TUM School of Life Sciences

Experimental Study on the Potential Role of Methionine as First-Limiting Amino Acid for Growing Fleckvieh (German Simmental) Bulls

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Vollständiger Abdruck der von der TUM School of Life Sciences

der Technischen Universität München zur Erlangung des akademischen Grades einer

Doktorin der Agrarwissenschaften (Dr. agr.)

genehmigten Dissertation.

Vorsitzender:	Prof. Dr. Kurt-Jürgen Hülsbergen
Prüfer der Dissertation:	1. Prof. Dr. Wilhelm Windisch
	2. apl. Prof. Dr. Michael W. Pfaffl

Die Dissertation wurde am 25.09.2023 bei der Technischen Universität München eingereicht und durch die TUM School of Life Sciences am 15.02.2024 angenommen.

LIST OF PUBLICATIONS

Publications in peer-reviewed journals

Inhuber, V., Windisch, W., Bächler, B., Schuster, M., Spiekers, H., & Ettle, T. (2021). Effects of supplementing a CP-reduced diet with rumen-protected methionine on Fleckvieh bull fattening. *Animal*, *15*(11), Article 100366. https://doi.org/10.1016/j.animal.2021.100366

Inhuber, V., Windisch, W., Kleigrewe, K., Meng, C., Bächler, B., Gigl, M., Steinhoff-Wagner, J., & Ettle, T. (2023). Effect of rumen-protected methionine on metabolic profile of liver, muscle and blood serum samples of growing German Simmental bulls fed protein-reduced diets. *Metabolites*, *13*(8), Article 946. https://doi.org/10.3390/metabo13080946

Abstracts and Articles in Conference Proceedings

Inhuber, V., Windisch, W., Spiekers, H., Ettle, T. (2019). Effect of supplemental rumen-protected methionine on zootechnical performance of bulls for fattening. *Adv. Anim. Biosci.* 10(3): 546.

Inhuber, V., Windisch, W., Spiekers, H., Ettle, T. (2019). Einfluss einer Ergänzung der Milchviehration mit pansengeschütztem Methionin und Lysin bei abgesenkter RNB auf Milchleistung und Futteraufnahme. In: Harms, K., Windisch, W. (2019): Nachhaltigere Tierernährung: Erfolgreiche Fütterung, Ökonomie, Biodiversität und Umwelt im Einklang. *Tagungsband der 57. Jahrestagung der Bayerischen Arbeitsgemeinschaft für Tierernährung (BAT) e.V.*. ISBN: 978-3-9816116-6-3, pp. 138-144.

Inhuber, V., Windisch, W., Spiekers, H., Ettle, T. (2019). Einfluss einer Ergänzung von Fresserrationen mit Futterharnstoff und pansengeschütztem Methionin auf die Zuwachsleistung. In: Hans Eisenmann-Zentrum (edt.): *10th HEFagrar Symposium, Biodiversität in der Agrarlandschaft: Messen - Fördern - Gestalten*. 26. September 2019. pp. 71-72.

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LIST OF ABBREVIATIONS

AA	Amino acid
CON	Control diet (standard diet according to GfE, 1995)
CP	Crude protein
Cys	Cysteine
DLG	Deutsche landwirtschaftliche Gesellschaft German Agricultural Society
DM	Dry matter
EC	European Commission
EU	European Union
EUROP	Carcass Classification System E, U, R, O, P
GLMM	General linear mixed model
INRA	Institut national de la recherche agronomique
IPC	Ideal protein concept
LfL	Bavarian State Research Centre for Agriculture
MET	Methionine
MP	Microbial protein
MS	Mass spectrometry
MUCP	Utilisable crude protein of microbial origin
NPN	Non-protein nitrogen
QC	Quality control
RED	Crude protein-reduced diet
RED+MET (RPMET)	Crude protein-reduced diet supplemented with rumen-protected methionine
SID	Standard ileal digestible
UCP	Utilisable crude protein
VDLUFA	Association of German Agricultural Analytic and Research Institutes e. V.

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SUMMARY

This doctoral project evaluated the potential role of methionine as the first-limiting amino acid for growth and its metabolic function in growing Fleckvieh bulls fed crude protein–reduced diets. This knowledge is pivotal for introducing the 'ideal protein concept' into beef cattle nutrition. By successfully implementing this nutritional concept and utilising marginal areas with difficult agroecological conditions, beef meat production should be more sustainable and contribute to global food security.

