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Inter-individual variation in nitrogen and phosphorus metabolism and excretions in lactating Holstein dairy cows

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Dedicated to my always supporting and loving family

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AA	amino acids
AD	apparent digestibility
ADDM	apparent digestibility of dry matter
ADH	antidiuretic hormone
ADP	apparent digestibility of phosphorus
AP	alkaline phosphatase
APE	atom percent excess
AQP	aquaporin
Arg	arginine
ATP	adenosine triphosphate
AUC	area under curve
BactN	bacterial nitrogen
BBMV	brush border membrane vesicles
BCS	body conditions score
BW	body weight
CCR/C-CR	renal creatinine clearance rate
CF	crude fiber
Cit	citrulline
CP	crude protein
CREACR	renal creatine clearance rate
D	dosage
DM	dry matter
DMI	dry matter intake
EB	energy balance
ECM	energy corrected milk yield
EE	ether extract
END	unbound endogenous nitrogen
FN	fecal nitrogen excretion
FP	fecal phosphorus excretion
GE	gross energy
GER	gastrointestinal tract urea entrance rate
GHG	greenhouse gas
Gln	glutamine
Gly	glycine
H_2PO^{4-}	monovalent dihydrogen phosphate
HMU	high milk urea concentration
HP	heat production
HPeff	high phosphorus utilization efficiency
HPO_4^{2-}	bivalent monohydrogen phosphate
ICP-OES	inductively coupled plasma-optical emission spectrometry
K_{Urea}	fractional ^{13}C urea turnover rate
LBW	live body weight
LMU	low milk urea concentration
LP	low protein
LPeff	low phosphorus utilization efficiency
LSM	least square means
mBW	metabolic body weight
ME	metabolizable energy
MEI	metabolizable energy intake
MPE	mol percent excess
MRT	mean residence time
MUN	milk urea nitrogen
NaP_i	sodium/inorganic phosphorus cotransporter
NDF	neutral detergent fiber
NEFA	non-esterified fatty acids
Neff/NUE	nitrogen utilization efficiency
NE_L	net energy for lactation
UT	urea transport proteins

NI	nitrogen intake
NP	normal protein
NPN	non-protein nitrogen
OMI	organic matter intake
Orn	ornithine
P-CR	renal phosphorus clearance rate
PD	purine derivatives
Peff	phosphorus utilization efficiency
Pi	inorganic phosphorus
PI	phosphorus intake
PiT	inorganic phosphorus transporter
PRR	renal phosphorus reabsorption rate
PU	plasma urea
Q	urea pool size
qPCR	quantitative real-time polymerase chain reaction
RAS	renin-angiotensin system
RDP	rumen degradable protein
RRR	renal urea reabsorption rate
RUN	ruminal undegraded feed nitrogen
SCC	somatic cell content
SCFA	short chain fatty acids
SE	standard error
SEM	standard error mean
Ser	serine
SLC20A	Solute Carrier Family 20
SLC34A	Solute Carrier Family 34
TG	triglycerides
UACR	renal uric acid clearance rate
UCR	renal urea clearance rate
UER	plasma urea entry rate
UHR	urea hydrolysis rate
UUN	urinary urea nitrogen
VFA	volatile fatty acids

Chapter 1: Extended introduction

The mitigation of nitrogen (N) and phosphorus (P) pollution derived from anthropogenic sources is one of the leading challenges of the 21st century. The global human population is estimated to peak near 11 billion people by the end of the century (UN, 2019), which subsequently will result in increased demands for food and water. Furthermore, the increased demand will likely lead to more intensive resource usage and environmental pollution of N and P (IPCC, 2019). Therefore, in order to minimize the risk towards human, animal, and environmental health, combating the harmful effects of N and P pollution on soil, air, and water quality, biodiversity, and the progressing climatic change caused by greenhouse gas (GHG) emissions is of great importance (Smirnov et al., 2016; Leip et al., 2015). Moreover, because global resources are limited and P resources have been estimated to be depleted within the next 50 to 100 years, a sustainable, efficient, and mindful use of available resources is needed (Cordell et al., 2009).

To this end, the European Green Deal aims to reduce GHG emissions by 55% from 1990 levels by 2030 and to achieve climate-neutrality of the European Union by 2050 in line with the obligations of the Paris Agreement (of the United Nations Framework Convention on Climate Change) (EU, 2021). The livestock sector constitutes a major source of N and P pollution that accounts for 23% of global nitrous oxide (N_2O) and 60% of global ammonia emissions (Uwizeye et al., 2020), both of which either directly or indirectly facilitate climate change (Hristov et al., 2011), as well as for more than 73% of agricultural P loads (Leip et al., 2015). Cattle farming contributes half of total N and P in animal manure (Liu et al., 2017) and 65% of GHG emissions derived from livestock production (Gerber et al., 2013); thus, requiring the development of holistic mitigation approaches in this field (IPCC, 2019).

Previous attempts at mitigating N and P pollution primarily focused on developing feeding strategies (Ouatahar et al., 2021). The most widely and intensively researched feeding strategy was to lower dietary N and P content in a manner that resulted in reduced N and P losses but concurrently improved feed utilization efficiency in dairy cows (Ouatahar et al., 2021). Predictive models revealed milk urea N (MUN) concentration linearly reflects urinary N emissions and an increase in milk P yield attenuates fecal P loss as more ingested P flows into milk production (Alvarez-Fuentes et al., 2016; Burgos et al., 2007). Regardless, a considerable portion of inter-individual variation in MUN concentration and milk P yield among lactating cows remains unexplained. Therefore, the present thesis focused on elucidating physiological and molecular mechanisms underlying these inter-individual differences in MUN and feed P utilization efficiency (Peff) with preference towards the latter due to dwindling global P reserves. Obtained results could prompt selective breeding for low MUN and high Peff cows as proposed earlier (Marshall et al., 2020; Alvarez-Fuentes et al., 2016) and used for mitigating

environmental concerns and improve feeding strategies; thus, enhancing the environmental sustainability of dairy industry.

Chapter 2: Literature review

2.1. Specific aspects of nitrogen and phosphorus digestibility in ruminants

2.1.1. Ruminal microbial protein synthesis

The difference between ruminants and nonruminants is characterized by a unique symbiotic relationship with ruminal-colonizing microorganisms that enable anaerobic fermentation, and thus allowing the utilization of an extended range of plant feed in ruminants. The level of microbial metabolic activity largely determines the level of energy and protein metabolism of the host and influences the respective feed conversion, animal performance, excretions of N and P, and methane emissions. Complex interacting and synchronized processes between N and energy metabolism within ruminal microbial community determine the quality and quantity of microbial protein synthesis (Walker et al., 2005; Wolin et al., 1997). The latter is affected by amount and structure of ruminal N and energy supply, but also P availability was found to be a limiting factor with respect to the level of microbial protein synthesis (Kebreab et al., 2005), demonstrating the connective link between N and P metabolism in ruminants.

2.1.1.1 Nitrogen

Ruminal ammonia, appearing mainly as ammonium at a physiological and mean pH of 6.5 (Abdoun et al., 2006), as well as preformed amino acids (AA) and peptides, serve as the predominant N source for *de novo* synthesis of microbial protein (Walker et al., 2005). On average, 50% of ruminal ammonia is derived from dietary protein N and non-protein N (NPN) supply (Abdoun et al., 2006). Dietary NPN sources are headed by exogenously supplemented urea, which in turn, is rapidly hydrolyzed to ammonia and carbon dioxide by the bacterial enzyme urease in order to become available for microbial N metabolism (Walker et al., 2005). Ruminal urea abundance has been described to be the rate-limiting factor determining urea conversion into ammonia, because microbial ureolytic activity exceeds the availability of ruminal urea in general (Patra and Aschenbach, 2018). The derivation of ammonia from dietary protein is preceded by a complex catabolic cascade from protein to ammonia involving proteolysis, peptidolysis, and deamination, which is largely driven by ruminal proteolytic bacteria (Brock et al. (1982); Figure 1). Although plant-derived proteinases contribute to the initial stages of protein breakdown in the rumen (Attwood, 2005), the localization and thus accessibility of plant-derived protein for catabolic proteolytic processes varies between different feedstuffs (Walker et al., 2005). Thus, the structure and type of dietary protein N plays a key role in determining the respective ruminal degradability (Rodriguez et al., 2007).

Overall, ammonia constitutes a sufficient N source for ruminal microbial growth and protein synthesis provided that availability of ruminal energy is not limiting *de novo* synthesis of AA and peptides (Leng and Nolan, 1984). However, the incorporation of preformed AA and peptides into microbial protein requires less ATP than the incorporation of ammonia N into

microbial protein (Walker et al., 2005). Therefore, preformed AA and peptide N constitute the preferred N sources for microbial protein synthesis (Walker et al., 2005). According to Satter and Roffler (1975), a ruminal ammonia content below 5 mg/100 mL of rumen fluid, usually reached with a dietary crude protein (CP) content between 12 and 13% in dry matter (DM), results in preformed AA, peptides, and ammonia being equally utilized as microbial N sources. However, ammonia contents exceeding 5 mg/100 mL rumen fluid elicit a preferred utilization of preformed AA and peptides as N sources causing an excessive hydrolysis of NPN and protein N to ammonia without microbial utilization (Satter and Roffler, 1975). As a consequence of the latter, a dietary CP content between 12 and 13% in DM was recommended to be adequate for maximizing microbial protein synthesis and microbial N utilization efficiency (Neff) in dairy cows (Satter and Roffler, 1975; Satter and Slyter, 1974).

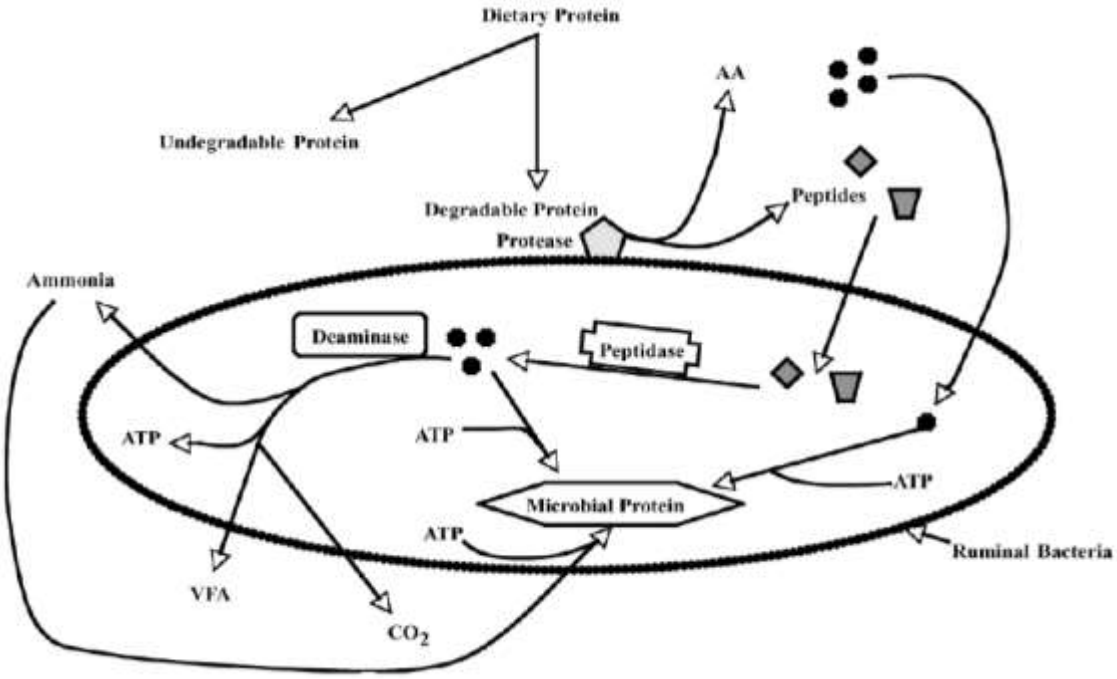

Figure 1. Schematic representation of dietary protein degradation in ruminal bacteria including symbolic illustrations of amino acids (black circles), peptides (grey squares) and degradable protein (pentagons). ATP = adenosine triphosphate; VFA = volatile fatty acids (Reprinted from Publication by Bach et al. 2005, Copyright (2021), with permission from Elsevier).

2.1.1.2 Energy

The availability of fermented energy constitutes the first limiting factor determining the level and efficiency of microbial growth (Lu et al., 2019; Zhou et al., 2015). Several studies highlighted an interaction between dietary energy and protein intake and stated that a constant and synchronized release of both should positively affect microbial protein yield (Castillo et al., 2000; Sinclair et al., 1995; Herrerasaldana et al., 1990). The availability of ATP as energy

source determines the fate of AA and N components once absorbed into microbial cells. For example, when ATP supply is high, they can be directly utilized for microbial protein synthesis but, if ATP supply is low, they are deaminated to ammonia (Lu et al., 2019; Bach et al., 2005; Tamminga, 1979) (Figure 1). Major factors influencing availability of fermented energy are the amount of ingested feed and diet quality as well as the structure of supplied carbohydrates (Hristov et al., 2005; Lee et al., 2003).

