.152
<i>g_Butyricoccus</i>	0.976	0.001	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000
<i>s_Bifidobacterium longum</i>	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>s_Lactobacillus amylovorus</i>	0.412	0.000	0.000	0.000	0.000	0.000	0.007	0.001	0.000	0.000	0.000
<i>s_Lactobacillus ingluviei</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.344	0.137	0.933	0.066	0.182
<i>g_Mediterraneibacter</i>	0.725	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>s_Streptococcus</i>											
<i>gallolyticus</i>	0.959	0.238	0.205	0.187	0.021	0.121	0.159	0.138	0.124	0.010	0.077
<i>f_Rikenellaceae</i>	0.797	0.276	0.000	0.000	0.000	0.000	0.131	0.000	0.000	0.000	0.000
<i>f_Eubacteriaceae</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.385	0.204	0.062	0.118	0.176
<i>f_Ruminococcaceae</i>	0.009	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.021	0.053	0.116
<i>o_Bacteroidales</i>	0.015	0.000	0.000	0.000	0.000	0.000	0.116	0.000	0.000	0.000	0.000
<i>f_Bacteroidaceae</i>	0.894	0.028	0.000	0.000	0.000	0.000	0.042	0.000	0.000	0.000	0.000
<i>o_Clostridiales</i>	0.064	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>c_Bacteroidia</i>	0.056	0.001	0.000	0.000	0.000	0.000	0.156	0.008	0.001	0.000	0.000
<i>o_Sphingobacteriales</i>	0.996	0.718	0.000	0.000	0.000	0.000	0.699	0.000	0.000	0.000	0.000

<i>f_Muribaculaceae</i>	0.805	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
<i>s_Olsenella umbonata</i>	0.005	0.000	0.000	0.033	0.028	0.450	0.016	0.014	0.508	0.566	0.036
<i>o_Bacteroidales incertae sedis</i>	0.716	0.120	0.378	0.076	0.098	0.020	0.247	0.661	0.151	0.200	0.057
<i>f_Lachnospiraceae</i>	0.000	0.000	0.000	0.002	0.001	0.000	0.509	0.043	0.010	0.034	0.120
<i>f_Erysipelotrichaceae</i>	0.005	0.001	0.002	0.098	0.015	0.067	0.687	0.874	0.349	0.720	0.417
<i>g_Blautia</i>	0.000	0.001	0.239	0.016	0.041	0.061	0.018	0.000	0.000	0.000	0.000
<i>f_Atopobiaceae</i>	0.001	0.000	0.000	0.001	0.000	0.000	0.095	0.163	0.975	0.831	0.844
<i>f_Spirochaetaceae</i>	0.876	0.459	0.104	0.011	0.004	0.004	0.273	0.044	0.004	0.002	0.004
<i>f_Marinilabiliaceae</i>	0.689	0.002	0.001	0.002	0.003	0.002	0.005	0.004	0.006	0.011	0.008
<i>g_Frisingicoccus</i>	0.000	0.002	0.087	0.117	0.062	0.248	0.000	0.000	0.000	0.000	0.000
<i>c_Cytophagia</i>	0.493	0.000	0.000	0.000	0.001	0.244	0.001	0.000	0.000	0.012	0.659
<i>s_Butyricimonas virosa</i>	0.000	0.000	0.000	0.275	0.562	0.967	0.025	0.329	0.000	0.000	0.000
<i>s_Phocaeicola coprocola</i>	0.877	0.647	0.890	0.843	0.878	0.977	0.786	0.973	0.905	0.746	0.894

<sup>5</sup>earlyC vs. earlyC Dunn's test

<sup>1</sup> Taxon	d42E – d70E	d42E – d98E	d42E - d112E	d42E - d140E	d70E – d098E	d70E - d112E	d70E - d140E	d112E – d98E	d98E - d140E	d112E - d140E
<b>Phyla</b>										
<i>Firmicutes</i>	0.821	0.015	0.030	0.062	0.031	0.061	0.118	0.824	0.608	0.824
<i>Bacteroidetes</i>	0.708	0.054	0.098	0.074	0.134	0.223	0.174	0.814	0.894	0.902
<i>Actinobacteria</i>	0.969	0.677	0.772	0.179	0.718	0.805	0.186	0.943	0.393	0.327
<i>Proteobacteria</i>	0.935	0.941	0.950	0.936	0.861	0.982	0.814	0.835	0.973	0.827
<i>Spirochaetes</i>	0.857	0.750	0.723	0.746	0.458	0.427	0.464	0.999	0.995	1.000
<b>Genera</b>										
<i>o_Bacteroidales</i>	0.001	0.047	0.003	0.001	0.229	0.774	0.915	0.377	0.271	0.860
<i>o_Bacteroidales incertae sedis</i>	0.562	0.860	0.950	0.528	0.379	0.489	0.162	0.911	0.668	0.579
<i>g_Blautia</i>	0.023	0.381	0.187	0.128	0.226	0.427	0.533	0.734	0.614	0.895
<i>g_Prevotella</i>	0.000	0.000	0.000	0.000	0.819	0.658	0.684	0.491	0.516	0.989
<i>g_Mediterraneibacter</i>	0.092	0.318	0.193	0.252	0.591	0.774	0.686	0.832	0.897	0.888
<i>g_Anaerostipes</i>	0.474	0.974	0.561	0.951	0.459	0.909	0.507	0.540	0.955	0.568
<i>g_Parabacteroides</i>	0.000	0.000	0.000	0.000	0.312	0.261	0.259	0.938	0.941	0.982
<i>g_Faecalicatena</i>	0.000	0.000	0.000	0.000	0.902	0.628	0.253	0.706	0.305	0.565
<i>g_Streptococcus</i>	0.480	0.756	0.268	0.417	0.814	0.827	0.958	0.543	0.759	0.862
<i>g_Frisingicoccus</i>	0.148	0.101	0.186	0.037	0.887	0.910	0.570	0.779	0.697	0.470
<i>g_Collinsella</i>	0.010	0.118	0.006	0.009	0.356	0.975	1.000	0.298	0.351	0.977
<i>o_Sphingobacteriales</i>	0.001	0.000	0.000	0.000	0.678	0.327	0.305	0.679	0.639	0.985
<i>f_Lachnospiraceae</i>	0.246	0.082	0.206	0.488	0.676	0.938	0.725	0.729	0.393	0.683
<i>g_Bacteroides</i>	0.000	0.000	0.000	0.000	0.344	0.144	0.174	0.653	0.747	0.911
<i>f_Bacteroidaceae</i>	0.000	0.000	0.000	0.000	0.892	0.911	0.980	0.781	0.890	0.910
<i>f_Rikenellaceae</i>	0.000	0.000	0.000	0.000	0.030	0.028	0.062	0.967	0.819	0.804
<i>g_Faecalibacterium</i>	0.001	0.006	0.000	0.006	0.637	0.885	0.621	0.522	0.985	0.513
<i>f_Eubacteriaceae</i>	0.735	0.382	0.572	0.691	0.667	0.836	0.964	0.809	0.694	0.883
<i>c_Cytophagia</i>	0.469	0.282	0.464	0.004	0.744	0.117	0.000	0.049	0.000	0.039
<i>c_Bacteroidia</i>	0.249	0.056	0.048	0.015	0.511	0.467	0.229	0.952	0.643	0.675
<i>g_Phocaeicola</i>	0.023	0.004	0.002	0.002	0.670	0.318	0.482	0.656	0.854	0.829

<i>f_Atopobiaceae</i>	0.833	0.077	0.144	0.137	0.140	0.241	0.227	0.824	0.828	0.967
<i>f_Muribaculaceae</i>	0.086	0.026	0.040	0.163	0.750	0.851	0.840	0.940	0.551	0.651
<i>g_Alistipes</i>	0.003	0.000	0.000	0.000	0.009	0.000	0.000	0.408	0.256	0.783
<i>o_Clostridiales</i>	0.410	0.950	0.930	0.794	0.451	0.465	0.212	0.970	0.721	0.689
<i>f_Spirochaetaceae</i>	0.495	0.085	0.028	0.029	0.431	0.187	0.185	0.725	0.720	0.986
<i>g_Barnesiella</i>	0.000	0.000	0.000	0.000	0.849	0.747	0.928	0.931	0.939	0.863
<i>g_Gemmiger</i>	0.088	0.202	0.163	0.198	0.874	0.885	0.854	0.959	1.000	0.965
<i>g_Butyricoccus</i>	0.469	0.743	0.480	0.741	0.786	0.994	0.789	0.792	1.000	0.795
<i>g_Butyricimonas</i>	0.001	0.000	0.000	0.000	0.003	0.000	0.000	0.627	0.259	0.553
<i>f_Marinilabiliaceae</i>	0.925	0.929	0.829	0.863	0.913	0.760	0.842	0.880	0.936	0.918
<i>f_Ruminococcaceae</i>	0.785	0.410	0.226	0.108	0.214	0.104	0.044	0.775	0.545	0.775
<i>g_Olsenella</i>	0.717	0.000	0.001	0.000	0.001	0.004	0.000	0.691	0.587	0.276
<i>f_Prevotellaceae</i>	0.444	0.942	0.905	0.902	0.506	0.554	0.568	0.953	0.951	0.977
<i>g_Clostridium</i>	0.872	0.771	0.428	0.023	0.869	0.539	0.024	0.698	0.043	0.098
<i>c_Clostridia</i>	0.011	0.140	0.010	0.365	0.360	0.990	0.142	0.363	0.636	0.136
<i>o_Erysipelotrichales</i>	0.088	0.158	0.903	0.565	0.889	0.150	0.505	0.258	0.661	0.653
<i>f_Lactobacillaceae</i>	0.997	0.731	0.845	0.970	0.728	0.837	0.966	0.464	0.655	0.888
<i>f_Oscillospiraceae</i>	0.110	0.443	0.740	0.398	0.576	0.275	0.620	0.758	0.960	0.745
<i>f_Tannerellaceae</i>	0.039	0.000	0.000	0.000	0.000	0.000	0.033	0.887	0.108	0.148
<i>f_Erysipelotrichaceae</i>	0.788	0.108	0.378	0.153	0.221	0.603	0.311	0.588	0.894	0.707
<i>g_Ligilactobacillus</i>	0.674	0.497	0.907	0.926	0.178	0.770	0.747	0.371	0.405	0.960
<i>g_Bifidobacterium</i>	0.615	0.054	0.057	0.475	0.323	0.337	0.913	0.980	0.409	0.438
<i>g_Gallibacterium</i>	0.980	0.472	0.734	0.612	0.463	0.729	0.616	0.801	0.870	0.868
<i>g_Enterococcus</i>	0.956	0.948	0.965	0.953	1.000	0.999	0.859	0.995	0.871	0.865
<i>g_Lactobacillus</i>	0.794	0.827	0.737	0.388	0.927	0.904	0.665	0.878	0.598	0.723
<i>g_Citrobacter</i>	0.641	0.809	0.957	0.915	0.946	0.823	0.867	0.919	0.938	0.985
<i>g_Limosilactobacillus</i>	0.826	0.756	0.592	0.765	0.401	0.922	0.974	0.250	0.351	0.950
<i>g_Schaalia</i>	0.773	0.982	0.781	0.797	1.000	1.000	1.000	1.000	1.000	1.000
<i>g_Turicibacter</i>	0.880	0.666	0.106	0.041	0.812	0.163	0.062	0.306	0.117	0.730
<b>Species</b>										
<i>s_Prevotella copri</i>	0.000	0.000	0.000	0.000	0.920	0.917	0.768	0.854	0.690	0.844
<i>g_Prevotella</i>	0.026	0.012	0.000	0.011	0.866	0.287	0.850	0.391	0.999	0.421
<i>s_Barnesiella</i>										
<i>intestinihominis</i>	0.000	0.000	0.000	0.000	0.849	0.747	0.928	0.931	0.939	0.863
<i>f_Prevotellaceae</i>	0.444	0.942	0.905	0.902	0.506	0.554	0.568	0.953	0.951	0.977
<i>s_Faecalibacterium</i>										
<i>prausnitzii</i>	0.001	0.006	0.000	0.004	0.623	0.715	0.719	0.372	0.906	0.460
<i>g_Faecalicatena</i>	0.000	0.000	0.000	0.000	0.902	0.628	0.251	0.706	0.303	0.563
<i>c_Clostridia</i>	0.011	0.140	0.010	0.365	0.360	0.990	0.142	0.363	0.636	0.136
<i>s_Bacteroides</i>										
<i>uniformis</i>	0.226	0.001	0.001	0.001	0.038	0.029	0.042	0.936	0.967	0.945
<i>s_Blautia wexlerae</i>	0.406	0.871	0.685	0.687	0.510	0.751	0.739	0.767	0.769	0.992
<i>f_Lactobacillaceae</i>	0.997	0.731	0.845	0.970	0.728	0.837	0.966	0.464	0.655	0.888
<i>s_Phocaeicola</i>										
<i>vulgatus</i>	0.011	0.002	0.001	0.002	0.758	0.324	0.596	0.616	0.889	0.782
<i>s_Gallibacterium</i>										
<i>anatis</i>	0.980	0.452	0.706	0.595	0.444	0.701	0.598	0.771	0.865	0.877
<i>f_Oscillospiraceae</i>	0.110	0.443	0.740	0.398	0.576	0.275	0.620	0.758	0.960	0.745
<i>f_Tannerellaceae</i>	0.039	0.000	0.000	0.000	0.000	0.000	0.033	0.887	0.108	0.148