An experimental feeding study was conducted to determine the specific role of methionine in growing Fleckvieh bulls. Growth and slaughter performance parameters and the amino acid blood profile were evaluated. Metabolomics analyses elucidated the metabolic responses of the bulls on supplemented rumen-protected methionine and crude protein–reduced diets. The results depict the effect of crude protein reduction and supplemental rumen-protected methionine on growth and slaughter performance and the single-cell metabolic response in growing Fleckvieh bulls.

A total of 69 growing-finishing Fleckvieh bulls (on average, 367 ± 25 kg initial bodyweight) were allocated to three different feeding groups: a positive control containing 13.7% crude protein (CON) and two negative control diets deficient in crude protein (RED; both 9.04%), with one of them being supplemented with rumen-protected methionine (RED+MET). The methionine concentrations in the diets were 2.11 g/kg, 1.56 g/kg and 2.11 g/kg, respectively (dry matter based). Metabolisable energy concentration was held equal between all feeding groups. The bulls were weighed at regular intervals during the experiment and directly before slaughter. Liver and muscle tissue and blood samples were taken, and carcasses were evaluated at slaughter. Zootechnical data were analysed using a general linear mixed model, with dietary treatment as a fixed effect and pen x treatment as a random effect. Statistical analysis of carcass quality was performed with a nonparametric post-hoc test. Metabolomics data were subject to bioinformatics analyses to detect pathway shifts. Different databases were used to reliably annotate significantly regulated metabolites.

The study outcomes are presented in two publications. The first publication covers growth and slaughter performance and the blood amino acid pattern. The results demonstrated that dietary crude protein reduction decreased feed intake. Hence, overall nutrient and metabolisable energy intake were lower in both RED and RED+MET bulls than in CON bulls. Consequently, the growth performance and carcass weights of RED and RED+MET bulls were lower than those of CON bulls. Adding rumen-protected methionine to the diet low in crude protein (RED+MET) elevated serum methionine levels but did not relieve the bulls from growth restriction.

Retrospective calculations revealed that pre-cecal digestible protein (and amino acid) intake was lower in both RED and RED+MET bulls, but intake relative to growth was not below retrospectively calculated

requirements. In conclusion, bulls that received crude protein–reduced diets were subject to a ruminal nitrogen deficit and, subsequently, a deficit of pre-cecal digestible protein. Bulls faced this nutritional situation with lower nutrient turnover (i.e., growth). Methionine did not appear to be the first-limiting amino acid for growth under these experimental conditions.

The second publication evaluated the effect of supplemented rumen-protected methionine on metabolic pathways in liver and muscle tissues and the blood serum of Fleckvieh bulls fed crude protein–reduced diets. Metabolomics analyses revealed that single-cell metabolism was nearly unaffected by a reduction in dietary crude protein. Fleckvieh bulls set maintenance as the prevailing priority of nutrient trafficking (homeostasis) at the expense of protein deposition (i.e., growth [homeorhesis]). This is in line with the performance results presented in the first publication. However, the addition of rumen-protected methionine significantly altered hepatic one-carbon metabolism. Bulls in the RED+MET group had higher cysteine glutathione disulfide synthesis than RED bulls ($p < 0.05$). Our experimental setup did not allow us to evaluate whether this was a 'push or pull' reaction on their metabolism.

The combined evaluation of the phenotype and metabolome results ('metabotype') indicated the metabolic process decoupling of maintenance and specific metabolic pathways, such as the activation of the antioxidant immune response. Metabotype analyses help to build a more precise picture of the metabolism and physiological adaptations of growing Fleckvieh bulls. This knowledge is crucial for adapting (amino acid) feeding recommendations and further approaching the 'ideal protein concept' for growing Fleckvieh bulls – thereby increasing the nitrogen efficiency of beef production, improving herd health and welfare and, ultimately, contributing to more sustainable beef supply chains.

ZUSAMMENFASSUNG

Im Rahmen dieses Promotionsprojekts werden die potenzielle Rolle von Methionin als erstlimitierende Aminosäure für das Wachstum und dessen spezifische Stoffwechselfunktion bei wachsenden Fleckviehbullen untersucht, die mit Rohprotein-reduziertem Futter gefüttert wurden. Die Rolle von Methionin ist entscheidend für die Entwicklung des *Idealprotein-Konzepts* für die Mastbullenfütterung. Durch die erfolgreiche Umsetzung dieses Fütterungskonzepts und die Nutzung von Regionen mit schwierigen agrarökologischen Bedingungen kann die Rindfleischproduktion nachhaltiger werden. Nicht zuletzt wird dadurch die globale Ernährungssicherung unterstützt.

