Aspects of Rumen Adaptation in Dairy Cattle

Morphological, functional, and gene expression changes of the rumen papillae and changes of the rumen microbiota during the transition period

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**Morphological, functional, and gene expression changes of
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Table of Contents

Chapter 1

General introduction

INTRODUCTION

Ruminant species (sheep, goats, and cows) have been domesticated 8,000 – 10,000 years ago (Bruford et al., 2003). This process was likely motivated by the recognition of the ruminant's capacity to digest feed inedible or unpalatable for humans but provide high quality protein and energy products fit for human consumption (meat and milk). In extensive farming systems, human edible feed input for milk or beef production is negligible, thus the efficiency (human edible food output/human edible feed input) of these system approaches infinity. However, in many modern farming systems ruminants are routinely fed human edible energy and protein sources (soybean meal, corn, and grains). Still, generally more high quality human edible energy and protein is produced than enters the system, especially for the dairy sector (Dijkstra et al., 2013). This capacity of the ruminant to 'upgrade' feed to food ensuring the ruminant's role in global food production in both developing and developed countries, can mainly be contributed to the presence of the reticulorumen. This part of the forestomach complex provides a dark, warm, wet, and anaerobic environment ideal for the rich microbiota community responsible for the fermentation of feed (Hobson and Steward, 1997).

Global developments in dairy can be typified by the post-Second World War developments in the Netherlands. The Dutch dairy sector comprised approximately 1.5 million head of (milk producing) cows between 1950 and 1960, comparable to the number of head between 2000 and 2015 (CBS, 2016). However, between 1950 and 2000 milk production (calculated as total recorded yearly milk production divided by average number of head of that year) increased from 3,800 kg/y to 7,400 kg/y (CBS, 2016). Currently, dairies with black-white Holstein-Friesian cows average 9,000 kg of milk/cow/y with the top dairies reaching 12,000 kg of milk/cow/y (CRV, 2015). Maintaining such high production levels requires a profound understanding of rumen physiology and microbial fermentation, which are essential for an efficient use of feed, a sustainable production without detrimental effects on animal health, and minimal environmental impact.

The Rumen Microbiota

The microbiota community comprises members of all 3 domains of life; e.g. bacteria, archaea, and eukaryota (protozoa, yeasts, and fungi). Sterile at birth, the rumen of the calf is immediately thereafter invaded by bacteria. After an initial bacterial colonization by ubiquitous environmental bacteria (for example *E. coli*), a rumen specific microbiota establishes in the subsequent weeks. These rumen specific bacteria, archaea, protozoa, yeasts and fungi are likely transferred to the calf via direct or indirect contact with saliva from ruminants with established rumen fermentation (Mizrahi, 2013). The fully developed rumen contains approximately 10^6 protozoa and 10^{10} bacteria per ml of rumen contents, with both protozoa and bacteria each accounting for 45% of the total microbial mass (Creevey et al., 2014; Weimer 2015). Genomic studies have shown that the rumen bacteria taxa are most numerous with 100s to 1000s possible species identified (McCann et al., 2014; Creevey et al., 2014; Weimer, 2015), depending on technique and sequencing depth used (Henderson et al., 2013). However, only a small fraction of these bacterial species are represented by a cultured isolate (~150 different cultures known; Creevey et al., 2014), thus the vast majority are essentially unknown except for their genetic fingerprint. Compared with the bacteria, the number of taxa of protozoa and archaea is far smaller, with 12 genera of protozoa and 34 species-level groups of archaea covering > 99% of their respective communities (Henderson et al., 2015). The large number of microbiota species suggests a substantial degree of redundancy for the capacity for degradation of different substrates, ensuring continuation of fermentation even with major shifts in rumen microbiota composition (Weimer, 2015).

Generally, microbial degradation of feed comprises the reduction of polysaccharides (e.g. cellulose, hemi-celluloses, pectins, and starch) to their constituent sugars. These are further metabolized yielding volatile fatty acids (**VFA**), primarily acetic (**Ac**), propionic (**Pr**), and butyric (**Bu**) acid, H_2, and CO_2. Removal of H_2 by archaeal methanogenesis is an essential process to maintain the fermentation processes (Baldwin and Allison, 1983). Ultimately, fermentation provides the energy for production of the microbial biomass. Microbial biomass is the primary protein source (Tamminga et al., 1994) for ruminants, whereas the VFA provide approximately 75% of total metabolic energy required by the animal (Bergman, 1990).

Anatomy of the Rumen and the Rumen Papillae

When fully developed, the forestomach-stomach complex occupies nearly the complete left half and extends well into the right half of the abdominal cavity. In dairy cows, the main chamber, the rumen, contains about 80% of the total volume, whereas the reticulum contains about 5%, with the remaining volume evenly split between the omasum and abomasum (Dyce et al., 1996). The reticulum and rumen form a single functional unit, the reticulorumen. The rumen is divided into a number of sub-compartments; e.g. the atrium ruminis, dorsal and ventral rumen sacs, and the caudodorsal and caudoventral blind rumen sacs. Typically, the rumen of a multiparous dry and gravid Holstein dairy cow holds 60 kg of contents, increasing to 68 kg one month after calving (Reynolds et al., 2004), and 80 kg or more later in lactation (Sutton et al., 2003).

Large areas of the rumen wall, mainly those which are in frequent or continuous contact with rumen fluid, are covered by rumen papillae (Figure 1A). Papillae are surface area increasing protrusions into the rumen lumen of varying size and shape, ranging from short and slender to long and leaf-like (Andersen et al., 1999; Reynolds et al., 2004). They typically have a length of 10 to 15 mm, a width of 1 to 5 mm and are 0.15 to 0.30 mm thick (Scott and Gardner, 1973; Dirksen et al., 1984; Graham et al., 2007). The rumen papillae, but also the non-papillated areas of the rumen wall, are covered by a stratified corneous epithelium (Figure 1B). The organization of this epithelium is comparable to that of skin, but it is thinner and in contrast to skin it does not contain any glands or hairs.

The lumen facing side of the epithelium, the stratum corneum, is in direct contact with rumen fluid and feed particles. It consists of keratinized cells, providing physical protection against tissue damage. The deeper layers, the strata granulosum and spinosum are not always clearly distinguishable from each other and in some cases appear to be completely absent (Dobson et al., 1956). The stratum granulosum cells are tightly connected and this layer is the main location of the epithelial permeability barrier (Graham and Simmons, 2005). The stratum spinosum cells are tightly interconnected by filaments providing strength to the epithelium (Dobson et al., 1956). The final layer, the stratum basale is mitotically the most active and responsible for continuous cell renewal (Mentschel et al., 2001). Over time, cells move from the stratum basale via the spinosum and granulosum to the stratum corneum. The stratum basale is tightly attached to a basal membrane, anchoring the epithelium to the supporting central core of connective tissue which contains a dense network of blood and lymphatic vessels. No muscle fibers are found in the papillae whereas elastic fibers penetrate only a short way into the papilla core from the rumen wall (Dobson et al., 1956). In contrast to the lumen

facing side of the stratum corneum, which is relatively smooth (Figure 1B), the basal side the epithelium is characterized by prominent folds (papillary bodies), especially at the tip of the papillae. Their prominence appears to vary according to the ration fed, and they generally are more pronounced with a higher energy intake (Dirksen et al., 1984).
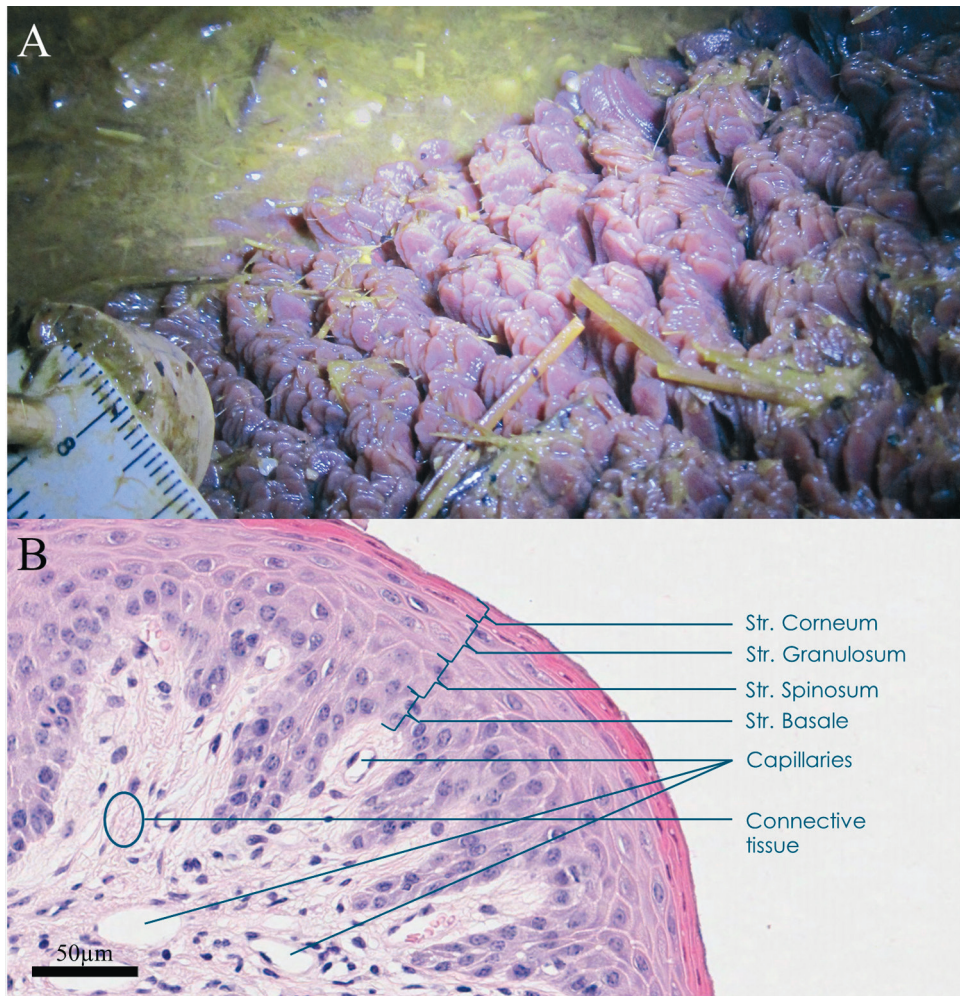
Figure 1. (A) Looking down through the cannula into a partially evacuated rumen, a section of wall of the ventral rumen sac, densely covered by papillae. Left side shows a section of a ruler (cm). Rumen fluid with suspended feed particles is visible in the top of the picture. Photograph courtesy of R. Goselink (Wageningen University & Research, the Netherlands); (B) Section of the tip of a rumen papilla, showing the stratification of the epithelium and the supporting connective tissue with capillaries. Hematoxylin-eosin stain, 4 μm section.

The epithelial permeability barrier does not allow ions and large molecules to enter paracellular space from the lumen and prevents invasion of bacteria (Graham and Simmons, 2005). Crucially, despite the multiple cell layers, the epithelium appears to function as a functional syncytium; i.e. a single functional layer. The cells are intimately connected through cell-to-cell gap junctions (Graham and Simmons, 2005), allowing small molecules and ions to pass from cell to cell without having to repeatedly cross the cell membrane. Furthermore, a distinct distribution of cellular transporters, ATP driven pumps, and ion channels has been demonstrated within the epithelium (Graham and Simmons, 2005; Kirat et al., 2006; Graham et al., 2007; Kirat et al., 2007). The idea of a functional syncytium with the differentiated arrangement of pumps and transporters is closely related to our current understanding of the absorption of VFA, electrolytes, and other metabolites.

Ruminal Absorption of VFA

The major VFA are weak acids with a pK_a ~4.8. Thus at physiological rumen fluid pH, 5.8 to 6.8, between 90 to 99% of the acid is in the dissociated form (**VFA⁻**), with the released H^+ buffered or eliminated. It has long been recognized that the VFA are absorbed through the rumen wall (Barcroft et al., 1944; Danielli et al., 1945), and that the capacity for absorption is related to the surface area of the papillae (Dirksen et al., 1984). The protonated, undissociated form (**HVFA**), in contrast to the dissociated form, can readily pass through the cell membrane and it has long been thought that passive diffusion was the main mode of absorption (Bugaut, 1987). However, both quantitative and qualitative constraints indicate that passive diffusion cannot be the sole route for absorption (Aschenbach et al., 2011). Firstly, only a small fraction of VFA is protonated, limiting the concentration gradient across the cell membrane which is all but impermeable to the dissociated form. Secondly, the differences in production rates (Ac > Pr > Bu; Sutton et al., 2003), lipophilic permeability (Bu > Pr > Ac) and intracellular metabolism (Bu >> Pr > Ac; Kristensen, 2005) should result in selective retention of Ac in the rumen when passive diffusion is the major absorption route. However, relatively similar absorption rates from incubation buffers using the washed rumen technique are observed for the different VFA (Dijkstra et al., 1993; Kristensen and Harmon, 2004), and the existence of facilitated absorption of both HVFA and VFA⁻ has been hypothesized and established since (Stevens et al., 1967).

The current model for VFA absorption has been reviewed by Leonhard-Marek et al. (2010) and Aschenbach et al. (2011). However, the mechanisms for absorption and their relative importance have still not been fully elucidated. The VFA can enter the epithelium in a number of ways (Figure 2). Firstly by passive diffusion of HVFA

through the lipid cell membrane, secondly by exchange of VFA$^-$ for HCO$_3^-$. Thirdly, a substantial but poorly characterized HCO$_3^-$ independent uptake of VFA$^-$ has been demonstrated (Aschenbach et al., 2009; Penner 2009), and fourthly, uptake of VFA$^-$ in conjunction with H$^+$ through a monocarboxylate transporter (**MCT**; Kirat et al., 2007). Efflux of VFA from the epithelium occurs at the basal side of the epithelium (stratum basale and deeper stratum spinosum cells) and largely though mechanisms similar to the uptake (Graham and Simmons, 2005; Aschenbach et al., 2011). Next to passive diffusion, VFA$^-$ can be exported in exchange for HCO$_3^-$ or exit together with H$^+$ through a basally located MCT (Graham et al., 2007). In addition, the VFA$^-$ can pass
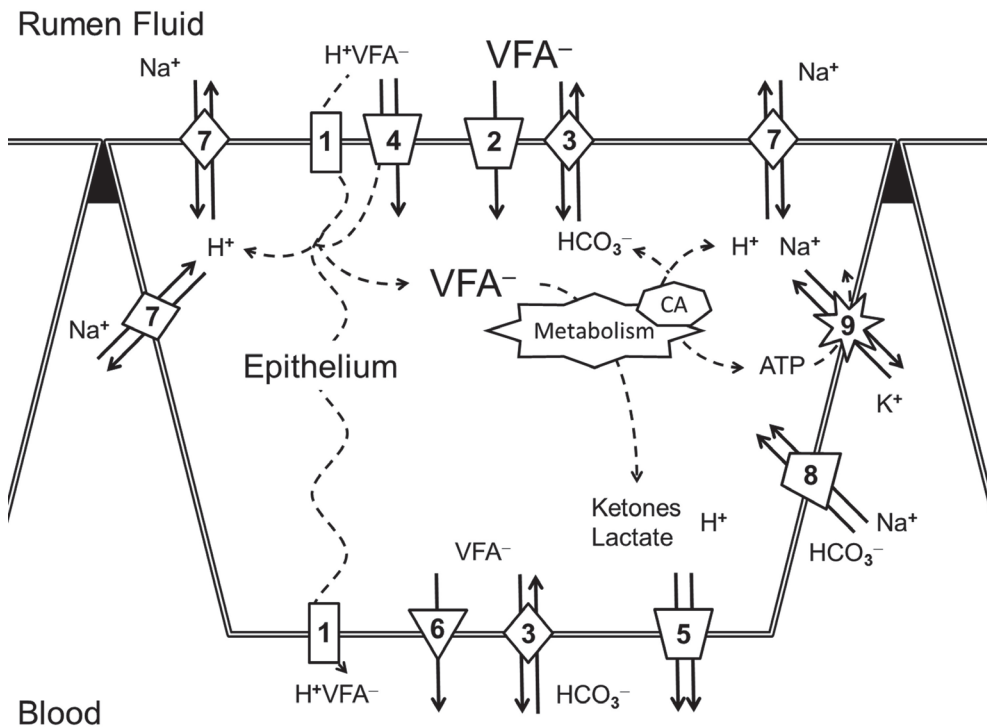
Figure 2. Absorption of VFA from the rumen fluid across the rumen epithelium. Uptake of VFA from the rumen fluid and export from the epithelium can occur through (1) passive diffusion of HVFA; (2) luminal HCO$_3^-$ independent uptake of VFA$^-$; (3) luminal and basolateral HCO$_3^-$ dependent uptake and export of VFA$^-$; (4) luminal mediated uptake of VFA$^-$ in conjunction with H$^+$; (5) basolateral mediated export of VFA$^-$ (and ketone bodies and lactate) in conjunction with H$^+$; (6) basolateral export of VFA$^-$ through a maxi-ion channel. Intracellular pH is maintained by export of H$^+$ through (7) luminal and basolateral Na$^+$/H$^+$ exchange; and (8) uptake of Na$^+$ and HCO$_3^-$ through co-transporters. Low intracellular Na$^+$, which drives a number of processes, is maintained by an ATP driven Na$^+$/K$^+$ exchanger (9). Intracellular metabolism of VFA can yield ketone bodies, lactate, and produces CO$_2$ which can be converted to H$^+$ and HCO$_3^-$ through carbon anhydrase (CA). Note: the epithelium comprises multiple cell layers which form a functional syncytium (behaves as a single layer). The multiple cell layers are not shown for clarity. Adapted from Aschenbach et al. (2011).

through a recently identified maxi-ion channel (Georgi et al., 2014), electrochemically driven by export of 3 Na^+ into intercellular space against 2 K^+ by a Na^+/K^+-ATPase (Graham and Simmons, 2005; Georgi et al., 2014).

Uptake of HVFA through diffusion and uptake through MCT potentially leads to acidification of the intracellular environment (Gäbel et al., 2002; Kirat et al., 2007; Aschenbach et al., 2011). Intracellular acidification is countered by moving H^+ into the basolateral space by the passive diffusion of HVFA. Alternatively, H^+ might be exported in conjunction with VFA^- by the MCT. The luminal and basolateral export of H^+ in exchange for Na^+ likewise provides a route. In addition, H^+ can be removed through intracellular buffering by HCO_3^-. Replenishment of the intracellular HCO_3^- pool is accomplished by basolateral exchange of VFA^- for HCO_3^- from the blood. In addition, HCO_3^- can be imported from the blood driven by the Na^+ gradient. Note that intracellular production of HCO_3^- by carbon anhydrase will not raise intracellular pH unless the equivalent amount of H^+ which is released is removed through one of the routes described above.

Buffering of VFA Produced in the Rumen and Rate of VFA Absorption

From the concentration of VFA in the rumen fluid and its pH it is clear that almost all dissociated H^+ is buffered or otherwise removed from the rumen fluid. The main buffering capacity is provided by HCO_3^- from saliva and from exchange across the rumen wall (Gäbel et al., 1991) and after reaction with H^+, H_2CO_3 is formed which rapidly dissociates into H_2O and CO_2. The CO_2 can be absorbed into the blood or removed through eructation, elevating the buffer efficiency of the HCO_3^- system to >90% (Kohn and Dunlap, 1998). Other major buffering components are $H_2PO_4^-$ (pK_a = 7.2) from saliva, and urea secreted by the rumen wall. Urea is rapidly converted to NH_3 by the rumen microbiota and protonates to NH_4^+. Both NH_3 and NH_4^+ can be absorbed by the rumen wall (Aschenbach et al., 2011). A quantitative estimation of the contributions of the different buffer sources is provided by Aschenbach et al. (2011) and Dijkstra et al. (2012). For a cow producing 100 mol of VFA/d and assuming a saliva production of 250 L/d (Dijkstra et al., 1992; Maekawa et al., 2002), about 40% of H^+ is buffered by $H_2PO_4^-$ (~5 mol/d) and HCO_3^- (~30 mol/d) from saliva, whereas HCO_3^- from the rumen wall accounts for ~45% assuming 50% of the VFA is absorbed as VFA^- in exchange for HCO_3^- (Gäbel et al., 1991; Penner et al., 2009). This example suggests buffering by NH_3 accounts for approximately 5% of total H^+ produced. However, the true contribution of urea and NH_3 to rumen pH buffering has yet to be elucidated (Aschenbach et al., 2011; Dijkstra et al., 2012).

Expression of Proteins and Genes Associated with VFA Absorption

Not all proteins and their genes associated with the VFA absorption model presented above have yet been fully elucidated (Connor et al., 2010; Penner et al. 2011). Candidates for the MCT located at the basal membrane, MCT1 (Graham and Simmons, 2005; Graham et al., 2007), and that located at the luminal side of the epithelium, MCT4 (Kirat et al., 2007), have been described (encoded by *MCT1* and *MCT4*, respectively). The exchangers which are likely responsible for the exchange of HCO_3^- against VFA^- at the luminal side have been identified as DRA (Downregulated-in-Adenoma; encoded by *DRA*) and PAT1 (Putative Anion Exchanger 1; encoded by *PAT1*) (Bilk et al., 2005), whereas the VFA^- for HCO_3^- exchanger at the basal side of the epithelium has been identified as AE2 (Anion Exchanger 2, encoded by *AE2*; Bilk et al., 2005; Connor et al., 2010). The cotransport of Na^+ and HCO_3^- from the blood at the basal side likely occurs through NBC1 (Na^+/HCO_3^- cotransporter 1, encoded by *NBC1*; Huhn et al., 2003). Essential for maintenance of the intracellular pH are the NHE (the Na^+/H^+ exchangers), of which three isoforms are expressed, NHE1, NHE2, and NHE3 (encoded by *NHE1*, *NHE2*, and *NHE3*, respectively). The NHE1 and NHE3 are located in the cell layers of the stratum granulosum closest to the rumen lumen, whereas NHE2 is located in the cytosol throughout the epithelium, arguably as a form of functional reserve (Zachos et al., 2005; Graham et al., 2007). Maintenance of the low intracellular Na^+ concentration is maintained through the ATP driven Na^+/K^+ exchanger ATP1A1 (ATPase Na^+/K^+ Transporting Subunit Alpha 1, encoded by *ATP1A1*; Connor et al., 2010). Currently, no candidate genes and proteins for the bicarbonate independent uptake for VFA^- have been proposed (Aschenbach et al., 2009; Penner et al., 2009). Likewise, the genetic and molecular nature of demonstrated maxi-ion channel remains, to date, elusive (Georgi et al., 2014).

RUMEN ADAPTATION IN DAIRY CATTLE

In dairy cattle the nutrient requirements change rapidly around calving. Typically, cows are dried off 6 to 8 weeks before expected calving date to allow the cow a period of recuperation before calving and onset of the new lactation (Goff and Horst, 1997). During the dry period nutrients are required for maintenance and fetal growth. After calving, milk production commences and nutritional demands increase accordingly. For a high producing dairy cow, energy requirement can increase by a factor 3 to 4 (Table 1) over the course of several weeks (CVB, 2011). Feed intake likewise increases during this period, generally by a factor of 2, whereas intake of rumen fermentable organic matter (**FOM**) increases more than the dry matter intake due to increase of the FOM density of the lactation ration compared with the dry period ration. Production rate of VFA

is estimated as 5 to 6 mol/kg of DM (Sutton et al., 2003; France and Dijkstra, 2005). Thus, daily VFA production increases from 60 to 132 mol/d. To maintain rumen pH at levels favorable for rumen fermentation (Gäbel et al., 2002; Dijkstra et al., 2012), and prevent a negative impact on production and health (Kleen et al., 2003; Enemark, 2008; Plaizier et al., 2012) increased capacity for clearance of the produced VFA and buffering of H^+ is essential, which is associated with morphological and functional changes (VFA absorption and metabolism) of the rumen papillae (Dirksen et al., 1984; Bannink et al., 2008; Martens et al., 2012).

Table 1. Estimated energy requirement, feed intake, and volatile fatty acid production in a dry and early lactation multiparous dairy cow

Item	Lactation stage[1]	
	Dry	Lactation
Milk production, kg/d	0	45
Energy requirement[2], NE_L MJ/d	55	184
DM intake[3], kg/d	12.0	24.0
FOM intake[4], kg/d	5.5	14.0
VFA production[5], mol/d	60	132

[1]Dry cow in the 9th month of gestation, lactating cow in the 1 month in lactation.
[2]According to CVB, 2011. Milk was assumed to contain 4.00% fat and 3.32% protein. 6.9 MJ NE_L = 1000 VEM.
[3]Dry period feed intake according to Hayirli et al., 2003. Lactation period feed intake calculated according to CVB, 2011.
[4]Rumen fermentable organic matter, calculated using CVB, 2011. Dry period ration comprised 4 kg grass silage, 4 kg corn silage, 4 kg wheat straw on a DM basis. Lactation ration comprised 6 kg grass silage, 6 kg corn silage, 12 kg of concentrate on a DM basis.
[5]Assuming 5 mol/kg of DM for the dry period ration, 6 mol/kg of DM for the lactation ration (Sutton et al., 2003).
NE_L = net energy for lactation; DM = dry matter; FOM = fermentable organic matter; VFA = volatile fatty acids

Morphological Adaptation of the Rumen

It has long been established that the onset of microbial fermentation and production of VFA, and not the physical nature of solid feed itself, are crucial for the development of the rumen papillae in calves (Warner et al., 1956; Flatt et al., 1958; Lane and Jesse, 1997). Simultaneously with the development of the rumen papillae, the capacity for absorption of VFA develops (Sutton et al., 1963). As in calves, changes in the plane of nutrition (changes in daily intake of FOM) have been shown to affect

rumen papillae dimensions in adult cows. Generally, an increase in FOM intake increases VFA production. The VFA can affect the proliferation of the rumen papillae directly through cell mitosis and apoptosis (Sakata and Tamate, 1978, 1979; Mentschel et al., 2001) and through growth factors of, e.g., the IGF axis (Mentschel et al., 2001; Steele et al., 2015). For example, in a classic study by Dirksen et al. (1984), energy intake (through FOM) of two dry non-gravid dairy cows was gradually increased from 47 to 86 MJ NE_L/d. The papillae surface area, length, and width showed a likewise gradual increase in dimensions. In adult dry and lactating cows, papillae dimensions are also related to the energy intake (Melo et al., 2013). Adapting a cow to the lactation ration starting two weeks before parturition resulted in an increase in the cross section of the papillae measured in histological preparations (Liebich et al., 1987), indicating proliferation of the papillae. More recently, Bannink et al. (2012) reported changes in the papillae epithelium associated with the postpartum concentrate build-up strategy which were interpreted as signs of rapid proliferation. However, results from other, more recent experiments into changes in papillae morphology were less conclusive. Two studies on the effects of dry period treatments, aimed at increasing energy intake, on rumen papillae did not report proliferative effects (Rabelo et al., 2001; Reynolds et al., 2004). Moreover, neither was an increase in papillae surface area postpartum observed (Reynolds et al., 2004). One of the explanations for not observing an increase in papillae surface during early lactation when compared to the dry period is that differences in papillae dimension are large between animals (Reynolds et al., 2004). Crucially, both these studies used an end point measurement for determining a treatment effect as rumen wall tissue was collected after sacrificing the animal. In contrast, the studies by Dirksen et al. (1984), Liebich et al. (1987), and Bannink et al. (2012) all used a repeated measurements approach, allowing the study of changes within the animal.

Despite the clear relationship between VFA production rate and development of the rumen papillae, our knowledge of macro- and microscopic morphological changes of the rumen papillae in dairy cows during the dry period and subsequent lactation is severely limited (Martens et al., 2012). As illustrated above, changes in feed intake, ration composition, and daily VFA production around calving are large and rapid. An important, as yet unsatisfactory answered question is whether the observed problems associated with a low rumen pH during the early lactation (Kleen et al., 2003; Plaizier et al., 2012) are the result of an untimely or inadequate adaptation of the rumen. Based on the work by Dirksen et al. (1984) and Liebich et al. (1987) it is suggested that the rumen papillae need 4 to 6 weeks to adapt to a ration change. However, Bannink et al. (2012) suggests a shorter adaptation period early postpartum leaving the matter open for discussion. Besides the work of Bannink et al. (2012), no experiments have examined whether, if at all, dairy cows can adapt differently to different early lactation nutritional management strategies (e.g. differences in rate of increase of concentrate

allowance), possibly hampering our capacity to optimize management of the dairy cow during the transition (Martens et al., 2012).

Changes in Rumen VFA Absorption Capacity

The fractional absorption rate of VFA (k_a**VFA**, fraction of VFA which is removed from the fluid per unit of time) can be interpreted as a measure of the VFA absorption capacity. The k_aVFA is affected by the carbon-chain length of the VFA (Bu > Pr > Ac), rumen fluid pH, VFA concentration, rumen fluid volume (Thorlacius and Lodge, 1973; Dijkstra et al., 1993), epithelial blood flow (Kristensen and Harmon, 2004; Storm et al., 2011), and epithelial capacity for facilitated transport (Penner et al., 2011; Schurmann et al., 2014). In addition, the rate of absorption of VFA has been shown to increase as papillae surface area increases (Dirksen et al., 1984; Melo et al., 2013). Conversely, in vitro studies using isolated sections of rumen wall suggested that the capacity for VFA uptake can increase independent of appreciable increases in papilla surface area (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014). Notwithstanding, dogma currently assumes that the capacity for VFA absorption is in large part dependent on the morphological change of the rumen papillae, specifically papillae surface area. However, the number of published experiments assessing both morphological changes of the papillae and functional changes in the VFA absorption (measured in vivo) in adult ruminants is very limited (Dirksen et al., 1984; Melo et al., 2013). Moreover, especially little is known about the changes in papillae morphology and the VFA absorption capacity during transition from the dry period to early lactation (Martens et al., 2012). Thus it is currently unknown whether stimulating proliferation of the rumen papillae during the late dry period is advantageous for early lactation VFA absorption capacity (which it generally is believed to do), which could possibly alleviate low rumen pH related problems. Likewise, the effects of various early lactation feeding strategies on rumen papillae morphology, and their relation with changes in VFA absorption capacity are currently unknown. Answering these questions will increase our knowledge about rumen adaptation and improve our base of knowledge for optimization of the management of the transition dairy cow and possibly reduce the problems observed during early lactation.

Changes in the Expression of Proteins and Genes Associated with VFA Absorption

In the developing rumen, a higher intake of FOM is associated with upregulation of VFA transporter genes (Yang et al., 2012; Metzler-Zebeli et al., 2013; Yan et al., 2014). However, in adult cows results are ambiguous. For example, feeding

high amounts of grain (Penner et al., 2009; Schlau et al., 2012; Steele et al., 2012) caused high rumen VFA concentrations and concurrent low pH values, but upregulation of VFA transporter genes was generally not observed. During the transition period, some changes in expression of genes associated with VFA transport, metabolism, and proliferation have been observed by Minuti et al. (2015) and Steele et al. (2015) but these studies did not examine VFA absorption in situ, leaving any relationships between gene expression and functional changes largely unknown. Moreover, few studies report changes in expression at the protein level in lieu of expression at mRNA level (Penner et al., 2009; Metzler-Zebeli et al., 2013; Minuti et al., 2015), further limiting functional interpretation of results. Thus, an integrated set of observations on changes in rumen morphology and function, and associated changes in gene expression in response to nutrition is currently still lacking.

To better understand underlying mechanisms for changes in VFA absorption capacity it is necessary to further study the genes and proteins involved. Finding an elevated gene expression independent of an increase in papillae surface area suggests that part of the adaptive response comprises an increase in efficiency (explaining the observations of Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014), whereas not finding such an elevated gene expression suggests that an increase in papillae dimensions is the major adaptive response. However, studying expression of genes alone is not satisfactory as this might also reflect an increase in turnover of protein. For increased efficiency of the tissue, an increase in the concentration of functional proteins would be desirable, thus requiring study of protein expression.

Changes in the Composition of the Rumen Microbiota Community

To meet the nutritional requirements of early lactation dairy cattle, high quality forages and concentrates (high FOM content) are offered, generally containing a substantial fraction of starch and sugar, and an increased fraction of protein compared with a typical dry period ration. In contrast, dry period rations generally contain predominantly lower quality forages (lower FOM, high fiber content, lower digestibility). The type and quantity of feed consumed leads to changes in the rate of substrate fermentation, rumen VFA concentrations, pH, and passage rate, which all interact to affect the composition of the microbiota community (Fernando et al., 2010; Belanche et al., 2012; McCann et al., 2014; Henderson et al., 2015). Various studies addressed this relationship between ration composition and the composition of the rumen microbiota (review by McCann et al., 2014). However, few studies (Mohammed et al., 2012; Pitta et al., 2014; Lima et al., 2015) focused on the transition period in dairy cattle. Unfortunately, these studies generally provide very little information about

feed intake and no studies have reported peripartum changes in papillae morphology, VFA absorption capacity, and their relationship to the changes in the microbiota. Whereas such studies contribute to our knowledge of 'who lives in the rumen' (McCann et al., 2014; Henderson et al., 2015), little is known about the effect of rate of change in ration composition on the rate of change in composition of the rumen microbiota, and if changes in microbiota composition coincide with changes in the capacity of the microbiota to degrade feed. Further understanding of these effects and changes may ultimately improve our management of the transition dairy cow.

OBJECTIVES AND OUTLINE

As outlined above, current knowledge on the adaptation of the rumen to the physiological and nutritional changes during the late dry period and early lactation is scarce. Therefore, the objective of this thesis was to study the adaptation of the rumen to ration changes during the dry period and early lactation. Changes in rumen papillae morphology, fractional rate of VFA absorption, and changes in the composition of the rumen microbiota were the primary targets for study. In addition, the expression of genes and proteins associated with absorption and metabolism of VFA by the rumen epithelium, as well as genes associated with tissue proliferation were studied to better understand the relationship between functional changes and morphological changes of the papillae. Furthermore, in situ incubations of selected substrates were performed to assess the degradation capacity of the microbiota. Uniquely, all these aspects were studied in parallel using a repeated measurement setup.

The central hypothesis of this thesis was that daily intake of FOM is positively related to the VFA production rate and thereby affects rumen papillae morphology and the capacity for VFA absorption and metabolism. It was hypothesized that a greater FOM intake would stimulate papillae growth resulting in a larger absorptive surface area, and that the larger surface area would coincide with a greater capacity for VFA absorption, measured as the fractional VFA absorption rate. Furthermore, the expression of genes and proteins associated with VFA absorption, metabolism and maintenance of intracellular pH was hypothesized to increase with greater FOM intake. Finally, the changes in ration composition and DMI associated with the changes in FOM intake were hypothesized to induce changes in the rumen microbiota. For the two experiments (the lactation experiment and dry period experiment) described in this thesis, manipulation of the concentrate provision in combination with free access (for ad libitum intake) to a basal mixed ration was used to create the treatment contrasts in FOM intake. The concentrates were fed using concentrate dispensers which could be

visited freely by the cow. This allowed for control of the daily intake of concentrate, meal size, and distribution of concentrate intake over the day.

During the lactation experiment the effect of transition from the dry period into the subsequent lactation, and the effect of early lactation concentrate build-up strategy on rumen adaptation were studied. In **Chapter 2**, the changes in morphology of rumen papillae during this experiment are reported, whereas the changes in VFA production rate and VFA absorption capacity are reported in **Chapter 3**. During the dry-period experiment, supplemental concentrate was fed during the late dry period in order to 'prepare' the rumen for lactation. Changes in rumen papillae morphology and VFA absorption capacity were studied and reported in **Chapter 4**. In **Chapter 5**, the changes in the expression of genes associated with VFA absorption and metabolism and tissue proliferation for both experiments and changes in the protein expression of key proteins are reported. The changes in composition and functionality of the rumen microbiota observed during the lactation experiment are reported in **Chapter 6**. Finally, **Chapter 7** comprises a general discussion of the results, suggestions for further research, and provides some recommendations for dairy practice.

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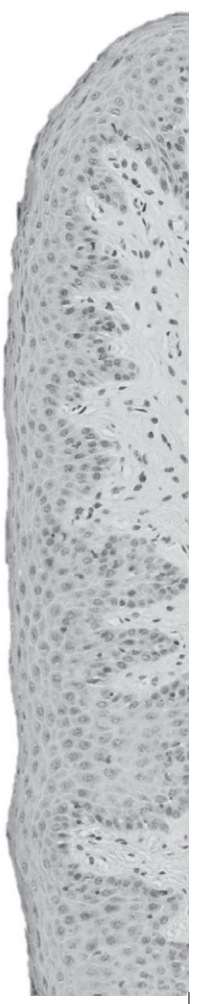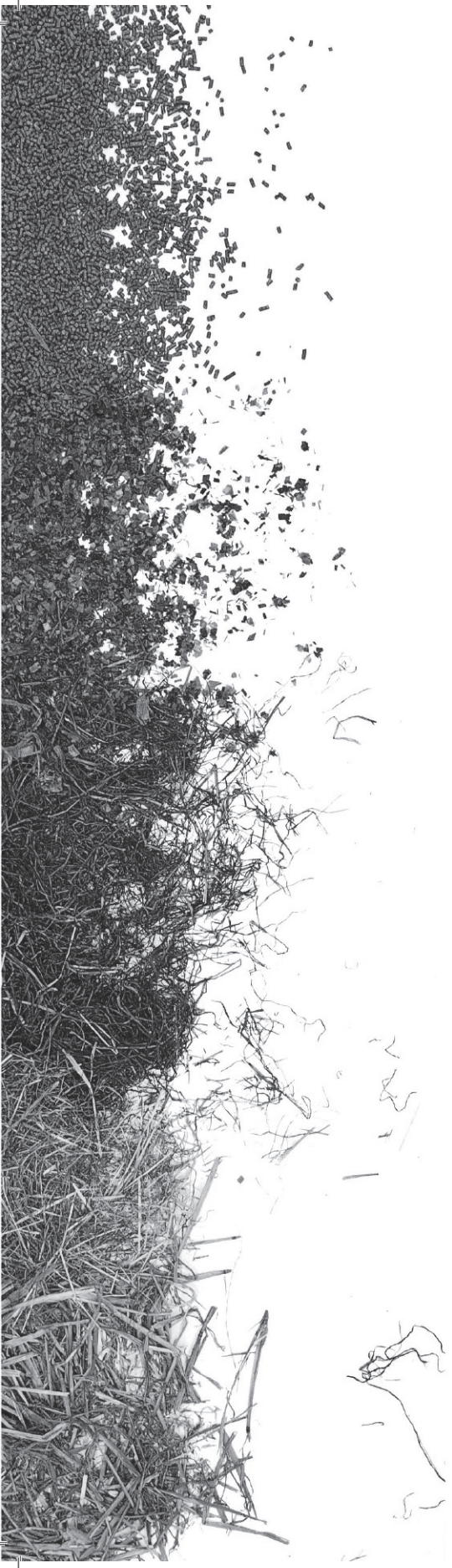
Chapter 1

Chapter 2

Morphological adaptation of rumen papillae during the dry period and early lactation as affected by rate of increase of concentrate allowance

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ABSTRACT

Knowledge of the morphological adaptation of rumen papilla, which plays an important role in volatile fatty acid absorption, in dry and early lactation dairy cattle is limited. Therefore, macro- and microscopic changes in papilla morphology during the dry period and lactation and the effect of rate of increase of concentrate allowance were studied. Samples were collected from 12 rumen-cannulated Holstein-Friesian dairy cows during a pretreatment period, 50, 30, and 10 d antepartum (the dry period) and 3 d postpartum (pp), and a treatment period, 9, 16, 30, 44, 60, and 80 d pp. Cows had free access to either a dry period ration [27% grass silage, 27% corn silage, 35% wheat straw, and 11% soybean meal on a dry matter (DM) basis] or a basal lactation ration (42% grass silage, 41% corn silage, and 17% soybean meal on a DM basis, and 0.9 kg of DM/d concentrate). Treatment consisted of either a rapid (1.0 kg of DM/d; RAP; n = 6) or gradual (0.25 kg of DM/d; GRAD; n = 6) increase of concentrate allowance (up to 10.9 kg of DM/d), starting at d 4 pp, aimed at creating a contrast in rumen-fermentable organic matter (FOM) intake. Papillae were collected from the ventral, ventral blind, and dorsal blind rumen sacs and measured digitally. Intake of DM (11.9 kg/d) and FOM (5.7 kg/d) did not change during the pretreatment period, but increased during the treatment period to 24.5 and 15.0 kg/d at 80 d pp, respectively. Concentrate treatment and sampling day interacted for FOM intake, which was 22% greater in RAP at 16 d pp compared with GRAD. Papilla surface area decreased during the pretreatment period by 19% to 28.0 mm^2 at 3 d pp, thereafter increasing to 63.0 mm^2 at 80 d pp. Concentrate treatment and sampling day interacted for surface area, which was greater in RAP compared with GRAD at 16 (46.0 vs. 33.2 mm^2), 30 (55.4 vs. 41.2 mm^2), and 44 (60.5 vs. 49.7 mm^2) days pp, showing that papillae can respond to a rapid rate of increase of FOM intake by increasing growth rate. Microscopic morphology was affected by sampling day, but neither by concentrate treatment nor by their interaction, with a decrease in papilla and epithelium thickness during the lactation. In conclusion, the rumen papillae respond to changes in FOM intake and the magnitude of this response depends on the rate of increase of FOM intake. This response in surface area of the rumen papillae potentially facilitates the absorption of the volatile fatty acids.

Key words: transition dairy cow, rumen papillae, rumen epithelium, rumen adaptation

INTRODUCTION

In ruminants, the reticulorumen is the major site for microbial fermentation of feed and, hence, production of VFA, which provide approximately 75% of total ME (Bergman, 1990). The luminal surface of the rumen wall is covered with papillae which increase the surface area for absorption of VFA. However, factors such as rumen VFA concentration and pH (Dijkstra et al., 1993), epithelial blood flow (Storm et al., 2012), and changes at the cellular level (Penner et al., 2011) may affect VFA absorption capacity as well. The absorption of VFA is seen as the primary process for maintaining rumen VFA concentration and pH within physiological limits, thereby supporting microbial fermentation of feed (Penner et al., 2009; Aschenbach et al., 2011; Dijkstra et al., 2012).

The proliferation of rumen papillae in young ruminants is triggered by the consumption of solid feed (Tamate et al., 1962; Sutton et al., 1963; Suárez et al., 2006), and thus associated with the intake of rumen fermentable organic matter (**FOM**) and its associated production of VFA. Furthermore, Dirksen et al. (1984) and Liebich et al. (1987) reported a positive relationship between the plane of nutrition and papilla surface area in the rumen. Therefore, it can be suggested that differences in surface area of rumen papilla are associated with differences in intake of FOM.

Currently, knowledge of macroscopic morphological changes of the rumen papillae (papilla surface area, length, and width) in dairy cows in the dry period until early lactation is limited (Shen et al., 2005; Malhi et al., 2013; Steele et al., 2015), or experiments have yielded inconclusive results (Reynolds et al., 2004). Likewise, knowledge about changes on the microscopic scale in rumen papilla (thickness of the papilla and epithelial layer) in the aforementioned period is limited as well. Diet- and time-related changes in thickness and organization of rumen epithelium have been reported by Dirksen et al. (1984), Liebich et al. (1987), Steele et al. (2011), and Bannink et al. (2012), but a comprehensive description of those changes during the dry period and early lactation is currently lacking (Martens et al., 2012). Therefore, the aim of the present experiment was to study macro- and microscopic morphological changes of rumen papillae from 50 d before expected calving date until 80 d postpartum in dairy cattle. After calving, 2 rates of increase of concentrate allowance were applied to create a contrast in FOM intake and evaluate its effect on morphological changes of the papillae. It was hypothesized that a rapid versus a gradual rate of increase of concentrate allowance creates a temporarily larger daily intake of FOM, inducing a greater rate of increase of surface area, length, and width of rumen papilla and a decrease in thickness of the papilla and its epithelial layer.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen UR and conducted under the Dutch Law on Animal Experiments.

Animals, Experimental Design and Management

Three months before the start of the experiment 12 first-parity Holstein-Friesian dairy cows were fitted with a rumen cannula (10 cm i.d., Bar Diamond Inc., Parma, ID). Eight weeks before the expected date of calving, cows were dried off and entered the experiment. The experiment had a randomized block design with repeated measurements. Cows were blocked by expected date of calving, and within each block cows were randomly assigned to either a rapid rate of increase of concentrate allowance or a gradual rate of increase of concentrate allowance. Samples were collected during the pretreatment period at 50, 30, and 10 d antepartum (**ap**) and 3 d postpartum (**pp**), as well as during the treatment period at 9, 16, 30, 44, 60, and 80 d pp. Cows were milked at 0530 and 1530 h, and milk yield was recorded daily. Each week milk samples from 4 consecutive milkings (same days every week) were analyzed for fat and protein content (ISO, 1999c; Qlip NV, Zutphen, the Netherlands). During the experiment, dry and lactating animals were housed in separate groups in a freestall barn with concrete slatted floor. Stalls were fitted with rubber mattresses covered with sawdust. On sampling days, cows were moved to a tiestall after the morning milking for the experimental procedures.

Rations and Experimental Treatments

Cows had free access to either a dry period ration or a basal lactation ration (Table 1) and free access to water throughout the experiment. Both rations had similar relative proportions of grass silage, corn silage and soybean meal (DM basis). In the dry period ration, however, chopped wheat straw was included to lower the energy density of the ration. The rations were mixed and fed once a day at ~1000 h. Concentrates were fed from calving onwards, with a daily allowance of 0.9 kg of DM/d, up to 3 d pp, irrespective of treatment. Thereafter, the concentrate treatment started and concentrate allowance was increased at either a rapid rate of 1.0 kg of DM/d (**RAP**) or a gradual rate of 0.25 kg of DM/d (**GRAD**). Maximum concentrate allowance was set at 10.9 kg of DM/d, irrespective of rate of increase, and was achieved at 13 and 43 d pp for RAP and GRAD, respectively.

Table 1. Basal ration composition, nutritional value, and chemical composition of rations and concentrate (g/kg of DM, unless stated otherwise)

	Basal ration		Concentrate[1]
	Dry period	Lactation	
Ingredients			
Grass silage[2]	273	419	
Corn silage[3]	270	414	
Soybean meal	108	167	
Wheat straw	349	0	
Chemical composition			
DM, g/kg of product	603	466	892
Crude ash	80	75	68
Crude protein	109	157	178
Crude fat	24	30	17
Starch	90	139	248
Sugars	44	67	95
NDF	553	392	252
ADF	325	235	115
ADL	32	16	8
Calculated value[4]			
NE_L, MJ/kg of DM	5.3	6.7	7.4
IDP	57	90	114
RDPB	3	14	10
FOM	455	561	682

[1]Concentrate composition (ingredients, g/kg): sugar beet pulp, 212; corn gluten, 200; barley, 150; wheat, 150; soybean meal, 120; citrus pulp, 100; molasses, 35; vinasse, 20; $CaCO_3$, 8; salt, 3; and mineral premix, 2.

[2]Chemical composition of grass silage (g/kg of DM): crude ash, 105; CP, 110; crude fat, 31; sugars, 113; NDF, 497; ADF, 302; and ADL, 18.

[3]Chemical composition of corn silage (g/kg of DM): crude ash, 44; CP, 64; crude fat, 31; starch, 330; NDF, 403; ADF, 231; and ADL, 17.

[4]Calculated values for grass and corn silage based on near-infrared spectrometry, Blgg AgroXpertus (Wageningen, the Netherlands). Calculated values for soybean meal and concentrate provided by Agrifirm Feed (Apeldoorn, the Netherlands). Calculated values for wheat straw obtained from CVB Feed Tables 2011 (CVB, 2011). NE_L values calculated according to the Dutch NE-system (van Es, 1978). IDP = intestinal digestible protein; RDPB = rumen-degradable protein balance; FOM = fermentable organic matter; all calculated according to the Dutch DVE/OEB-system (Tamminga et al., 1994).

Daily intake of the dry period ration or the basal lactation ration (kg/d) was measured individually using feed bins (Insentec, Marknesse, the Netherlands), with a maximum stocking density of 2 cows/bin. Cows had access to all feed bins. Concentrate was fed using a concentrate dispenser (Manus VC5, DeLaval, Steenwijk, the Netherlands) and made the individual daily allowance available in equal portions over six 4-h periods and recorded the quantity actually dispensed (kg/d). Concentrate and ration ingredient samples were taken once a week. One sample of concentrate and one of each ration ingredient was immediately used for DM determination by forced-air oven drying (105°C, 24 h); the remaining samples were stored at –20°C pending analyses. If necessary, basal ration formulation (on product basis) was adjusted for changes in ration ingredient DM content; DM content of concentrate was constant throughout the experiment.

After forced-air oven drying (60°C, 24 h) and determination of DM (ISO, 1999b), feed samples were ground (1-mm screen) and pooled (each pool containing samples of 4 consecutive weeks) before determination crude ash (ISO, 2002), crude fat (ISO, 1999a), starch (ISO, 2004), and sugars (van Vuuren et al., 1993). Crude protein was calculated from nitrogen (N × 6.25) obtained by the Kjeldahl method (ISO, 2005). The NDF (with heat stable α-amylase), ADF, and ADL were determined according to van Soest et al. (1991) using an Ankom 2000 Fiber Analyzer (Ankom Technology Corp., Macedon, NY). The NE_L was calculated according to van Es (1978); intestinal digestible protein, RDP balance, and FOM were calculated according to Tamminga et al. (1994).

Rumen Fluid Sampling and Papillae Collection

Approximately 1 h after last access to the feed bins and, during lactation, the concentrate dispenser, a 200-mL rumen fluid sample was taken from the ventral rumen sac using a stainless steel sampling probe (2-mm pore size). Immediately after collection, rumen fluid pH was measured and a 600-µL aliquot was taken, acidified with 600 µL of 5% H_3PO_4 (vol/vol), and subsequently stored at –20°C pending analysis for acetic, propionic, and butyric acid concentration by GC as described by van Gastelen et al. (2015). Thereafter, rumen content solids were removed by hand and rumen liquid using a 1-L wide-mouth plastic jar. The rumen compartments were visually and manually inspected for remaining contents to ensure complete evacuation. The rumen contents were stored in an insulated tub and covered to prevent cooling until return into the rumen. Prior to the collection of rumen papillae, the rumen was washed twice with 10 L of tap water at 39°C. After introduction, the warm water was manually scooped against

the rumen wall of all compartments. After each wash, water was removed using a 1-L wide-mouth plastic jar.

Biopsies were taken from 3 sites in the rumen (Dirksen et al., 1984; Lesmeister et al., 2004; Bannink et al., 2012): the ventral rumen sac, directly opposite of the rumen cannula, 15 cm cranial of the caudal coronary pillar, and 20 cm below the dorsal limit of the papillae mat; the caudoventral blind rumen sac, opposite the rumen cannula, halfway between the dorsal and ventral limit of the caudoventral blind rumen sac, and 15 cm caudal of the ventral coronary pillar; and the caudodorsal blind rumen sac, opposite the rumen cannula halfway between the dorsal and ventral limit of the caudodorsal blind rumen sac, and 15 cm caudal of the dorsal coronary pillar (Dyce et al., 1996). Papillae were extracted from the rumen wall using forceps (No. 631319, Stuemer, Würzburg, Germany) and gently rinsed in 0.9% NaCl solution before storage in buffered 4% formalin solution. In line with Odongo et al. (2006) and Steele et al. (2011, 2015), at least 6 intact papillae per site per cow per sampling day were collected. Papillae damaged by the biopsy forceps were easily recognized as damaged papillae, as they showed distinctive circular cut marks.

Macroscopic Measurements

For macroscopic measurements (per site per cow per sampling day), all papillae were photographed using a digital camera (Casio Exilim EX-Z70, Casio Computer Co., Tokyo, Japan), including a ruler in each photograph. The one-sided surface area of all papillae was measured using the Analyze Particles feature of ImageJ (Version 1.44n, National Institutes of Health, Bethesda, MD), comparable to the Geographical Information System (GIS) method described by Scocco et al. (2012). Length and width of all papillae was measured using ImageScope (Version 11.2.0.780, Aperio Technologies, Leica Biosystems, Nußloch, Germany). Papilla length was measured from the tip to the base of the papilla along its axis, papilla width was measured halfway of and perpendicular to the papilla length.

Microscopic Measurements

Five papillae per site per cow per sampling day (Odongo et al., 2006; Steele et al., 2011, 2015) were processed (minimum of 6 mo after collection and storage in 4% buffered formalin) for histological examination (Veterinary Pathology, Faculty of Veterinary Medicine, Utrecht University). After stepwise dehydration and paraffin infiltration, papillae were mounted parallel to each other before being fully embedded

in paraffin. Longitudinal sections, 4-μm thick, perpendicular to the flat surface (median sagittal plane), were made halfway across the papilla width (coronal plane). Two consecutive sections were mounted on glass slides and stained with hematoxylin and eosin (Dobson et al., 1956; Andersen et al., 1999). Slides were digitized at 40× magnification (Department of Pathology, Utrecht Medical Center) using an Aperio ScanScope slide scanner (Aperio Technologies, Leica Biosytems). Complementary software was used for measurements (ImageScope Version 11.2.0.780, Aperio Technologies, Leica Biosytems). Starting at the top-left of the slide, each pair of papilla sections was evaluated for impairing artifacts and the first 3 suitable papillae were used for subsequent assessments.
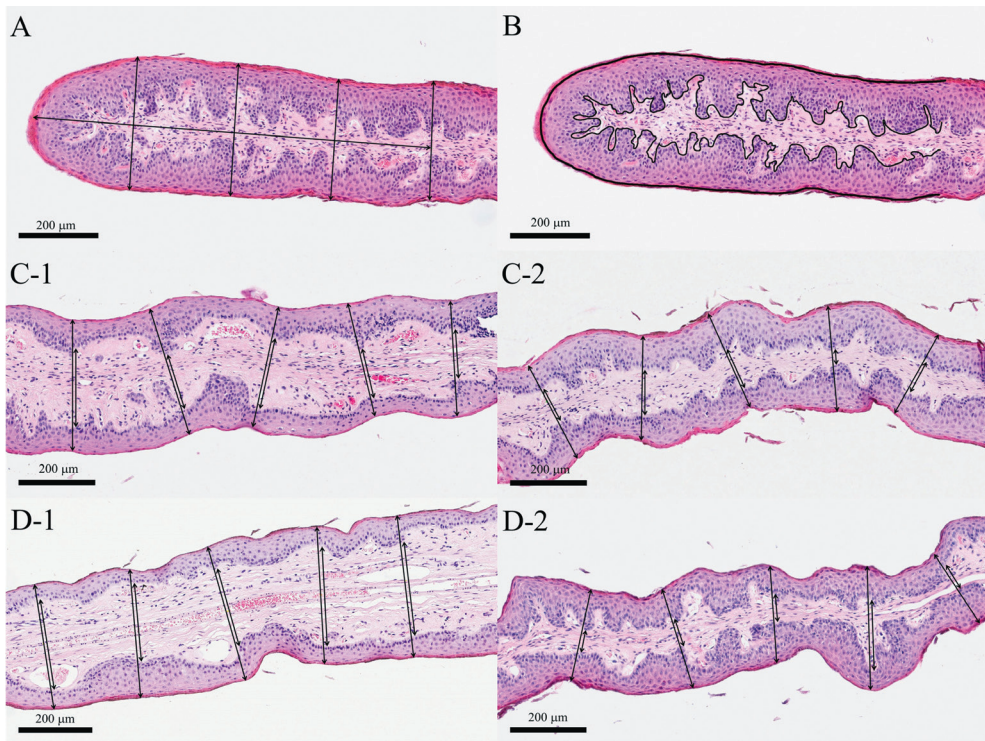
Figure 1. (A) Total thickness of the papilla at the tip (measured perpendicular to papilla length, indicated by vertical arrows) at 250, 500, 750, and 1,000 μm from the tip. (B) Length of the luminal boundary of the stratum corneum (thick outer line) and the length of the basal boundary of the stratum basale (thin inner line) in 1,000 μm of papilla length, starting from the papilla tip. (C and D) Total thickness of the papilla in the middle and thickness of the interstitium (both measured perpendicular to papilla length, approximately halfway the papilla length), measured 5 times with 250-μm intervals over a total length of 1,000 μm. A and B represent papillae at 16 d postpartum; C1 and C2 represent papillae at 10 d antepartum for future gradual (0.25 kg of DM/d; GRAD) and rapid (1.0 kg of DM/d; RAP) rate of increase of concentrate allowance groups, respectively; D1 and D2 represent papillae at 16 d postpartum for GRAD and RAP groups, respectively. All papillae shown were from the ventral rumen sac, 4-μm thick section, with hematoxylin-eosin stain.

1.   Total thickness of the papilla (measured perpendicular to papilla length) at 250, 500, 750, and 1,000 μm from the tip (Figure 1A).
2.   Length of the luminal boundary of the stratum corneum and the length of the basal boundary of the stratum basale in 1,000 μm of papilla length, starting from the papilla tip (Figure 1B).
3.   Total thickness of the papilla and thickness of the interstitium (both measured perpendicular to papilla length) was measured approximately halfway the papillae length, each 5 times with 250-μm intervals in both directions over a total length of 1,000 μm (Figure 1C and D).

Calculations and Statistical Analysis

Daily DMI was calculated after correction for the weekly variation in DM content of ration components. For each sampling day, the reported DM and nutrient intakes are the averages of intakes recorded during the 3 preceding days.