The fermentation of structural carbohydrates, such as cellulose and hemicellulose, provides ATP for microbial activity and short chain fatty acids (SCFA), the major energy source for the host for a long-term period post-feeding (Leedle et al., 1986). However, cellulolytic fermenting processes shift the ruminal SCFA pattern towards acetate in expense of propionate formation (Bannink et al., 2008), thus releasing more molecular hydrogen than the synthesis of propionate (Dijkstra et al., 2011). Accordingly, acetate formation has been shown to increase microbial interspecies hydrogen transfer between cellulolytics and methanogens (Morgavi et al., 2010; Wolin et al., 1997), thus justifying a high fiber diet to be associated with increased ruminal methanogenesis and loss of energy (Vaidya et al., 2020; Niu et al., 2016; Morgavi et al., 2010). Non-structural carbohydrates, containing soluble sugars and starch, show a high fermentation rate that initially delivers higher levels of ATP post-feeding compared to structural carbohydrates but provide almost no ATP in the long-term (Leedle et al., 1986). Moreover, the fermentation of non-structural carbohydrates favors the ruminal synthesis of propionate and reduces hydrogen availability for hydrogenotrophic methanogenesis, the dominate pathway of methanogens (Vaidya et al., 2020). Nevertheless, a high fermentation rate and rapid synthesis of SCFA decrease ruminal pH to a greater extent and thus increase the risk of ruminal acidosis (Hünerberg et al., 2015), affecting microbial community structure (Sato, 2016) and concomitantly impairing microbial proteolytic activity (Bach et al., 2005). Considering this information, combining structural and non-structural carbohydrate sources results in a constant degradation and release of fermented energy (Hoover and Stokes, 1991), ameliorating incorporation of N into microbial *de novo* synthesized protein.

2.1.1.3 Phosphorus

Ruminal P supply constitutes one limiting factor determining the level of microbial synthesis of ATP, nucleic acids, and phospholipids (Komisarczuk et al., 1987; Bucholtz and Bergen, 1973), and is essentially involved into ruminal microbial protein synthesis, cellulose, and DM digestibility (Kincaid and Rodehutscord, 2005; Breves and Höller, 1987). Accordingly, Komisarczuk et al. (1987) demonstrated that insufficient ruminal P content impairs microbial protein synthesis. The infusion of a P-deficient (0 mg/d) compared to a P-containing (120 mg/d) buffer solution into rumen fluid for 11 days resulted in a reduction of microbial protein synthesis by up to 45% with declining ruminal P supply (Komisarczuk et al., 1987).

Dietary P is predominantly ingested in the form of plant-derived phytates (> 65%; *myo*-inositol-1,2,3,4,5,6-hexakisdihydrogenphosphat), which vary in amount and location among feedstuffs as well as in accessibility for enzymatic degradation (Humer and Zebeli, 2015; Kiarie and Nyachoti, 2010). Additionally, the enzymatic activity of plant intrinsic phytases (*myo*-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases), which hydrolyze phytate P, have been described to vary greatly among different plant species (Humer and Zebeli, 2015). However, the latter is of less importance for ruminants (Haese et al., 2017), since primarily ruminal bacterial phytases (Yanke et al., 1998), followed by large intestinal microbial phytases, are capable of sufficiently catalyzing phytate P hydrolysis along the rumen-intestinal tract (Humer and Zebeli, 2015). Along with nonruminants, endogenous mucosal phytases located in the small intestine are negligibly involved in phytate P catabolism (Humer et al., 2015; Humer and Zebeli, 2015).

Overall, the efficiency of ruminal phytate hydrolysis remains controversial. Ray et al. (2013) found dietary phytate to be almost completely hydrolyzed by ruminal bacterial phytases, concluding that the chemical form of P ingested has less of an impact on digestibility of P than the amount of dietary P intake. The latter has been supported by Morse et al. (1992), reporting phytate hydrolysis to be greater than 99% in lactating cows. However, Kincaid et al. (2005) found supplementation of exogenous phytases to feed rations containing corn or barley increased phytate degradability by 4.8% and 7% in lactating cows, respectively. Kincaid et al. (2005) assumed complete phytate hydrolysis might be prevented by limiting microbial exposure, depending on feed characteristics and ruminal passage rate. Accordingly, Klop et al. (2013) showed that a low dietary protein supply impaired P digestibility in dairy cows, which in turn diminished ruminal microbial growth and microbial P incorporation.

2.1.1.4 The mutual microbial interaction determining efficiency and emissions

The aforementioned contributing factors emphasize the mutual interacting relationships between ruminal N, P, and energy metabolic pathways among the microbial community (Kincaid and Rodehutscord, 2005; Wolin et al., 1997). Maximizing efficiency of microbial protein synthesis demands for a balanced, constant release and sufficient availability of N, P, and energy positively affecting feed digestibility and feed conversion into milk (Guinguina et al., 2019; Castillo et al., 2000). Approximately 80% of ruminal microbial protein is digested within small intestine (Lu et al., 2019) with two thirds of total intestinally absorbed AA originating from microbial protein (Pathak, 2008). Accordingly, Xue et al. (2020) showed the level and pattern of ruminal microbial protein to influence milk protein yield, milk production, and animal health (Diether and Willing, 2019; Cant et al., 2018). Moreover, milk protein and milk P yield are phenotypically and genetically positively correlated (Visentin et al., 2019; Klop et al., 2014),

supporting that microbial protein synthesis affects Peff and milk P yield as well (Kebreab et al., 2005).

On commercial farms, dairy cows are commonly fed N and P in excess of the recommendations by the NRC (2001) in order to avoid potential nutrient deficiencies impairing milk production. However, if ruminal ingested dietary N and P exceed the capacity of microbial incorporation, high levels of ruminal ammonia and non-absorbable phytate P cause increasing urinary N and fecal P losses, respectively (Chadwick et al., 2018; Lapierre et al., 2005; Tamminga, 1992). Kauffman and St-Pierre (2001) showed that elevating dietary N intake from 445 to 566 g/d in dairy cows yielding 28 kg of milk per day doubled urinary N excretions (97 vs. 188 g/d), while milk N (147 vs. 157 g/d) and fecal N excretion (181 vs. 198 g/d) only slightly increased. Data from Kauffman and St-Pierre (2001) also emphasized the relative distribution of ingested N shifted from fecal N (40.9 vs. 35%) and milk N (33 vs. 28%) towards urinary N excretion (22 vs. 33%) with increasing dietary N intake. Knowlton and Herbein (2002) increased dietary P content from 0.34% to 0.67% in feed DM, resulting in a 17% increase in fecal P loss per dietary P intake (50% vs. 67%), whereas milk P yield decreased from 54% to 26% of dietary P intake. Since percentage of urinary P loss amounted 0.4% and 2.4% of dietary P intake, respectively, urinary P loss seems to be of negligible magnitude. Given this information, these elevated N and P emissions from cattle husbandry are associated with negative environmental effects as discussed in the introductory section (Leip et al., 2015). Furthermore, feeding P and N in excess of requirements attenuates Peff and feed Neff for milk synthesis (Dijkstra et al., 2013a; Pfeffer et al., 2005), which is particularly important with respect to dwindling global P reserves (Cordell et al., 2009). Diether and Willing (2019) suggest that an excess of dietary N supply affects long-term nutritional patterns and may have an impact on animal health caused by the interactions between microbial and host metabolism.

During the past years, several feeding strategies have been developed to reduce the environmental impact by cattle husbandry. The most widespread and intensively researched mitigation strategy is the reduction of dietary N and P content relative to dietary energy content in order to enhance microbial and host Neff and Peff and its conversion towards milk production (Guo et al., 2019; Klop et al., 2013; Dijkstra et al., 2011). However, the amount and source of fermented energy largely impact the level of methane emitted and ruminal pH as previously discussed. Therefore, the challenge remains to develop mitigation strategies reconciling the reduction of N and P excretions and methane emissions at a high level of milk production while not impairing animal health (Ouatahar et al., 2021; Dijkstra et al., 2011). An indispensable and unique key mechanism in ruminants to be taken into account is the ability of recycling endogenous N and P into the digestive tract. Particularly in times of low or even insufficient dietary N and P supply, these recycling mechanisms are of great importance to maintain a high

level of microbial protein synthesis and to ensure animal survival (Batista et al., 2017; Abdoun et al., 2006).

2.1.2. Endogenous recycling of urea and phosphorus

2.1.2.1. Urea

Lapierre et al. (2005) described N absorption to primarily occur in the rumen and intestines as either ammonia or free AA in dairy cows, with absorption of N ranging between 29-83% (mean 58%) and 27-108% (mean 57%) of dietary ingested N, respectively. On average, 50% (up to 80%) of ruminal ammonia N, and between 20% and 40% of intestinally absorbed ammonia N, result from endogenously secreted urea N (Abdoun et al., 2006; Lapierre et al., 2005; Lapierre and Lobley, 2001). The endogenous secretion of urea N accounts for the occasionally noted excess of absorbed ammonia N beyond ingested dietary N (Lapierre et al., 2005). Lipophilic ammonia is effectively absorbed by passive non-ionic diffusion from the rumen-intestine into the portal vein following the concentration gradient (Abdoun et al., 2006; Tan and Murphy, 2004). However, according to the Henderson-Hasselbalch equation, at a physiological rumen pH 6-7, 98.7% to 99.9% of ammonia is ionized to ammonium (Abdoun et al., 2006). Since the ruminal membrane is 175 times less permeable for ammonium than for ammonia, apical K^+-channels function as active carriers for ammonium (Abdoun et al., 2005). Additionally, absorption of non-ionized SCFA and bicarbonate have been found to serve as proton donors that promote the formation of intracellular ammonium, and thus the absorption of ammonia as well (Bodeker et al., 1992). Absorbed ammonia reacts with alpha-ketoglutarate, converts into glutamine and alanine within glucose-alanine cycle (Nelson and Cox, 2013) to be safely transported within bloodstream.

In order to avoid hyperammonemia, a condition associated with toxic pathophysiological effects on various organs, essentially all ammonia and ammonium is removed and detoxified to urea by the liver (Parker et al., 1995). Hepatic ureagenesis from ammonia occurs concurrently with hepatic catabolism of AA to aspartate in order to supply the secondary N atom necessary for urea synthesis (Parker et al., 1995) (Figure 2). On average, 45% of absorbed AA from the portal vein are removed by the liver, depending on the AA, converted into urea or used for hepatic anabolic processes (Lapierre et al., 2005). However, it remains to be clarified whether the liver passively distributes AA to peripheral tissues or actively controls the fate of the absorbed AA (Lapierre et al., 2005). Overall, 43% to 123% (mean 88%) of ingested N is hepatically converted into urea (Lapierre and Lobley, 2001). Thus, on average only 12% of ingested N is directly available for anabolic processes such as the synthesis of hepatic proteins, peptides, free AA, and other N-containing metabolites such as creatine (Nelson and Cox, 2013). To summarize, the liver functions as a central hub of N

metabolism that determines the fate of absorbed dietary ingested N for either anabolic purposes or urea synthesis.

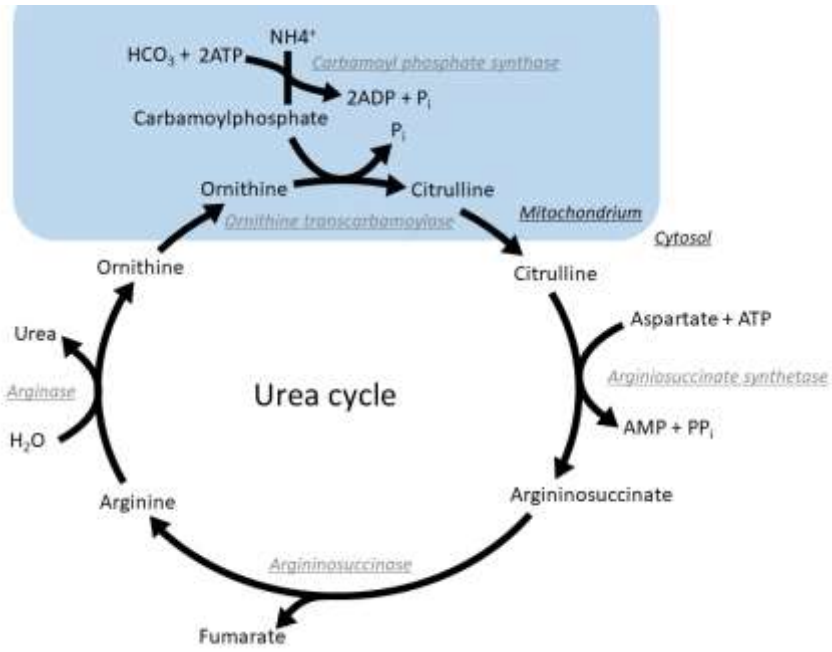

Figure 2. Schematic presentation of hepatic urea synthesis in ruminants following the description by Nelson and Cox (2013).