<i>s_Ligilactobacillus salivarius</i>	0.674	0.497	0.907	0.926	0.178	0.770	0.747	0.371	0.405	0.960
<i>s_Bifidobacterium pseudolongum</i>	0.644	0.065	0.122	0.521	0.181	0.303	0.919	0.819	0.223	0.371
<i>g_Butyricoccus s_Bifidobacterium longum</i>	0.466	0.740	0.477	0.737	0.786	0.994	0.789	0.792	1.000	0.795
<i>s_Lactobacillus amylovorus</i>	0.858	0.908	0.588	0.675	0.762	0.722	0.860	0.480	0.601	0.882
<i>s_Lactobacillus ingluviei</i>	0.798	0.614	0.548	0.308	0.834	0.798	0.581	0.928	0.793	0.819
<i>g_Mediterraneibacter s_Streptococcus gallolyticus</i>	0.882	0.532	0.606	0.940	0.219	0.946	0.964	0.120	0.286	0.889
<i>s_Streptococcus gallolyticus</i>	0.434	0.590	0.607	0.808	0.865	0.850	0.646	0.987	0.818	0.845
<i>f_Rikenellaceae</i>	0.993	0.967	0.324	0.849	1.000	0.350	0.838	0.356	0.863	0.514
<i>f_Rikenellaceae</i>	0.000	0.000	0.000	0.000	0.030	0.028	0.062	0.967	0.819	0.804
<i>f_Eubacteriaceae</i>	0.735	0.382	0.572	0.691	0.667	0.836	0.964	0.809	0.694	0.883
<i>f_Ruminococcaceae</i>	0.785	0.410	0.226	0.108	0.214	0.104	0.044	0.775	0.545	0.775
<i>o_Bacteroidales</i>	0.001	0.047	0.003	0.001	0.229	0.774	0.915	0.377	0.271	0.860
<i>f_Bacteroidaceae</i>	0.000	0.000	0.000	0.000	0.892	0.911	0.980	0.781	0.890	0.910
<i>o_Clostridiales</i>	0.410	0.950	0.930	0.794	0.451	0.465	0.212	0.970	0.721	0.689
<i>c_Bacteroidia</i>	0.249	0.056	0.048	0.015	0.511	0.467	0.229	0.952	0.643	0.675
<i>o_Sphingobacteriales</i>	0.001	0.000	0.000	0.000	0.678	0.327	0.305	0.679	0.639	0.985
<i>f_Muribaculaceae s_Olsenella umbonata</i>	0.086	0.026	0.040	0.163	0.750	0.851	0.840	0.940	0.551	0.651
<i>o_Bacteroidales incertae sedis</i>	0.973	0.001	0.001	0.000	0.001	0.001	0.000	0.948	0.213	0.182
<i>f_Lachnospiraceae</i>	0.562	0.860	0.950	0.528	0.379	0.489	0.162	0.911	0.668	0.579
<i>f_Lachnospiraceae</i>	0.246	0.082	0.206	0.488	0.676	0.938	0.725	0.729	0.393	0.683
<i>f_Erysipelotrichaceae</i>	0.788	0.108	0.378	0.153	0.221	0.603	0.311	0.588	0.894	0.707
<i>g_Blautia</i>	0.030	0.354	0.207	0.150	0.265	0.436	0.540	0.745	0.652	0.872
<i>f_Atopobiaceae</i>	0.833	0.077	0.144	0.137	0.140	0.241	0.227	0.824	0.828	0.967
<i>f_Spirochaetaceae</i>	0.495	0.085	0.028	0.029	0.431	0.187	0.185	0.725	0.720	0.986
<i>f_Marinilabiliaceae</i>	0.925	0.929	0.829	0.863	0.913	0.760	0.842	0.880	0.936	0.918
<i>g_Frisingicoccus</i>	0.148	0.101	0.186	0.037	0.887	0.910	0.570	0.779	0.697	0.470
<i>c_Cytophagia s_Butyricimonas virosa</i>	0.469	0.282	0.464	0.004	0.744	0.117	0.000	0.049	0.000	0.039
<i>s_Phocaeicola coprocola</i>	0.001	0.000	0.000	0.000	0.007	0.001	0.000	0.626	0.260	0.568
<i>s_Phocaeicola coprocola</i>	0.752	0.907	0.407	0.600	0.880	0.741	0.883	0.638	0.765	0.895

<sup>5</sup>lateC vs. lateC Dunn's test

<sup>1</sup> Taxon	d1L – d28L	d1L – d42L	d1L – d70L	d1L – d98L	d1L - d112L	d1L - d140L	d28L – d42L	d28L – d70L	d28L – d98L	d28L - d112L	d28L - d140L
<b>Phyla</b>											
<i>Firmicutes</i>	0.312	0.948	0.939	0.201	0.001	0.000	0.279	0.276	0.016	0.000	0.000
<i>Bacteroidetes</i>	0.043	0.005	0.002	0.000	0.000	0.000	0.554	0.338	0.059	0.000	0.000
<i>Actinobacteria</i>	0.002	0.004	0.000	0.006	0.000	0.000	0.813	0.814	0.767	0.163	0.180
<i>Proteobacteria</i>	0.522	0.414	0.307	0.636	0.935	0.856	0.970	0.818	0.815	0.554	0.670
<i>Spirochaetes</i>	0.878	0.877	1.000	0.746	0.037	0.016	0.996	0.898	0.936	0.086	0.030
<b>Genera</b>											
<i>o_Bacteroidales</i>	0.175	0.000	0.001	0.001	0.000	0.000	0.035	0.054	0.064	0.000	0.000
<i>o_Bacteroidales incertae sedis</i>	0.668	0.098	0.000	0.000	0.223	0.004	0.028	0.000	0.000	0.085	0.001

<i>g_Blautia</i>	0.000	0.000	0.000	0.000	0.527	0.962	0.977	0.570	0.115	0.000	0.000
<i>g_Prevotella</i>	0.000	0.000	0.001	0.019	0.435	0.164	0.662	0.149	0.016	0.000	0.000
<i>g_Mediterraneibacter</i>	0.012	0.152	0.600	0.060	0.000	0.000	0.344	0.072	0.000	0.000	0.000
<i>g_Anaerostipes</i>	0.001	0.000	0.067	0.820	0.225	0.160	0.760	0.276	0.003	0.000	0.000
<i>g_Parabacteroides</i>	0.001	0.000	0.001	0.015	0.932	0.022	0.938	0.939	0.477	0.002	0.000
<i>g_Faecalicatena</i>	0.000	0.000	0.001	0.336	0.047	0.174	0.895	0.412	0.001	0.000	0.000
<i>g_Streptococcus</i>	0.814	0.966	0.070	0.017	0.000	0.000	0.862	0.030	0.006	0.000	0.000
<i>g_Frisingicoccus</i>	0.000	0.000	0.000	0.000	0.002	0.187	0.565	0.686	0.027	0.002	0.000
<i>g_Collinsella</i>	0.000	0.005	0.111	1.000	0.027	0.029	0.550	0.089	0.001	0.000	0.000
<i>o_Sphingobacteriales</i>	0.828	0.328	0.930	0.133	0.000	0.000	0.547	0.952	0.276	0.000	0.000
<i>f_Lachnospiraceae</i>	0.000	0.000	0.000	0.000	0.001	0.001	0.841	0.440	0.598	0.012	0.009
<i>g_Bacteroides</i>	0.001	0.004	0.154	0.205	0.111	0.001	0.653	0.085	0.047	0.000	0.000
<i>f_Bacteroidaceae</i>	0.593	0.190	0.001	0.000	0.000	0.000	0.527	0.007	0.000	0.000	0.000
<i>f_Rikenellaceae</i>	0.878	0.509	0.085	0.001	0.000	0.000	0.378	0.054	0.000	0.000	0.000
<i>g_Faecalibacterium</i>	0.181	0.422	0.948	0.232	0.037	0.006	0.635	0.162	0.007	0.000	0.000
<i>f_Eubacteriaceae</i>	0.001	0.000	0.007	0.002	0.000	0.000	0.278	0.664	0.905	0.001	0.049
<i>c_Cytophagia</i>	0.883	0.454	0.012	0.000	0.000	0.004	0.372	0.009	0.000	0.000	0.002
<i>c_Bacteroidia</i>	0.078	0.020	0.023	0.000	0.000	0.000	0.656	0.662	0.028	0.000	0.000
<i>g_Phocaeicola</i>	0.341	0.417	0.449	0.668	0.977	0.293	0.926	0.895	0.679	0.354	0.023
<i>f_Atopobiaceae</i>	0.023	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.176	0.265
<i>f_Muribaculaceae</i>	0.976	0.130	0.000	0.000	0.000	0.000	0.147	0.000	0.000	0.000	0.000
<i>g_Alistipes</i>	0.000	0.000	0.000	0.000	0.016	0.552	0.200	0.476	0.860	0.084	0.000
<i>o_Clostridiales</i>	0.047	0.004	0.000	0.000	0.000	0.000	0.547	0.000	0.005	0.000	0.001
<i>f_Spirochaetaceae</i>	0.729	0.948	0.596	0.477	0.212	0.027	0.804	0.883	0.736	0.088	0.010
<i>g_Barnesiella</i>	0.371	0.041	0.191	0.010	0.509	0.976	0.364	0.797	0.141	0.911	0.356
<i>g_Gemmiger</i>	0.000	0.000	1.000	0.636	0.200	0.198	0.385	0.000	0.000	0.000	0.000
<i>g_Butyricoccus</i>	0.509	0.822	0.005	0.010	0.000	0.000	0.319	0.000	0.000	0.000	0.000
<i>g_Butyricimonas</i>	0.219	0.000	0.000	0.000	0.001	0.940	0.007	0.000	0.000	0.077	0.274
<i>f_Marinilabiliaceae</i>	0.909	0.677	0.074	0.012	0.001	0.012	0.611	0.054	0.008	0.000	0.009
<i>f_Ruminococcaceae</i>	0.030	0.026	0.000	0.000	0.000	0.000	0.993	0.002	0.003	0.001	0.020
<i>g_Olsenella</i>	0.000	0.000	0.000	0.000	0.015	0.094	0.727	0.722	0.406	0.005	0.001
<i>f_Prevotellaceae</i>	0.205	0.160	0.159	0.066	0.037	0.011	0.955	0.942	0.642	0.496	0.287
<i>g_Clostridium</i>	0.545	0.044	0.434	0.499	0.338	0.126	0.175	0.879	0.945	0.833	0.493
<i>c_Clostridia</i>	0.002	0.000	0.000	0.000	0.000	0.000	0.678	0.156	0.014	0.018	0.001
<i>o_Erysipelotrichales</i>	0.006	0.000	0.003	0.013	0.016	0.019	0.037	0.882	0.887	0.803	0.791
<i>f_Lactobacillaceae</i>	0.014	0.000	0.000	0.000	0.000	0.000	0.071	0.005	0.231	0.037	0.022
<i>f_Oscillospiraceae</i>	0.037	0.000	0.000	0.000	0.000	0.000	0.197	0.134	0.076	0.026	0.108
<i>f_Tannerellaceae</i>	0.599	0.776	0.320	0.000	0.000	0.000	0.388	0.105	0.000	0.000	0.000
<i>f_Erysipelotrichaceae</i>	0.020	0.003	0.016	0.010	0.083	0.223	0.726	0.936	0.881	0.706	0.424
<i>g_Ligilactobacillus</i>	0.244	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.006	0.000	0.000
<i>g_Bifidobacterium</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.950	0.339	0.806	0.856	0.967
<i>g_Gallibacterium</i>	0.543	0.000	0.000	0.001	0.000	0.000	0.003	0.000	0.028	0.003	0.010
<i>g_Enterococcus</i>	0.042	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.002	0.000	0.000
<i>g_Lactobacillus</i>	0.415	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000
<i>g_Citrobacter</i>	0.498	0.010	0.001	0.001	0.001	0.001	0.148	0.018	0.019	0.028	0.019
<i>g_Limosilactobacillus</i>	0.141	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000
<i>g_Schaalia</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.291	0.294	0.979	0.302	0.292