For all papilla variables, averages were calculated per site per cow per sampling day [an average of 11.2 (SD = 2.9) papillae per site per cow per sampling day were collected]. Papilla surface area was calculated as $2 \times$ one-sided surface area as measured. The thickness of the epithelial layer was calculated from the difference in thickness of the total papilla and thickness of the interstitium. Average of papilla surface area growth (mm^2/d) and daily change in FOM intake (kg/d) were calculated by dividing the difference in surface area and FOM intake by the difference in days for 2 consecutive sampling days. Total VFA concentration in rumen fluid was calculated as the sum of acetic, propionic, and butyric acid.

Papilla variables were assumed to be related to sampling day, biopsy site, and treatment. Milk production and feed intake were assumed to be related to sampling day and treatment, and milk fat and protein content were assumed to be related to sample week and treatment. Data on papilla variables were analyzed using the MIXED procedure in SAS 9.2 (SAS Institute Inc., Cary, NC) with the model (Littell et al., 2006):

$$Y_{ijkl} = \mu + T_i + S_k + D_l + (T \times D)_{il} + C_j + D(C)_{jl} + e_{ijkl},$$

where variable Y_{ijkl} was dependent on μ as the average experimental value and fixed main effects of concentrate treatment T_i (i = RAP, GRAD), sampling day D_l (l = –50,…, 80), site S_k (k = ventral rumen sac, caudoventral blind rumen sac, caudodorsal blind rumen sac), and fixed interaction $(T \times D)_{il}$. All other interactions were not significant and

removed from the model. Cows, as experimental units, were represented with random effect C_j ($j = 1,\ldots, 12$). Furthermore, random effects of sampling day within cows $D(C)_{jl}$ were allowed, avoiding pseudo-replication due to multiple biopsy sites S_k. Errors e_{ijkl} were assumed to be autocorrelated for repeated observations on the same cow and site using a spatial power covariance structure over sampling days (Littell et al., 1998). As concentrate treatment was only applicable to the treatment period (sampling d 9 to 80), the design could not be treated as a complete factorial design with respect to concentrate treatment and sampling day. The mixed model, however, was formulated as if a complete factorial design was in place, allowing treatment groups to differ before the treatment period. This structure was exploited by specifying custom hypotheses tests (ignoring default tests for main effects for sampling day, treatment, and their interaction, as made by PROC MIXED). Custom CONTRAST statements were constructed for: (1) the pretreatment period (sampling d –50 to 3), to compare the 2 future treatment groups, thereby evaluating the success of the random treatment allocation [neither differences between groups were expected, nor found ($P \geq 0.268$)]; (2) testing the treatment main effect, T_i (averaged over sampling days), over the treatment period; (3) testing the sampling day main effect, D_l (averaged over treatments), over the total experimental period; and (4) testing the interaction between treatment and sampling day, $(T \times D)_{il}$, by testing sampling day by sampling day difference between concentrate treatment groups over the treatment period. To test specific hypotheses, CONTRAST and ESTIMATE statements were formulated to separate means. For analyses of feed intake, rumen pH and VFA, weekly milk production, milk fat and protein contents, papilla surface area by site, change in FOM intake, and change in papilla surface area, a simpler mixed model was used: fixed effect S_k and random effect $D(C)_{jl}$ were removed, cow replaced site(cow) as subject of repeated measurement, and, where appropriate, D_l was replaced by $Week_t$. All results are reported as least squares means with their standard error unless indicated otherwise. Significance of effect was declared at $P < 0.050$ and trends at $0.050 \leq P < 0.100$.

RESULTS

All cows completed the experiment. One cow in group GRAD calved earlier than expected and missed sampling at 10 d ap; otherwise all samples could be collected. Average (SD) actual sampling days were 46.3 (5.1), 26.5 (5.7), and 8.6 (3.4) d ap for the pretreatment and 3.0 (0.0) d pp and 9.0 (0.0), 16.1 (0.3), 30.2 (0.3), 44.2 (0.4), 60.0 (0.9), and 79.3 (2.2) d pp for the treatment period. One animal was treated for mastitis (from RAP) and 7 were treated for (chronic) endometritis (4 from RAP, 3 from GRAD). All animals reacted favorably to veterinary treatment and followed their experimental treatments and sampling schedule as planned. The rapid or gradual rate of increase of concentrate allowance was readily accepted by all cows.

Intake of DM and Macronutrients

Intake of DM and macronutrients is presented in Table 2; in addition, daily DMI is shown in Figure 2A. Total DMI was affected by sampling day ($P < 0.001$). During the pretreatment period total DMI remained similar over the sampling days (P = 0.277), and during the pretreatment period future RAP and GRAD groups showed no differences in total DMI (P = 0.416) or, consequently, in intake of FOM, starch, sugars, and NE_L ($P \geq 0.325$; data not shown).

Table 2. Least squares means of feed intake[1] (kg/d unless otherwise noted) for rapid rate of increase (1.0 kg of DM/d) of concentrate allowance (RAP; n = 6) and gradual rate of increase (0.25 kg DM/d) of concentrate allowance (GRAD; n = 6) treatments

| | Sampling day relative to calving | | | | | | | | | | | Fixed effects[2] | | |
| | Pretreatment period | | | | Treatment period | | | | | | | | | |
Item	-50	-30	-10[3]	3	9	16	30	44	60	80	SE[4]	T	D	T × D
Total dry matter intake														
RAP	12.4	12.2	12.0	11.1	15.8	19.8	22.3	23.6	23.1	24.2	0.54	0.757	<0.001	0.116
GRAD		12.2	12.0	11.1	15.8	17.6	22.5	24.6	25.0	24.7				
Basal ration DMI														
RAP	12.4	12.2	12.0	11.1	11.3	8.9	11.3	12.7	12.1	13.1	0.54	0.002	<0.001	<0.001
					*	***	***		†					
GRAD		12.2	12.0	11.1	14.0	14.0	15.2	13.8	14.0	13.7				
Fermentable OM intake														
RAP	5.6	5.6	5.4	6.0	9.3	12.3	13.8	14.4	14.2	14.8	0.27	0.574	<0.001	0.001
						***			†					
GRAD		5.6	5.4	6.0	8.9	10.1	13.3	14.9	15.2	15.1				
Starch intake														
RAP	1.0	1.1	1.2	1.5	2.7	3.6	4.0	4.2	4.0	4.2	0.07	0.108	<0.001	<0.001
					†	***	†		*					
GRAD		1.1	1.2	1.5	2.4	2.8	3.7	4.3	4.4	4.3				
Sugar intake														
RAP	0.6	0.6	0.5	0.7	1.1	1.6	1.7	1.8	1.8	1.9	0.03	0.217	<0.001	<0.001
						**								
GRAD		0.6	0.5	0.7	1.1	1.2	1.6	1.9	1.9	1.9				
NE$_L$ intake, MJ/d														
RAP	65.9	65.5	64.5	71.2	108.5	141.0	158.0	166.2	163.0	169.9	3.17	0.814	<0.001	0.004
									†					
GRAD		65.5	64.5	71.2	105.9	119.2	155.0	172.3	175.4	173.3				

[1]Reported mean is calculated over the 3 d preceding the sampling day.

[2]T = treatment, P-value for treatment (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d −50 to 80); T × D = P-value for treatment period (d 9 to 80).

[3]Group GRAD: n = 5, 1 cow had no measurement at d −10 due to early calving.

[4]Standard error for LSM by sampling day; n = 12.

†$P < 0.100$, *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$, significance of difference in LSM of RAP or GRAD for the same sampling day and variable.
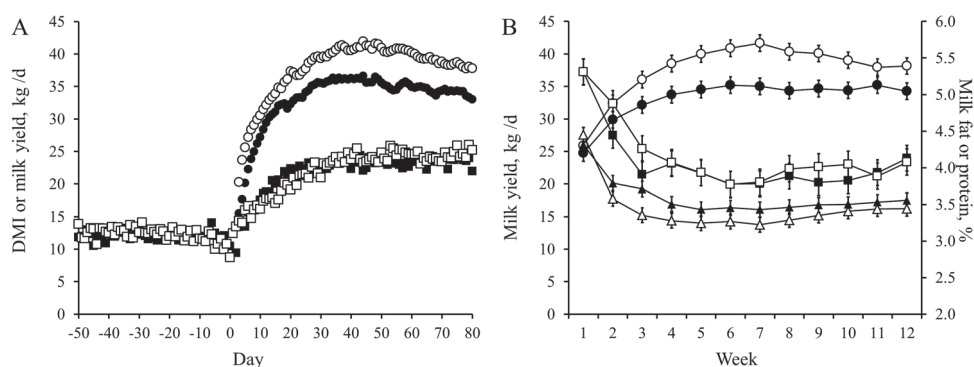
Chapter 2

Figure 2. (A) Average DMI (kg/d; ■□) or milk production (kg/d; ●○) for a rapid (1.0 kg of DM/d; RAP, ■●, n = 6) or gradual (0.25 kg of DM/d; GRAD, □○, n = 6) rate of increase of concentrate allowance postpartum. (B) Average milk production (kg/d; ●○), milk fat content (%; ■□), or milk protein content (%; ▲△) by lactation week for a rapid (1.0 kg of DM/d; RAP, ●■▲, n = 6) or gradual (0.25 kg of DM/d; GRAD, ○□△, n = 6) rate of increase of concentrate allowance postpartum. Values represent average of 4 consecutive milkings; 2 milkings/d. Day = day relative to calving. Values represent LSM ± SE.

In the treatment period, total DMI increased from 3 to 30 d pp for group RAP and tended to increase further to 80 d pp ($P = 0.062$). For group GRAD, DMI increased to 44 d pp ($P \leq 0.004$), remaining similar thereafter. Total DMI was neither affected by the concentrate treatment nor by an interaction between concentrate treatment and sampling day. Concentrate treatment, sampling day, and their interaction affected DMI of the basal ration, which was higher for group GRAD at 9, 16, and 30 d pp, respectively ($P \leq 0.015$). In group RAP, DMI from the basal ration was 21.2% lower ($P \leq 0.006$) at 16 d pp compared with 9 or 30 d pp, whereas this decrease was not observed in group GRAD ($P \geq 0.156$).

In the treatment period, FOM intake was affected by sampling day ($P < 0.001$); in contrast to total DMI, an interaction between concentrate treatment and sampling day was observed ($P < 0.001$). Intake of FOM was 21.8% greater in group RAP than group GRAD at 16 d pp ($P < 0.001$). From 30 d pp onwards, FOM intake was similar for RAP and GRAD, but at 60 d pp tended to be greater for GRAD than RAP ($P = 0.062$). The intake of starch, sugar, and NE_L was affected by sampling day ($P < 0.001$), and an interaction between concentrate treatment and sampling day ($P \leq 0.004$) was observed. Intake of starch, sugar, and NE_L was greater for group RAP at 16 d pp ($P \leq 0.001$), and starch intake tended to be greater at 9 and 30 d pp ($P \leq 0.083$) compared with group GRAD. At 60 d pp, starch intake was higher ($P = 0.014$) and NE_L tended ($P = 0.055$) to be higher in GRAD compared with RAP.

Chapter 2

Rumen Content Mass, VFA Concentration, and pH

Rumen content mass (solids and liquid) was affected by sampling day, increasing from 64.5 ± 2.8 kg during the pretreatment to 77.2 ± 2.8 kg during the treatment period ($P < 0.001$), but was not affected by concentrate treatment ($P = 0.826$) nor its interaction with sampling day ($P = 0.154$). Total VFA (mM) and rumen fluid pH (Figure 3A) were affected by sampling day ($P < 0.001$). Total rumen VFA concentration increased during the pretreatment period ($P = 0.011$), whereas pH decreased ($P < 0.001$). Total VFA averaged 79.5 ± 5.2 mM during the dry period, –50 to –10 d ap, increasing to 96.4 ± 5.2 mM at 3 d pp ($P = 0.002$), whereas fluid pH averaged 6.71 ± 0.09 during the dry period and decreased to 6.27 ± 0.09 at 3 d pp ($P < 0.001$). In the treatment period, total VFA increased to 120 ± 5.2 mM at 44 d pp ($P < 0.001$), whereas pH tended to decrease to 6.07 ± 0.09 at 44 d pp ($P = 0.097$); thereafter both remained similar up to 80 d pp. Neither total VFA nor pH were affected by concentrate treatment ($P \geq 0.538$) nor its interaction with sampling day ($P \geq 0.289$).
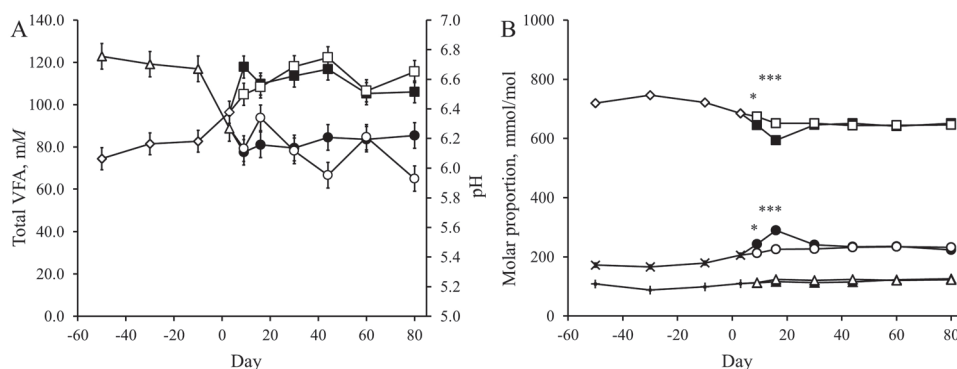
Figure 3. (A) Average of total VFA concentration (mM; ◇■□) or pH (△●○) in the pretreatment period (◇△; n = 12) and in the treatment period for a rapid (1.0 kg of DM/d; RAP, ■●, n = 6) and gradual (0.25 kg of DM/d; GRAD, □○, n = 6) rate of increase of concentrate allowance postpartum. (B) Molar proportions (mmol/mol) of acetic (◇■□), propionic (×●○), or butyric acid (+▲▲) in the pretreatment period (◇×+; n = 12) and in the treatment period for a rapid (1.0 kg of DM/d; RAP, ■●▲, n = 6) and gradual (0.25 kg of DM/d; GRAD, □○△, n = 6) rate of increase of concentrate allowance postpartum. Day = day relative to calving. Values represent LSM ± SE. *$P < 0.050$, ***$P < 0.001$, significance of difference in LSM of RAP or GRAD.

The molar proportions of acetic, propionic, and butyric acid in the rumen fluid (Figure 3B) were affected by sampling day ($P < 0.001$). Molar proportion of acetic acid was larger ($P < 0.001$) during the pretreatment period compared with the treatment

period (718 ± 7 vs. 645 ± 7 mmol/mol, respectively), whereas the molar proportions of propionate and butyrate were smaller ($P < 0.001$) during the pretreatment period (181 ± 7 and 101 ± 3 mmol/mol, respectively) compared with the treatment period (236 ± 7 and 119 ± 3 mmol/mol, respectively). An interaction was observed between concentrate treatment and sampling day for molar proportions of acetic and propionic acid ($P \leq$ 0.003), but not for butyric acid ($P = 0.501$). The molar proportion of acetic acid in group RAP was 4.5 and 8.7% lower at 9 and 16 d pp, respectively, compared with group GRAD, with an opposite effect for molar proportion of propionic acid.

Macroscopic Rumen Papillae Assessment

Papilla surface area (Table 3) was affected by sampling day ($P < 0.001$). During the pretreatment period, papilla surface area decreased by 18.6% ($P = 0.018$), caused by a 17.8% decrease ($P < 0.001$) in papilla width. The future RAP and GRAD groups showed neither a difference in papilla surface area ($P = 0.520$) nor in length or width ($P \geq 0.268$; data not shown) in the pretreatment period. More specifically, at 3 d pp, the day before starting the concentrate treatment, papilla surface area was similar for RAP and GRAD ($P = 0.306$), as were papilla length and width ($P \geq 0.156$). During the treatment period, papilla surface area increased by 125% from 28.0 mm^2 at 3 d pp to 63.0 mm^2 at 80 d pp ($P < 0.001$). Concentrate treatment interacted with sampling day for papilla surface area ($P = 0.011$) and from 16 through 44 d pp papilla surface area was larger in group RAP ($P \leq 0.013$) compared with GRAD.

Papilla length increased by 39% ($P < 0.001$) and width by 50% ($P < 0.001$) from 3 to 80 d pp. Papilla length tended to be affected by concentrate treatment ($P = 0.058$) without concentrate treatment interacting with sampling day, whereas papilla width was affected by concentrate treatment ($P = 0.041$) and by an interaction between concentrate treatment and sampling day ($P = 0.020$). Group RAP had wider papillae at 16 and 30 d pp ($P \leq 0.035$) and tended to have wider papillae at 44 d pp ($P = 0.053$) when compared with GRAD.

Between sampling sites, papilla surface area differed (Figure 4; $P = 0.001$). Papillae from the ventral rumen sac (46.0 ± 1.9 mm^2) had a larger surface area ($P \leq$ 0.017) compared with the ventral blind (39.3 ± 1.9 mm^2) and dorsal blind (41.8 ± 1.9 mm^2) rumen sacs, which did not differ ($P = 0.160$). All sites were affected by sampling day ($P < 0.001$) and showed a similar development of papilla surface area.

Chapter 2

Table 3. Least squares means of papilla surface area, length, and width for rapid rate of increase (1.0 kg of DM/d) of concentrate allowance (RAP; n = 6) and gradual rate of increase (0.25 kg of DM/d) of concentrate allowance (GRAD; n = 6) treatments

Item	Sampling day relative to calving										SE[3]	Fixed effects[1]			
	Pretreatment period				Treatment period							T	D	S	T × D
	-50	-30	-10[2]	3	9	16	30	44	60	80					
Papilla surface area,[4] mm^2						**	**	*							
RAP	34.4	31.7	30.2	28.0	35.2	46.0	55.4	60.5	64.7	66.1	2.03	0.015	<0.001	0.001	0.011
GRAD					28.6	33.2	41.2	49.7	58.4	59.9					
Papilla length, mm						*	**	†							
RAP	7.8	7.5	7.8	7.5	8.4	9.1	9.7	10.1	10.5	10.6	0.25	0.058	<0.001	<0.001	0.199
GRAD					7.4	7.8	8.7	9.2	10.3	10.3					
Papilla width, mm						*	**	†							
RAP	2.8	2.5	2.4	2.3	2.7	3.0	3.4	3.6	3.6	3.4	0.10	0.041	<0.001	0.183	0.020
GRAD					2.2	2.5	2.9	3.2	3.6	3.5					

[1]T = treatment, P-value for treatment period (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d −50 to 80); S = sampling site; T × D = P-value for treatment period (d 9 to 80).

[2]Group GRAD: n = 5; 1 cow no measurement at d −10 due to early calving.

[3]Standard error for LSM by sampling day, n = 12.

[4]Average number of papillae collected per site per cow per sampling day was 11.2 (SD = 2.9). Mean of coefficients of variation were 0.22, 0.14, and 0.17 for papilla surface area, length, and width, respectively.

†$P < 0.100$, *$P < 0.050$, **$P < 0.010$, significance of difference in LSM of RAP or GRAD for the same sampling day and variable.

Table 4. Least squares means of total papilla thickness at the tip and at the middle, epithelium thickness at the papilla middle, and ratio of stratum basale to stratum corneum for rapid rate of increase (1.0 kg of DM/d) of concentrate allowance (RAP; n = 6) and gradual rate of increase (0.25 kg of DM/d) of concentrate allowance (GRAD; n = 6) treatments

Item	Sampling day relative to calving										SE³	Fixed effects¹			
	Pretreatment period			Treatment period								T	D	Site	T × D
	-50	-30	-10²	3	9	16	30	44	60	80					
Total papilla thickness tip, mm															
RAP	0.33	0.34	0.35	0.35	0.36	0.34	0.33	0.31	0.32	0.30	0.013	0.378	<0.001	0.003	0.399
GRAD	0.35	0.35	0.35	0.35	0.39	0.36	0.37	0.33	0.33	0.32					
Papilla thickness middle, mm															
RAP	0.25	0.26	0.28	0.27	0.27	0.25	0.23	0.23	0.22	0.22	0.009	0.306	<0.001	0.026	0.497
GRAD	0.28	0.26	0.28	0.27	0.29	0.28	0.27	0.23	0.23	0.22					
Epithelial thickness middle, µm															
RAP	66	68	74	72	73	71	69	65	68	66	2.12	0.944	0.002	0.002	0.805
GRAD	74	68	72	72	73	72	73	65	65	62					
Ratio of length stratum basale to stratum corneum															
RAP	1.71	1.79	1.84	1.76	1.95	1.71	1.67	1.58	1.68	1.63	0.035	0.739	<0.001	<0.001	0.330
GRAD	1.93	1.79	1.84	1.76	1.93	1.80	1.78	1.60	1.63	1.58					

¹T = treatment, P-value for treatment period (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d –50 to 80); S = sampling site; T × D = P-value for treatment period (d 9 to 80).

²Group GRAD: n = 5; 1 cow no measurement at d –10 due to early calving.

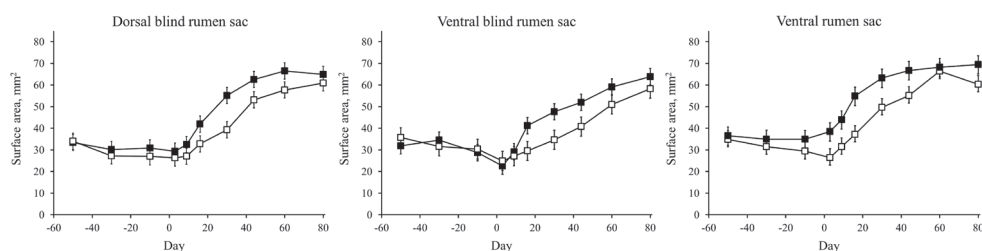³Standard error for LSM by sampling day; n = 12.

Figure 4. Papilla surface area (mm²) at the 3 rumen sampling sites for a rapid (1.0 kg of DM/d; RAP, ■, n = 6) and gradual (0.25 kg of DM/d; GRAD, □, n = 6) rate of increase of concentrate allowance postpartum. Day = day relative to calving. Values represent LSM ± SE.

Concentrate treatment affected the papilla surface area in the ventral rumen sac and the dorsal blind rumen sac ($P ≤ 0.031$), but did not affect the area in the ventral blind rumen sac ($P = 0.105$). A tendency for an interaction between concentrate treatment and sampling day was observed for papilla surface area in the ventral rumen sac ($P = 0.068$), but not in the ventral blind rumen sac ($P = 0.140$) and the dorsal blind rumen sac ($P = 0.159$).

Microscopic Rumen Papillae Assessment

Thickness of the rumen papillae at the tip and in the middle, thickness of the epithelium in the middle, and stratum basale-to-stratum corneum ratio (Table 4) were affected by sampling day ($P ≤ 0.002$). During the pretreatment period total papilla thickness at the middle, epithelial thickness at the middle, and stratum basale-to-stratum corneum ratio increased ($P ≤ 0.027$); the future RAP and GRAD groups showed no differences for any of the microscopic papilla variables ($P ≥ 0.289$; data not shown). In the treatment period, total papilla thickness at the tip and at the middle, epithelial thickness at the middle, and stratum basale-to-stratum corneum ratio decreased ($P ≤ 0.002$). Papilla thickness at the tip and the stratum basale-to-stratum corneum ratio were higher at 9 d pp compared with 3 d pp ($P < 0.001$). Neither concentrate treatment ($P ≥ 0.306$) nor an interaction between concentrate treatment and sampling day affected the microscopic papilla variables ($P ≥ 0.330$).

Dynamics in FOM Intake and of the Papillae Surface Area

Change in daily FOM intake (Figure 5A) was affected by sampling day ($P < 0.001$). During the pretreatment period, variation in change in daily FOM intake was

49

minimal (P = 0.195) and averaged 0.01 ± 0.03 kg/d, and the future treatment groups did not reveal differences in change in daily FOM intake (P = 0.713; data not shown). In the treatment period, the change in daily FOM intake was greatest between 3 to 9 d pp for RAP and GRAD (P ≤ 0.004). Thereafter, the change in daily FOM intake decreased and was approximately zero between 44 to 60 d pp and onwards. Change in daily FOM intake was not affected by concentrate treatment (P = 0.124), but an interaction between concentrate treatment and sampling day existed (P < 0.001). The change in daily FOM intake was greater in group RAP than group GRAD between 3 to 9 d pp (0.59 vs. 0.46 ± 0.03 kg/d, respectively; P < 0.001), and between 9 to 16 d pp (0.42 vs. 0.17 ± 0.03 kg/d, respectively; P = 0.018). Between 16 to 30 d pp, the change in daily FOM intake decreased to 0.10 ± 0.03 kg/d for group RAP and was lower than that for group GRAD with 0.23 ± 0.03 kg/d (P = 0.018). In group GRAD, change in daily FOM intake decreased from 0.23 ± 0.03 kg/d at 16 to 30 d to 0.02 ± 0.03 kg/d between 44 and 60 d pp (P < 0.001), remaining similar thereafter (P = 0.619).
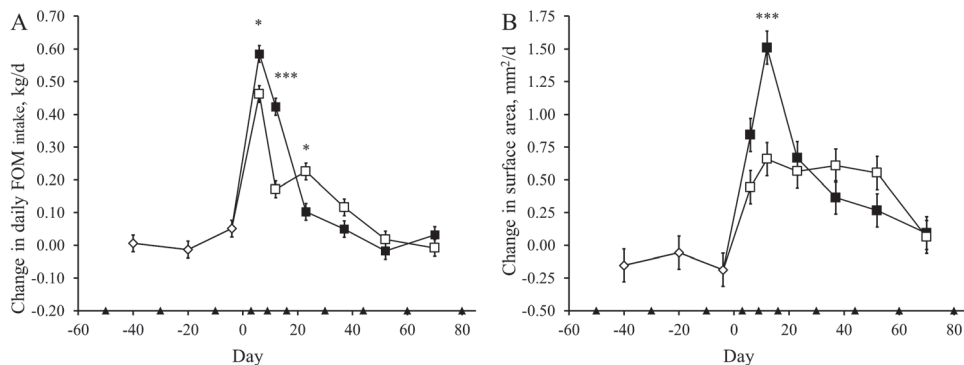
Figure 5. (A) Change in daily fermentable organic matter (kg/d; FOM) intake in the pretreatment period (◇; n = 12), and in the treatment period for a rapid (1.0 kg of DM/d; RAP, ■, n = 6) and gradual (0.25 kg of DM/d; GRAD, □, n = 6) rate of increase of concentrate allowance. (B) Change in papilla surface area (mm^2/d) for the pretreatment (◇) and the treatment period (RAP, ■, n = 6; GRAD, □, n = 6). Change in daily FOM intake and change in papilla surface area is calculated for the period between 2 subsequent sampling days (sampling days indicated by ▲). Day = day relative to calving. Values represent LSM ± SE. *P < 0.050, ***P < 0.001, significance of difference in LSM of RAP or GRAD.

Change in papilla surface area (Figure 5B) was affected by sampling day (P < 0.001). During the pretreatment period, change in papilla surface area averaged –0.13 ± 0.13 mm^2/d, and the future treatment groups did not reveal differences in in change in papilla surface area (P = 0.335). The treatment period showed an interaction between concentrate treatment and sampling day (P = 0.013), but overall change in papilla surface area was not affected by concentrate treatment (P = 0.169). Change in papilla

surface area was greater ($P < 0.001$) between 9 to 16 d pp in group RAP compared with GRAD, at 1.51 versus 0.66 ± 0.13 mm²/d respectively. After this peak for group RAP, change in papilla surface area gradually declined to 0.09 mm²/d between 60 to 80 d pp. In group GRAD, change in papilla surface area was similar ($P = 0.868$) from 3 to 9 d pp through the 44 to 60 d pp interval, averaging 0.57 ± 0.13 mm²/d, and growth declined to 0.06 ± 0.13 mm²/d between 60 to 80 d pp ($P = 0.001$).

DISCUSSION

The present study provides an overview of macroscopic and microscopic morphological changes of the rumen papillae in adult dairy cows during the dry period and subsequent 80 d of lactation and presents the first data showing the effect of postpartum concentrate build-up on rumen morphology. During the dry period, papilla surface area decreased and then increased rapidly again from d 3 pp onwards. In line with our hypothesis, the rapid rate of increase of concentrate allowance created a temporary difference in FOM intake, affecting rumen papilla development. Rumen papilla surface area and width increased at a higher rate in the group with the rapid rate of increase of concentrate allowance. In contrast to our expectations, neither the thickness of the papillae nor the thickness of the epithelium was affected by the concentrate treatment.

In the present experiment, rumen papillae surface area and papilla width showed a 19% reduction in some 50 d during the pretreatment period, whereas DM and FOM intake did not change. Earlier work suggests rapid rumen papilla atrophy is possible, with an approximate 65% reduction in surface area over 14 d (Dirksen et al., 1984). However, the 65% reduction coincided with an approximate 50% decrease in NE_L intake (86 to 41 MJ/d) suggesting the rate of atrophy is influenced by the magnitude in ration change. In contrast to the limited papilla atrophy during the dry period, large and rapid changes in papilla surface area occur during early lactation. Depending on concentrate treatment, papilla surface area increased by 9 to 16 d to approximately the surface area at 50 d ap and increased by a further 85% by 80 d pp. The time required for rumen papilla adaptation to reach a steady state after transition to a higher plane of nutrition varies. Six to 8 wk are often cited, based on the work of Dirksen et al. (1984) and Liebich et al. (1987), whereas Bannink et al. (2008) concluded less than 4 wk of adaptation is required, although these studies base their conclusions on different measurements and experimental conditions. In the present study, papilla surface area, length, and width appear to reach a steady state between 60 and 80 d pp. However, it is evident that papilla surface area increases rapidly when daily FOM intake increases rapidly. Therefore, discussing rumen adaptation in dairy cattle in terms of change per day in morphological variables under study compared with change per day in ration

variables (such as FOM) seems more appropriate. The initial increase in change of daily FOM intake between 3 and 9 d pp coincided with growth of the rumen papillae. This implies a capacity for a quick initiation of a response by the rumen papillae to an increase in FOM intake. However, in group RAP, daily change in surface area almost doubled between 9 and 16 d pp compared with 3 to 9 d pp, whereas the change in daily FOM intake peaked between 3 to 9 d pp, indicating a lag effect in the maximum response of papilla surface area to ration changes. In group GRAD, the initial peak in change of daily FOM intake between 3 to 9 d pp was not followed by a peak in change in papilla surface area, but the papilla surface area showed almost linear growth up to 60 d pp. Daily change in FOM intake from 30 to 44 and 44 to 60 d pp, however, was much smaller than that from 9 to 16 and from 16 to 30 d pp, also indicating a lag effect in the response of the rumen papillae in group GRAD. Obviously, the rumen papillae show a capacity for rapid growth, but the maximum response is delayed when compared with the changes in FOM. It remains unclear, however, why the capacity for rapid growth is not used later in lactation (especially in GRAD and to lesser extent in RAP) to reach a steady state in rumen papilla surface area in relation to the FOM intake earlier, as FOM intake was similar from 44 d pp onwards.

The lack of effect of rate of increase of concentrate allowance on histological variables suggests that rumen papillae proliferate in a similar manner when faced with a rapid or gradual rate of increase of concentrate allowance (Bannink et al., 2012) and, arguably, maintain a similar functional capacity per unit of surface area. In line with results of Steele et al. (2011) and Bannink et al. (2012), papilla thickness and the thickness of the epithelial layer in the middle decreased during the treatment period, although Steele et al. (2015) did not report a difference in epithelium thickness in the middle of the papillae when cows transitioned to a lactation ration. The thinner epithelium was maintained at the end of the experimental period, whereas the papilla surface area remained similar from 60 d onward. This suggests a thinner epithelium is also advantageous after absorption of VFA into the epithelium at the luminal side, either passively or by mediated transport (Aschenbach et al., 2011). Rumen papilla epithelium might be regarded as a functional syncytium, with cell-cell gap junctions allowing rapid diffusion between the apical and basal cell layers aided by a distinct distribution of pumps on the luminal and basal side of the epithelium (Graham and Simmons, 2005; Graham et al., 2007). A thinner epithelium reduces the distance VFA have to diffuse across before transfer from the epithelium into the blood, arguably enhancing the VFA absorption capacity.

A higher ratio between the stratum basale and stratum corneum indicates more extensive folding of the basal membrane and reflects the extent of rete-peg formation in the epithelium. The increase in this ratio at 9 d pp is in accordance with Dirksen et al.

(1984), Liebich et al. (1987), and Steele et al. (2015), who showed similar changes after switching cows to a higher plane of nutrition. Arguably, this increase is associated with the onset of papilla growth seen at this time (Figure 5B) and might reflect a high rate of cellular proliferation at the papilla tip, as suggested by Steele et al. (2015). The process responsible for the later decrease in the ratio remains unclear but might be associated with restructuring of the epithelium. The temporary larger stratum basale-to-stratum corneum ratio at 9 d pp might also have functional consequences. It can be speculated that more extensive folding of the stratum basale increases the capacity to maintain a large concentration gradient of VFA across the epithelium by increasing the capacity for efflux of VFA over the basal membrane to the capillaries with which it is closely associated (Dobson et al., 1956).

In the present study, manipulating the rate of increase of concentrate allowance created a difference in daily FOM intake as well as a greater intake of starch, sugar, and NE_L at 16 d pp in group RAP. In general, higher supply of nutrients in early lactation increases daily milk production (Kokkonen et al., 2004). Therefore, in the present experiment we expected, but did not observe, an interaction between sampling day and concentrate treatment for milk production, with a greater milk production and earlier peak in production for cows in group RAP than in group GRAD. We fed the basal diet and concentrate separately, and intake of the basal diet (mainly silage) was greater in the GRAD than the RAP group. Similarly, Ingvartsen et al. (2001) did not observe positive effects of faster concentrate supply in early lactation on milk yield with separate feeding of silage and concentrate. They attributed this lack of response to a decline in silage intake with fast concentrate supply, whereas a complete diet feeding resulted in highest milk production in their trial. It should also be stressed that unintended systematic differences in milk production capacity between treatment groups, despite the random assignment of cows, cannot be ruled out because treatment groups were relatively small (n = 6) and cows were blocked on expected calving date before random assignment to either one of the treatment groups.

The effects of the dry period ration, basal lactation ration, and concentrate treatment on rumen fluid affected total VFA concentration, VFA molar proportions, and pH of the rumen fluid, which is in agreement with earlier work (Dijkstra, 1994; Sutton et al., 2003; Bannink et al., 2008). All 3 major VFA likely affect rumen papillae development (Sander et al., 1959; Sakata and Tamate, 1979; Suarez et al., 2006), with butyric acid probably having the most pronounced effect (Sakata and Tamate, 1978; Shen et al., 2005; Malhi et al., 2013). Although the production rate of the different VFA influences their molar proportions in the rumen fluid (Dijkstra, 1994), no conclusions on proliferative effects of any specific VFA can be drawn based on VFA concentration and molar proportion alone.

In conclusion, our study clearly shows the relation between changes in FOM intake and papilla morphology (papilla length, width, thickness, surface area, and epithelium) in Holstein-Friesian dairy cows, and these results were obtained under experimental conditions and with rations that reflect common dairy practice. During the 8-wk dry period, papilla surface area decreased, but, within 1 to 2 wk pp, surface area returned to the level at the start of the dry period and continued to increase up to 8 to 9 wk pp. Rate of increase of concentrate allowance after calving affected rate of increase of papilla surface area, indicating rumen papillae can mount a variable response to the level of increase of daily FOM intake.

ACKNOWLEDGMENTS

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Chapter 2

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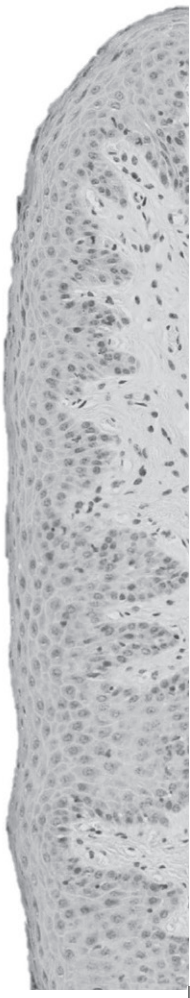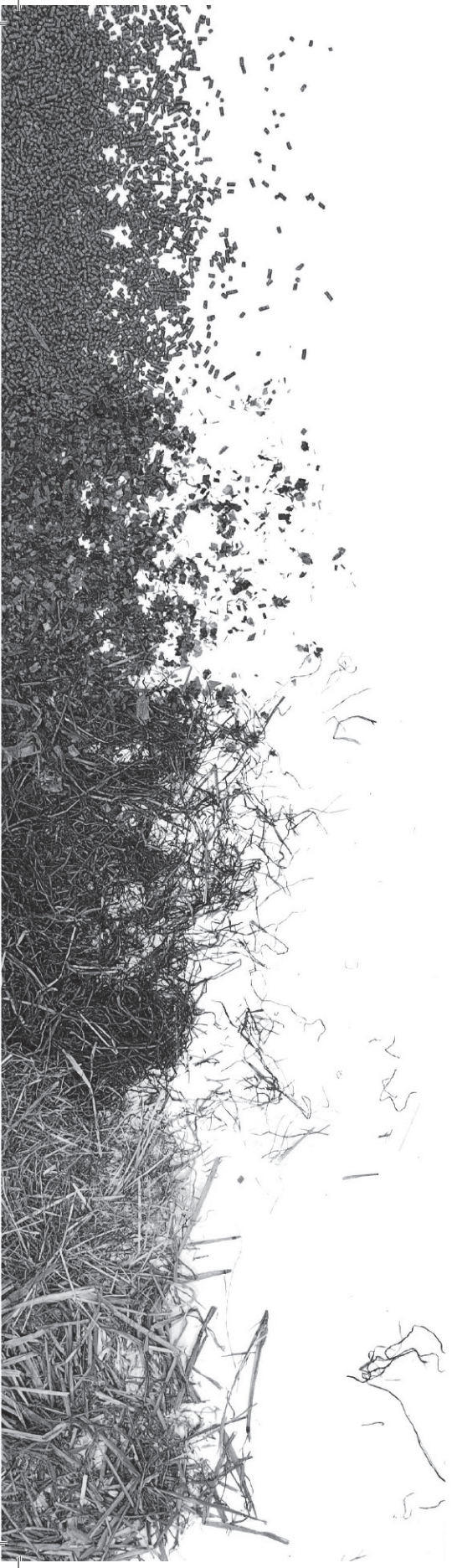
Chapter 3

Changes in ruminal volatile fatty acid production and absorption rate during the dry period and early lactation as affected by rate of increase of concentrate allowance

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ABSTRACT

The aim of the present experiment was to study changes in volatile fatty acid (VFA) production using an isotope dilution technique, and changes in VFA fractional absorption rate (k_aVFA) using a buffer incubation technique (BIT) during the dry period and early lactation, as affected by the postpartum (pp) rate of increase of concentrate allowance. The current results are complementary to previously reported changes on rumen papillae morphology from the same experiment. From 50 d antepartum to 80 d pp, VFA production rate was measured 5 times and k_aVFA was measured 10 times in 12 rumen-cannulated Holstein-Friesian cows. Cows had free access to a mixed ration, consisting of grass and corn silage, soybean meal, and (dry period only) chopped straw. Treatment consisted of either a rapid (RAP; 1.0 kg of DM/d; n = 6) or gradual (GRAD; 0.25 kg of DM/d; n = 6) increase of concentrate allowance (up to 10.9 kg of DM/d), starting at 4 d pp, aimed at creating a contrast in rumen-fermentable organic matter intake. For the BIT, rumen contents were evacuated, the rumen washed, and a standardized buffer fluid introduced [120 mM VFA, 60% acetic (Ac), 25% propionic (Pr), and 15% butyric (Bu) acid; pH 5.9 and Co-EDTA as fluid passage marker]. For the isotope dilution technique, a pulse-dose of ^{13}C-labeled Ac, Pr, and Bu and Co-EDTA as fluid passage marker was infused. The rate of total VFA production was similar between treatments and was 2 times higher during the lactation (114 mol/d) than the dry period (53 mol/d). Although papillae surface area at 16, 30, and 44 d pp was greater in RAP than GRAD, Bu and Ac production at these days did not differ between RAP and GRAD, whereas at 16 d pp RAP produced more Pr than GRAD. These results provide little support for the particular proliferative effects of Bu on papillae surface area. Similar to developments in papillae surface area in the dry period and early lactation, the k_aVFA (per hour), measured using the BIT, decreased from 0.45 (Ac), 0.53 (Pr) and 0.56 (Bu) at 50 d antepartum to 0.28 (Ac), 0.34 (Pr) and 0.38 (Bu) at 3 d pp. Thereafter, k_aVFA (/h) rapidly increased up to 0.67 (Ac), 0.79 (Pr), and 0.79 (Bu) at 80 d pp. Although papillae surface area was greater at 16, 30, and 44 d pp in RAP than GRAD, no differences in k_aVFA between RAP and GRAD were observed during these days showing papillae surface area is not the limiting factor for k_aVFA during early pp adaptation.

Key words: transition dairy cow, volatile fatty acid absorption, volatile fatty acid production, rumen papillae, rumen adaptation

Chapter 3

INTRODUCTION

After calving, feed intake as well as ration quality generally increases. Consequently, the production of VFA from the microbial fermentation of OM in the rumen increases (Bergman, 1990). This results in a rise in VFA concentration and a decrease in pH. To maintain favorable conditions for rumen fermentation, the removal of VFA from the rumen is an essential process (Penner et al., 2009; Aschenbach et al., 2011; Dijkstra et al., 2012). Removal of VFA occurs by passage with the rumen fluid, but mainly across the rumen epithelium (Gäbel et al., 2002; Aschenbach et al., 2011), either through passive diffusion or facilitated transport (Aschenbach et al., 2009, 2011). The VFA fractional absorption rate is affected by the carbon-chain length of the VFA, rumen fluid pH, VFA concentration, rumen fluid volume (Thorlacius and Lodge, 1973; Dijkstra et al., 1993), epithelial blood flow (Storm et al., 2011), and epithelial capacity for facilitated transport (Penner et al., 2011; Schurmann et al., 2014). The rate of absorption of VFA has been shown to increase as papillae surface area increases (Dirksen et al., 1984; Melo et al., 2013), and papillae surface area has been shown to increase when animals were transitioned to a higher plane of nutrition (Dirksen et al., 1984; Liebich et al., 1987; Bannink et al., 2012). However, evidence also exists that, in response to a higher plane of nutrition, VFA absorption rate can increase independent from changes in papillae surface area (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014). These adaptive responses, possibly aided by an increased blood flow (Reynolds et al., 2003; Storm et al., 2011), would result in an increase of the VFA absorption capacity after calving. However, our knowledge of the changes in the VFA fractional absorption rate measured in vivo concomitant with nutritionally induced changes in papillae surface area is limited (Dirksen et al., 1984; Martens et al., 2012). Moreover, no such studies cover both the dry period and early lactation.

The aim of the present experiment was to study changes in daily VFA production rate and VFA fractional absorption rate in the rumen of dairy cows from the start of the dry period up to 80 d postpartum (**pp**), and to evaluate the effect of the pp rate of increase of concentrate allowance on VFA production and absorption. Previously, in the same experiment, it was shown that the rumen papillae surface area increased with the pp increase in intake of rumen-fermentable organic matter (**FOM**), and this surface area increase was faster with a more rapid rate of increase of concentrate allowance (Dieho et al., 2016). Therefore, daily VFA production rate and VFA fractional absorption rate were expected to increase from the dry period to early lactation and were also expected to increase faster with a more rapid rate of increase of concentrate allowance.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen UR and conducted under the Dutch Law on the Animal Experiment.

Animals, Experimental Design and Housing

Full details on the animals, experimental design, housing, feeding, feed and milk sampling, and feed and milk chemical analyses have been presented by Dieho et al. (2016). Briefly, 12 rumen-cannulated, first-parity Holstein-Friesian dairy cows were dried-off and entered the experiment 8 wk before the expected calving date. The experiment had a randomized block design with repeated measurements. Prior to the start of the experiment, cows were blocked by expected calving date and, within each block, cows were randomly assigned to either a rapid or a gradual rate of increase of concentrate allowance pp. The ruminal fractional absorption rate of VFA was measured using a buffer incubation technique during the pretreatment period at 50, 30, and 10 d antepartum (**ap**) and 3 d pp, as well as during the treatment period on 9, 16, 30, 44, 60, and 80 d pp. The ruminal VFA production and absorption rates were measured using an isotope dilution technique on 50 and 10 d ap, and on 16, 44, and 80 d pp. Dry and lactating animals were housed in separate groups in a freestall barn, with lactating cows from both treatments sharing the same pen, and were moved to a tiestall for the duration of the experimental procedures.

Rations and Experimental Treatments

Cows had free access to water and to either a dry period ration or basal lactation ration consisting of grass and corn silage, soybean meal, and (dry period only) chopped wheat straw (Dieho et al., 2016). Rations were freshly mixed and fed once a day throughout the experiment. The dry period ration (603 g of DM/ kg) provided 5.3 MJ of NE_L (calculated according to the Dutch NE-system; van Es, 1978) and 455 g of FOM (calculated according to the Dutch DVE/OEB-system; Tamminga et al., 1994) per kilogram of DM. The basal lactation ration (466 g of DM/kg) provided 6.7 MJ of NE_L and 561 g of FOM per kilogram of DM.

From calving up to 3 d pp, 0.9 kg of DM/d concentrate was fed; thereafter the concentrate treatment started, in which concentrate allowance increased at either a rapid rate of 1.0 kg of DM/d (**RAP**), or a gradual rate of 0.25 kg of DM/d (**GRAD**). Maximum concentrate allowance was 10.9 kg of DM/d, irrespective of rate of increase,

achieving the maximum concentrate allowance at 13 and 43 d pp for RAP and GRAD, respectively. Daily intake of the dry period ration or the basal lactation ration was measured individually using feed-bins (Insentec, Marknesse, the Netherlands), and concentrate was fed using a dispenser (Manus VC5, DeLaval, Steenwijk, the Netherlands) with individual daily allowance available in equal portions over six 4-h periods, recording the quantity actually dispensed (kg/d). During visits cows were shielded from herdmates and the concentrate was fed as a series of small portions, effectively preventing the possibility of other cows stealing concentrate. The concentrate (892 g of DM/kg) provided 7.4 MJ of NE_L and 682 g of FOM per kilogram of DM.

Measurement of Production and Absorption of VFA

Isotope Dilution Technique. The isotope dilution technique (IDT) was used to measure the ruminal VFA production rate based on the fractional clearance rate of 1-^{13}C-VFA and the VFA pool size; in addition, the fractional fluid passage rate was measured using Co-EDTA as fluid passage marker (France and Dijkstra, 2005). The measurements were made under the assumption that the rumen fermentation and rumen fill were in steady state (constant fractional passage rate and fractional rate of VFA production and absorption), which was established by feeding small meals once every hour before introduction of the stable isotopes. For each cow the hourly meal size (kg) was calculated from the intake of the dry period ration or the basal lactation ration recorded for the 3 d preceding the measurement. The amount of concentrate to be fed each hour was calculated from the allowance for the day of the measurement (for GRAD: 0.2 kg/h at 16 d pp, and 0.5 kg/h at 44 and 80 d pp; for RAP: 0.5 kg/h at 16, 44, and 80 d pp). At 0600 h, the first meal was fed and a pH logger was introduced in the rumen (15-s reading interval; model T4, Dascor Inc., Escondido, CA; Penner et al., 2006). At 0700 h, and every hour up to 1400 h, subsequent meals were fed.

At 1100 h, a 200-mL rumen fluid sample was taken from the ventral rumen sac followed by the quantitative introduction of 2 L of water at 39°C containing 30 g of Co-EDTA (Udén et al., 1980) and 1-^{13}C-Na-VFA salts into the ventral rumen sac. The quantity of 1-^{13}C-Na-VFA introduced varied between the pretreatment and treatment period, and between treatments at 16 d pp, but was calculated to achieve an initial ^{13}C enrichment of 1.30% of total C for the acetic (**Ac**), propionic (**Pr**), and butyric (**Bu**) acid pools. For 1-^{13}C-Na-acetate 1,000 to 1,400 mg, for 1-^{13}C-Na-propionate 500 to 1,000 mg, and for 1-^{13}C-Na-butyrate 400 to 900 mg was introduced (99% enriched, Sigma-Aldrich, Zwijndrecht, the Netherlands). The Co-EDTA and 1-^{13}C-Na-VFA were dissolved in water just before introduction into the rumen (Sutton et al., 2003). At 1400 h, a 200-mL rumen fluid sample was taken from the ventral rumen sac and a

600-µL aliquot was taken (acidified with 600 µL 5% vol/vol H_3PO_4, with 19.68 mM isocaproic acid as internal standard) for measurement of the VFA concentration, and a 10-mL aliquot was taken for measurement of the Co concentration. Samples were stored at –20°C pending analysis. Subsequently, the pH logger was retrieved and the buffer incubation technique (**BIT**) procedure was started.

Buffer Incubation Technique. The BIT was used to measure the fractional rate of absorption of Ac, Pr, and Bu as well as fractional fluid passage rate under standardized conditions in an empty washed rumen (Dijkstra et al., 1993). At the start of each measurement, 0 to 1 h after last access to feed, the rumen contents were completely evacuated, stored in an insulated tub to prevent cooling, and weighed. After evacuating approximately half of the rumen contents, a sample of whole rumen content (stored at −20°C) was taken for determination of DM content. After evacuation, the rumen was washed twice with 10 kg of tap water at 39°C and subsequently with 5.1 kg (5.0 L) of buffer solution. The wash buffer fluid in the rumen was completely removed using the vacuum of a hand-held milking system. A visual and manual inspection of the rumen was performed to confirm complete removal of rumen contents and fluid. Then, 46.7 kg (46.0 L) of buffer solution (39°C, pH 5.9) was introduced into the empty rumen using an electric bailing pump (2,200 L/h, Hozelock, Birmingham, UK). The buffer solution (adapted from Dijkstra et al., 1993) was freshly prepared immediately before each assessment and contained 39 mM Na_2HPO_4, 70 mM $NaHCO_3$, 14 mM NaCl, 4.6 mM KCl, 0.9 mM $CaCl_2$, 0.7 mM $MgCl_2$, and 20 mM NaOH, with 72 mM Ac, 30 mM Pr, and 18 mM Bu, with 0.17 mM Co-EDTA (0.07 g/L) as a marker of fluid outflow rate. Buffer fluid (200 mL) was sampled immediately before introduction into the rumen and after 60 min of incubation in the rumen. Then the buffer fluid was recovered using the vacuum system, and a visual and manual inspection of the rumen was performed to confirm complete recovery. The recovered buffer fluid was weighed before returning the rumen contents. In the buffer fluid samples, pH was measured immediately after collection and aliquots taken as described earlier. Complete emptying of the rumen before buffer introduction and at the end of the procedure was assumed for the calculations.

Chemical Analyses

The concentration of Ac, Pr, and Bu in the rumen fluid taken during the IDT was measured by gas chromatography as described by van Gastelen et al. (2015). The concentration of Ac, Pr, and Bu in the buffer fluid samples taken during the BIT measurements was also measured by gas chromatography with slight modifications. Briefly, after thawing (at ambient temperature, 20°C) and 5 min of centrifugation

(14,000 × g), 0.1 µL of supernatant was injected into the gas chromatograph (Trace GC Ultra, Thermo Scientific, Milan, Italy). The inlet temperature was 260°C with a split ratio of 1:9. Hydrogen gas flowed (25 kPa, constant pressure) through a capillary column (Agilent HP-FFAP, Agilent Tech., Santa Clara, CA; 30 m length, 0.53 mm i.d., 1 µm film) to a flame-ionization detector (260°C). Initial column temperature was 80°C for 1 min, increasing with 20°C/min to 120°C, and subsequently with 6.1°C/min to 205°C and held for 2 min. Quantification was based on a reference solution after internal standard correction.

For ^{13}C enrichment in the rumen fluid samples, Ac, Pr, and Bu were separated using a gas chromatograph and subsequently ^{13}C was measured by continuous flow isotope ratio mass spectrometry (Finnigan Delta V Plus, Thermo Scientific, Bremen, Germany). After thawing (at ambient temperature, 20°C) and 5 min of centrifugation (14,000 × g), 1.0 µL of supernatant was injected into the gas chromatograph (Trace GC Ultra, Thermo Scientific, Milan, Italy). The inlet temperature was 225°C with a split ratio of 1:5, with helium carrier gas flow set to 2.5 mL/min (vacuum compensated) through a capillary column (Agilent HP-FFAP, Agilent Tech.; 30 m length, 0.32 mm i.d., 0.25 µm film) with the column outlet fitted to a combustion interface (Thermo Finnigan GC Combustion III, Thermo Scientific, Bremen, Germany) that was connected to the isotope ratio mass spectrometer. Initial column temperature was 110°C for 2 min, increasing 18°C/min to 200°C, and held for 2 min.

Cobalt was determined in rumen and buffer fluid after thawing (at ambient temperature, 20°C) and 10 min of centrifuging (5,000 × g) using an atomic absorption spectrophotometer (AA240FS, Varian Inc., Palo Alto, CA) at 240.7 nm (Udén et al., 1980).

Calculations and Statistical Analysis

Total VFA concentration was defined as the sum of Ac, Pr, and Bu. Fractional absorption rate (k_a; per hour) of Ac (k_a**Ac**), Pr (k_a**Pr**), and Bu (k_a**Bu**), fractional fluid passage rate (k_l; per hour), and net influx of water (I; L/h) for the BIT were calculated using the following equations (Dijkstra et al., 1993):

$$\text{Co(t)} = \text{Co(0)} \exp (-k_l \times t),$$

$$V(\text{t}) = [V(0) - I/k_l] \exp (-k_l \times t) + I/k_l,$$

$$\text{VFA(t)} = \text{VFA(0)} \exp [-(k_l + k_a) \times t],$$

where Co(t) is the amount of cobalt in the rumen (g), V(t) is rumen fluid volume (L), VFA(t) is the amount of VFA (Ac, Pr, or Bu) in the rumen (mmol; all after 60 min incubation), and Co(0), V(0), and VFA(0) denote the amount of Co, the volume, and the amount of VFA in the rumen immediately after introduction of the solution in the rumen, respectively. For the BIT, pool size of Co and VFA at the start and at the end of the incubation were known, and the equations were solved to calculate k_l, k_a, and I. Average VFA fractional absorption rate, k_aVFA, was calculated as the average of k_aAc, k_aPr, and k_aBu. The k_aVFA represents the average fractional absorption rate during the 1-h incubation period.

For the IDT, the k_a, k_l, and I were calculated by solving the equations used for the BIT. Fractional clearance rate, k_c (per hour), was calculated by addition of k_l and k_a. For k_a, VFA(0) was the amount of ^{13}C added to the rumen at t = 0 h (for Ac, Pr, and Bu) and VFA(t) was the amount of ^{13}C at t = 3 h (for Ac, Pr, and Bu). For k_l, Co(0) was the amount of Co added at t = 0 h, and Co(t) the amount of Co at t = 3 h. The amount of ^{13}C VFA(t) at t = 3 h (for Ac, Pr, and Bu) was calculated from the rumen fluid volume, the VFA concentration in the rumen fluid, and the ^{13}C-enrichment (corrected for the natural ^{13}C enrichment). The rumen fluid volume, V(t), was calculated from the rumen content mass and the DM content of the mid-rumen samples, and V(0) was assumed to be equal to V(t). Rate of VFA production (for Ac, Pr, and Bu; mol/d) was calculated from kc and amount of VFA in the rumen. Amount of VFA in the rumen was assumed to be constant during the IDT and calculated from the rumen fluid volume, V(t), and the average VFA concentration in the rumen fluid at t = 0 h and t = 3 h. Rumen fluid pH data, logged during the steady-state feeding and IDT, was averaged per minute before analysis.

All variables were assumed to be related to sampling day and treatment. Data were analyzed using the MIXED procedure in SAS 9.2 (SAS Institute Inc., Cary, NC) with the model (Littell et al., 2006):

$$Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ij},$$

where variable Y_{ij} was dependent on μ as the average experimental value and fixed main effects of concentrate treatment T_i (i = RAP, GRAD), of sampling day D_j (j = –50, –30, –10, 3, 9, 16, 30, 44, 60, and 80 for BIT, and j = –50, –10, 16, 44, and 80 for IDT), and fixed interaction $(T \times D)_{ij}$. Errors e_{ij} were assumed to be autocorrelated for repeated observations on the same cow, using a spatial power covariance structure over sampling days (Littell et al., 1998). As described in detail by Dieho et al. (2016), the experimental design could not be treated as a complete factorial design with respect to concentrate treatment and sampling day. Therefore custom CONTRAST statements

Chapter 3

were constructed for (1) the pretreatment period (sampling d –50 through 3 or –50 and –10, if applicable), to compare the 2 future treatment groups, thereby evaluating the success of the random treatment allocation [no differences between treatment groups were found at the end of the pretreatment period ($P \geq 0.19$), except for the fractional fluid passage rate during the BIT ($P = 0.03$)]; (2) the treatment main effect, T_i, over the treatment period; (3) the sampling day main effect, D_j, over the total experimental period; and (4) the interaction between treatment and sampling day, $(T \times D)_{ij}$, by testing sampling day by sampling day difference between concentrate treatment groups over the treatment period (Dieho et al., 2016). Specific hypotheses for separating means were tested by formulating CONTRAST and ESTIMATE statements. The strength of estimated squared coefficients of correlation (r^2) was interpreted as weak if $r^2 < 0.50$, moderate if $0.50 \leq r^2 < 0.70$, strong if $0.70 \leq r^2 < 0.90$, and very strong if $0.90 \leq r^2 \leq 1.00$. All results are reported as least squares means with their standard error unless indicated otherwise. Significance of effect was declared at $P < 0.05$ and trends at $0.05 \leq P < 0.10$.