Distribution and catabolic fate of urea can be investigated by measuring the enrichments of intravenously applied isotope-labeled urea such as $^{15}N_2$ or ^{13}C urea as well as $^{13}CO_2$, the metabolic product of microbial ^{13}C urea hydrolysis (Hristov et al., 2019). Spek et al. (2013a) elucidated the influence of dietary N intake on rumino-hepatic urea circulation in dairy cows through the usage of an intravenously ^{13}C urea bolus. The latter revealed that the absolute ^{13}C urea transfer into the rumen-intestinal tract decreased by 20% (225 vs. 180 g urea N per day) when dietary N intake was reduced from 485 g to 356 g per day. However, the relative urea fraction transferred into the rumen-intestinal tract from whole plasma urea increased from 74% to 85% with decreasing dietary N intake (Spek et al., 2013a), buffering asynchronous dietary N and energy supply (Reynolds and Kristensen, 2008). Additionally, Marini and Van Amburgh (2005) demonstrated that ruminal bacterial N incorporation derived from endogenous urea increased from 2 g to 11 g per day as dietary N content decreased from 3.4% to 1.45% in DM. Finally, according to Lapierre et al. (2004), 52% of whole recycled urea is used for anabolic purposes, 39% is returned to the urea cycle, and 9% is lost in feces, increasing proportionally with dietary N intake and amount of absolute recycled urea (Wickersham et al., 2008). Remaining plasma urea, not secreted into the rumen-intestinal tract, is predominantly excreted with urine (mean 20%) and secreted with milk (mean 0.6%), while a negligible part is lost via skin and breath (Spek et al., 2013a).

In general, endogenous N, mainly urea N, is secreted along the length of the rumen-intestinal tract (Figure 3). However, Marini et al. (2008) estimated, if feeding a dietary N content of 24.2 g per kg feed organic matter, the majority (57%; 10.54 g N/kg organic matter intake (OMI)) of endogenous N enters the rumen compared to the small intestine (17%; 3.10 g N/kg OMI) and large intestine (26%; 5.00 g N/kg OMI) (Figure 3). Thus, major anabolic N conversion is attributed to the forestomach, whereas the majority of N absorption occurs at the small intestine (57%; 18.99 g N/kg OMI), followed by the rumen (29%; 9.57 g N/kg OMI) and large intestine (14%; 4.54 g N/kg OMI) (Figure 3).

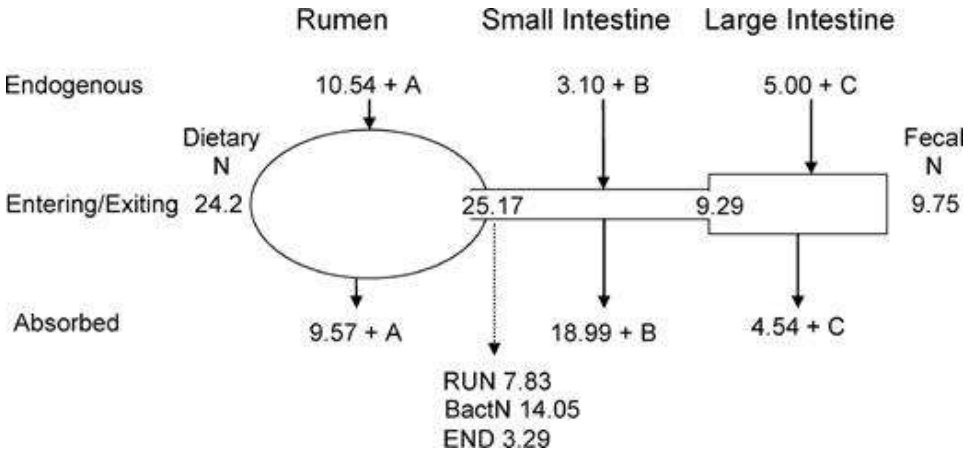

Figure 3. Nitrogen fluxes (in g of N per kg OMI) along the rumen-intestinal tract of dairy cows fed 24.2 g of N/kg feed OM. N flow into the small intestine contains ruminal undegraded feed N (RUN), bacterial N (BactN), and unbound endogenous N (END). Amounts of endogenous N entering or escaping the digestive tract (g of N/ kg OMI) constitute minimal estimated amounts with potential additional contributions indicated by the letters A, B, and C (Reprinted from Publication by Marini et al. 2008, Copyright (2021), with permission from Oxford University Press).

Salivary urea secretion accounts for 10 to 40% of whole ruminal urea entry (Stewart and Smith, 2005) and is strongly related to plasma urea concentration (Muscher et al., 2010). Moreover, since the dietary neutral detergent fiber fraction stimulates chewing activity, dietary fiber supply determines the amount of daily saliva production (Dix et al., 2013). Urea transfer from plasma to saliva is driven by urea transport protein (UT)-B located in the ductal system of salivary glands, which expression was found to be non-adaptable to dietary N intake (Ludden et al., 2009). Thus, with increased plasma urea concentrations owed to increasing dietary N intake, urea transfer capacity from UT-B becomes saturated indicating a limitation of adapting capabilities of salivary secretion (Muscher et al., 2010).

Urea transfer across the ruminal wall is found to be predominantly transcellular by simple lipid phase diffusion largely driven by the rumen-plasma concentration gradient (Jin et al., 2018; Abdoun et al., 2006). However, besides simple diffusion, UT-B was found to contribute to urea transfer localized in the ruminal papillae epithelial layer as well as in the

basolateral membrane of rumen enterocytes (Coyle et al., 2016; Stewart and Smith, 2005). Moreover, Walpole et al. (2015) found aquaporins (AQP), in particular AQP3, -7 and -10 to be expressed in the rumen wall and stated both, UT-B and AQP, to contribute to ruminal urea transport.

Besides the rumen-plasma urea concentration gradient, further dietary and ruminal factors have been revealed to be affecting urea transfer. Ureolytic activity of rumen wall-adherent bacteria rapidly convert secreted urea into ammonia and ammonium (Patra and Aschenbach, 2018). Consequently, urea hydrolysis near the rumen wall facilitates the driving force of the urea concentration gradient in the rumen, and subsequently, the absorption of ammonia and ammonium across the rumen wall into plasma, particularly in the presence of low dietary N supply (Patra and Aschenbach, 2018). Since ruminal concentrations of SCFA and CO_2 promote intracellular ammonia uptake (Bodeker et al., 1992) as mentioned above, butyrate and CO_2 were shown to upregulate bacterial ureolytic activity and thus epithelial permeability to urea (Abdoun et al., 2010). In addition, Patra and Aschenbach (2018) assumed an enhanced absorption of ammonia near the rumen wall contributed to regulating local pH, since a low pH is known to promote ruminal urea transfer. However, if dietary N supply generally exceeds the capacity of microbial N incorporation, high intra-ruminal ammonia concentrations may impair bacterial ureolytic activity in the long-term and urea influx (Cheng and Wallace, 1979). Nonetheless, these inhibitory effects of increasing ruminal ammonia concentrations with a higher dietary N intake are countered by linearly increasing plasma urea concentrations that promote the urea concentration gradient in the rumen. Consequently, the absolute amount of urea transfer into the rumen is positively related to dietary N supply owed to the maintained plasma-ruminal urea concentration gradient (Spek et al., 2013a). Conversely, the inhibition by increasing ruminal ammonia concentrations causes a decrease in relative urea transfer of whole urea synthesis as shown by Spek et al. (2013a).

2.1.2.2. Phosphorus

In general, salivary secretion of endogenous inorganic P (P_i) largely determines ruminal P availability (> 50%) regardless of dietary P intake (Pfeffer et al., 2005), but becomes even more important for microbial P incorporation with decreasing dietary P intake (Kincaid and Rodehutscord, 2005). Salivary P concentration is positively related to plasma P concentration, but salivary P is concentrated by a factor of 16 (Breves and Schröder, 1991). Thus, under condition of a sufficient dietary fiber supply (Dix et al., 2013; Huber et al., 2007), salivary P secretion accounts for 70-80% of total endogenous P secretion (Horst, 1986). According to Puggaard et al. (2011), salivary P secretion is not down regulated with low dietary P intake, but instead is maintained on a high level by recycling absorbed P at the expense of body P reserves prioritizing, the protection of rumen function from negative effects of depleted P

intake. Negative effects on rumen function consider P as a limiting factor for microbial growth as well, although less important compared to salivary bicarbonate secretion, as pH buffer neutralizing ruminal SCFA (Counotte et al., 1979). Approximately 80-85% of body P reserves are found in the skeleton, stored as hydroxyapatite (Grünberg, 2014) in conjunction with calcium, and are primarily resorbed to overcome long-term P depletion. The other 15-20% of body P reserves localized in plasma and soft tissue exist as bivalent monohydrogen phosphate (HPO_4^{2-}) and monovalent dihydrogen phosphate ($H_2PO_4^-$), both P_i, or as organic P bound to C-containing components and are more labile for short-term mobilization (Gidlund et al., 2015; Horst, 1986; Braithwaite, 1980). However, a high level of endogenous P secretion may exceed intestinal absorption processes and cause endogenous fecal P loss (Dou et al., 2002; Valk et al., 2002) ranging between 66 to 75% of whole fecal P (Pfeffer et al., 2005).

In line with nonruminants, the small intestine accounts for the majority of P absorption with P_i being absorbed paracellularly by passive diffusion or transcellularly involving active transport proteins (Puggaard et al., 2011). The passive paracellular diffusion is known to predominate with high luminal P concentrations whereas active transport mechanisms were shown to be saturable, and thus are of greater importance with low intestinal P flow (Wilkens and Muscher-Banse, 2020).

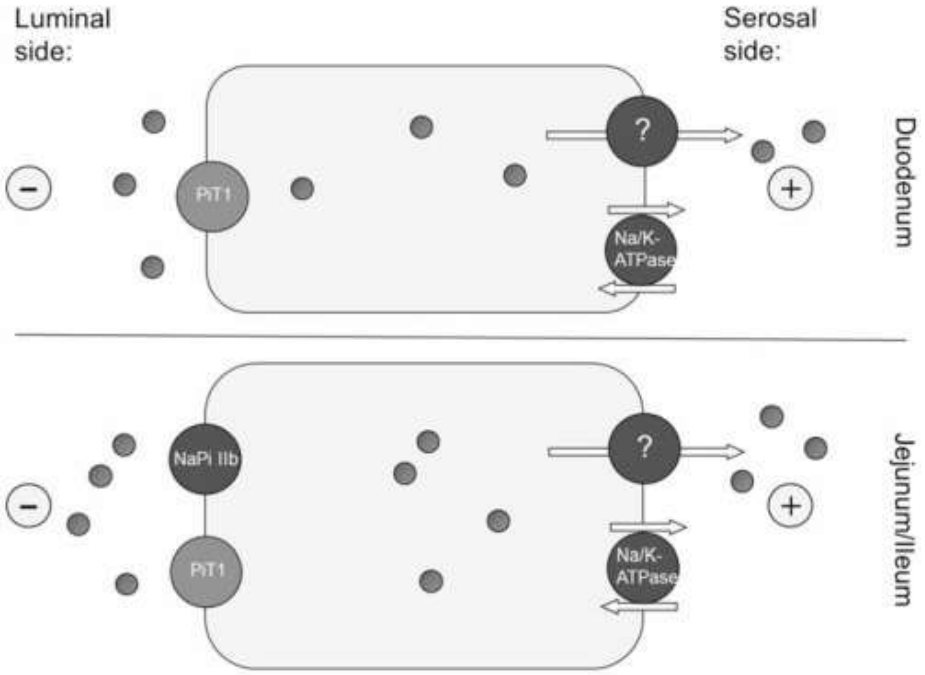

Figure 4. Intestinal P_i absorption by transport proteins localized in duodenal, jejunal and ileal enterocytes of ruminants (This figure was published by Wilkens and Muscher-Banse 2020 (https://doi.org/10.1017/S1751731119003197), Copyright Elsevier (2021) (https://creativecommons.org/licenses/by-nc-nd/4.0/)).

Within the duodenal lumen, P_i appears primarily as $H_2PO_4^-$, which facilitates the passive transfer across the intestinal wall from the mucosal to the serosal side since P_i is found as HPO_4^{2-} within the bloodstream (Pfeffer et al., 2005). However, with increasing distance to the stomach, intraluminal pH climbs from 2.4 to 8 at the terminal ileum, reducing the ratio between $H_2PO_4^-$ and HPO_4^{2-} (Pfeffer et al., 2005). Thus, passive absorption of P_i primarily occurs at the upper part of the small intestine within the first 0.05 to 0.6 m of the duodenum and represents the dominate P_i transport mechanism in this intestinal section (Pfeffer et al., 2005). Duodenal transcellular absorption of P_i into the duodenal brush border membrane vesicles (BBMV) has been shown to be H^+-dependent and Na^+-sensitive and is mediated by apical localized P_i transporter (PiT)-1 (*SLC20A1*) (Wilkens and Muscher-Banse, 2020) and basolateral Na^+/K^+-ATPase (Figure 4). Nevertheless, the majority of whole intestinal transcellular P_i absorption is mediated by Na^+-dependent and H^+-sensitive Na^+/P_i cotransporter (NaP_i) IIb transport proteins expressed on the apical side of enterocytes in the distal jejunum and ileum, yet nearly absent in the duodenal and proximal jejunal mucosa (Wilkens and Muscher-Banse, 2020; Foote et al., 2011) (Figure 4).