<i>g_Turicibacter</i>	0.888	0.530	0.112	0.022	0.075	0.068	0.435	0.080	0.016	0.059	0.051
<b>Species</b>											
<i>s_Prevotella copri</i>	0.000	0.000	0.000	0.003	0.661	0.229	0.613	0.113	0.005	0.000	0.000
<i>g_Prevotella</i>	0.006	0.026	0.323	0.323	0.570	0.337	0.684	0.115	0.110	0.000	0.000
<i>s_Barnesiella</i>											
<i>intestinihominis</i>	0.371	0.041	0.191	0.010	0.509	0.976	0.364	0.797	0.141	0.911	0.356
<i>f_Prevotellaceae</i>	0.205	0.160	0.159	0.066	0.037	0.011	0.955	0.942	0.642	0.496	0.287
<i>s_Faecalibacterium</i>											
<i>prausnitzii</i>	0.110	0.292	0.907	0.322	0.035	0.007	0.627	0.148	0.006	0.000	0.000
<i>g_Faecalicatena</i>	0.000	0.000	0.001	0.337	0.047	0.176	0.894	0.418	0.001	0.000	0.000
<i>c_Clostridia</i>	0.002	0.000	0.000	0.000	0.000	0.000	0.678	0.156	0.014	0.018	0.001
<i>s_Bacteroides</i>											
<i>uniformis</i>	0.061	0.124	0.641	0.397	0.418	0.159	0.835	0.230	0.394	0.004	0.001
<i>s_Blautia wexlerae</i>	0.004	0.017	0.586	0.886	0.028	0.103	0.728	0.000	0.002	0.000	0.000
<i>f_Lactobacillaceae</i>	0.014	0.000	0.000	0.000	0.000	0.000	0.071	0.005	0.231	0.037	0.022
<i>s_Phocaeicola vulgatus</i>	0.260	0.526	0.755	0.872	0.077	0.045	0.742	0.131	0.169	0.002	0.003
<i>s_Gallibacterium</i>											
<i>anatis</i>	0.330	0.000	0.000	0.001	0.000	0.000	0.011	0.002	0.068	0.004	0.027
<i>f_Oscillospiraceae</i>	0.037	0.000	0.000	0.000	0.000	0.000	0.197	0.134	0.076	0.026	0.108
<i>f_Tannerellaceae</i>	0.599	0.776	0.320	0.000	0.000	0.000	0.388	0.105	0.000	0.000	0.000
<i>s_Ligilactobacillus</i>											
<i>salivarius</i>	0.245	0.000	0.000	0.000	0.000	0.000	0.029	0.000	0.006	0.000	0.000
<i>s_Bifidobacterium</i>											
<i>pseudolongum</i>	0.182	0.003	0.210	0.001	0.000	0.002	0.134	0.926	0.086	0.009	0.100
<i>g_Butyricoccus</i>	0.525	0.823	0.005	0.010	0.000	0.000	0.332	0.000	0.001	0.000	0.000
<i>s_Bifidobacterium</i>											
<i>longum</i>	0.101	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.010	0.000	0.000
<i>s_Lactobacillus</i>											
<i>amylovorus</i>	0.011	0.000	0.000	0.000	0.000	0.000	0.014	0.003	0.070	0.009	0.005
<i>s_Lactobacillus</i>											
<i>ingluviei</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.007	0.010	0.019	0.014	0.033
<i>g_Mediterraneibacter</i>	0.012	0.071	0.266	0.084	0.000	0.000	0.600	0.250	0.000	0.000	0.000
<i>s_Streptococcus</i>											
<i>gallolyticus</i>	0.138	0.138	0.000	0.000	0.000	0.000	1.000	0.010	0.042	0.008	0.020
<i>f_Rikenellaceae</i>	0.878	0.509	0.085	0.001	0.000	0.000	0.378	0.054	0.000	0.000	0.000
<i>f_Eubacteriaceae</i>	0.001	0.000	0.007	0.002	0.000	0.000	0.278	0.664	0.905	0.001	0.049
<i>f_Ruminococcaceae</i>	0.030	0.026	0.000	0.000	0.000	0.000	0.993	0.002	0.003	0.001	0.020
<i>o_Bacteroidales</i>	0.175	0.000	0.001	0.001	0.000	0.000	0.035	0.054	0.064	0.000	0.000
<i>f_Bacteroidaceae</i>	0.593	0.190	0.001	0.000	0.000	0.000	0.527	0.007	0.000	0.000	0.000
<i>o_Clostridiales</i>	0.047	0.004	0.000	0.000	0.000	0.000	0.547	0.000	0.005	0.000	0.001
<i>c_Bacteroidia</i>	0.078	0.020	0.023	0.000	0.000	0.000	0.656	0.662	0.028	0.000	0.000
<i>o_Sphingobacteriales</i>	0.828	0.328	0.930	0.133	0.000	0.000	0.547	0.952	0.276	0.000	0.000
<i>f_Muribaculaceae</i>	0.976	0.130	0.000	0.000	0.000	0.000	0.147	0.000	0.000	0.000	0.000
<i>s_Olsenella umbonata</i>	0.000	0.000	0.000	0.000	0.019	0.352	0.885	0.705	0.589	0.022	0.000
<i>o_Bacteroidales</i>											
<i>incertae sedis</i>	0.668	0.098	0.000	0.000	0.223	0.004	0.028	0.000	0.000	0.085	0.001
<i>f_Lachnospiraceae</i>	0.000	0.000	0.000	0.000	0.001	0.001	0.841	0.440	0.598	0.012	0.009
<i>f_Erysipelotrichaceae</i>	0.020	0.003	0.016	0.010	0.083	0.223	0.726	0.936	0.881	0.706	0.424
<i>g_Blautia</i>	0.000	0.000	0.000	0.000	0.155	0.554	0.533	0.739	0.353	0.000	0.000
<i>f_Atopobiaceae</i>	0.023	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	0.176	0.265
<i>f_Spirochaetaceae</i>	0.729	0.948	0.596	0.477	0.212	0.027	0.804	0.883	0.736	0.088	0.010
<i>f_Marinilabiliaceae</i>	0.909	0.677	0.074	0.012	0.001	0.012	0.611	0.054	0.008	0.000	0.009
<i>g_Frisingicoccus</i>	0.000	0.000	0.000	0.000	0.002	0.187	0.565	0.686	0.027	0.002	0.000

<i>c_Cytophagia</i>	0.883	0.454	0.012	0.000	0.000	0.004	0.372	0.009	0.000	0.000	0.002
<i>s_Butyricimonas virosa</i>	0.260	0.000	0.000	0.000	0.001	0.968	0.004	0.000	0.000	0.051	0.275
<i>s_Phocaeicola coprocola</i>	0.884	0.725	0.038	0.000	0.006	0.718	0.526	0.016	0.000	0.003	0.523

<sup>1</sup> Taxon	5lateC vs. lateC Dunn's test									
	d42L - d70L	d42L - d98L	d42L - d112L	d42L - d140L	d70L - d98L	d70L - d112L	d70L - d140L	d112L - d98L	d98L - d140L	d112L - d140L
<b>Phyla</b>										
<i>Firmicutes</i>	0.980	0.219	0.001	0.000	0.240	0.001	0.000	0.061	0.002	0.260
<i>Bacteroidetes</i>	0.731	0.223	0.002	0.000	0.424	0.009	0.000	0.084	0.006	0.334
<i>Actinobacteria</i>	0.594	0.945	0.073	0.079	0.535	0.289	0.311	0.067	0.076	0.980
<i>Proteobacteria</i>	0.796	0.833	0.495	0.624	0.626	0.280	0.438	0.653	0.853	0.822
<i>Spirochaetes</i>	0.899	0.947	0.079	0.028	0.782	0.046	0.018	0.129	0.044	0.757
<b>Genera</b>										
<i>o_Bacteroidales</i>	0.922	0.857	0.004	0.000	0.920	0.003	0.000	0.002	0.000	0.269
<i>o_Bacteroidales incertae sedis</i>	0.000	0.000	0.707	0.311	0.859	0.000	0.001	0.000	0.003	0.144
<i>g_Blautia</i>	0.537	0.093	0.000	0.000	0.391	0.000	0.000	0.003	0.000	0.550
<i>g_Prevotella</i>	0.400	0.066	0.000	0.000	0.498	0.000	0.000	0.001	0.000	0.661
<i>g_Mediterraneibacter</i>	0.460	0.000	0.000	0.000	0.011	0.000	0.000	0.022	0.015	0.894
<i>g_Anaerostipes</i>	0.114	0.000	0.000	0.000	0.139	0.001	0.000	0.115	0.079	0.899
<i>g_Parabacteroides</i>	0.981	0.379	0.001	0.000	0.419	0.001	0.000	0.021	0.000	0.014
<i>g_Faecalicatena</i>	0.311	0.000	0.000	0.000	0.022	0.000	0.000	0.002	0.012	0.633
<i>g_Streptococcus</i>	0.050	0.011	0.000	0.000	0.768	0.000	0.001	0.002	0.004	0.964
<i>g_Frisingicoccus</i>	0.273	0.003	0.000	0.000	0.089	0.010	0.000	0.465	0.016	0.107
<i>g_Collinsella</i>	0.297	0.006	0.000	0.000	0.122	0.000	0.000	0.024	0.026	0.996
<i>o_Sphingobacteriales</i>	0.454	0.708	0.000	0.000	0.208	0.000	0.000	0.001	0.000	0.116
<i>f_Lachnospiraceae</i>	0.602	0.764	0.004	0.003	0.836	0.001	0.001	0.001	0.001	0.920
<i>g_Bacteroides</i>	0.197	0.138	0.000	0.000	0.875	0.002	0.000	0.003	0.000	0.126
<i>f_Bacteroidaceae</i>	0.047	0.002	0.000	0.000	0.365	0.002	0.000	0.043	0.005	0.508
<i>f_Rikenellaceae</i>	0.324	0.008	0.000	0.000	0.144	0.000	0.000	0.034	0.001	0.296
<i>g_Faecalibacterium</i>	0.383	0.029	0.002	0.000	0.274	0.048	0.009	0.479	0.179	0.587
<i>f_Eubacteriaceae</i>	0.100	0.209	0.026	0.431	0.751	0.000	0.011	0.000	0.030	0.211
<i>c_Cytophagia</i>	0.092	0.000	0.000	0.039	0.017	0.004	0.739	0.698	0.040	0.012
<i>c_Bacteroidia</i>	0.995	0.083	0.001	0.000	0.086	0.002	0.000	0.191	0.028	0.414
<i>g_Phocaeicola</i>	0.968	0.800	0.442	0.031	0.833	0.474	0.039	0.682	0.085	0.259
<i>f_Atopobiaceae</i>	0.098	0.078	0.589	0.455	0.953	0.019	0.011	0.014	0.007	0.836
<i>f_Muribaculaceae</i>	0.015	0.000	0.000	0.000	0.256	0.054	0.184	0.585	0.941	0.698
<i>g_Alistipes</i>	0.616	0.140	0.001	0.000	0.374	0.009	0.000	0.121	0.000	0.002
<i>o_Clostridiales</i>	0.006	0.038	0.000	0.006	0.605	0.493	0.967	0.169	0.628	0.458
<i>f_Spirochaetaceae</i>	0.678	0.508	0.169	0.022	0.889	0.047	0.005	0.029	0.004	0.479
<i>g_Barnesiella</i>	0.660	0.663	0.244	0.039	0.312	0.626	0.174	0.071	0.009	0.483
<i>g_Gemmiger</i>	0.000	0.000	0.000	0.000	0.663	0.203	0.201	0.556	0.554	1.000
<i>g_Butyricicoccus</i>	0.014	0.024	0.000	0.000	0.907	0.149	0.152	0.090	0.093	1.000
<i>g_Butyricimonas</i>	0.334	0.288	0.441	0.000	0.946	0.067	0.000	0.049	0.000	0.002
<i>f_Marinilabiliaceae</i>	0.289	0.058	0.002	0.059	0.675	0.072	0.683	0.312	0.997	0.308

<i>f_Ruminococcaceae</i>	0.002	0.003	0.001	0.019	0.913	0.862	0.550	0.766	0.623	0.419
<i>g_Olsenella</i>	0.978	0.675	0.001	0.000	0.690	0.001	0.000	0.000	0.000	0.578
<i>f_Prevotellaceae</i>	0.964	0.691	0.528	0.309	0.713	0.564	0.328	0.887	0.580	0.728
<i>g_Clostridium</i>	0.249	0.189	0.297	0.631	0.903	0.902	0.622	0.858	0.540	0.706
<i>c_Clostridia</i>	0.353	0.045	0.056	0.003	0.388	0.425	0.073	0.926	0.413	0.368
<i>o_Erysipelotrichales</i>	0.084	0.014	0.010	0.010	0.671	0.648	0.651	0.982	0.983	0.988
<i>f_Lactobacillaceae</i>	0.440	0.779	0.883	0.774	0.185	0.604	0.767	0.660	0.480	0.880
<i>f_Oscillospiraceae</i>	0.878	0.763	0.533	0.817	0.895	0.718	0.980	0.793	0.939	0.740
<i>f_Tannerellaceae</i>	0.507	0.000	0.000	0.000	0.002	0.000	0.000	0.163	0.586	0.484
<i>f_Erysipelotrichaceae</i>	0.759	0.806	0.379	0.165	0.918	0.652	0.376	0.562	0.316	0.728
<i>g_Ligilactobacillus</i>	0.248	0.745	0.143	0.212	0.550	0.893	0.957	0.370	0.497	0.906
<i>g_Bifidobacterium</i>	0.365	0.708	0.820	0.962	0.119	0.165	0.337	0.920	0.797	0.847
<i>g_Gallibacterium</i>	0.726	0.679	0.981	0.844	0.346	0.736	0.547	0.692	0.847	0.856
<i>g_Enterococcus</i>	0.230	0.798	0.464	0.390	0.599	0.890	0.900	0.882	0.856	0.985
<i>g_Lactobacillus</i>	0.617	0.753	0.767	0.656	0.271	0.855	0.928	0.438	0.305	0.888
<i>g_Citrobacter</i>	0.638	0.622	0.774	0.655	0.986	0.944	0.986	0.937	0.982	0.959
<i>g_Limosilactobacillus</i>	0.311	0.397	0.342	0.481	0.945	0.957	0.926	0.965	0.960	0.957
<i>g_Schaalia</i>	1.000	0.861	1.000	1.000	0.810	1.000	1.000	0.881	0.834	1.000
<i>g_Turicibacter</i>	0.432	0.077	0.327	0.300	0.429	0.902	0.877	0.534	0.591	0.955