RESULTS

One cow (RAP) calved early and missed the sampling day at 10 d ap, otherwise all measurements were completed as planned. One cow was treated for mastitis (from RAP) and 7 for (chronic) endometritis (4 from RAP and 3 from GRAD). All cows fully recovered after veterinary treatment, and all cows completed the experiment. Actual sampling days (mean ± SD) for the pretreatment period were 46.3 ± 5.1, 26.5 ± 5.7, and 8.6 ± 3.4 d ap, and 3.0 ± 0.0 d pp and for the treatment period 9.0 ± 0.0, 16.1 ± 0.3, 30.2 ± 0.3, 44.2 ± 0.4, 60.0 ± 0.9, and 79.3 ± 2.2 d pp. Repeated rumen evacuations and buffer incubations during the experiment had no adverse effects on feed intake and milk production.

Feed Intake and Rumen Contents

Feed intake is presented in detail by Dieho et al. (2016). Intake of DM (11.9 ± 0.54 kg/d; Figure 1) and FOM (5.7 ± 0.27 kg/d) did not vary during the pretreatment period, and increased during the treatment period to 24.5 ± 0.54 and 15.0 ± 0.27 kg/d at 80 d pp, respectively. Concentrate treatment and sampling day interacted for FOM intake but not for DMI. Intake of FOM was 21.8% larger for RAP compared with GRAD at 16 d pp and was 12.3 ± 0.27 and 10.1 ± 0.27 kg/d, respectively.
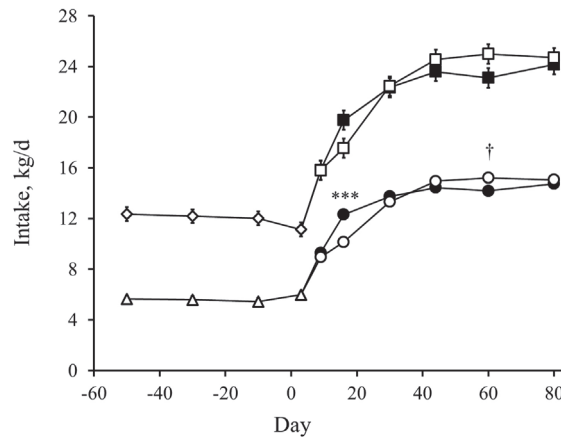
Figure 1. Dry matter intake (kg/d; ◇■□) and fermentable organic matter intake (kg/d; △●○) in the pretreatment period (◇△; n = 12), and in the treatment period for a rapid (1.0 kg DM/d; ■●, n = 6) and gradual (0.25 kg DM/d; □○, n = 6) rate of increase of concentrate allowance postpartum. Values represent LSM ± SE. ***P < 0.001, †P < 0.10, significance of difference in LSM of RAP or GRAD. Adapted from Dieho et al. (2016).

During the pretreatment period, the average mass of the rumen contents was 64 ± 2.8 kg containing 133 ± 3.6 g of DM/kg. During the treatment period, mass of the rumen contents increased to 77 ± 2.8 kg containing 140 ± 3.6 g of DM/kg at 80 d pp (P < 0.01). Neither concentrate treatment (P = 0.79) nor the interaction between concentrate treatment and sampling day (P = 0.20) affected mass of the rumen contents. The DM fraction of the rumen contents tended (P = 0.06) to be affected by sampling day, but was not affected (P = 0.26) by the interaction between concentrate treatment and sampling day. The DM fraction of the rumen content mass was slightly higher for GRAD compared with RAP (144 vs. 136 ± 3.6 g of DM/kg; P = 0.02; data not shown).

71

VFA Production Rate

Rumen Fluid Composition and pH. The total VFA concentration in rumen fluid (Table 1) was affected by sampling day ($P < 0.01$), increasing from on average 77 mM during the pretreatment period to on average 120 mM during the treatment period. Sampling day affected the fractions of Ac, Pr, and Bu of total VFA ($P < 0.01$), with the fraction of Ac being higher and that of Pr being lower in the pretreatment period (on average 71.9 and 17.8 mol/100 mol, respectively) compared with the treatment period (on average 64.0 and 24.0 mol/100 mol, respectively), which was also reflected by the ratio between the fraction of Ac and Pr. Although total VFA concentration was neither affected by concentrate treatment nor by the interaction between concentrate treatment and sampling day; the fractions of Ac and Pr, but not of Bu, were affected by the interaction ($P \leq 0.02$). At 16 d pp, the fraction of Ac was smaller ($P < 0.01$) and the Pr fraction greater ($P < 0.01$) in RAP compared with GRAD. This was also reflected in the Ac-to-Pr ratio, which was smaller for RAP at 16 d pp compared with GRAD. Rumen fluid pH was affected by sampling day ($P < 0.01$), decreasing from pH 6.72 during the pretreatment period to 6.17 during the treatment period. The fraction of time of rumen fluid below pH 6.3 (below pH 6.3, proportion of Ac, Pr, and Bu produced from starch and sugar, and NDF digestion change; Erdman, 1988; Bannink et al., 2008) and pH 5.8 (associated with subacute rumen acidosis; Zebeli et al., 2012) was affected by sampling day ($P \leq 0.03$), with a larger fraction of time below the threshold values during the treatment period than the pretreatment period. Concentrate treatment and sampling day interacted for fraction of time below pH 5.80 ($P = 0.02$). At 16 d pp, fluid pH was 18 min/h below pH 5.80 for RAP compared with 2 min/h for GRAD ($P = 0.01$).

Table 1. Rumen fluid composition and pH from the VFA production rate measurement using the isotope dilution technique for acetic (Ac), propionic (Pr), and butyric acid (Bu) during the pretreatment and treatment period with a rapid (1.0 kg of DM/d; RAP, n = 6) and a gradual (0.25 kg of DM/d; GRAD, n = 6) rate of increase of concentrate allowance postpartum

| | Sampling day relative to calving | | | | | | Fixed effects[1] | | |
| | Pretreatment period | | Treatment period | | | | | | |
Item	-50	-10[2]	16	44	80	SE[3]	T	D	T × D
Total VFA,[4] mM									
RAP	70	79	118	125	115	4.6	0.76	<0.01	0.93
GRAD	74	87	121	122	120				
Fraction Ac, mol/100 mol			***						
RAP	70.8	72.8	60.2	64.4	64.7	0.73	0.09	<0.01	<0.01
GRAD	71.4	72.3	66.3	64.1	64.2				
Fraction Pr, mol/100 mol			***						
RAP	18.0	17.2	28.8	24.2	22.8	0.73	0.03	<0.01	<0.01
GRAD	17.9	18.1	21.9	23.2	23.2				
Fraction Bu, mol/100 mol									
RAP	11.2	10.0	11.0	11.4	12.5	0.35	0.06	<0.01	0.15
GRAD	10.7	9.6	11.8	12.7	12.6				
Ratio Ac : Pr			***						
RAP	4.0	4.3	2.1	2.7	2.9	0.13	0.11	<0.01	<0.01
GRAD	4.1	4.0	3.1	2.8	2.8				
Average rumen fluid pH									
RAP	6.75	6.81	6.06	6.17	6.18	0.069	0.24	<0.01	0.28
GRAD	6.62	6.69	6.34	6.11	6.18				
Time pH < 6.30, min/h									
RAP	5	0	47	37	30	5.1	0.53	<0.01	0.12
GRAD	8	7	23	41	40				
Time pH < 5.80, min/h			**						
RAP	0	0	18	7	12	2.6	0.03	0.03	0.02
GRAD	0	0	2	11	5				

[1]T = treatment, P-value for treatment period (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d –50 to 80); T × D, P-value for treatment period (d 9 to 80).

[2]Group GRAD: n = 5; 1 cow no measurement at d –10 due to early calving.

[3]Standard error for LSM, pooled by sampling day, n = 12.

[4]Sum of Ac, Pr, and Bu.

***P < 0.001, **P < 0.01, significance of difference in LSM of RAP or GRAD for the same sampling day and variable.

VFA Fractional Clearance Rate and VFA Production Rate. Only the fractional clearance rate of Ac (k_cAc) was affected by sampling day (P < 0.01), decreasing from 0.60 to 0.49/h during the pretreatment period (P = 0.02), increasing to 0.60/h at

16 d pp (P = 0.02), and tending to further increase to 0.68/h at 80 d pp (P = 0.09; Table 2). The k_cPr and k_cBu were not affected by sampling day ($P \geq 0.14$), averaging 0.66 and 0.47/h, respectively. The k_c of Ac, Pr, and Bu were neither affected by the concentrate treatment nor by the interaction between concentrate treatment and sampling day ($P \geq 0.40$).

Table 2. Fractional rate (per hour) of VFA clearance (k_c), fluid passage (k_l), and absorption (k_a) from the VFA production rate measurement using the isotope dilution technique for acetic (Ac), propionic (Pr), and butyric acid (Bu), and fluid inflow, during the pretreatment and treatment period with a rapid (1.0 kg of DM/d; RAP, n = 6) and a gradual (0.25 kg of DM/d; GRAD, n = 6) rate of increase of concentrate allowance postpartum

| | Sampling day relative to calving | | | | | | Fixed effects[1] | | |
| | Pretreatment period | | Treatment period | | | | | | |
Item	-50	-10[2]	16	44	80	SE[3]	T	D	T × D
k_cAc, /h									
RAP	0.63	0.51	0.58	0.61	0.68	0.032	0.99	<0.01	0.73
GRAD	0.57	0.47	0.62	0.57	0.68				
k_cPr, /h									
RAP	0.77	0.64	0.63	0.67	0.72	0.039	0.62	0.16	0.74
GRAD	0.68	0.57	0.64	0.59	0.71				
k_cBu, /h									
RAP	0.56	0.48	0.48	0.46	0.49	0.033	0.40	0.14	0.56
GRAD	0.50	0.44	0.45	0.37	0.48				
k_l, /h									
RAP	0.18	0.18	0.20	0.19	0.18	0.016	0.45	0.03	0.09
GRAD	0.21	0.17	0.23	0.16	0.22				
Fluid inflow, L/h									
RAP	8.2	9.0	12.8	12.2	11.6	0.81	0.43	<0.01	0.24
GRAD	10.9	9.6	13.2	11.7	14.6				
k_aAc, /h									
RAP	0.46	0.33	0.38	0.43	0.50	0.027	0.5	<0.01	0.89
GRAD	0.36	0.30	0.39	0.41	0.46				
k_aPr, /h									
RAP	0.59	0.46	0.43	0.48	0.54	0.032	0.29	0.07	0.72
GRAD	0.46	0.40	0.41	0.43	0.49				
k_aBu, /h									
RAP	0.38	0.30	0.28	0.28	0.31	0.027	0.12	0.20	0.47
GRAD	0.29	0.27	0.22	0.21	0.26				

[1]T = treatment, P-value for treatment period (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d –50 to 80); T × D, P-value for treatment period (d 9 to 80).
[2]Group GRAD: n = 5; 1 cow no measurement at d –10 due to early calving.
[3]Standard error for LSM, pooled by sampling day, n = 12.

Total VFA production rate was affected by sampling day ($P < 0.01$) but was neither affected by concentrate treatment nor by the interaction between concentrate treatment and sampling day ($P \geq 0.45$; Table 3). Total VFA production rate increased from on average 53 mol/d during the pretreatment to 103 mol/d at 16 d pp ($P < 0.01$), and increased further to 116 mol/d at 44 d pp (tendency only; $P = 0.06$) and 122 mol/d at 80 d pp ($P < 0.01$), when compared with 16 d pp. Production rates of Ac and Bu were only affected by sampling day, with an increased production rate during the treatment period. However, the production rate of Pr was affected by an interaction between concentrate treatment and sampling day ($P = 0.02$), with a 35% higher production rate on 16 d pp for RAP (34 mol/d) compared with GRAD (22 mol/d; $P < 0.01$). The fractions of Ac (60.0 vs. 68.0 mol/100 mol) and Pr (31.1 vs. 23.2 mol/100 mol) differed ($P < 0.01$) on 16 d pp for RAP and GRAD, respectively, whereas on all other days the fractions of Ac, Pr, and Bu were not affected by treatment (data not shown). Sampling day, concentrate treatment, and their interaction did not affect VFA production expressed per unit intake of DM or FOM.

Fractional Rate of Fluid Passage and of VFA Absorption

IDT. The fractional fluid passage rate (k_l; Table 2) was affected by sampling day, with the highest fluid passage rate at 16 d pp. Concentrate treatment did not affect k_l ($P = 0.45$) and a tendency was observed for an interaction between concentrate treatment and sampling day ($P = 0.09$). The inflow of water into the rumen was affected by sampling day ($P < 0.01$) and was lower during the pretreatment (9.4 L/h) compared with the treatment period (12.7 L/h). Concentrate treatment and interaction between concentrate treatment and sampling day did not affect water inflow ($P \geq 0.24$). The fractional absorption rate (k_a; Table 2) was affected by sampling day for Ac ($P = 0.01$) but not for Bu ($P = 0.20$), whereas it tended to affect k_a of Pr ($P = 0.07$). Both k_aAc and k_aPr decreased from 50 to 10 d ap ($P \leq 0.04$). The k_aAc averaged 0.36/h in the pretreatment period, increasing by 26% during the treatment period to 0.48/h at 80 d pp ($P = 0.02$). The k_aAc was greater during the treatment period (averaging 0.43/h) compared with the pretreatment period ($P = 0.01$). The fractional absorption rates of Ac, Pr, and Bu were neither affected by concentrate treatment nor by the interaction between concentrate treatment and sampling day ($P \geq 0.12$).

BIT. The fractional fluid passage rate (k_l, Table 4) was affected by sampling day ($P < 0.01$), generally increasing during the pretreatment period and decreasing during the treatment period. Concentrate treatment affected k_l ($P < 0.01$), and was on average greater in GRAD (0.20/h) compared with RAP (0.13/h). Concentrate treatment interacted with sampling day for k_l, with a higher fractional passage rate for

Chapter 3

75

GRAD at 16, 30, and 80 d pp compared with RAP ($P \leq 0.05$). Net influx of water was affected by sampling day ($P < 0.01$) and generally was higher during the treatment period. Net influx of water was neither affected by concentrate treatment ($P = 0.36$) nor the interaction between concentrate treatment and sampling day ($P = 0.30$).

Fractional absorption rates (k_a) of Ac, Pr, and Bu (Table 4) were affected by sampling day ($P < 0.01$), and decreased during the pretreatment period by approximately 35% to 0.28, 0.34, and 0.38/h for Ac, Pr, and Bu at 3 d pp, respectively ($P \leq 0.01$). During the treatment period, the k_a of Ac, Pr, and Bu increased to 0.67, 0.79, and 0.79/h at 80 d pp, respectively ($P < 0.01$). The k_a of Ac, Pr, and Bu were neither affected by concentrate treatment ($P \geq 0.85$) nor by the interaction between sampling day and concentrate treatment ($P \geq 0.40$). Similarly, the pH of the buffer fluid at the end of the incubation period was only affected by sampling day. It decreased during the pretreatment period from pH 7.11 at 50 d ap to pH 7.01 at 3 d pp ($P = 0.03$), increasing during the treatment period to pH 7.24 at 80 d pp ($P < 0.01$).

Table 3. Total VFA production rate, production rate of acetic (Ac), propionic (Pr), and butyric acid (Bu), and VFA production per unit of feed intake calculated from the VFA production rate measurements using the isotope dilution technique during the pretreatment and treatment period with a rapid (1.0 kg of DM/d; RAP, n = 6) and a gradual (0.25 kg of DM/d; GRAD, n = 6) rate of increase of concentrate allowance postpartum

| | Sampling day relative to calving | | | | | | Fixed effects[1] | | |
| | Pretreatment period | | Treatment period | | | | | | |
Item	-50	-10[2]	16	44	80	SE[3]	T	D	T × D
Total VFA production rate, mol/d									
RAP	52	49	108	117	119	3.8	0.64	<0.01	0.45
GRAD	49	58	98	115	126				
Ac production rate, mol/d									
RAP	36	35	65	76	79	2.6	0.33	<0.01	0.79
GRAD	34	41	67	76	83				
Pr production rate, mol/d		***							
RAP	11	10	34	31	29	1.3	0.03	<0.01	<0.01
GRAD	10	12	22	28	32				
Bu production rate, mol/d									
RAP	5	4	10	10	11	0.4	0.76	<0.01	0.63
GRAD	5	5	9	10	11				
VFA production, mol/kg of DM									
RAP	4.6	4.2	5.5	5.0	4.9	0.32	0.99	0.11	0.96
GRAD	4.2	4.8	5.6	4.7	5.1				
VFA production, mol/ kg of FOM[4]									
RAP	10.2	9.3	8.8	8.1	8.1	0.68	0.77	0.22	0.89
GRAD	9.2	10.7	9.7	7.7	8.3				

[1]T = treatment, *P*-value for treatment period (d 9 to 80); D = sampling day, *P*-value for pretreatment and treatment period (d –50 to 80); T × D, *P*-value for treatment period (d 9 to 80).
[2]Group GRAD: n = 5, one cow no measurement at d –10 due to early calving.
[3]Standard error for LSM, by sampling day, n = 12.
[4]FOM = fermentable organic matter (Tamminga et al., 1994).
***$P < 0.001$, significance of difference in LSM of RAP or GRAD for the same sampling day and variable.

Chapter 3

Table 4. Fractional rate of absorption (k_a) of acetic (Ac), propionic (Pr), and butyric acid (Bu), fractional fluid passage rate (k_l), buffer fluid end pH, and net influx of water, measured using the buffer incubation technique in the empty washed rumen during the pretreatment and treatment period with a rapid (1.0 kg DM/d; RAP, n = 6) and a gradual (0.25 kg/d; GRAD, n = 6) rate of increase of concentrate allowance postpartum.

Item		Pretreatment period				Treatment period						SE[3]	Fixed effects[1]		
	-50	-30	-10[2]	3	9	16	30	44	60	80		T	D	T × D
k_aAc, /h														
RAP	0.44	0.31	0.36	0.28	0.38	0.55	0.52	0.61	0.47	0.64	0.032	0.92	<0.01	0.40
GRAD	0.46	0.32	0.33	0.28	0.34	0.51	0.44	0.61	0.60	0.70				
k_aPr, /h														
RAP	0.52	0.38	0.43	0.34	0.45	0.62	0.63	0.74	0.60	0.77	0.035	0.94	<0.01	0.45
GRAD	0.53	0.39	0.40	0.34	0.41	0.58	0.53	0.73	0.72	0.82				
		†												
k_aBu, /h														
RAP	0.56	0.44	0.49	0.38	0.46	0.62	0.63	0.74	0.61	0.78	0.033	0.85	<0.01	0.40
GRAD	0.57	0.45	0.46	0.38	0.44	0.58	0.52	0.72	0.72	0.81				
Buffer fluid end pH														
RAP	7.09	6.97	7.00	6.96	7.11	7.12	7.17	7.26	7.26	7.25	0.033	0.60	<0.01	0.22
GRAD	7.13	7.04	7.02	7.05	7.03	7.21	7.21	7.27	7.33	7.23				
				*		***	*			*				
k_l, /h														
RAP	0.13	0.17	0.12	0.22	0.23	0.09	0.19	0.10	0.14	0.05	0.018	<0.01	<0.01	0.02
GRAD	0.14	0.23	0.14	0.30	0.27	0.22	0.26	0.14	0.18	0.13				
Net influx water, L/h														
RAP	2.3	4.6	3.0	5.0	6.5	3.4	6.6	3.9	7.0	3.5	0.51	0.36	<0.01	0.30
GRAD	3.6	5.0	3.2	4.8	5.0	3.9	7.1	5.2	7.6	4.9				

[1]T = treatment, P-value for treatment period (d 9 to 80); D = sampling day, P-value for pretreatment and treatment period (d −50 to 80); T × D, P-value for treatment period (d 9 to 80).

[2]Group GRAD: n = 5; 1 cow no measurement at d −10 due to early calving.

[3]Standard error for LSM, pooled by sampling day, n = 12.

†P < 0.10, *P < 0.05, ***P < 0.001, significance of difference in LSM of RAP or GRAD for the same sampling day and variable.

DISCUSSION

The present study provides a comprehensive overview of changes in ruminal VFA production rate and VFA fractional absorption rate in dairy cows during the dry period and the first 80 d of lactation. In addition, the effect of the postpartum rate of increase of concentrate allowance on VFA production and absorption was examined. The current results are complementary to previously reported changes on rumen papillae morphology from the same experiment (Dieho et al., 2016).

VFA Production

In line with our expectations, daily VFA production and rumen fluid VFA concentration increased with increased DM and FOM intake after onset of lactation. Concentration of a VFA in rumen fluid is mainly a reflection of its rate of production and rate of clearance, and to lesser degree rumen fluid volume. However, fractions of the VFA produced (mol/100 mol) are not sensitive to the volume of rumen fluid. In line with results of Sutton et al. (2003), fractions of Ac and Pr of VFA produced were strongly related (Ac: r^2 = 0.85; Pr: r^2 = 0.95) to fractions of Ac or Pr in rumen fluid, whereas this relationship for Bu was weak (r^2 = 0.17; Figure 2). These relationships were established for dry period and lactation rations with daily feed intakes ranging from 11 to 25 kg of DM, confirming that fractions of Ac and Pr in rumen fluid are good indicators of the proportion in which they are produced. For Bu, the poor relationship and regression parameters indicate Bu production to be lower than that expected based on the fraction of Bu in rumen fluid. The fraction of Ac and Pr in the rumen fluid is slightly underestimated by the fraction in which Ac and Pr were produced, whereas Bu is overestimated.

In contrast to Sutton et al. (2003), who continuously infused ^{14}C-labeled VFA for 22 h into the rumen, we introduced ^{13}C-labeled VFA and fluid passage marker as a pulse dose at the start of the IDT measurements. We neither disturbed normal rumen function nor stratification by mixing the rumen contents by hand, and therefore a delay in the start of the clearance of ^{13}C-labeled VFA and fluid passage marker may have occurred (Teeter and Owens, 1983) leading to possible underestimation of VFA clearance and, thus, production rates in our experiment. The net total VFA production per kilogram of DM pp (on average 5.1 mol/ kg of DM) was somewhat lower than that reported by Sutton et al. (2003; 6.2 mol/kg of DM for a 60% concentrate, 40% roughage diet), but within the range reported by Siciliano-Jones and Murphy (1989; range = 4.8 to 6.3 mol/kg of DM in two 80% concentrate 20% roughage and two 20% concentrate, 80% roughage treatments). In contrast, the net VFA production per

kilogram of DM calculated from Markantonatos et al. (2009; 2.6 and 2.9 mol/kg of DM for the dry period and lactation, respectively) is substantially lower than in our study and the studies of Sutton et al. (2003) and Siciliano-Jones and Murphy (1989).
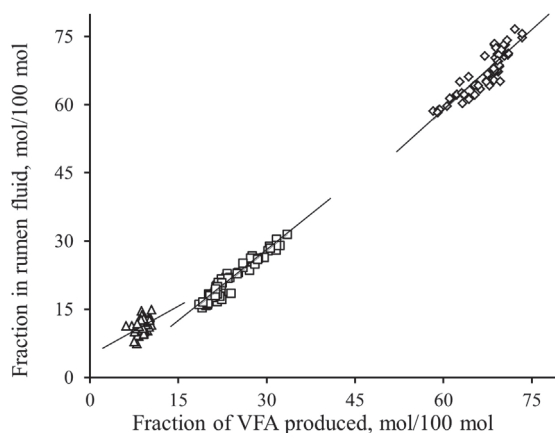

Figure 2. Relationship between the fraction of acetic (Ac), propionic (Pr), and butyric acid (Bu) of total VFA produced (mol/100 mol) and fraction of Ac, Pr, or Bu of total VFA concentration in rumen fluid (mol/100 mol) during the isotope dilution technique, for Ac [$\Diamond$; solid line: b_0 = -14.3 ± 4.62, b_1 = 1.21 ± 0.069, squared correlation (r^2) = 0.85, P < 0.01], Pr ($\square$; solid line: b_0 = -4.0 ± 0.78, b_1 = 1.07 ± 0.032, r^2 = 0.95, P < 0.01), and Bu ($\triangle$; solid line: b_0 = 4.7 ± 1.94, b_1 = 0.75 ± 0.216, r^2 = 0.17, P < 0.01) (n = 12). b_0: intercept; b_1: slope of the regression equation.

Based on previous results from buffer incubation studies (Sutton et al., 1963; Thorlacius and Lodge, 1973; Dijkstra et al., 1993), the lower fractional absorption rate of Bu compared with Ac observed in the present experiment using the IDT was not expected. The use of a pulse-dose, which simultaneously enriches Ac, Pr, and Bu and does not allow for quantification of the C fluxes between Ac, Pr, and Bu, may explain the low fractional absorption rate of Bu. Interconversion of VFA would result in a net efflux of C from the Ac pool, and net influx of C into the Pr and in particular into the Bu pool (Sutton et al., 2003; France and Dijkstra, 2005; Markantonatos et al., 2008). The clearance rate of Ac during the IDT is therefore overestimated, as ^{13}C not only leaves the Ac pool through absorption across the rumen wall and passage with the fluid but also by conversion to other VFA. In contrast, the fractional clearance rates of Pr and in particular of Bu are likely underestimated, as there is a net flow of ^{13}C from Ac into the Pr and Bu pools. Due to the difference in ^{13}C pool sizes of Ac and Bu added at t = 0 h, a net flow of 10 to 30% 13C from Ac to Bu (Sutton et al., 2003; Markantonatos et al., 2008) is sufficient to replenish 33 to 100% of the initial ^{13}C pool of Bu, explaining

a significant part of the low fractional rate of absorption of Bu. A difference in ^{13}C flow from Ac to Bu for RAP and GRAD might mask the treatment effect on Bu production, although differences in ration composition were limited compared with the earlier studies suggesting a difference in ^{13}C flow was likewise limited.

All major VFA promote rumen papillae development in vivo (Sutton et al., 1963; Sakata and Tamate, 1979; Suárez et al., 2006), but Bu is generally believed to have a particular proliferative effect (Mentschel et al., 2001; Malhi et al., 2013; Kowalski et al., 2015). Given the greater papillae surface area in early lactation (16, 30, and 44 d pp) associated with a rapid compared with a gradual increase in concentrate allowance (Dieho et al., 2016), it was expected that Bu production would be larger at 16 and possibly 44 d pp in RAP compared with GRAD. However, neither the Bu production nor its concentration and proportion in the rumen fluid at 16 d pp appeared to be affected by rate of increase of concentrate allowance, which did result in a greater starch and sugar intake for RAP (Dieho et al., 2016). In contrast, the production, concentration and molar proportion of Pr in the rumen fluid was higher in RAP at 16 d pp. Whereas this experiment did not aim to examine the specific effects of Pr or Bu on papillae proliferation and no causal relationships can be made, a larger Pr production rate coinciding with papillae proliferation suggests that Bu is not the sole VFA implicated with this adaptive process.

Fractional Absorption Rate of VFA

For the BIT, the k_aAc, k_aPr, and k_aBu approximately doubled from the end of the dry period (10 d ap) to the end of the experimental period (80 d pp), supporting our expectations based on the observed increase in papillae surface area. For the IDT, however, changes in k_a of VFA were less pronounced, showing only modest increases of 55% for Ac, 20% for Pr, and no increase for Bu. Major methodological differences exist between the BIT and IDT. Basically, BIT reflects the capacity of the rumen to absorb VFA under standardized conditions, whereas IDT reflects absorption of VFA during the actual physiological conditions at time of measurement. The k_a of VFA is affected by factors including concentration of VFA in rumen fluid, VFA carbon-chain length, fluid pH, and rumen fluid volume (Sutton et al., 1963; Thorlacius and Lodge, 1973; Dijkstra et al., 1993). A standardized buffer fluid was used for the BIT to study changes in k_a of VFA related to differences in papillae surface area, rather than related to other factors. For the current experiment, buffer fluid VFA concentrations and pH were chosen to represent rumen fluid typically observed in lactation dairy cows (Rabelo et al., 2003; Abrahamse et al., 2008; Steele et al., 2012). The total VFA concentration, type of VFA, and initial pH measured in the BIT buffer resembled those measured in

the rumen fluid pp (Table 1). Therefore, comparable k_a of VFA for the BIT and IDT during the lactation were expected. However, k_aAc, k_aPr, and k_aBu with the BIT were approximately 20, 25, and 45% larger than those found with the IDT, respectively. In the dry period there seems to be more agreement between the methods, as k_aAc (BIT = 0.40/h; IDT = 0.36/h) and k_aPr (BIT = 0.47/h; IDT = 0.48/h) were rather similar between both methods, although not for k_aBu (BIT = 0.52/h; IDT = 0.31/h). The good agreement in fractional absorption rate of Ac or Pr between both methods during the dry period was unexpected. The conditions for absorption of VFA from the rumen fluid during the IDT in the dry period (higher pH, lower VFA concentration) were less favorable compared with the buffer fluid, suggesting higher k_a of VFA would be found for the BIT. However, as discussed in section VFA Production, it is likely that the IDT overestimates fractional clearance rate of Ac and underestimates that of Bu, resulting in corresponding over- or underestimation of the fractional absorption rates. During the dry period this might explain the similar k_aAc for the BIT and IDT, and explain part of the difference in k_aBu.

Overall, the fractional absorption rates measured during the BIT had weak relationships with those measured during the IDT (k_aAc, r^2 = 0.19; k_aPr, r^2 = 0.08; k_aBu, r^2 = 0.01; Figure 3), and fractional absorption rates during BIT were usually higher than those during IDT. The correlation between the BIT and IDT, when determined separately for the dry period and lactation period, marginally improved for Pr only, increasing to r^2 = 0.21 and r^2 = 0.16, respectively. The generally higher k_a of VFA measured using the BIT may also result from a more efficient mixing of the buffer fluid during the incubation. Feed particles, which physically inhibit fluid flow, were absent and frequent rumen contractions caused turbulence within the buffer fluid which could easily be observed, reducing intraruminal differences or gradients in VFA concentration. During lactation, the greater rumen fill would have impaired free movement of rumen fluid to greater extent than during the dry period. However, during the dry period, the greater k_aVFA of the BIT is better explained by the higher VFA concentration and lower pH compared with the IDT. The capacity of the rumen epithelium to absorb VFA appears to have been used to a larger extent with BIT than IDT. These results, and differences in VFA concentration observed within the rumen (Tafaj et al., 2006), supports the concept that movement of rumen fluid and transport of VFA to the rumen wall may significantly influence VFA absorption (Storm and Kristensen, 2010). This may also imply that subacute rumen acidosis is not just related to a lack of intrinsic capacity of the rumen epithelium for VFA absorption, but is also related to limitations of intraruminal movement of VFA toward the rumen epithelium.
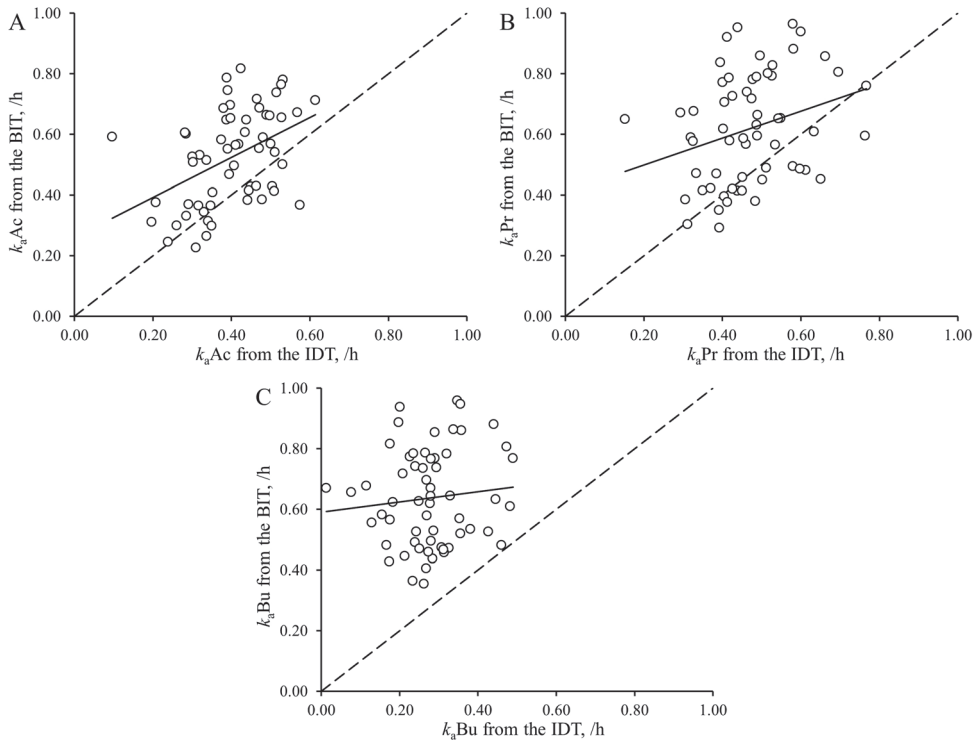
Figure 3. Relationships between the fractional absorption rate (k_a, per hour) obtained using the isotope dilution technique (IDT) in the naturally filled rumen and the buffer incubation technique (BIT) in the empty washed rumen, for (A) acetic acid [k_aAc; solid line: $b_0 = 0.26 \pm 0.074$, $b_1 = 0.66 \pm 0.180$, squared correlation (r^2) = 0.19, $P < 0.01$], (B) propionic acid (k_aPr; solid line: $b_0 = 0.41 \pm 0.095$, $b_1 = 0.44 \pm 0.196$, $r^2 = 0.08$, $P = 0.03$), (C) butyric acid (k_aBu; solid line: $b_0 = 0.59 \pm 0.064$, $b_1 = 0.17 \pm 0.216$, $r^2 = 0.01$, $P = 0.43$). Dashed line indicates unity. b_0: intercept; b_1: slope of the regression equation.

Rumen Papillae Surface Area and VFA Fractional Absorption Rate

Although a larger rumen papillae surface area has been shown to coincide with a higher rate of VFA absorption (Dirksen et al., 1984; Gaebel et al., 1987; Melo et al., 2013), a limited amount of in vivo data are available examining this relation in dairy cows during the transition period (Bannink et al., 2012; Martens et al., 2012). The previously reported results from the present experiment show that papillae surface area decreased during the dry period and increased after calving (Dieho et al., 2016). Generally, the observed increase in papillae surface area coincides with an increase in VFA absorption capacity during the dry period up to 80 d pp in the present experiment and confirms earlier reports (Dirksen et al., 1984; Gaebel et al., 1987; Melo et al., 2013). However, in contrast to the greater rumen papillae surface area at 16, 30, and 44 d pp

with a rapid increase of concentrate allowance pp (Dieho et al., 2016), the measured k_a of VFA was not affected by the rate of increase of concentrate allowance, neither for the BIT (16, 30 and 44 d pp) nor the IDT (16 and 44 d pp). An increased papillae surface area without a concomitant rise in fractional absorption of VFA was unexpected. It may suggest a diminished capacity of VFA absorption per unit of surface area; however, this is not supported by the literature (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014). Also, a decrease in true rumen surface area, despite growth of individual papillae, is unlikely, as papillae density does not differ between the dry period and early lactation (Reynolds et al., 2004). However, epithelial blood flow is reported to affect VFA absorption (Storm et al., 2011), and blood flow rather than papillae surface area might be a limiting factor for the increase in VFA absorption with rapid increase of concentrate allowance during early lactation. The large increase in splanchnic blood flow after calving (80% increase by 11 d pp, compared with 9 d ap; Reynolds et al., 2003) might be a factor explaining the rapid increase in k_aVFA between 10 d ap and 16 d pp in the present experiment. A greater capacity for VFA metabolism by the rumen epithelium might also aid k_aVFA by decreasing intracellular VFA concentrations, although metabolism of Ac and Pr is limited and inhibited by Bu (Kristensen, 2005).

Papillae surface area and morphology differ markedly between individual cows even on the same ration and with similar feed intake (Reynolds et al., 2004; Dieho et al., 2016). The repeated measurement approach used in the present study allows determination of the relationship between observed papillae surface area and VFA fractional absorption rate for individual cows. This relationship per cow ranged from weak (minimum r^2 = 0.32) to strong (maximum r^2 = 0.87), with generally a lower correlation for individual cows in RAP compared with cows in GRAD (data not shown). Using the mixed-model approach for multiple experiments described by St-Pierre (2001), repeated measurement data from multiple cows were adjusted for the random effect of cow. For the entire experimental period, 50 d ap to 80 d pp, papillae surface area was moderately related to k_aVFA obtained using the BIT (r^2 = 0.50; Figure 4A). However, for both the pretreatment period (Figure 4B) and treatment period (Figure 4C) the relationship was weak (pretreatment period, r^2 = 0.23; treatment period, r^2 = 0.38).
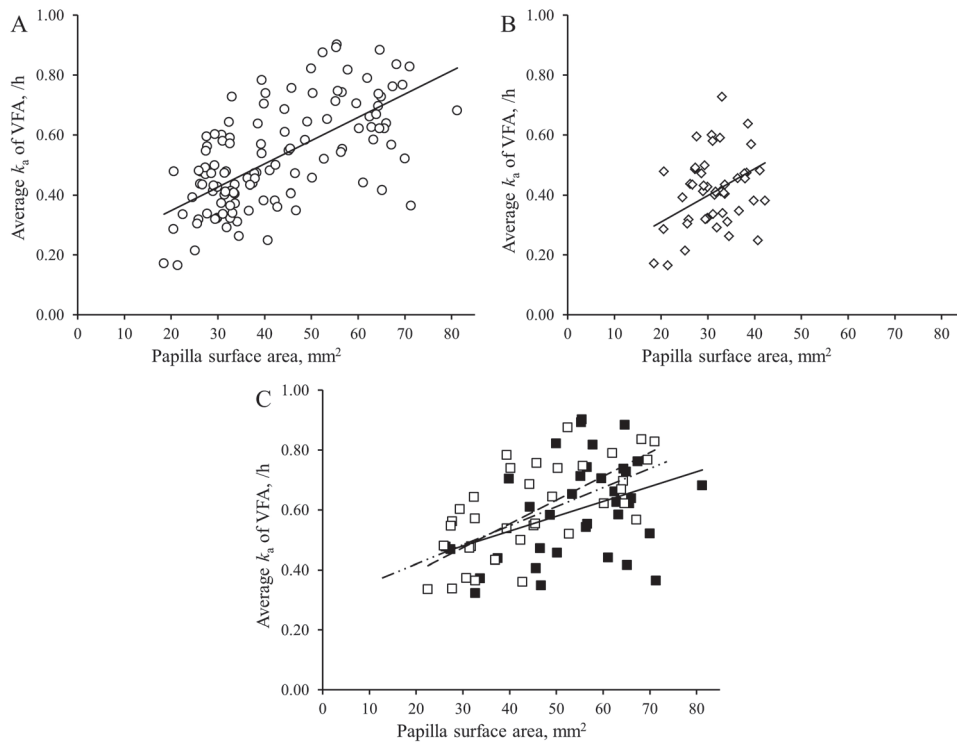
Figure 4. Relationship between rumen papillae surface area (mm^2) and rumen fractional absorption rate of VFA (k_a of VFA, per hour; average of k_a of acetic, propionic, and butyric acid) obtained using the buffer incubation technique for (A) the entire experimental period [O; solid line: $b_0 = 0.19 \pm 0.033$, $b_1 = 0.0078 \pm 0.0007$, squared correlation (r^2) = 0.50, $P < 0.01$; n = 12], (B) the pretreatment period (◇; solid line: $b_0 = 0.13 \pm 0.077$, $b_1 = 0.0089 \pm 0.0024$, $r^2 = 0.23$, $P < 0.01$; n = 12), and (C) the treatment period for cows receiving a rapid rate of increase of concentrate allowance postpartum (1.0 kg DM/d; ■; solid line: $b_0 = 0.33 \pm 0.095$, $b_1 = 0.0050 \pm 0.0017$, $r^2 = 0.20$, $P < 0.01$; n = 6), a gradual rate of increase of concentrate allowance postpartum (0.25 kg DM/d; □; dashed line: $b_0 = 0.24 \pm 0.095$, $b_1 = 0.0081 \pm 0.0017$, $r^2 = 0.40$, $P < 0.01$; n = 6), and both concentrate treatments combined (dash-and-dotted line: $b_0 = 0.29 \pm 0.047$, $b_1 = 0.0063 \pm 0.0010$, $r^2 = 0.38$, $P < 0.01$; n = 12). b_0: intercept and b_1: slope of the regression equation.

The moderate relationships between papillae surface area and VFA fractional absorption rate for the entire experimental period contrasts with the weak relationships between papillae surface area and VFA fractional absorption rate during the period with rapid changes in FOM intake. This suggests that, besides papillae surface area, other factors should be considered as indicators for rumen wall adaptation and concomitant VFA absorption during the transition period in dairy cattle.

CONCLUSIONS

The ruminal VFA fractional absorption rate measured under standardized (BIT) and physiological (IDT) conditions decreased during the dry period and increased after calving, and generally followed a pattern similar to changes in papillae surface area. However, unlike papillae surface area, the VFA fractional absorption rate was not affected by rate of increase of concentrate allowance pp. This suggests only a limited effect of papillae surface area on fractional VFA absorption rate during the transition period in dairy cattle. Fractional absorption rates pp obtained with the BIT were in general greater than those obtained with the IDT, suggesting the presence of rumen contents affects VFA fractional absorption rate. The increased papillae surface area with a faster rate of increase of concentrate allowance in early lactation coincided with a higher Pr production rate, whereas the production rate of Ac and Bu did not differ.
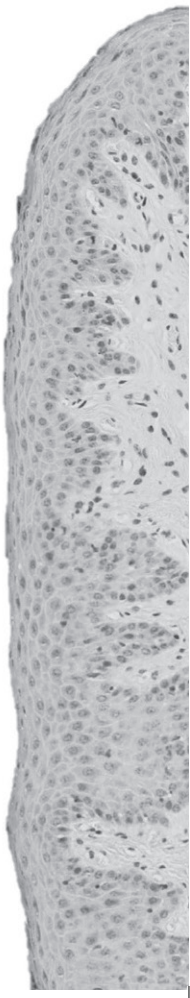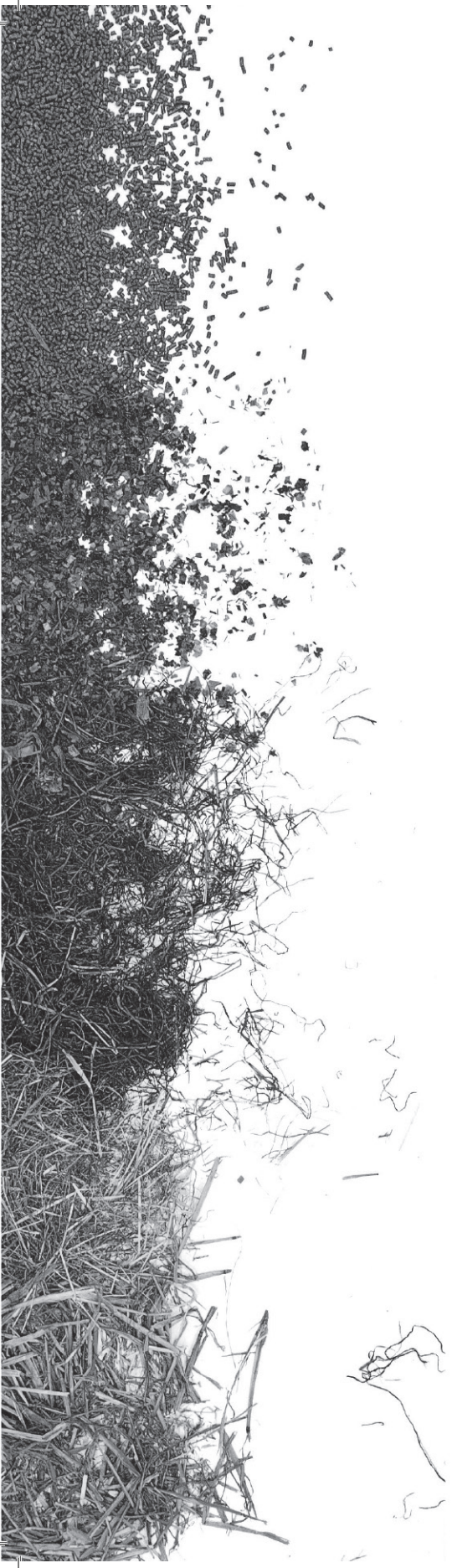
ACKNOWLEDGMENTS

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Chapter 4

The effect of supplemental concentrate fed during the dry period on morphological and functional aspects of rumen adaptation in dairy cattle during the dry period and early lactation

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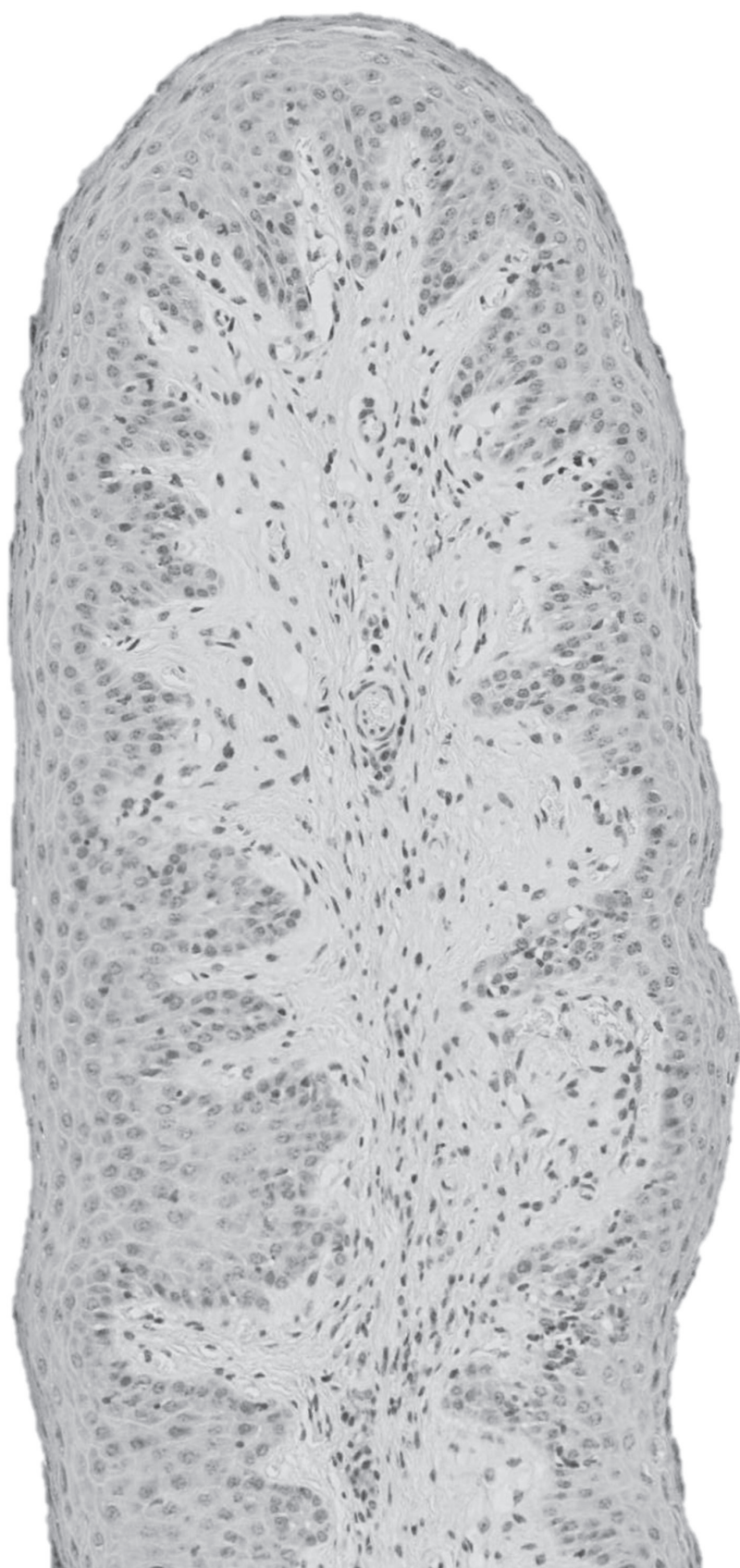
ABSTRACT

Ten rumen-cannulated Holstein-Friesian cows were used to examine the effect of feeding supplemental concentrate during the dry period on rumen papillae morphology and fractional absorption rate (k_a) of volatile fatty acids (VFA) during the dry period and subsequent lactation. Treatment consisted of supplemental concentrate [3.0 kg of dry matter (DM)/d] from 28 d antepartum (ap) until the day of calving, whereas control did not receive supplemental concentrate. Cows were fed for ad libitum intake and had free access to the dry period ration (27% grass silage, 28% corn silage, 35% wheat straw, and 11% soybean meal on a DM basis) and, from calving onward, to a basal lactation ration (42% grass silage, 42% corn silage, and 16% soybean meal on a DM basis). From 1 to 3 d postpartum (pp), all cows were fed 0.9 kg DM/d of concentrate, which increased linearly thereafter to 8.9 kg of DM/d on d 11 pp. At 28, 18, and 8 d ap, and 3, 17, 31, and 45 d pp, rumen papillae were collected and k_aVFA was measured in all cows. On average, 13.8 (standard deviation: 3.8) papillae were collected each from the ventral, caudodorsal, and caudoventral rumen sacs per cow per day. The k_aVFA was measured by incubating a standardized buffer fluid (45 L), containing 120 mM VFA (60% acetic, 25% propionic, and 15% butyric acid) and Co-EDTA as fluid passage marker, in the evacuated and washed rumen. Treatment did not affect ap or pp DM and energy intakes or milk yield and composition. Treatment increased papillae surface area, which was 19 and 29% larger at 18 and 8 d ap compared with 28 d ap, respectively. Surface area increased, mainly due to an increase in papillae width. However, treatment did not increase k_aVFA at 18 and 8 d ap compared with 28 d ap. In the control group, no changes in papillae surface area or k_aVFA were observed during the dry period. In the treatment group, papillae surface area decreased between 8 d ap and 3 d pp, whereas no decrease was observed for control. From 3 to 45 d pp, papillae surface area and k_aVFA increased for all cows by approximately 50%, but the ap concentrate treatment did not affect k_aVFA pp. In conclusion, the efficacy of supplemental concentrate during the dry period to increase papillae surface area and k_aVFA in preparation for subsequent lactation is not supported by the present study. Current observations underline the importance of functional measurements in lieu of morphological measurements to assess changes in the adapting rumen wall.

Key words: transition dairy cow, rumen adaptation, rumen papillae, rumen epithelium, VFA absorption

95

INTRODUCTION

After calving, fermentable organic matter (**FOM**) intake of cows increases rapidly, resulting in a more than 2-fold increase in the production of VFA at maximal feed intake postpartum (Bergman, 1990). To maintain ruminal conditions favorable for fermentation, buffering and clearance of the produced VFA is essential (Penner et al., 2009; Aschenbach et al., 2011; Dijkstra et al., 2012). Clearance of VFA from the rumen is the result of either passage or absorption across the rumen epithelium (Gäbel et al., 2002; Aschenbach et al., 2011). Earlier work has shown that the rate of absorption of VFA depends on the pH of the rumen fluid, on the concentration and type of VFA (Thorlacius and Lodge, 1973; Dijkstra et al., 1993), and on the surface area of the rumen papillae (Dirksen et al., 1984; Melo et al., 2013).

Increased FOM intake results in an increase in VFA production and thereby in a proliferation of the rumen papillae and epithelium, through a direct effect on cell mitosis and apoptosis (Mentschel et al., 2001) and through growth factors of, for example, the IGF axis (Steele et al., 2015). Although growth can be rapid, the process of proliferation may take several weeks to complete (Dirksen et al., 1985; Bannink et al., 2012; Dieho et al., 2016a). It can be hypothesized that a mismatch between the rate of increase in VFA production and development of the papillae is a factor underlying problems with maintaining optimal rumen conditions for fermentation during early lactation (Dirksen et al., 1985; Krause and Oetzel, 2006; Aschenbach et al., 2011). Stimulation of the surface area of rumen papillae during the dry period may be an effective strategy to prevent high VFA concentrations and concomitant low pH rumen during early lactation (Liebich et al., 1987). However, an increase in papilla surface area does not necessarily result in a concomitant increase in fractional rate of VFA absorption (Dieho et al., 2016b). This finding casts doubt on the efficacy of increasing papillae surface area on VFA absorption capacity in the late dry period. In addition, to date, the efficacy of increasing papilla surface area during the late dry period on VFA absorption capacity during early lactation has not been supported by in vivo measurements.

The aim of the present experiment was therefore to examine the effect of feeding supplemental concentrate during the last 4 wk of the dry period on rumen papillae morphology and fractional absorption rate of VFA (k_a**VFA**) during the dry period and early lactation. Feeding supplemental concentrate during the dry period was expected to increase papillae surface area and k_aVFA in that period and to positively affect surface area and k_aVFA in the early weeks of subsequent lactation.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen University & Research and conducted under the Dutch Law on Animal Experiments.

Animals, Experimental Design and Management

Nine weeks before the expected calving date, 10 rumen-cannulated (10 cm i.d., Bar Diamond Inc., Parma, ID; cannulated during the first lactation) Holstein-Friesian dairy cows [second (n = 8) and fourth (n = 2) lactation] entered the randomized block design with repeated measurements experiment. Cows were paired based on parity, expected calving date, and milk yield of the previous lactation. Within pairs, cows were randomly assigned to either a control (**CON**, n = 5) or a supplemental concentrate treatment group (**SUP**, n = 5). Samples were collected 28, 18, and 8 d antepartum (**ap**), and at 3, 17, 31, and 45 d postpartum (**pp**). Dry and lactating animals were housed in separate groups in a freestall barn with a slatted concrete floor. Stalls were fitted with rubber mattresses covered with sawdust. On sampling days, before morning feeding, cows were moved to a tiestall at ~0830 h for the experimental procedures. Cows were milked at 0530 and 1530 h. Milk production was recorded at each milking and, each week, samples from 4 consecutive milkings (same days every week) were analyzed for fat and protein contents (ISO, 1999; Qlip NV, Zutphen, the Netherlands). During the dry period, cows were weighed at weekly intervals, and during lactation, BW was automatically recorded in the milking parlor.

Rations and Chemical Analysis

Throughout the dry period, all cows had free excess to the dry period ration (Table 1). From 28 d ap onward, the cows allocated to SUP were fed 3 kg DM/d of supplemental concentrate. From the day of calving onward, all cows had free access to the lactation ration (Table 1), and daily concentrate allowances were identical for both treatment groups.

Table 1. Basal ration composition, nutritional value and chemical composition of rations and concentrate (g/kg of DM unless stated otherwise)

	Basal ration		
	Dry-period	Lactation	Concentrate[1]
Ingredients			
Grass silage[2]	270	416	
Corn silage[2]	278	424	
Soybean meal	107	160	
Wheat straw	345	0	
Chemical composition			
DM, g/kg of product	503	409	890
Crude ash	81	78	70
Crude protein	109	151	162
Crude fat	21	32	23
Starch	111	165	201
Sugar	41	60	137
NDF	531	394	212
ADF	331	240	126
ADL	35	17	16
Calculated value[3]			
NE_L, MJ/kg of DM	5.3	6.6	7.5
IDP	56	87	103
RDPB	3	13	1
FOM	476	547	685

[1]Concentrate composition (ingredients, g/kg of DM): sugar beet pulp, 214; corn gluten, 211; barley, 175; citrus pulp, 160; wheat, 69; rapeseed meal, 63; soybean meal, 56; molasses, 40; salt, 7; $CaCO_3$, 3; and mineral premix, 2.

[2]Chemical composition of grass silage (g/kg of DM): crude ash, 111; CP, 102; crude fat, 36; sugar, 96; NDF, 525; ADF, 326; and ADL, 19. Chemical composition of corn silage (g/kg of DM): crude ash, 50; CP, 68; crude fat, 31; starch, 382; NDF, 377; ADF, 217; and ADL, 20.

[3]Calculated values for grass and corn silage based on near-infrared spectrometry (Blgg AgroXpertus, Wageningen, the Netherlands). Calculated values for soybean meal and concentrate provided by Agrifirm Feed (Apeldoorn, the Netherlands). Calculated values for wheat straw obtained from CVB Feed Tables (CVB, 2011). NE_L values were calculated according to the Dutch NE-system (van Es, 1978). IDP = intestinal digestible protein; RDPB = RDP balance; FOM = fermentable OM; all calculated according to the Dutch DVE/OEB system (Tamminga et al., 1994).

All cows were fed for ad libitum intake (minimum 10% refusals). From calving to 3 d pp, cows received 0.9 kg DM/d of concentrate. Thereafter, concentrate allowance increased at a rate of 1.0 kg of DM/d to a maximum of 8.9 kg of DM/d on 11 d pp.

The maximum concentrate allowance was maintained until the end of the experiment. Concentrate composition (Table 1) did not differ for the ap and pp periods. Daily intake (kg/d) of the rations was measured individually using computerized feed bins with automatic weighing (Insentec, Marknesse, the Netherlands). Maximum stocking density was 2 cows per feed bin, with cows having access to all feed bins. Feed was prepared daily and fed at 1000 h. All cows had free access to water throughout the experiment. The individual daily concentrate allowance was made available in equal portions over six 4-h periods using a concentrate dispenser (Manus VC5, DeLaval, Steenwijk, the Netherlands). During the visits, concentrate was fed in a series of small portions and cows were shielded from herdmates, effectively preventing other cows stealing concentrate. Roughage and concentrate samples were taken once a week, stored at −20°C pending analysis, and analyzed as described by Dieho et al. (2016a). Additional weekly roughage samples were used for determination of DM content by forced-air oven drying. If necessary, the basal ration formulation (on a product basis) was adjusted for changes in ration ingredient DM content.