In the large intestinal section, the amount of secreted endogenous P may exceed absorption capacity, highlighting the leading role of small intestinal P absorption for whole P absorption capacity (Pfeffer et al., 2005) (Figure 5). According to Pfeffer et al. (2005), whole intestinal P absorption efficiency may range between 35% and 75%, depending on dietary P supply, digestibility of P, and respective physiological status. Remaining non-absorbed luminal P is excreted in feces, consisting of endogenous P, non-absorbable dietary P, and inevitable P losses of metabolic or microbial origin (Pfeffer et al., 2005). Finally, the amount and composition of fecal P has a great impact on the environmental fate of fecal P loss (Pagliari and Laboski, 2012).

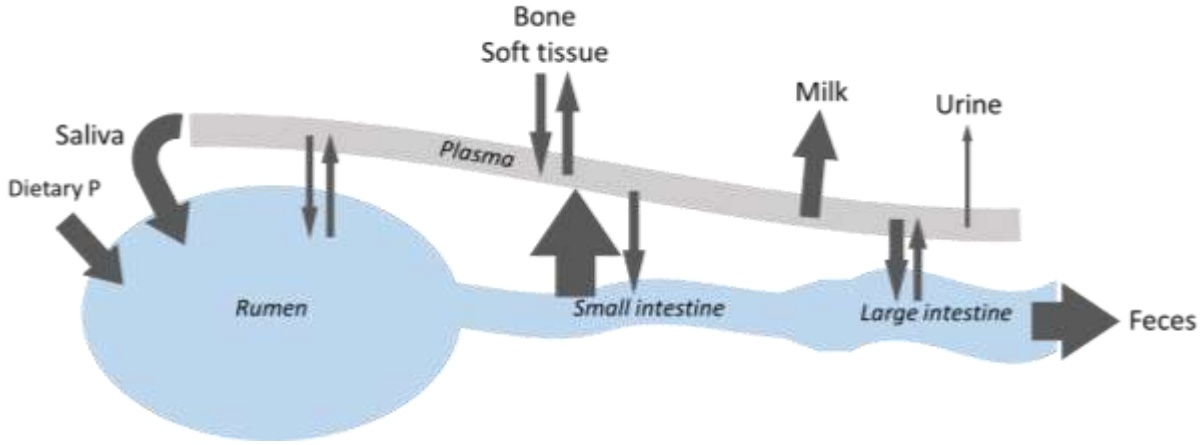

Figure 5. Schematic presentation of phosphorus flow in dairy cattle inspired by Goselink et al. (2015).

2.2. Renal function and excretions of nitrogen and phosphorus

Proper renal function is crucial in regulating water, electrolyte, and N homeostasis in mammals. Plasma water and small solutes are selectively ultra-filtrated across the glomerular membrane (Arif and Nihalani, 2013). The subsequent countercurrent multiplication within the nephric tubular system, involving alternating water-permeable and water-impermeable sections, allows for facilitated water reabsorption with concurrent urinary accumulation of osmolytically active uremic substances ((Weiner et al., 2015; Sands and Layton, 2009); Figure 6). Urine volume and composition are primarily regulated by the renin-angiotensin system (RAS) that is activated by a decrease in blood volume, blood pressure, or renal filtrate rate (Hoorn et al., 2020).

The RAS stimulates the synthesis of angiotensin II, eliciting the release of the steroid hormone aldosterone by the adrenal cortex and vasopressin, also called antidiuretic hormone (ADH), by the posterior pituitary lobe (Hoorn et al., 2020). The simultaneous actions of aldosterone and ADH emphasize the close osmolytic relationship between renal water, minerals, and urea reabsorption that constitutes the driving force of the countercurrent multiplication (Hoorn et al., 2020). Aldosterone stimulates coupled renal reabsorption of Na^+ and secretion of K^+ through the incorporation of Na^+- (ENaC) and K^+-channels (ROMK) along the apical membrane and of Na^+-Cl^--transporters at the distal renal tubule (Hoorn et al., 2020; Sands and Layton, 2009) (Figure 6). Thus, aldosterone indirectly causes water reabsorption into the renal interstitium according to the osmotic pressure.

Antidiuretic hormone ameliorates water and mineral permeability by inducing the apical accumulation of AQP2 along the collecting duct and the expression of AQP3 and AQP4 at the basolateral membrane (Sands and Layton, 2009). Furthermore, ADH promotes the transcription of Na^+-K^+-$2Cl^-$ cotransporter (NKCC2/BSC1), hence enhancing Na^+Cl^- reabsorption at the thick ascending limb in dependence to ROMK activity increasing interstitial osmolality (Sands and Layton, 2009). Concerning UT, ADH upregulates the apical expression of UT-A1 and UT-A3 (*SLC14A2*) at the terminal collecting duct (Klein et al., 2011). However, whether UT-A2, localized at the descending limp of Henle's loop (Figure 6), and UT-B1 are ADH-independent or –dependent remains uncertain (Weiner et al., 2015; Sands and Layton, 2009). UT-B1 is found in erythrocytes and along the descending renal vasa recta and accumulates reabsorbed urea within the bloodstream. Thus, UT-B1 participates in maintaining osmotic equilibrium between plasma and interstitium and enables intrarenal urea recycling (Weiner et al., 2015; Bankir and Trinh-Trang-Tan, 2000). Finally, between 30 and 50% of primarily ultrafiltrated urea is excreted in urine (Weiner et al., 2015; Klein et al., 2011).

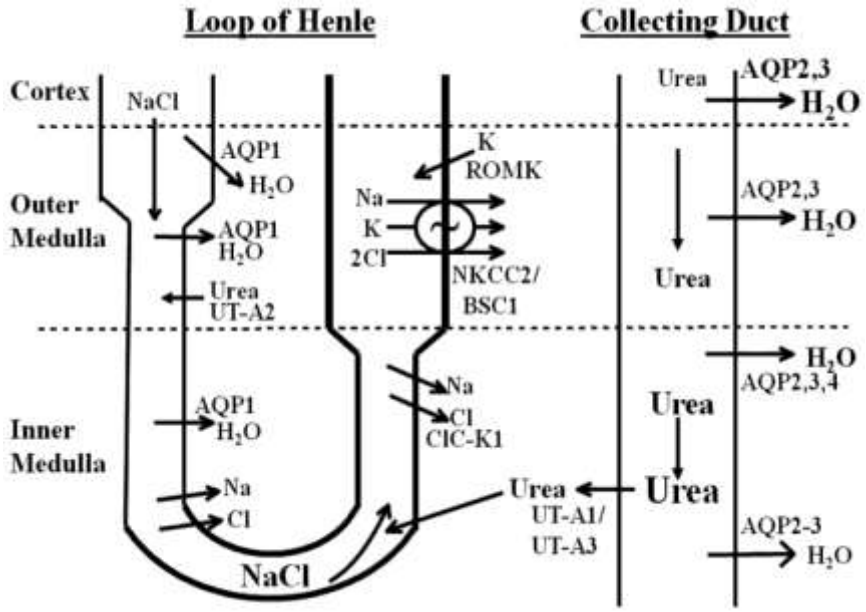

Figure 6. Molecular localizations of water, urea, and mineral transport proteins within the renal tubular system (Loop of Henle and Collecting Duct) involved in renal countercurrent multiplication determining final urinary water and urea excretion (Reprinted from Sands and Layton 2009, Page 19, Copyright (2021), with permission from Elsevier).

Major factors influencing urinary volume and N content include water intake in proportion to metabolic demand, water expense for milk production or temperature regulation, and dietary mineral and N intake (Bannink et al., 1999). An increase of dietary Na content from 3 to 19 g/kg DM resulted in a 4-times higher daily urine excretion (18.2 vs. 67.7 kg/d) and an 8% higher urinary N excretion (189 vs. 205 g/d) without affecting urinary urea excretion (Spek et al., 2012). Spek et al. (2012) speculated that an increased water intake (61.7 vs. 115.7 kg/d) with a simultaneous decrease in ADH levels resulted in a lower renal water reabsorption. However, Bannink et al. (1999) postulated water intake to only indirectly influence urine volume in lactating cows, rather the osmolytic effects of urinary Na, K, and N content more heavily influence urine volume.

Dietary N intake is generally known as a major factor determining urinary N and urea excretions and thus constitutes an important component manipulating general N emissions. As shown in goats by Elfers et al. (2014) and Starke et al. (2012), dietary N restriction leads to an increase of ADH level causing a higher accumulation of AQP2 and UT-A1 at the renal collecting duct and subsequently a higher water and urea reabsorption. Urinary urea constitutes the major urinary N-metabolite with proportionate fluctuations in whole urinary N between 52 and over 93% (3 to 19.2 urea N g/L; Dijkstra et al. (2013b); Bristow et al. (1992)). Thus, a reduction in urinary urea N excretion owed to a lower dietary N intake is associated with lower N emissions. Additionally, due to an increasing renal recovering of urea, dietary N

restriction increases the chance of reabsorbed urea to be recycled into the rumen and reutilized for microbial protein synthesis, thus improving microbial and host Neff (Starke et al., 2012; Rojen et al., 2011).

Besides urea, other urinary N-metabolites have been described to be osmotically effective, and depending on urinary N composition, may differ in environmental decomposition and N footprint (Dijkstra et al., 2013b). Whitehead et al. (1989) demonstrated different urinary N components vary in ammonia volatilization (urea > allantoin > creatinine > creatine > hippuric acid). Contents of creatinine and creatine, as metabolites of muscle metabolism, account for 3.1% and 2.5% of whole urinary N excretion, respectively, on average (Dijkstra et al., 2013b; Bristow et al., 1992). Since creatinine is not reabsorbed along the nephric tubular system, creatinine may be utilized as an accurate marker for urine volume and renal glomerular filtration rate, and thus enables the estimation of renal clearance rate of other urinary metabolites as well (Tas and Susenbeth, 2007). The urinary purine derivatives (PD) allantoin, uric acid, and (hypo-) xanthine account for 2.2-14.2%, 0.6-1.9%, and 0.3-0.7% of whole urinary N excretions, respectively (Dijkstra et al., 2013b). Urinary contents of PD derived from catabolism of microbial nucleic acids rapidly respond to changes in level of duodenal PD flow (within 2-3 h) (Chen et al., 1997). Therefore, the quantitative urinary excretion of PD (Shingfield, 2000) or uric acid alone (Johnson et al., 1998) constitutes a reliable marker for estimating duodenal microbial N flow in ruminants. However, according to Tas and Susenbeth (2007) urinary PD may also originate from body tissue and rumen undegraded feed nucleic acids; thus impairing accuracy and sensitivity of urinary PD as a marker of microbial N flow. Urinary hippuric acid excretion contributes between 3.4 and 8% of whole urinary N and is attributed to the detoxification of plant-derived benzoic acid (Dijkstra et al., 2013b). Several studies assigned urinary hippuric acid or benzoic acid, as a product of hippuric acid hydrolysis, an inhibitory effect on environmental N_2O formation (Bertram et al., 2009; Kool et al., 2006; van Groenigen et al., 2006). Dijkstra et al. (2013b) suggested that inhibition of N_2O formation was caused by the antimicrobial feature of benzoic acid. However, Clough et al. (2009) and Ciganda et al. (2019) could not confirm previous results and assumed that several environmental factors influence N_2O formation, such as type of soil and soil pH, mask the potential inhibitory effects of urinary hippuric acid.

With regard to urinary P excretions, between 98 and 99% of primary glomerular P ultrafiltrate was shown to be reabsorbed along the proximal renal tubular section (Widiyono et al., 1998). Consequently, urinary P excretions constitute less than 1% of dietary P intake and thus is considered of minor importance in environmental P pollution relative to fecal P excretion. Forster et al. (2013) and Villa-Bellosta et al. (2009) described NaP_i IIa, and IIc to absorb the largest portion of ultrafiltrated urinary P and have been exclusively identified in the

renal proximal tubular system (Figure 7). Furthermore, both PiT-1 and PiT-2 have been detected in renal tissue (Forster et al., 2013) with PiT-2 being localized at the renal brush border membrane of proximal tubules (Villa-Bellosta et al., 2009). Controversially, renal basolateral P extrusion has not yet been clarified (Wilkens and Muscher-Banse, 2020) (Figure 7).