Es wurde eine experimentelle Fütterungsstudie durchgeführt, um die spezifische Rolle von Methionin bei heranwachsenden Fleckviehbullen zu evaluieren. Bewertet wurden Wachstums- und Schlachtleistungsparameter sowie das Aminosäure-Blutprofil. Mit Metabolom-Analysen wurden die Stoffwechselreaktionen der Fleckviehbullen auf die Zugabe von pansengeschütztem Methionin in rohproteinreduzierten Rationen im Detail untersucht. Die Ergebnisse zeigen die Auswirkungen einer Rohproteinreduzierung und einer Supplementierung von pansengeschütztem Methionin auf das Wachstum und die Schlachtleistung sowie auf die Zellstoffwechselreaktionen bei wachsenden Fleckviehbullen.

Es wurden 69 Fleckviehbullen (durchschnittlich 367 ± 25 kg Ausgangskörpergewicht) in 3 Fütterungsgruppen eingeteilt: eine Positivkontrolle mit 13,7 % Rohprotein (CON) und 2 Negativkontrollen mit reduziertem Rohproteingehalt (RED; beide 9,04 %; eine davon wurde mit pansengeschütztem Methionin (RED + MET) ergänzt). Die Methionin-Konzentrationen in den Rationen betrugen 2,11 g/kg, 1,56 g/kg und 2,11 g/kg (Trockenmasse-Basis). Die Energiekonzentration (metabolische Energie) wurde in allen Fütterungsgruppen gleich gehalten. Während des Experiments und direkt vor der Schlachtung wurden die Bullen in regelmäßigen Abständen gewogen. Es wurden Blut- sowie Leber- und Muskelgewebeproben entnommen und die Schlachtkörper wurden bei der Schlachtung untersucht sowie bewertet. Die zootechnischen Daten wurden unter Verwendung eines allgemeinen linearen gemischten Modells analysiert, wobei die Ration ein fixer Effekt und die Bucht-x-Ration ein zufälliger Effekt war. Die statistische Analyse der Schlachtkörperqualität wurde mit einem nichtparametrischen Post-hoc-Test durchgeführt. Die Metabolom-Daten wurden zur Bestimmung von Veränderungen in den physiologischen Stoffwechselwegen bioinformatischen Analysen unterzogen. Zur zuverlässigen Annotation signifikant regulierter Metaboliten wurden verschiedene Datenbanken herangezogen.

Die Studienergebnisse werden in zwei Publikationen vorgestellt. Die erste Veröffentlichung befasst sich mit Wachstum und Schlachtleistung sowie mit dem Aminosäureprofil im Blut. Die Ergebnisse zeigen, dass die Reduzierung des Rohproteins die Futteraufnahme signifikant verringert hat. Daher war die Gesamtnährstoff- und Energieaufnahme sowohl bei RED als auch bei RED + MET niedriger als bei CON.

Folglich waren die Wachstumsleistung und die Schlachtkörpergewichte von RED und RED + MET geringer als bei den CON-Bullen. Die Zugabe von pansengeschütztem Methionin zur Rohprotein-reduzierten Ration (RED + MET) erhöhte die Methionin-Konzentration im Serum, kompensierte jedoch nicht die durch die Rohprotein-Reduktion induzierte Wachstumsdepression.

Retrospektive Berechnungen zeigten, dass die Aufnahme von präcecal verdaulichem Protein (und Aminosäuren) sowohl bei RED als auch bei RED + MET geringer war, die Aufnahme im Verhältnis zum Wachstum jedoch nicht unter dem retrospektiv berechneten Bedarf lag. Zusammenfassend lässt sich sagen, dass Bullen, die eine Rohprotein-reduzierte Ration erhielten, einem Stickstoffdefizit im Pansen und in der Folge einem Defizit an verdaulichem Protein zur Absorption im Dünndarm ausgesetzt waren. Die Fleckviehbullen begegneten der Rohprotein-reduzierten Ration mit einem geringeren Nährstoffumsatz (Wachstum). Methionin war unter diesen experimentellen Bedingungen nicht die erstlimitierende Aminosäure für das Wachstum der Fleckviehbullen.