Collection of Rumen Papillae and Measurements

During all sampling days, the rumen evacuation and papillae collection and processing were performed as described by Dieho et al. (2016a). Briefly, a rumen fluid sample was taken from the ventral rumen sac between 0.5 and 3 h (average 1 h) after last access to feed to determine pH and VFA concentration. Thereafter, rumen contents were completely evacuated and stored in a covered, insulated tub. Then, the rumen was washed twice with 10 L of tap water at 39°C. Subsequently, papillae were collected from the ventral rumen sac (**VRS**), the caudoventral blind rumen sac (**VBS**), and caudodorsal blind rumen sac (**DBS**). An average of 13.8 (SD 3.8; minimum of 8) of intact papillae per site per cow per sampling day were collected. Collected papillae were gently rinsed in 0.9% NaCl and stored in buffered 4% formaldehyde solution pending processing.

Macroscopic measurements on digital photographs of the papillae (one-sided surface area, length, and width) were made as described by Dieho et al. (2016a). Briefly, all papillae (per site per cow per sampling day) were photographed (Casio Exilim EX-Z70, Casio Computer Co., Tokyo, Japan), including a ruler in each photograph. The surface area of all papillae was measured using the 'Analyze Particles' feature of ImageJ (version 1.44n, National Institutes of Health, Bethesda, MD). Length (tip to base) and width (measured at the halfway point of the tip-to-base length) of all papillae was measured using ImageScope (version 11.2.0.780, Aperio Technologies, Leica Biosystems, Nußloch, Germany). Total papilla surface area was calculated as $2 \times$ one-sided surface area as measured.

Five papillae per site per cow per sampling day (in line with Odongo et al., 2006; Steele et al., 2015; Dieho et al., 2016a) were processed for histology (minimum of 3 wk after collection and storage in buffered 4% formaldehyde) and digitized, and measurements of papillae and epithelium thickness were made, as adapted from Dieho et al. (2016a). Briefly, total thickness of the papilla and thickness of the interstitium (both measured perpendicular to papilla length) were measured at 250, 500, 750, 1,000, and 1,250 µm from the tip of the papillae, and 5 times at approximately half of the papilla length at 250-µm intervals over a total length of 1,000 µm, using ImageScope (version 11.2.0.780, Aperio Technologies). Papillae and epithelium thicknesses measured at the tip and halfway the papillae length were averaged.

Measurement of Fractional Absorption Rate of VFA

The fractional absorption rate of VFA was measured using a buffer incubation technique (**BIT**) in an empty washed rumen. The BIT procedure, buffer fluid composition, sample processing, and analysis was described in detail in Dieho et al. (2016b). Briefly, 50.0 L of standardized buffer fluid at 39°C and pH 5.9, containing 120 mM VFA [60% acetic (**Ac**), 25% propionic (**Pr**), and 15% butyric (**Bu**) acid] and 0.17 mM Co-EDTA as fluid passage marker (Udén et al., 1980), was prepared immediately before use. After the rumen evacuation and collection of rumen papillae as described above, 5.0 L of buffer fluid was used to wash the rumen wall. All fluid was subsequently removed from the rumen using a vacuum system, followed by introduction of the remaining 45.0 L of buffer fluid. After 60 min, the remaining fluid was completely recovered using the vacuum system and weighed. Thereafter, rumen contents were returned to the respective cow. For determination of pH and VFA concentrations, buffer fluid was sampled immediately before introduction into the rumen and after 60 min of incubation in the rumen. Concentration of VFA in rumen and buffer fluid was determined by GC using hydrogen as carrier gas, and the concentration of Co in the buffer fluid was determined by atomic-absorption spectrophotometry (Dieho et al., 2016b). The fractional absorption rate (k_a, /h) of Ac (k_a**Ac**), Pr (k_a**Pr**), and Bu (k_a**Bu**), and the fractional fluid passage rate (k_p, /h) were calculated as described by Dijkstra et al. (1993).

Blood Sampling and Analysis

Before starting the evacuation of the rumen contents, blood samples were collected from the tail vein using a vacuum sample tube (Vacuette, Greiner BioOne, Kremsmünster, Austria) containing either NaF for glucose, or heparin for nonesterified

fatty acids (**NEFA**) and BHB. Immediately after collection, samples were stored on ice for a maximum of 3 h before centrifuging (10 min at 3,000 × *g*). Blood plasma aliquots were stored at –20°C pending analysis of glucose, NEFA, and BHB concentration by the Veterinary Diagnostic Laboratory (Utrecht University, the Netherlands) as described by van Knegsel et al. (2007).

Statistical Analysis

All variables were assumed related to sampling day and treatment. For each sampling day, the reported DMI and nutrient intake are the averages of intakes recorded during the 3 preceding days. All morphological measurements were averaged per site per cow per sampling day before statistical analysis. Data describing rumen papillae were analyzed using the MIXED procedure (Littell et al., 2006) in SAS (version 9.3; SAS Institute Inc., Cary, NC) with the model

$$Y_{ijkl} = \mu + T_i + S_k + D_l + (T \times D)_{il} + D(C)_{jl} + e_{ijkl},$$

where variable Y_{ijkl} was dependent on μ as the average experimental value and fixed main effects of concentrate treatment T_i (i = CON, SUP), sampling day D_l (l = −28, ..., 45), site S_k (k = VRS, VBS, DBS), and interaction $(T \times D)_{il}$. Random effects of sampling day within cows $D(C)_{jl}$ were included, avoiding pseudo-replication due to multiple biopsy sites S_k. Errors e_{ijkl} were assumed to be auto-correlated for repeated observations on the same cow and site, using a spatial power covariance structure over sampling days (Littell et al., 1998). All other data for which sampling site within the rumen was not applicable were analyzed with a reduced model by removing S_k and $D(C)_{jl}$ and with repeated measurements on the same cow (Dieho et al., 2016b). Where appropriate, D_l was replaced by *Week*₁. Contrast statements were constructed for testing the (1) effect of dry period versus lactation; (2) treatment main effect, T_i (averaged over sampling days); (3) sampling day main effect, D_l (averaged over treatments); and (4) interaction between treatment and sampling day, $(T \times D)_{il}$. Treatment effects on individual sampling days were tested using the SLICE option when an interaction $(T \times D)_{il}$ was observed. All morphological papillae variables are reported as the least squares means of the 3 sampling sites for each treatment and sampling day combination. All results are reported as least squares means with standard errors unless indicated otherwise. Significance of effect was declared at $P < 0.05$ and trends at $0.05 \leq P < 0.10$.

RESULTS

Nine out of 10 cows completed the experiment successfully. One cow (SUP) calved earlier than expected (stillbirth), and missed sampling at 8 d ap. This cow was treated for retained fetal membranes, endometritis, and subsequently for a displaced abomasum that required surgery. At this point, the cow was removed from the experiment to aid recovery. All data relating to this cow were excluded from the analysis. Four other cows received veterinary treatment. One cow was treated for coliform mastitis (CON), 2 for (chronic) endometritis (both CON), and 1 for ketosis (SUP). These animals reacted favorably to treatment and completed the experiment as planned. Average (SD) actual sampling days were 29.1 (1.8), 18.9 (2.0), and 8.6 (1.9) d ap, and 3.2 (0.4), 17.7 (1.0), 31.3 (0.5), and 45.6 (1.3) d pp. No difference in actual sampling days was detected between CON and SUP (P = 0.85).

Feed Intake, Production, BW, and Blood Metabolites

The daily allowances of the supplemental concentrate were completely consumed during the dry period. Total DMI (Table 2; Figure 1A) increased from, on average, 12.2 kg/d during the dry period to 22.3 kg/d at 45 d pp (P < 0.01). No interaction between treatment and sampling day (P = 0.58) was observed for DMI, and it was similar for CON and SUP throughout the experimental period (P = 0.56). Basal ration DMI was numerically lower from 18 d ap to 3 d pp in SUP (9.2 kg/d) than in CON (11.8 kg/d), but neither an effect of treatment (P = 0.14) nor an interaction between treatment and sampling day (P = 0.78) was observed. Despite the greater contents of FOM, starch, sugar, and NE_L, and lesser content of NDF in the concentrate versus the basal dry period ration, only the intake of sugar was affected. For sugar intake, we observed an interaction between treatment and sampling day (P < 0.01), with approximately double intake for SUP (0.8 kg/d) compared with CON (0.5 kg/d) at 18 and 8 d ap.

During lactation, daily intake of NE_L increased from 66.5 MJ/d during the dry period to 154.0 MJ/d at 45 d pp (P < 0.01). Total DMI and intakes of FOM, starch, sugar, and NE_L were greater during lactation compared with the dry period (P < 0.01), whereas intakes of basal ration and NDF were similar during the dry period and lactation ($P \geq$ 0.37).
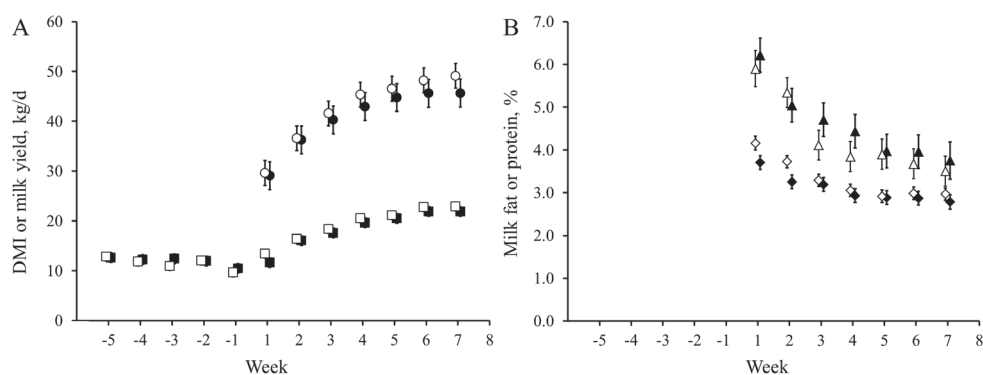
Figure 1. (A) Weekly average of DMI ($\square\blacksquare$) and daily milk production ($\bigcirc\bullet$), and (B) weekly average of milk fat ($\triangle\blacktriangle$) and milk protein ($\diamondsuit\blacklozenge$) content of 4 consecutive milking sessions for the control (no concentrate during the dry period; $\square\bigcirc\triangle\diamondsuit$, n = 5) and for the dry period treatment with supplemental concentrate (3.0 kg of DM/d concentrate during the dry period; $\blacksquare\bullet\blacktriangle\blacklozenge$, n = 4), and the subsequent lactation period. Values represent LSM ± SE (values are slightly offset for clarity).

The intake of supplemental concentrate during the dry period had no effect on subsequent milk yield or milk composition. Daily milk production (Figure 1A) increased from wk 1 to 7 from an average of 29.3 to 47.4 ± 1.86 kg/d ($P < 0.01$), but we observed neither a carry-over effect of treatment ($P = 0.59$) nor an interaction between treatment and week ($P = 0.98$). The milk fat and protein contents (Figure 1B) were not affected by treatment ($P \geq 0.27$) and we did not detect an interaction between treatment and week ($P \geq 0.21$). Daily milk yield expressed as fat- and protein-corrected milk did not show differences between CON and SUP (data not shown).

Body weight (Figure 2) was not affected by treatment ($P = 0.67$), and no interaction between treatment and week was observed ($P = 0.97$). Body weight did not change during the dry period ($P = 0.15$), averaging 816 ± 25.6 kg. After calving, BW decreased from 737 ± 25.6 kg during wk 1 pp to 698 ± 25.6 kg during wk 7 pp ($P < 0.01$).

Blood glucose (Table 3) was not affected by treatment or sampling day, and we found no interaction between treatment and sampling day ($P \geq 0.32$). Blood glucose was higher during the dry period compared with lactation ($P < 0.01$). In contrast, NEFA and BHB concentrations were higher during the lactation compared with the dry period ($P < 0.01$). Mean NEFA and BHB concentrations increased ($P \leq 0.01$) from 0.30 and 0.53 mM during the dry period to peak values of 1.43 and 1.16 mM at 3 d pp, respectively. We detected no interactions between treatment and sampling day for either blood NEFA or BHB ($P \geq 0.21$).

103
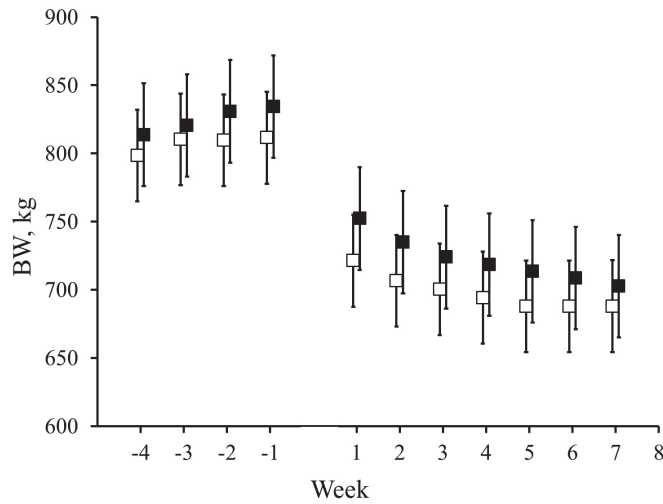
Figure 2. Weekly average of bodyweight (BW) for the control (no concentrate during the dry period; □, n = 5) and for the dry period treatment with supplemental concentrate (3.0 kg of DM/d concentrate during the dry period; ■, n = 4), and the subsequent lactation period. Values represent LSM ± SE (values are slightly offset for clarity).

Morphological Changes of the Rumen Papillae

Papillae surface area (Table 4) changed during the experimental period ($P <$ 0.01) and was greater during lactation than the dry period ($P < 0.01$). An interaction between treatment and sampling day was observed ($P = 0.04$). Papillae surface area increased by 10.1 mm^2 from 28 to 8 d ap ($P < 0.01$) for SUP but subsequently decreased by 6.9 mm^2 between 8 d ap and 3 d pp ($P = 0.02$). Papillae surface area for CON remained similar during the dry period ($P = 0.64$) and did not change from 8 d ap to 3 d pp ($P = 0.37$). During lactation, from 3 to 45 pp, papillae surface area increased by 51 and 43% for CON and SUP ($P < 0.01$), respectively. An interaction between treatment and sampling day was observed ($P = 0.03$) for the change in papillae surface area relative to 28 d ap. Papillae surface area increased for SUP at 18 and 8 d ap, whereas a numerical decrease was observed for CON on these sampling days. These differences in papillae development between treatment groups disappeared from 3 d pp onward. Changes in papillae surface area during lactation were greater than those during the dry period (P < 0.01).

Table 2. Least squares means of feed intake[1] (kg/d unless otherwise noted) for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP, n = 4[2])

Item	Dry period			Lactation				SE[4]	Fixed effects[3]		
	−28	−18	−8	3	17	31	45		T	D	T × D
Total DMI											
CON	13.1	11.0	12.4	12.9	17.7	20.8	23.0	0.87	0.56	<0.01	0.58
SUP	12.5	12.3	12.2	10.2	17.4	20.5	21.5				
Basal ration DMI											
CON	13.1	11.0	12.4	12.1	8.8	11.8	14.0	0.86	0.14	<0.01	0.78
SUP	12.5	9.3	9.2	9.2	8.5	11.5	12.5				
Fermentable OM intake											
CON	6.2	5.2	5.9	7.0	10.9	12.6	13.8	0.46	0.82	<0.01	0.30
SUP	5.9	6.5	6.4	5.8	10.8	12.5	13.0				
NDF intake											
CON	7.0	5.9	6.6	5.1	5.4	6.6	7.4	0.40	0.27	<0.01	0.80
SUP	6.6	5.6	5.5	3.8	5.3	6.5	6.9				
Starch intake											
CON	1.4	1.2	1.4	2.0	3.2	3.8	4.1	0.13	0.98	<0.01	0.30
SUP	1.4	1.6	1.7	1.7	3.2	3.7	3.9				
Sugar intake											
CON	0.5	0.4	0.5	0.8	1.7	1.9	2.1	0.05	0.30	<0.01	<0.01
SUP	0.5	0.8 ***	0.8 **	0.7	1.7	1.9	2.0				
NE$_L$ intake, MJ/d											
CON	68.9	57.4	64.8	82.9	124.0	144.2	158.8	5.4	0.78	<0.01	0.37
SUP	65.9	70.9	71.0	68.0	122.3	142.3	149.1				

[1]Reported mean is calculated over the 3 d preceding the sampling day.
[2]Group SUP: n = 4 (one cow was removed from the experiment).
[3]T = Treatment; D = sampling day; T × D = interaction between treatment and sampling day.
[4]SE for LSM by sampling day; n = 9.
**P < 0.01, ***P < 0.001, significance of difference in LSM of CON and SUP for the same sampling day and variable.

Chapter 4

105

Changes in papillae surface differed between sites (P = 0.01). The greatest changes in surface area were observed for the DBS, mainly during lactation. Surface area in the DBS increased, on average, 10.6 mm^2 from 3 to 45 d pp, compared with 5.6 and 4.6 mm^2 for the VRS and VBS ($P \leq 0.02$), respectively. Changes in papillae surface area were similar in the VRS and VBS (P = 0.61). Papillae length was affected by sampling day ($P < 0.01$) and was greater during lactation than during the dry period ($P < 0.01$). It also tended to increase between 28 and 8 d ap in SUP (P = 0.05) but not in CON (P = 0.35).

After calving, papillae length increased ($P < 0.01$). The length of papillae differed (P = 0.02) for the sites, with an average length of 9.6, 9.1, and 8.4 mm for the VRS, VBS, and DBS, respectively. Papillae width was affected by sampling day ($P < 0.01$), treatment ($P < 0.01$), and the interaction between treatment and sampling day ($P < 0.01$) and width was greater during lactation than the dry period ($P < 0.01$). Papillae width was greater in CON at 28 d ap than in SUP. However, at 18 and 8 d ap and at 3 d pp, this difference disappeared and returned from 17 d onward. Compared with 28 d ap, papillae width had increased by 24% in SUP by 8 d ap, whereas no changes in width were observed in CON.

Papillae were generally thicker for CON than for SUP (P = 0.01), and thickness was affected by sampling day ($P < 0.01$), showing a temporary peak at 17 d pp. An interaction between treatment and day was observed (P = 0.04) and papillae were thinner at 18 and 8 d ap and 3 d pp in SUP compared with CON. In contrast to thickness of the complete papillae, thickness of the epithelial layer was not affected by sampling day (P = 0.15) and we observed no interaction between treatment and sampling day (P = 0.19). Thickness of the epithelium was greater, on average, for CON than for SUP (P = 0.02). Papillae thickness and epithelium thickness were similar during the dry period and lactation ($P \geq 0.49$)

Rumen Fluid Composition and Fractional VFA Absorption Rate

An interaction between treatment and sampling day ($P \leq 0.03$) was observed for total VFA concentration in, and pH of, the rumen fluid (Figure 3A). At 18 d ap, total VFA concentration was 33 mM higher ($P < 0.01$) for SUP (120 ± 5.5 mM) compared with CON (87 ± 5.5 mM). For SUP, total VFA concentration subsequently decreased (P = 0.02) to a nadir (91 ± 5.5 mM) at 3 d pp, whereas for CON it tended to increase during this period (P = 0.08).

Table 3. Least squares means of blood glucose, nonesterified fatty acids (NEFA), and b-hydroxybutyrate (BHB) concentrations for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP; n = 4[1])

Item	Sampling day relative to calving								Fixed effects[2]		
	Dry period		Lactation								
	-28	-18	-8	3	17	31	45	SE[3]	T	D	T × D
Glucose, mM											
CON	3.58	3.52	3.52	3.42	3.22	3.20	3.32	0.157	0.95	0.32	0.98
SUP	3.78	3.63	3.58	3.18	3.13	3.23	3.35				
NEFA, mM											
CON	0.21	0.39	0.41	1.20	0.85	0.64	0.50	0.108	0.57	<0.01	0.21
SUP	0.28	0.22	0.30	1.66	0.79	0.82	0.58				
BHB, mM											
CON	0.49	0.53	0.52	1.13	0.89	0.68	0.50	0.125	0.54	<0.01	0.98
SUP	0.58	0.51	0.54	1.16	0.95	0.74	0.80				

[1]Group SUP: n = 4 (1 cow was removed from the experiment).
[2]T = Treatment; D = sampling day; T × D = interaction between treatment and sampling day.
[3]SE for LSM by sampling day; n = 9.

Chapter 4

Table 4. Least squares means of papillae surface area, length, width, and thickness, and of thickness of the epithelial layer for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP, n = 4[1])

Item	Sampling day relative to calving							SE[3]	Fixed effects[2]			
	Dry period			Lactation					T	D	S	T × D
	-28	-18	-8	3	17	31	45					
Papillae surface area, mm[2]												
CON	41.5	39.4	38.5	40.7	47.6	56.3	61.6	2.10	0.29	<0.01	0.29	0.04
SUP	34.7	41.3	44.8	37.9	42.7	48.5	54.4					
Change[4] in papillae surface area, mm[2]		**	***									
CON	NA	-2.1	-3.0	-0.8	6.0	14.8	20.1	1.66	0.04	<0.01	0.01	0.03
SUP	NA	6.7	10.1	3.3	8.0	13.8	19.8					
Papillae length, mm												
CON	8.4	8.2	8.1	8.2	9.1	9.5	10.5	0.26	0.38	<0.01	0.02	0.23
SUP	8.5	8.9	9.3	8.4	9.3	9.6	10.4					
Papillae width, mm	*				†	*	*					
CON	2.6	2.6	2.6	2.6	2.8	3.3	3.3	0.07	<0.01	<0.01	0.58	0.01
SUP	2.1	2.4	2.6	2.5	2.5	2.7	2.9					
Papillae thickness[5], mm		*	*	***								
CON	298	289	294	291	303	273	279	7.2	0.01	<0.01	0.78	0.04
SUP	275	255	255	234	290	282	266					
Epithelium thickness[5], mm												
CON	70	67	71	70	72	66	66	2.3	0.02	0.15	0.11	0.19
SUP	63	61	59	56	70	68	66					

[1] Group SUP: n = 4, one cow was removed from the experiment.
[2] T = Treatment; D = sampling day; S = sampling site within the rumen. T × D = interaction between treatment and sampling day.
[3] SE for LSM by sampling day; n = 9.
[4] Relative to d -28.
[5] Average of thickness at papillae tip and halfway the papillae length.
† $P < 0.10$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significance of difference in LSM of CON and SUP for the same sampling day and variable.

When cows were lactating, total VFA concentration tended to be higher compared with that during the dry period (P = 0.05). Changes in rumen fluid pH were the inverse of the VFA concentration changes during the dry period and lactation, and pH was lower during the lactation compared with the dry period (P < 0.01). An interaction between treatment and sampling day was observed (P < 0.01), and pH was lower for SUP at 18 d ap (6.43 ± 0.09), and higher at 3 (6.72 ± 0.09) and 17 d pp (6.43 ± 0.09) (P ≤ 0.03) compared with CON (6.83, 6.13, and 5.95 ± 0.09 for 18 d ap, 3 and 17 d pp, respectively).
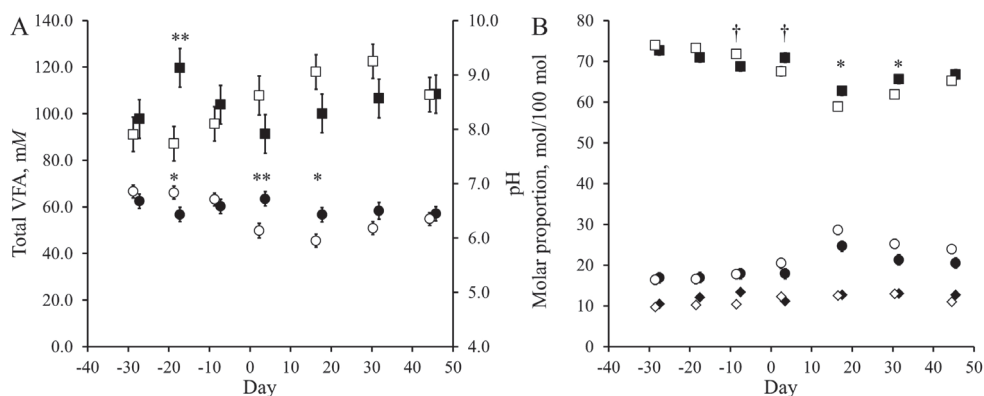
Figure 3. (A) Total VFA concentration (□■) and pH (○●), and (B) molar proportion of acetic (□■), propionic (○●), and butyric acid (◇◆) of total VFA in rumen fluid for the control (no concentrate during the dry period; □○◇, n = 5) and for the dry period treatment with supplemental concentrate (3.0 kg of DM/d concentrate during the dry period; ■●◆, n = 4), and the subsequent lactation period. Values represent LSM ± SE. †P < 0.10, *P < 0.05, **P < 0.01, significance of differences in LSM of CON and SUP for the same sampling day and variable (values are slightly offset for clarity).

Molar proportions (mol/100 mol) of Ac, Pr, and Bu (Figure 3B) were affected by sampling day (P ≤ 0.01). Generally, the proportion of Ac decreased (P < 0.01) after calving, from 72 ± 0.8 mol/100 mol during the dry period to 65 ± 0.8 mol/100 mol during lactation. Molar proportions of Pr and Bu increased accordingly (P < 0.01) from 17 ± 0.9 and 11 ± 0.5 mol/100 mol during the dry period to 23 ± 0.9 and 12 ± 0.5 mol/100 mol during lactation, respectively. An interaction between treatment and sampling day was observed for the molar proportion of Ac (P = 0.01), which tended to be lower at 8 d ap and higher at 3 d pp (P = 0.06) for SUP compared with CON. At 17 and 31 d pp, molar proportion of Ac was higher in SUP compared with CON (P ≤ 0.03). No interaction between treatment and sampling day was observed for the molar proportions of Pr (P = 0.45) and Bu (P = 0.16).

109

Table 5. Least squares means of the fractional rate of absorption (k_a) of acetic (Ac), propionic (Pr), and butyric acid (Bu), fractional fluid passage rate (k_f), and buffer fluid end pH measured using the buffer incubation technique in the empty washed rumen, for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP, n = 4[1])

Item	Sampling day relative to calving							SE[3]	Fixed effects[2]		
	Dry period			Lactation							
	-28	-18	-8	3	17	31	45		T	D	T × D
k_aAc, /h											
CON	0.26	0.28	0.30	0.27	0.34	0.39	0.45	0.027	0.53	<0.01	0.91
SUP	0.31	0.31	0.34	0.31	0.31	0.41	0.42				
k_aPr, /h											
CON	0.34	0.35	0.37	0.34	0.39	0.48	0.55	0.030	0.33	<0.01	0.94
SUP	0.39	0.40	0.43	0.39	0.39	0.51	0.53				
k_aBu, /h											
CON	0.39	0.40	0.42	0.39	0.42	0.48	0.56	0.028	0.30	0.01	0.73
SUP	0.46	0.43	0.48	0.46	0.41	0.52	0.55				
k_f, /h											
CON	0.16	0.14	0.12	0.23	0.18	0.27	0.23	0.020	0.14	0.03	0.06
SUP	0.16	0.16	0.16	0.22	0.20	0.17	0.13				
Buffer fluid end pH											
CON	7.14	7.11	6.86	7.03	7.05	7.27	7.35	0.056	0.02	<0.01	0.10
SUP	7.17	7.18	7.18	7.10	7.15	7.23	7.28				

[1]Group SUP: n = 4, one cow was removed from the experiment.
[2]T = Treatment; D = sampling day; T × D = interaction between treatment and sampling day.
[3]SE for LSM by sampling day; n = 9.

The k_aAc, k_aPr, k_aBu, and k_l (Table 5) were affected by sampling day ($P \leq 0.03$). The fractional rates of absorption remained similar throughout the dry period ($P \geq 0.45$) but increased between 3 and 45 d pp by 31% for k_aBu, 49% for k_aPr, and 50% for k_aAc ($P < 0.01$). Neither an effect of treatment ($P \geq 0.30$) nor an interaction between treatment and sampling day was observed ($P \geq 0.73$) for k_aAc, k_aPr, or k_aBu. The k_aAc and k_aPr were greater ($P < 0.01$), and the k_aBu tended to be greater ($P = 0.09$) during the lactation than the dry period. The k_l was lower ($P < 0.01$) during the dry period (0.15/h) compared with lactation (0.20/h). A tendency for an interaction between treatment and sampling day was observed ($P = 0.06$). The k_l was greater for CON on 31 and 45 d pp compared with SUP. The pH of the buffer fluid at the end of the incubation period was affected by sampling day ($P < 0.01$), with the lowest values occurring around calving. Buffer fluid end pH was also higher when cows were in lactation compared with the dry period ($P < 0.01$), and was generally higher for SUP compared with CON ($P = 0.02$), most notably at 8 d ap.

DISCUSSION

The present study provides an overview of the morphological changes of the rumen papillae and changes in ruminal k_aVFA in periparturient dairy cows with or without supplemental concentrate during the dry period. In line with our hypothesis, dry period concentrate supplementation increased papillae surface area and width. However, by 3 d pp, these differences had disappeared. Contrary to our expectation, concentrate supplementation during the dry period did not affect k_aVFA during the dry period or during early lactation.

Feed Intake, Production, BW, and Blood Metabolites

Unexpectedly, the feeding of supplemental concentrate did not increase DMI during the dry period. Inclusion of supplemental concentrate increased the NFC content and lowered the NDF content per kilogram of total diet DM consumed. Generally, a higher NFC (Holcomb et al., 2001; Rabelo et al., 2003; Overton and Waldron, 2004) and lower NDF (Hayirli et al., 2002) content increases dry period feed intake. In addition, concentrate is not expected to replace a roughage based ration on a kilogram per kilogram DM basis (NRC, 2001; CVB, 2011). However, a near-complete substitution did occur in SUP during the dry period. Possible explanations might be found in the daily energy intake and composition of the rations in relation to energy requirements. Energy requirement during the last month of gestation approximates 60 (CVB, 2011) to 66 MJ of NE_L/d (NRC, 2001) for a dairy cow (740 kg of BW).

Energy intake (71 MJ of NE_L/d, average of 18 and 8 d ap) by SUP thus exceeded requirements by 5 to 11 MJ of NE_L/d during the last 4 wk of the dry period, whereas energy intake was close to requirement for CON (61 MJ of NE_L/d, average of 18 and 8 d ap).The excess energy intake of SUP and glycogenic character of the concentrate might have increased blood leptin and insulin levels, which are known to depress feed intake (Ingvartsen and Andersen, 2000; Ingvartsen and Boisclair, 2001; Chilliard et al., 2005). Greater palatability of the concentrate (no refusals of concentrate were observed) might suggest that intake of the dry period ration was relatively decreased. Doepel et al. (2002) likewise did not find an increased DMI with rations with increased nutrient density in the dry period. Intake of DM during the dry period was comparable to that reported by that reported by Holcomb et al. (2001), Doepel et al. (2002), and Rabelo et al. (2003). The characteristic periparturient reduction in feed intake was not yet apparent at 8 d ap. However, inspection of daily feed intake records from 8 d ap to 3 d pp showed this decrease for both SUP and CON, with an average intake of 9.9 and 9.2 kg of DM/d during 3 to 1 d ap, respectively. In contrast to Doepel et al. (2002), but in line with Holcomb et al. (2001), Ingvartsen et al. (2001), and Reynolds et al. (2004), no effect of ap treatment on pp feed intake was found and feed intake was, compared with CON, only numerically lower in SUP at 3 d pp. Treatment contrasts (when compared on a NE_L or CP basis) as applied by Doepel et al. (2002) were greater than in the present study and might explain the different effects of the ap treatment on pp DMI. Although DMI was not affected by supplemental concentrate, sugar intake was approximately doubled and FOM intake was 25 and 9% greater (numerically only) with SUP than CON at 18 and 8 d ap, respectively. Supplemental concentrate reduced rumen pH, increased VFA concentrations, and induced rumen papillae growth during the dry period.

In line with Holcomb et al. (2001), Ingvartsen et al. (2001), Doepel et al. (2002), and Rabelo et al. (2003; multiparous cows only), feeding concentrate during the dry period had no effect on milk yield or milk fat and protein contents in the subsequent lactation. The numerical increase in BW of SUP compared with CON during the dry period agrees with the numerically higher energy intake of SUP. The decrease in BW after calving corresponds with the negative energy balance typically seen during early lactation. The treatment did not affect ap and pp blood NEFA, BHB, or glucose concentrations, which were within the range reported by earlier studies (VandeHaar et al., 1999; Holcomb et al., 2001; Kokkonen et al., 2004). Notably, no blood BHB concentrations above the clinical ketosis threshold (> 3.0 mM) were found, whereas 8 out of 63 collected samples were above the subclinical ketosis threshold (> 1.2 mM; McArt et al., 2012); namely, 4 samples from CON (3 cows at 3 d pp, 1 cow at 17 d pp) and 4 from SUP (1 cow at 3 d pp, and 1 cow at 3, 17, and 45 d pp). Therefore, supplemental concentrate during the dry period did not appear to affect the

milk production and metabolic status of cows in the subsequent lactation, although caution is warranted given the relatively small number of cows used.

Morphological Changes of the Rumen Papillae

Despite the limited effect of supplemental concentrate on nutrient intake during the dry period, papillae surface area was increased, supporting the earlier studies in dry cows by Dirksen et al. (1984) and Liebich et al. (1987). In contrast, Andersen et al. (1999), Rabelo et al. (2001), and Reynolds et al. (2004) did not report proliferative effects of dry period treatments on rumen papillae. However, clear differences in approach and methodology can be identified. Andersen et al. (1999) applied a 'VFA load strategy', feeding 3.9 kg of DM rolled barley in the morning and grass silage in the afternoon compared with a control ration of grass silage and 0.8 kg of concentrate divided between the morning and afternoon. No differences in nutrient intake existed and the treatment was applied for 4 wk before collecting papillae. This strategy succeeded in affecting rumen fluid composition, increasing the total VFA, Pr, and Bu concentrations in the VFA load group, but did not affect rumen papillae morphology. Reynolds et al. (2004) fed 0.80 kg of DM/d of supplemental barley or 0.75 kg of DM/d of supplemental soybean protein compared with a nonsupplemented dry period ration starting 6 wk before calving. Unexpectedly, the barley treatment coincided with a smaller papillae width and surface area compared with nonsupplemented cows, which was attributed to genetic or previous environmental influences. It can be argued that the treatment contrasts imposed by Andersen et al. (1999) and Reynolds et al. (2004) were too minor to induce papillae proliferation. Rabelo et al. (2001) fed low- (6.3 MJ of NE_L/kg of DM) or high-energy rations (6.9 MJ of NE_L/kg of DM) with a marked difference in concentrate to roughage ratio for 3 wk before collection of papillae, resulting in a treatment contrast (0.6 MJ of NE_L/kg of DM) comparable to that in the current experiment. In contrast to the present study, they found only a tendency for longer papillae in the ventral rumen when feeding the high-energy ration. In addition, Reynolds et al. (2004) did not observe a change in papillae dimensions (length, width, surface area) when comparing the dry period (21 and 7 d ap) with early lactation (10 and 22 d pp). Crucially, Andersen et al. (1999), Rabelo et al. (2001), and Reynolds et al. (2004) all made end-point measurements compared with repeated measurements on the same animal, as was done in the present study and by Dirksen et al. (1984), Liebich et al. (1987), and Dieho et al. (2016a). Substantial variation in papillae morphology between animals is commonly found (Reynolds et al., 2004; Dieho et al., 2016a), and this underlines the importance of a repeated-measurements approach for studying changes in rumen morphology over time. A minimum of 8 papillae per site per cow per sampling day were used for macroscopic measurements, and 5 papillae per site per cow

per sampling day were used for microscopic measurements; these numbers are in general agreement with previous work (Odongo et al., 2006; Steele et al., 2011, 2015). The large variation in papillae morphology between individual animals is also illustrated by the present study, where papillae surface area appeared to be (numerically) 20% greater for CON compared with SUP at 28 d ap, the first day of the treatment. This was likely the result of inherent differences between individual cows. A treatment × sampling day interaction indicated differences in surface area development between CON and SUP. After correction for differences in papillae characteristics of individual cows, concentrate supplementation during the dry period increased papillae surface area compared with the control. In addition, the increase in papillae surface area during lactation (independent of treatment during dry period) was also clearly shown. This experiment thus confirms the repeated-measurements approach as viable for studying morphological changes to the rumen wall.

The observed postpartum increase in papillae size generally corresponded to earlier work (Liebich et al., 1987; Dieho et al., 2016a). However, the decrease in papillae surface area between 8 d ap and 3 d pp in SUP was not expected and contrasts with data reported by Liebich et al. (1987), where the gains in papillae size made during the dry period carried over into the lactation. No decrease was found for CON between 8 d ap and 3 d pp, and Dieho et al. (2016a) found only a numerical (2.2 mm^2) reduction between 10 d ap and 3 d pp. Papillae atrophy when cows switch to a lower feeding level (Dirksen et al., 1984). The decrease in papillae surface area in SUP between 8 d ap and 3 d pp may therefore be explained by the peripartum depression in FOM intake, as observed at 3 d pp compared with 8 d ap. The reduction in papillae surface area was most pronounced in the ventral blind rumen sac. Capillary blood flow in the ventral parts of the rumen is also reported to be approximately twice that of the dorsal regions (von Engelhardt and Hales, 1977), and is therefore suggested to be responsible for a major part of total VFA absorption capacity. We can therefore speculate that this active part of the rumen mucosa is more sensitive to changes in rumen fluid composition (decreased total VFA, increase in pH) as observed for SUP around calving. Alternatively, it can be speculated that the larger papillae induced by the supplemental concentrate are more sensitive to the peripartum decrease in feed intake. Increasing the concentrate allowance between 8 d ap and 3 d pp might have prevented the decrease, as this likely maintained the higher VFA concentrations and lower pH.

Rumen Fluid Composition and Fractional VFA Absorption Rate

Feeding supplemental concentrate increased rumen fluid VFA concentration, lowered pH, and tended to affect molar proportions of Ac, Pr, and Bu during the dry

period, as expected (Rabelo et al., 2001; Bannink et al., 2006). However, treatment effects on VFA concentration and pH were only apparent at 18 d ap and not at 8 d ap. At 18 d ap, this might be explained by a numerically higher FOM intake for SUP. At 8 d ap, FOM intake was similar to that at 18 d ap for SUP, but rumen fluid pH was higher and VFA concentration lower than at 18 d ap. This seems to suggest an adaptive response in SUP. However, only minor numerical increases in the capacity for VFA absorption between 18 and 8 d ap were observed, offering little support for an adaptive response. The difference in pH between SUP and CON at 3 d pp might be explained by the numerical difference in FOM intake. However, the difference in pH between SUP and CON at 17 d cannot be explained by a difference in FOM intake—no such difference was observed at 17 d pp. Whereas this arguably better buffering capacity of SUP at 3 and 17 d pp might be attributed to the dry period treatment, the pH and VFA concentration data should be interpreted with caution. Sampling of rumen fluid was not frequent (once a day) and not strictly standardized relative to last access to feed, meal size, and meal composition. Thus, observed differences in pH and VFA concentrations are not fully representative for the daily averages of treatment effects.

The k_aAc, k_aPr, and k_aBu from the dry period and 3 d pp in the present study largely agree with those reported earlier by Dieho et al. (2016b) for similar sampling days using an identical incubation technique and buffer composition. The k_aAc, k_aPr, and k_aBu observed in the present study from 17 d pp onward are lower than those reported by Dieho et al. (2016b), but both our current and previous studies show a clear increase in k_aVFA early postpartum. Greater intake of DM and FOM as reported by Dieho et al. (2016b) compared with the present study might have affected k_aVFA. In contrast to our expectations, the k_aVFA for SUP did not increase with the increase in papillae surface area during the dry period. This apparently contradicts the classic study by Dirksen et al. (1984), who observed an increase in VFA absorption in dry cows concomitant with increase in papillae surface area. The contradiction with Dirksen et al. (1984) is likely the result of differences in treatment contrasts. Dirksen et al. (1984) nearly doubled the NE_L intake from 47 to 86 MJ/d. In order of magnitude, this is similar to the difference between the dry period and lactation intake in the present experiment, for which a clear increase in k_aVFA was found. It should be noted that the BIT technique reflects the capacity of the rumen to absorb VFA under standardized conditions. Fractional absorption rates of VFA obtained using the BIT technique do not necessarily reflect those during the actual physiological conditions at time of measurement (Dieho et al., 2016b).

In our previous experiment (Dieho et al., 2016b) in early lactation, we observed a similar lack of increase in k_aVFA with a temporary increase in rumen papillae surface area in response to a more rapid increase of the rate of increase of concentrate allowance.

115

It is unlikely that the lack of effect of greater papillae surface area on k_aVFA can be explained by a decreased capacity for k_aVFA per unit of surface area (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014). However, epithelial blood flow has been shown to affect VFA absorption, and this might be a limiting factor for VFA absorption (Storm et al., 2011). Two independent mechanisms might be involved. First, a limited epithelial blood flow likely coincides with an increase in blood VFA concentration in the papilla capillaries. This decreases the concentration gradient between blood and VFA in the epithelium, and the intracellular accumulation of VFA in turn limits the concentration gradient with the rumen fluid (Storm et al., 2011). Second, exchange of VFA⁻ (dissociated VFA) for HCO_3^- has been shown to play an important role in VFA absorption and is arguably responsible for >50% of VFA uptake (Aschenbach et al., 2011). The major part of the HCO_3^- required for this exchange derives from extracellular (extra-epithelial) sources (Aschenbach et al., 2009) and is therefore not associated with formation of HCO_3^- by carbon-anhydrase from CO_2. A limited supply of HCO_3^- via the epithelial blood flow might therefore limit the capacity for VFA uptake. The large increase in epithelial blood flow after calving (Reynolds et al., 2003) might both aid maintenance of concentration gradients and increase the supply of HCO_3^-, thereby increasing the capacity for VFA absorption as observed. However, ruminal blood flow is also subjected to diurnal variation and postprandial effects such as rumen fluid composition (von Engelhardt and Hales, 1977; Storm and Kristensen 2010). Future studies are required to fully elucidate the role of epithelial blood flow and HCO_3^- on the ruminal VFA absorption capacity.

CONCLUSIONS

The dry period and lactation feed and energy intakes, blood glucose, NEFA, and BHB concentrations, and milk yield and milk composition were not affected by feeding supplemental concentrate during the dry period. Feeding supplemental concentrate during the dry period increased papillae surface area during the dry period without a concomitant increase in k_aVFA. The increased papillae surface area in the dry period was not maintained into the subsequent lactation period, and k_aVFA in the lactation period was not affected by the dry period concentrate supplement. Thus, our results indicate that feeding supplemental concentrate during the dry period, although increasing papillae surface area during this period, did not positively affect papillae surface area and k_aVFA during subsequent early lactation.

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Chapter 4

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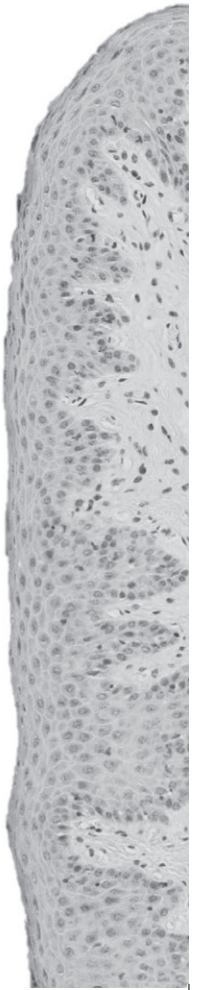
Chapter 4

Chapter 5

Changes in rumen epithelium gene and protein expression during the transition period

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ABSTRACT

We previously reported two experiments with rumen-cannulated Holstein-Friesian dairy cows showing that during the transition period, rumen papillae surface area and fractional absorption rate of volatile fatty acids (VFA) increase after calving. However, supplemental concentrate during the dry period and rate of increase of concentrate allowance during lactation affected papillae surface area, but not VFA absorption. Here we report the changes in gene and protein expression in rumen papillae related to tissue proliferation and VFA utilization. The lactation experiment treatment consisted of a rapid [RAP; 1.0 kg of dry matter (DM)/d; n = 6] or gradual (GRAD; 0.25 kg of DM/d; n = 6) increase of concentrate allowance (up to 10.9 kg of DM/d), starting at 4 d postpartum (pp). The dry period experiment treatment consisted of 3.0 kg of DM/d of concentrate (SUP; n = 4) or no concentrate (CON, n = 5) during the last 28 d of the dry period. Real-time qPCR analysis of rumen papillae showed that the expression of apoptosis related genes was neither affected by day nor its interaction with treatment for both experiments. Expression of epithelial transporter genes was not affected by day or treatment in the lactation experiment, except for *NBC1*. In the dry period experiment, expression of *MCT1*, *NBC1*, *DRA*, *NHE2*, *NHE3*, and *UT-B* generally decreased after calving. A day and treatment interaction was observed for *ATP1A1* in the dry period experiment, with greater expression at 18 and 8 d antepartum for SUP than CON. Generally, expression of VFA metabolism-related genes was not affected by day or its interaction with treatment. In the lactation experiment, immunoblotting of five selected genes showed protein expression of DRA and PCCA was greater at 16 d pp compared with 3 and 44 d pp. Expression of NHE2 was greater, and that of ATP1A1 lower, at 16 and 44 d pp compared with 3 d pp, suggest alterations in intracellular pH regulation and sodium homeostasis. MCT1 and PCCA protein were upregulated by RAP from 3 to 16 d pp, indicating modulations in VFA metabolism. Our data suggests that VFA absorption and metabolism capacity changed little per unit of surface area during the transition period, and that changes in the mitosis rate rather than apoptosis rate are the major response to increased ruminal VFA production. Only for PCCA, a significant but weak correlation between the examined gene and protein expression levels was observed, indicating that care must be taken when interpreting results obtained at either level.

Key words: rumen epithelium, gene expression, protein expression, VFA absorption and metabolism, rumen adaptation.

Chapter 5

125

INTRODUCTION

Absorption of VFA by the rumen depends on several factors including rumen fluid pH, VFA concentration, type of VFA, and surface area of the rumen papillae (Dirksen et al., 1984; Dijkstra et al., 1993; Melo et al., 2013). Absorption occurs both through passive diffusion and protein-mediated transport (Aschenbach et al., 2011). The latter involves a range of bicarbonate dependent and bicarbonate independent mechanisms, monocarboxylate transporters, and anion channels (Aschenbach et al., 2011). Most of the absorbed VFA are removed by the blood, with the remainder metabolized in the rumen epithelium (Bergman, 1990; Kristensen and Harmon, 2004). An increased intake of rumen fermentable OM (**FOM**) results in greater VFA production (Sutton et al., 2003; France and Dijkstra, 2005) and stimulates enlargement of the ruminal absorptive area and concomitantly the capacity for VFA absorption (Dirksen et al., 1984; Melo et al., 2013; Dieho et al., 2016ab; Dieho et al., 2017). This coordinated response of the rumen epithelia, including adaptation on the molecular level, allows maintenance of its normal function and of intraruminal conditions within physiological boundaries (Penner et al., 2011; Bannink et al., 2012; Steele et al., 2015).

Upregulation of transporter genes associated with a higher plane of nutrition has been reported in ruminants (Yang et al., 2012; Metzler-Zebeli et al., 2013; Yan et al., 2014) and also during the transition period changes in expression of some genes associated with VFA transport, metabolism, and tissue proliferation have been observed (Minuti et al., 2015; Steele et al., 2015). However, using high grain or concentrate challenge models with adult cows, Penner et al. (2009) and Steele et al. (2012) generally did not observe upregulation of transporter genes. Unfortunately, only a few of these studies coincided with in vivo measurements on VFA absorption (Penner et al., 2009), or papillae surface area (Metzler-Zebeli et al., 2013), and consequently any relationships between gene expression and functional changes (the capacity for VFA absorption and metabolism) remain largely unknown. Moreover, few studies report changes in expression at the protein level in lieu of expression at mRNA level (Penner et al., 2009; Metzler-Zebeli et al., 2013; Minuti et al., 2015), further limiting functional interpretation of results. Thus, an integrated set of observations on changes in rumen morphology and function, and associated changes in gene and protein expression, in response to nutrition is still lacking.

We recently reported results of two experiments (Dieho et al., 2016a; Dieho et al., 2017) showing that the large postpartum increase in daily FOM intake, and thus VFA production, in dairy cattle after calving coincided with an increase in rumen papillae surface area and fractional VFA absorption rate (k_a**VFA**; Dieho et al., 2016b; Dieho et al., 2017). Moreover, papillae surface area increased when feeding supplemental

concentrate during the dry period compared with non-supplemented cows (Dieho et al., 2017), and increased faster when increasing the daily concentrate allowance postpartum (**pp**) at a rapid compared with at a gradual rate (Dieho et al., 2016a). However, the k_aVFA was not affected by the concentrate treatments (Dieho et al., 2016b; Dieho et al., 2017). The aim of this study was to test for these two experiments, the hypothesis that changes in the expression of genes in rumen papillae involved in tissue proliferation reflect the observed changes in papillae morphology. In addition, we hypothesized that the expression of genes and selected proteins involved in the absorption and metabolism of VFA in rumen epithelial tissue increased with greater daily VFA production and VFA absorption rates.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen University & Research and conducted under the Dutch Law on the Animal Experiment.

Experimental Design, Management, and Sample Collection

Samples originating from two experiments were used which have been reported in detail (Dieho et al., 2016a; Dieho et al., 2017). Brief summaries of these experiments are presented below.

Lactation Experiment. Effect of Rate of Increase of Concentrate Allowance during Early Lactation. The experiment conducted by Dieho et al. (2016a) with experimental treatments during the early lactation period after calving will be referred to as the *lactation experiment.* Twelve dry rumen-cannulated Holstein-Friesian dairy cows entered the experiment 8 wk before the expected date of calving. Cows had free access to a mixed basal ration, consisting of grass silage, corn silage, soybean meal and (dry period only) chopped wheat straw. The dry period ration contained 603 g of DM/kg, providing 5.3 MJ NE_L (calculated according to van Es, 1978) and 455 g FOM (calculated according to Tamminga et al., 1994) per kg of DM. The basal lactation ration contained 466 g of DM/kg, providing 6.7 MJ NE_L and 561 g FOM per kg of DM. After calving, all cows were fed 0.9 kg of DM/d concentrate up to 3 d pp. Treatment consisted of either a rapid (1.0 kg of DM/d; **RAP**; n = 6) or gradual (0.25 kg of DM/d; **GRAD**; n = 6) increase of concentrate allowance (up to 10.9 kg of DM/d), starting at 4 d pp. The concentrate contained 892 g of DM/kg, providing 7.4 MJ NE_L and 682 g FOM per kg of DM. The dry period and basal lactation rations were fed once a day for ad libitum intake and cows

were milked twice daily. Daily individual intake of the mixed ration and concentrate was recorded. Rumen papillae were collected for epithelial mRNA and protein expression analysis during the pretreatment period at 10 d antepartum (ap) and 3 d pp, and during the treatment period at 9, 16, and 44 d pp.

Dry Period Experiment: Effect of Supplemental Concentrate During the Dry Period. The experiment conducted by Dieho et al. (2017) with experimental treatments during the dry period will be referred to as the *dry period experiment.* Nine weeks before the expected calving date, ten dry rumen-cannulated Holstein-Friesian dairy cows entered the experiment. Cows were randomly assigned to a control (**CON**) or treatment group (**SUP**). From the start of the experiment up to calving, all cows had free access to a dry period ration consisting of grass silage, corn silage, soybean meal and chopped wheat straw. Treatment started 28 d ap and consisted of supplemental concentrate (3.0 kg of DM/d) until day of calving (SUP) or no supplemental concentrate (CON). After calving, both CON and SUP had free access to a basal lactation ration, consisting of grass silage, corn silage, and soybean meal. All cows were fed 0.9 kg of DM/d concentrate up to 3 d pp, which thereafter increased with 1.0 kg of DM/d to a maximum of 8.9 kg of DM/d from 11 d pp onwards. The dry period ration contained 503 g of DM/kg, providing 5.3 MJ NE_L and 476 g FOM per kg of DM. The basal lactation ration contained 409 g of DM/kg, providing 6.6 MJ NE_L and 547 g FOM per kg of DM. The concentrate contained 890 g DM/kg (fed during both the dry period and lactation), providing 7.5 MJ NE_L and 685 g FOM per kg DM. The dry period and basal lactation rations were fed once a day for ad libitum intake and cows were milked twice daily. Daily individual intake of the mixed ration and concentrate were recorded. Rumen papillae were collected for epithelial mRNA and protein expression analysis 28, 18 and 8 d ap and 3, 17, 31 and 45 d pp.

Rumen Papillae Collection. In both experiments, papillae were collected according to the procedure described by Dieho et al. (2016a). Briefly, on sampling days, papillae were collected from each cow from a completely evacuated and washed rumen. Papillae were collected from the ventral rumen sac (**VRS**), ventral blind rumen sac (**VBS**), and dorsal blind rumen sac (**DBS**). After gently rinsing with 0.9% NaCl (Kirat et al., 2006), papillae (150-300 mg/sample) were placed in sterile cryogenic vials (Cryo.S, Greiner Bio-One GmbH, Kremsmünster, Austria) snap frozen in liquid nitrogen and stored at -80°C until processing.

RNA Extraction and Real Time Quantitative PCR

Frozen rumen papillae were ground in liquid nitrogen with a pestle and mortar. The RNA was extracted using Trizol Reagent (ThermoFisher Scientific, Bleiswijk, the Netherlands) and subsequently subjected to an on-column DNase digestion to eliminate possible DNA contamination (NucleoSpin RNA II kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany). Total RNA concentrations and purity were determined by optical density measurement using a NanoDrop ND-1000 (ThermoFisher Scientific). An aliquot of 250 ng total RNA was reverse transcribed with Superscript III (ThermoFisher Scientific) in the presence of random hexamers (Roche, Almere, the Netherlands) and dNTPs (Roche) at 25°C for 5 min, 50°C for 1 h, and, to inactivate the enzyme, at 70°C for 15 min. Real-time quantitative PCR was carried out in a ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA), and SensiMix SYBR Low-ROX mix (Bioline UK Ltd., London, UK) was employed on the basis of the manufacturers conditions. Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 5 s, and 72°C for 5 s. A final melting protocol with ramping from 65 to 95°C with 0.5°C increments of 5 s each was performed, confirming the presence of a single amplicon. Agarose gel electrophoresis revealed single bands of expected mobility. The primers used were designed with Primer Express Software (ThermoFisher Scientific), and recommended primer sets spanning at least one intron were selected (Table 1). Quantitative mRNA measurement was performed by establishing a linear calibration curve using 10-fold serial dilutions of cDNA template for corresponding genes. Amplification efficiency ranged between 90% and 100% (Table 1). As internal standards, expression of housekeeping genes importin 8 (*IPO8*), eukaryotic translation elongation factor 2 (*eEF2*), almodulin 2 (*CALM2*), and keratin 6 (*KRT6*) were analyzed. NormFinder (Andersen et al., 2004) identified *KRT6* as the most stable reference gene in both experiments. For this reason, absolute expression levels of genes of interest were normalized using their corresponding values of *KRT6*, which is thought to be a marker of keratinizing stratified squamous epithelia (Moll et al., 2008).

Chapter 5

Table 1. Primers used for real-time quantitative PCR.