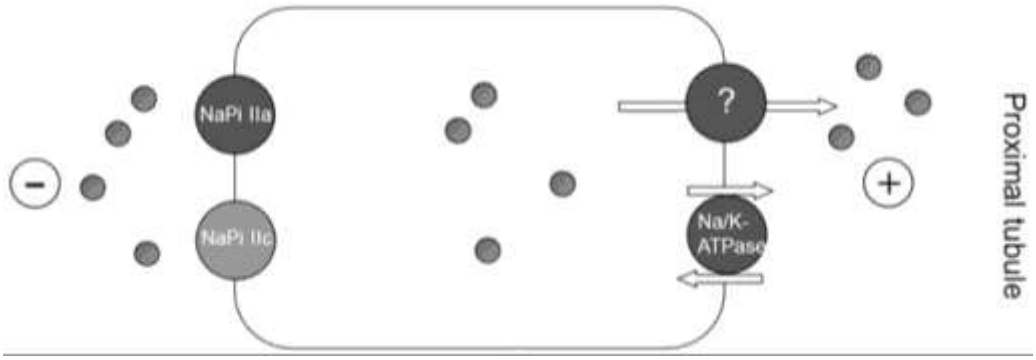

Figure 7. Renal P_i reabsorption by transport proteins localized in the renal proximal tubular section (This figure was published by Wilkens and Muscher-Banse 2020 (https://doi.org/10.1017/S1751731119003197), Copyright Elsevier (2021) (https://creativecommons.org/licenses/by-nc-nd/4.0/)).

2.3. Milk nitrogen and phosphorus secretion

2.3.1. Synthesis, transport and predictive modelling of emissions

Milk N yield is primarily attributed to milk protein synthesis (95%; Cerbulis and Farrell (1975)) and to a minor extent to the milk NPN fraction (5%; Depeters and Ferguson (1992)). MUN content, accounting for 50% of milk NPN yield (Depeters and Ferguson, 1992), represents a sink for plasma urea concentration (R^2 = 0.84; Broderick and Clayton (1997)). Diffusion of urea along the mammary ducts from plasma to milk, and vice versa, follows a diurnal dynamic responding to feed intake and milking frequency (Spek et al., 2016; Gustafsson and Palmquist, 1993). Gustafsson and Palmquist (1993) described the peak in plasma urea occurs 1.5 to 2 h after the ruminal ammonia peak. Accordingly, MUN concentration seems to mirror the level of ingested N exceeding ruminal microbial N incorporation (R^2 = 0.77), and thus, ruminal ammonia concentration (R^2 = 0.57) favoring hepatic urea synthesis (Broderick and Clayton, 1997). Moreover, since milk protein content positively responds to the efficiency of ruminal microbial protein synthesis (Cant et al., 2018), the level of MUN concentration is negatively related to milk protein content (Marshall et al., 2020; Beatson et al., 2019). As a consequence, MUN concentration is affected by all the factors influencing the efficiency of ruminal microbial protein synthesis (Spek et al., 2013b) as discussed in chapter 2.1. Because CP intake constitutes the major influencing factor of ruminal microbial protein synthesis, plasma urea, and MUN content (R^2 = 0.778; Nousiainen et al. (2004)), MUN concentration has been

identified as reliable indicator assessing dietary protein supply in ruminants (Huhtanen et al., 2015; Nousiainen et al., 2004; Jonker et al., 2002).

Several studies have shown MUN concentration to be appropriate in predicting urinary urea and urinary N excretions (Broderick, 2003; Jonker et al., 1998; Ciszuk and Gebregziabher, 1994) as well as resultant ammonia emissions (Burgos et al., 2010; van Duinkerken et al., 2005). The ratio between MUN concentration and urinary urea excretion is primarily determined by dietary factors such as dietary CP and salt intake (Spek et al., 2013a). Increasing the dietary CP content by 30% (11.7 vs. 15.4% in DM) decisively increases both MUN concentration (4.5 vs. 8.4 MUN mg/dL) and urinary urea N excretions (31 vs. 76 g/d), but shifts the ratio towards urinary urea excretions due to a decrease in renal urea reabsorption (Spek et al., 2013a). Conversely, increasing dietary Na content from 3 g to 19 g per kg DM reduces MUN concentration by 21% (12.5 vs. 9.9 mg of N/dL) without affecting urinary urea excretion, indicating an enhancement in renal urea reabsorption (Spek et al., 2012). Apart from dietary factors, several animal-related aspects have been discussed that influence the relationship between MUN content and urinary urea excretion as well. Kauffman and St-Pierre (2001) and Hojman et al. (2005) recommended the inclusion of body weight in predictive modelling of urinary urea excretions because body weight has been shown to be positively associated with urinary urea excretions and negatively with MUN concentration. However, the relationship between body weight and MUN concentration is considerably influenced by parity, as Hojman et al. (2005) observed primiparous cows displayed a lower MUN concentration than multiparous dairy cows.

In regards to milk P secretion, Shennan and Peaker (2000) and Mather (2011) defined two major secretory routes of P_i across the apical alveolar membrane: either as a monolayer of phospholipids coating milk lipid droplets during budding (10%) or as part of Golgi vesicles secreted via exocytosis (90%) (NRC, 2001). Within Golgi vesicles, 78% of P is present as ionized P released during casein synthesis and UDP hydrolyzing lactose synthesis (Klop et al., 2014; NRC, 2001; Shennan and Peaker, 2000). The remaining 22% of P within Golgi vesicles constitute colloidal calcium phosphate nanoclusters esterified with the protein matrix of casein micelles (Bijl et al., 2013; Holt, 2004; NRC, 2001), which accounts for 82% of whole milk protein distribution (Cerbulis and Farrell, 1975). Controversially, less is known about P_i uptake into the mammary alveolar cell. According to Huber et al. (2007), P_i uptake from milk into mammary alveolar cells is mediated by NaP_i IIb transport proteins, which were identified immunohistochemically in the apical membrane (Huber et al., 2007). Virkki et al. (2007) also assumed a basolateral uptake of P_i from plasma to be mediated by PiT proteins.

As a consequence of the aforementioned milk synthesis relationships, Klop et al. (2014) and Visentin et al. (2019) delivered clear evidence of milk P secretion to be phenotypically and

genetically correlated with milk protein synthesis and milk N yield. Thus, milk production constitutes a further connective link between N and P metabolism in dairy cows. Bijl et al. (2013) stated milk P yield to be predominantly determined by the secretion of casein protein, and thus is directly related to efficiency of ruminal microbial protein synthesis (Cant et al., 2018). Even at low P intake (0.31 vs. 0.39% P in DM) over a long period of two years, dairy cows maintain milk P yield in expense of bone P body reserves as shown by Wu et al. (2001). Similarly, Wang et al. (2014), feeding three different dietary P contents (0.37%, 0.47% and 0.57%) one full lactation period, reported no differences in milk yield among experimental groups. A higher milk P yield relative to the amount of P ingested, paralleled by a larger transfer of metabolic P into milk, results in less fecal P excretion (Klop et al., 2013; Valk et al., 2002). Thus, Alvarez-Fuentes et al. (2016) observed milk yield, including milk P yield, is negatively related to fecal P excretion, specifying an increase of 1 kg milk production per day reduces fecal P excretion by 0.48 g per day independently from P intake.

2.3.2. Individual variation in milk urea and milk phosphorus secretion

Despite the intensive studying of dietary and animal-related factors affecting MUN and milk P concentrations and secretions, a considerable portion of underlying metabolic backgrounds causing individual variations among dairy cows remains unknown (Spek et al., 2016; Klop et al., 2013; Aguilar et al., 2012). Beatson et al. (2019) calculated a heritability of MUN concentration of h^2 = 0.24 for Holstein dairy cows; thus, indicating a genetic component that contributes to MUN concentration and the potential for selective breeding to modify MUN concentration. Based on the latter, Marshall et al. (2020) found a positive correlation between MUN breeding value and MUN concentration (R^2 = 0.67), and between MUN breeding value and urinary urea concentration (R^2 = 0.46); thus, concluding a selection for low MUN cows will reduce urinary N emissions.

Visentin et al. (2019), Toffanin et al. (2015) and van Hulzen et al. (2009) recorded a moderate heritability of milk P concentration ranging between h^2 = 0.15 and 0.21 in dairy cows. However, Visentin et al. (2019) assumed heritability of milk P to be indirectly influenced by the heritability of milk protein content (h^2 = 0.39), since milk P and milk protein secretion have been described to be genetically correlated (r = 0.91; Toffanin et al. (2015)). Beside the genetic correlation between milk P and protein yield, Toffanin et al. (2015) also found milk P and milk fat secretion to be genetically correlated (r = 0.83) owed to milk fat synthesis involving P as monolayer of phospholipids. Finally, it remains to be elucidated whether selective breeding for high milk P secretion in dairy cows (Visentin et al., 2019; van Hulzen et al., 2009) contributes to reducing fecal P loss as postulated by Alvarez-Fuentes et al. (2016).

Taken the given information, it has been proposed that the selection of animals for low MUN concentration and high milk P secretion would reduce N and P emissions and thus enhances environmental sustainability of dairy husbandry. However, underlying metabolic backgrounds causing these inter-individual differences in MUN and milk P secretion and associated effects on N and P metabolism and excretions are almost unknown and demanding for further enlightenment.

Chapter 3: Aims and experimental approaches

Over the past decades, a plethora of studies have primarily focused on dietary strategies that reduce N and P losses while concomitantly enhancing feed utilization efficiency in dairy cows (Ouatahar et al., 2021). Models that predict urinary N emissions based on MUN concentration have been identified (Burgos et al., 2007; Ciszuk and Gebregziabher, 1994). Moreover, an increase in milk P yield has been postulated to linearly reduce fecal P loss since more ingested feed P is channeled into milk synthesis (Alvarez-Fuentes et al., 2016). However, owed to the complexity of the mutual interactions between N, P, and energy metabolism in ruminal microbes and the host, the challenge remains to develop holistic approaches reconciling the mitigation of N and P excretions. Moreover, a considerable portion of individual variations in MUN and milk P secretion among dairy cows has not been fully clarified. Based on the current knowledge from literature, it appears that a combinatory effect between animal genetic differences and dietary ration composition account for the variability in MUN and milk P yield. However, the metabolic reasons causing the individual cow differences in MUN and milk P secretion, as well as the potentially associated differences in urinary and fecal N and P excretions despite feeding the same feed, are largely unknown. Therefore, the overall objective of the present studies was to elucidate the physiological and molecular mechanisms underlying varying MUN secretion and Peff of dairy cows. Decreasing MUN levels ought to reduce the environmental pollution elicited by fecal and urinary N excretions in parallel. Furthermore, increasing the Peff should reduce fecal P loss and conserve dwindling global P reserves. To approach the overall objective, two animal experimental trials were conducted to address the following specific aims:

(1) The first specific aim was to elucidate mechanisms regulating urea metabolism and N excretions in dairy cows with divergent MUN concentrations fed two different CP levels. To this end, 20 German Holstein dairy cows with comparable milk yield and stage of lactation but divergent MUN concentration at the last five milk quality records were selected from commercial farms. Pairs of cows were transported to the Research Institute for Farm Animal Biology (FBN), Dummerstorf, Germany and assigned to a high and a low MUN concentration group. Cows were fed two isoenergetically formulated rations differing in CP content. On each diet, cows underwent a four-week experimental protocol that included the implementation of

indirect calorimetry based on respiratory measurements, a 4-day N balance trial and a ^{13}C urea isotope tracer study.

(2) The second specific aim was to investigate physiological and molecular mechanisms affecting P and N excretions in dairy cows differing in Peff. For this, 20 Holstein dairy cows were fed a ration containing the lowest P and CP levels as recommended by the NRC (2001). After three weeks on the experimental diet, a 4-day P- and N balance study was conducted and, based on the respective Peff, cows were retrospectively grouped into either a high or low group. Cows were slaughtered to obtain tissue from kidney, jejunal mucosa, and the mammary gland. Tissue mRNA was isolated and the expression of P transport proteins was analyzed by real-time PCR.

The results from these studies could benefit selective breeding and precision feeding strategies that help to mitigate the environmental N and P footprint resulting from cattle husbandry.

Chapter 4 - Publication I: Differences between Holstein dairy cows in renal clearance rate of urea affect milk urea concentration and the relationship between milk urea and urinary nitrogen excretion

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Chapter 6: General discussion

Inter-individual variation in milk urea nitrogen concentration and associated effects on environmental nitrogen pollution

Milk urea N (MUN) concentration is positively related to plasma (Spek et al., 2016) and urinary urea N (UUN) concentration (Ciszuk and Gebregziabher, 1994); the latter accounting for 73% of whole urinary N (Dijkstra et al., 2013b). Thus, MUN concentration has been recognized as a reliable indicator for assessing appropriateness of dietary N supply and predicting urinary N emissions in dairy cows (Burgos et al., 2007; Nousiainen et al., 2004). Apart from environmental aspects, MUN concentration may additionally serve as indicator for fertility of the cow and affects milk manufacturing characteristics. Rajala-Schutz et al. (2001) demonstrated that increasing MUN concentration compromised fertility in dairy cows, which is worth considering given that infertility is one of the most widespread reasons for involuntary culling (Dallago et al., 2021). Dairy cows with MUN concentrations below 10 mg/dL or in the range between 10 and 12.7 mg/d show a 2.4 and 1.4 times higher fertility, respectively, than dairy cows with MUN concentrations above 12.7 mg/dL (Rajala-Schutz et al., 2001). However, if these differences in fertility are directly attributable to differences in individual plasma urea concentration or rather reflect differences in availability of nutrients in the diet (Rodney et al., 2018; Broderick and Clayton, 1997) requires further investigation. Furthermore, MUN concentration, or rather the enzymatic conversion into ammonia, may have an impact on further manufacturing processes such as fermented food products and cheese. Ammonia increases the pH, thereby diminishing acidifying effects of lactic cultures in general (Pernoud et al., 2004), while the MUN content influences acidification kinetics and final chemical composition and texture in cheese manufacturing (Martin et al., 1997).