In der zweiten Veröffentlichung wurde die Wirkung von Rohprotein-reduziertem Futter sowie pansengeschütztem Methionin auf Stoffwechselwege in Leber- und Muskelgewebe sowie im Blutserum von Fleckviehbullen untersucht. Metabolom-Analysen ergaben, dass der Zellstoffwechsel durch eine Reduzierung des Rohproteins in der Nahrung nahezu unbeeinflusst blieb. Die Fleckviehbullen stellten die Erhaltung des Stoffwechsels (Homöostase) auf Kosten des Proteinansatzes, d. h. des Wachstums (Homöorhese), in den Vordergrund. Dies steht im Einklang mit den in der ersten Veröffentlichung dargestellten Leistungsergebnissen. Allerdings beeinflusste die Zugabe von pansengeschütztem Methionin den One-Carbon-Stoffwechsel in der Leber. Die Bullen der RED+MET-Gruppe hatten eine höhere ($p < 0,05$) Cystein-Glutathion-Disulfid-Synthese als RED-Bullen. Mit diesem Versuchsaufbau war es jedoch nicht möglich, zu beurteilen, ob es sich hierbei um eine Push-oder eine Pull-Reaktion ihres Stoffwechsels handelte.

Die kombinierte Evaluierung der Phänotyp- und der Metabolom-Ergebnisse (*Metabotyp*) zeigte die Entkopplung des Stoffwechselprozesses von Erhaltungs- und spezifischen Stoffwechselwegen (z.B. Aktivierung der antioxidativen Immunantwort).

Metabotyp-Analysen tragen dazu bei, ein genaueres Bild des Stoffwechsels und der physiologischen Anpassungen wachsender Fleckviehbullen zu gewinnen. Dieses Wissen ist entscheidend, um die (Aminosäure-) Fütterungsempfehlungen anzupassen und das Idealprotein-Konzept für wachsende Fleckviehbullen weiterzuentwickeln.

1 GENERAL INTRODUCTION

Beef supply chains have gained significant public attention in global agricultural production. This is mainly due to beef's lower feed-to-food transformation efficiency compared to poultry and swine (Gerber et al., 2015). It has been estimated that cattle contributes 56%–60% of the nitrogen excretions by all livestock species (Oenema, 2006). Cattle's efficiency in utilising dietary protein to produce human-edible protein depends on the digestibility of protein and the respective amino acid (AA) pattern. A deficient AA supply decreases performance, whereas an oversupply of AA results in excess nitrogen excretion into the environment (van Milgen & Dourmad, 2015). The 'ideal protein concept' (IPC) describes a very high-quality dietary protein that delivers an AA profile as close as possible to animals' metabolic requirements. Mitchell (1962) developed this concept decades ago, and it has been well established in poultry and pig nutrition. The initial step is identifying (performance) limiting essential AAs. Dietary supplementation of these single AAs then allows for an overall reduction in dietary crude protein (CP), which results in less nitrogen excretions. Hence, precision livestock feeding is possible, but a deep understanding of animals' AA requirements is warranted (Cappelaere et al., 2021; Chalvon-Demersay et al., 2021; van Milgen & Dourmad, 2015). Still, transferring the IPC to beef cattle nutrition is not simply possible.

With the aid of rumen microbiota, cattle have the exclusive ability to digest cell wall–rich feedstuffs, such as grass and straw, to produce energy and synthesise endogenous protein (Thrän & Moesenfechtel, 2022, Chapter 5.2.2). They make use of dietary protein and non-protein nitrogen (NPN) compounds (e.g., urea) to produce endogenous protein (referred to as microbial protein [MP]). In more detail, MP results from a two-step process: first, rumen microbiota break down dietary protein into peptides and AA and subsequently *de novo* synthesise protein using these breakdown products and NPN compounds. Due to these transformation processes, the AA pattern of MP substantially differs from that of dietary protein. Next to MP, rumen-bypass protein (RBP) and endogenous secretions contribute to the duodenal flow of AA for absorption, referred to as utilisable CP (UCP; Clark et al., 1992; Richardson & Hatfield, 1978). The AA pattern of UCP is challenging to predict. It does not necessarily represent the ideal AA pattern the host animal requires since there is no direct feedback mechanism (Lapierre & Lobley, 2001). Consequently, applying the IPC in beef cattle nutrition must account for rumen transformation processes. It must overcome the limitation of the AA pattern incongruence between UCP and animal requirements. Identifying limiting essential AAs is logically the pivotal step to successfully implementing the IPC in beef cattle nutrition.