Gene	Forward primer	Reverse primer	Efficiency (%)	Accession
KRT6	5'-CAACATCTCCGTGGTCCAGT-3'	5'-AACTGAAGCACCTCCAAGG-3'	92	NM_001257404
eEF2	5'-TCACTGACACCCGAAGGAT-3'	5'-ATGAAATTCAAGTCGTTCTCTGAAAG-3'	91	NM_001075121
CALM2	5'-GAATTAGTCCGAGTGGAGCGA-3'	5'-TCTTTGAATTCTGCAATCTGCTCT-3'	90	NM_001242587
IPO8	5'-TGCCATGGTATTTCTCCTCAAA-3'	5'-ACAATCCTGCTGAAAAACCTCATAT-3'	95	NM_001206120
MCT1	5'-AACACTGTGCAGGAACTTTACTTTTC-3'	5'-TGCCAGCGGTCGTCTCTTAT-3'	90	NM_001037319
MCT4	5'-TGTGTGTGAATCGCTTTGGC-3'	5'-CCAAACCCAAGCCAGTAAGGA-3'	90	NM_001109980
NBC1	5'-CAACCACGGCAAGAAAGCTC-3'	5'-AACGGTGCAACAAACCAACC-3'	95	NM_174605
PAT1	5'-TACCACAGGGCCTTGCCTAT-3'	5'-CTGCCCACCATCACAGACAT-3'	94	NM_001076852
DRA	5'-CCTAGCAGGCAGACTTTCCA-3'	5'-GCGATCAACTCCTGATTGCC-3'	95	NM_001083676
AE2	5'-CGACACTTGGTGCGGAAGGA-3'	5'-TTCAGCTCCACAAACACCTCA-3'	91	NM_001205664
NHE1	5'-GACTACCAACACGTGCGCATA-3'	5'-CAGCAGCCCCACCACAAT-3'	95	NM_174833
NHE2	5'-GTTGACGTGTTTGCTGGCAT-3'	5'-GATCACGCGGATGTTGTGTG-3'	94	XM_604493
NHE3	5'-CCCGGCAGGAGTACAAACAT-3'	5'-TTGGCCGACTTGAAGGACTC-3'	93	NM_001192154
UT-B	5'-TGGCACTCACCTGCAAACCC-3'	5'-TGGCAATCCGACCACAGCCAT-3'	97	NM_001008666
ATP1A1	5'-GAGATTACCCCCTTCCTGATATTT-3'	5'-TGGATCATACCAATCTGTCCATAG-3'	91	NM_001076798
PCCA	5'-GGGTTACCCTCTCAGGCACAA-3'	5'-CAACAGACGGCAAACCAAAA-3'	96	NM_001083509
HMGCS1	5'-CAGCTCTTTGAGGAGTCTGGG-3'	5'-GGCATACGGTCCATCCCAAG-3'	98	NM_001206578
HMGCS2	5'-GCAACACTGACATTGAGGGC-3'	5'-ACCAGTGCATAGCGACCATC-3'	99	NM_001045883
BDH1	5'-GCAAAAGGCCGCGTTGTTA-3'	5'-GTCAGAGAAAGCCTCCACCC-3'	91	NM_001034600
BDH2	5'-TGGCTCAGAAATCTGGCAAC-3'	5'-AACTGTTCCTGGACACACACA-3'	92	NM_001034488
ACSS1	5'-GCGGGTTACCTGCTCTATGC-3'	5'-TAGCTGTTGTCCCGTGATCCA-3'	94	NM_174746
OXCT1	5'-CTAGCACACGTCACCATACCA-3'	5'-ACCCAAAACCACCAACCAGTAT-3'	94	NM_001076070
PPARa	5'-GGATGTCCCATAACGCGATT-3'	5'-GGTCATGTCTCACACGTAAGGATT-3'	93	NM_001034036
BAX1	5'-GGAGCTGCAGAGGGATGATCG-3'	5'-CCTTGAGCACCAGTTTGCTG-3'	98	NM_173894
XIAP	5'-TCCAGCCATGGCAGATTACG-3'	5'-TTAGCCCTCCTCCACAGTGA-3'	100	NM_001205592
BCL2	5'-GAGTTCGGAGGGGTCATGTG-3'	5'-TGAGCAGTGCCTTCAGAGAC-3'	90	NM_001166486

KRT6 = keratin 6A-C; eEF2 = eukaryotic translation elongation factor 2; CALM2 = calmodulin 2; IPO8 = importin 8; MCT1 = SLC16A1, monocarboxylic acid transporter 1; MCT4 = SLC16A3, monocarboxylic acid transporter 4; NBC1 = SCL4A4, sodium bicarbonate cotransporter 1; PAT1 = SLC26A6, Putative Anion Exchanger 1; DRA = SLC26A3, Anion Exchanger member 3; AE2 = SLC4A2, Anion Exchanger member 2; NHE1 = SLC9A1, Na$^+$/H$^+$ antiporter member 1; NHE2 = SLC9A2, Na$^+$/H$^+$ antiporter member 2; NHE3 = SLC9A3, Na$^+$/H$^+$ antiporter member 3; UT-B = SLC14A1, urea transporter; ATP1A1 = Na$^+$/K$^+$ transporting ATPase subunit a1; PCCA = propionyl-CoA carboxylase alpha subunit; HMGCS1 = 3-hydroxy-3-methylglutaryl-CoA synthase 1; HMGCS2 = 3-hydroxy-3-methylglutaryl-CoA synthase 2; BDH1 = 3-hydroxybutyrate dehydrogenase, type 1; BDH2 = 3-hydroxybutyrate dehydrogenase, type 2; ACSS1 = acyl-CoA synthetase short-chain family member 1; OXCT1 = 3-oxoacid CoA-transferase 1; PPARa = peroxisome proliferator-activated receptor alpha; BAX1 = BCL2 associated X protein, apoptotic activator; XIAP = X-linked inhibitor of apoptosis; BCL2 = Apoptosis regulator BCL2, upregulation blocks apoptosis.

Western Blotting

Protein expression of PCCA, MCT1, DRA, NHE2 and ATP1A1 was assessed in the VBS of the lactation experiment by Western blot analysis. The VBS site was chosen since it was the most ventrally located sampling site, associated with the highest blood flow (von Engelhardt and Hales, 1977). Sampling days 3, 16 and 44 d pp were chosen as these captured the start, peak, and end of the differences in concentrate treatment between RAP and GRAD respectively (Dieho et al., 2016a). Ground rumen biopsies were homogenized in a lysis buffer (50 m*M* Tris-HCl pH 8.0, 150 m*M* NaCl, l,1% (v/v) NP-40, 1 m*M* phenylmethylsulfonyl fluoride) by rotating end-over-end at 4°C for 30 min, followed by centrifugation at 14,000 × g at 4°C for 10 min to remove insoluble material. Subsequently, protein concentrations were measured using a Coomassie Plus 'Bradford' assay kit (ThermoFisher Scientific) with bovine serum albumin as a standard. Protein (40 μg) was dissolved in a reducing buffer (150 m*M* Tris-HCl pH 8.5, 2% lithiumdodecylsulfate, 10% glycerol, 0.5 m*M* EDTA, 0.2 m*M* SERVA Blue G, 0.15 m*M* Phenol Red), size fractionated by electrophoresis on precast NuPage Novex 4-12% Bis-Tris gels (ThermoFisher Scientific) and electroblotted to Immobilon-FL PVDF membranes (0.45 μm; Li-Cor Biosciences, Bad Homburg, Germany) at a constant 80 V for 90 min. Membranes were blocked with Blocking Solution (Odyssey; Li-Cor Biosciences, Bad Homburg, Germany) diluted 1:1 in PBS for 1 h at room temperature, and probed overnight (4°C) with either polyclonal rabbit anti-PCCA or anti-DRA antibody (LifeSpan Biosciences, Seattle, WA), polyclonal chicken anti-MCT1 (Merck Millipore, Amsterdam, the Netherlands), polyclonal goat anti-NHE-2 (Santa Cruz Biotechnology, Heidelberg, Germany), or monoclonal mouse anti-ATP1A1 antibody (Abcam, Cambridge, UK), all diluted 1:2,000 in PBS containing 0.1% Tween-20. Subsequently, blots were incubated with IRDye-800-conjugated donkey polyclonal secondary antibody (Li-Cor Biosciences, diluted 1:15,000 in PBS) for 30 min at room temperature. Finally, positive bands were detected using an Odyssey infrared imaging system (Li-Cor Biosciences). As a loading control, blots were reprobed with monoclonal mouse anti-ACTB (Abcam, 1:5,000) and visualized with IRDye-680-conjugated donkey anti-mouse antibody (Li-Cor Biosciences, 1:20,000). The size of the detected bands were conform to those described for MCT1 (Koho et al., 2005), DRA (Hayashi and Yamashita, 2012), NHE2 (Lam et al., 2009), ATP1A1 (Hickey and Buhr, 2012), and PCCA (Ballhausen et al., 2009). Band density was measured with the software program ImageJ (http://rsb.info.nih.gov/ij/index.html) and normalized to ACTB.

Chapter 5

Calculations and Statistical Analysis

The gene expression data were $\log_{10}$ transformed before analysis to achieve normal distribution. Gene and protein expression data was analyzed using the MIXED procedure (Littell et al., 2006) in SAS 9.3 (SAS Institute Inc., Cary, NC) with the model:

$$Y_{ijkl} = \mu + T_i + S_k + D_l + (T \times D)_{il} + D(C)_{jl} + e_{ijkl},$$

where variable Y_{ijkl} was dependent on μ as the average experimental value and fixed main effects of concentrate treatment T_i (i = RAP, GRAD for the lactation experiment or CON, SUP for the dry period experiment, respectively), sampling day D_l (gene expression data, l = –10, ..., 44 for the lactation experiment, and -28, ..., 45 for the dry period experiment; protein expression data, l = 3, 16, and 44 d pp for the lactation experiment), site S_k (k = VRS, VBS, DBS; not applicable for protein expression data) and interaction $(T \times D)_{il}$. Random effects of sampling day within cows, $D(C)_{jl}$ were included, avoiding pseudo-replication due to multiple biopsy sites S_k (Dieho et al., 2016a). Errors e_{ijkl} were assumed to be auto-correlated for repeated observations on the same cow and site, using a spatial power covariance structure over sampling days (Littell et al., 1998). For the gene expression data from the lactation experiment, CONTRAST statements were formulated adapted from Dieho et al. (2016a) to test for T_i and $(T \times D)_{il}$ as the study design could not be treated as fully factorial. All other data for which sampling site within the rumen was not applicable, were analyzed with a reduced model by removing S_k and $D(C)_{jl}$, with repeated measurements on the same cow. To test specific hypotheses, CONTRAST or ESTIMATE statements were formulated to separate means. All results are reported as least squares means with their standard error unless indicated otherwise. Significance of effect was declared at P < 0.05 and trends at $0.05 \leq P$ < 0.10.

RESULTS

During the lactation experiment, one cow (RAP) calved early, missing sampling at 10 d ap, otherwise all samples were collected (Dieho et al., 2016a). During the dry period experiment, one cow (SUP) calved early (stillbirth), and subsequently developed endometritis and a displaced abomasum requiring surgery. To aid recovery, the animal was removed from the experiment and all data relating to this cow were excluded from analysis (Dieho et al., 2017).

Feed Intake, Morphological and Functional Adaptation of the Rumen

For the lactation experiment, feed intake and morphological adaptation of the rumen has been reported in detail by Dieho et al. (2016a), and changes in k_aVFA and daily VFA production by Dieho et al. (2016b). Briefly, after calving, both DMI and FOM intake increased for RAP and GRAD, and the rapid increase in concentrate allowance resulted in a greater FOM intake at 16 d pp in RAP than GRAD. Papillae surface area increased after calving and was greater during the lactation than the dry period, and surface area increased faster for RAP compared with GRAD, and was greater at 16 and 44 d pp for RAP than GRAD (Figure 1A). The k_aVFA increased after calving and was greater during the lactation than the dry period (Figure 1A), however, despite the greater papillae surface area in RAP than GRAD, no differences in k_aVFA between RAP and GRAD were observed at 16 and 44 d pp. Total daily VFA production increased after calving and was numerically greater for RAP than GRAD at 16 d pp, whereas propionate production was greater RAP than GRAD on that day.
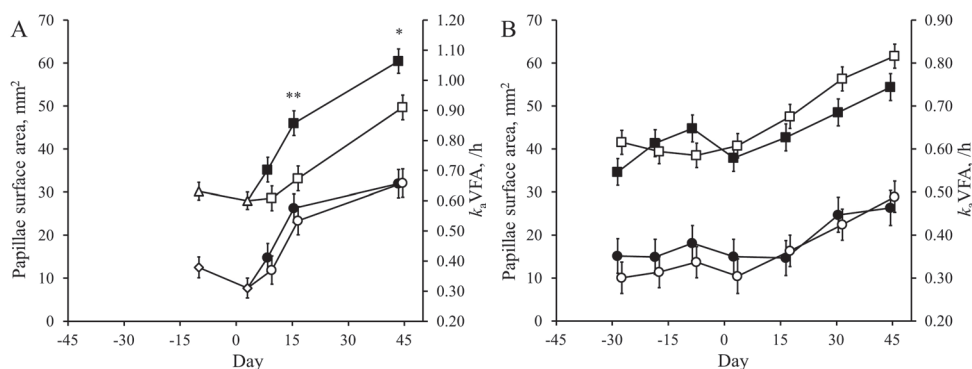
Figure 1. (A) Rumen papilla surface area (mm²; △■□) and fractional rate of VFA absorption (k_aVFA, per hour; ◇●○) during the pretreatment period (◇△; n = 12), and the treatment period for a rapid (1.0 kg of DM/d; ■, n = 6) and gradual (0.25 kg of DM/d; □, n = 6) rate of increase of concentrate allowance postpartum. Adapted from Dieho et al. (2016ac). (B) Rumen papilla surface area (mm²; ■□) and fractional rate of VFA absorption (/h; ●○) for a control dry period ration (no concentrate, □○; n = 5) and dry period ration with supplemental concentrate (3.0 kg DM/d concentrate, ■●; n = 4), and the subsequent lactation. Adapted from Dieho et al. (2016b). Day = day relative to calving. Values represent LSM ± SE (values are slightly offset for clarity). *P < 0.05, **P < 0.01, significance of differences in LSM of CON and SUP for the same sampling day and variable.

For the dry period experiment, feed intake, morphological adaptation of the rumen, and changes in k_aVFA have been reported by Dieho et al. (2017). Feeding supplemental concentrate during the dry period was reflected by a consistent numerically

greater FOM intake at 18 and 8 d ap for SUP compared with CON. After calving, DMI and FOM intake increased for both SUP and CON, compared with the dry period. Papillae surface area increased for SUP but not for CON during the dry period (Figure 1B). However, the increase in surface area for SUP was temporary and was not present anymore from 3 d pp onwards. No differences in k_aVFA between SUP and CON were observed during the dry period or lactation. In line with the lactation experiment, papillae surface area and k_aVFA increased after calving and were greater during the lactation than the dry period.

Expression of Genes Associated with Tissue Proliferation

In both experiments, expression of the apoptotic inhibitors *XIAP* and *BCL2*, and expression of the apoptotic activator *BAX1*, were neither affected by sampling day nor by an interaction between sampling day and treatment (Table 2 and 3). The expression of *XIAP* was not affected by the treatments. The expression of *BCL2* was not affected by concentrate treatment in the lactation experiment, and tended (P = 0.08) to be lower in SUP compared with CON in the dry period experiment. The expression of *BAX1* tended to be greater (P = 0.08) for RAP than GRAD in the lactation period experiment, and was greater (P = 0.01) for SUP than CON in the dry period experiment.

Expression of Genes Associated with VFA Absorption, Intracellular pH, and Urea Transport

During the lactation experiment, except for a postpartum increase for Na^+/HCO_3^- cotransporter *NBC1* (P = 0.04) no effects of sampling day ($P \leq 0.10$) were observed on the expression of the membrane transporters *ATP1A1*, *MCT1*, *MCT4*, *DRA*, *PAT1*, *AE2*, *NHE1*, *NHE2*, *NHE3* and *UT-B* (Table 4). The concentrate treatment during early lactation did not affect the expression of most of the absorption related genes. The expression of monocarboxylate transporter *MCT4* tended (P < 0.08) to be greater with RAP than with GRAD, and expression of VFA⁻/HCO_3^- exchanger *PAT1* tended to be greater (P = 0.05) for RAP than GRAD. No interaction between treatment and sampling day was observed for the absorption-related genes.

Table 2. Least squares means of $\log_{10}$ transformed expression of genes in the rumen papillae associated with tissue proliferation during the lactation experiment with a rapid rate of increase (RAP; 1.0 kg of DM/d; n = 6) of concentrate allowance or gradual rate of increase (GRAD; 0.25 kg of DM/d; n = 6) of concentrate allowance postpartum. Expression was normalized against *Keratin-6*.

Gene		Sampling day relative to calving					SE[3]	Fixed effects[1]			
		Pretreatment period		Treatment period				T	D	S	T × D
		-10[2]	3	9	16	44					
BAX1	RAP	-1.08	-1.03	-1.10	-1.03	-1.11	0.025	0.08	0.83	<0.01	0.16
	GRAD	-1.17	-1.21	-1.13	-1.14	-1.12					
BCL2	RAP	-1.36	-1.38	-1.39	-1.35	-1.45	0.027	0.89	0.84	<0.01	0.55
	GRAD	-1.43	-1.40	-1.41	-1.40	-1.39					
XIAP	RAP	-0.79	-0.84	-0.88	-0.85	-0.85	0.023	0.30	0.92	<0.01	0.60
	GRAD	-0.91	-0.91	-0.88	-0.91	-0.88					

[1]T = treatment, *P*-value for treatment period (d 9 to 44); D = sampling day, *P*-value for pretreatment and treatment period (d –10 to 44); T × D = *P*-value for treatment period (d 9 to 44).

[2]Group GRAD: n = 5, 1 cow had no measurement at d –10 due to early calving.

[3]Standard error for LSM by sampling day; n = 12.

Chapter 5

Table 3. Least squares means of $\log_{10}$ transformed expression of genes in the rumen papillae associated with tissue proliferation during the dry period experiment for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP, n = 4[1]). Expression was normalized against *Keratin-6*

Gene		Sampling day relative to calving							SE[3]	Fixed effects[2]			
		Dry period			Lactation					T	D	S	T × D
		-28	-18	-8	3	17	31	45					
BAX1	CON	-0.91	-0.96	-0.91	-0.91	-0.93	-0.97	-0.94	0.024	0.01	0.27	<0.01	0.22
	SUP	-0.88	-0.80	-0.85	-0.86	-0.88	-0.95	-0.88					
BCL2	CON	-1.71	-1.73	-1.72	-1.69	-1.73	-1.77	-1.77	0.038	0.08	0.65	<0.01	0.98
	SUP	-1.79	-1.78	-1.73	-1.77	-1.73	-1.85	-1.79					
XIAP	CON	-0.85	-0.88	-0.88	-0.84	-0.93	-0.94	-0.94	0.033	0.64	0.29	<0.01	0.77
	SUP	-0.91	-0.87	-0.87	-0.82	-0.85	-0.97	-0.89					

[1]Group SUP: n = 4, 1 cow was removed from the experiment.
[2]T = treatment; D = sampling day; T × D = interaction between treatment and sampling day.
[3]Standard error for LSM by sampling day; n = 9.

During the dry period experiment, sampling day affected the mRNA levels of the Na^+/H^+ exchangers *NHE2* and *NHE3* ($P \leq 0.03$), but not *NHE1* ($P = 0.98$; Table 5). The expression of *NHE2* decreased towards calving, peaked at 3 d pp, and decreased again afterwards, whereas that of *NHE3* was lower during the lactation period compared with the dry period. The expression of *NBC1* was affected by sampling day ($P < 0.01$), and decreased after calving compared with the dry period. In addition, a tendency ($P = 0.06$) for an interaction between sampling day and treatment was observed, with greater expression of *NBC1* for SUP at 17 d pp compared with CON. The expression of *PAT1* and *AE2* was not affected ($P \geq 0.36$) by treatment, sampling day, or their interaction, whereas expression of VFA^-/HCO_3^- exchanger *DRA* was affected by sampling day ($P = 0.04$), and decreased after calving compared with the dry period. The expression of *MCT1,* but not *MCT4*, and that of urea transporter *UT-B* were affected by sampling day ($P \leq 0.04$), showing both a peak at 3 d pp. For *ATP1A1*, encoding the α1-subunit of Na^+/K^+-ATPase, an interaction between treatment and sampling day was observed ($P = 0.02$), with greater mRNA expression at 18 and 8 d ap in SUP compared to CON. A treatment effect was observed for *MCT4*, *NBC1*, *DRA*, and *NHE3* ($P \leq 0.03$), generally showing a greater expression for SUP than CON.

Expression of Genes Associated with VFA Metabolism

During the lactation experiment (Table 6), the expression of HMG-CoA synthase 1 *HMGCS1* and succinyl-CoA:3-oxoacid CoA transferase *OXCT1* tended to be affected by sampling day ($P = 0.07$). However, there was no clear pattern of change in expression over sampling days. A treatment effect was observed only for the expression of peroxisome proliferator activated receptor alpha *PPARα* and 3-hydroxybutyrate dehydrogenase type 2 *BDH2* ($P \leq 0.04$) with greater expression in cows from RAP compared with GRAD, with the expression level of *PPARα* and *BDH2* already being greater during the pretreatment period in RAP compared with GRAD. No interaction between treatment and sampling day was observed for any of the measured VFA metabolism-related genes.

Chapter 5

137

Table 4. Least squares means of $\log_{10}$ transformed expression of genes in the rumen papillae associated with VFA absorption and maintenance of intracellular pH during the lactation experiment with a rapid rate of increase (RAP; 1.0 kg of DM/d; n = 6) of concentrate allowance or gradual rate of increase (GRAD; 0.25 kg of DM/d; n = 6) of concentrate allowance postpartum. Expression was normalized against *Keratin-6*

Gene		Sampling day relative to calving						Fixed effects[1]			
		Pretreatment period		Treatment period			SE[3]	T	D	S	T × D
		-10^2	3	9	16	44					
ATP1A1	RAP	0.69	0.64	0.62	0.64	0.61	0.028	0.19	0.74	<0.01	0.36
	GRAD	0.60	0.59	0.59	0.55	0.60					
MCT1	RAP	1.52	1.57	1.57	1.62	1.53	0.032	0.33	0.48	<0.01	0.58
	GRAD	1.46	1.55	1.54	1.53	1.53					
MCT4	RAP	0.26	0.26	0.18	0.28	0.19	0.039	0.08	0.25	<0.01	0.20
	GRAD	0.19	0.02	0.09	0.15	0.19					
NBC1	RAP	-0.69	-0.72	-0.74	-0.77	-0.59	0.064	0.26	0.04	<0.01	0.39
	GRAD	-0.77	-0.94	-0.87	-0.74	-0.67					
PAT1	RAP	0.02	0.10	0.08	0.14	0.03	0.032	0.05	0.39	0.01	0.41
	GRAD	-0.04	0.06	0.04	-0.02	0.01					
DRA	RAP	0.24	0.28	0.36	0.37	0.38	0.044	0.41	0.10	<0.01	0.72
	GRAD	0.18	0.37	0.30	0.32	0.39					
AE2	RAP	0.24	0.21	0.15	0.17	0.10	0.029	0.48	0.28	<0.01	0.82
	GRAD	0.14	0.12	0.12	0.12	0.14					
NHE1	RAP	0.30	0.28	0.23	0.25	0.22	0.031	0.95	0.88	<0.01	0.90
	GRAD	0.24	0.20	0.25	0.21	0.23					
NHE2	RAP	0.77	0.82	0.84	0.85	0.86	0.028	0.35	0.14	<0.01	0.49
	GRAD	0.74	0.84	0.86	0.78	0.82					
NHE3	RAP	0.88	0.90	0.87	0.86	0.86	0.037	0.91	0.86	0.78	0.86
	GRAD	0.80	0.85	0.86	0.81	0.91					
UT-B	RAP	0.63	0.71	0.72	0.77	0.72	0.034	0.40	0.30	<0.01	0.78
	GRAD	0.65	0.76	0.70	0.70	0.70					

[1] T = treatment, *P*-value for treatment period (d 9 to 44); D = sampling day, *P*-value for pretreatment and treatment period (d −10 to 44); T × D = *P*-value for treatment period (d 9 to 44).

[2] Group GRAD: n = 5, 1 cow had no measurement at d −10 due to early calving.

[3] Standard error for LSM by sampling day; n = 12.

138

Table 5. Least squares means of log$_{10}$ transformed expression of genes in the rumen papillae associated with VFA absorption and maintenance of intracellular pH during the dry period experiment with the control (CON; no concentrate during the dry period; n = 5) and supplemental concentrate treatment (SUP; 3.0 kg of DM/d concentrate during the dry period; n = 4). Expression was normalized against *Keratin-6*.

Gene		Sampling day relative to calving							SE[3]	Fixed effects[2]			
		Dry period			Lactation					T	D	S	T × D
		-28	-18	-8	3	17	31	45					
			**	***			†						
ATP1A1	CON	0.52	0.50	0.50	0.61	0.59	0.58	0.56	0.024	0.01	0.07	<0.01	0.02
	SUP	0.60	0.63	0.66	0.63	0.62	0.50	0.63					
MCT1	CON	1.52	1.48	1.47	1.69	1.53	1.55	1.57	0.034	0.25	0.02	<0.01	0.17
	SUP	1.55	1.61	1.58	1.66	1.63	1.46	1.53					
MCT4	CON	0.06	0.05	0.06	0.02	-0.01	0.02	0.02	0.042	<0.01	0.81	<0.01	0.90
	SUP	0.10	0.25	0.17	0.11	0.15	0.10	0.16					
NBC1	CON	-0.92	-0.96	-0.87	-1.08	-1.36	-1.08	-1.08	0.051	0.03	<0.01	<0.01	0.06
	SUP	-0.87	-0.82	-0.80	-1.17	-0.97	-1.07	-1.06					
PAT1	CON	-0.07	-0.15	-0.08	0.05	-0.04	-0.06	-0.04	0.035	0.49	0.36	<0.01	0.37
	SUP	-0.05	-0.02	-0.01	-0.02	-0.05	-0.12	0.02					
DRA	CON	0.48	0.40	0.39	0.38	0.26	0.28	0.28	0.045	0.03	0.04	<0.01	0.78
	SUP	0.48	0.53	0.40	0.53	0.40	0.34	0.38					
AE2	CON	0.10	0.11	0.19	0.26	0.17	0.09	0.06	0.051	0.76	0.38	<0.01	0.91
	SUP	0.15	0.16	0.18	0.18	0.10	0.04	0.10					
NHE1	CON	0.21	0.17	0.15	0.20	0.17	0.17	0.20	0.035	0.12	0.98	<0.01	0.80
	SUP	0.20	0.25	0.26	0.21	0.27	0.18	0.23					
NHE2	CON	0.82	0.79	0.74	0.85	0.80	0.76	0.79	0.030	0.10	0.03	<0.01	0.92
	SUP	0.86	0.90	0.81	0.93	0.85	0.79	0.79					
NHE3	CON	0.82	0.79	0.74	0.72	0.64	0.60	0.62	0.038	0.01	<0.01	<0.01	0.97
	SUP	0.92	0.93	0.88	0.77	0.74	0.69	0.69					
UT-B	CON	0.66	0.62	0.56	0.73	0.66	0.61	0.63	0.031	0.96	0.04	<0.01	0.36
	SUP	0.64	0.72	0.65	0.71	0.62	0.54	0.59					

[1]Group SUP: n = 4, 1 cow was removed from the experiment.

[2]T = treatment; D = sampling day; T × D = interaction between treatment and sampling day.

[3]Standard error for LSM by sampling day; n = 9.

†$P < 0.10$, **$P < 0.01$, ***$P < 0.001$, significance of difference in LSM of CON or SUP for the same sampling day and gene.

Chapter 5

139

Table 6. Least squares means of $\log_{10}$ transformed expression of genes in the rumen papillae associated with VFA metabolism during the lactation experiment with a rapid rate of increase (RAP; 1.0 kg of DM/d; n = 6) of concentrate allowance or gradual rate of increase (GRAD; 0.25 kg of DM/d; n = 6) of concentrate allowance postpartum. Expression was normalized against *Keratin-6*

Gene		Pretreatment period		Sampling day relative to calving Treatment period			SE[3]	Fixed effects[1] T	D	S	T × D
		-10[2]	3	9	16	44					
ACSS1	RAP	-0.07	-0.09	-0.11	-0.02	-0.13	0.040	0.29	0.26	<0.01	0.45
	GRAD	-0.12	-0.23	-0.22	-0.10	-0.09					
PPARa	RAP	0.74	0.67	0.69	0.74	0.70	0.041	0.02	0.96	<0.01	0.13
	GRAD	0.62	0.61	0.62	0.58	0.59					
HMGCS1	RAP	0.95	1.08	0.98	0.92	0.83	0.036	0.12	0.07	<0.01	0.25
	GRAD	0.87	0.87	0.84	0.87	0.82					
HMGCS2	RAP	2.28	2.35	2.37	2.43	2.34	0.037	0.38	0.10	<0.01	0.56
	GRAD	2.19	2.36	2.35	2.32	2.35					
BDH1	RAP	1.33	1.42	1.42	1.48	1.40	0.033	0.19	0.10	<0.01	0.44
	GRAD	1.27	1.37	1.39	1.38	1.38					
BDH2	RAP	0.10	0.11	0.14	0.21	0.08	0.035	0.04	0.36	<0.01	0.16
	GRAD	0.02	0.01	0.07	0.07	0.03					
PCCA	RAP	0.21	0.19	0.22	0.23	0.16	0.024	0.20	0.77	<0.01	0.46
	GRAD	0.14	0.18	0.19	0.16	0.16					
OXCT1	RAP	-0.85	-0.90	-0.90	-0.80	-0.99	0.027	0.30	0.07	<0.01	0.11
	GRAD	-0.93	-1.00	-0.93	-0.93	-0.94					

[1]T = treatment, *P*-value for treatment period (d 9 to 44); D = sampling day, *P*-value for pretreatment and treatment period (-10 to 44 d); T × D = *P*-value for treatment period (d 9 to 44).

[2]Group GRAD: n = 5, 1 cow had no measurement at d -10 due to early calving.

[3]Standard error for LSM by sampling day; n = 12.

Table 7. Least squares means of $\log_{10}$ transformed expression of genes in the rumen papillae associated with VFA metabolism during the dry period experiment for the control (no concentrate during the dry period; CON, n = 5) and supplemental concentrate treatment (3.0 kg of DM/d concentrate during the dry period; SUP, n = 4[1]). Expression was normalized against *Keratin-6*

Gene		Sampling day relative to calving							SE[3]	Fixed effects[2]			
		Dry period			Lactation					T	D	S	T × D
		-28	-18	-8	3	17	31	45					
ACSS1	CON	0.07	-0.05	-0.01	-0.14	-0.09	-0.05	-0.08	0.046	<0.01	0.24	<0.01	0.76
	SUP	0.07	0.15	0.13	-0.01	0.05	0.01	0.02					
PPARa	CON	0.45	0.42	0.46	0.52	0.47	0.46	0.46	0.054	<0.01	0.99	<0.01	0.97
	SUP	0.30	0.34	0.34	0.30	0.33	0.25	0.32					
HMGCS1	CON	0.79	0.78	0.79	0.96	0.91	0.85	0.85	0.035	0.61	0.13	<0.01	0.06
	SUP	0.77	0.92	0.90	0.84	0.87	0.74	0.80					
HMGCS2	CON	2.24	2.23	2.14	2.40	2.28	2.29	2.28	0.039	0.78	0.01	<0.01	0.63
	SUP	2.22	2.29	2.23	2.42	2.33	2.17	2.26					
BDH1	CON	1.24	1.20	1.15	1.33	1.18	1.20	1.22	0.042	0.03	0.16	<0.01	0.83
	SUP	1.28	1.35	1.27	1.39	1.31	1.20	1.26					
BDH2	CON	-0.18	-0.22	-0.20	-0.15	-0.21	-0.20	-0.18	0.029	<0.01	0.36	<0.01	0.54
	SUP	-0.09	-0.02	-0.10	0.00	-0.09	-0.14	-0.12					
PCCA	CON	0.20	0.18	0.14	0.25	0.18	0.18	0.20	0.032	0.18	0.34	<0.01	0.78
	SUP	0.19	0.23	0.22	0.28	0.27	0.13	0.22					
OXCT1	CON	-0.71	-0.75	-0.68	-0.80	-0.79	-0.79	-0.80	0.034	0.01	0.15	<0.01	0.72
	SUP	-0.71	-0.60	-0.63	-0.76	-0.69	-0.71	-0.70					

[1]Group SUP: n = 4, 1 cow was removed from the experiment.

[2]T = treatment; D = sampling day; T × D = interaction between treatment and sampling day.

[3]Standard error for LSM by sampling day; n = 9.

Chapter 5

141

During the dry period experiment (Table 7), sampling day affected (P = 0.01) only the expression of HMG-CoA synthase 2 (*HMGCS2*), showing a peak at 3 d pp (Table 5). The mRNA abundance of acetyl-CoA synthetase 1 *ACSS1*, *BDH1*, *BDH2*, and *OXCT1* was greater ($P \leq 0.03$), and that of *PPARα* was lower (P = 0.02), in SUP compared with CON. A tendency for a treatment and sampling day interaction was only observed for expression of *HMGCS1* (P = 0.06), with expression being greater in SUP compared to CON at 18 and 8 d ap, but lower in SUP compared with CON at 3 d pp.

Expression of Genes at Different Sites in the Rumen

In both experiments, site of collection of the papillae affected the expression of all genes examined, except for *NHE3* during the lactation experiment (Table 8). The expression of tissue proliferation genes was generally highest in VBS for the lactation experiment, but not for the dry period experiment. In the dry period experiment, expression of VFA transporter genes was generally lowest in VBS. Such a pattern did not occur in the lactation experiment. Similarly, expression of VFA metabolism genes was generally highest in VRS for the dry period experiment, but this was not the case for the lactation experiment.

Expression of Proteins Associated with VFA Absorption and Metabolism

An interaction was observed for treatment and sampling day for the ruminal expression of MCT1 protein (P = 0.02; Figure 2). Expression of MCT1 increased by 32% in RAP from 3 to 16 d pp (P = 0.01) but at 44 d pp did not differ from 3 d and 16 d pp (P = 0.12), whereas expression of MCT1 did not change in GRAD ($P \geq 0.16$). Expression of DRA was affected by sampling day ($P < 0.01$) and peaked at 16 d pp with an increase of 32% compared to 3 d and 44 d pp ($P < 0.01$). Expression of DRA was neither affected by treatment (P = 0.37) nor by the treatment and sampling day interaction (P = 0.96). Expression of NHE2 was affected by sampling day ($P < 0.01$) and increased by 113% from 3 to 16 d pp ($P < 0.01$), remaining similar for 16 and 44 d pp (P = 0.82). The NHE2 content was not affected by treatment (P = 0.74) or interaction between treatment and sampling day (P = 0.44).

Table 8. Least squares means of $\log_{10}$ transformed expression of genes associated with VFA absorption, VFA metabolism, and tissue proliferation in rumen papillae obtained from 3 different sites for the lactation experiment (treatment comprised either a rapid or gradual rate of increase of concentrate allowance post-partum) and the dry period experiment (treatment comprised either supplemental concentrate during the last 4 weeks of the dry period or no concentrate supplement). Expression was normalized against *Keratin-6*

| | Sampling site in the rumen[1,2] | | | | | | | |
| | Lactation experiment | | | | Dry period experiment | | | |
	VRS	VBS	DBS	SE	VRS	VBS	DBS	SE
Tissue proliferation								
BAX1	-1.32[a]	-1.02[b]	-0.99[b]	0.016	-0.85[b]	-0.98[a]	-0.88[b]	0.017
BCL2	-1.56[a]	-1.09[b]	-1.53[a]	0.019	-1.64[c]	-1.79[b]	-1.83[a]	0.016
XIAP	-1.02[a]	-0.55[b]	-1.03[a]	0.016	-1.03[a]	-0.81[b]	-0.83[b]	0.015
VFA transporters								
ATP1A1	0.59[b]	0.73[c]	0.51[a]	0.015	0.60[b]	0.40[a]	0.74[c]	0.017
MCT1	1.65[c]	1.45[a]	1.52[b]	0.021	1.67[c]	1.47[a]	1.54[b]	0.018
MCT4	0.12[a]	0.19[b]	0.23[b]	0.021	0.10[b]	0.22[c]	-0.05[a]	0.024
NBC1	-0.98[b]	-0.40[a]	-0.87[b]	0.033	-1.12[b]	-1.13[b]	-0.78[a]	0.029
PAT1	0.02[b]	0.02[b]	0.08[a]	0.020	-0.06[b]	-0.16[b]	0.08[a]	0.017
DRA	0.32[b]	0.44[c]	0.19[a]	0.017	0.63[b]	0.29[a]	0.27[a]	0.026
AE2	0.13[b]	0.22[a]	0.09[b]	0.024	0.22[a]	0.06[c]	0.12[b]	0.022
NHE1	0.16[a]	0.21[b]	0.35[c]	0.016	0.33[c]	0.17[b]	0.12[a]	0.019
NHE2	0.80[b]	0.94[c]	0.72[a]	0.018	0.93[c]	0.68[a]	0.85[b]	0.025
NHE3	0.86	0.85	0.87	0.024	0.82[b]	0.78[b]	0.66[a]	0.028
UT-B	0.67[a]	0.82[b]	0.63[a]	0.019	0.65[b]	0.50[a]	0.76[c]	0.020
VFA metabolism								
ACSS1	0.31[c]	-0.40[a]	-0.26[b]	0.027	0.24[b]	-0.10[a]	-0.13[a]	0.085
PPARa	0.71[b]	0.53[a]	0.72[b]	0.023	0.69[c]	0.14[a]	0.33[b]	0.024
HMGCS1	1.02[c]	0.70[a]	0.98[b]	0.020	1.00[c]	0.71[a]	0.82[b]	0.014
HMGCS2	2.46[c]	2.13[a]	2.41[b]	0.020	2.48[c]	2.25[b]	2.09[a]	0.021
BDH1	1.35[a]	1.31[a]	1.49[b]	0.019	1.32[b]	1.18[a]	1.27[b]	0.022
BDH2	0.02[b]	0.37[c]	-0.13[a]	0.023	-0.01[b]	-0.20[a]	-0.20[a]	0.018
PCCA	0.11[a]	0.29[b]	0.15[a]	0.016	0.22[b]	0.07[a]	0.32[c]	0.015
OXT1	-0.86[b]	-0.85[b]	-1.05[a]	0.019	-0.87[a]	-0.65[b]	-0.65[b]	0.019

[1]Fixed effect for site was $P < 0.01$ for all genes except *NHE3* ($P = 0.78$) during the lactation experiment.
[2]VRS = ventral rumen sac; VBS = ventral blind rumen sac; DBS = dorsal blind rumen sac.
[a,b,c]Significance of differences in LSM of the site within gene and experiment, $P < 0.05$.

Expression of ATP1A1 was affected by an interaction between treatment and sampling day ($P \leq 0.03$). For RAP, ATP1A1 tended to decrease from 3 to 16 d pp ($P = 0.06$), whereas for GRAD a decrease was observed from 3 to 16 d pp ($P < 0.01$). The expression of ATP1A1 was similar at 16 and 44 d pp ($P \geq 0.18$) for both RAP and GRAD. Expression of ATP1A1 at 44 d pp was greater for RAP compared with GRAD ($P = 0.01$). The expression of PCCA, a subunit of propionyl-CoA carboxylase involved in mitochondrial metabolism of propionate, was affected by sampling day only ($P = 0.02$) and increased from 3 to 16 d pp by 21% ($P < 0.01$), while PCCA expression at 44 d pp did not differ from either 3 or 16 d pp ($P \geq 0.14$). The expression of PCCA with RAP increased from 3 to 16 d pp ($P < 0.01$) and decreased from 16 to 44 d pp ($P = 0.05$), whereas there were no changes in the expression with GRAD ($P \geq 0.34$).
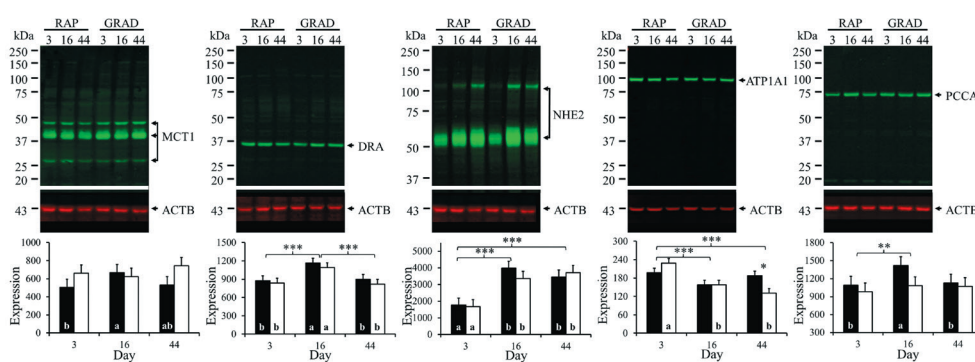

Figure 2. Western blot rumen epithelial protein expression of VFA transporters DRA and MCT1, the Na^+/H^+ antiporter NHE2, ATP driven Na^+/K^+ exchanger ATP1A1, and propionate metabolism associated PCCA with a rapid rate of increase (1.0 kg of DM/d; RAP) of concentrate allowance (black bar; n = 6) and gradual rate of increase (0.25 kg of DM/d; GRAD) of concentrate allowance (white bar; n = 6) treatments. Values represent LSM ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of difference in LSM by day (bars indicate respective days), or RAP or GRAD for the same sampling day; [abc]indicate differences between days within treatment group, $P < 0.05$. Expression is normalized against beta actin, ACTB.

DISCUSSION

The present experiments provide a unique combination of results on morphological changes of the rumen papillae and in vivo changes in fractional VFA absorption and production rate, and the concomitant changes in expression of selected genes involved in VFA absorption, VFA metabolism and epithelial proliferation. Several of the genes examined were affected by the transition from the dry period ration to the lactation ration, or by the treatments applied during either the dry period or early lactation. However, an interaction between sampling day and treatment was

only observed for a few genes. Generally, more lactation stage related effects on gene expression were observed for the dry period experiment when compared with the lactation experiment, which might be explained by the greater number of sampling days (8 vs. 5) and broader timespan (28 d ap to 45 d pp vs. 10 d ap to 44 d pp) under investigation. The level of expression of the genes compares well between experiments, indicating consistency in methodology and allowing for direct comparison of the experiments but differed between sites for nearly all genes. The ventral part of the rumen is thought to be primarily responsible for the absorption of VFA (von Engelhardt and Hales, 1977; Storm et al., 2011). It could, therefore, be hypothesized that the VRS, and especially the VBS, would have a greater level of expression of genes associated with VFA transport and metabolism than the DBS. Whereas this was generally the case for the lactation experiment, no such pattern was observed for the dry period experiment. When comparing the experiments, there also appears to be little consistency in ranking of the expression levels between sites.

Expression of Genes Associated with Tissue Proliferation

Generally, tissue proliferation coincides with both an increase in mitosis and apoptosis (Raab et al., 1998; Gui and Shen, 2016). Mitosis in the rumen epithelium has been shown to increase in response to a higher VFA concentration (Sakata and Yajima, 1984). For apoptosis, the effects of VFA appear to depend on type of VFA. Propionate stimulates mitosis and simultaneously increases rate of apoptosis, which might be interpreted as a sign of extensive tissue remodeling (Mentschel et al., 2001). However, butyrate appears to suppress an increase in apoptosis rate while eliciting a similar increase in mitosis rate as propionate, possibly explaining increased tissue growth with butyrate compared with propionate (Mentschel et al., 2001).

In the present experiments, the concentration and molar proportions of propionate and butyrate increased after calving, as well as the production rates of the VFA, associated with a large increase in papillae surface area (Dieho et al., 2016ab, 2017). However, the expression levels of *BCL2* and *XIAP*, both inhibitors of apoptosis, and the expression of apoptotic activator *BAX1* were not affected by the transition from the dry period into lactation for both experiments. These observations suggest that after calving the suppressive effects of the increased butyrate concentration on the apoptosis rate are balanced against the stimulating effect of increased propionate concentration on apoptosis rate. In the lactation experiment, the propionate concentration but not the butyrate concentration was higher in RAP than GRAD at 9 and 16 d pp (Dieho et al., 2016a), whereas daily propionate but not butyrate production was greater at 16 d pp in RAP than GRAD (Dieho et al., 2016b). In line with the greater propionate

concentration and production, expression of *BAX1* tended to be greater in RAP than GRAD, which suggests a greater apoptosis rate. The papillae surface area increased faster in RAP than GRAD which suggests an even greater increase in mitosis rate and extensive remodeling of the epithelium (Gui and Shen, 2016). However, expression of *BAX1* was already numerically greater for RAP than GRAD during the dry period, indicating that the observed treatment effect might be due to inherent differences between the groups.

In the dry period experiment, propionate concentration and particularly butyrate concentration was higher in SUP than CON at 18 and 8 d pp. However, the greater expression of *BAX1* and the tendency towards lower expression of *BCL2* in SUP compared with CON indicated increased apoptosis rate, which would contradict expectations based on the greater butyrate concentration (Mentschel et al., 2001). In conclusion, it is difficult to consistently explain the observed changes in papillae surface area from the changes in expression of apoptosis related genes. This suggests that the proliferation of papillae in transition dairy cattle is primarily the result of fluctuations in mitosis rate while maintaining apoptosis rate at a rather stable level.

Expression of Genes Associated with VFA Absorption, Intracellular pH, and Urea Transport

It was hypothesized that the large increase in daily VFA production during lactation would increase the expression of genes associated with VFA transport and maintenance of intracellular pH (Penner et al., 2009), and due to greater protein intake, urea transport. In contrast to our hypothesis, effects of sampling day were not observed during the lactation experiment, except for *NCB1* which increased after calving. In contrast, expression of genes generally decreased after calving during the dry period experiment. Assuming that changes in gene expression coincide with equivalent effects on protein expression and function, this suggests that per unit of surface area the capacity for transport is not affected (lactation experiment) or decreases (dry period experiment). This suggestion implies that the large increase in k_aVFA observed postpartum is mainly the result from the increase in papillae surface area. In contrast to this relationship between k_aVFA and papillae surface in the transition period, increases in papillae surface area resulting from dietary treatment (most notably at 16 pp in the lactation experiment and at 18 and 8 d ap during the dry period experiment) were not associated with elevated k_aVFA. The absence of such an association suggests involvement of extra-epithelial processes, such as in epithelial blood flow (Storm et al., 2011). These results contrasts with earlier findings from in vitro measurements suggesting an increase in VFA absorption capacity independent of surface area (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014).

Although the importance of protein-mediated VFA uptake relative to passive diffusion is still uncertain, a number of pathways have been established (Aschenbach et al., 2011). Two major isoforms of monocarboxylate transporters (MCT1, Kirat et al., 2006; Graham et al., 2007; MCT4, Kirat et al., 2007) have been demonstrated in the rumen epithelium. Results from the present experiments show that *MCT1* expression is markedly greater than *MCT4* expression, confirming earlier reports on transition dairy cattle (Minuti et al., 2015). The transition from the dry period to lactation only increased *MCT1* expression at 3 d pp in the dry period experiment. In contrast, Minuti et al. (2015) observed a tendency for increase in *MCT1* from 10 d ap to 28 d pp. Other experiments likewise report conflicting results on the expression of *MCT1*. Upregulation of *MCT1* was reported in associating with a large increase in grain content of the ration (Metzler-Zebeli et al., 2013), whereas dietary NFC source (starch, sugar, or lactose; Oba et al., 2015) and animal susceptibility to acidosis (Schlau et al., 2012) were not associated with differences in *MCT1* expression. Protein expression of MCT1 increased from 3 to 16 d pp with RAP but not with GRAD. Although expression of *MCT1* was not affected by treatment or day, the absence of changes in gene expression from 3 to 44 d pp with GRAD, and the numerical increase in gene expression from 3 to 16 d pp followed by a decline again to 44 d pp, are in line with the observed changes in MCT1 protein expression. The enhanced protein expression of MCT1 at 16 d pp might indicate a greater capacity for export of VFA from the epithelium to the blood, an adaptation in line with the (numerically) greater VFA production rate (Dieho et al., 2016b). The subsequent numerical decrease in transport capacity per unit of surface area by 44 d pp might be compensated by the larger papillae surface area at 44 d pp. However, the greater expression of MCT1 did not affect the k_aVFA in RAP compared with GRAD (Dieho et al., 2016b).

As exchangers of VFA$^-$/HCO$_3^-$, the apically located DRA and PAT1 (Bilk et al., 2005) play a central role in the absorption of dissociated VFA and buffering of the rumen fluid (Penner et al., 2011; Aschenbach et al., 2011) whereas the basally located Na$^+$/HCO$_3^-$ cotransporter NBC1 plays a role in replenishing the epithelial HCO$_3^-$ pool (Connor et al., 2010). Oba et al. (2015) reported the expression of *PAT1* to be affected by NFC source (starch, sugar, or lactose), and the large changes in NFC intake due to lactation stage or treatment in the present experiment were therefore expected to induce effects on *PAT1* expression. However, *PAT1* was not affected by lactation stage or treatment in both experiments. In case of *DRA*, results from both the experiments were not consistent. Expression of *DRA* did not change after calving in the lactation experiment, but decreased after calving in the dry period experiment, despite cows receiving similar rations in both experiments. In contrast to *PAT1*, NFC source did not affect expression of *DRA* (Oba et al., 2015), which again appears not in line with the present results. Although expression of *DRA* was not affected by sample day in the

lactation period experiment, the protein expression of DRA in the lactation experiment indicates that the temporary upregulation at 16 d pp occurred at the protein translation level. The subsequent decrease in VFA^-/HCO_3^- exchange capacity per unit of surface area by 44 d pp might be due to the larger papillae surface area at 44 d pp.

The Na^+/H^+ exchanger isoforms 1 to 3 play a central role in the regulation of intracellular pH (Zachos et al., 2005). The NHE1 are concentrated in the stratum granulosum, whereas NHE2 is located in the cytosol in all cell layers, arguably as a form of functional reserve (Graham et al., 2007). During both experiments similar expression levels were found for *NHE2* and *NHE3*, which were expressed at ~4 times the level of *NHE1* (before log^{10} transformation). However, Etschmann et al. (2009) reported that *NHE1* was by far the most expressed in rumen epithelium cell cultures from sheep, which might reflect in vitro - in vivo differences. In our experiments (Dieho et al., 2016a; Dieho et al., 2017), the treatments and particularly the transition into lactation increased starch and sugar intake. Still, the NHEs were not affected by sampling day or treatment during the lactation experiment, whereas during the dry period experiment expression of NHE2 and NHE3 decreased after calving. This contrasts with Oba et al. (2015), who reported an increase in expression of *NHE1* and *NHE2* in rumen epithelium of dry non-gravid dairy cows upon daily ruminal dosing of 3.0 kg of DM of sucrose or lactose, compared with starch. Similar to DRA, expression of NHE2 was greater at 16 and 44 d pp compared with 3 d pp, without concomitant changes in gene expression. The greater protein expression of NHE2 suggests that the rumen epithelium increases its back-up capacity for maintaining intracellular pH during lactation (Graham et al., 2007), where rumen pH is known to fluctuate to greater extent than during the dry period.

In the dry period experiment we observed a peak in the expression of *UT-B* at 3 d pp, which might be associated with the abrupt transition to the lactation ration (higher CP than dry period ration) on the day of calving. Numerically, a similar increase may be noticed between 10 d ap to 3 d pp during the lactation experiment, where cows were also abruptly transitioned to a similar lactation ration. During the first weeks of lactation concentrate allowance and consequently CP intake increased further, but did not affect *UT-B* expression. Previous results are likewise ambiguous. In transition dairy cattle, Minuti et al. (2015) found similar *UT-B* expressions between 14 d ap and 10 d pp, which thereafter increased at 28 d pp. Simmons et al. (2009) demonstrated a greater expression of *UT-B* for a concentrate rich ration compared with a forage rich ration (both 13% CP) in Limousine steers, whereas Røjen et al. (2011) did not find an effect of dietary CP content (13 vs. 17%) on *UT-B* expression in dairy cows.

The ATP driven Na^+/K^+ exchanger, of which ATP1A1 is a subunit, maintains low intracellular Na^+ and high K^+ concentrations thereby providing a driving force for

a number of exchangers and co-transporters (Aschenbach et al., 2011). The absence of changes in *ATP1A1* expression in the lactation experiment is in line with the general absence of effects observed for the other transporters, most notably the *NHE*s. Similarly, the greater expression levels for *ATP1A1* in SUP in the dry period experiment correspond to the generally greater expression levels of the other transport associated genes, including the *NHE*s. However, the apparent effect of treatment on *ATP1A1* at 18 and 8 d ap is at odds with the lack of effect of the transition into lactation, and also with the outcome of earlier experiments which did not show an increase in expression of *ATP1A1* with increased concentrate feeding (Penner et al., 2009; Metzler-Zebeli et al., 2013). The observed decrease in expression of ATP1A1 protein during the lactation whereas no concomitant change in expression of *ATP1A1* was observed, suggests that protein expression is regulated after transcription. The lower expression of ATP1A1 protein suggests an alteration in rumen sodium homeostasis after calving by decreasing the capacity for Na^+/K^+ exchange.

During the dry period experiment, the expression of *ATP1A1*, *MCT4*, *NBC1*, *DRA*, and *NHE3* was, on average, greater for SUP than CON. This suggests a positive effect of concentrate supplementation during the dry period on rumen buffering and VFA uptake capacity. Moreover it suggests that such changes are carried over into lactation. However, no differences between treatments in k_aVFA were observed at any moment during the experiment (Dieho et al., 2017), indicating that caution is warranted when basing conclusions on tissue function on gene expression results. In addition, as discussed earlier, changes in gene and protein expressions generally did not correspond, and protein expression data did also not consistently correspond with the observed k_aVFA, again indicating that caution is warranted when interpreting such data. It is also hard to explain why the relatively limited effect of supplemental concentrate during the dry period experiment on feed intake, rumen VFA production and rumen fluid composition (Dieho et al., 2017) would have a significant and long lasting effect on on gene expressions, whereas the much larger differences in feed intake, VFA production, and rumen fluid composition due to transition into lactation had not. It seem more plausible that the results reflect an undesired, unbalanced distribution of cows as was observed for the papillae surface area during this experiment (Dieho et al., 2017). Such inherent differences in gene expression, despite feeding similar rations, have been suggested earlier (Schlau et al., 2013).

Expression of Genes Associated with VFA Metabolism

Increases in VFA production were hypothesized to coincide with increases in expression of genes associated with rumen epithelial metabolism (Penner et al., 2011).

Chapter 5

However, the large increase in daily VFA production due to increased intake of FOM (Dieho et al., 2016b; Dieho et al., 2017) had minor effects on the expression of genes associated with epithelial metabolism of VFA when comparing the dry period and lactation for both experiments. This effect, however, might in part be explained by the increase in tissue mass associated with the increase in papillae size.

During both experiments, the treatments had pronounced effects on the composition of the rumen fluid (Dieho et al., 2016a; Dieho et al., 2017). The concentration of butyrate was greater at 18 and 8 d ap, and 3 d pp for SUP than CON, whereas both the propionate production and concentration was greater for RAP than GRAD at 16 d pp. The mitochondrial enzyme HMGCS2, and the 3-hydroxybutyrate dehydrogenases BDH1 and BDH2 catalyze the synthesis of BHBA (Lane et al., 1997; Penner et al., 2011; Oba et al., 2015), whereas OXCT1 is associated with catabolism of ketone bodies. Butyrate is considered the main substrate for ketogenesis (Kristensen and Harmon, 2004) therefore an upregulation of the corresponding genes was expected due increased butyrate availability after calving for both experiments, and during the dry period for SUP. Likewise, PCCA, involved in the metabolism of propionate, was also expected to be upregulated after calving due to increased production of propionate, particularly for RAP. However, only *HMGCS1* but not *HMGCS2* appears to reflect the temporarily greater butyrate concentrations in SUP at 18 and 8 d ap, compared with CON. The general absence of a treatment and lactation stage effects in the present experiments is supported by results from trials where cows were fed high concentrate rations for 3-4 weeks (Penner at al., 2009; Steele et al., 2012). No changes in expression were observed for *HMGCS2* and *PPARα* with a weekly sampling regime (Steele et al., 2012) and for *HMGCS2*, *PPARα*, *BDH1*, *BDH2*, *ACSS1*, and *PCCA* with sampling after 4 weeks of feeding a high concentrate versus a high forage ration (Penner et al., 2009). It should be noted that during the dry period experiment, consistent differences between SUP and CON in *ACSS1*, *PPARα*, *BDH1*, *BDH2*, and *OXCT1* throughout the experimental period were observed. As discusses previously, these consistent patterns suggest inherent differences between individuals.

Expression of *HMGCS2* is under the control of PPARα (Steele et al., 2011), thus the greater expression of *PPARα* in SUP is at odds with the lack of a treatment effect on expression of *HMGCS2*. Another contrast between the expressions of these genes has been reported by Minuti et al. (2015), who observed an increase in *HMGCS2* but not *PPARα*. The greater levels of expression of *HMGCS2* and *BDH2* than *PCCA* agree with the extensive metabolism of butyrate and limited metabolism of propionate by the epithelium (Kristensen and Harmon, 2004), which corresponds to earlier studies (Naeem et al., 2012; Minuti et al. 2015). In contrast to the absence of changes in expression of *PCCA* during the lactation experiment, expression of PCCA protein was

greater for RAP compared with GRAD at d 16 pp, which is in line with the elevated propionic acid concentration and production with RAP, suggesting regulation at the protein translation level.