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**Species**


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<i>s_Prevotella copri</i>	0.365	0.032	0.000	0.000	0.376	0.000	0.000	0.000	0.000	0.519
<i>g_Prevotella</i>	0.297	0.291	0.002	0.001	1.000	0.085	0.036	0.083	0.034	0.808
<i>s_Barnesiella</i>										
<i>intestinihominis</i>	0.660	0.663	0.244	0.039	0.312	0.626	0.174	0.071	0.009	0.483
<i>f_Prevotellaceae</i>	0.964	0.691	0.528	0.309	0.713	0.564	0.328	0.887	0.580	0.728
<i>s_Faecalibacterium</i>										
<i>prausnitzii</i>	0.354	0.028	0.001	0.000	0.275	0.028	0.005	0.356	0.122	0.595
<i>g_Faecalicatena</i>	0.309	0.000	0.000	0.000	0.022	0.000	0.000	0.002	0.012	0.633
<i>c_Clostridia</i>	0.353	0.045	0.056	0.003	0.388	0.425	0.073	0.926	0.413	0.368
<i>s_Bacteroides</i>										
<i>uniformis</i>	0.391	0.619	0.010	0.002	0.833	0.147	0.042	0.066	0.015	0.666
<i>s_Blautia wexlerae</i>	0.001	0.011	0.000	0.000	0.667	0.205	0.434	0.042	0.138	0.749
<i>f_Lactobacillaceae</i>	0.440	0.779	0.883	0.774	0.185	0.604	0.767	0.660	0.480	0.880
<i>s_Phocaecicola vulgatus</i>	0.258	0.340	0.008	0.004	0.904	0.188	0.131	0.135	0.086	0.884
<i>s_Gallibacterium</i>										
<i>anatis</i>	0.698	0.674	0.846	0.861	0.324	0.863	0.528	0.493	0.846	0.704
<i>f_Oscillospiraceae</i>	0.878	0.763	0.533	0.817	0.895	0.718	0.980	0.793	0.939	0.740
<i>f_Tannerellaceae</i>	0.507	0.000	0.000	0.000	0.002	0.000	0.000	0.163	0.586	0.484
<i>s_Ligilactobacillus</i>										
<i>salivarius</i>	0.248	0.745	0.143	0.212	0.550	0.893	0.957	0.370	0.497	0.906
<i>s_Bifidobacterium</i>										
<i>pseudolongum</i>	0.106	0.864	0.356	0.915	0.070	0.006	0.083	0.476	0.954	0.452
<i>g_Butyricicoccus</i>	0.014	0.024	0.000	0.000	0.903	0.148	0.151	0.089	0.092	1.000
<i>s_Bifidobacterium</i>										
<i>longum</i>	0.591	0.627	0.157	0.259	0.208	0.588	0.718	0.032	0.066	0.856
<i>s_Lactobacillus</i>										
<i>amylovorus</i>	0.778	0.795	0.921	0.825	0.461	0.821	0.918	0.703	0.557	0.901
<i>s_Lactobacillus</i>										
<i>ingluviei</i>	0.975	0.971	0.958	0.881	0.942	0.955	0.925	0.963	0.946	0.951
<i>g_Mediterraneibacter</i>	0.650	0.000	0.000	0.000	0.002	0.000	0.000	0.078	0.055	0.883
<i>s_Streptococcus</i>										
<i>gallolyticus</i>	0.008	0.035	0.007	0.016	0.669	1.000	0.848	0.665	0.855	0.852
<i>f_Rikenellaceae</i>	0.324	0.008	0.000	0.000	0.144	0.000	0.000	0.034	0.001	0.296

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<i>f_Eubacteriaceae</i>	0.100	0.209	0.026	0.431	0.751	0.000	0.011	0.000	0.030	0.211
<i>f_Ruminococcaceae</i>	0.002	0.003	0.001	0.019	0.913	0.862	0.550	0.766	0.623	0.419
<i>o_Bacteroidales</i>	0.922	0.857	0.004	0.000	0.920	0.003	0.000	0.002	0.000	0.269
<i>f_Bacteroidaceae</i>	0.047	0.002	0.000	0.000	0.365	0.002	0.000	0.043	0.005	0.508
<i>o_Clostridiales</i>	0.006	0.038	0.000	0.006	0.605	0.493	0.967	0.169	0.628	0.458
<i>c_Bacteroidia</i>	0.995	0.083	0.001	0.000	0.086	0.002	0.000	0.191	0.028	0.414
<i>o_Sphingobacteriales</i>	0.454	0.708	0.000	0.000	0.208	0.000	0.000	0.001	0.000	0.116
<i>f_Muribaculaceae</i>	0.015	0.000	0.000	0.000	0.256	0.054	0.184	0.585	0.941	0.698
<i>s_Olsenella umbonata</i>	0.864	0.751	0.009	0.000	0.935	0.005	0.000	0.003	0.000	0.233
<i>o_Bacteroidales incertae sedis</i>	0.000	0.000	0.707	0.311	0.859	0.000	0.001	0.000	0.003	0.144
<i>f_Lachnospiraceae</i>	0.602	0.764	0.004	0.003	0.836	0.001	0.001	0.001	0.001	0.920
<i>f_Erysipelotrichaceae</i>	0.759	0.806	0.379	0.165	0.918	0.652	0.376	0.562	0.316	0.728
<i>g_Blautia</i>	0.737	0.091	0.000	0.000	0.204	0.000	0.000	0.007	0.000	0.437
<i>f_Atopobiaceae</i>	0.098	0.078	0.589	0.455	0.953	0.019	0.011	0.014	0.007	0.836
<i>f_Spirochaetaceae</i>	0.678	0.508	0.169	0.022	0.889	0.047	0.005	0.029	0.004	0.479
<i>f_Marinilabiliaceae</i>	0.289	0.058	0.002	0.059	0.675	0.072	0.683	0.312	0.997	0.308
<i>g_Frisingicoccus</i>	0.273	0.003	0.000	0.000	0.089	0.010	0.000	0.465	0.016	0.107
<i>c_Cytophagia</i>	0.092	0.000	0.000	0.039	0.017	0.004	0.739	0.698	0.040	0.012
<i>s_Butyricimonas virosa</i>	0.325	0.264	0.448	0.000	0.945	0.066	0.000	0.044	0.000	0.001
<i>s_Phocaeicola coprocola</i>	0.220	0.004	0.047	0.999	0.301	0.785	0.223	0.620	0.004	0.050

<sup>1</sup>Phyla and their corresponding genera and species are indicated and only those taxa with maximum relative abundance > 1% in atleast 1 group are shown

<sup>2</sup>earlyC group weaned at 7 weeks of age (experimental days 28–42)

<sup>3</sup>lateC group weaned at 17 weeks of age (experimental days 98–112)

<sup>4</sup>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

<sup>5</sup>Pairwise comparisons were done using Dunn's test and  $p \leq 0.05$  indicates significant effect

**Table S2.** Average relative concentrations ( $\mu\text{mol/L}$ ) of plasma metabolites in early- and late-weaned calves.

<sup>1</sup> Metabolites	<sup>2</sup> earlyC							<sup>3</sup> lateC							<sup>4</sup> p-value (FDR-adjusted)
	d1E	d28E	d42E	d70E	d98E	d112E	d140E	d1L	d28L	d42L	d70L	d98L	d112L	d140L	
Carnitine	10.15	14.81	10.82	8.38	8.84	8.84	8.38	9.49	15.87	14.77	12.63	11.93	9.85	10.39	< 0.001
Propionyl carnitine	0.16	0.25	0.60	0.79	0.48	0.40	0.32	0.16	0.30	0.38	0.57	0.67	0.56	0.38	< 0.001
Propenoyl carnitine	0.02	0.04	0.04	0.04	0.04	0.04	0.04	0.02	0.04	0.04	0.04	0.04	0.04	0.04	< 0.001
Valeryl carnitine	0.07	0.10	0.14	0.22	0.21	0.19	0.16	0.08	0.12	0.14	0.15	0.14	0.22	0.17	< 0.001
Nonacylcarnitine	0.04	0.05	0.04	0.05	0.05	0.05	0.05	0.04	0.05	0.04	0.05	0.05	0.05	0.05	< 0.001
Hexadecanoyl carnitine	0.08	0.09	0.07	0.06	0.06	0.06	0.06	0.08	0.09	0.09	0.07	0.06	0.06	0.06	< 0.001
Hydroxyhexa decadienyl carnitine	0.04	0.09	0.09	0.09	0.09	0.09	0.09	0.04	0.08	0.08	0.08	0.08	0.08	0.09	< 0.001
Octadecenoyl carnitine	0.07	0.05	0.06	0.05	0.04	0.05	0.04	0.07	0.05	0.07	0.05	0.05	0.04	0.05	< 0.001
Arginine	203.00	237.83	192.21	143.66	151.33	151.04	144.89	222.71	237.62	216.30	182.43	165.57	152.48	141.95	< 0.001
Glutamate	153.75	103.55	92.25	98.71	108.06	97.13	89.77	156.99	89.14	85.19	90.95	100.72	104.12	91.25	< 0.001
Glycine	626.50	441.42	424.00	422.36	447.78	445.26	437.93	611.82	413.00	442.26	408.14	376.90	344.26	385.95	< 0.001
Histidine	101.35	116.58	91.39	66.53	66.80	72.86	78.48	98.47	123.80	121.90	98.60	95.55	84.50	77.50	< 0.001
Lysine	282.95	266.46	184.50	151.34	156.04	158.58	167.59	260.91	275.81	229.43	199.48	183.38	184.26	188.32	< 0.001
Methionine	50.06	48.62	29.18	24.43	30.65	31.22	33.80	44.14	46.46	41.03	35.83	33.22	31.67	33.05	< 0.001
Ornithine	98.14	93.25	135.26	72.18	74.74	78.65	78.82	92.47	90.53	102.20	96.49	107.20	85.06	78.37	< 0.001
Phenylalanine	105.70	93.82	89.21	71.54	72.77	68.94	68.58	103.97	94.65	84.07	77.56	79.28	80.83	75.03	< 0.001
Proline	200.35	152.23	91.11	73.12	78.72	80.95	83.86	193.64	149.21	120.14	93.96	87.02	86.44	76.60	< 0.001
Serine	202.05	142.67	107.78	81.35	85.74	87.46	90.82	196.05	142.67	115.75	99.97	98.40	79.21	88.30	< 0.001
Threonine	156.27	125.34	90.88	71.30	93.26	95.08	99.81	163.48	118.55	110.18	108.16	111.85	98.54	89.99	< 0.001
Tyrosine	86.85	93.35	77.57	56.51	63.63	62.67	67.40	89.15	93.96	81.99	75.93	76.98	73.06	61.57	< 0.001
Valine	276.35	248.25	333.08	278.44	286.22	285.85	281.59	270.64	255.90	283.65	321.29	341.52	322.83	314.05	< 0.001
Acetyl ornithine	1.15	0.63	1.48	4.66	6.56	6.85	7.21	1.36	0.65	1.04	1.75	1.72	4.56	7.03	< 0.001
Asymmetric dimethylarginine	2.09	1.59	1.51	1.49	1.51	1.51	1.52	1.99	1.60	1.53	1.54	1.34	1.40	1.48	< 0.001
Carnosine	9.83	10.02	11.62	10.82	12.47	13.95	15.04	10.09	9.98	11.06	13.44	14.62	15.21	14.74	< 0.001
Creatinine	81.70	67.25	65.93	63.75	55.76	57.86	62.23	78.00	66.70	60.58	57.62	54.43	64.99	64.57	< 0.001
Dihydroxy phenylalanine	0.00	0.12	0.11	0.12	0.10	0.10	0.11	0.00	0.09	0.09	0.09	0.10	0.09	0.10	< 0.001
Dopamine	0.00	2.00	2.37	2.69	2.76	2.92	2.51	0.00	2.31	2.11	2.97	2.54	3.10	2.78	< 0.001
Histamine	0.00	0.21	0.22	0.21	0.19	0.21	0.21	0.00	0.25	0.28	0.26	0.28	0.27	0.29	< 0.001
Sarcosine	7.58	6.53	2.62	7.50	6.79	3.93	1.07	8.09	7.57	5.44	3.30	2.30	1.94	1.33	< 0.001
Symmetric dimethylarginine	1.05	0.67	0.62	0.63	0.63	0.65	0.64	0.99	0.66	0.65	0.66	0.58	0.60	0.63	< 0.001
Spermine	0.00	0.79	1.63	1.11	1.47	1.82	0.91	0.03	1.52	1.09	0.88	1.13	1.45	1.33	< 0.001
trans-4-hydroxyproline	88.72	73.10	47.50	38.44	46.28	49.43	51.19	91.78	65.39	60.70	52.96	44.95	45.25	41.99	< 0.001
Taurine	116.14	50.94	51.58	37.56	26.71	30.79	34.63	84.45	50.26	57.43	45.67	48.25	42.98	37.95	< 0.001
SM (OH) C14:1	2.92	3.64	3.94	5.15	6.56	6.71	7.72	3.36	3.64	4.08	5.42	6.23	6.68	6.77	< 0.001
SM (OH) C16:1	1.14	1.34	2.26	4.62	5.84	5.82	6.45	1.30	1.36	1.88	3.37	4.47	5.51	5.81	< 0.001

SM (OH) C22:1	4.63	9.79	10.76	6.87	7.79	7.72	8.19	5.19	8.93	10.03	10.02	10.40	10.44	8.60	< 0.001
SM (OH) C22:2	2.44	3.30	3.71	3.01	3.48	3.88	4.45	2.76	3.00	3.44	3.83	4.51	4.87	4.15	< 0.001
SM (OH) C24:1	0.47	0.62	0.77	0.79	0.95	0.92	0.91	0.52	0.60	0.66	0.82	0.96	1.07	0.95	< 0.001
SM C18:1	2.82	3.99	4.06	3.34	4.06	4.26	4.84	3.30	3.61	3.84	4.23	4.99	5.13	4.19	< 0.001
SM C22:3	0.05	0.17	0.17	0.01	0.06	0.07	0.02	0.05	0.25	0.22	0.11	0.07	0.07	0.02	< 0.001
SM C24:1	12.78	14.19	13.43	6.18	5.85	6.43	6.67	14.29	13.48	12.34	9.79	9.66	9.53	6.99	< 0.001
SM C26:0	0.10	0.09	0.13	0.17	0.23	0.23	0.22	0.10	0.10	0.11	0.16	0.18	0.23	0.23	< 0.001

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**<sup>1</sup>Metabolites**

**<sup>5</sup>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-28E; 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-42L; 70L-42L; 98L-42L; 70L-70E; 98E-70E
Propenylcarnitine	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
Valeryl carnitine	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-140L; 42E-140L; 42L-140L; 70E-140L; 70L-140L; 98E-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; 42E-42E; 98E-42E; 98E-42L
Nonacylcarnitine	1E-112E; 1L-112E; 1E-140E; 1L-140E; 70E-1E; 98E-1E; 98E-1L
Hexadecanoyl carnitine	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
Hydroxyhexa decadienylcarnitine	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
Octadecenoyl carnitine	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; 70E-42L; 98E-42L; 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-42L; 98E-42L; 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; 42L-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
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; 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
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-112L; 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
Acetylmornithine	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-70L; 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; 98L-28E; 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; 42E-140L; 42L-140L; 70L-140L; 98L-140L; 70E-1E; 98E-1E; 98L-1E; 70L-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
Dihydroxy phenylalanine	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;