Beatson et al. (2019) and Miglior et al. (2007) described MUN concentration in Holstein dairy cows to be of moderate heritability. Hence, selectively breeding dairy cows for low MUN may potentially contribute to reducing urinary N emissions derived from the dairy industry (Marshall et al., 2020). Aguilar et al. (2012) already speculated dairy herds may genetically and phenotypically diverge in MUN concentration, albeit not in UUN excretion, since less urea transfer to urine may cause an elevation in MUN concentration and secretion. As a consequence, Aguilar et al. (2012) advised against applying target MUN concentrations to evaluate N supply and urinary N emissions across dairy herds before calibration to the specific herd. Indeed, in line with previous studies (Spek et al., 2013b; Ciszuk and Gebregziabher, 1994), results of the present work (**Chapter 4**) revealed MUN concentration to be positively correlated with UUN and urinary N excretions taking into account variation in dietary crude protein (CP) content. We found that a reduction in dietary CP supply from 15.9 to 13.8% of dry

matter (DM) considerably decreases renal urea clearance rate (UCR) and thus overall UUN and urinary N excretions. However, with regard to individual MUN concentrations under the same feeding conditions, our findings also support the assumptions by Aguilar et al. (2012) mentioned above. High and low MUN cows did not differ in either urinary N excretions, UUN output, or ammonia emissions recorded during respiratory measurements. The renal clearance rates of creatinine (CCR), reflecting the overall renal glomerular filtration rate, and UCR were greater in low MUN compared to high MUN cows. Hence, and in agreement with Aguilar et al. (2012), differences in MUN concentration and plasma urea concentration were attributed to a different renal performance. Interestingly, MUN secretion and UUN excretion were positively correlated at the lower dietary CP level but negatively correlated at the higher CP level. Thus, the scope of differences in renal function between groups seems to be amplified the more N is ingested. Contrasting our study, Marshall et al. (2020) investigated the N metabolism in grazing dairy cows and stated MUN breeding value and UUN excretion to be linearly correlated (R^2 = 0.46). Regardless, owed to the experimental conditions, neither the exact pasture composition nor the CP intake were monitored (Marshall et al., 2020). Thus, data generated by Marshall et al. (2020) may only be applicable on pastoral dairy husbandry and not reflective of the general potential of selectively breeding based on MUN concentrations across different husbandry systems. Besides, the authors stated that cows with low and high MUN breeding values do not differ in total amount of N excreted with urine or feces (Marshall et al., 2021). However, differences in urinary excretion patterns and urinary urea concentrations between single urination events might be of relevance for defining the extent of N emissions deriving from pastoral systems (Marshall et al., 2021).

Recently, Souza et al. (2021) confirmed UUN excretion to remain unaffected with varying MUN secretion or concentration under constant feeding conditions. However, Souza et al. (2021) described MUN concentration to be only slightly related to kidney transport activity; concluding renal function does not affect differences in MUN concentration, but argued the lack of relationship might be a result of inadequate statistical power. In turn, variation in MUN concentration has been ascribed to differences in fractional urea transfer from plasma into the rumen-intestinal tract such that urea transfer is negatively related to MUN concentration (Souza et al., 2021). As outlined in chapter 2.3.1, the MUN concentration has been assumed to mirror the level of ruminal N entry exceeding microbial N capturing (Broderick and Clayton, 1997). Conversely, milk protein content positively responds to the N utilization efficiency (Neff) and level of ruminal microbial protein synthesis (Cant et al., 2018). Accordingly, and referring to the present study, reducing the CP level by 2.1% of DM did not alter milk protein percentage or yield, but concomitantly abated MUN yield. These findings were attributed to a greater rumen-intestinal urea clearance rate and microbial urea hydrolysis rate (UHR) of endogenous

urea affecting an increase in Neff with the lower CP supply. Moreover, the latter results suggest microbial capturing of dietary supplemented N and of endogenous urea N in the rumen becomes more efficient with lowering the dietary CP level. Nevertheless, we could neither detect any considerable differences in plasma urea transfer into the rumen-intestinal tract nor in microbial UHR between high and low MUN cows. Moreover, experimental MUN groups in the present trial did not differ in either milk protein percentage, in milk protein yield or in Neff despite fed the same dietary CP and energy level. Thus, even if relatively more plasma urea would be transferred into the rumen-intestinal tract, the aforementioned information indicates that the absolute level of microbial protein synthesis and microbial endogenous urea N incorporation is comparable between MUN groups. Consequently, the latter evidences that the lower MUN concentrations in the present study are not attributable to higher fractional plasma urea transfer into the rumen-intestinal tract.

Partially contradictory results between our work and the study by Souza et al. (2021) regarding the mechanisms affecting individual differences in MUN concentration might be owed to the utilization of different urea isotope tracer methods. For example in the present study, a ^{13}C urea isotope tracer was administered as an intravenous bolus (Spek et al., 2013a). We applied a two-exponential curve fitting of the plasma ^{13}C urea enrichment curve to calculate the respective urea turnover rate, urea pool size and distribution (Wolfe and Chinkes, 2005). In contrast, Souza et al. (2021) performed a continuous intravenous infusion of $^{15}N^{15}N$ urea isotope tracer followed by the analysis of $^{15}N^{15}N$ urea enrichments in plasma, urine and feces to assess tracer distribution (Lobley et al., 2000). However, the more likely explanation is that the different results between our study and Souza et al. (2021) occurred due to differences in experimental grouping and the composition of the diet. Souza et al. (2021) primarily worked with regression analysis ranking MUN concentration by size. In contrast, our experimental cows were selected in pairs and grouped according to their MUN concentration at the beginning of the experiment. Moreover, our MUN groups were fed two different dietary crude protein (CP) levels in a cross-over design to promote the occurrence of metabolic differences. Although Souza et al. (2021) fed a CP level of 17.6% in DM, relative urea transfer into the rumen-intestinal tract amounted on average 69.9% and up to 81.2% of plasma urea entry rate, which seems to be relatively high when compared with our results. We can only speculate if these differences in fractional urea transfer into the rumen-intestinal tract is a result of the lower CP levels applied in our study or composing different feed ingredients. For example, Broderick et al. (2015) demonstrated soybean meal increased ruminal CP degradation, ruminal ammonia content, and shifted ruminal short chain fatty acids (SCFA) patterns and abundance compared to canola meal. Ellis et al. (2012) noted that dietary monensin shifted the ruminal SCFA pattern towards propionate and ascribed this result as antimicrobial effects on specific ruminal

microbial species. Consequently, both the supplementation of dietary soybean meal and monensin, as used by Souza et al. (2021), potentially modifies microbial energy metabolism and N capturing, thus indirectly affecting rumen-intestinal urea clearance rate. However, we did not include soybean meal or monensin in our ration formulations.

One peculiarity of our study was the analysis of excretions and the percentages of urinary non-urea N-compounds in total urine N; revealing low MUN cows excrete more urinary creatine regardless of dietary CP level and more urinary uric acid on the low CP diet than high MUN cows. Concomitantly, CCR, renal clearance rate of uric acid and creatine increased with a reduction in MUN concentration. Gardiner et al. (2018) demonstrated non-urea urinary N-metabolites affect N_2O production when applied on soil. Furthermore, non-urea urinary N-metabolites vary considerably in their respective contribution to the N_2O emission factor, defined as the percentage of applied N that is emitted as N_2O, as follows: uric acid > creatine/creatinine (Gardiner et al., 2018). Thus, it might be tempting to speculate that high MUN cows reveal lower N_2O emissions from urine patches than low MUN cows, as already discussed in chapter 4. However, UUN constitutes the major urinary N metabolite (52-94%; Dijkstra et al. (2013b)) and contributes to N_2O to a comparable extent as non-urea urinary N-metabolites (Gardiner et al., 2018). Thus, Gardiner et al. (2018) concluded varying amounts of non-urea N-metabolites in cattle urine do not affect overall N_2O emissions derived from urine patches. The situation with ammonia emissions is similar to that with N_2O emissions. Eight days after urine samples from cattle have been applied on sandy clay-loam soil, Whitehead et al. (1989) found 15% of UUN and under 1% of creatine N was volatilized to ammonia. Applying this information to our results suggests that MUN groups, although not experimentally confirmed, may not differ significantly in either urinary deriving N_2O or ammonia emissions since MUN groups did not vary in overall urinary N or UUN excretions. In addition, we found MUN groups not to alter in fecal N loss. Thus, the latter supports MUN groups neither to vary in terms of urinary N emissions nor in fecal N emissions, whereas a reduction in CP level by 2.1% in DM revealed urinary N load to be efficiently attenuated by 41% and fecal N load tended to decrease by 9%.

To summarize, the present work demonstrated inter-individual variation in MUN concentration despite cows being fed the same feed ration, and we attribute this variation to differences in renal clearance rates of urea and other N-metabolites. Furthermore, selective breeding for low MUN concentration does not alleviate UUN, urinary and fecal N excretions and therefore not the resulting N_2O and ammonia emission potentials. Thus, the results of our study support the recommendation by Aguilar et al. (2012) who proposed the calibration of a target MUN concentration on the individual herd before utilizing MUN concentration as an indicator for urinary N emissions or dietary N supply. Moreover, as previously suspected

(Ariyarathne et al., 2021; Huhtanen et al., 2015; Marini and Van Amburgh, 2003), the potential overall effect of selecting for low MUN concentration appears to be negligible on attenuating urinary and fecal N excretions, particularly if compared with the mitigating potential of reducing dietary CP content. Therefore, current knowledge suggest that optimizing dietary protein supply rather than selective breeding on low MUN has greater potential on attenuating environmental pollution from N derived from cattle husbandry.

Despite the research of us and others, several questions remain to be answered in next future. Physiological mechanisms affecting a higher UCR despite a lower plasma urea concentration in low MUN cows remain unknown. Thus, we can only speculate if differences in the expression of urea transport proteins or aquaporins within the renal tissue, osmolytic interactions of different metabolites within the renal tubular system or hepatic metabolic processes are involved. Moreover, it remains to be evaluated, if the results from late-lactating non-pregnant cows in our study (on average 292 days in milk) are applicable to different stages of lactation. Energy and N metabolism vary throughout lactation period (Gross et al., 2011) and across parity (Wathes et al., 2007), which in turn may impact the applicability of our results. For example, around parturition dairy cows experience a metabolic transition accompanied by a negative energy balance and the mobilization of body fat and protein reserves (Gross et al., 2011). Interestingly, in line with Hojman et al. (2004) and Godden et al. (2001), but contrasting results by Johnson and Young (2003), dairy cows with greater MUN yield had considerably higher milk fat percentages in our study. Johnson and Young (2003) and Hojman et al. (2004) argued different results are affected by nutritional differences among studies. However, the specific metabolic backgrounds leading to a positive or negative relationship between MUN and milk fat percentage remain unknown and require further research.

Inter-individual differences in phosphorus utilization efficiency and potential mitigating effects on environmental phosphorus pollution

An elevation in P utilization efficiency (Peff) is associated with a reduction in relative amount of feed P required for milk production and a corresponding decrease in fecal and urinary P excretions (Alvarez-Fuentes et al., 2016; Valk et al., 2002). Visentin et al. (2019) and van Hulzen et al. (2009) reported milk P concentration and secretion to be of moderate heritability as well as phenotypically and genetically correlated with milk protein yield (Visentin et al., 2019) and milk fat yield (Toffanin et al., 2015). Thus, selection for high milk P secretion, or rather high Peff in light of dwindling global P reserves (Cordell et al., 2009), may potentially reduce the overall environmental P load derived from cattle husbandry and the amount of mineral P included in feed. With respect to the given information, selective breeding for a high Peff appears to be ecologically and economically reasonable. Initially, results of our study (**Chapter**

5) validated that milk P secretion is strongly related to milk fat and protein yield and thus associated with an increase in Neff (Visentin et al., 2019; Toffanin et al., 2015; Klop et al., 2014). Concentrations of milk components constitute the main osmolytic drivers of water influx into micellar cells, thereby regulating the final volume of milk production (Mather, 2011). Consequently, the amount of milk components determines the final milk production and, in turn, milk yield mutually defines the amount of milk component secretion. Besides, we analyzed the expression of inorganic P transport (PiT)-1 proteins and sodium/inorganic phosphorus cotransport (NaP_i) IIb proteins in mammary gland tissue; revealing high Peff cows tending to express more NaP_i IIb proteins compared to low Peff cows. So far, NaP_i IIb has been localized in the apical membrane of mammary alveolar cells (Huber et al., 2007), suggesting NaP_i IIb to be at least strongly involved in transferring inorganic P from milk into mammary alveolar cell. The latter appears to be facilitated with increasing P requirement for milk production and Peff.