Correlation between Gene and Protein Expression Level

Although it is generally recognized that changes in gene expression levels not necessarily indicate similar changes in protein expression, previous studies by Penner et al. (2009), Schlau et al. (2012), and Yang et al. (2012) (among others), on gene expression in rumen epithelium assume that greater gene expression levels represent equivalent functional changes in the relevant characteristics. Few studies report the relationship between gene and protein expression levels in rumen papillae, although the explanatory power of gene expression level alone without concomitant protein expression or functional measurements is known to be limited (Connor et al., 2010). To further examine the validity of the assumption that gene expression is related to protein expression, the correlation between the gene and protein expression of MCT1, DRA, NHE2, ATP1A1, and PCCA in the ventral blind rumen sac was determined (Figure 3). The relationship between gene and protein expression of MCT1, DRA, NHE2 and ATP1A1 was not significant ($P \geq 0.32$). Only for PCCA a significant ($P = 0.02$) but weak ($r^2 = 0.16$) correlation was observed. These results suggest that, at least in the fully developed rumen, care should be taken to interpret gene expression levels at the protein expression level.
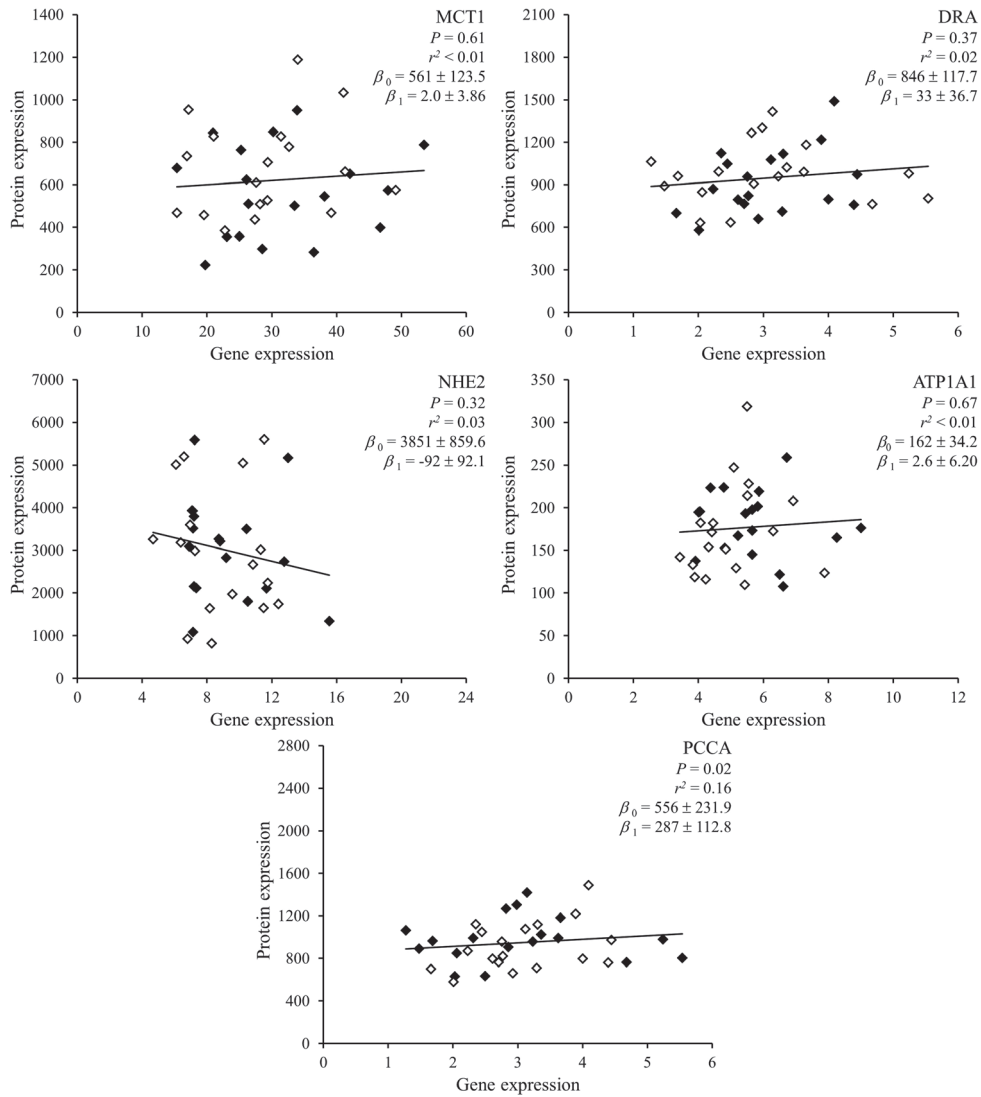
Chapter 5

151

Figure 3. Relationship between gene and protein expression in rumen papillae collected from the ventral blind rumen sac during the lactation experiment for a rapid (1.0 kg DM/d; ◆, n = 6) and gradual (0.25 kg DM/d; ◇, n = 6) rate of increase of concentrate allowance postpartum. Gene and protein expression data are not transformed. Solid line represents linear regression of all data points, β_0: intercept ± SE, and β_1: slope of the regression equation ± SE.

CONCLUSIONS

The present results show minor changes in the expression of genes associated with rumen epithelial transport and metabolism of VFA in dairy cows during the transition period. The results, therefore, indicate that the capacity for VFA absorption and metabolism of the epithelium changes little per unit of surface area, and that the major response to the increase in daily VFA production after calving is tissue proliferation. Furthermore, expression of apoptosis related genes was not affected by the transition into lactation. This indicates that an increase in mitosis rate, and not downregulation of apoptosis rate, drives papillae tissue proliferation. In contrast to the expression at the gene level, expression of selected proteins involved in the absorption and metabolism of VFA in rumen epithelial tissue was affected during early lactation. The differences between gene and protein expression and the generally non-significant and weak correlation between the examined gene and protein expression levels, indicate that care must be taken when interpreting results obtained at either level.

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Chapter 5

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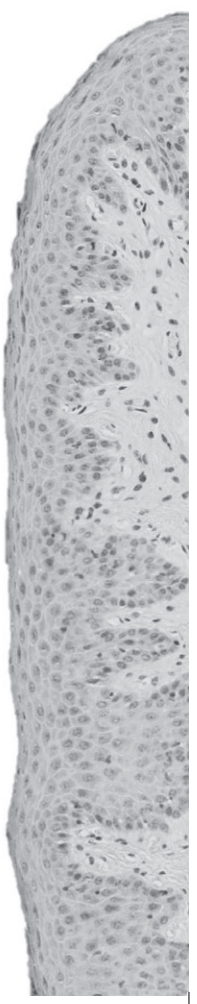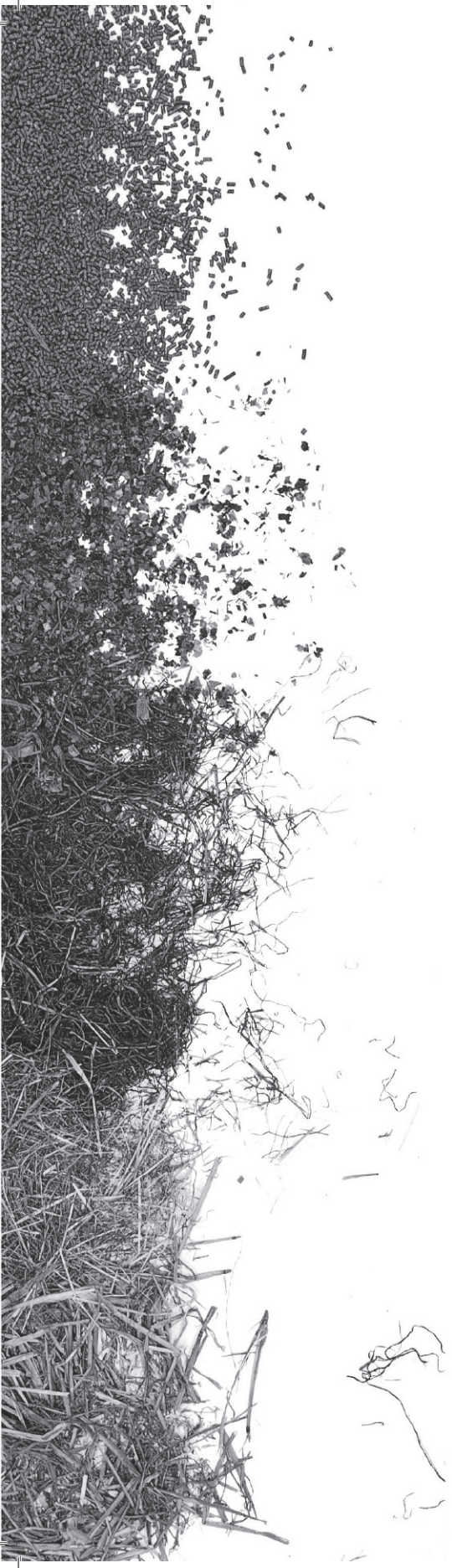
Chapter 5

Chapter 6

Changes in rumen microbiota composition and in situ degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance

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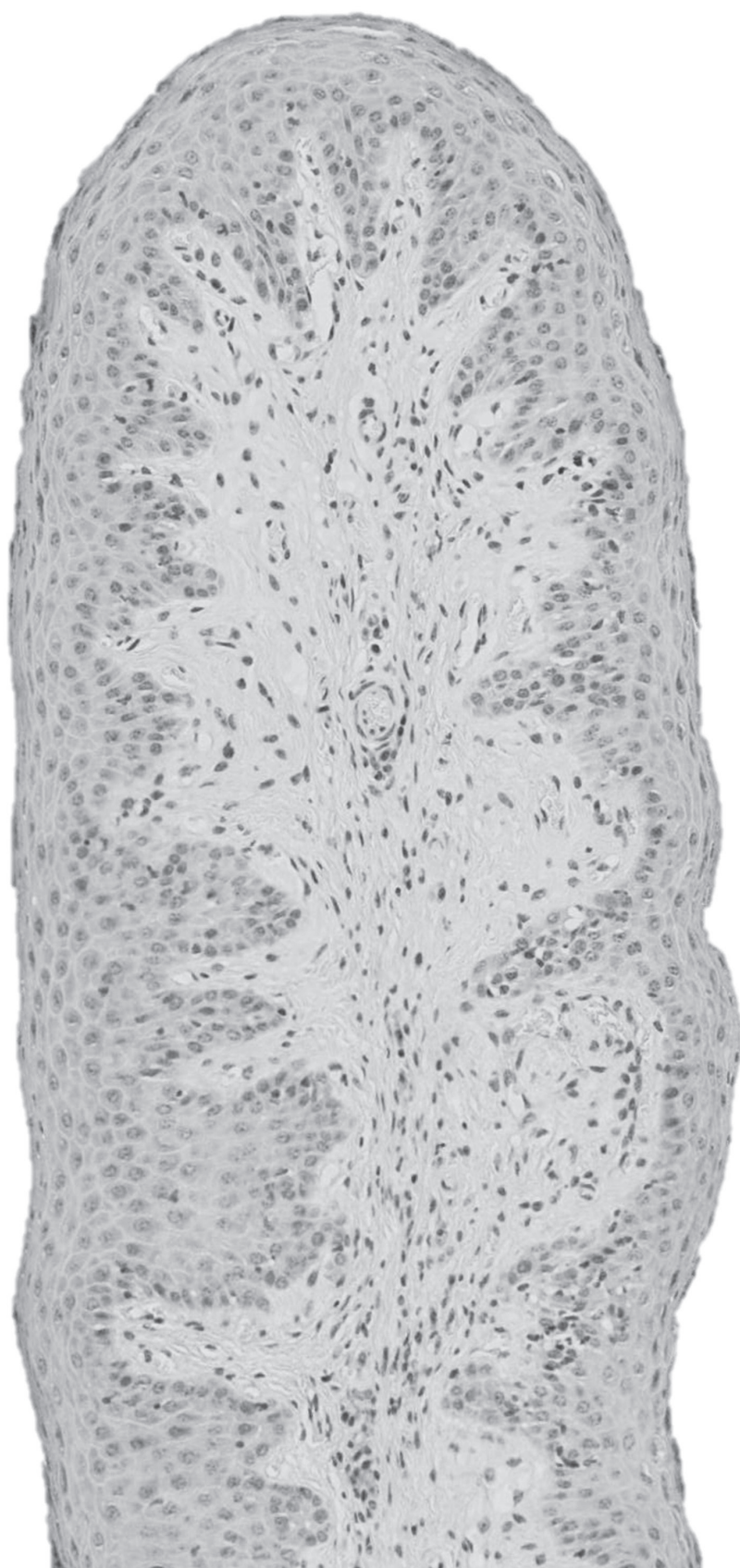
ABSTRACT

Changes in rumen microbiota and in situ degradation kinetics were studied in 12 rumen-cannulated Holstein-Friesian dairy cows during the dry period and early lactation. The effect of a rapid (RAP) or gradual (GRAD) postpartum (pp) rate of increase of concentrate allowance was also investigated. Cows were fed for ad libitum intake and had free access to a mixed ration consisting of chopped wheat straw (dry period only), grass silage, corn silage, and soybean meal. Treatment consisted of either a rapid (1.0 kg of DM/d; n = 6) or gradual (0.25 kg of DM/d; n = 6) increase of concentrate allowance (up to 10.9 kg of DM/d), starting at 4 d pp. In whole rumen contents, bacterial community composition was assessed using samples from 50, 30 and 10 d antepartum (ap), and 3, 9, 16, 30, 44, 60, and 80 d pp, and protozoal and archaeal community composition using samples from 10 d ap, and 16 and 44 d pp. Intake of fermentable organic matter, starch and sugar was temporarily greater in RAP than GRAD at 16 d pp. Bacterial community richness was higher during the dry period than during the lactation. A rapid increase in concentrate allowance decreased bacterial community richness at 9 and 16 d pp compared with a gradual increase in concentrate allowance, whereas from 30 d pp onwards richness of RAP and GRAD was similar. In general, the relative abundances of *Clostridiales* and *Aeromonadales* were greater, and those of *Bacteroidales*, *Fibrobacterales* and *Spirochaetales* smaller, during the lactation compared with the dry period. An interaction between treatment and sampling day was observed for some bacterial community members, and most of the protozoal and archaeal community members. Transition to lactation increased the relative abundance *Epidinium* and *Entodinium*, but reduced the relative abundance of *Ostracodinium*. Archaea from genus *Methanobrevibacter* dominated during both the dry period and lactation. However, during lactation the abundance of the methylotrophic *Methanomassiliicoccaceae* and *Methanosphaera* increased. The in situ degradation of organic matter, neutral detergent fiber, starch, and crude protein was neither affected by treatment nor by transition from the dry period to lactation. Results show that the composition of the rumen microbiota can change quickly from the dry period to the lactation period, in particular with a rapid increase in fermentable substrate supply pp, but this was not associated with changes in rumen degradation kinetics.

Key words: transition period, dairy cattle, rumen microbiome or microbiota, rumen adaptation

Chapter 6

161

INTRODUCTION

Ruminants are characterized by the presence of a highly developed forestomach, the reticulorumen, which harbors a complex microbial ecosystem. Rumen microbiota ferment organic matter yielding microbial biomass and VFA as fermentation end-products. These cover a significant part of the energy (Bergman, 1990) and protein (Tamminga et al., 1994) requirement of the ruminant host. The rumen microbiota is highly diverse, especially within the domain bacteria (Edwards et al., 2004; Kim et al., 2011), and its composition depends on the type and quantity of substrates ingested, and on host species and its physiological status (McCann et al., 2014; Henderson et al., 2015). The type and quantity of substrate consumed leads to changes in the rate of substrate fermentation, rumen VFA concentrations, pH, passage rate, and the composition of the microbiota (Fernando et al., 2010; Belanche et al., 2012). However, the capacity to ferment a wide range of substrates is likely to be maintained despite changes in microbiota community composition as a high degree of functional redundancy and resilience coincides with the diversity of the microbiota (Henderson et al., 2015; Weimer, 2015).

In many dairy systems, the transition from dry period to lactation is associated with large dietary changes. Typically, a dry cow has a low DMI and a fiber-rich ration of relatively poor digestibility, whereas during early lactation DMI increases and rations are rich in readily fermentable carbohydrates (structural and non-structural). Various studies addressed the interrelationship between ration composition and the composition of the rumen microbiota (reviewed by McCann et al., 2014). However, few studies (Mohammed et al., 2012; Pitta et al., 2014; Lima et al., 2015) focused on the transition period in dairy cattle, and knowledge of rumen microbiota changes during this period is limited. In addition, the idea of functional redundancy requires the study of changes in the microbiota together with measurements of substrate degradation capacity by the microbiota to understand the functional impact of any changes in microbiota community composition. This combination of measurements in the transition period is rarely reported.

Therefore, the primary aim of the present experiment was to study the effects of ration changes associated with transition from the dry period to lactation, and the effect of rate of concentrate build-up treatments during the early lactation, on shifts in the ruminal microbiota and on in situ degradation characteristics of OM, starch, NDF, and CP. Transition from the dry period to lactation was hypothesized to have a persistent effect on the rumen microbiota up to the conclusion of the experiment, with an additional transient effect of the concentrate build-up strategy on the ruminal microbiota. In view of functional redundancy of rumen microbial community, effects

of dry versus lactation period and of concentrate strategy on the capacity of the rumen microbiota to degrade OM, starch, NDF and CP were expected to be minor only.

MATERIALS AND METHODS

The experimental procedures were approved by the Animal Care and Ethics Committee of Wageningen UR and conducted under the Dutch Law on the Animal Experiment.

Animals, Experimental Design, Rations, and Treatment

The experiment has been described in detail by Dieho et al. (2016a). Briefly, twelve rumen-cannulated, first parity Holstein-Friesian dairy cows were dried-off 8 weeks prior to expected calving date and entered the experiment. Samples were collected during the pretreatment period at 50, 30, and 10 d antepartum (**ap**) and 3 d postpartum (**pp**), and during the treatment period at 9, 16, 30, 44, 60, and 80 d pp. In situ degradation series started 14 d ap (pretreatment), and at 18, 46, and 62 d pp (treatment period).

Cows received either a dry-period ration or basal lactation ration that was fed once a day for ad libitum intake, and had free access to water. The dry period ration (603 g of DM/kg) consisted of 27% grass silage, 27% maize silage, 11% soybean meal, and 35% chopped wheat straw (DM basis), containing 920 g OM, 553 g NDF, 325 g ADF, 32 g ADL, 109 g CP, 90 g starch, and 44 g sugar per kg of DM, providing 5.3 MJ NE_L (van Es, 1978) and 455 g rumen fermentable organic matter (**FOM**; Tamminga et al., 1994) per kg of DM. The basal lactation ration (466 g of DM/kg) consisted of 42% grass silage, 41% maize silage, and 17% soybean meal (DM basis), containing 925 g OM, 392 g NDF, 235 g ADF, 16 g ADL, 157 g CP, 139 g starch, and 67 g sugar per kg of DM, providing 6.7 MJ NE_L and 561 g FOM per kg of DM. From 0 to 3 d pp, 0.9 kg of DM/d concentrate was fed. Thereafter concentrate treatment started consisting of either a rapid (**RAP**; 1.0 kg of DM/d) or a gradual (**GRAD**; 0.25 kg of DM/d) increase of concentrate allowance up to 10.9 kg of DM/d, irrespective of rate of increase. The concentrate (892 g of DM/kg) comprised 212 g sugar beet pulp, 200 g maize gluten, 150 g barley, 150 g wheat, 120 g soybean meal, 100 g citrus pulp, 35 g molasses, 20 g vinasse, 8 g chalk, 3 g salt, and 2 g mineral premix per kg DM and contained 932 g OM, 252 g NDF, 115 g ADF, 8 g ADL, 178 g CP, 248 g starch, and 95 g sugar per kg of DM providing 7.4 MJ NE_L and 682 g FOM per kg of DM. Daily intake of dry-period or basal lactation ration and concentrate (kg/d) was measured individually.

Chapter 6

The concentrate was individually fed using a concentrate dispenser (Manus VC5, DeLaval, Steenwijk, the Netherlands), and made available in equal portions over six 4-h periods. Daily management and housing conditions, and feed intake measurements, feed sampling, and chemical analyses of feed samples are described in detail by Dieho et al. (2016a).

Rumen Content Sampling

Rumen contents were completely evacuated on all sampling days (Dieho et al., 2016a). Just before starting the evacuation a rumen fluid sample was taken from the ventral rumen sac for determination of pH, and VFA concentration and composition. After evacuation of approximately half of the rumen contents, a sample of whole rumen content was taken (Henderson et al. 2015), immediately placed on ice and stored at -20°C until further processing. After freeze-drying, samples were ground to pass a 1 mm screen (100AN, Peppink Mills BV, Olst, the Netherlands).

Profiling Rumen Bacterial Community

The DNA extraction and profiling is described in detail in Supplemental Methods S1. Briefly, purified bacterial DNA was extracted from 50 mg of freeze dried whole rumen content (Zoetendal et al., 2006; van den Bogert et al., 2013) using a modified version of the QIAamp DNA Stool Mini Kit protocol (Ambion, Waltham, MA). The quantity and quality of obtained DNA was assessed using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, PA) and electrophoresis on a 1% (w/v) agarose gel. For 16S rRNA gene based microbial composition profiling, barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated using a 2-step PCR strategy that reduces the impact of barcoded primers on the outcome of microbial profiling (Berry et al., 2011; Tian et al., 2016). Purified PCR products were mixed in equimolar amounts and resulting pools underwent adaptor ligation followed by sequencing on the MiSeq platform with addition of 20% PhiX (GATC-Biotech, Konstanz, Germany). The 16S rRNA gene sequencing data was analyzed using NG-Tax (Ramiro-Garcia et al., 2016). Operational taxonomic units (**OTU**s) were defined using an open reference approach, and taxonomy was assigned to those OTUs using the SILVA 16S rRNA gene reference database (Quast et al., 2013).

Profiling Rumen Protozoal and Archaeal Communities

Freeze dried samples of whole rumen content from 10 d ap, and 16 and 44 d pp, were shipped to AgResearch (Palmerston North, New Zealand) for processing as part of the Global Rumen Census project (Henderson et al., 2015). Briefly, after DNA extraction using the PCQI method described by Rius et al. (2012), and PCR amplification described by Henderson et al. (2015), archaeal 16S rRNA and protozoal 18S rRNA genes were sequenced using 454 GS FLX Titanium chemistry (Eurofins MWG Operon, Ebersberg, Germany). Pyrosequence data were processed using QIIME (version 1.8; Caporaso et al., 2010) and grouped into OTUs sharing over 99% sequence similarity for archaea and 100% for protozoa. Sequence data were assigned to phylogenetic groups using BLAST (Altschul et al., 1990) against RIM-DB version 13_11_13 (Seedorf et al., 2014) for archaea and an AgResearch in-house database (Kittelmann and Janssen, 2011) for protozoa. Archaea were assigned at the species level, protozoa at the genus level.

In Situ Degradation

The fractional degradation rate of OM, starch, NDF, and CP was evaluated four times in the same cows from each treatment group (three each) using an all-out in situ method adapted from Tas et al. (2006). In short, for each incubation series, four polyamide bags (8.5 × 16.5 cm; 40 μm pore size; PA 40/30, Nybolt, Zurich, Switzerland) each containing 3.0 g soybean hulls and 3.0 g ground corn (both ground to pass a 3 mm screen; 5.7 g of DM per bag), were incubated for 72, 48, 24, 8, and 4 h. After recovery from the rumen, the bags were immediately placed in iced water. After gently rinsing with tap water, the bags were washed for 40 min using 58 L cold water with a wool program without centrifuging (Öko Turnamat 2800, AEG Hausgeräte, Nürnberg, Germany) including 4 non-incubated bags (0 h) for determination of the washable (**W**) fraction. In addition, four bags were incubated for 336 h in each cow for the determination of the undegradable (**U**) fraction. After washing, bags were stored at -20°C before forced air oven drying (60°C, overnight). After drying, samples were acclimatized to air and weighed. Residues were pooled per incubation time for each cow and incubation series, and ground to pass a 1 mm screen (ZM1, Retch GmbH, Haan, Germany). Residues were analyzed for DM (ISO, 1999), ash (ISO, 2002), starch (ISO, 2004), N (ISO, 2005), and for NDF (with heat stable α-amylase; van Soest et al., 1991) using an ANKOM 2000 Fiber Analyzer (ANKOM Technology Corp., Macedon, NY). The fractional degradation rate (k_d, /h) of the potentially degradable fraction of starch and N was calculated according to the first-order model of Robinson et al. (1986):

$$Rt = U + D \times exp\ (-k_d \times t)$$

where Rt is the residual at time t, U the rumen undegradable fraction, and D the potentially degradable fraction ($D = 1 - W - U$) and t the time of incubation (h). For starch, the U-fraction was assumed to be zero. For NDF and OM the model included a lag time (L, h) with:

$$Rt = U + D\ (t \leq L)$$

and

$$Rt = U + D \times exp\ [-k_d \times (t\text{-}L)]\ (t > L)$$

For NDF, the W-fraction was assumed to be zero.

Calculations and Statistical Analysis

The similarity in bacterial community composition between samples was assessed by principal coordinate analysis using the weighted UniFrac metrics (Lozupone et al., 2007) in QIIME (v1.2; Caporaso et al., 2010). The PD-whole tree (Faith, 1992) richness metric of the bacterial community was calculated ten times for each sample using 5,000 randomly selected reads. Sample averages of the PD-whole tree richness metric were analyzed. Bacterial taxa were summarized at the order level, and bacterial taxa with a relative abundance of > 1.0% in more than > 20.0% of the samples at the genus or family rank were considered 'major' taxa and summarized separately. All other taxa were grouped under 'Others'. The association scores between the major taxa and rumen fluid and feed intake variables (reported by Dieho et al., 2016ab) were explored using regularized canonical correlation analysis (González et al., 2012) and visualized as a heatmap as described by Henderson et al. (2015).

All feed and rumen fluid variables, protozoal, archaeal, and major bacterial taxa, and in situ incubation variables were assumed to be related to sampling day and treatment. Data were analyzed using a MIXED model (Littell et al., 2006) in SAS 9.2 (SAS Institute Inc., Cary, NC):

$$Y_{ij} = \mu + T_i + D_j + (T \times D)_{ij} + e_{ij},$$

where variable Y_{ij} was dependent on μ as the average experimental value and fixed main effects of concentrate treatment T_i (i = RAP, GRAD), sampling day D_j (j = −50, ..., 80 for bacteria; or −10, 16, and 44 for protozoa and archaea; or −14, ..., 62 for the in

166

situ incubations), and fixed interaction $(T \times D)_{ij}$. Errors e_{ij} were assumed to be auto-correlated for repeated observations on the same cow, using a spatial power covariance structure over sampling days (Littell et al., 1998). As the concentrate treatment was only applicable to the treatment period, custom CONTRAST statements were constructed as described by Dieho et al. (2016b) to test for treatment effect and the interaction between treatment and day over the treatment period. Means were separated by formulating CONTRAST or ESTIMATE statements. All results are reported as least squares means with their SE unless indicated otherwise. Significance of effect was declared at $P \leq 0.05$ and as trend between $0.05 < P \leq 0.10$.

RESULTS

One cow (RAP) calved early, and missed sampling at 10 d ap, otherwise all sampling was completed as planned. Details on actual sampling days and animal health have been reported by Dieho et al. (2016a).

Feed Intake and Rumen Fluid Composition

Feed intake and rumen fluid composition have previously been reported in detail by Dieho et al. (2016a). Briefly, DMI approximately doubled from the dry period to 44 d pp (Figure 1A), and was neither affected by treatment nor was an interaction observed between treatment and sampling day. Intake of FOM increased from 5.6 kg/d during the dry period to 14.4 kg/d from 44 d pp onwards and was affected by an interaction between treatment and sampling day, with a greater intake for RAP at 16 d pp compared with GRAD. The abrupt changeover at calving from the dry period ration to the lactation ration resulted in a temporary decrease in NDF intake after calving, whereas intake of CP, starch and sugar all increased (Figure 1B and 1C). An interaction between treatment and sampling day was observed for CP, starch, and sugar intake, with greater intake of CP, starch and sugar for RAP compared with GRAD, most notably around 16 d pp.
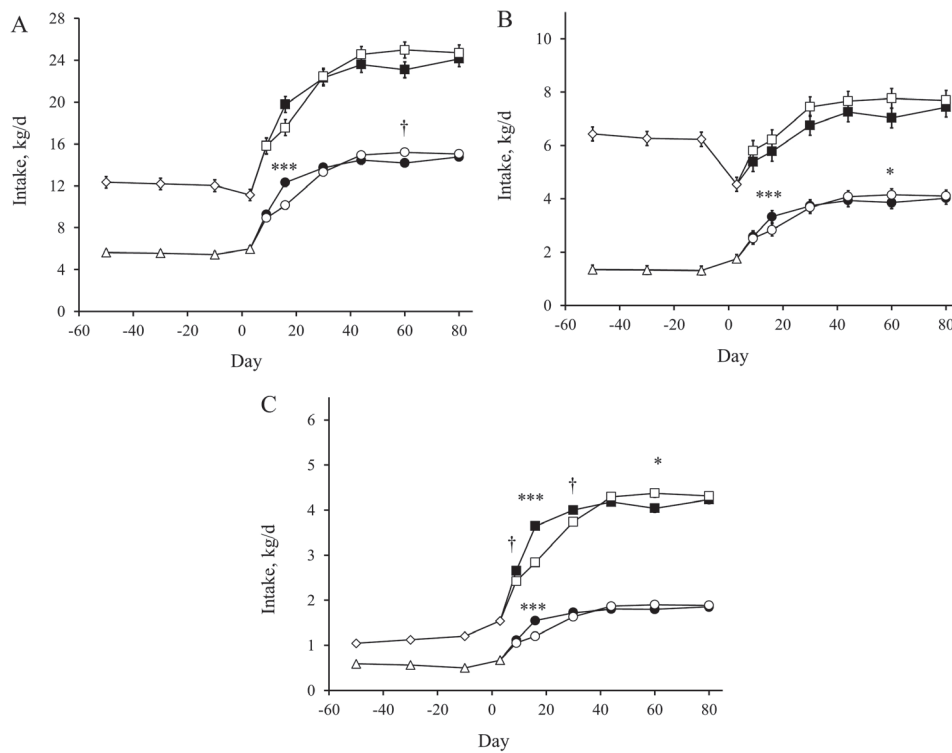
Chapter 6

Figure 1. (A) Dry matter ($\diamond\blacksquare\square$) and fermentable organic matter ($\triangle\bullet\bigcirc$) intake; (B) intake of NDF ($\diamond\blacksquare\square$) and CP ($\triangle\bullet\bigcirc$); and (C) intake of starch ($\diamond\blacksquare\square$) and sugar ($\triangle\bullet\bigcirc$) in the pretreatment period ($\diamond\triangle$; n = 12), and the treatment period with a rapid (RAP; 1.0 kg of DM/d; $\blacksquare\bullet$, n = 6) and gradual (GRAD; 0.25 kg of DM/d; $\square\bigcirc$, n = 6) rate of increase of concentrate allowance postpartum. Values represent LSM ± SE; ***P < 0.001, *P < 0.05, †*P* < 0.10, significance of difference in LSM of RAP or GRAD. Adapted from Dieho et al. (2016a).

The total VFA concentration and the pH in rumen fluid were affected by sampling day and the treatment and sampling day interaction, but not by treatment (Dieho et al., 2016a). Total rumen fluid VFA concentration increased from approximately 80 m*M* during the dry period to 110 m*M* during the lactation, whereas pH decreased after calving from approximately 6.7 during the dry period to 6.2 during the lactation. The molar proportion of acetic acid decreased from 73% during the dry period to 65% during lactation, whereas the molar proportions of propionic and butyric acid increased from 17% and 10% during the dry period to 23% and 12% during lactation, respectively. An interaction between treatment and sampling day was observed for the molar proportions of acetic and propionic acid, but not butyric acid. At 9 and 16 d pp the molar proportion of propionic acid was higher in RAP (24 and 29%) compared

with GRAD (21 and 23%), whereas the molar proportion of acetic acid was lower in RAP (64 and 59%) compared with GRAD (67 and 65%) during these days.

Rumen Bacterial Community Composition and Richness

Differences in the bacterial community composition were visualized using principal coordinate analysis (Figure 2). Samples taken during the dry period cluster together indicating a high degree of similarity, and they were clearly separated from the samples taken during the treatment period. Samples taken 3 d pp already showed a divergence from dry period samples. Samples taken during the treatment period showed less clear grouping, regardless of sampling day or treatment. Samples from RAP appear to diverge more rapidly from the dry period cluster after the start of the treatment.
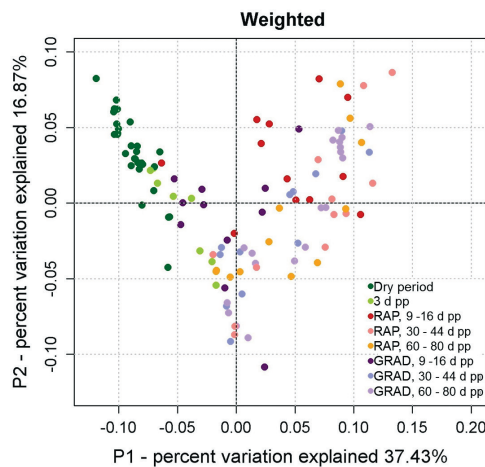

Figure 2. Principal coordinate analysis of the similarity of the bacterial community composition using weighted UniFrac distance metrics during the pretreatment period [50, 30 and 10 d antepartum, and 3 d postpartum (pp); dark and light green dots, respectively] and the treatment period with a rapid (RAP; 1.0 kg of DM/d; red/orange dots) and a gradual (GRAD; 0.25 kg of DM/d; purple/violet dots) rate of increase of concentrate allowance postpartum.

The bacterial community richness (Figure 3) decreased over time ($P < 0.01$) and was lower during lactation when compared with the dry period for both RAP and GRAD ($P < 0.01$). During the dry period, community richness of RAP and GRAD was similar. Postpartum, an interaction between sampling day and treatment was observed

(*P* < 0.01). Richness was lower for RAP at 9 and 16 d pp compared with GRAD (*P* = 0.02). However, from 30 d pp onwards richness became similar again for RAP and GRAD (*P* ≥ 0.12).
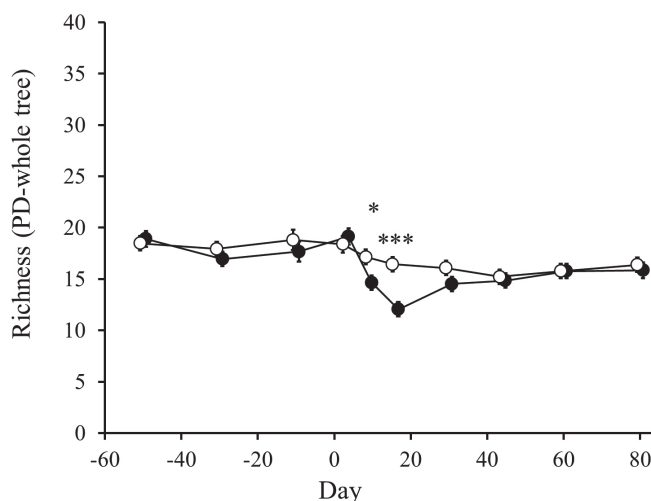

Figure 3. Bacterial community richness (■□) during the pretreatment and treatment period with a rapid (RAP; 1.0 kg of DM/d; ■, n = 6) and gradual (GRAD; 0.25 kg of DM/d; □, n = 6) rate of increase of concentrate allowance postpartum. Values represent LSM ± SE (values are slightly offset for clarity). ***P* < 0.001, *P* < 0.05, significance of difference in LSM of RAP or GRAD.

The bacterial community was dominated by orders *Clostridiales* and *Bacteroidales* throughout the experiment (Table 1), both comprising a relative abundance of approximately 40%. The orders *Clostridiales*, *Bacteroidales*, *Fibrobacterales*, *Aeromonadales* and *Spirochaetales* were not affected by treatment or treatment by day interaction. The relative abundance of *Clostridiales* was generally lower, and that of *Bacteroidales* generally greater, in the lactation compared with the dry period (*P* < 0.01). The *Fibrobacterales* comprised 11.4% of the community during the dry period, but its relative abundance was lower during the lactation (*P* < 0.01), decreasing to 4.4% from 44 d pp onwards. In contrast, relative abundance of the *Aeromonadales* was low (0.9%) during the dry period but was higher during the lactation (*P* < 0.01), increasing to 9.0% from 16 d pp onwards. Only the *Erysipelotrichales* were affected by treatment (*P* = 0.03), with a relative abundance of 4.2% in RAP averaged over the treatment period compared with 1.6% for GRAD. The relative abundance of the *Spirochaetales* was low throughout the experiment, decreasing from 1.7% during the dry period to 1.4% during the lactation (*P* < 0.01). The relative abundance of all other orders of bacteria combined comprised

5.0% and 3.3% of the community during the pretreatment and treatment periods, respectively.

Of the major taxa at the family and genus level (Table 1), *Prevotella* had the highest relative abundance during the dry period (11.6%) increasing ($P < 0.01$) to 21.8% during lactation. The RC9 gut group and unclassified *Christensenellaceae* were prominent community members during the dry period with a relative abundance of 11.6 and 9.5%, respectively. During lactation however, the relative abundance of these two taxa was lower ($P < 0.01$), rapidly decreasing to on average 3.6 and 3.2% during the lactation period, respectively. Likewise, *Fibrobacter* decreased ($P < 0.01$) from 8.4% during the dry period to 4.7% during the lactation period. In contrast, the relative abundances of *Acetitomaculum* and especially the unclassified *Succinivibrionaceae* increased ($P < 0.01$) from the dry period to the lactation period (3.2 to 7.4% and 0.9 to 7.8%, respectively). The relative abundances of several major taxa were not markedly affected by sampling day, notably *Butyrivibrio* (8.0% on average over the experimental period; $P = 0.11$) and the unclassified *Prevotellaceae* (4.2% on average; $P = 0.07$). For 4 out of 22 major taxa an interaction between treatment and sampling day was observed ($P = 0.01$). The relative abundance of *Oribacterium* showed a temporary peak for RAP at 16 d pp compared to GRAD, whereas the relative abundances of the unclassified *Ruminococcaceae*, *Saccharofermentans*, and *Xylanibacter* were lower in RAP compared with GRAD at 16 d pp. The unclassified *Erysipelotrichaceae* tended to be affected by treatment only, with numerically higher relative abundances in RAP during the treatment period (3.6% average) compared with GRAD (1.5%).

Chapter 6

Table 1. Relative abundance of bacterial community members (% of total community) at the order, family, and genus level during the pretreatment and treatment period with a rapid (RAP; 1.0 kg of DM/d; n = 6) and a gradual (GRAD; 0.25 kg of DM/d; n = 6) rate of increase of concentrate allowance postpartum.

Taxonomic rank[1]			Group	Day[2]										SE[4]	Fixed effects[3]		
Order	Family	Genus		Pretreatment				Treatment							T	D	T×D
				-50	-30	-10	3	9	16	30	44	60	80				
Clostridiales (sum of all)			RAP	**43.8**	**41.7**	**45.4**	**45.9**	**35.6**	**34.0**	**38.2**	**38.2**	**38.4**	**38.6**	**1.91**	**0.39**	**<0.01**	**0.60**
			GRAD	**42.7**	**38.8**	**49.2**	**40.4**	**40.4**	**39.6**	**37.3**	**38.8**	**38.1**	**41.8**				
	Christensenellaceae	Unclassified	RAP	10.6	10.0	9.4	7.5	3.0	1.2	2.2	2.3	2.7	2.8	0.76	0.43	<0.01	0.48
			GRAD	8.3	9.7	9.3	6.2	3.5	3.4	2.4	2.7	2.3	2.6				
	Lachnospiraceae	Unclassified	RAP	3.4	2.5	3.8	2.6	2.8	2.4	2.9	3.1	3.0	3.0	0.27	0.73	<0.01	0.74
			GRAD	3.5	3.2	4.6	2.5	3.2	2.5	2.8	2.9	2.7	3.4				
		Acetitomaculum	RAP	3.7	2.7	3.9	4.7	6.4	6.2	9.3	8.2	7.9	7.1	1.00	0.37	<0.01	0.30
			GRAD	3.8	2.0	2.7	4.5	6.9	7.9	7.4	9.2	8.2	9.5				
		Butyrivibrio	RAP	8.2	7.9	9.2	9.7	6.9	6.6	7.1	8.1	9.0	9.5	1.06	0.68	0.11	0.95
			GRAD	6.8	7.5	6.9	7.2	6.6	6.8	8.4	8.5	8.7	10.5				
		Incertae Sedis	RAP	1.3	1.6	1.4	2.0	3.1	2.6	2.1	1.8	1.6	1.4	0.37	0.85	<0.01	0.51
			GRAD	2.2	1.0	1.7	2.1	2.4	1.9	2.7	2.1	1.8	1.5				
		Oribacterium	RAP	0.1	0.0	0.0	0.2	0.3	3.6	1.6	1.4	1.1	1.3	0.40	0.00	<0.01	<0.01
			GRAD	0.1	0.0	0.0	0.1	0.0	0.6	0.5	0.9	1.1	0.7				
	Ruminococcaceae	Unclassified	RAP	4.9	5.3	6.5	6.9	3.4	1.4	3.1	2.4	2.4	3.1	0.64	0.01	<0.01	<0.01
			GRAD	5.9	5.9	6.6	5.3	4.7	5.4	3.3	3.1	3.5	3.4				
		Ruminococcus	RAP	1.3	2.8	2.0	2.0	1.4	1.5	1.8	1.5	1.1	1.9	0.42	0.44	0.27	0.69
			GRAD	1.3	1.8	2.2	1.9	2.5	2.1	1.7	1.2	1.4	1.7				
		Saccharofermentans	RAP	2.9	2.9	3.4	3.4	2.7	1.7	1.3	1.6	1.8	1.6	0.27	0.00	<0.01	0.01
			GRAD	3.5	3.3	4.0	2.7	3.5	2.5	2.0	1.7	1.7	1.8				
	Veillonellaceae	Unclassified	RAP	1.0	0.8	1.0	1.4	0.8	1.5	1.7	1.6	2.0	1.8	0.34	0.20	0.25	0.24
			GRAD	0.8	0.8	1.0	0.8	1.0	1.0	0.9	1.1	1.3	1.4				

172

Table 1. Continued

Order	Family	Genus	Group	Day										SE³	Fixed effects		
				-50	-30	-10	3	9	16	30	44	60	80		T	D	T×D
Bacteroidales (sum of all)			**RAP**	**35.0**	**39.1**	**34.7**	**39.2**	**36.9**	**39.0**	**40.8**	**39.8**	**39.4**	**42.1**	**1.09**	**0.35**	**<0.01**	**0.46**
			GRAD	**35.5**	**40.8**	**34.9**	**39.6**	**39.6**	**40.4**	**41.5**	**40.8**	**42.9**	**40.0**				
	BS11 gut group	Unclassified	RAP	1.3	2.0	0.7	1.1	0.3	0.2	0.7	0.6	0.7	0.5	0.21	0.34	<0.01	0.09
			GRAD	0.8	1.4	1.0	0.8	0.6	0.6	0.7	0.4	0.7	0.5				
	Prevotellaceae	Unclassified	RAP	3.3	3.1	2.6	3.7	4.4	5.0	5.1	4.0	4.6	5.2	0.83	0.98	0.07	0.88
			GRAD	3.3	3.6	2.4	3.5	3.3	4.0	5.3	5.0	5.3	5.4				
		Prevotella	RAP	9.3	13.0	9.4	16.1	17.6	25.4	22.9	24.2	22.2	24.7	1.92	0.55	<0.01	0.52
			GRAD	11.3	12.9	13.8	19.5	18.7	21.4	21.1	23.4	24.8	22.8				
		Xylanibacter	RAP	0.6	1.6	0.9	1.1	1.5	0.8	1.0	1.0	1.3	1.3	0.28	0.00	0.01	0.00
			GRAD	0.8	1.2	0.8	1.8	2.2	2.5	1.7	1.3	1.3	1.7				
	Rikenellaceae	RC9 gut group	RAP	11.4	11.4	12.9	9.0	4.6	1.2	2.3	2.1	2.0	1.8	0.96	0.31	<0.01	0.28
			GRAD	10.3	13.5	10.2	7.7	4.7	3.9	3.3	2.5	2.7	2.1				
	S24-7	Unclassified	RAP	5.5	3.6	3.9	4.2	5.6	2.7	4.8	4.1	4.2	4.3	0.80	0.76	0.01	0.79
			GRAD	4.0	3.1	1.5	3.1	5.3	4.2	5.0	4.5	4.2	3.5				
Fibrobacterales (sum of all)			**RAP**	**12.9**	**12.1**	**11.5**	**8.2**	**9.9**	**5.7**	**2.8**	**3.5**	**5.2**	**4.7**	**0.91**	**0.45**	**<0.01**	**0.53**
			GRAD	**11.8**	**13.1**	**6.8**	**7.7**	**9.0**	**6.3**	**6.6**	**4.0**	**4.6**	**4.4**				
	Fibrobacteraceae	Unclassified	RAP	1.8	3.0	3.9	1.9	1.9	0.7	0.1	0.3	0.3	0.3	0.41	0.29	<0.01	0.43
			GRAD	2.7	3.3	2.2	2.2	1.6	1.2	1.4	0.4	0.4	0.4				
		Fibrobacter	RAP	10.8	8.8	7.7	6.1	7.6	4.3	2.6	3.0	4.6	4.2	1.03	0.63	<0.01	0.78
			GRAD	8.8	9.6	4.7	5.6	7.0	4.8	4.9	3.4	4.0	3.8				
Aeromonadales (sum of all)			**RAP**	**1.0**	**1.0**	**1.1**	**0.1**	**10.2**	**10.8**	**10.1**	**10.0**	**8.1**	**8.6**	**1.43**	**0.20**	**<0.01**	**0.54**
			GRAD	**0.9**	**0.5**	**0.7**	**5.2**	**4.1**	**8.3**	**7.7**	**10.5**	**8.2**	**7.7**				
	Succinivibrionaceae	Unclassified	RAP	1.0	1.0	1.1	0.1	10.2	10.7	10.1	10.0	8.1	8.6	2.02	0.21	<0.01	0.54
			GRAD	0.9	0.5	0.7	5.2	4.1	8.3	7.7	10.5	8.1	7.7				

Chapter 6

173

Table 1. Continued

Order	Family	Genus	Group	Day										SE[3]	Fixed effects		
				-50	-30	-10	3	9	16	30	44	60	80		T	D	T × D
Erysipelotrichales (sum of all)			**RAP**	**0.4**	**0.4**	**0.6**	**0.1**	**2.7**	**6.2**	**4.5**	**4.8**	**4.3**	**2.9**	**1.03**	**0.03**	**0.20**	**0.17**
			GRAD	**0.6**	**0.5**	**0.4**	**0.4**	**0.6**	**0.6**	**2.3**	**2.0**	**2.5**	**1.9**				
	Erysipelotrichaceae	Unclassified	RAP	0.4	0.4	0.6	0.1	2.4	3.9	3.7	4.7	4.1	2.7	1.32	0.07	0.36	0.51
			GRAD	0.6	0.4	0.4	0.3	0.4	0.5	2.2	1.8	2.4	1.8				
Spirochaetales (sum of all)			**RAP**	**1.6**	**1.3**	**1.9**	**1.7**	**1.7**	**0.9**	**0.8**	**1.3**	**1.4**	**1.3**	**0.22**	**0.48**	**<0.01**	**0.40**
			GRAD	**2.2**	**1.5**	**1.9**	**2.4**	**2.3**	**1.3**	**1.5**	**1.0**	**0.8**	**1.4**				
	Spirochaetaceae	*Treponema*	RAP	1.3	1.1	1.8	1.4	1.4	0.8	0.7	1.3	1.2	1.3	0.30	0.45	0.04	0.48
			GRAD	1.8	1.4	1.5	2.3	2.0	1.2	1.4	1.0	0.8	1.4				
Others[5] (sum of all)			**RAP**	**5.3**	**4.5**	**4.1**	**4.7**	**2.9**	**3.5**	**2.8**	**2.3**	**3.1**	**2.3**	**0.36**	**0.16**	**<0.01**	**0.37**
			GRAD	**6.3**	**4.8**	**5.0**	**4.5**	**4.3**	**3.5**	**3.1**	**2.8**	**3.0**	**2.8**				

[1]Bacterial taxa with a relative abundance of > 1.0% in > 20.0% of the samples at the genus or family rank were considered 'major' taxa and displayed. Taxa summarized at the order level include all the major taxa displayed at family or genus level as well as all those not displayed individually.

[2]Sampling day relative to calving.

[3]T = treatment, *P*-value for treatment period (d 9 to 80); D = sampling day, *P*-value for pretreatment and treatment period (d −50 to 80); T × D, *P*-value for treatment period (d 9 to 80).

[4]Standard error for LSM, pooled by sampling day; n = 12.

[5]Summarizes the abundance of all bacterial taxa at the order level when not listed above.

174

Rumen Protozoal and Archaeal Community Composition

Rumen protozoal communities were more diverse during the dry period (10 d ap) compared with the lactation period (16 and 44 d pp; Figure 4). *Epidinium* and *Ostracodinium* were the dominant protozoal genera during the dry period with a relative abundance of 40.7 and 21.9%, respectively. The relative abundances of *Eudiplodinium* (11.0%), *Anaplodinium-Diplodinium* (9.7%), and the holotrich *Isotricha-Dasytricha* (8.3%) were similar to each other at 10 d ap. During lactation the relative abundance of *Epidinium* increased ($P < 0.01$), dominating the protozoal community with an average relative abundance of 68.5% at 44 d pp. Likewise, *Entodinium* increased relative to the dry period ($P < 0.01$) to 16.3% at 44 d pp. In contrast, *Ostracodinium*, *Eudiplodinium*, *Anaplodinium-Diplodinium*, and *Eremoplastron-Diploplastron* all decreased ($P < 0.01$) during lactation to relative abundancies between 1.4 to 3.3% at 44 d pp.
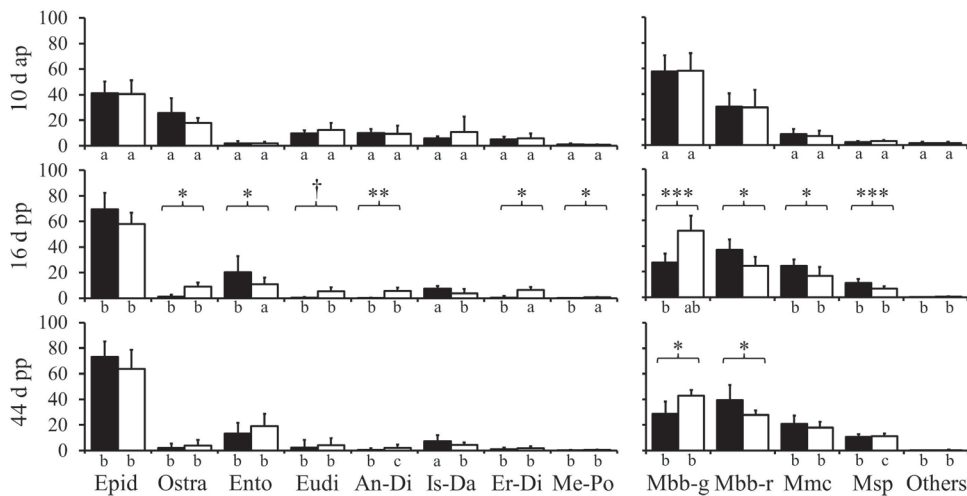
Figure 4. Least squares means of relative abundances of rumen protozoal (left chart) and archaeal (right chart) community members expressed as a fraction (%) of the total community during the pretreatment period (10 d antepartum) and the treatment period (16 and 44 d postpartum) with a rapid (RAP; 1.0 kg of DM/d; black bars) and gradual (GRAD; 0.25 kg of DM/d; white bars) rate of increase of concentrate allowance postpartum. Abbreviations: Epid, *Epidinium*; Ostra, *Ostracodinium*; Ento, *Endodinium* spp.; Eudi, *Eudiplodinium*; An-Di, *Anaplodinium-Diplodinium*; Is-Da, *Isotricha-Dasytricha*; Er-Di, *Eremoplastron-Diploplastron*; Me-Po, *Metadinium-Polyplastron*; Mbb-g, *Methanobrevibacter gottschalkii* clade; Mbb-r, *Methanobrevibacter ruminantium* clade; Mmc, *Methanomassiliicoccaceae*; Msp, *Methanosphaera*; Others, sum of remaining archaeal taxa. †$P < 0.10$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, significance of difference in LSM of RAP or GRAD for the same sampling day and taxon; [abc]Indicates differences between days within treatment group, $P \leq 0.05$; error bars represent standard deviations.

175

An interaction between sampling day and treatment was observed for a number of protozoal genera. The relative abundance of *Ostracodinium*, *Eudiplodinium*, *Anaplodinium-Diplodinium*, *Eremoplastron-Diploplastron*, and *Metaplodinium-Polyplastron* at 16 d pp was lower in RAP compared with GRAD. In contrast, the relative abundance of *Entodinium* was greater in RAP than GRAD by 16 d pp. By 44 d pp no more differences were found in the relative abundance of the protozoal community members between treatments.

The relative abundance of the *Methanobrevibacter* (**Mbb.**) *gottschalkii* (57.8%) and *ruminantium* (29.8%) clades dominated the archaeal community at 10 d ap (Figure 4). In contrast to the relative abundance of *Mbb. ruminantium*, which did not change throughout the experiment (P = 0.63), that of *Mbb. gottschalkii* decreased during the lactation (P < 0.01) in particular with RAP for which an interaction (P = 0.02) between sampling day and treatment was observed. The relative abundances of *Methanomassiliicoccaceae* and *Methanosphaera* increased (P < 0.01) from 7.8 and 2.8% at 10 d ap to 19.5 and 11.0% at 44 d pp, respectively. For all these taxa an interaction between treatment and sampling day was observed ($P \leq$ 0.04). During the dry period no differences were observed between the treatment groups. At 16 d pp, the relative abundance of *Mbb. gottschalkii* was lower, and that of *Mbb. ruminantium*, *Methanomassiliicoccaceae* and *Methanosphaera* higher with RAP than GRAD. By 44 d pp differences between RAP and GRAD were only observed for *Mbb. gottschalkii* and *ruminantium*.

In Situ Fractional Degradation Rate

The fractional degradation rates of starch, NDF, CP, and OM (Table 2) were neither affected by starting day of the in situ incubation ($P \geq$ 0.18) nor by treatment ($P \geq$ 0.24), and no interaction was observed between both factors ($P \geq$ 0.12). The k_d of starch tended to be higher during lactation compared with the dry period (P = 0.07). A similar numerical change is suggested by the k_d of CP (P = 0.12), whereas no such change is suggested by the k_d of NDF and OM.

Table 2. Fractional degradation rate (k_d) of starch, NDF, CP, and OM determined with nylon bag incubations of soybean hulls and corn, during the pretreatment (-14 d relative to calving) and treatment period (18, 46, and 62 d relative to calving) with a rapid (RAP; 1.0 kg of DM/d; n = 3) and a gradual (GRAD; 0.25 kg of DM/d; n = 3) rate of increase of concentrate allowance postpartum

Item	Sampling day relative to calving[1]				SE[3]	Fixed effects[2]		
	-14	18	46	62		T	D	T × D
k_d of starch[4], /h								
RAP	0.046	0.065	0.064	0.049	0.0059	0.24	0.19	0.19
GRAD	0.054	0.054	0.076	0.075				
k_d of NDF[5], /h								
RAP	0.023	0.014	0.024	0.013	0.0035	0.48	0.18	0.64
GRAD	0.029	0.018	0.022	0.021				
k_d of CP[6], /h								
RAP	0.023	0.039	0.031	0.033	0.0047	0.47	0.30	0.12
GRAD	0.031	0.024	0.051	0.042				
k_d of OM[7], /h								
RAP	0.029	0.028	0.033	0.021	0.0042	0.32	0.52	0.46
GRAD	0.034	0.026	0.037	0.036				

[1]Day of start of incubation.
[2]T = Treatment, *P*-value for treatment period (d 18 to 62); D = Day of start of incubation, *P*-value for pretreatment and treatment period (d -14 to 62); T × D, *P*-value for treatment period (d 18 to 62).
[3]Standard error for LSM, pooled by sampling day; n = 6.
[4]Starch: washout (W)-fraction 0.25 ± 0.01.
[5]NDF: lag, 2.4 ± 1.13 h; undegradable (U)-fraction, 0.03 ± 0.003.
[6]CP: W-fraction, 0.19 ± 0.01, U-fraction, 0.05 ± 0.037.
[7]OM: W-fraction 0.16 ± 0.06, U-fraction, 0.02 ± 0.006.

Chapter 6

DISCUSSION

Ration Effects on Bacterial Community Richness

Richness of the bacterial community is known to fluctuate throughout the lactation cycle (Jewell et al., 2015), and older cows have been reported to host a richer and more diverse microbiota than heifers (Lima et al., 2015). The first weeks after calving coincide with a large increase in DMI and in increase in dietary FOM content, which is typically achieved by an inclusion of grains or pelleted concentrates in the ration. By comparing the bacterial community at 7 d ap and 7 d pp, Lima et al. (2015) showed that richness and diversity decreased after calving, both in primiparous and multiparous cows. However, Wang et al. (2012) did not observe changes in richness and diversity during the transition period in samples taken at weekly intervals from 21 d ap to 21 d pp. In the present study, we observed a sharp but temporary decrease in richness early postpartum with the rapid increase in concentrate allowance. Similar to Lima et al. (2015), this change in composition occurred rapidly, indicating little time is required for bacterial community adaption to manifest. Although the decrease in richness was much less pronounced with a gradual increase in concentrate allowance, the richness during lactation was still lower compared with the richness in the dry period.

The decrease in richness with a rapid increase in concentrate allowance (RAP) was not associated with an overgrowth of the rumen bacterial community by a small number of taxa as evidenced by the relative abundances of the major bacterial taxa. This suggests that a range of bacterial taxa can capitalize on the rapid increase in readily fermentable OM, thereby reducing the relative abundance of other taxa below detection limit. With a gradual increase in concentrate allowance (GRAD), changes were more gradual especially after 9 d pp, allowing the minor species to maintain their presence at detectable levels. The recovery in richness might then be explained by the cessation of rapid concentrate build-up after 16 d pp which might have removed the competitive advantage.

Simultaneous with the increase in readily fermentable OM, changes in the rumen fluid might further impact the relative abundance of taxa, both positively and negatively. As reported by Dieho et al. (2016b), the greater intake of FOM, starch, and sugar in RAP compared with GRAD at 16 d pp increased daily VFA production, particularly production of propionic acid, and resulted in a decrease in pH, especially the time rumen fluid pH was below 5.8. At 44 d pp, average rumen fluid pH was similar for RAP and GRAD, and also the time rumen fluid pH was below 5.8 did not differ. This suggests that cows in RAP had the capacity to limit further decreases in pH, which might also be advantageous in the recovery of bacterial richness.