Respective feeding trials must comply with two main conditions: first, protein supply must be deficient, while other nutrients and metabolisable energy must be supplied in sufficient amounts. Second, the AA

in question must be supplemented into the diet by adding it in a rumen-protected form or infusing it postruminally to prevent it from being transformed in the rumen. Relief from growth limitation as an effect of AA supplementation displays its limitation potential. However, this effect is only pronounced until the next critical AA becomes limiting (D'Mello, 2003, Chapter Ruminants; Storm & Ørskov, 1979).

Various protein evaluation systems worldwide try to assess the metabolic AA requirements of both beef cattle and dairy cows. Over the last decades, progress has been made in precise AA nutrition in dairy cows. However, knowledge on limiting AAs in beef cattle, especially for the modern Fleckvieh breed genotype, still needs to be improved (Inhuber, Windisch, Bächler, et al., 2021). Some older sophisticated research results in growing-finishing beef cattle, applying postruminal infusion techniques, suggest the importance of methionine (MET) for both growth performance and (immuno-/redox-) metabolism of growing cattle (Froidmont et al., 2002; R. Greenwood & Titgemeyer, 2000; Richardson & Hatfield, 1978). Protein evaluation systems, however, generally focus on the role of AAs as protein building blocks (i.e., growth). However, AAs are also precursors to energy and functional molecules, and hence, they modulate specific metabolic pathways (Chalvon-Demersay et al., 2021). Recent research by Beaumont et al. (2022) and Chalvon-Demersay et al. (2021) in broiler and piglet nutrition have demonstrated the relevance of including the functional metabolic role of AAs in determining metabolic requirements, ultimately improving animal health in high-performing animals.

Therefore, this doctoral thesis aimed to evaluate the potential role of MET as the first-limiting AA in growing Fleckvieh bulls through a comprehensive feeding trial combined with metabolomics analyses in selected target tissues. The next subsection (Chapter 1.1) provides insight into the environmental discussion around beef cattle production, focusing on the role of nitrogen. This chapter is followed by an explanatory section on the nitrogen flux in ruminants (Chapter 1.2) and, subsequently, an overview of different protein evaluation systems (Chapter 1.3).

1.1 Beef Cattle Production in the Global Discussion: Food Security, Environment and Social Perception

The global population is projected to grow to 9.7 billion by 2050 (Food and Agriculture Organization of the United Nations et al., 2018; UN Department for Economic and Social Affairs, 2023). Hence, on the one hand, the consumption of animal protein (meat, eggs and dairy) is increasing worldwide (Kearney, 2010), but on the other hand, the agricultural livestock systems are challenged by increasing environmental issues, such as greenhouse gas emissions, the loss of natural ecosystems, deteriorating biodiversity and disruption of nutrient cycles (Intergovernmental Panel on Climate Change, 2019; Thompson et al., 2023). Although livestock production itself has contributed to these changes, as indeed have other industries, it can form part of the solution to improve public health and environmental

resilience (Leroy et al., 2023). However, livestock production must align with the agroecological framework comprehensively, considering local circumstances (Thompson et al., 2023). In this context, beef cattle (i.e., ruminants) are particularly important. First, their unique digestive qualification to use high-roughage, non-human-edible biomass enables them to develop in conditions where other livestock species, such as poultry and swine, are excluded (Gerber et al., 2015) and thereby enlarges the terrestrial basis for more sustainable food production (P. Greenwood, 2021; Thrän & Moesenfechtel, 2022). Second, cattle upcycle nonedible biomass into an edible high-value protein source (P. Greenwood, 2021) while recycling nutrients back into the soil and sequestering carbon. This puts them into an intrinsic connection with crop production. Recapitulating, livestock, especially beef cattle (ruminants), efficiently drive bioeconomic circularity (Thompson et al., 2023). Livestock integration into soybean production systems, for instance, has shown to be beneficial for system stability and profitability, depending on overall management (de Albuquerque Nunes et al., 2021).