Depleting dietary P intake is the most effective method for attenuating fecal P excretions while simultaneously ameliorating Peff (Klop et al., 2013; Knowlton and Herbein, 2002; Wu et al., 2001). Even a long-term reduction in dietary P content (0.48 vs. 0.38% in DM) over two consecutive years did not show an impairment to either milk production or fertility in dairy cows yielding 10,167 kg of milk per year (Wu and Satter, 2000). However, Wu et al. (2001) documented feeding dairy cows a dietary P content of 0.31% compared to 0.39% in DM over three years elevated milk production (11,909 vs. 13,038 kg/year), but at the expense of bone P concentration. Accordingly, Puggaard et al. (2011) stated ruminal P supply prioritizes mobilization of body P reserves with depleted dietary P intake, likely to maintain high levels of microbial protein and related milk protein synthesis (Cant et al., 2018). In our experiment, we fed a dietary P content of 0.37% in DM (on average 64.2 g P intake/d for 23 L milk /d), which is in the lower range of the NRC (2001) recommendations and above the recommendations by GfE (2001) (57 g/d P intake) and INRA (Boudon et al. (2018); 36.4 g/d P intake). However, across experimental groups we found a mobilization of body P storages reflected by negative P balances. A high Peff was associated with elevated milk protein and P yields and increased resorption of body P reserves (Puggaard et al., 2011); thus, negative P balances in our study became even more negative with increasing Peff. Furthermore, observed body P mobilization is of particular interest since late-lactation cows, such as those utilized in this study, were shown to restore, rather than to mobilize, body P reserves in order to compensate for body P storages partially resorbed during early lactation (Knowlton et al., 2001). Also, Knowlton and Herbein (2002) confirmed that reducing dietary P level from 0.51% to 0.34% in DM during early lactation elicits a resorption of body P reserves until the fifth week post-partum without compensation until week 11. Thus, present knowledge indicates that a long-term inadequate

dietary P supply, associated with mobilization of P storages without restoration, compromises animal health and longevity of dairy cows, which is even reinforced with elevation in milk P yield. The strong relationship between P and Ca bone storage (Moreira et al., 2009) suggests that a low dietary P intake and bone P mobilization potentially increases the occurrence of hypophosphatemia, as well as of hypocalcemia and milk fever, particularly when considering that an insufficient dietary P intake may compromise overall feed intake (Grünberg et al., 2019). Both, hypophosphatemia and hypocalcemia have been considered some of the most common risk factors associated with downer cow syndrome and thus involuntary culling around parturition (Grünberg, 2014; DeGaris and Lean, 2008).

Interestingly, compared to low Peff cows, we found high Peff cows responded with upregulated renal P reabsorption rates and thus reduced urinary P losses to compensate for P deficiency. Furthermore, we detected high Peff cows to have an amplified expression of PiT-2 transport proteins in renal cortex tissue compared to low Peff cows, while the expressions of PiT-1, NaP_i-IIa and NaPi-IIc proteins were comparable. These results suggest that the expression of PiT-2 transport protein to be decisively involved in regulating individual renal P reabsorption in primary urine. The latter is particular of interest as previously NaP_i IIa and IIc proteins were described to reabsorb the largest portion of inorganic P within the proximal renal tubular section (Forster et al., 2013; Villa-Bellosta et al., 2009); however, less has been so far reported about the partition of PiT-2 proteins indicating more research in this field to be necessary.

As mentioned previously, an increase in milk protein yield indicates a higher level of ruminal microbial protein synthesis (Cant et al., 2018) and thus a greater microbial P incorporation. However, Valk et al. (2002) argued mobilization of body P reserves is associated with endogenous P secretion into the rumen-intestinal tract that subsequently may increase microbial P availability and concomitantly fecal P loss. Endogenous P sources account for up to 75% of whole fecal P excretion (Pfeffer et al., 2005). Consequently, albeit a potentially higher microbial P incorporation in high Peff cows, an even higher body P mobilization may intensify fecal P load and diminish the apparent digestibility of P by 13% compared to low Peff cows. However, based on our data we cannot fully exclude that - vice versa - the lower apparent P digestibility would account for the more negative P balance in high Peff cows. The actual reason for the higher P efficiency is certainly hard to determine because P digestibility, P mobilization, P secretion into the rumen-intestinal tract, and milk P yield are imbedded in the same regulatory circuit. Regardless, a lower apparent digestibility of feed DM in high Peff cows compared with low Peff cows suggests true feed digestibility mutually interacts with true P digestibility to contribute to higher fecal P losses. Moreover, whether the magnitude of fecal P loss is attributable to fecal inorganic or organic P loss remains unknown, but should be

considered in future studies, as the fecal P composition determines the environmental fate of P excreted (Pagliari and Laboski, 2012).

To summarize, present results emphasize selectively breeding dairy cows for a high Peff is economically and ecologically justified. However, although located in the lower range of NRC (2001) and above recommendations by GfE (2001) and INRA (Boudon et al., 2018), dietary P supply in this study appeared to be not sufficient. Thus, we found a mobilization of body P reserves and an amplification of fecal P loss, both reinforced with facing increasing P requirements for milk synthesis. Consequently, initial environmental advantages of a higher Peff in dairy cows are impaired by higher fecal P losses compared with low Peff cows. Furthermore, long-term mobilization of body P storages might have negative effects on animal health and longevity. To this end, current data suggest that the recommendations by NRC (2001), GfE (2001) and INRA (Boudon et al., 2018) on dietary P supply should be used as a baseline, but producers should frequently validate and adjust dietary P supply for their specific herds based on the milk P yield by analyzing fecal P loss within the herd.

Further questions remain regarding the generalization and transferability of this data across different stages of lactation, particularly since P metabolism may differ across lactation period and stage of pregnancy (Pfeffer et al., 2005; Knowlton et al., 2001). Experimental cows in the current study were between second and fourth lactation. Hence, we cannot exclude certain circumstances such as the nutritional profile or subclinical diseases that occurred during previous lactations and previous farm management practices that may have influenced the size of body P storages. Considering the aforementioned information, long-term studies feeding a dietary P supply at the lower range of NRC (2001) to high and low Peff cows, while accounting for potential effects of lactation and pregnancy on P metabolism, appear to be necessary. Finally, it remains to be clarified whether a long-term dietary P level at the lower range of NRC (2001) would result in adaptations to the rumen-intestinal P absorption capacity and in expression of P transport proteins in rumen-intestinal, renal and mammary gland tissue in dairy cows.

Conclusion

In order to counter climate change and anthropogenic environmental pollution, selective breeding for low MUN concentrations and a high Peff in dairy cows has been hypothesized to abate N and P losses derived from cattle husbandry. However, albeit varying in MUN concentration and secretion, experimental cows in our work did not differ in urinary N excretions, which is likely attributable to differences in renal function and specific clearance rates of urea and other N-metabolites. Consequently, based on present results a targeted

selection for low MUN concentration seems not to modify environmental N pollution, especially when the mitigation potential of depleting dietary CP supply is considered.

Contradictory, selection based on high Peff has been shown to be economically and ecologically justified as it is associated with a lower relative amount of feed P required for milk production while concurrently facilitating Neff and milk protein and fat yield. However, an inappropriate dietary P supply in regards to the respective milk P yield stimulated the resorption of body P storages, which amplifies with increasing P requirements for milk synthesis. Besides a potential long-term impairment of animal health and longevity, mobilization of body P reserves was paralleled by intensified fecal P load, thus compromising initial ecological benefits of a high Peff. To summarize, recent results emphasize that selectively breeding for high Peff is reasonable, but dietary P supply should be geared towards the respective demands and frequently verified by fecal P load.

Overall, our study contributes to the evaluation of the potential of selectively breeding for low MUN concentration and high Peff in dairy cows as a tool to aid in emission mitigation by capitalizing on individual metabolic differences among dairy cows. Although some limitations require further research, the present thesis offers practical recommendations for a more sustainable dairy industry. Moreover, present results enable the establishment of new directions in the scientific field of ruminant physiology to develop new emission mitigation strategies and to improve existing ones.

Chapter 7: Summary / Zusammenfassung

Summary:

Inter-individual variation in nitrogen and phosphorus metabolism and excretions in lactating Holstein dairy cows

Cattle husbandry constitutes a major source of anthropogenic nitrogen (N) and phosphorus (P) losses associated with negative effects on human, animal and environmental health. Thus, particularly in light of a growing global human population and dwindling global resources, there is a demand to develop holistic emission mitigation approaches that concurrently promote a sustainable and efficient handling of available resources. Initial mitigating attempts primarily focused on nutritional strategies such as reducing N and P content of feed, while maintaining a constant energy level, in order to ameliorate utilization efficiency of ingested N and P for milk production in dairy cows. Milk urea N (MUN) concentration was shown to linearly correlate with urinary urea excretions, thus enabling the prediction of urinary N emissions and the assessment of dietary N supply. A higher P utilization efficiency (Peff), defined as the ratio between milk P secretion and P ingested, was assumed to reduce the fecal P load in dairy cows, since more P is channeled into milk.

So far, variation in MUN concentration and Peff appear to be a result of a combinatory effect between animal genetic and feed composition. Thus, selectively breeding for low MUN concentration and high Peff dairy cows may potentially contribute attenuating urinary N and fecal P load and facilitating a more sustainable dairy industry. However, a significant portion of inter-individual variation in MUN concentration and Peff and underlying physiological mechanisms are largely unknown. Thus, the objective of the present thesis was to elucidate physiological and molecular mechanisms affecting inter-individual variation in MUN concentration and Peff in lactating Holstein dairy cows. Results are implicated to contribute in assessing the mitigation potential of selectively breeding on MUN concentration and Peff, while concomitantly precising feeding strategies to attenuate the environmental N and P footprint of cattle husbandry.

The first study focused on clarifying physiological mechanisms involved in the regulation of urea metabolism and associated N excretions in dairy cows with divergent MUN concentrations. For this purpose, 20 German Holstein dairy cows were purchased in pairs, comprising one cow high (277 mg/L; n = 10) and one low (189 mg/L; n = 10) in MUN concentration, but were comparable in lactation period, body weight and milk production. After transported to the institutional barn of the Research Institute for Farm Animal Biology (FBN, Dummerstorf, Germany), cows were fed two different planes of dietary crude protein (13.8% vs. 15.9% in dry matter). After a two-week feed adaptation period within a loose housing

system, we conducted a 2-day indirect calorimetry and a 4-day N balance with ^{13}C urea isotope tracer study in tie stalls. Our study revealed, MUN groups did not differ in either fecal N, urinary urea or urinary N excretions despite fed the same feed composition. The latter results have been attributed to differences in renal performance concerning the overall renal glomerular filtration rate and specifically the renal clearance rate of urea. Interestingly, high MUN cows excreted less urinary creatine independent of protein feeding and less urinary uric acid on the low protein diet than low MUN cows. However, since urinary urea accounts for 75% of whole urinary N and MUN groups did not differ in overall urinary N excretion, we concluded high and low MUN cows not to differ in terms of nitrous oxide or ammonia emissions. In contrast, a reduction in dietary crude protein level considerably attenuated urinary N excretions with simultaneously improving N utilization efficiency for milk production.

The second study aimed to elucidate underlying physiological and molecular mechanisms affecting P and N excretions of dairy cows differing in Peff. To this end, 20 German Holstein dairy cows were fed a feed ration comparable in dietary P (0.37% in dry matter) and crude protein (14% in dry matter) content, both located in the lower range of recommendations by NRC (National Research Council; Nutrient requirements of dairy cattle, 2001). After a three-week feed adaptation in a loose housing system we implied a 4-day P- and N balance study in tie-stalls. Cows were retrospectively grouped into a high (40%; *n* = 10) and a low Peff (33%; *n* = 10) group according to the results obtained from the P balance study. Finally, dairy cows were slaughtered to obtain tissue samples from jejunal mucosa, renal cortex and mammary gland with subsequently analyzing the expression of P transport proteins encoding mRNA by quantitative real-time PCR. Initially, our work revealed Peff and milk P yield to be strongly related to milk fat and milk protein yield. High Peff cows had an enhanced renal P reabsorption rate compared to low Peff cows, which was associated with reduced urinary P losses. The latter was paralleled by an upregulation of inorganic P transport protein -2 expression in renal tissue suggesting to be decisively involved in affecting individual differences in urinary P excretion. However, with respect to the much greater fecal P excretion, differences in urinary P excretion seem to be negligible. High Peff cows had a lower P apparent digestibility and a more negative P balance than low Peff cows, which has been attributed to a higher mobilization of body P reserves. Thus, taking the given results, in the presence of an insufficient P supply microbial protein synthesis in the rumen and related milk synthesis seem to be prioritized in expense of body P reserves. Beyond that, we found high Peff cows to excrete more fecal P relative to P ingested than low Peff cows compromising initial ecological benefits of a high Peff.