Taurine	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-42E; 70E-42L; 98E-42L; 98L-42L; 70L-70E; 112L-112E; 1E-112E; 1L-112E; 28E-112E; 28L-112E; 42E-112E; 42L-112E; 70L-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; 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; 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-70L; 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; 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
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; 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; 98E-70L; 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-70L; 98L-98E
SM (OH) C22:2	1E-112E; 1L-112E; 28E-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; 42L-140E; 70E-140E; 98E-140E; 1E-140L; 1L-140L; 28E-140L; 28L-140L; 42E-140L; 42L-140L; 70E-140L; 70L-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; 42E-1E; 42L-1E; 70E-1E; 70L-1E; 98E-1E; 98L-1E; 42E-1L; 70E-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-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; 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; 70L-42L; 98E-42L; 98L-42L; 70L-70E; 98E-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; 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; 98L-140L; 70E-1E; 98E-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

<sup>1</sup>Metabolites list and their corresponding class is indicated and only those metabolites with VIP >

1, FDR < 0.001, ANOVA are shown

<sup>2</sup>earlyC group weaned at 7 weeks of age (experimental days 28–42)

<sup>3</sup>lateC group weaned at 17 weeks of age (experimental days 98–112)

<sup>4</sup>P-values were obtained using one-way Analysis of Variance (ANOVA) and FDR-adjusted  $p \leq 0.05$  is considered significant

<sup>5</sup>Pairwise comparisons were done using Tukey's HSD test

**Table S3.** Spearman's rank correlations between morphometric variables of calves, differential faecal microbial genera and plasma metabolites of weaning groups.

		d70				
		LWG	LW (kg)	Wither height (cm)	Hip height (cm)	
Faecal bacterial genera	<i>f_Rikenellaceae</i>	-0.52	---	---	---	
	<i>g_Parabacteroides</i>	0.68	---	---	---	
	<i>g_Mediterraneibacter</i>	0.59	---	0.55	0.63	
	<i>g_Prevotella</i>	0.54	---	---	0.51	
	<i>g_Blautia</i>	0.60	---	0.50	0.51	
	<i>o_Bacteroidales incertae sedis</i>	0.60	0.55	0.61	0.68	
	<i>f_Lachnospiraceae</i>	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	---	---	---	
	Acetylmethionine	-0.52	---	---	---	
	trans-4-hydroxyproline	---	0.51	---	---	
	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	LW (kg)	Heart girth (cm)	Hip height (cm)	Body length (cm)
Faecal bacterial	<i>f_Rikenellaceae</i>	---	-0.59	-0.54	---	---
	<i>f_Atopobiaceae</i>	---	0.53	---	---	---
	<i>g_Olsenella</i>	0.55	---	---	---	---

	<i>o_Bacteroidales incertae sedis</i>	---	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	---	---	---
	Acetyloronithine	---	-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	---	---	---

LWG = Live weight gain

LW = Live weight

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

## **2.4 Further Publications**

Title: Evolution of rumen and oral microbiota in calves is influenced by age and time of weaning

Nida Amin, Sarah Schwarzkopf, Asako Kinoshita, Johanna Tröscher-Mußotter, Sven Dänicke, Amélia Camarinha-Silva, Korinna Huber, Jana Frahm & Jana Seifert

Published in Animal Microbiome Volume 3, Article number: 31 (2021)

### **3. General Discussion**

This thesis aimed at gaining a comprehensive understanding of the influence of two different weaning ages and mothers' parity on development of various organs and metabolic features. As seen in the data presented in the included manuscripts, many metabolites in blood and urine changed abruptly when weaning is performed at the age of 7 weeks. This might put a lot of strain on organs and their metabolic functions. Many of these metabolite concentrations were still changing weeks after weaning was completed, which indicated that metabolic adaptations were not done quickly and rather needed time to develop. Delaying weaning to 17 weeks of age provided calves with this time and led to smoother changes in many analyzed metabolite concentrations in blood and urine. Therefore, it possibly reduced stress associated with weaning (Weary et al., 2008). Thereby, delaying weaning supported animal welfare, which becomes increasingly important to consumers (Alonso et al., 2020).

#### **3.1 Limitations of the Study**

Studying weaning at different ages is a challenging research approach. Many management decisions influence the weaning process and its effects on the individual calf. On farms, weaning is often performed alongside other potential stressors like changing the barn or social companions, which can influence the process of weaning as well (Weary et al., 2008). In the current study, weaning was combined with transportation into another barn. Therefore, the composition of the animal group often changed over time. The change of social companion as well as change of the living environment could have influenced individual calf's development and welfare. However, both could not have been avoided due to technical and organizational reasons on the farm.

Generally, concentrations of metabolites are influenced by many external (nutrition, environment) and internal (genetic) factors, which should always be considered when interpreting these. Furthermore, analysis of blood, urine and feces can give a momentary insight, but neither can it be evaluated where the metabolites were derived from, nor how concentrations might change shortly before or after sampling. Different possible sources should be considered. Concentrations in blood might not completely reflect the intracellular metabolism as excretion and removal by various tissues are often unknown. Therefore, no direct information about organs and their metabolic functions can be obtained. It is only possible to hypothesize about possible sources and metabolic uses of each metabolite. The feedstuff itself can be a source of some metabolites, such as non-esterified fatty acids (NEFA), cholesterol (**Manuscript 1**) and highly digestible proteins in MR (**Manuscript 3**). Feedstuff composition can influence many of the analyzed indicators, despite the calf's age and maturation, especially bacterial composition in the gut (**Manuscript 3**) and parameters of rumen function (**Manuscript 2**). It is challenging to separate the influence of feedstuff (external) and maturation (internal) on different metabolite concentrations. Therefore, the influence of external factors should always be kept in mind when interpreting indicators for metabolic functions. This is especially important when not much research is done about a given metabolite, which is the case for many metabolites analyzed in the metabolomics kit used in the present study (**Manuscript 3**).

Time of sampling might influence the concentration of analyzed metabolites. Some blood parameters like glucose, NEFA, cholesterol and urea concentrations are influenced by circadian rhythms (Shehab-El-Deen et al., 2010). To minimize this influence, blood samples were always collected between eight and ten o'clock am. As mentioned before (**Manuscript 1**), sampling relative to feed intake could have influenced blood metabolite concentrations as well. This should be controlled in future studies.

### **3.2 Indicators of Liver Metabolic Maturation**

As discussed before in the related manuscripts, some of the measured metabolites indicated an underdeveloped capacity of liver functions in early-weaned calves, at least until 5 months of age. Early weaning decreased serum glucose concentration from 5.5 mmol/L to 4.8 mmol/L on day 42 and further to 4.1 mmol/L on day 70. These post-weaning concentrations were up to standard values of healthy non-lactating cows, which are 3.4 – 4.2 mmol/L (Bertoni and Trevisi, 2013). On experimental days 98, 112 and 140 serum glucose concentrations increased again to 4.7 – 4.9 mmol/L. Late-weaned calves did not experience such a steep decrease in glucose concentrations. Their serum glucose concentrations decreased smoothly from 5.2 – 5.8 mmol/L before weaning to 4.8 mmol/L at the end of the experiment. Therefore, both groups showed the same serum glucose concentrations at day 140 when weaning was completed in both. Similarly, in female Holstein calves weaned with 4 or 8 weeks of age blood glucose concentrations decreased with weaning and were around 4.12 mmol/L at 9 – 11 weeks of age. Afterwards, glucose concentration increased again to 4.72 mmol/L at 13 weeks of age (Quigley et al., 1991). Similar changes in plasma glucose concentrations were found in male calves weaned with 6 weeks of age. They had high glucose concentration before weaning (5.05 – 5.54 mmol/L) as well, which decreased to 3.49 (2 weeks after weaning) and 4.18 mmol/L (7 weeks after weaning). At 19 weeks of age, their plasma glucose concentration increased to 4.90 mmol/L (Haga et al., 2008), which is similar to the concentration the calves in the current study had at 21 weeks of age. At 16 weeks of age, female Holstein calves had blood glucose concentrations between 5.00 and 5.5 mmol/L independent of being weaned at 6 weeks of age or still receiving MR (Quigley and Bernard, 1992). Overall, early weaning seemed to reduce blood glucose concentrations, which increased again in the weeks after weaning. Contrary, weaning with greater age and feeding higher amount of MR led to higher plasma glucose

concentrations (Ballou, 2012; Kamiya et al., 2009; Kesser et al., 2017). The increase of blood glucose concentrations in the weeks after early weaning supports the hypothesis that these higher concentrations were of importance for growing calves. They might have had a need for higher blood glucose concentrations than adult cows, which could not have been achieved by the liver after early weaning. A sufficient gluconeogenesis might have been reached around the age of 17 weeks only. As propionate is the main substrate for gluconeogenesis (Aschenbach et al., 2010) another reason for the lower glucose concentrations after early weaning might be an inability of production or absorption of propionate in these calves. This will be discussed in detail in Chapter 3.2.1.1.

Low glucose concentrations resulted in low insulin concentrations in early-weaned calves as well, which indicated a catabolic state as it is an important anabolic hormone. This catabolic state, in which body proteins (proteolysis) and lipids (lipolysis) were used to gain energy, was pointed out by other indicators as well. BHB concentrations increased after early weaning, which might have originated from proteolysis and lipolysis and reduced cholesterol synthesis, as the same precursor metabolite was used (Cerqueira et al., 2016). Low insulin concentrations might have contributed to the lower cholesterol concentrations in early-weaned calves as well, as it activates an enzyme which is important for cholesterol biosynthesis (Harris et al., 2000). Increased concentrations of creatinine in urine during early weaning could have resulted from elevated proteolysis, whereas creatinine concentrations did not change significantly during the late weaning period. Overall, the important functions of the liver regarding energy metabolism might not be sufficient for early-weaned calves. Therefore, the liver appeared to be an important organ that needed time and adequate nutrient supply to develop.

Feeding greater amounts of MR and prolonging weaning age might support liver maturation in calves. With increased availability of nutrients from MR, liver weight relative to BW increased in Holstein bull calves (Bartlett et al., 2014; Diaz et al., 2001; Kamiya et al., 2009). When

slaughtered at 65 kg BW, calves that received the amount of MR of 1% of BW had a liver weight of 2.2 % of empty BW, whereas calves that received the amount of MR of 4% of BW had a liver weight of 3.1 % of empty BW (Diaz et al., 2001). Ad libitum amount of MR during the first 5 weeks of life increased the expression of hepatic mRNA encoding the cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme of gluconeogenesis, compared to calves which received 6 L MR per day (Schäff et al., 2016). A greater concentrate intake of 2 kg per day compared to 1 kg per day did not influence the expression of the liver gluconeogenic enzymes, cytosolic and mitochondrial PEPCK and pyruvate carboxylase, in calves fed restricted amounts of MR (2 – 6 L MR/day; Lohakare et al., 2012). Overall, this indicated the limited possibility to force the shift to a glucogenic metabolism through solid feedstuff and the importance for MR nutrients for liver development.

As nutritional experiences in early life can be responsible for later diseases (Hales and Barker, 2001; Waterland and Garza, 1999), low blood glucose concentrations in early-weaned calves might led to an impaired liver metabolism in adult life. As glucose from hepatic gluconeogenesis is the main energy source for many cells and tissues like the brain, erythrocytes or ovaries (Aschenbach et al., 2010), this might reduce performance in dairy cows. This might be the explanation for the association between low glucose concentrations at 180 days of age with an increased age at first breeding in nulliparous Holstein-Friesian heifers (Brickell et al., 2009).

### **3.2.1 Further Insight in Liver Metabolism through Metabolomics**

As only a small fraction of the metabolism is reflected in the conventionally used indicators, a greater overview of potentially involved metabolites is needed to gain better understanding of the metabolic development of calves. The metabolomics approach used in the present study quantifies many metabolites at once belonging to 5 compound classes: acylcarnitines (40),

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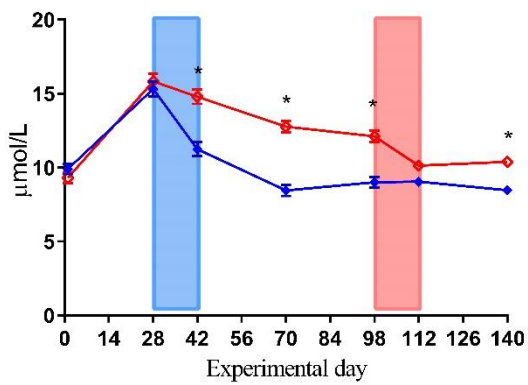
amino acids (19), glycerophospho- and sphingolipids (76 phosphatidylcholines, 14 lysophosphatidylcholines, 15 sphingomyelins), biogenic amines (19) and monosaccharides (1) (AbsoluteIDQ p180 Kit, Biocrates Life Science AG, Innsbruck, Austria). Therefore, it allows insight in a variety of metabolic pathways associated with energy metabolism, amino acid metabolism, lipid metabolism and pro-inflammatory signaling. Metabolites are products of complex interactions between different molecular levels like genome, transcriptome and proteome (Goldansaz et al., 2017; Schalch Junior et al., 2022) and derived from many possible sources like feedstuff or various metabolic pathways (Courant et al., 2014; Schalch Junior et al., 2022). As discussed before, metabolite concentrations in blood can give no complete insight into the intracellular metabolism. Therefore, they can only be used to postulate a hypothesis about organ functions. The metabolomics approach allows to detect many metabolites at once which might be regulated by mothers' parity or weaning age. Therefore, it supports generating new hypotheses for biomarkers and pathways involved in growing calves' metabolism. Not much research is done regarding this subset of metabolites, especially in calves.

It is important to note that most of the measured metabolites in this metabolomics approach were found in milk, especially amino acids (Wu et al., 2011; Xu et al., 2021). Therefore, they might be found in MR consisting mostly of milk components as well. As discussed before, feedstuff can be an external source of metabolites (Courant et al., 2014) and the targeted metabolomics approach is unable to distinguish between sources of analysed metabolites (Ramsay et al., 2012). Therefore, the significantly higher concentration of many metabolites in late-weaned calves might result directly from their MR intake. However, their availability might promote calves' development, growth and health as discussed later.