From a global perspective, beef supply chains are responsible for approximately 40% of all livestock emissions (life-cycle approach; Gerber et al., 2015). There are fibre-rich plant resources that humans cannot (or choose not to) consume which can be converted into high quality food. This conversion causes unavoidable losses of nitrogen, methane, and carbon dioxide into the environment. The carbon footprint of beef cattle with 1,000 g daily live weight gain is 55 kg CO_2equ/kg edible protein, whereas growing broiler chickens with an average daily live weight gain of 60 g only have a carbon footprint of 3 kg CO_2equ/kg edible protein. These values may be significantly influenced by various production factors (e.g., animal disease and reproduction), but they give a good general ranking (Thrän & Moesenfechtel, 2022, Chapter 5.2.2).

Within animal protein production systems, it is not only due to the impact on the environment that, but beef cattle production also emerges as a major public attention (Gerber et al., 2015), losing the original public cherishment of red meat as valuable food (Leroy & Hite, 2020). Next to generalised environment arguments that do not consider pivotal subtleties, such as the consideration of the positive impact of livestock on other ecosystem services than food production (e.g., soil health and carbon sequestration; Thompson et al., 2023), health arguments with negative connotations (Leroy et al., 2023) are often presented in mass media communications (Leroy & Hite, 2020). Some authors believe that animal-source foods are intrinsically unhealthy, unsustainable and unethical (Barnard & Leroy, 2020; Deckers, 2013) and even consider red meat as far more harmful than other foods. This perception is driven by several sensationalist media communications. Particularly, the proposals in the EAT-Lancet report have influenced public perception of meat consumption and even impacted policy levels. The nonprofit organisation EAT, placing itself in a network of innovative food and food alternative multinationals (Leroy & Hite, 2020), has suggested a ‘Great Food Transformation’ (Lucas & Horton, 2019) and proposes the ‘planetary health diet’, which is nearly vegetarian and allows for a vegan option (Willett et al., 2019). A

plant-based diet, however, may lead to a shortage of protein intake (quantity and quality; Steenson & Buttriss, 2020), along with a decrease in some micronutrients (e.g., zinc, iron, vitamin B12; Leroy & Cofnas, 2020; Thompson et al., 2023) which are already limiting nutrients in many world regions (Ederer & Leroy, 2023).

Beef production, among livestock systems in general, has become subject to simplification, reductionism, and zealotry. To counteract this progressive development, 'The Dublin Declaration of Scientists on the Societal Role of Livestock' (2023) gives a voice to scientists worldwide who conduct serious and reliable research 'to achieve a balanced view of the future of animal agriculture'.

Within this context, the objective of beef cattle nutritionists is to continuously improve feeding concepts that overcome the limitations of ruminal transformation processes and thereby further drive beef production efficiency. In particular, protein transformation processes (i.e., the breakdown of dietary protein and subsequent *de novo* MP synthesis) need to be exceedingly evaluated and strategically manipulated to further increase feed-to-food transformation efficiency.

1.2 Nitrogen and Amino Acid Flux in Cattle

The ruminal transformation of (non-human-edible) biomass into high-value nutrients for the animal and, subsequently, into human-edible protein relies on a multitude of complex metabolic processes. Ruminants require predominantly energy and protein and, of course, other nutrients, such as minerals and vitamins. A four-compartment stomach dominates the ruminant digestive system, where the rumen plays the central role. Figure 1 shows a simplified model of nitrogen flux in cattle.