In conclusion, results suggest reducing dietary crude protein supply rather than a selectively breeding for low MUN concentration to have a significant potential on mitigating N

emissions deriving from cattle husbandry. In contrast, a selective breeding for high Peff and associated Neff was shown to be ecologically and economically reasonable. However, an insufficient dietary P supply stimulated the mobilization of body P reserves. A long-term mobilization of P reserves may negatively affect animal health and longevity and intensifies fecal P load. Thus, selective breeding for high Peff is reasonable, but dietary P supply should be geared towards the respective milk P yield and should be frequently verified by fecal P load within herd.

Zusammenfassung

Interindividuelle Variation im Stickstoff- und Phosphormetabolismus und in den Ausscheidungen von laktierenden Holstein Milchkühen

Die Rinderhaltung ist eine der Hauptquellen für anthropogene Stickstoff (N) und Phosphor (P) -Verluste, die sich negativ auf die Gesundheit von Mensch, Tier und Umwelt auswirken. Vor allem angesichts einer wachsenden Weltbevölkerung und schwindender globaler Ressourcen besteht daher die Notwendigkeit, ganzheitliche Ansätze zur Emissionsminderung zu entwickeln, die gleichzeitig einen nachhaltigen und effizienten Umgang mit den verfügbaren Ressourcen fördern. Erste Versuche zur Emissionsminderung konzentrierten sich zunächst auf Ernährungsstrategien wie die Reduzierung des N- und P-Gehalts im Futter bei gleichbleibendem Energieniveau, um die Nutzungseffizienz des aufgenommenen N und P für die Milchproduktion bei Milchkühen zu verbessern. Es hat sich gezeigt, dass die Milchharnstoff-N (MUN)-Konzentration linear mit den Urinharnstoffausscheidungen korreliert, was eine Vorhersage der N-Emissionen mit dem Urin und die Bewertung der diätetischen N-Versorgung ermöglicht. Es wurde angenommen, dass eine höhere P-Nutzungseffizienz (Peff), definiert als das Verhältnis zwischen der P-Sekretion in der Milch und dem aufgenommenen P, die fäkale P-Last bei Milchkühen verringert, da mehr P in die Milch geleitet wird.

Bisher scheinen die Unterschiede in der MUN-Konzentration und in der Peff das Ergebnis eines kombinierten Effekts zwischen der genetischen Ausstattung der Tiere und der Futterzusammensetzung zu sein. Die selektive Zucht auf Milchkühe mit niedriger MUN Konzentration und hoher Peff könnte also dazu beitragen, die N- und P-Last im Urin und im Kot zu verringern und eine nachhaltigere Milchwirtschaft zu fördern. Ein erheblicher Teil der interindividuellen Schwankungen der MUN-Konzentration und der Peff sowie die zugrundeliegenden physiologischen Mechanismen sind jedoch weitgehend unbekannt. Ziel der vorliegenden Arbeit war es daher, physiologische und molekulare Mechanismen aufzuklären, die die interindividuelle Variation der MUN-Konzentration und der Peff bei laktierenden Holstein Milchkühen beeinflussen. Die Ergebnisse sollen dazu beitragen, das Potenzial einer selektiven Zucht auf MUN-Konzentration und Peff zu bewerten und gleichzeitig

Fütterungsstrategien zu entwickeln, um den N- und P-Fußabdruck der Rinderhaltung in der Umwelt zu verringern.

Die erste Studie hatte zum Ziel, physiologische Mechanismen, die an der Regulierung des Harnstoffmetabolismus und der damit verbundenen N-Ausscheidungen bei Milchkühen mit unterschiedlichen MUN-Konzentrationen beteiligt sind, aufzuklären. Zu diesem Zweck wurden 20 deutsche Holstein-Milchkühe paarweise gekauft, wobei eine Kuh eine hohe (277 mg/L; *n* = 10) und das Partnertier eine niedrige (189 mg/L; *n* = 10) MUN-Konzentration aufwies, aber hinsichtlich Laktationsdauer, Körpergewicht und Milchproduktion vergleichbar waren. Nach dem Transport in den Stall des Forschungsinstituts für Nutztierbiologie (FBN, Dummerstorf, Deutschland) wurden die Kühen auf zwei unterschiedlichen Rohproteinniveaus gefüttert (13,8% vs. 15,9% in der Trockenmasse). Nach einer zweiwöchigen Futteradaptationsperiode in einem offenen Laufstallsystem führten wir eine zweitägige indirekte Kalorimetrie und anschließend eine viertägige N-Bilanz- inklusive einer ^{13}C-Harnstoff-Isotopen-Tracer-Studie durch. Unsere Ergebnisse zeigen, dass die MUN-Gruppen sich weder in den N-Ausscheidungen im Kot noch in den Harnstoff- oder N-Ausscheidungen im Urin unterschieden, obwohl sie das gleiche Futter erhielten. Die letztgenannten Ergebnisse wurden auf eine unterschiedliche Nierenleistung in Bezug auf die allgemeine glomeruläre Filtrationsrate der Nieren und insbesondere auf die renale Clearance-Rate von Harnstoff zurückgeführt. Interessanterweise schieden Kühe mit hoher MUN-Konzentration unabhängig von der Eiweißfütterung weniger Kreatin und bei eiweißarmer Ernährung weniger Harnsäure mit dem Urin aus als Kühe mit niedriger MUN-Konzentration. Da jedoch 75% des gesamten N im Urin auf Harnstoff entfallen und sich die MUN-Gruppen in der Gesamt-N-Ausscheidung mit dem Urin nicht unterschieden, kamen wir zu dem Schluss, dass sich Kühe mit hohem und niedrigem MUN-Gehalt in Bezug auf die potentiellen Lachgas- oder Ammoniakemissionen nicht unterscheiden. Im Gegensatz dazu führte eine Verringerung des diätetischen Rohproteingehalts zu einer beträchtlichen Verringerung der N-Ausscheidungen im Urin bei gleichzeitiger Verbesserung der N-Nutzungseffizienz für die Milchproduktion.

Die zweite Studie zielte darauf ab, die zugrunde liegenden physiologischen und molekularen Mechanismen aufzuklären, die die P- und N-Ausscheidungen von Milchkühen mit unterschiedlicher Peff beeinflussen. Zu diesem Zweck wurden 20 deutsche Holstein Milchkühe mit einer Futterration gefüttert, die einen vergleichbaren Gehalt an P (0,37% in der Trockenmasse) und Rohprotein (14% in der Trockenmasse) aufwies. Beide Gehalte liegen im unteren Bereich der NRC-Empfehlungen (National Research Council; Nutrient requirements of dairy cattle, 2001). Nach einer dreiwöchigen Futteradaptation in einem offenen Laufstallsystem führten wir eine 4-tägige P- und N-Bilanzstudie in Anbindung durch. Die Kühe wurden entsprechend den Ergebnissen der P-Bilanzstudie nachträglich in eine Gruppe mit

hoher (40%; *n* = 10) und niedriger Peff (33%; *n* = 10) eingeteilt. Schließlich wurden die Milchkühe geschlachtet, um Gewebeproben aus der Jejunalschleimhaut, der Nierenrinde und der Milchdrüse zu gewinnen und anschließend die Expression der für P-Transportproteine kodierenden mRNA mittels quantitativer Echtzeit-PCR zu analysieren. Unsere Resultate zeigten, dass Peff und der P-Ertrag in der Milch stark mit dem Milchfett- und dem Milcheiweißertrag zusammenhängen. Kühe mit hoher Peff wiesen im Vergleich zu Kühen mit niedriger Peff eine höhere renale P-Rückresorptionsrate auf, was mit geringeren P-Verlusten im Urin einherging. Letzteres ging mit einer vergrößerten Expression des anorganischen P-Transportproteins-2 im Nierengewebe einher, was darauf hindeutet, dass es maßgeblich an den individuellen Unterschieden in der P-Ausscheidung im Urin beteiligt ist. Im Hinblick auf die viel größere fäkale P-Ausscheidung scheinen die Unterschiede in der P-Ausscheidung im Urin jedoch vernachlässigbar zu sein. Kühe mit hoher Peff hatten eine niedrigere scheinbare P Verdaulichkeit und eine negativere P-Bilanz als Kühe mit niedriger Peff, was auf eine höhere Mobilisierung der körpereigenen P-Reserven zurückgeführt wurde. Anhand der vorliegenden Ergebnisse lässt sich vermuten, dass bei unzureichender P-Versorgung die mikrobielle Proteinsynthese im Pansen und die damit verbundene Milchsynthese auf Kosten der Körper-P-Reserven priorisiert wird. Darüber hinaus haben wir festgestellt, dass Kühe mit hoher Peff im Verhältnis zum aufgenommenen P mehr P mit dem Kot ausscheiden als Kühe mit niedriger Peff, was die anfänglichen ökologischen Vorteile einer hohen Peff beeinträchtigt.

Zusammenfassend lässt sich sagen, dass die Ergebnisse darauf hindeuten, dass eine Verringerung der diätetischen Rohproteinzufuhr ein erhebliches Potenzial zur Verringerung der N-Emissionen aus der Rinderhaltung hat, wohingegen eine Selektion auf niedrige MUN-Konzentrationen kaum Beiträge leisten kann. Im Gegensatz dazu erwies sich eine etwaige Zucht auf Kühe mit hoher Peff und einhergehender Neff als ökologisch und wirtschaftlich sinnvoll. Eine unzureichende diätetische P-Versorgung stimuliert jedoch die Mobilisierung der körpereigenen P-Reserven. Eine langfristige Mobilisierung von P-Reserven kann sich negativ auf die Gesundheit und Langlebigkeit der Tiere auswirken und erhöht die P-Last im Kot. Daher ist eine selektive Zucht auf eine hohe Peff sinnvoll, aber die P-Versorgung mit dem Futter sollte sich an der jeweiligen Milch-P-Leistung orientieren und regelmäßig anhand der fäkalen P-Last innerhalb der Herde überprüft werden.

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List of publications

Peer-reviewed publications

Müller, C.B.M., Kuhla, B. (2021): Holstein dairy cows with high phosphorus utilization efficiency fed a low phosphorous diet secreted less phosphorus with urine but more with milk and feces. Sci. Total Environ. 788:147813.

Müller, C. B. M., Görs, S., Derno, M., Tuchscherer, A., Wimmers, K., Zeyner, A., Kuhla, B. (2021): Differences between Holstein dairy cows in renal clearance rate of urea affect milk urea concentration and the relationship between milk urea and urinary nitrogen excretion. Sci. Total Environ. 755:143198.

Peer-reviewed abstracts

Müller, C.B.M., Görs S., Tuchscherer A., Reinsch N., Wimmers K., Kuhla B. (2020): Nitrogen intake, metabolism and excretions of dairy cows with divergent milk urea concentrations. Proceedings of the Society of Nutrition Physiology 29:24.

Carolin B.M. Müller, Björn Kuhla (2019): Influence of dietary protein content on nitrogen and methane emissions and urea recycling in dairy cows with divergent milk urea content. 12. Doktorandensymposium and DRS Präsentationsseminar “Biomedical Sciences”:24.

Oral presentations

Carolin Müller (2021): Stickstoff- und Phosphor-Umweltbelastung (Promotion Pitch). KlimAgrar (unter-2-grad.de), Transferwerkstatt für Doktoranden.

Carolin B.M. Müller, Solvig Görs, Michael Derno, Armin Tuchscherer, Klaus Wimmers, Annette Zeyner, Björn Kuhla (2020): Differences between Holstein dairy cows in renal clearance rate of urea affect milk urea concentration and the relationship between milk urea and urinary nitrogen excretion. Day of the Doctoral Student 2020, Leibniz Institute for Farm Animal Biology.

Carolin Müller (2020): Holstein dairy cow urea renal clearance rate, milk urea concentration, urinary nitrogen excretion. KlimAgrar (unter-2-grad.de), Arbeitstagung Tierwohl in der Nutztierhaltung.

Müller, C.B.M., Görs S., Tuchscherer A., Reinsch N., Wimmers K., Kuhla B. (2020): Nitrogen intake, metabolism and excretions of dairy cows with divergent milk urea concentrations. Proceedings of the Society of Nutrition Physiology 29:24.

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Carolin B.M. Müller (2018): Influence of dietary protein content on nitrogen and methane emissions and urea recycling in dairy cows with divergent milk urea content. Day of the Doctoral Student 2018, Leibniz Institute for Farm Animal Biology.

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Declaration of independence and conflict of interest

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe. Es besteht kein Interessenskonflikt durch die finanzielle Unterstützung der Arbeiten.

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Carolin Beatrix Maria Müller