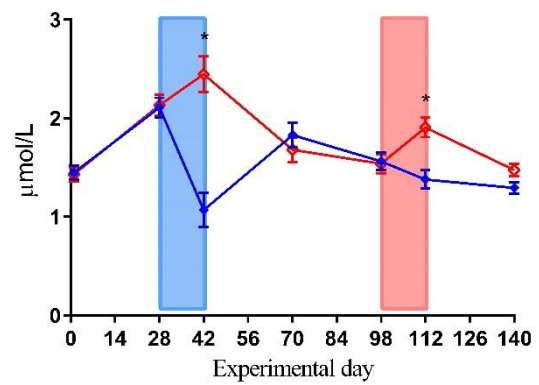
To investigate the metabolic changes directly after early weaning, the differences in metabolite concentrations between the two weaning groups were tested on experimental day 42. Early-weaned calves were currently weaned and received no more MR from this day onwards. They

consumed 1168 g DM concentrate on an average on this day. Late-weaned calves obtained their energy and nutrients mostly from MR, as they still received MR and on average consumed 1180 g DM MR. Furthermore, they consumed less than their allowance of concentrate feed on this day (850 g DM). Ten of the measured metabolites were significantly different between the two groups on day 42. These metabolites are hexoses, the amino acids ornithine, proline, methionine and histidine, the acylcarnitines propionylcarnitine (C3), acetylcarnitine (C2) and carnitine (C0), the biogenic amines alpha-amino adipic acid (alpha-AAA) and trans-4-hydroxyproline. Concentrations of these metabolites during the experiment and their roles in metabolism are discussed in the following chapters. Visualization of concentrations over time were done using GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA; Figure 1). To increase the knowledge about these metabolites and support their use as novel indicators, conventionally examined metabolites with a known link to liver metabolism were combined with the metabolites which were significantly different between the two groups on day 42 (Figure 2). A correlation analysis was done with R (R, 2022) and figures were produced using the *corrplot* package (R package '*corrplot*', 2021). As it was stated before that the weaning groups might have been in different metabolic states on this day, this correlation was done separately for each group. As anticipated, these correlations showed different patterns. Overall, 13 correlation coefficients were significant and indicated the same direction in early- and late-weaned calves, whereas 41 correlation coefficients indicated different directions or significance. This might hint at generally different metabolic performances for both groups.

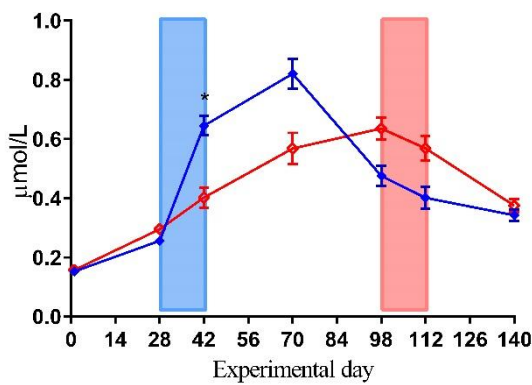
A Carnitine



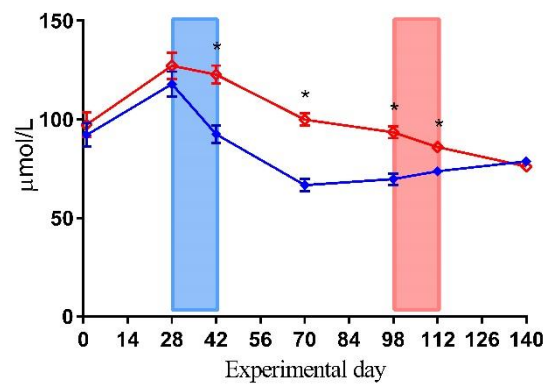
B Acetylcarnitine



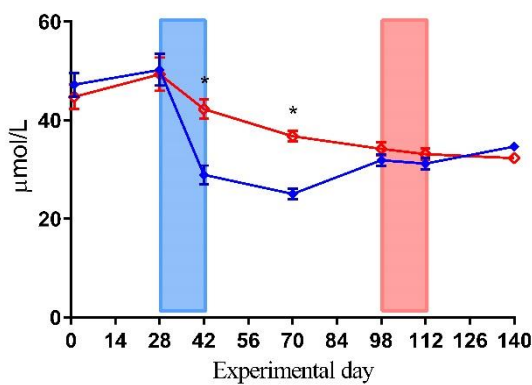
C Propionylcarnitine



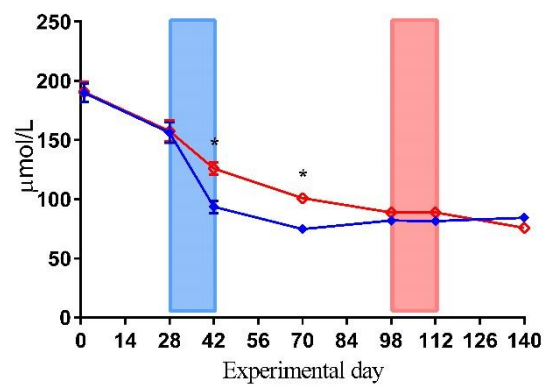
D Histidine



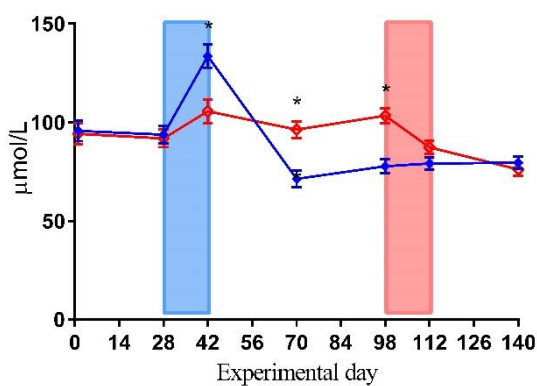
E Methionine



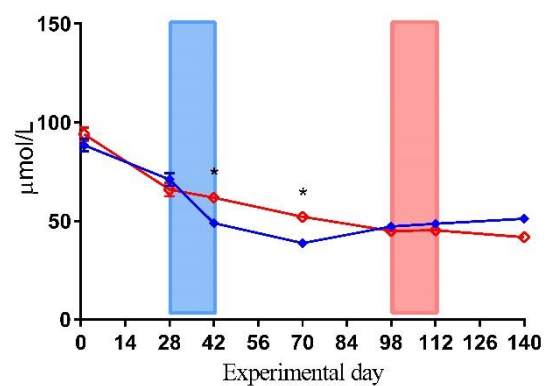
F Proline



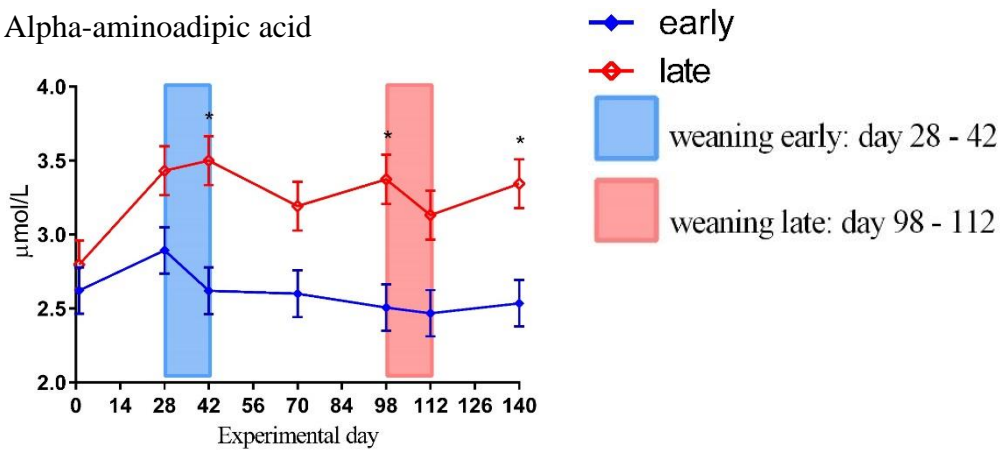
G Ornithine



H Trans-4-hydroxyproline



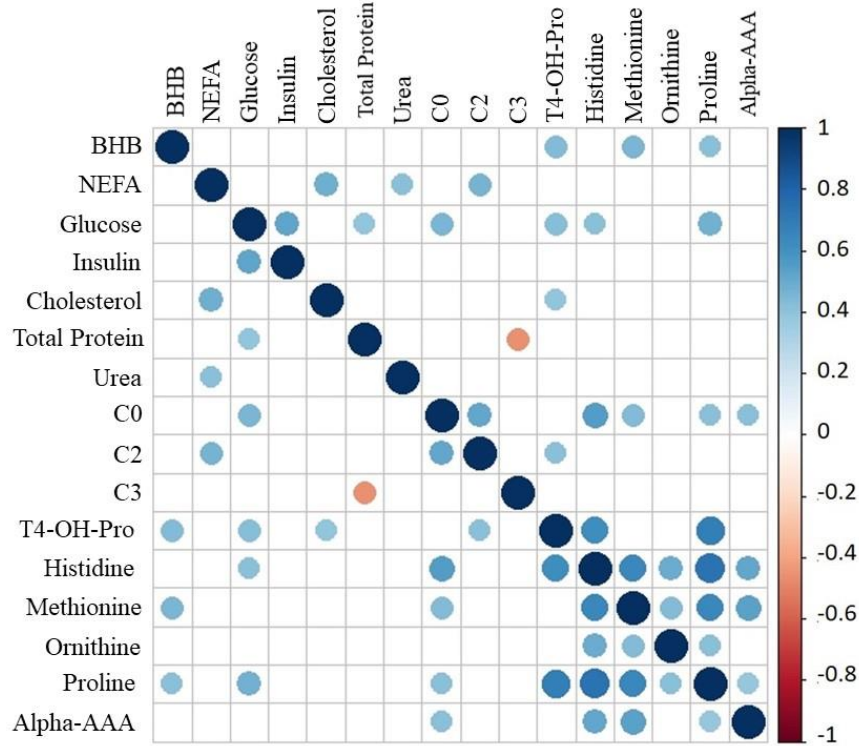
I Alpha-aminoadipic acid



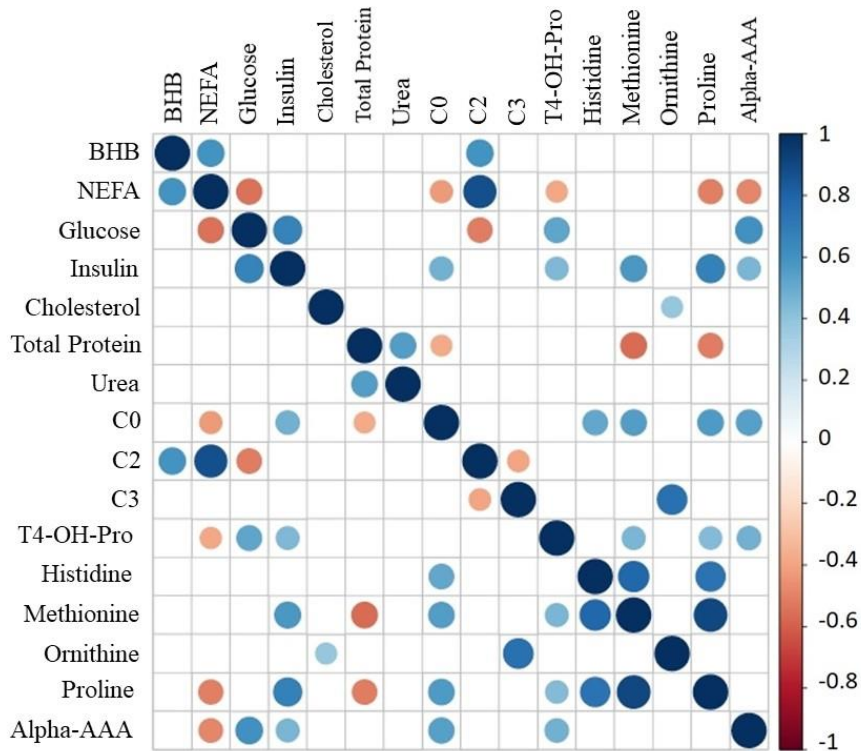
**Figure 1.** Plasma concentrations of some selected metabolites over time. Shown are carnitine (A), acetylcarnitine (B), propionylcarnitine (C), histidine (D), methionine (E), proline (F), ornithine (G), trans-4-hydroxyproline (H) and alpha-aminoadipic acid (I). Early-weaned calves (n= 31) were weaned gradually between days 28 and 42 of the trial. Late-weaned calves (n = 28) were weaned gradually between days 98 and 112 of the trial. Data shown as LSMeans  $\pm$  SE.

\*indicate a significant difference between weaning groups at the given experimental day (p < 0.05)

A



B



**Figure 2.** Correlation between some selected metabolites on experimental day 42 in early-weaned (A) and late-weaned (B) calves. Early-weaned calves were weaned gradually between days 28 and 42 of the trial. Late-weaned calves were weaned gradually between days 98 and 112 of the trial. Only correlations with a significance level  $p < 0.05$  are shown. Size

of dots indicates p value; big dots indicate low p value. Colors of dots indicate the value of the Pearson correlation coefficient. Red dots show  $0 > r > -1$ ; blue dots show  $0 < r < 1$ .

Early-weaned calves n = 31; late-weaned calves n = 28.

BHB = beta-hydroxybutyrate; NEFA = non-esterified fatty acids; C0 = carnitine;

C2 = acetylcarnitine; C3 = propionylcarnitine; T4-OH-Pro = trans-4-hydroxyproline;

Alpha-AAA = alpha-aminoadipic acid.

### 3.2.1.1 Gluconeogenesis in Early Weaned Calves

As discussed before (Chapter 3.2) the lower serum glucose concentrations after early weaning might have indicated the inability of hepatic gluconeogenesis to compensate the lack of MR glucose, while a higher glucose concentration might be beneficial for growing calves. As propionate is the main substrate for gluconeogenesis (Aschenbach et al., 2010), the lower serum glucose concentrations might have been the result of lower production or absorption of propionate in the rumen as well. Both hypotheses will be discussed in this chapter.