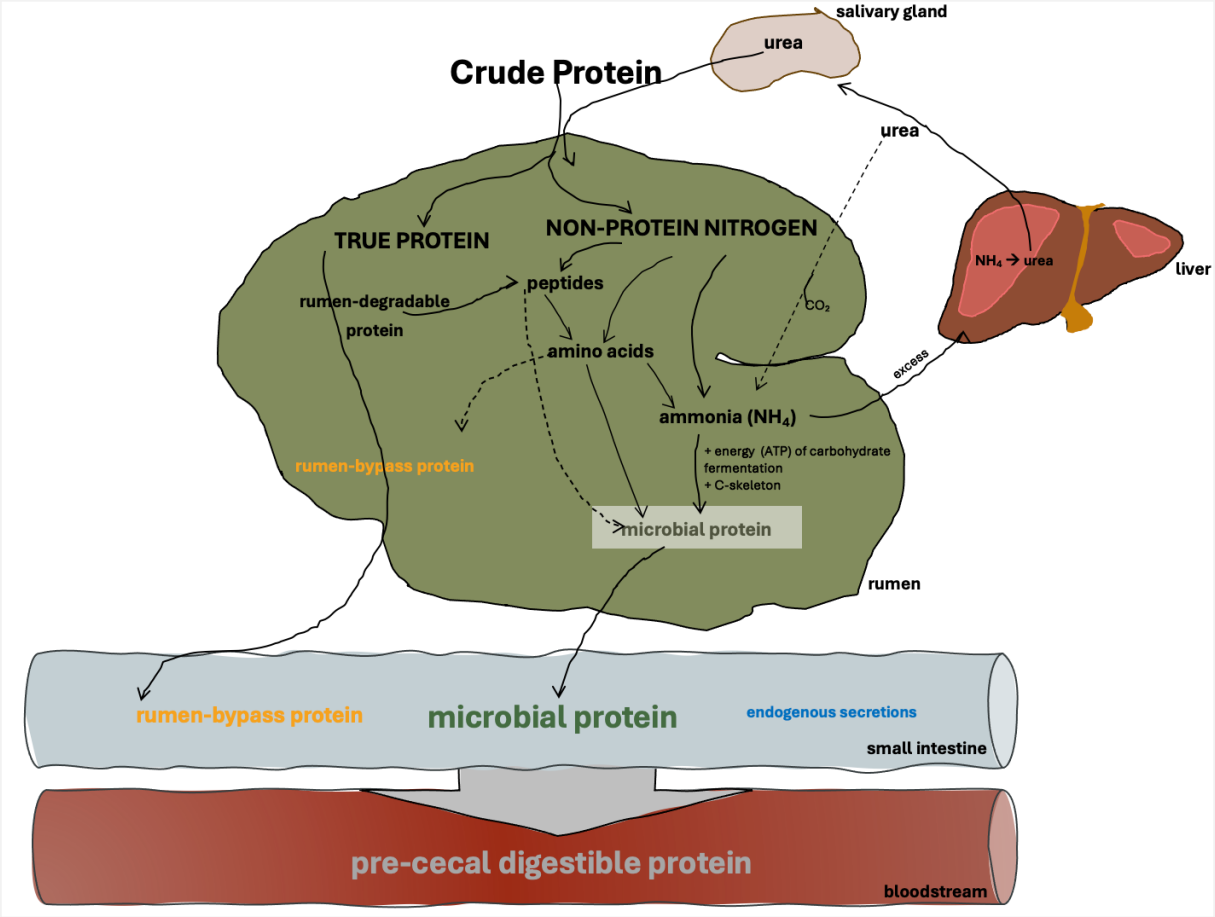

Figure 1: Schematic depiction of nitrogen flux in the rumen and subsequent protein digestion.

Dietary CP (nitrogen x 6.25) can be differentiated between true protein and NPN (Schwab & Broderick, 2017). Rumen microbiota breaks down (rumen-degradable) true protein into peptides, single AAs, and ammonia. Non-protein nitrogen, either from feed or recycled endogenously in the form of urea, serves as a nitrogen source for microbiota to *de novo* synthesise peptides and AAs – which, together with the ones derived from true feed protein – build the base for MP synthesis (Clark et al., 1992; D'Mello, 2003; Lapierre & Lobley, 2001). Microbial CP then flows to the small intestine together with true protein resistant to microbial degradation (RBP) and endogenous protein secretions, delivering AA for absorption (Sok et al., 2017).

Microbial protein accounts for more than 50% of the duodenal CP flow (Clark et al., 1992), implying the relevance of precise knowledge on its AA composition. Clark et al. (1992) is widely used – for example, in the National Research Council (NRC, 2001) to assess the AA composition of the duodenal MP flow. However, these data are quite old, used studies from sheep (n = 7 studies) and ruminants (n = 11 studies) and only considered fibre-associated bacteria. Sok et al. (2017) updated the AA composition of rumen bacteria and included protozoa in their extensive analyses. They showed that the true protein proportion of microbial CP should be 82.4% instead of 80% (e.g., NRC, 2001). Furthermore, they differentiated

between fibre-associated bacteria, particle-associated bacteria, and protozoa. AA analyses of these fractions indicated significant differences between protozoa and bacteria for four essential and six nonessential AAs. Within bacterial fractions, AA concentrations also differed significantly. The authors calculated the AA composition of microbial CP, applying a ratio of 16.5% of protozoa and 83.54% for bacteria distributed in a 40/60 ratio of fibre-associated and particle-associated bacteria (33.4% and 50.1% of the total obtained from Brito et al., 2007, Brito and Broderick, 2007, and Reynal and Broderick, 2005). Sok et al. (2017) published the AA composition of MP for future reference with updated values based on their equations.