Alternatively, it can be hypothesized that the driving force behind the differences in richness between RAP and GRAD at 9 and 16 d pp is the difference in daily FOM intake, in particular that of starch and sugar intake, rather than the actual amount of FOM consumed. If the absolute level of FOM, starch, and sugar intake drives the decrease in richness, a continued decreased richness would be expected for RAP after 16 d pp. In addition, a gradual decrease in richness for GRAD at d 44 to the same reduced level of bacterial richness as in RAP at d 16 should be observed, as intake of the rapidly fermentable OM at d 44 in GRAD is equal to that at d 16 in RAP. However, such decreases in richness were not observed. Richness actually recovered after 16 d pp in RAP and only a small decline in richness in GRAD up to d 44 occurred. Therefore it can be suggested that a rapid (in several days only) increase of intake of readily fermentable OM to a level above a certain threshold, and not the daily intake level as such, is required to decrease bacterial richness.

Ration Effects on the Relative Abundance of Bacterial Community Members

With a few exceptions discussed in more detail below, most of the major taxa only showed rather low association scores (-0.45 < association < 0.45) between their relative abundance and measures of daily feed intake, ration composition and rumen fluid composition, indicating limited effect of DMI and ration composition (Figure 5). The genera *Acetitomaculum* and *Prevotella* showed a strong positive association with DMI and the fractions of FOM, starch, sugar, and CP in the rations, reflecting the increase in their relative abundance after calving. As shown by Dieho et al. (2016b), treatment RAP resulted in a temporary peak in the production rate of propionate. This was reflected by a higher molar proportion and concentration of propionate in the rumen fluid. In turn, this has a strong positive association with the abundance of *Prevotella*, unclassified *Succinivibrionaceae*, and *Oribacterium*, which are known producers of propionate (Stewart et al., 1997). In contrast, the RC9 gut group, unclassified *Christensenellaceae*, and to lesser degree *Fibrobacter* and other unclassified *Fibrobacteraceae*, unclassified *Ruminococcaceae*, and *Saccharofermentans* showed a strong positive association with the fraction of NDF in the ration, reflecting their higher relative abundance during the dry period. *Fibrobacter* and the *Ruminococcaceae* are known cellulose degraders and the abundance of substrate, fibrous quality of the feed, low fractional passage rates, and higher ruminal pH likely all favor their presence during the dry period (Stewart et al., 1997; Ransom-Jones et al., 2012). This also explains their strong positive association with the molar proportion of acetate and negative association with the molar proportion of propionate. Despite their abundance, little is known about the RC9 gut group or the *Rikenellaceae* and their function is unclear, warranting further exploration of these taxa.

Chapter 6

179

In comparison to some studies, where bacterial communities were dominated by *Bacteroidales*, and specifically its genus *Prevotella* (Jami et al., 2013; Pitta et al., 2014), in the present study multiple orders of bacteria have a sizable relative abundance. Such more diverse bacterial communities appear to be more common globally (Henderson et al., 2015), and is likewise reported by Wang et al. (2012), Petri et al. (2013) and Lima et al. (2015). Nevertheless, *Prevotella* also had the highest relative abundance during lactation in the present study, as is typically observed.
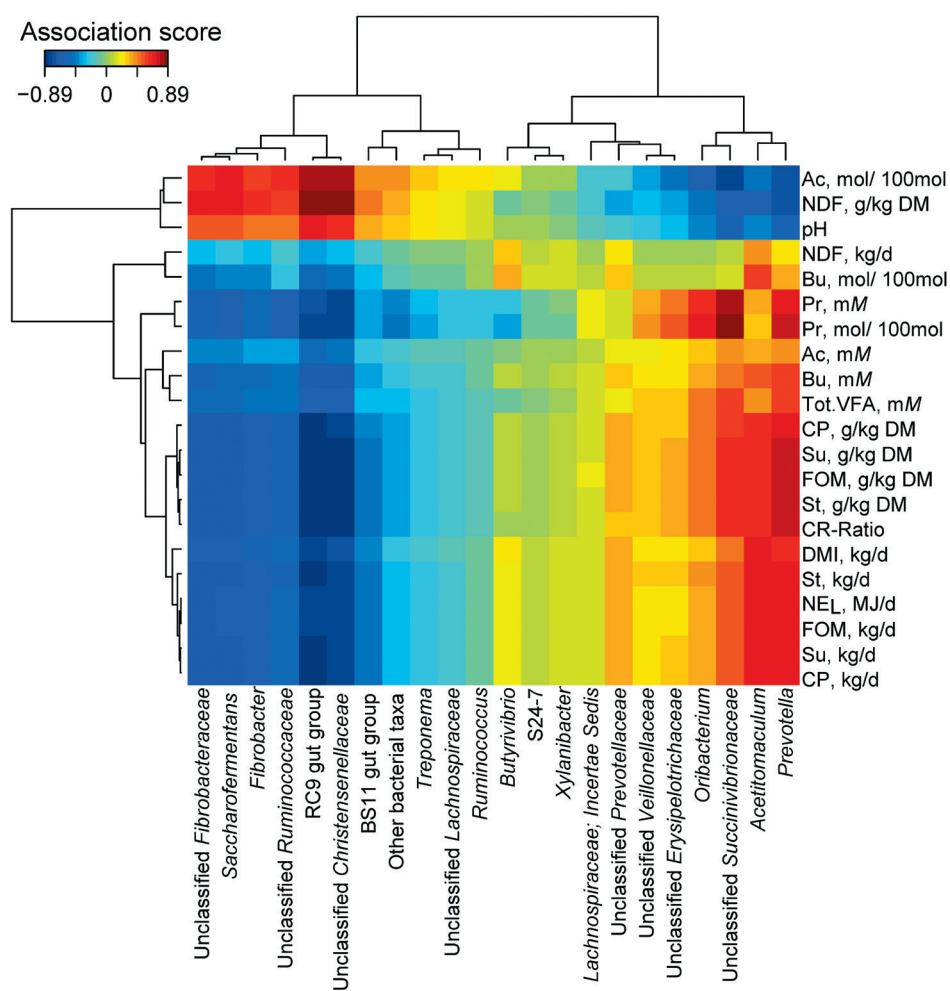
Figure 5. Heatmap of the associations between bacterial community composition and rumen fluid composition and feed intake variables using regularized canonical correlation analysis. Abbreviations: Ac, acetic acid; Pr, propionic acid; Bu, butyric acid; CR-Ratio, concentrate to roughage ratio; FOM, fermentable organic matter; St, Starch; Su, Sugar.

Despite the clear effect of the concentrate treatment on the richness of the rumen bacterial community, at order level only the relative abundance of *Erysipelotrichales* was affected by concentrate treatment. Similarly, at family and genus level the relative abundance of only a few of the major taxa was affected by the interaction between sampling day and treatment. This contrasts with the effect of the ration changes associated with transition from the dry period to lactation on the relative abundance of these taxa. Due to the similarity of a large part of the rumen bacterial community in RAP and GRAD, differences in capacity for degradation are also likely limited. Indeed, no effect of treatment or of period by treatment interaction on fractional degradation rates of several substrates was observed. The rapid changes in relative abundance of *Prevotella*, the RC9 gut group and unclassified *Christensenellaceae* with onset of feeding the lactation ration suggests that the bacterial community can adapt quickly.

Changes in Rumen Protozoal and Archaeal Communities

The dominance of *Epidinium* during lactation is unusual, as often *Entodinium* is found to be most abundant in both forage and concentrate dominated rations (Hristov et al., 2001; Henderson et al., 2015). Whereas both genera have similar growth rates in vitro (Dehority, 2003), their respective capacities for degradation of substrates differ. Members of *Entodinium* are known to be able to rapidly engulf starch particles (Williams and Coleman, 1992). The increase in their relative abundance after calving, and higher abundance in RAP at 16 d pp, likely reflects their capacity to capitalize on the increased fractions of starch in the rations. *Epidinium* likewise engulfs starch, albeit more slowly than *Entodinium* (Williams and Coleman, 1992), but in contrast to *Entodinium*, *Epidinium* has a high cellulase activity and actively invades plant tissues. In addition, *Epidinium* degrades pectins, hemicelluloses, and xylans (non-starch polysaccharides) faster than *Entodinium* (Williams and Coleman, 1992). Several of the concentrate ingredients (citrus pulp, sugar beet pulp) contain a substantial amount of non-starch polysaccharides (and a relatively small amount of NDF). *Epidinium* can capitalize on the pp increase in the fractions of starch, pectins, hemicelluloses, and xylans, explaining its increase despite the decrease in the fraction of cellulose. However, the lower rumen fluid pH coinciding with RAP (Dieho et al., 2016b), which likely negatively affects *Epidinium* more than *Entodinium* (Williams and Coleman, 1992), might explain the lack of a treatment effect at 16 d pp. The capacity of *Epidinium* to invade plant tissue might partly explain the overall relatively high abundance of *Epidinium* as both rumen fluid and feed particles were sampled in the present study. A major part of the samples collected by Henderson et al. (2015) likewise consisted of whole rumen contents, but they did not observe a majority of *Epidinium* in the protozoal community. The relatively high abundance of *Ostracodinium* as observed

during the dry period with forage dominated rations, and low relative abundance in concentrate dominated rations corresponds with earlier reports (Hristov et al., 2001; Henderson et al., 2015). Like *Epidinium*, *Ostracodinium* has a large capacity to degrade cellulose, explaining its relatively high abundance during the dry period where there was an abundance of substrate and a relatively high rumen fluid pH, and similarly explains the significantly higher abundance at 16 pp with RAP compared with GRAD. The near disappearance of *Ostracodinium* after calving, in particular at d 44 pp, can be associated with the increase in fermentation rate of the ration and the concomitant decrease in pH (Goad et al., 1998; Hristov et al., 2001).

In line with previous studies (e.g., Henderson et al., 2015), in the present study rumen archaea were found to be much less diverse than rumen bacteria. This likely reflects the narrow range of substrates they use. The dominance of the hydrogen utilizers *Mbb. gottschalkii* and *Mbb. ruminantium* clades within the archaeal community is commonly observed (Janssen and Kirs, 2008; Henderson et al., 2015). The decrease in the relative abundance of *Mbb. gottschalkii* likely represents a greater sensitivity to the changes in feed intake and rumen environment (pH, H_2 concentration), particularly in RAP up to 16 d pp. However, after having reached the maximum concentrate allowance, ration changes in RAP were minimal, and differences in rumen fluid VFA concentration and pH between RAP and GRAD disappeared. This suggest that the selective pressure on both clades might be similar from 16 d pp onwards, resulting in an apparent persistence of the treatment effect. The postpartum increase in methylotrophic *Methanomassiliicoccaceae* and treatment effect at 16 d pp is likely the result of the concentrate intake during lactation. These archaea metabolize methylamines (Poulsen et al., 2013), which increase in concentration with an increased intake of grains (Ametaj et al., 2010).

Functional Changes in the Rumen Microbiota

From a rumen function perspective, it is important to evaluate whether the loss of richness and changes in the relative abundance of individual taxa during the first weeks of lactation also affected the degradation of rumen substrates. The concept of a functional redundant rumen microbiome (Weimer, 2015), together the present observation of a continuous sizable relative abundance of many of the major bacterial taxa suggests that the microbiota community had a continuous capacity to degrade a wide range of substrates throughout the whole experimental period. Predicting the degradation capacity of a microbiota community based on its composition remains difficult as the metabolic capabilities have not been assessed in culture for most of the microbiota (Creevey et al., 2014). Therefore in situ incubations were used to assess

the degradation capacity in the present study. Ground corn and soybean hulls were used as substrate for in situ incubations for their high starch and NDF contents, respectively. However, CP content was low in both substrates (97 and 118 g/kg of DM, respectively). Consequently, the relatively small amounts of substrate incubated might have been affected by contamination with microbial protein (Vanzant et al., 1998). The fractional degradation rates observed for CP should therefore be treated with caution. The fractional degradation rates of starch are comparable to the 5.5%/h for ground corn reported by Offner et al. (2003). The wash-out fraction was somewhat smaller than that observed by Offner et al. (2003), but differences in sample processing (screen size used for grinding) and washing technique are likely factors that may explain this difference (Vanzant et al., 1998).

The digestion of NDF has been shown to be reduced at lower pH values (Calsamiglia et al., 2008). Likewise, the rate of degradation was found to be lower at a higher concentrate inclusion rate which coincided with a lower pH (Stensig and Robinson, 1997). The numerically lower NDF degradation rate at 18 d pp compared with the dry period may reflect an effect of the reduced rumen pH in RAP in the present study. However, the lack of a clear lactation or treatment effect on the fractional degradation rate of OM, NDF, and starch in the nylon bags suggests that functionally the microbiota communities did not differ throughout the whole experiment. This might indicate that the composition of the microbiota of lactating cows on high concentrate ration (approximately 60%) enables it to degrade the selected substrates in a lower pH and higher VFA concentration environment in vivo at a similar rate as cow on a forage dominated ration during the dry period with a high pH and low VFA concentration environment. Alternatively, the variation in in situ incubation results may have been too large to establish a treatment or lactation stage effect. Overall, in the present experiment the loss in bacterial richness and the changes in relative abundance of individual taxa in response to dietary changes were not associated with changes in rumen degradation kinetics.

Chapter 6

CONCLUSIONS

Richness of the rumen bacterial community decreased during the transition from the dry period to lactation. Furthermore, a rapid increase in concentrate allowance temporarily decreased richness of the bacterial community compared with a gradual increase to the same maximum daily concentrate allowance. Principle coordinate analysis and changes in the relative abundances of bacterial taxa show that adaptation of the community composition to changes in the ration occurs rapidly. The relative abundances of most major bacterial taxa were affected by the transition to lactation but few were affected by the rate of increase of the concentrate allowance. The relative abundances of rumen protozoal taxa changed after calving, and were affected by the concentrate treatment. However, differences between treatments groups disappeared again when concentrate intake became similar. The archaeal community was likewise affected by both the transition to lactation and the treatment. The observed changes in rumen microbiota composition including changes in bacterial community richness did not appear to affect the fractional degradation rate of NDF, starch, CP, and OM measured in situ using a nylon bag technique.

ACKNOWLEGDMENTS

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Chapter 6

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SUPPLEMENTAL METHODS S1

DNA Extraction for Profiling Rumen Bacterial Populations

Using a method adapted from Zoetendal et al. (2006) and van den Bogert et al. (2013), DNA was extracted. In short, 50 mg freeze dried rumen content was mixed in a microfuge tube containing 180 mg macaloid suspension (Zoetendal et al., 2006), 0.1 mm zirconium beads, and 50 µL 10% SDS (Invitrogen, Carlsbad, CA). The solution was mixed with 500 µL acid phenol (Invitrogen, Thermo Fisher Scientific, Waltham, MA), followed by three Fastprep (Bertin Technologies, Montigny le Bretonneux, France) treatments at 5.5 m/s for 45 s with cooling on ice for 90 s between treatments. The sample was centrifuged at 13,400 *g* at 4°C for 15 min, after which the nucleic acids in the aqueous phase were purified by consecutive extraction with phenol:chloroform:isoamylalcohol (25:24:1) and chloroform:isoamylalcohol (24:1). Phases were separated by centrifugation (13,400 *g* at 4°C, 5 min) using Phase Lock Gel tubes (5 Prime, Hamburg, Germany). Subsequently, 300 µL of the aqueous phase was treated with 3 µL RNAse A (10 mg/mL; Qiagen GmbH, Hilden, Germany) and incubated at 37°C for 15 min. Subsequent steps employed a modified version of the QIAamp DNA Stool Mini Kit protocol: 22.5 µL proteinase K (20 mg/mL; Ambion, Waltham, MA) and 300 µL buffer AL were added to the sample followed by incubation at 70°C for 10 min. After addition of 300 µL ethanol (VWR, Amsterdam, The Netherlands), the sample was transferred to a QIAamp column and centrifuged (13,000 *g*, 1 min). DNA pellets were washed with AW1 and AW2 buffer according to manufacturer's instructions. Total DNA was eluted in 30 µL Nuclease Free Water (Promega, Leiden, Netherlands). DNA quantity and quality were assessed using NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, PA) and electrophoresis on a 1% (w/v) agarose gel.

Profiling Rumen Bacterial Populations

For 16S rRNA gene based microbial composition profiling, barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated using a 2-step PCR strategy that reduces the impact of barcoded primers on the outcome of microbial profiling (Berry et al., 2011). The first PCR was performed in a total volume of 50 µl containing 1 × HF buffer (Finnzymes, Vantaa, Finland), 1 µl dNTP Mix (10 m*M*; Promega, Leiden, The Netherlands), 1 U of Phusion Hot Start II High-Fidelity DNA polymerase (Fynnzymes, Thermo Fisher Scientific, Waltham, MA), 500 nM of the 27F-DegS primer (van den Bogert et al., 2011; van den Bogert et al., 2013) that was appended with Universal Tag (UniTag) 1 at the 5' end, 500 nM of an equimolar mix of two reverse primers [338R I

and II (van den Bogert et al., 2011) based on three previously published probes (EUB 338 I, II and III (Guss et al., 2011)], that were 5'-extended with UniTag 2 (Table S1), and 0.2-0.4 ng/µl of template DNA. The sequence of the UniTags were selected to have a GC content of ~66%, minimal tendency to form secondary structures including hairpin loops, heterodimers, and homodimers; analyzed by the IDTDNA Oligoanalyzer 3.1 (Integrated DNA Technologies, Leuven, Belgium), no matches in 16S rRNA gene databases [based on results of the 'TestProbe' tool offered by the SILVA rRNA database project (Quast et al., 2013) using the SSU r117 database], and no perfect matches in genome databases based on results of the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), which uses the Primer3 program (Rozen and Skaletsky, 1999) using 'Genomes (chromosomes from all organisms)' from all Bacteria available on July 8th, 2014 as a reference database. Appending the UniTag sequences on the 5' end of the 16S rRNA gene targeted primers creates target sites for incorporation of sample specific barcodes during the second PCR using primers targeted to the UniTag sequences. PCRs were performed with a SensoQuest Labcycler (SensoQuest, Göttingen, Germany) using an amplification program that consisted of an initial denaturation at 98°C for 30 s, 25 cycles of: denaturation at 98°C for 10 s, annealing at 56°C for 20 s and elongation at 72°C for 20 s, and a final extension at 72°C for 10 min (van den Bogert et al., 2013). The size of the PCR products (~380 bp) was confirmed by agarose gel electrophoresis using 5 µL of the amplification-reaction mixture on a 2% (w/v) agarose gel containing 1 × SYBR Safe (Invitrogen, Carlsbad, CA). The second PCR was performed in a total volume of 100 µl containing 1 × HF buffer, dNTP Mix 2 U of Phusion Hot Start II High-Fidelity DNA polymerase, 500 nM of a forward and reverse primer equivalent to the Unitag1 and UniTag2 sequences, respectively, that were each appended with an 8 nucleotide sample specific barcode (Ramiro-Garcia et al., 2016) at the 5' end.

Testing of different volumes of PCR product from the first PCR as template source for the second PCR revealed that 5 µL PCR product of the first reaction was sufficient to generate PCR product for further analysis (data not shown) employing the same amplification program as above, but with an annealing temperature set to 52°C and a reduced number of 5 cycles.

Incorporation of the sample specific barcodes, yielding a PCR product of ~400 bp, was confirmed by gel electrophoresis using 5 µL of the amplification-reaction mixture on a 2% (w/v) agarose gel containing 1 × SYBR Safe (Invitrogen, Carlsbad, CA). Control PCR reactions were performed alongside each separate amplification without addition of template, and consistently yielded no product.

PCR products were purified with HighPrepTM (Magbio Genomics, Rockville, MD) using 20 µl Nuclease Free Water (Promega) for elution and quantified using a Qubit in combination with the dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). Purified

PCR products were mixed in equimolar amounts. Pools underwent adaptor ligation followed by sequencing on the MiSeq platform with addition of 20% PhiX (GATC-Biotech, Konstanz, Germany). The 16S rRNA gene sequencing data was analyzed using NG-Tax (Ramiro-Garcia, et al., 2016). Paired-end libraries were filtered to contain only read pairs with perfectly matching barcodes, and those barcodes were used to de-multiplex reads by sample. Finally operational taxonomic units (OTUs) were defined using an open reference approach, and taxonomy was assigned to those OTUs using a SILVA 16S rRNA gene reference database (Quast et al., 2013). Microbial composition plots were generated using a workflow based on QIIME (v1.2, Caporaso et al., 2010).

Table S1. Adaptors and primers used in this study

Primer[a]	Primer sequence (5'-3')[b]	Reference
UniTag1-27F-DegS	*gagccgtagccagtctgc-*GTTYGATYMTGGCTCAG[c]	van den Bogert et al., 2011
UniTag2-338R-I	*gccgtgaccgtgacatcg-*GCWGCCTCCCGTAGGAGT[c]	Daims et al., 1999
UniTag2-338R-II	*gccgtgaccgtgacatcg-*GCWGCCACCCGTAGGTGT[c]	Daims et al., 1999

[a]Primer names may not correspond to original publication.
[b]M = A or C; R = A or G; W = A or T; Y = C or T.
[c]UniTag sequences are indicated in lower case italic.

Chapter 6

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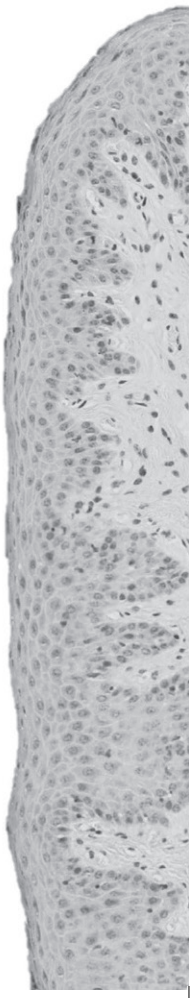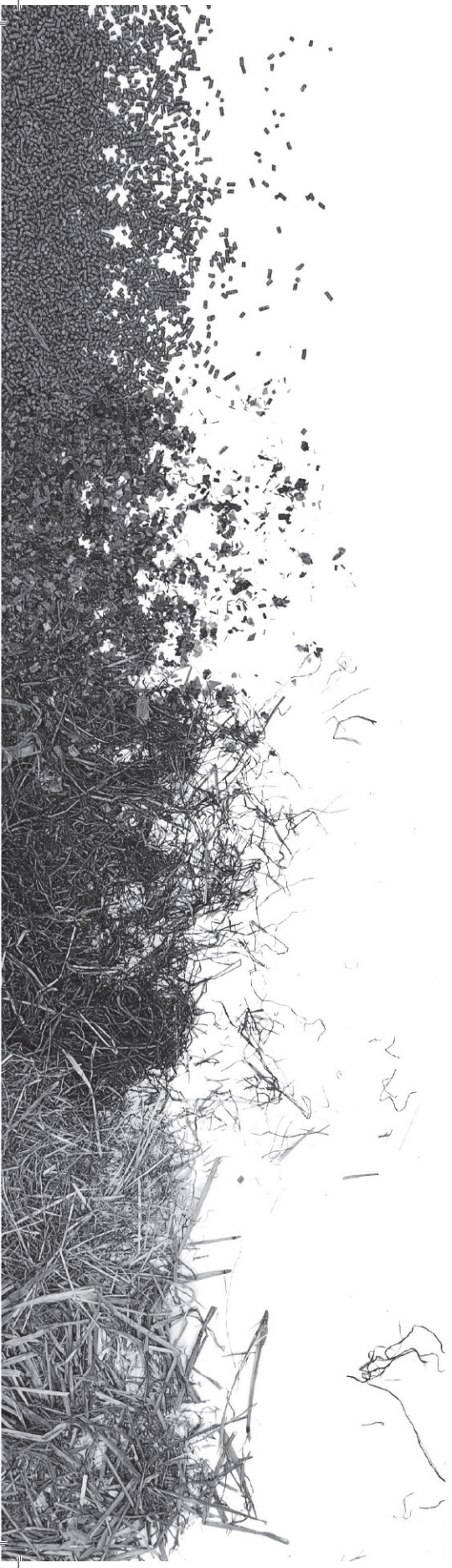
Chapter 7

General discussion
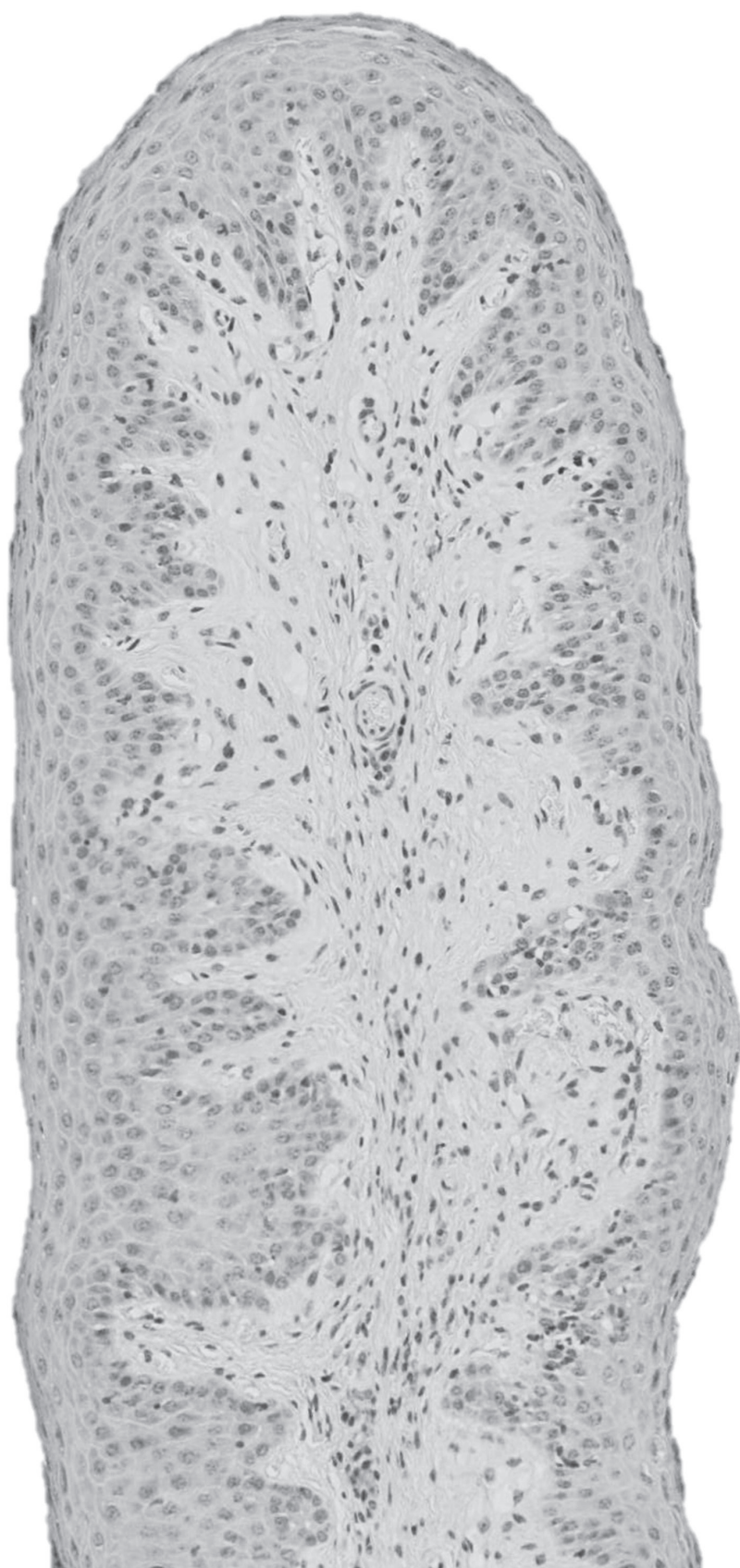
INTRODUCTION

To date, knowledge of the adaptation of the rumen in dairy cattle to the physiological and nutritional changes during the late dry period and early lactation was scarce (Chapter 1). With this thesis, by presenting a unique set of observations on multiple aspects of rumen adaptation, a major step has been taken filling this knowledge gap. Further understanding of these adaptation processes may ultimately improve our management of the transition dairy cow.

The aim of this chapter is firstly to discuss rumen adaptation based on both experiments described in this thesis. Secondly, measurement of the volatile fatty acid (**VFA**) absorption as affected by method (natural filled rumen and buffer incubation in empty washed rumen, respectively) and experimental design is discussed. Thirdly, variation between individual animals in VFA absorption capacity and its effects on the rumen environment is discussed. Finally, general conclusions are presented and some recommendations for further research and the implications of the work in this thesis are provided.

ASPECTS OF RUMEN ADAPTATION IN DAIRY CATTLE

The central hypothesis of the present thesis was that the daily intake of fermentable organic matter (**FOM**) in dairy cattle is positively related to the volatile fatty acid production, thereby affecting rumen papillae morphology and thus the capacity for VFA absorption and metabolism (Chapter 1). As hypothesized, the increase in daily FOM intake after calving was found to increase daily VFA production (Chapter 3), papillae surface area (Chapter 2, 4), and fractional VFA absorption rate (k_a**VFA**; Chapter 3, 4). Moreover, as hypothesized, a greater FOM intake during early lactation due to a rapid instead of gradual rate of increase of concentrate allowance (lactation experiment) was found to numerically increase total VFA production, increase propionate production and coincide with a greater papillae surface area (Chapter 2) but not a greater k_aVFA (Chapter 3). Similarly, it was hypothesized that feeding a supplement of concentrate during the last 4 weeks of the dry period (dry period experiment) would increase FOM intake and thereby the papillae surface area and k_aVFA. The concentrate supplement resulted in a numerical increase in FOM intake only, but did increase papillae surface area (Chapter 4). Again, contrary to the hypothesis, k_aVFA was not affected during the dry period by feeding concentrate (Chapter 4). Furthermore, the expression of genes at the mRNA and protein level associated with VFA absorption, metabolism and maintenance of intracellular pH was hypothesized to increase with greater FOM intake, thereby enhancing the capacity for VFA absorption of the epithelium covering the papillae. In

Chapter 7

contrast to the hypothesis, gene mRNA expression was generally not found to increase with greater FOM intake during the transition into lactation, or the concentrate treatments (Chapter 5). Protein expression was generally affected by the transition into lactation, but contrary to the hypothesis greater FOM intake did not consistently increase protein expression. Expression of some proteins was found to be affected by the treatment during the lactation experiment (Chapter 5). Finally, the changes in ration composition and dry matter intake, resulting in the changes in FOM intake, were hypothesized and found to induce changes in the rumen microbiota (Chapter 6).

VFA Driving Rumen Morphological Adaptation

The importance of ruminal VFA production for the development of rumen papillae in juveniles is beyond doubt (Chapter 1). The observations from the present experiments and earlier studies (Dirksen et al., 1984; Liebich et al., 1987) strongly support the idea that, in general, FOM intake and therefore daily production of VFA (Chapter 3; Sutton et al., 2003; France and Dijkstra, 2005) is also the main driving force for the development of the rumen papillae in the adult ruminant. However, several observations in the present thesis suggest that not only FOM intake and total VFA production as such, but also qualitative changes in ration composition (due to differences in the fraction of concentrate) and the associated changes in rumen fluid VFA concentration and VFA profile may influence rumen papillae proliferation. Two examples of such qualitative changes are, firstly, that during the dry period experiment the supplemental concentrate did not affect daily FOM intake but did result in a changed ration composition and increased total VFA concentration (Chapter 4). Secondly, during the lactation experiment the rapid concentrate treatment resulted in a greater FOM intake at 16 d postpartum (**pp**), resulting in a numerical increase in total daily VFA production and a greater daily propionate production compared with the gradual concentrate treatment.

Potential differences in the role of acetic (**Ac**), propionic (**Pr**), and butyric (**Bu**) acid in promoting papillae development have been a matter of debate for several decades. Often, Bu is credited with special proliferative properties and is frequently used in treatments in rumen studies (Mentschel et al., 2001; Shen et al., 2005; Malhi et al., 2013; Kowalski et al., 2015). Papers on the special proliferative effects of Bu often refer to the work of Sakata and Tamate (1978; 1979). They reported that signs of proliferation, based on the mitotic index, appeared stronger with Bu than with Ac and Pr. However it was also reported by Sakata and Tamate (1978; 1979) that all three major VFA stimulated proliferation. More recently, Mentschel et al. (2001) reported similar increases in mitosis rate with Pr and Bu, but found lower apoptosis rates with Bu

than Pr in the examined rumen papillae. Conversely, Shen et al. (2005) found increased rumen papillae proliferation when supplementing Bu but also a large increase in dead and dying cells in the rumen epithelium. Interpretation of these results is complicated by the fact that the effect of Bu is generally not studied independently of the effect of the total amount of VFA supplied. In addition, Bu is often elevated to un-physiologically high levels. Thus whereas generally positive effects of Bu supplementation on papillae development are reported, such effects might also be induced with supplementation of Ac or Pr. Indeed, Suárez et al. (2006) using principle component analysis found that in calves the three major VFA had similar loadings, indicating they all had a similar stimulating effect on papillae development.

In the lactation experiment (Chapter 3) it is shown that a numerically greater total VFA production rate, and greater production rate of Pr but not of Bu, coincided with faster papillae growth at 16 d pp. Of the dry period experiment (Chapter 4), no VFA production rates have been reported. However, the numerically greater FOM intake due to feeding the supplemental concentrate during the dry period and observations on the VFA yield per kg of FOM (~10 mol/ kg FOM; Chapter 3) suggest that VFA production rate was up to 25% greater at 18 d antepartum (**ap**). Whereas this had little effect on the molar proportions of Ac, Pr, and Bu, it clearly increased papillae surface area. Based on Mentschel et al. (2001), a dominant role of Bu in tissue proliferation suggests an increase in apoptosis inhibiting genes and a decrease in apoptosis stimulating genes. However, this was not observed during the dry period or the lactation period experiments (Chapter 5). Although it was not the aim of the present thesis to further examine the role of individual VFA types in the rumen adaptation process, these data indicate that the importance of Bu may be overrated and trials are needed which are designed to compare any differential effects of each of the major VFA on papillae morphology.

Macroscopic Adaptation of the Rumen Papillae

The data presented in this thesis show that during both the dry period as well as during the subsequent lactation, papillae surface area is remarkably sensitive to changes in daily FOM intake and daily VFA production. In earlier work in dairy cattle (Andersen et al., 1999; Rabelo et al., 2001; and Reynolds et al., 2004) no such treatment or lactation stage effects were found. As discussed in Chapter 4, using a repeated measurements approach instead of end-point measurements is a methodological difference which might explain these apparently different outcomes. The choice for repeated measurements was based on the work of Dirksen et al. (1984), Liebich et al. (1987), and Bannink et al. (2012) who also successfully used this approach to study changes in papillae morphology.

Chapter 7

Total rumen surface area (total number of papillae in the rumen × average papillae surface area) could not be measured or estimated during the experiments. Obviously, papillae density (number of papillae per unit of surface area) contributes to the total surface area of the rumen wall but density does not appear to be affected by daily FOM intake in adolescent animals and feedlot steers (Lane and Jesse, 1997; Schurmann et al., 2014; Kern et al., 2016). Similarly, in dairy cows during the transition period no differences in papillae density were found, although after calving papillae density showed a numerical decrease (Reynolds et al., 2004). This numerical decrease was associated with an increase in total rumen weight and increased weight of the rumen contents, and might be explained by increased spacing of papillae due to stretching of the rumen wall (Reynolds et al., 2004). These findings imply that development of new papillae is limited. The fraction of the rumen wall covered by papillae also contributes to total rumen surface area but could also not be measured or estimated during the experiments. However, judged by the dorsal limit of papillae coverage in the ventral rumen sac which was visible during the experimental procedures, papillae coverage appeared not to change from the dry period to lactation (personal observation). It can be hypothesized that this results from the ventral and lateral compression of the rumen by the gravid uterus, which could maintain rumen fluid at a level comparable to that during the lactation, despite the smaller rumen content mass during the dry period than the subsequent lactation (Chapter 3). In conclusion, it seems likely that an increase of total rumen surface area, and thereby arguably an increase in absorption capacity, is mainly caused by an increase in size of the individual papillae instead of an increase in number of papillae or coverage of the rumen wall by papillae.

Both experiments have shown that the response of the rumen papillae to a rapid (lactation experiment) or instantaneous (dry period experiment) increase in FOM intake is not linear. During the lactation experiment, a pronounced peak in growth rate of the papillae was observed between 9 and 16 d pp (Chapter 2), whereas feeding concentrate from 28 to 18 d ap (Chapter 4) showed a 19% increase in papillae surface area, in contrast to 18 to 8 d ap which showed a further 10% increase, compared with 28 d ap. After a period of rapid growth, papillae keep slowly growing until equilibrium with the daily FOM intake and VFA production appears to be achieved (Chapter 2, 3). As was observed during the lactation experiment, papillae can continue to proliferate despite negligible changes in FOM intake. There papillae surface area of the group with a gradual increase in concentrate allowance increased with a further 10 mm^2 from 44 to 80 d pp whereas FOM intake was similar (14.9 and 15.1 kg, respectively). Results of earlier studies suggested that morphological adaptation of the rumen takes 4-6 weeks (Dirksen et al., 1985) or a shorter 2-3 weeks (Bannink et al., 2012). However, daily feed intake and therefore daily VFA production increased in a non-linear fashion over the course of several weeks during early lactation (Park et al., 2010; Chapter 2, 4).

The results from the lactation experiment (Chapter 2) clearly show that the rate of proliferation of the papillae varies according to the rate at which FOM intake changes. Therefore it seems not appropriate to discuss rumen adaptation during the transition period in terms of 'time required for adaptation' but rather whether the rate of change in FOM intake is met by an appropriate response of the papillae.

Similar to the capacity for rapid proliferation, clues were found that atrophy of the rumen papillae may progress at a rapid rate. At 63 d ap, 7 d before drying off and the start of the lactation experiment, rumen papillae were sampled. Papillae surface area was 41.4 mm^2 at 63 d ap, decreasing by 7.0 mm^2 to 34.4 mm^2 at 50 d ap, and by a further 6.4 mm^2 to 28.0 mm^2 at 3 d pp. This suggests that a major part of papillae atrophy associated with the dry period occurred around dry-off. It can be hypothesized that most surface area was lost in the week after the switch to the dry period ration was made as these rations typically consist predominantly of forage with a relatively low FOM content (in the Netherlands), reducing FOM intake. However, individual daily feed intake of the lactation ration before dry-off was not recorded. A substantial 15% decrease in papillae surface area was also observed between 8 d ap and 3 d pp during the dry period experiment for the group fed supplemental concentrate during the dry period, which could be explained by the decreased periparturient FOM intake (Chapter 3). In literature, a great reduction in papillae size was observed by Dirksen et al. (1984), where papillae surface area decreased by 70% over the course of two weeks after abruptly switching from a daily ration of 2 kg hay and 10.5 kg concentrate to a ration consisting of 5.5 kg hay and 6 kg straw. These cases of papillae atrophy can thus be associated with a decrease in FOM intake, and associated VFA production. This apparent sensitivity of the rumen papillae for decreases in FOM intake can have practical ramifications. It is known that severe cases of mastitis, endometritis, and digestive problems such as (subacute) rumen acidosis or a displaced abomasum coincide with a decrease in feed intake for several days. This may also result in atrophy of the rumen papillae and reduced VFA absorption capacity of the rumen and possibly a greater risk for rumen acidosis when feed intake recovers. In addition, transition from a mixed ration to pasture has been associated with a reduction in papillae surface area and greater risk for subacute rumen acidosis (Schären et al., 2016). Indeed, Albornoz et al. (2013) reported that 5 d of severe feed restriction (feeding 25% of voluntary feed intake) reduced k_aVFA. Unfortunately, Albornoz et al. (2013) did not report papillae surface area, and the decrease in k_aVFA might also be explained by a reduction in epithelial blood flow (discussed below). Finally, considering that the majority of papillae atrophy occurs during the first weeks of the dry period, it is unlikely that a shorter dry period (van Knegsel et al., 2014) than those in the present experiments will prevent such atrophy of the rumen papillae.

The postpartum development of papillae surface area during the dry period experiment was markedly slower than that during the lactation experiment (Figure 1A). All cows participating in the lactation experiment were in first lactation at the moment of drying off, whereas 8 cows in the dry period experiment were in 2nd lactation and 2 in 4th lactation at the moment of drying off. This apparently subdued response in the dry period experiment, despite subjecting the cows to the same rapid rate of increase of concentrate allowance as in the lactation experiment might indicate that the adaptive capacity of the cows diminishes with age. Alternatively, average papillae surface area was already greater during the dry period which also may have limited the response after calving.
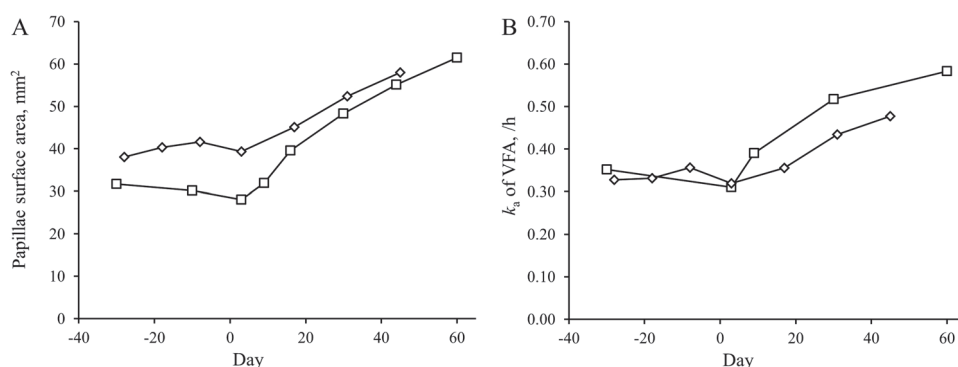
Figure 1. (A) Papillae surface area during the dry period and subsequent lactation for the lactation (□; n = 12; Chapter 2) and dry period experiments (◇; n = 9; Chapter 4). (B) Fractional absorption rate of VFA (k_a of VFA) obtained using the buffer incubation technique in an empty washed rumen during the dry period and early lactation. Absorption rates obtained during the lactation experiment (□; Chapter 3) are from those measurements which were not preceded by a VFA production measurement, from the dry period experiment (◇; Chapter 4) all measurements are shown. Values represent the LSM. Day = day relative to calving.

Histological Adaptation of the Rumen Papillae

Histological study of the rumen papillae aimed to examine whether the organization of epithelium covering the papillae changed in response to changes in FOM intake during the transition period. Generally, results from the present thesis suggest that the epithelium covering the papillae is histologically similar during the dry period and lactation, and is little affected by the rate at which the papillae grow due to applied treatments. Although statistically significant, changes in the thickness of the epithelial layer remained small during the lactation experiment, and were independent of the applied treatments (Chapter 2). During the dry period experiment, thickness of the epithelium was not affected by sampling day (Chapter 4). Steele et al. (2015)

likewise found limited changes in the epithelial layers in transition dairy cows. However, Bannink et al. (2012) observed that rapid growth of papillae coincided with a thinner epithelial layer, which they associated with a greater potential for VFA absorption. The observed changes in thickness of the epithelial layer during the lactation experiment (maximum difference between any two sampling days was 14 µm; Chapter 2) were relatively small compared with the thickness of individual cells (approximately 8 µm for stratum granulosum and 25 µm for stratum basale cells; unpublished data). The relevance of such limited changes in epithelium thickness can hence be questioned, also given that the epithelial layer likely functions as a functional syncytium (behaving as a single functional layer; Graham and Simmons, 2005).

During the lactation experiment the stratum corneum was not measured, but it was during the dry period experiment (unpublished data). The thickness of the stratum corneum averaged 11.2 µm (sd 1.84 µm; minimum, 7.6 µm; maximum, 17.3 µm; range of average by sampling day, 10.5 to 11.6 µm) and was not affected by treatment, sampling day or an interaction between these two ($P \geq 0.65$), which corresponds with observations of Melo et al. (2013) and Steele et al. (2015). It was found that the stratum corneum of papillae collected in the dorsal blind sac was thicker than of papillae collected from the ventral rumen sac and the ventral blind rumen sac (12.1 vs. 10.7 and 10.7 µm, respectively), possibly due to more intensive contact with the rumen mat which might increase the requirement for a physical protective layer.

An important difference in the adaptive response of the epithelium seems to exist between cows during the transition period and cows subjected to treatments which aimed to induce (subacute) rumen acidosis (Steele et al., 2011; Steele et al., 2015). The latter usually comprises a change from forage to a grain dominated ration. With rumen acidosis, hyper- or parakeratinization of the rumen epithelium is reported as a pathological change (Hinders and Owen, 1965; Steele et al., 2009). Furthermore, changes in permeability of the epithelium which could allow (toxic) substances from the rumen fluid to enter the blood through gaps in the apically located permeability barrier were reported (Steele et al., 2011). Steele et al. (2011) also reported a decrease in thickness of the epithelium during a SARA challenge, as well as a more extensive sloughing of the stratum corneum and decreased demarcation between the epithelial strata (Steele et al., 2009; Steele et al., 2011). This contrasts with the observations made in the present experiments where increase in FOM intake and rate of growth was rapid, but did not coincide with signs of pathology of the rumen epithelium such as discussed above.

In conclusion, the limited changes in the histological morphology of the epithelium do not suggest that the potential capacity for absorption of VFA is affected during the transition period or during periods of rapid tissue proliferation.

Chapter 7

Functional Adaptation of the Rumen: Fractional Absorption Rate of VFA

During both experiments reported in this thesis, the transition from the dry period to lactation coincided with an increase in the k_aVFA measured using the buffer incubation technique (Figure 1B; Chapter 3, 5), in line with the hypotheses. However, as discussed in detail previously (Chapter 3, 5) the concentrate treatments resulted in differences in papillae surface area but they did not affect k_aVFA during the dry period or lactation. As discussed in the section *Macroscopic Adaptation of the Rumen Papillae*, it is very likely that total rumen surface area was greater due the larger papillae as well and hence does not appear to offer an explanation for the contrast between the morphological and k_aVFA changes. As discussed in *Histological Adaptation of the Rumen Papillae*, the limited changes in the histological morphology of the epithelium covering the rumen papillae does not give an indication that the larger papillae are less capable of absorbing VFA. In addition, as discussed earlier (Chapter 1, 3, 5), several studies suggest that the rumen epithelium can increase its capacity for absorption and metabolism of VFA without an apparent increase in surface area, indicating an increased concentration of transporter proteins and enzymes per unit of surface area. This was explored by studying gene and protein expression (Chapter 5) as the observed greater daily VFA production and capacity for VFA absorption was expected to coincide with greater level of expression. However, for both experiments in this thesis, the expression of genes associated with rumen epithelial transport and metabolism (mainly of VFA) was generally not greater after the transition into lactation. In contrast, during the dry period experiment even an apparent downregulation of a number of genes rather than an increase was observed. In general, the gene expression data thus suggest that the capacity for VFA absorption and metabolism of the epithelium changes little per unit of surface area or is even slightly reduced. Moreover, gene expression levels were generally not affected by an interaction between the treatments and time. The gene expression data, therefore, do not provide an explanation for the apparently decreased capacity for VFA uptake per unit of surface area, which the combination of greater papillae surface area and similar k_aVFA suggests.

The protein expression data from the Western blots (Chapter 5) did show lactation stage effects. A temporary increase in expression of the VFA^-/HCO_3^- exchanger DRA in the papillae at 16 d pp compared with 3 and 44 d pp was observed, suggesting a greater capacity for VFA absorption per unit of surface area. In addition, expression of Na^+/H^+ exchanger type 2 was greater at 16 and 44 d pp compared with 3 d pp, suggesting greater capacity for H^+ elimination. However, these observations do not seem to offer an explanation for the contrast between changes in papillae surface area and k_aVFA. Conversely, Laarman et al. (2015) reported recently that transition into lactation did not coincide with changes in expression of VFA transporter proteins. Monocarboxylate

transporter MCT1 was not affected by a treatment or lactation stage effect but an interaction was observed showing a temporary increase at 16 d pp compared with 3 and 44 d pp for the rapid concentrate treatment. This suggests an increase in VFA absorption capacity per unit of surface area. Hence these observations do not offer an explanation for the contrast between changes in papillae surface area and k_aVFA. However, since the role of MCT1 is to export VFA from the epithelium to blood, the impact on VFA absorption is unclear. This leads me to conclude that a factor not related with papillae morphology or capacity of the epithelium for VFA absorption or metabolism is likely to explain the contrast in the results. In other words, the papillae surface area is not the limiting factor for k_aVFA, but more likely a prerequisite.

Epithelial blood flow [photographs by Dobson et al. (1956) show the dense web of capillaries in the papilla] is a factor known to affect the absorption capacity of the epithelium for VFA (von Engelhardt and Hales, 1977; Storm et al., 2011). Visceral blood flow has been found to increase after calving (Reynolds et al., 2004) and with greater daily DMI (Ellis et al., 2016). This might explain why the increase in papillae surface area after calving could coincide with an increase in k_aVFA, but might also present a mechanism explaining why the temporary greater papillae surface area early pp due to the rapid increase in concentrate allowance did not coincide with a greater k_aVFA. Saturation with VFA of the blood in the capillaries in the papillae might restrict the movement of VFA from the epithelium to the blood, in turn increasing intracellular VFA concentrations and reducing the efficiency for VFA absorption. In addition, it can be hypothesized that the amount of HCO_3^- available for the bicarbonate-dependent uptake of VFA is limited by epithelial blood flow, as the HCO_3^- is believed to predominantly derive from extra-epithelial sources (Aschenbach et al., 2011). This would also limit the capacity for VFA uptake irrespective of papillae surface area. Future efforts are required to further elucidate the role of epithelial blood flow for VFA absorption.

Similarly as for the papillae surface area, a difference seems to exist between the experiments for the changes in k_aVFA after calving. Whereas the k_aVFA was similar during the dry period for both experiments, after calving the increase in k_aVFA was more gradual during the dry period experiment compared with the lactation experiment (Figure 1B). As noted earlier, cows were older during the dry period experiment. But besides age as such, two additional factors might explain the slower increase in k_aVFA during the dry period experiment. Firstly, the cows were much heavier, and secondly, milk production (FPCM, kg/d) was markedly higher during the dry period experiment (Figure 2 A and B). Both peripheral and mammary blood flow therefore needed to be greater for these animals. It can be speculated that if the cardiac output of these animals does not increase concomitantly, preferential channelling of blood to the udder and peripheral tissues might limit blood flow through the viscera and epithelia. As noted

Chapter 7

205

above, visceral blood flow has been found to increase postpartum and with greater daily DMI (Reynolds et al., 2004; Ellis et al. 2016). Since DMI during the lactation was marginally lower for the dry period experiment than the lactation experiment (Chapter 2, 4) this might also have limited the k_aVFA.
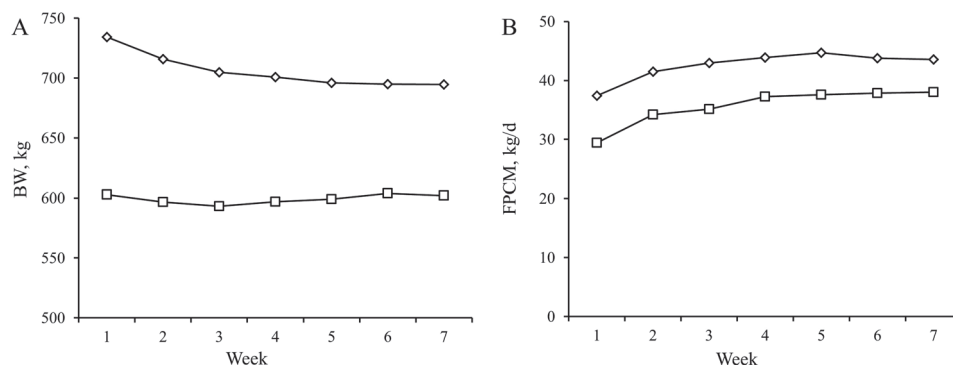

Figure 2. (A) Bodyweight (BW) of the cows during the lactation (□; Chapter 2) and dry period experiments (◇; Chapter 4). (B) Fat and protein corrected milk (FPCM) production of the cows during the lactation (□) and the dry period experiment (◇). Values represent the average of all cows.

Adaptation of the Rumen Microbiota

Changes in the rumen microbiota during the lactation experiment have been extensively discussed in Chapter 6. Briefly, rumen bacterial community composition was stable during the dry period and changed rapidly after calving during the lactation experiment. In addition, the rumen protozoal and archaeal communities changed markedly after calving. However, the capacity of the microbiota for the degradation of starch and NDF appeared not to be affected. Richness is a clear indicator of changes in the composition of the bacterial community, although rather intangible in terms of consequence for rumen fermentation due to the redundancy of the microbiota (Weimer, 2015). Richness decreased during the transition from the dry period to lactation. More interesting is that a rapid increase in concentrate allowance temporarily decreased richness of the bacterial community compared with a gradual increase to the same maximum daily concentrate allowance. This novel finding indicates that next to ration composition and feed intake in general, also the rate at which dietary changes occur affects rumen microbiota (Chapter 6). The temporary nature of the decrease in richness at 9 and 16 d pp indicates that a substantial number of bacterial taxa were more successful in capitalizing on the rapid increase in FOM between 3 and 16 d pp than other taxa. However, after 16 d pp, when the maximum daily concentrate allowance was

fed, further changes in FOM intake were limited and occurred gradually. This arguably reduced the competitive edge for the outcompeting taxa and allowed for the recovery of the temporarily outcompeted taxa.

As for the lactation experiment, rumen content samples were collected during the dry period experiment and used for profiling of the microbiota (Textbox 1; unpublished). The postpartum rate of concentrate build-up during the dry period experiment was similar to the treatment with the rapid build-up in the lactation experiment (1.0 kg of DM/d). Although a lower maximum concentrate allowance of 8.9 kg of DM/d (compared to 10.9 kg of DM/d) was used during early lactation, a very similar decrease in richness pp was observed (Figure 3) with a similar recovery to a level slightly lower than during the dry period. Also feeding concentrate during the dry period in the dry period experiment appeared to induce a temporary numerical decrease in richness. These results thus confirm the temporary decrease in richness observed during the lactation experiment.

Textbox 1. Effect of Supplemental Concentrate Fed during the Dry Period on Bacterial Community Richness in Dairy Cattle during the Dry Period and subsequent Lactation

Samples of whole rumen contents were collected during the dry period experiment (Chapter 4) using the technique described in Chapter 6. The rumen content samples were freeze-dried, ground to pass a 1 mm screen (100AN, Peppink Mills BV, Olst, the Netherlands), and subsequently shipped to AgResearch (Palmerston North, New Zealand). Samples were prepared and the bacterial community profiled as described by Henderson et al. (2015) using 454 GS FLX Titanium chemistry at Eurofins MWG Operon (Ebersberg, Germany). Pyrosequence data were analyzed using QIIME version 1.8 (Caporaso et al., 2010) and grouped into operational taxonomic units sharing over 97% sequence similarity. Sequences were assigned to phylogenetic groups by BLAST (Altschul et al., 2010) using the Greengenes database (v. 13.5; McDonald et al., 2012). The Chao1 metric was calculated using QIIME 1.8. Data were analyzed using the MIXED model using SAS 9.3 (SAS Institute Inc., Cary, NC) described in Chapter 4. To test specific hypotheses, CONRAST and ESTIMATE statements were constructed as implied by the text. In- or exclusion of the outliers (3) did not affect the interpretation of the results, outliers were included in the used dataset. Results are presented in Figure 3, below.
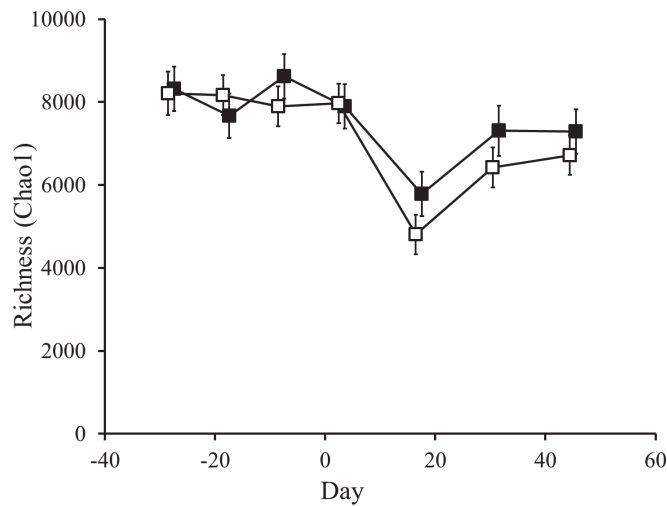
Chapter 7

Figure 3. Bacterial community richness for the control (no concentrate during the dry period; CON; □; n = 5) and for the dry period treatment with supplemental concentrate (3.0 kg of DM/d concentrate during the dry period; SUP; ■, n = 4) from 28 d ap up to calving, and the subsequent lactation period. During the lactation daily concentrate allowance for all cows increased from 0.9 kg of DM/d at 0 to 3 d pp, by 1.0 kg of DM/d starting 4 d pp up to 8.9 kg of DM/d, which was maintained up to 45 d pp. Values represent LSM ± SE (values are slightly offset for clarity). Day 0 = day of calving.

Averaged over the dry period, richness was similar (P = 0.50) for the dry period treatment with supplemental concentrate (3.0 kg of DM/d concentrate during the dry period; **SUP**) and for the control (no concentrate during the dry period; **CON**), and likewise for the subsequent lactation (P = 0.23). Richness changed over the course of the experiment ($P < 0.01$), but no interaction between treatment and day (P = 0.48) was observed. Between 28 and 18 d ap, richness in SUP remained similar (P = 0.23), but tended to increase between 18 and 8 d ap (P = 0.08). Richness in CON did not change during the dry period ($P \geq 0.57$). After calving, from 3 to 17 d pp, richness decreased ($P < 0.01$) by 27% (SUP) to 40% (CON), and both increased again thereafter ($P \leq 0.03$). Averaged over 31 and 45 d pp, richness of SUP was similar to that on 3 d pp (P = 0.38), whereas it was lower for CON (P =0.02).