Propionate is the precursor for C3 as well. It is activated by coenzyme A building propionyl-CoA, which is further conjugated with C0 inside the mitochondria producing C3, which can be released into the cytosol (Roe et al., 2000). As potential fiber-degrading bacteria appeared increasingly in calves' feces with age in both weaning groups, the production of propionate through microbial fermentation in the rumen is likely established in these calves. Besides providing glucose for energy, gluconeogenesis has the important function to regulate the prevalence of glucogenic precursors in the blood. Systemic accumulation of propionate led to propionic acidemia, which can be fatal (Aschenbach et al., 2010). Under circumstances of reduced or impaired gluconeogenesis, the body has to eliminate propionate into other non-toxic compounds. This might be achieved through the formation of C3, which was higher in early-weaned calves on experimental days 42. The entry of propionate into gluconeogenesis depends on three mitochondrial enzymes (Aschenbach et al., 2010). One of these is PEPCK which is located in the cytosol (PEPCK-C) and the mitochondria (PEPCK-M) (Aschenbach et al., 2010) and works as a rate-limiting enzyme for gluconeogenesis in the liver (Girard et al., 1992). The specific effects of feedstuff on the development of PEPCK expression remained unclear, but there seemed to be an influence of MR intake. Despite the possible increase of substrate for this enzyme, the gene expression of PEPCK did not increase with increasing concentrate intake (1 kg concentrate/day vs. 2 kg concentrate/day) in 11 and 22 weeks old calves fed limited amount

of MR for the first 11 weeks (Haga et al., 2008; Lohakare et al., 2012). *Ad libitum* MR intake increased hepatic mRNA expression of PEPCK compared to restricted feeding (Schäff et al., 2016). In calves fed a high amount of MR (MR powder 2% of weekly measured BW) the abundance of PEPCK mRNA increased with age during the first 6 weeks of life (Hammon et al., 2005), whereas PEPCK activity decreased with age during the first 19 weeks of life in calves which were fed limited amounts of MR powder (300 – 600 g/day depending on the age) and were weaned at 6 weeks of age (Haga et al., 2008). Downregulation of PEPCK, when not enough glucogenic precursors are available, is a protective mechanism as this enzyme might drain oxaloacetate from the tricarboxylic acid cycle (TCA; Aschenbach et al., 2010). Hypothetically, this might have been the case in early-weaned and restrictedly fed calves as well, in which enough propionate was available, but the immature liver was not able to use all for gluconeogenesis. Hence, PEPCK might have been downregulated to ensure the functioning of the TCA. Therefore, C3 concentration increased during and after early-weaning, but did not change during late weaning (Figure 1C) as these calves were able to use more propionate in gluconeogenesis.

Alpha-AAA decreased after early-weaning and was, hence, lower in early-weaned calves on experimental day 42 (Figure 1I). Not much research was done regarding this metabolite, especially not in ruminants. In humans, the alpha-AAA concentration was positively associated with obesity (Lee et al., 2019) and diabetes (Wang et al., 2013). Contrary, feeding alpha-AAA to obese mice reduced BW, decreased fat accumulation and lowered fasting glucose (Xu et al., 2019). Overall, a role in glycolipid metabolism and adipogenesis seemed likely (Lee et al., 2019). Concentrations were higher in late-weaned calves on experimental days 42, 98 and 140 (Figure 1I). As these animals were growing greater and likely accumulated more body fat than early-weaned calves, this supports the role of alpha-AAA in adipogenesis. This is also supported by the negative correlation with NEFA in late-weaned calves ( $p = 0.009$ ;  $r = - 0.485$ ;

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Figure 2B). In early-weaned calves correlation between alpha-AAA and NEFA was not significant ( $p = 0.072$ ; Figure 2A), but negative as well ( $r = -0.352$ ). In late-weaned calves, alpha-AAA was correlated with glucose ( $p < 0.001$ ;  $r = 0.605$ ) and insulin ( $p = 0.012$ ;  $r = 0.467$ ) as well (Figure 2B), which supported its role in glucose metabolism. As late-weaned calves were stated as more mature and less metabolically challenged before, this metabolite might be a predictor for metabolic health as well.

### 3.2.1.2 Catabolism in Early Weaned Calves

As stated before, early-weaned calves were in a catabolic state, in which proteolysis and lipolysis provide substrates for energy production. Therefore, they used a higher amount of body proteins and fats for energy production compared to late-weaned calves at this age, which is reflected in an increased BHB concentration after weaning. As these concentrations decreased again with age, rumen wall ketogenesis might not have been the only source of BHB in early-weaned calves (**Manuscript 1**).

C0 and related acylcarnitines shuttle long-chain fatty acids from the cytoplasm into the mitochondrial matrix. In the mitochondrial matrix they split again in their precursor molecules. The acyl-CoA enters the TCA for  $\beta$ -oxidation and ATP production. Therefore, acylcarnitines are important for the onset of  $\beta$ -oxidation in the mitochondria (Rinaldo et al., 2002). C0 is synthesized in liver and kidneys only (Ringseis et al., 2018). Therefore, liver and kidneys are the sources of C0 in the blood, especially under circumstances that require energy production through fatty acid oxidation. Other organs must acquire C0 from the blood (Schooneman et al., 2013). Hence, more than one specific organ influences blood acylcarnitine concentrations. It is not clear to what extent circulating acylcarnitines represent the tissue acylcarnitine metabolism. Recent studies emphasize that caution should be taken when interpreting metabolite blood

concentrations only, because these might be influenced by the metabolism of many organs especially the liver and muscles (Schären et al., 2021; Yang et al., 2019).

C0 is synthesized endogenously from N-trimethyllysine, which in turn is derived from the breakdown of specific lysine residues. These are structural proteins like myosin and calmodulin (Ringseis et al., 2018). Alpha-AAA is a product of lysine degradation as well (Wang et al., 2013). Therefore, the positive correlation between alpha-AAA and C0 in early- ( $p = 0.031$ ;  $r = 0.416$ ) and late-weaned calves ( $p = 0.002$ ;  $r = 0.548$ ) might be biologically plausible (Figure 2).

Well-fed animals, that were deprived of energy, increased free C0 concentrations in blood likely to supply tissues with sufficient C0 for the transport of long-chain fatty acids into mitochondria for the increased  $\beta$ -oxidation (Ringseis et al., 2018). Similarly, Kenéz et al. (2018a) found several acylcarnitines to be higher in *ad libitum* MR fed calves compared to restrictedly fed calves. Lower concentrations of acylcarnitines in weaned and restrictedly fed calves might have originated either from their usage in energy production in the mitochondria or from a restricted capacity of liver synthesis. The reduced concentration of C0 in early-weaned calves (Figure 1A) might have derived from the enhanced use in the transport of fatty acids into the mitochondria to generate energy or to eliminate propionate through the formation of C3. Similarly, postpartum cows with a high mobilization of body fat showed reduced levels of C0 and elevated levels of C2 compared to low mobilizing cows (Humer et al., 2016). In humans, reduced levels of C0 and increased levels of C2 during diabetic ketosis have been reported (Genuth and Hoppel, 1979). C2 was elevated in postpartum cows with a high BCS and positively correlated with BHB (Ghaffari et al., 2019), which showed the excess of fatty acid  $\beta$ -oxidation relative to oxidation in the TCA and oxidative phosphorylation. This positive correlation was detected in late-weaned calves as well ( $p < 0.001$ ;  $r = 0.601$ ; Figure 2B), but not in early-weaned calves ( $p = 0.354$ ;  $r = 0.185$ ; Figure 2A). Late-weaned calves also showed a negative correlation of C2 and glucose ( $p = 0.005$ ;  $r = -0.513$ ; Figure 2B), whereas in early-

weaned calves it was not significant ( $p = 0.108$ ;  $r = 0.316$ ; Figure 2A). This supports the hypothesis that in the presence of sufficient glucose supply in a non-catabolic state, acetyl entered the TCA as acetyl-CoA and did not form C2. This is in accordance with what others measured in postpartum cows (Ghaffari et al., 2019; Humer et al., 2016). In the early-weaned calves of the current study the metabolic situation seemed to be different. C2 concentrations decreased during early weaning and therefore was lower on day 42 compared to late-weaned calves (Figure 1B). Contrary to late-weaned calves, early-weaned calves showed no correlation between C2 and BHB or glucose on day 42 (Figure 2A). The reduced concentrations of C2 indicated that lipolysis or proteolysis might not have been elevated as much to produce an excess of acetyl-CoA which has to be eliminated. Concentrations of C2 might decrease because of the use of acetyl-CoA in the TCA for energy production. Therefore, this might have been the main source of energy for these early-weaned calves. No significant change of C2 concentration could be detected in the late weaning period (Figure 1B), which indicates that they did not mobilize body fat during weaning.

Methionine, histidine and proline were significantly lower after early weaning, whereas ornithine increased during early weaning and decreased afterwards. Methionine, histidine and proline were positively correlated with LWG (**Manuscript 3**), which highlighted their importance in anabolic metabolism and growth (Lent-Schochet et al., 2019). Proline is an important component of collagen in calf skin (Hörlein et al., 1978; Wu et al., 2011). Besides their role in anabolic metabolism, these amino acids protect cells against oxidative stress through the reduction of free radicals (Krishnan et al., 2008; Lent-Schochet et al., 2019; Wu et al., 2011). Therefore, an increased concentration in the blood could mean that they were available for growth or to resist oxidative stress. Low concentrations of these amino acids were linked to health problems in humans (Lent-Schochet et al., 2019). Proline and histidine were lower in cows with ketosis compared to healthy cows (Sun et al., 2014; Wang et al., 2016).

Therefore, the lower concentrations of those amino acids in early-weaned calves might have been due to metabolic stress of weaning. As discussed before, protein levels might be highly influenced by feedstuff and higher in late-weaned calves as MR contained a high amount of easily digestible protein and amino acids (Wu et al., 2011).

We found a positive correlation between ornithine and LWG and heart girth (**Manuscript 3**). Therefore, the elevated ornithine concentrations after early weaning might have resulted from a reduced growth and corresponding cell proliferation. Ornithine serves as the precursor for biosynthesis of polyamines, with the rate-limiting step being catalyzed by ornithine decarboxylase (ODC). Cell proliferation is associated with enhanced ODC activity, which necessitates an increased use of ornithine (Pegg, 1986). Ornithine plays a critical role in mitochondrial metabolic processes and the functioning of the liver as well (Sivashanmugam et al., 2017). It is an intermediate in the urea cycle and a substrate for the production of proline (Sivashanmugam et al., 2017). As both metabolites were not correlated with ornithine in late-weaned calves (Figure 2B) and only a small positive correlation with proline was detected in early-weaned calves ( $p=0.033$ ,  $r = 0.412$ ; Figure 2A), these metabolic pathways might not have been the reason for the elevated ornithine levels.

Concentrations of trans-4-hydroxyproline decreased during early weaning and were higher in calves that still received MR on day 42. In horses, a higher trans-4-hydroxyproline concentration was a positive sign for health and insulin sensitivity (Kenéz et al., 2018b). Dairy cows fed high grain diets (Pacífico et al., 2020) or experiencing subacute ruminal acidosis had lower plasma trans-4-hydroxyproline concentrations (Humer et al., 2018). As it was suggested before (**Manuscript 2**) that early-weaned calves might have suffered from ruminal acidosis, this metabolite might have been an indicator of metabolic health in calves as well. Trans-4-hydroxyproline is formed from proline (Wu et al., 2011), which might explain the positive correlation between those metabolites in both weaning groups (early  $p < 0.001$ ,  $r = 0.688$ ; late

$p = 0.021$ ,  $r = 0.434$ ; Figure 2). Furthermore, hydroxyproline is a substrate for the synthesis of pyruvate and glucose (Wu et al., 2011). This might be the reason for the positive correlation of glucose and trans-4-hydroxyproline in both early ( $p = 0.028$ ;  $r = 0.421$ ; Figure 2A) and late-weaned calves ( $p = 0.004$ ;  $r = 0.527$ ; Figure 2B). A deficiency of this metabolite might play an important role in low glucose concentrations after early weaning.

### 3.3 Conclusions and Prospects

Nutritional restrictions during prenatal and postnatal life are challenging for calves' maturation. This study provided new insights in metabolic consequences and alterations through mothers' parity and two different weaning ages.

As all three included manuscripts highlight the importance of the maturation of liver metabolism for a smooth transition to the ruminant status, indicators for this should be considered in future research about weaning calves successfully. The capacity for gluconeogenesis is a very important metabolic feature for ruminants and was likely not sufficient for early-weaned calves. Therefore, this should be considered in the evaluation of rearing regimens. For basic research it might be useful to measure gluconeogenesis *in vivo* in calves through isotope techniques.

Interdisciplinary research is needed to gain more knowledge about certain metabolites analyzed in this study. Hence, we combined data derived from metabolomics with growth parameters and data from microbiome analysis. This approach might be useful regarding other data from the fields of physiology, behavior or genetics. To investigate the metabolic source of various metabolites a metabolomics analysis in organ samples like liver and muscle might be considered. Further research is needed, though, to understand the origin and function of each metabolite.

As C0 and alpha-AAA were still significantly different between weaning groups on day 140, when all calves were weaned, there might have been a permanent shift in certain involved pathways through weaning age. The nutritional experience through different weaning ages might have persistent effects through *metabolic imprinting*. Further research is needed to test the lasting effects of different weaning ages and mothers' parity. Therefore, all animals were monitored in an ongoing observational study. However, results from these ongoing studies are not part of the thesis.

### **3.3.1 Conclusions regarding Weaning age**

Based on the results of this study, weaning calves at the age of 7 weeks is not recommended. These calves were in a catabolic status for at least 4 weeks after weaning and several biomarkers indicated metabolic stress, inflammation and immaturity of various organs and their metabolic functions. Overall, the results of the performed experiment indicate that weaning at the age of 7 weeks leads to abrupt and possibly stressful metabolic changes. Therefore, weaning at 7 weeks of age can be considered too early and inferior compared to a weaning age of 17 weeks.

Weaning at the age of 17 weeks led to less abrupt metabolic alterations and a possible smoother maturation of various organs such as liver, kidney, rumen and gastrointestinal tract. Overall, the results indicated that maturation was influenced mostly by age and could not be forced through feedstuff and weaning. As a consequence, weaning might have been less stressful for late-weaned calves. This rearing regime might have supported a greater animal welfare. There was a growing interest in animal welfare in consumer, which was reflected by a willingness to pay higher prices for products produced under conditions that support animal welfare. Therefore, higher prices for milk might be one strategy to counteract the greater costs for MR during calf rearing.

Nevertheless, late-weaned calves experienced significant changes in many blood metabolite concentrations during weaning as well, which might have reflected metabolic stress. Furthermore, the pH in saliva, which indicates the buffer capacity, increased until experimental day 140, unaffected by weaning age or mothers' parity. Therefore, the ability to use ruminal fermentation without the risk of ruminal acidosis might not have been established until the age of 21 weeks. Overall, this indicated that even weaning at 17 weeks of age might have been too early and ideally might be extended. It might be useful in future research to test the effects of an even greater weaning age.

### **3.3.2 Conclusions regarding Mothers' Parity**

Calves that were born to primiparous mothers and weaned early showed reduced back length and lower leptin concentrations. However, weaning at a greater age was advantageous to correct the imprinted changes in body proportions and leptin metabolism. Therefore, prolonging the MR feeding period was of particular importance for calves born to young primiparous mothers, as they might especially benefit from a greater weaning age. Hence, when advising skeptical farmers about delaying weaning age, particular emphasis should be put on this rearing regimen for calves born to primiparous mothers.

For scientific researchers, these results indicated that a mothers' parity should be considered in studies regarding calf rearing. As mothers' parity affected calves and the possible outcome of a rearing regimen, it should be kept in mind when conducting an experiment and interpreting results, especially about body proportions and leptin metabolism.

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## 5. Summary

Dairy farmers aim for a young age at first calving for economic reasons. As these young heifers are still growing during pregnancy, they might compete with their calf for nutrients. Therefore, mothers' parity and related nutritional supply during pregnancy might influence early development. Calves are born as functional monogastric animals that rely on milk or MR for nutrition. To reach the ruminant status and use solid feed as a nutritional source, many digestive, endocrine and biochemical functions need to change. Therefore, weaning is a crucial and potentially stressful event. The average weaning age of female dairy calves is 6 – 11 weeks, which is accompanied with stress that might negatively impair the immune system, growth and gastrointestinal development. Hence, delaying weaning potentially benefits calves' health and development. It improves growth, reduces distress associated with weaning and possibly increases age at removal from herd.

The thesis at hand aimed to identify the effects of two weaning ages and mothers' parity on development and adaption to weaning. By means of an animal experiment including fifty-nine female Holstein calves, the effects of weaning early (7 weeks of age) and late (17 weeks of age) on growth, behavior, rumen and gastrointestinal development and adaption of liver and kidney metabolism were tested. In addition, the effect of different weaning ages on calves born to primiparous and multiparous cows was taken into consideration. Samples were taken continuously beginning at the age of 8 days (experimental day 1) to 21 weeks of age (experimental day 140). The analyses and results are described in three peer-reviewed scientific papers and summarized below.

Growth performance was evaluated via live weight, live weight gain, withers and hip height, heart girth, back and body length. Energy metabolism and liver function was quantified by measuring serum concentrations of glucose, insulin, urea, cholesterol, leptin, beta-hydroxybutyrate and non-esterified fatty acids (**Manuscript 1**). Animal behavior was observed

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divided in resting, chewing and active behavior to draw conclusion about signs of hunger and stress as well as rumen development. Rumen development and corresponding metabolic and adaptive changes in acid-base metabolism were evaluated by measuring pH in urine, feces and saliva and nitrogen-containing metabolites in urine (**Manuscript 2**). The microbiome in fecal samples was analyzed to assess maturation of the gastrointestinal tract. To gain insight to metabolic adaptations a broad set of metabolites was measured. These included amino acids, biogenic amines, acylcarnitines and sphingomyelins (**Manuscript 3**).

Delaying weaning to 17 weeks of age had positive effects on body growth and adaptation of energy and acid-base metabolism (**Manuscript 1; Manuscript 2**). The pH of saliva increased over time during the experiment unaffected by weaning age or mothers' parity (**Manuscript 2**). As an alkaline pH in saliva is important for its buffering capacity, this highlights the importance to delay weaning. Gastrointestinal development was not impaired by prolonged MR intake, but rather reached a functional status at the end of the trial. Factors of rumen development (**Manuscript 2**) and microbiota in feces (**Manuscript 3**) adapted quickly and indicated an age-dependent maturation. The analyzed metabolites indicated distinct effects of early weaning on various metabolic pathways (**Manuscript 3**). Particularly, liver gluconeogenesis seemed to be insufficient for early-weaned calves.

Leptin concentrations and back length was reduced in early-weaned calves born to primiparous mothers, but not in late-weaned calves from primiparous mothers (**Manuscript 1**). Therefore, calves born to primiparous cows particularly benefit from delaying weaning.

The results indicated that weaning at the age of 7 weeks is not recommended, as it led to abrupt and possibly stressful metabolic changes and impaired maturation of various organs and their metabolic functions. The current study showed that delaying weaning to 17 weeks of age enables a smooth transition of various physiological functions to the ruminant status. Therefore, it seemed to reduce the metabolic problems and stress associated with weaning. Further research

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is needed about weaning, taking rearing costs and later milk performance and health into consideration.

## 6. Zusammenfassung

Milchbauern streben aus wirtschaftlichen Gründen ein junges Erstkalbealter an. Da diese jungen Färsen während der Trächtigkeit noch wachsen, könnten sie mit ihrem Kalb um Nährstoffe konkurrieren. Daher können die Parität der Mütter und die damit verbundene Nährstoffversorgung während der Trächtigkeit die frühe Entwicklung beeinflussen. Kälber werden als funktionell monogastrische Tiere geboren, die auf Milch oder Milchaustauscher zur Ernährung angewiesen sind. Um den Status eines Wiederkäuers zu erreichen und festes Futter als Nahrungsquelle zu verwenden, müssen sich viele digestive, endokrine und biochemische Funktionen ändern. Daher ist das Absetzen ein kritisches und potenziell stressiges Ereignis. Das durchschnittliche Absetzalter weiblicher Milchkälber beträgt 6 – 11 Wochen, was mit Stress einhergeht, der das Immunsystem, das Wachstum und die Magen-Darm-Entwicklung negativ beeinträchtigen kann. Daher kommt ein Aufschieben des Absetzens möglicherweise der Gesundheit und Entwicklung der Kälber zugute. Es verbessert das Wachstum, reduziert die mit dem Absetzen verbundene Belastung und erhöht möglicherweise das Alter bei der Entfernung aus der Herde.

Die vorliegende Arbeit zielte darauf ab, die Auswirkungen von zwei Absetzalterstufen und der Parität der Mütter auf die Entwicklung und Anpassung an die Entwöhnung zu identifizieren. Mittels eines Tierversuchs mit neunundfünfzig weiblichen Holstein-Kälbern wurden die Auswirkungen eines frühen (7 Wochen) und späten (17 Wochen) Absetzens auf Wachstum, Verhalten, Pansen- und Magen-Darm-Entwicklung und Anpassung des Leber- und Nierenstoffwechsels getestet. Darüber hinaus wurde der Einfluss unterschiedlicher Absetzalter auf Kälber von primiparen und multiparen Kühen berücksichtigt. Proben wurden kontinuierlich im Alter von 8 Tagen (Versuchstag 1) bis zum Alter von 21 Wochen (Versuchstag 140) genommen. Die Analysen und Ergebnisse werden in drei begutachteten wissenschaftlichen Arbeiten beschrieben und im Folgenden zusammengefasst.

Die Wachstumsleistung wurde anhand von Lebendgewicht, Lebendgewichtszunahme, Widerrist- und Hüfthöhe, Brustumfang, Rücken- und Körperlänge bewertet. Der Energiestoffwechsel und die Leberfunktion wurden durch Messung der Serumkonzentrationen von Glukose, Insulin, Harnstoff, Cholesterin, Leptin, Beta-hydroxybutyrate und nicht-veresterten Fettsäuren quantifiziert (**Manuskript 1**). Das Verhalten der Tiere wurde beobachtet unterteilt in Ruhe-, Kau- und Aktivverhalten, um Rückschlüsse auf Anzeichen von Hunger und Stress sowie der Pansenentwicklung zu ziehen. Die Pansenentwicklung und entsprechende metabolische und adaptive Veränderungen im Säure-Basen-Stoffwechsel wurden durch Messung des pH-Wertes in Urin, Kot und Speichel sowie stickstoffhaltiger Metaboliten im Urin bewertet (**Manuskript 2**). Das Mikrobiom in Kotproben wurde analysiert, um die Reifung des Magen-Darm-Trakts zu beurteilen. Um Einblicke in metabolische Anpassungen zu erhalten, wurde eine umfassende Anzahl an Metaboliten gemessen. Dazu gehörten Aminosäuren, biogene Amine, Acylcarnitine und Sphingomyeline (**Manuskript 3**).

Ein späteres Absetzen im Alter von 17 Lebenswochen wirkt sich positiv auf das Körperwachstum und die Anpassung des Energie- und Säure-Basen-Stoffwechsels aus (**Manuskript 1; Manuskript 2**). Der pH-Wert des Speichels stieg während des Experiments im Laufe der Zeit an, unabhängig vom Absetzalter oder der Parität der Mutter (**Manuskript 2**). Da ein alkalischer pH-Wert im Speichel für dessen Pufferkapazität wichtig ist, unterstreicht dies, wie wichtig es ist, das Absetzen zu verzögern. Die gastrointestinale Entwicklung war durch eine längere Milchaustauscheraufnahme nicht beeinträchtigt, sondern erreichte am Ende der Studie einen funktionellen Status. Faktoren der Pansenentwicklung (**Manuskript 2**) und die Mikrobiota im Kot (**Manuskript 3**) passten sich schnell an und deuteten auf eine altersabhängige Reifung hin. Die analysierten Metaboliten wiesen auf ausgeprägte Effekte eines frühen Absetzens auf verschiedene Stoffwechselwege hin (**Manuskript 3**). Insbesondere die Gluconeogenese in der Leber schienen bei früh abgesetzten Kälbern unzureichend zu sein.

Die Leptinkonzentrationen und die Rückenlänge waren bei früh abgesetzten Kälbern primiparer Mütter reduziert, nicht jedoch bei spät abgesetzten Kälbern primiparer Mütter (**Manuskript 1**). Daher profitieren Kälber von primiparen Kühen besonders von einem späteren Absetzen.

Die Ergebnisse zeigten, dass ein Absetzen im Alter von 7 Wochen nicht empfohlen werden kann, da es zu abrupten und möglicherweise belastenden Stoffwechselveränderungen und einer gestörten Reifung verschiedener Organe und deren Stoffwechselfunktionen führte. Die aktuelle Studie zeigte, dass die Verzögerung des Absetzens auf ein Alter von 17 Wochen einen gleichmäßigen Übergang verschiedener physiologischer Funktionen in den Wiederkäuerstatus ermöglicht. Deshalb schien es die mit dem Absetzen verbundenen Stoffwechselprobleme und den Stress zu reduzieren. Weitere Forschung über das Absetzen, unter Berücksichtigung der AufzuchtKosten und späteren Milchleistung und Gesundheit, ist erforderlich.

## **7. Annexes**

### **7.1 Acknowledgements**

Many people contributed to my personal and academical development during the last years.

First of all, I would like to thank my supervisor Prof. Dr. Korinna Huber for the opportunity to perform my doctoral thesis at the Department of Functional Anatomy of Livestock. Her encouragement and trust were the basis for enthusiastic work throughout this project. I am very grateful for a supervisor whose doors were always open for support while encouraging me to think independently.

I wish to express my gratitude to Dr. Jana Frahm and Prof. Dr. Dr. Sven Dänicke for their willingness to welcome me into this project and offer the opportunity to work with these data. Many thanks to them for their supervision of my doctoral thesis. Their continuous and constructive advice was crucial for the progress.

I would like to thank the team of Friedrich-Löffler-Institut in Braunschweig for the professional support during the experiment and their great work with the animals and in the lab. I am very thankful for everybody who made my stays there delightful and enjoyable. Many thanks to Dr. Asako Kinoshita, Dr. Susanne Kersten, Alva Voigt, Dr. Katharina Bünemann, Susanne Kononov and Jennifer Meyer for a great time during work and leisure time.

Moreover, I want to thank all my colleagues from the Department of Functional Anatomy of Livestock, with whom I had the pleasure to work during the last years. Special thanks go to my fellow doctoral candidates, Fernando Gonzalez, Sandra Grindler, Sonja Bäßler and Arezou Abdi. They made conferences, trips and academic discussions rich and joyful.

Sincere thanks go to all the co-authors who contributed to the included manuscripts with constructive support and discussions.

Further, I would like to thank my family. Special thanks go to my boyfriend who supported me unconditionally and always made sure I had enough time to work on this thesis. I am grateful for my father, who always supported my interests and now takes care of me from heaven.

Finally, I would like to mention the calves we worked with. They had no choice but to participate in this experiment. Nevertheless, I know, these animals were treated with respect and I am grateful that we had the opportunity to perform this project with them. I am convinced we will get the best out of the data and their sacrifice was useful for other calves, who hopefully will benefit from the gained knowledge.

## 7.2 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 "Mother's parity and weaning age influence the transition from liquid to solid feed in female Holstein calve" 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, 31.01.2023

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Place, Date

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Signature