ABSORPTION OF VFA FROM THE NATURAL FILLED RUMEN AND FROM STANDARDIZED BUFFERS

The k_aVFA obtained using the buffer incubation technique appeared to be affected by the preceding VFA production measurements (Chapter 3). The k_aVFA measured after a VFA production measurement was ~20% higher than expected based on the interpolation of the k_aVFA measurements which were not preceded by a VFA production measurement (Figure 4). As buffer was standardized with regard to fluid volume, pH, and VFA concentration, which are all known to affect the k_aVFA (Dijkstra et al., 1993), this suggests that a physiological difference in the cows was responsible for this difference.
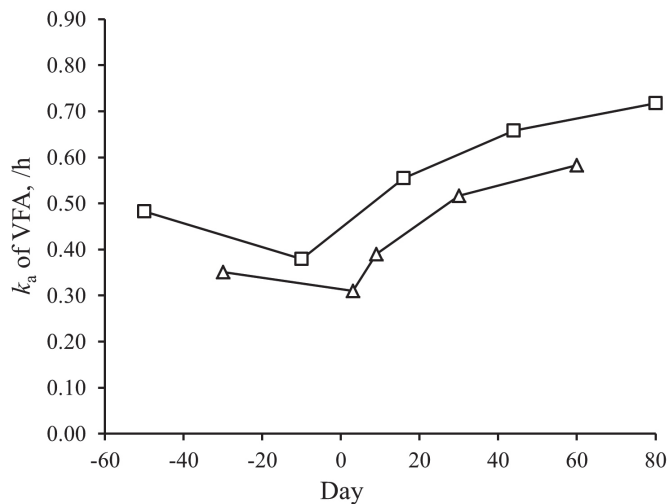
Figure 4. Fractional absorption rate of VFA (k_a of VFA) obtained using the buffer incubation technique in an empty washed rumen during the dry period and early lactation. Values indicated by □ derive from buffer incubations which were conducted immediately following a VFA production measurement. For the VFA production measurement cows were fed small meals over the course of 8 hours to stimulate steady state rumen fermentation with the actual measurement covering the last 3 hours. Values indicated by △ were not preceded by a VFA production measurement. Values represent LSM ± SE of all cows from the lactation experiment (n=12; Chapter 3).

Feeding might have affected the k_aVFA measurements in several ways. Feed intake in the hours preceding the k_aVFA measurements conducted in the morning was likely limited due to limited access (milking) and cows anticipating the delivery of fresh feed. Low feed intake might have reduced the visceral blood flow (Ellis et al., 2016). The frequent feeding during the VFA production measurements might have increased

visceral blood flow (Storm and Kristensen, 2010), and thereby have stimulated VFA absorption (Storm et al., 2011). Alternatively, the frequent feeding might have increased rumen motility during the buffer incubations, continuously mixing the buffer fluid thereby aiding intra-ruminal equilibration of the VFA. However, rumen contractions, which caused visible turbulence in the buffer fluid, were also frequent during the buffer incubations which were not preceded by the frequent feeding (personal observation). The increased k_aVFA after frequent feeding suggests a greater capacity for VFA absorption in the hours after feed intake. This adaptive response would be a desirable effect as during the first hours after feeding high VFA concentrations and low pH occur due to more FOM (in particular the rapidly fermentable fraction). Further exploration of the relationship between time of feeding, meal size, and the capacity for VFA absorption and mechanisms involved might offer new incentives and clues for the optimization of VFA absorption and rumen pH management.

As discussed in Chapter 3, the rumen contents affect the intra-ruminal movement of VFA. The fluid in the ventral rumen is likely more readily mixed than the fluid in the dorsal regions of the rumen due to the absence of the rumen mat. This allows for a more rapid equilibration of VFA concentration in the rumen fluid aiding VFA absorption (Tafaj et al., 2004; Storm and Kristensen, 2010). The formation of the rumen mat is considered essential for optimal rumen functioning (Zebeli et al., 2012). Ration composition, feed particle size, and fiber content affect the stratification of feed in the rumen and mat formation (Tafaj et al., 2004; Storm and Kristensen, 2010). In the rumen mat, VFA concentration is generally higher, and pH lower, than the fluid in the ventral rumen (Zebeli et al., 2012). As hypothesized and studied by Storm and Kristensen (2010), structure of the rumen mat might also affect the intraruminal fluid dynamics, rumen mat VFA concentration and pH, and VFA clearance. Using dairy cows which were fed 20 kg/d of DM of a mixed ration containing 30% grass hay, 35% corn silage, 20% rolled barley, and 11% rapeseed cake (DM basis) but with differences in particle size, Storm and Kristensen (2010) found some indications that intra-ruminal fluid dynamics were affected by particle size. However, they did not find differences in pH and VFA concentration in the rumen mat and ventral rumen fluid with the different particle size rations. In addition, ruminal absorption of VFA calculated from arterial and venous blood samples appeared not to be affected. Nonetheless, observations on ruminal CO_2 dynamics suggest that feed particle size does have an effect on rumen pH, with larger feed particles allowing for easier escape and eructation of CO_2 than small feed particles (Laporte-Uribe, 2016). The effect of particle size in the ration on rumen fluid dynamics thus appears still inconclusive. Further investigation of the hypothesis that inclusion of larger feed particles in the ration results in a more 'open' structure of the rumen mat, allowing for a more effective flow of rumen fluid might be worthwhile as different rations and ranges of particle size might have different effects.

Versatility of VFA absorption

Historically the passive diffusion of undissociated VFA (**HVFA**) has been seen as the most important component of the total VFA absorption (Aschenbach et al., 2011). Currently it is estimated that ~50% of the VFA are absorbed in association with H^+, either passively as HVFA or via an apical monocarboxylate transporter (**MCT**; Aschenbach et al., 2011; Dijkstra et al., 2012). The remainder of the VFA is assumed to be absorbed via bicarbonate-dependent and bicarbonate-independent routes, where the HCO_3^- required for this absorption is predominantly supplied by the blood (Aschenbach et al., 2011; Dijkstra et al., 2012). In case of the buffer incubations employed for the measurement of the k_a VFA there might be major shifts in the route and form [undissociated or dissociated (**VFA$^-$**)] through which VFA are absorbed relative to absorption from the natural filled rumen. The major difference between absorption from the natural filled rumen, where fermentation continuously produces HVFA, and absorption from the buffer is that in the latter no new HVFA is produced and VFA predominantly are present as VFA$^-$.

The buffer salts used for preparation of the buffer were, for the main part, $NaHCO_3$, Na_2HPO_4, and $NaOH$ (Chapter 3). During preparation of the buffer, these salts were dissolved in water and pure HVFA was added. The dissolved HCO_3^-, OH^-, and HPO_4^{2-} captured H^+ from the HVFA, resulting in the formation of H_2CO_3, H_2O, and $H_2PO_4^-$. Dissociation of H_2CO_3 in H_2O and CO_2, and escape of CO_2 from the buffer permanently removed H^+ from the buffer. Similarly, the strong alkaline OH^- permanently removed H^+. From the buffer composition (Chapter 3), it can be calculated that 75% of potential H^+ from the HVFA is permanently removed in this way. The remaining H^+ is buffered by the VFA$^-$ itself and HPO_4^{2-}, with a very small fraction of H^+ in solution (since pH was 6.0; Henderson-Hasselbalch equation). This buffer was subsequently infused into the rumen.

During the buffer incubation, the continued influx of HCO_3^- with the saliva over the course of the incubation would have removed part of the remaining H^+ pool in the buffer as it is a stronger base (pK_a = 10.3) than both VFA$^-$ (pK_a = 4.8) and $H_2PO_4^-$ (pK_a = 7.2). Additional HCO_3^- is introduced into the rumen by the exchange of VFA$^-$ for HCO_3^- by the rumen epithelium. This influx of HCO_3^-, by capturing H^+, greatly limits the amount of VFA which can be absorbed as HVFA. However, the end pH of ~7.2 (Chapter 3) indicates that the uptake via VFA$^-$/HCO_3^- exchange is likewise limited, because then a more alkaline buffer fluid might be expected. This suggests that with the buffers used for the present experiment, the epithelium predominantly absorbs VFA$^-$ via the bicarbonate-independent route. Alternatively, intracellular formation of H^+ and HCO_3^- by carbon-anhydrase and apical exchange of H^+ for Na^+ might introduce

Chapter 7

211

additional H$^+$ to the rumen fluid. However, a net movement of H$^+$ into the rumen appears contra-intuitive as in the natural filled rumen, the rumen has an important role in removing or neutralizing H$^+$ in the rumen fluid (Aschenbach et al., 2011). In conclusion, the high k_aVFA observed with the buffer incubations suggest that the mechanisms for VFA absorption are not only versatile but also have a high capacity under widely different physiological situations.

VARIATION BETWEEN INDIVIDUALS IN k_AVFA AND EFFECTS ON THE RUMEN ENVIRONMENT

It is known that there is large variation between animals in the susceptibility for (experimentally induced) subacute rumen acidosis (**SARA**; Bevans et al, 2005; Krause and Oetzel, 2006; Dohme et al., 2008; Penner et al., 2009). In a study by Penner et al. (2009), sheep were administered an oral drench of glucose (dose based on bodyweight) aimed at inducing subacute rumen acidosis. Post hoc, those animals with the longest time of rumen pH below 5.8 were classified as 'responders', and thereby defined as most susceptible to SARA, or as 'non-responders' with the shortest time of pH below pH 5.8, and thereby least susceptible to SARA. Using the in vitro Ussing-chamber technique, it was found that 'non-responders' had a greater capacity for uptake of Ac and Bu (Pr was not measured). The authors concluded that a main part of the variation between individuals in susceptibility for SARA can be explained by the capacity for Ac and Bu uptake. In the recent study by Gao and Oba (2016), mid-lactation dairy cows were adapted to a high grain ration (30% forage), and like Penner et al. (2009) animals were divided into high or low SARA risk groups based on rumen pH measurements. In contrast to Penner et al. (2009) no differences in VFA absorption were found by Gao and Oba (2016) with the VFA absorption measured using the Co-EDTA/*n*-valerate method in the natural filled rumen instead of in vitro Ussing-chamber measurements.

Results from the experiments described in this thesis can also be used to explore differences in the capacity for VFA absorption between animals. A novel approach is to classify cows not based on rumen pH, but based on the k_aVFA. Two points of interest are (1) whether the k_aVFA during the dry period is indicative for that during lactation period; and (2) whether the k_aVFA during lactation affects rumen VFA concentrations and pH. It can be hypothesized that cows with a higher k_aVFA during the dry period likewise have a higher k_aVFA during lactation. In addition, it can be hypothesized that cows with a higher k_aVFA during the lactation are able to maintain lower rumen VFA concentrations and higher pH than cows with a lower k_aVFA during lactation. Finally, cows with a higher k_aVFA during lactation can be hypothesized to have a greater

absorptive surface area, reflected by a greater papillae surface area, or a greater expression of genes and protein associated with VFA transport and metabolism.

During the lactation experiment VFA production was repeatedly measured (Chapter 3). For these measurements steady state rumen fermentation conditions were promoted, rumen fluid was sampled for determination of VFA, and pH was continuously measured in situ. Immediately after completion of the VFA production measurements, rumen papillae were collected and the k_aVFA was measured using the buffer incubation technique in vivo (Chapter 2, 3). Data from these k_aVFA measurements were used to classify cows as either HIGH or LOW cows during the dry period or lactation as described in Textbox 2, and data from the preceding VFA production measurements were used to explore the effects of a greater k_aVFA.

Textbox 2. Classification and Analysis of HIGH or LOW k_aVFA Cows

The cows were divided into 3 groups of 4 according to either (A) the average k_aVFA obtained during the dry period (using the buffer incubation technique), or (B) the average k_aVFA obtained during early lactation (up to 80 d pp). The 4 cows with the highest k_aVFA during either the dry period or early lactation were classified as $HIGH_{DRY}$ or $HIGH_{LAC}$, respectively. The 4 cows with the lowest k_aVFA were classified as LOW_{DRY} or LOW_{LAC}, respectively. The data from the 4 remaining cows with intermediate k_aVFA were not used for this analysis to maximize separation between HIGH and LOW. The $HIGH_{DRY}$ and LOW_{DRY} groups each contained 3 cows which would receive the rapid rate of increase of concentrate allowance treatment, minimizing interference from treatment effects. Likewise, $HIGH_{LAC}$ and LOW_{LAC} groups each contained 3 cows from the rapid treatment group. Data were analyzed using the MIXED procedure (Littell et al., 2006) in SAS 9.3 (SAS Institute Inc., Cary, NC) using a model similar to that used for the analysis of the VFA production variables in Chapter 3, employing a compound symmetry covariance structure for the repeated observations on the same cow. CONTRAST statements were constructed to test (1) averaged over the dry period, the difference in k_aVFA between cows classified as HIGH or LOW; and (2) averaged over the lactation period, the difference between cows classified as HIGH or LOW for all variables presented in Figure 5 panels A to F, or mentioned in the text. In addition, differences between HIGH and LOW were tested for each of the sampling days.

Chapter 7

The k_aVFA of cows classified as $HIGH_{DRY}$ differed (P = 0.02) during the dry period (Figure 5A) from those classified as LOW_{DRY}, but did not differ during early lactation (up to 80 d pp; P = 0.69). In contrast, the k_aVFA of cows classified as $HIGH_{LAC}$ (Figure 5B) did not differ from LOW_{LAC} during the dry period (P = 0.69), but was higher during lactation (P < 0.01) where the k_aVFA tended (P = 0.06) to be higher at 16 d pp and was higher (P < 0.01) at 44 d pp. Papillae surface area (Figure 5C) and VFA production rate (Figure 5D) did not differ ($P \geq 0.21$) between $HIGH_{LAC}$ and LOW_{LAC} averaged over the dry period, or the lactation. Whereas papillae surface area was similar ($P \geq 0.40$) for $HIGH_{LAC}$ and LOW_{LAC} at all sampling days, cows in LOW_{LAC} had a higher (P = 0.03) VFA production rate at 50 d ap than $HIGH_{LAC}$. More importantly, no differences in VFA production were found between $HIGH_{LAC}$ and LOW_{LAC} at 16 or 44 d pp ($P \geq 0.14$), whereas at 80 pp VFA production rate was higher (P < 0.01) for $HIGH_{LAC}$ than LOW_{LAC}. The rumen fluid VFA concentration (Figure 5E) and rumen fluid pH (Figure 5F) did not differ ($P \geq 0.11$) between $HIGH_{LAC}$ and LOW_{LAC} averaged over the dry period or the lactation, whereas at 44 d pp total VFA concentration was higher in LOW_{LAC} than $HIGH_{LAC}$ (P < 0.01) and rumen fluid pH tended (P = 0.10) to be lower in LOW_{LAC} than $HIGH_{LAC}$.

This analysis of differences between individual cows indicates that a higher k_aVFA relative to the daily VFA production is beneficial for maintaining a lower VFA concentration and a higher pH in rumen fluid. However, contrary to the hypothesis, a high k_aVFA during the dry period did not coincide with a high k_aVFA during the subsequent lactation. Instead, it appears that the cows with a high k_aVFA during the early lactation period can better be described as having a greater capacity for adaptation, i.e. that the k_aVFA increases more rapidly and to a higher maximum rate after calving. Also contrary to the hypothesis, a higher k_aVFA during the early lactation period did not coincide with a concomitant greater papillae surface area. Because the papillae surface area during the dry period was similar, the greater increase in k_aVFA of $HIGH_{LAC}$ can hence not be attributed to a greater increase in papillae size compared to size during the dry period. Traditionally total papillae surface area is considered to be the major factor for k_aVFA. This suggests that the cows in $HIGH_{LAC}$ had a larger total rumen surface area caused by denser papillae coverage and/or more extensive papillae coverage of the rumen. This is not supported by the dry period k_aVFA which was not greater in $HIGH_{LAC}$, unless it is assumed that the total number of papillae can increase after calving in addition to the increase in surface area. As discussed earlier, this seems unlikely.
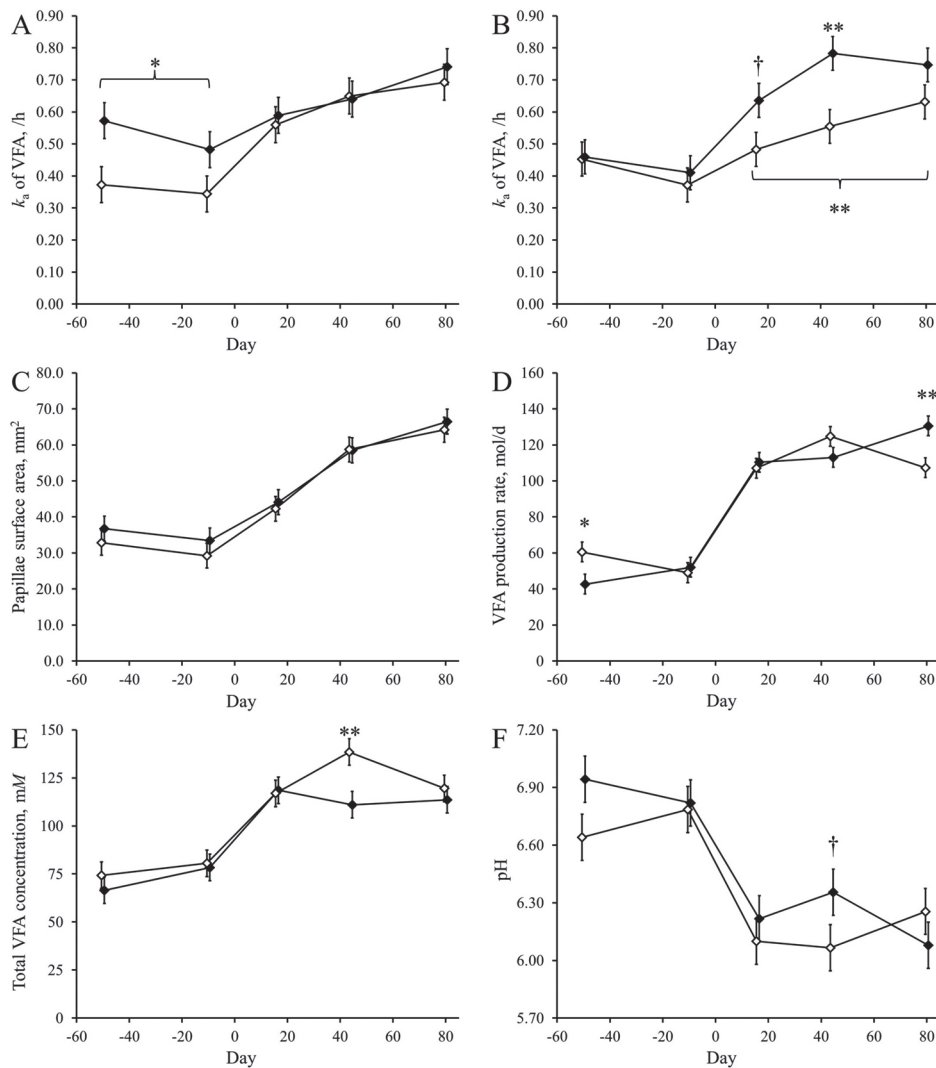
Figure 5. (A) Fractional absorption rate of VFA (k_a of VFA) of cows with a high (HIGH$_{DRY}$, ◆, n = 4) or low (LOW$_{DRY}$, ◇; n = 4) k_aVFA during the dry period; (B) cows with a high (HIGH$_{LAC}$, ◆, n = 4) or low (LOW$_{LAC}$, ◇; n = 4) k_aVFA during lactation. For HIGH$_{LAC}$ (◆) or LOW$_{LAC}$ (◇) papillae surface area, ruminal VFA production rate, rumen fluid VFA concentration and pH are presented (C to F). The papillae surface area data derived from Chapter 2. The VFA production rate, concentration of VFA and the pH in the rumen fluid were obtained under steady state rumen fermentation conditions during the VFA production measurements (Chapter 3). The k_aVFA was measured using a standardized buffer in the empty washed rumen (Chapter 3). Values represent LSM ± SE (values are slightly offset for clarity). †$P < 0.10$, *$P < 0.05$, **$P < 0.01$, significance of differences between HIGH and LOW. Day 0 = day of calving.

Chapter 7

Alternatively, the k_aVFA might be greater in HIGH$_{LAC}$ due to an increase in absorption capacity per unit of surface area (Sehested et al., 2000; Etschmann et al., 2009; Schurmann et al., 2014) due to a greater blood flow (Storm et al., 2011), or a higher concentration of VFA transporters (Aschenbach et al., 2011). The latter suggestion is not supported by the expression of VFA transporter genes or genes associated with VFA metabolism (Chapter 5) as no differences between HIGH$_{LAC}$ and LOW$_{LAC}$ during lactation were observed ($P \geq 0.12$; data not shown). Specifically, the gene expression of the monocarboxylate transporters *MCT1* and *MCT4* and HCO$_3^-$/VFA$^-$ exchangers *PAT1* and *DRA* were examined ($P \geq 0.41$), as were the 3-hydroxybutyrate dehydrogenases *BDH1* and *BDH2*, and the 3-hydroxy-3-methylglutaryl-CoA synthase 2 *HMGCS2* ($P \geq$ 0.35). The protein expression of DRA, MCT1, NHE2, PCCA, and ATP1A1 (Chapter 5) likewise did not show any differences between HIGH$_{LAC}$ and LOW$_{LAC}$ ($P \geq 0.18$). Similarly, expression of genes involved in intra-cellular pH regulation, VFA metabolism and absorption, which included most genes examined in Chapter 5, was not found to differ between acidosis risk groups in the study of Gao and Oba (2016). Likewise, Schlau et al. (2013) did not find differences in gene expression of VFA transporters and pH regulators (*MCT1*, *PAT1*, *DRA*, *NHE1*, *NHE2*) between acidosis-resistant and acidosis-susceptible steers that were fed a high grain ration, except for *NHE3*.

In conclusion, these results suggest that a greater k_aVFA can contribute to lower rumen fluid VFA concentrations and pH, and that this greater k_aVFA may be attributed to extra-epithelial causes, including blood flow. As suggested by Penner et al. (2009), these between-animal differences and their underlying mechanisms offer an opportunity to further decrease observed problems with low rumen pH. Future research efforts might focus on identifying the mechanisms explaining the difference in k_aVFA of these animals, and whether these differences can be induced through nutrition.

CONCLUSIONS AND RECOMMENDATIONS

The objective of this thesis was to study the adaptation of the rumen to ration changes during the dry period and early lactation. Changes in rumen papillae morphology, fractional rate of VFA absorption, and changes in the composition of the rumen microbiota were the primary targets for study. In addition, the expression of genes and proteins associated with absorption and metabolism of VFA by the rumen epithelium, as well as genes associated with tissue proliferation, were studied to better understand the relationship between functional changes and morphological changes of the papillae. Furthermore, in situ incubations of selected substrates were performed to assess the degradation capacity of the microbiota. Based on the work presented in this thesis the following conclusions can be drawn and recommendations can be given:

Conclusions and Recommendations

❖ An increase in daily FOM intake, and thus daily VFA production, coincided with an increase in rumen papillae surface area. Papillae growth can be stimulated during the dry period and early lactation. The rate of papillae growth depends on the rate of increase in daily FOM intake.

❖ Papillae surface area decreases rapidly in respons to a decrease in daily FOM intake.

❖ No evidence was found for the special proliferative properties of butyric acid under the imposed experimental conditions. This indicates that the importance of butyric acid as promotor of the epithelial proliferation might be overrated. In contrast, evidence was found that the role of propionate might be underrated.

❖ Changes in daily VFA production resulted in limited changes in the histomorphology of the epithelium covering the rumen papillae and expression of genes associated with VFA absorption and metabolism. This suggests that the capacity for absorption and metabolism of VFA per unit of papillae surface area is similar during the dry period and subsequent lactation, and is not affected by the rate of tissue proliferation.

❖ The ruminal fractional absorption rate of VFA measured under standardized conditions decreased during the dry period and increased after calving, and generally followed a pattern similar to changes in papillae surface area. However, unlike papillae surface area, the fractional absorption rate of VFA was not affected by the rate of increase of concentrate allowance after calving. Likewise, supplementation of concentrate during the dry period stimulated papillae growth but did not increase the fractional absorption rate of VFA.

❖ The contrast in response of rumen papillae surface area development and the fractional absorption rate of VFA to the concentrate treatments indicates that papillae surface area is not the limiting factor for the VFA absorption capacity. Considering that the capacity for absorption and metabolism of VFA per unit of papillae surface area appears similar, an extra-epithelial factor, likely visceral blood flow, limits VFA absorption.

❖ The capacity of the dairy cow rumen to adapt to the ration changes associated with the transition period indicates that the commonly observed negative impact of low rumen fluid pH and high VFA concentrations on milk production and cow health are primarily a management issue. The greater fractional absorption rate of VFA from the incubated buffer fluid compared with that from the natural filled rumen indicates that in daily practice it is unlikely that the capacity for absorption is the limiting factor in controlling rumen fluid VFA concentration and pH.

Chapter 7

❖ The differences between gene and protein expression, and the generally non-significant and weak correlation between the examined gene and protein expression levels, indicate that care must be taken when interpreting results obtained at either level.

❖ The rumen microbiota changes rapidly, concomitant with the changes in FOM intake and ration composition. The observed changes in the rumen microbiota and richness of the bacterial community did not appear to affect the capacity of the microbiota to degrade feed. The transient depression in bacterial community richness with a rapid, but not a gradual, rate of increase of concentrate allowance postpartum indicates that the rate of changes in ration composition and feed intake has a greater effect than the change in ration composition and feed intake level as such.

❖ The weak to moderate relationship between papillae surface area and fractional absorption rate of VFA indicates that morphological changes cannot directly be interpreted at a functional level.

❖ The large variation in fractional absorption rate of VFA between individual cows and its apparent effect on rumen fluid VFA concentration and pH under similar nutritional conditions merits further study.

❖ The capacity of the rumen to adapt after calving and the limited beneficial effects of supplementing concentrate during the dry period indicate that dry period feeding strategies can best be optimized for the prevention of periparturient metabolic disorders including hypocalcaemia and ketosis.

❖ The frequency and time of feeding appears to affect fractional absorption rate of VFA. Thus, management of time and frequency of feeding offers an opportunity to further control rumen fluid VFA concentration and pH.

❖ The physical presence of digesta affected fractional absorption rate of VFA, and may have affected intra-ruminal equilibration of VFA concentration. Intra-ruminal fluid dynamics and its effect on equilibration of VFA concentration warrants further study.

❖ The observed differences in age, body condition, and/or milk yield of the cows participating in the experiments might be associated with the differences in development of the papillae surface area and fractional absorption rate of VFA during early lactation. Further study of these possible associations might result in opportunities to optimize rumen adaptation at the individual cow level.

❖ Although large differences in daily VFA production and absorption as well as papillae surface area were observed, gene expression at the mRNA level showed

minor differences. Measurement of changes in gene expression at the mRNA level only is not recommended as indicator of changes in VFA absorption and metabolism in rumen epithelium of transition dairy cattle.

Chapter 7

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Chapter 7

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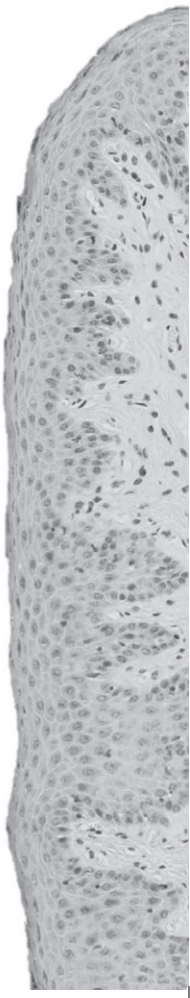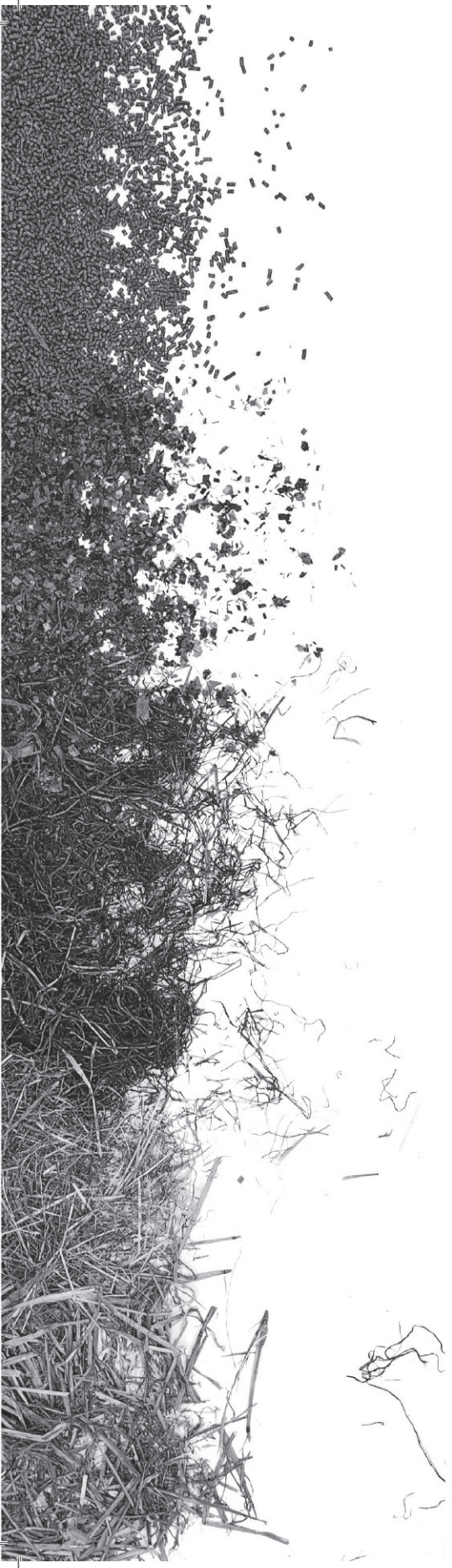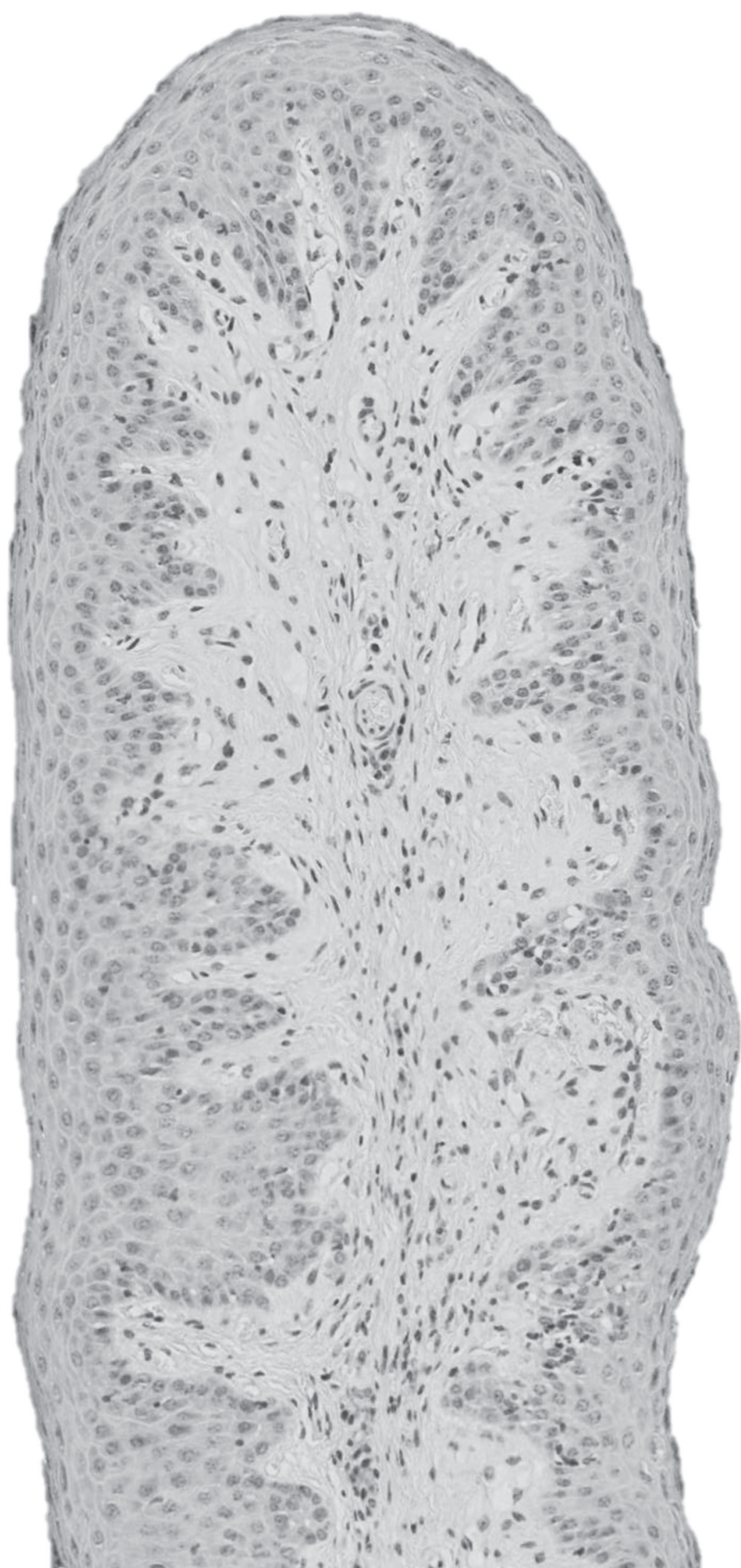
Summary

SUMMARY

In dairy cattle the nutrient requirements change rapidly around calving. During the dry period nutrients are required for maintenance, recovery from the previous lactation, and fetal growth. After calving, milk production commences and the energy requirements can increase by a factor 3 to ~184 MJ net energy for lactation (NE_L/d) during the first weeks of lactation, compared with the dry period, whereas feed intake doubles to ~24 kg dry matter (DM)/d compared with the dry period. In addition, high quality lactation rations are fed, usually containing a sizable portion of concentrate, thereby increasing fermentable organic matter (FOM) intake to ~14 kg/d. As a result, daily volatile fatty acid (VFA) production by the rumen microbiota increases from ~60 mol/d during the dry period to ~132 mol/d during early lactation. To maintain rumen pH at levels favorable for microbial fermentation, and prevent a negative impact on production and health, clearance of the produced VFA is essential. This mainly occurs through absorption over the rumen wall. The increase in capacity of the rumen for absorption of VFA is associated with morphological and functional changes of the rumen papillae which cover the rumen wall. However, current knowledge of these changes as they occur around calving is scarce (Chapter 1). Increasing our understanding of the adaptation of the rumen can provide new insights to optimize dairy cattle nutrition and thereby health, welfare, and production.

The objective of this thesis was to study the adaptation of the rumen to ration changes during the dry period and early lactation. Changes in rumen papillae morphology, fractional absorption rate of VFA (k_aVFA), and changes in the composition of the rumen microbiota were the primary targets for study. In addition, the expression of genes and proteins associated with absorption and metabolism of VFA by the rumen epithelium were studied to better understand the relationship between functional changes and morphological changes of the papillae. Uniquely, all these aspects were studied in parallel in the same dairy cows during the dry period and early lactation using a repeated measurement setup. Two experiments were conducted. In the lactation experiment, the effect of transition from the dry period to the subsequent lactation, and the effect of early lactation concentrate build-up strategy on the adaptation of the rumen were studied. In the dry period experiment, the effect of feeding supplemental concentrate during the late dry period in order to 'prepare' the rumen for the lactation was studied. Treatments of both experiments were aimed at creating a difference in FOM intake (kg/d) and thereby VFA production (mol/d), as VFA production was hypothesized to affect rumen papillae development and thereby the capacity for VFA absorption.

During the lactation experiment, intake of FOM did not change during the dry period (5.7 kg/d), but increased during the subsequent lactation to 15.0 kg/d at 80 d postpartum (pp). In addition, the rapid increase in concentrate allowance resulted in a temporarily 22% greater FOM intake compared with a gradual increase at 16 d pp (Chapter 2). The total production rate of VFA, measured using an isotope dilution technique (Chapter 3), was affected by these changes in FOM intake and increased 2.3 fold to 123 mol/d after calving, compared with the dry period (53 mol/d). The temporarily greater FOM intake with the rapid increase in concentrate allowance at 16 d pp coincided with a 54% greater propionate production (34 mol/d) compared with a gradual increase in concentrate allowance, whereas acetate (66 mol/d) and butyrate (10 mol/d) production were not affected. Papillae surface area (Chapter 2) decreased by 19% between 50 d antepartum (ap) and 3 d pp to 28.0 mm^2, but increased during early lactation to 63.0 mm^2. Papillae surface area increased faster with the rapid increase in concentrate allowance and surface area was 38, 34 and 22% larger at 16, 30, and 44 d pospartum respectively, than with a gradual rate of increase of concentrate allowance. Histology (Chapter 2) revealed that rumen papillae and epithelium thickness decreased slightly after calving, but were not affected by the concentrate treatment. Feeding concentrate during the dry period did not affect daily FOM intake (6.0 kg/d) but did increase VFA concentration in the rumen fluid by 21 mM to 121 mM, and increased papillae surface by 29% (Chapter 4). However, the increased papillae surface area in the dry period was not maintained to the subsequent lactation period. After calving, papillae surface area increased by 50% to 58.0 mm^2 at 45 d pp. The postpartum development of the rumen papillae was not affected by the treatment during the dry period. These results indicate that rumen papillae respond to changes in FOM and VFA production intake during the dry period and early lactation, and that the magnitude of this response depends on the rate of change in FOM intake.

During both experiments, k_aVFA was measured using a buffer incubation technique in an empty washed rumen. During the lactation experiment (Chapter 3), in accordance with the developments in papillae surface area, the k_aVFA decreased during the dry period from 0.48/h at 50 d ap to 0.34/h at 3 d pp. During the subsequent lactation, it increased rapidly to 0.56/h at 16 d pp and further to 0.72/h at 80 d pp. However, the greater papillae surface area due to the rapid increase in concentrate did not coincide with a greater k_aVFA. During the dry period experiment (Chapter 4), k_aVFA increased after calving by 50% to 0.48/h at 45 d pp, but the increase in papillae surface area due to supplemental concentrate during the dry period did not affect the k_aVFA during the dry period (0.36/h) or the subsequent lactation. These results indicate that papillae surface area is not the limiting factor for k_aVFA.

Changes in the expression of genes were studied at the mRNA level in papillae tissue from both experiments (Chapter 5). The expression of apoptosis related genes was not affected by sampling day or its interaction with treatment for both experiments, suggesting papillae proliferation during the transition period was mainly the result of an increased mitosis rate. The limited changes in the expression of genes associated with rumen epithelial transport and metabolism of VFA in dairy cows during the transition period do not suggest that these capacities of the epithelium increased per unit of surface area. Thus the major response to the increase in daily VFA production after calving was tissue proliferation. In addition, papillae from the lactation experiment were used to study expression at the protein level using immunoblotting. Results showed that expression of several proteins changed during early lactation indicating modulation of intracellular pH regulation and sodium homeostasis, and VFA metabolism. Only for one gene, a significant but weak correlation between the examined mRNA and protein expression levels was observed, indicating that care must be taken when interpreting results obtained at either level.

Ration changes associated with the transition from the dry period to lactation affected the rumen microbiota during the lactation experiment (Chapter 6). The rapid increase in concentrate allowance postpartum temporarily decreased bacterial community richness by as much as 30% compared with a gradual increase in concentrate. This transient depression in bacterial community richness with a rapid, but not a gradual, rate of increase of concentrate allowance pp indicates that the rate of change in ration composition and feed intake has a greater effect than the change in ration composition and feed intake level as such. The relative abundances of most major bacterial taxa were affected by the transition to lactation, but few were affected by the rate of increase of the concentrate allowance. The relative abundances of rumen protozoal taxa changed after calving, and were affected by the concentrate treatment. However, differences between treatments groups disappeared again when concentrate intake became similar. The archaeal community was likewise affected by both the transition to lactation and the treatment. The observed changes in rumen microbiota composition, including changes in bacterial community richness, did not appear to affect the fractional degradation rate of NDF, starch, CP, and OM measured in situ using a nylon bag technique.

The results in the present thesis show that morphologically and functionally the rumen papillae can adapt rapidly to the changes in FOM intake and daily VFA production associated with the transition from the dry period into the subsequent lactation. However, the contrast in response of rumen papillae surface area development and the fractional absorption rate of VFA to the concentrate treatments indicates that papillae surface area is not the limiting factor for VFA absorption. This proposition is further supported by the limited histological changes of the rumen epithelium and

limited changes in gene expression. Considering that the capacity for absorption and metabolism of VFA per unit of papillae surface area remains similar, an extra-epithelial factor, likely visceral blood flow, limits VFA absorption. The capacity of the rumen to adapt after calving and the limited beneficial effect of supplementing concentrate during the dry period indicate that dry period feeding strategies can best be optimized for the prevention of periparturient diseases.

Acknowledgments

Dit dankwoord gaat niet beginnen met de dooddoener dat deze thesis niet zonder hulp van anderen tot stand kon zijn gekomen. Dat is vanzelfsprekend. Ik wil graag beginnen met het opdragen van dit werk aan opa en oma Kapsenberg (Kappie voor intimi). Opa, oma, de noviteit van een promoverend kleinkind mag er bij de 4^e dan wel een beetje vanaf zijn (Lydia, Ties, en Inge, ik sluit me graag bij jullie aan), toch hoop ik dat ook dit werkje een plek in de boekenkast krijgt. Opa, collega dierenarts, het was na het zien van een spoedoperatie tijdens een logeerpartij in Sluis dat ik besloot: dat wil ik ook, ik wil dierenarts worden! Zonder dat moment zou dit boekje er zeker niet zijn geweest. Oma, als alumnus van de Landbouwhogeschool Wageningen is dit boekje er ook zeker voor jou!

Het echte dankwoord begint natuurlijk bij mijn promotor en college van co-promotoren. Wouter, tijdens ons welkomsgesprek hield je een gloedvol betoog over het belang van kwaliteit boven kwantiteit. Wat betreft de kwantiteit... ik weet het niet of dit boekje nog binnen de marges valt maar wat betreft kwaliteit... ik denk dat dat nog wel goed zit :) Bedankt voor het promoten van de informele sfeer bij ANU, ik voelde me daar erg thuis.

André, Jan, en Thomas, jullie bedankt voor alle support en begeleiding afgelopen jaren. Van proefopzet tot statistiek en interpretatie van de data, we hebben er heel wat over gediscussieerd. Het was voor jullie vast niet altijd makkelijk met zo'n veearts (met mening) uit Utrecht, maar jullie input heb ik altijd zeer gewaardeerd! Met jullie drie totaal verschillende schrijfstijlen: het minimalistische, het zakelijke, en het prozaïsche, was het soms een hele klus om een eigen weg in het schrijven van de artikelen te vinden maar ze zijn er! Zeker ook dankzij jullie heb ik het hele traject met veel plezier doorlopen en we zullen elkaar zeker in de toekomst nog vaak tegenkomen.

De uitvoering van de experimenten had nooit gekund zonder de medewerking van de Dairy Campus en studenten van de WUR, Van Hall, en Diergeneeskunde. Martin en alle dierverzorgers van de Waiboerhoeve, bedankt voor de samenwerking tijdens de proeven! Arie, jij hartelijk bedankt voor de hulp bij het uitvoeren van de in-sacco's. Ilse en Rosaline, bedankt voor alle hulp met het verzamelen en het meten van de penspapillen. Sytske, je recordtijd pens-evacueren moet nog steeds verbeterd worden! Degefa, thank you for your help with the hundreds of nylon bags. Danjella, bedankt voor je inzet en veel succes als herkauwerdierenarts!

Het uitvoeren van experimenten is één ding, maar het staat of valt allemaal bij de voorbereiding en de lab-analyses. Ik geloof dat ik het voltallige ANU lab wel met mijn experimenten heb vermaakt. Saskia, Michel, Jane-Martine, Adriaan, Xuan, en Erika, dank voor de goede samenwerking! Tom and Gemma, thank you for your collaboration with the microbiota projects. Jurgen, David, en Leo, jullie bedankt voor alle inzet rondom de genenpaper. De interpretatie van de resultaten was niet gemakkelijk,

maar het staat als een huis en geeft eens goed tegengas... top! Betty en Yvon, zonder jullie ondersteuning en hulp bij alle administratie zou het leven van een PhD een stuk onaangenamer zijn. Dank daarvoor (en voor het voor de 27e keer doorsturen van, ik noem maar wat... de ANU-PhD guide ;)!

Melanie, je was dan formeel wel geen collega maar de samenwerking was er niet minder om! Altijd fijn om met een nog enthousiastere collega koeien-arts uren te kunnen filosoferen over pensadaptatie, weidegang, en vetzuren! Hopelijk creëren we in de toekomst nog gelegenheden om samen te werken!

When spending 5 years at ANU you see a lot of colleagues come and go. Thank you all for your contribution to ANU, during office hours or thereafter, it makes it an excellent place to work and you are a great group of people to work with! Twee collega's wil ik toch in het bijzonder bedanken. Geronda, heel erg bedank voor de samenwerking, gezelligheid, en je input als writing-buddy op al mijn schrijfsels! Een leuk verhaaltje maken van een artikel valt me niet altijd mee, bedank voor je hulp daarbij. Fijn dat je als paranimf bij mijn promotie aanwezig bent. Enne, het onderzoek is inderdaad vaak samen te vatten met... 2 woorden, 9 letters! Pierre, not only did we have many scientific (and many more not-so scientific) discussions (think about it!!), you also turned out to be a great partner in crime when it came to organizing stuff for ANU, creating havoc, and otherwise enlivening the situation in the odd circumstance that there was a risk for boredom. Next to that, you turned out to be a great friend, spinning partner (TSUNAMI!!!), and race biking companion (but running... no... there are limits). Glad to have you as paranymph during the defence. Thanks!

Naast ANU heb ik tijdens mijn promotieonderzoek ook 3 jaar bij Carus door kunnen brengen. Rob en Frits, bedankt voor jullie uitnodiging om het Carus team te komen versterken. Het was een mooie aanvulling op het werk als promovendus en goede gelegenheid om mijn achtergrond als dierenarts te blijven benutten. Geurt, bedankt voor de goede samenwerking! Ik ben blij dat ik mijn bijdrage aan het ontwikkel-traject en het MT-team heb kunnen leveren. Ries en alle dierverzorgers, jullie bedankt voor de samenwerking bij de diverse proeven, het onderwijs, en de trainingen. Keep up the good work!

De laatste paragraaf is natuurlijk voor mijn familie en vrienden. Dank voor alle support, afleiding, en gezonde desinteresse in het onderwerp. Eén penswand enthousiasteling per familie is echt meer dan genoeg! Ties en Inge, heerlijk om met jullie alle promotie-frustraties te kunnen delen. Ties, Ties K., en Gert... rondje Zeist? Pa, ma, en Mirjam, ik ben nu wel een beetje uitgestudeerd.... Dank voor jullie steun, het heeft zijn doel bereikt!

Lieve Amanda, dank je voor al je liefde en support. Als ik weer eens 's nachts zo nodig moest piekeren over wat er allemaal nog gedaan moest worden of hoe iets te fixen, helpt echt niets beter als jouw coaching: kan en ga je er nú (2 uur 's nachts) iets aan doen? Nee? Ga dan gewoon slapen! Gelukkig is dit boekje nu afgerond en heb ik weer meer tijd voor al onze projecten. Eens denken... de garage verbouwen? tuin renoveren? fietsenhok vernieuwen? Ow wacht even, we doen ze gewoon naast ons meeste recente projectje... Ylva!

Kasper

About the author

Curriculum vitae
Overview of scientific publications
Training and supervision plan

CURRICULUM VITAE

Kasper Dieho was born on March 29, 1981 and grew up in Alphen aan den Rijn, the Netherlands. After finishing secondary school (Scala College, Alphen aan den Rijn, the Netherlands) in 2000, he studied at Utrecht University, obtaining his doctorandus degree in Veterinary Medicine in 2004. In 2007, he graduated as a veterinarian with a specialisation in large animal veterinary medicine. After graduating, Kasper worked as a ruminant practitioner and teacher at the Department of Farm Animal Health (Utrecht University) and subsequently the Universitaire Landbouwhuisdieren Praktijk (Harmelen, the Netherlands). Starting November 2010, Kasper worked on the 'Weerbaar Vee' project at the Animal Health Service (Deventer, the Netherlands). After completion of phase one of 'Weerbaar Vee', Kasper started his PhD at Wageningen University & Research (Wageningen, the Netherlands) in November 2011. From November 2012 to November 2015, next to his responsibilities as PhD-candidate, Kasper took up the position of veterinarian and policy officer at Research Facility 'Carus' (Wageningen University & Research), and was a member of its management team. In November 2016, Kasper joined Cargill as Technical Application Specialist Ruminants for Western Europe at Cargill Premix and Nutrition (Provimi).

OVERVIEW OF SCIENTIFIC PUBLICATIONS

Peer-reviewed scientific publications related to this thesis

Dieho, K., B. van der Bogert, G. Henderson, A. Bannink, J. Ramiro-Garcia, H. Smidt, and J. Dijkstra. 2017. Changes in rumen microbiota composition and in situ degradation kinetics during the dry period and early lactation as affected by rate of increase of concentrate allowance. Journal of Dairy Science *Accepted for publication*

Dieho, K., J. Dijkstra, G. Klop, J. T. Schonewille, and A. Bannink. 2017. The effect of supplemental concentrate fed during the dry period on morphological and functional aspects of rumen adaptation in dairy cattle during the dry period and early lactation. Journal of Dairy Science 100:343-356. http://dx.doi.org/10.3168/jds.2016-11575

Dieho, K., A. Bannink, I. A. L. Geurts, J. T. Schonewille, G. Gort, and J. Dijkstra. 2016. Morphological adaptation of rumen papillae during the dry period and early lactation as affected by rate of increase of concentrate allowance. Journal of Dairy Science 99:2339-2352. http://dx.doi.org/10.3168/jds.2015-9837.

Dieho, K., J. Dijkstra, J. T. Schonewille, and A. Bannink. 2016. Changes in ruminal volatile fatty acid production and absorption rate during the dry period and early lactation as affected by rate of increase of concentrate allowance. Journal of Dairy Science 99:5370-5384. http://dx.doi.org/10.3168/jds.2015-10819.

Dieho. K., J. van Baal, L. Kruijt, A. Bannink, J.T. Schonewille, D. Carreño, W.H. Hendriks, and J. Dijkstra. Changes in rumen epithelium gene and protein expression during the transition period. Journal of Dairy Science *Submitted*

Other peer-reviewed scientific publications

Klop, G., A. Bannink, K. Dieho, W.H. Hendriks, and J. Dijkstra. 2017. Enteric methane production in lactating dairy cows with continuous feeding of essential oils or rotational feeding of essential oils and lauric acid. Journal of Dairy Science *Accepted for publication*

Klop, G., A. Bannink, K. Dieho, W.J.J. Gerrits, and J. Dijkstra. 2016. Short communication: Using diurnal patterns of ^{13}C enrichment of CO_2 to evaluate the effects of nitrate and docosahexaenoic acid on fiber degradation in the rumen of lactating dairy cows. Journal of Dairy Science 99:7216-7220. http://dx.doi.org/10.3168/jds.2016-11148

Schären, M., G. M. Seyfang, H. Steingass, K. Dieho, J. Dijkstra, L. Hüther, J. Frahm, A. Beineke, D. von Soosten, U. Meyer, G. Breves, and S. Dänicke. 2016. The effects of a ration change from a total mixed ration to pasture on rumen fermentation, volatile fatty acid absorption characteristics, and morphology of dairy cows. Journal of Dairy Science 99:3549–3565. http://dx.doi.org/10.3168/jds.2015-10450

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

Velkers, F. C., K. Dieho, F. W. M. Pecher, J. C. M. Vernooij, J. H. H. van Eck and W. J. M. Landman. 2011. Efficacy of allicin from garlic against *Ascaridia galli* infection in chickens. Poultry Science 90:364-368. http://dx.doi.org/10.3382/ps.2010-01090

Contributions to conferences, symposia and other scientific output

Dieho, K., J. Dijkstra, J.T. Schonewille, and A. Bannink. 2016. PRELIMINARY RESULTS. Feeding supplemental concentrate during the dry period: can we prepare the rumen for the lactation ration? Proceedings of the 41th Animal Nutrition Research Forum. Wageningen, the Netherlands

Dieho, K., A. Bannink, J.T. Schonewille, and J. Dijkstra. 2015. Effect of lactation stage and rate of increase of concentrate allowance on rumen adaptation in dairy cows. Book of abstracts of 2015 Joint Annual Meeting of ADSA-ASAS. Orlando, CA, USA

Dieho, K., A. Bannink, J.T. Schonewille, and J. Dijkstra. 2015. Adaptation of the rumen in transition dairy cattle: does function follow form? Proceedings of the WIAS Science Day. Wageningen, the Netherlands

Dieho, K., A. Bannink, J.T. Schonewille, and J. Dijkstra. 2014. Morphological aspects of rumen adaptation in dairy cattle during the dry period and lactation. Proceedings of the WIAS Science Day. Wageningen, the Netherlands

Dieho, K., A. Bannink; J.T. Schonewille, and J. Dijkstra. 2014. Morphological aspects of rumen adaptation in dairy cattle during the dry period and lactation. Proceedings of the 39th Animal Nutrition Research Forum. Utrecht, the Netherlands

TRAINING AND SUPERVISION PLAN

Completed in fulfilment of the requirements for the Education Certificate
of the Wageningen Institute of Animal Sciences

The Basic Package (3.0 ECTS[1])	Year
WIAS Introduction Course	2011
Philosophy and Ethics of Science	2013

Scientific Exposure (15.3 ECTS)

International conferences

International Veterinary Congress "Getting ready for a sustainable and healthy future", Rotterdam, the Netherlands	2010
Annual Centre for Animal Nutrition (CAN) International Dairy Nutrition Symposium, Wageningen, the Netherlands	2011, 2012, 2014, 2015
Joint Annual Meeting (JAM) of the American Dairy Science Association (ADSA) and American Society of Animal Science (ASAS), Orlando, Florida, USA	2015
16th International Conference on Production Diseases in Farm Animals, Wageningen, the Netherlands	2016

Seminars and workshops

GD-Boerderij "Gezonde Melkveehouderij" Congres, Zwolle, the Netherlands	2015
Annual WIAS Science Day, Wageningen, the Netherlands	2012, 2013, 2014, 2015
Annual Animal Nutrition Research (ANR) Forum, Wageningen, Utrecht, or Ghent	2012, 2014, 2015, 2016
Wetenschappelijke bijeenkomst VGH-KNMVD, Utrecht, the Netherlands	2012, 2014

Presentations

International Veterinary Congress (oral presentation)	2010
ANR Forum (oral presentation)	2014
WIAS Science Day (oral presentation)	2014
GD-Boerderij "Gezonde Melkveehouderij" Congres (oral presentation)	2015
WIAS Science Day (poster)	2015
JAM ADSA-ASAS (poster)	2015
Symposium Veefokkers, Universiteit Utrecht (oral presentation)	2015
CAN Symposium "Nutrition and Animal Health" (oral presentation)	2015
ANR Forum (oral presentation)	2016

[1]one ECTS credit equals a study load of approximately 28 hours

In-Depth Studies (7.8 ECTS)

Disciplinary and interdisciplinary courses

Cursus Rundveevoeding, Wageningen Business School	2008
Statistics for the Life Sciences, WIAS	2012
Advances in Feed Evaluation Science, Wageningen Business School	2013
Advanced Statistics of Experimental Design, WIAS	2013
Nutrient Dynamics, Animal Nutrition Group, Wageningen University and Research	2015

Statutory Courses (3.0 ECTS)

Use of Laboratory Animals (Article 9 certification)	2011

Professional Skills Support Courses (5.1 ECTS)

Teaching and Supervising Thesis Students	2012
Information Literacy and Endnote	2012
Course Techniques for Scientific Writing,	2014
Workshop Appreciative Inquiry	2014
Writing Grant Proposals	2015

Research Skills Training (2.9 ECTS)	**Year**
Preparing PhD Research Proposal	2012
Feline Husbandry, Handling, and Experimental Techniques	2013
Endoscopy in Research	2015

Didactic Skills Training (14.3 ECTS)

Supervising practicals and lectures

Principles Animal Nutrition, guest lecture	2012
Principles of Animal Nutrition, practicals	2012
Animal Nutrition and Physiology, practicals	2012
Toegepaste Dierbiologie, practicals	2012, 2013, 2014, 2015, 2016
Nutrient Dynamics, practicals	2015, 2016

Supervising theses

MSc Thesis, 3×	2012, 2013, 2014
BSc Thesis, 2×	2012, 2013
MSc Research Internship	2015

Tutorship

Inleiding in de Dierwetenschappen	2012, 2013, 2014

Management Skills Training (6.0 ECTS)

Organisation of seminars and courses

150th Anniversary Symposium "Duurzaam Duet" of the KNMvD, Utrecht, the Netherlands	2012

Membership of boards and committees

WAPS Council Member - Education Committee	2012
Management Team member CARUS	2012-2015

Education and Training Total   **57.7 ECTS[1]**

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[1] one ECTS credit equals a study load of approximately 28 hours

COLOPHON