1.3 Protein Evaluation Systems

The definition of a cattle nutrition model is the 'integrated set of equations and transfer coefficients that describe their various physiological functions' (Tedeschi, 2019). On the one hand, these models use prediction equations for tissue requirements (maintenance, growth and tissue reserves), which have to be based on frequently updated data from the currently used genotypes. Honig, Inhuber, Spiekers, Windisch, Götz, and Ettle (2020), for instance, determined the carcass tissue composition of modern Fleckvieh bulls, depending on varying dietary energy concentrations. These data serve as an important base to evaluate changes in modern genotypes as compared to prior genotypes of the Fleckvieh breed and, subsequently, to adapt feeding recommendations (energy and protein) accordingly.

On the other hand, nutrition models try to predict nutrient supply (dry matter [DM] intake, dietary carbohydrate and protein fraction pool) and their respective characteristics (digestibility, passage rate, microbial growth potential, intestinal digestion and absorbed nutrients; Tedeschi et al., 2015). The AA flow in the small intestine of ruminants consists of three origins: first, the flow of ruminal bypass protein. The prediction of AA supply derived from RBP strongly depends on feed component analysis. It must be considered that there is high variation in rumen-bypass content in forages. This is due to many factors, such as soil conditions, maturity, species, fertilisation regime and proteolysis during ensiling (Lee et al., 2008). Two forage types which are mainly used for ruminants in central Europe are grass and maize silage, which differ substantially in their rumen-bypass concentrations and their respective AA patterns. The second AA flow is that of endogenous secretions, which only contributes to a small part of the protein flux at the small intestine, and the third is MP flow, the major part of intestinal AA flow (Clark et al., 1992; Richardson & Hatfield, 1978). Microbial protein is the result of two rumen-fermentation process steps. First, dietary CP is broken down by rumen microbiota, and subsequently, these reaction products (AA, peptides) are used for *de novo* microbial synthesis. This leads to a biologically inevitable mismatch in the AA pattern of dietary protein and actual requirements by the host ruminant. The amount

of AAs relevant to the animal, however, is the sum of AAs actually absorbed from the small intestine into the bloodstream (D' Mello, 2003; Inhuber, Windisch, Bächler, et al., 2021).

Protein evaluation systems must consider both the microbial degradation of dietary CP and subsequent *de novo* protein synthesis in the rumen (D'Mello, 2003; Lapierre et al., 2018; Lapierre & Lobley, 2001). Therefore, the core part of these calculation models aims at precisely modelling ruminal nitrogen transformation. These models try to identify AA limitations, which are caused by the gap between AA flow towards the small intestine (pre absorption) and the actual metabolic requirements of the host ruminant (post absorption). Knowledge on limiting essential AAs then allows for supplementation of rumen-protected AAs and therefore approaches the 'ideal protein'.

Mathematical modelling certainly entails limitations. Correct and precise modelling of the metabolism is a very sophisticated approach because body composition brought about by fat and protein deposition must be precisely predicted. Net AA requirements, for instance, must be generated from protein requirements that use respective AA profiles of body tissues. In this context, the genotype of a certain breed plays an important role. For central European conditions, Honig, Inhuber, Spiekers, Windisch, Götz, and Ettle (2020), Honig, Inhuber, Spiekers, Windisch, Götz, Schuster, and Ettle (2022) and Honig, Inhuber, Spiekers, Windisch, Götz, Strauß, and Ettle (2022) modelled the growth and tissue pattern of modern-type Fleckvieh bulls. The outcomes clearly indicated that modern-type Fleckvieh bulls grow faster than bulls of prior genotypes. Even though empty body tissue distribution has not changed substantially between genotypes at the same bodyweight, modern genotypes demonstrate greater growth potential (Honig, Inhuber, Spiekers, Windisch, Götz and Ettle et al., 2020). These observations serve as important information for the development of feeding recommendations for modern-genotype Fleckvieh bulls. However, in central Europe, with Fleckvieh as the predominant breed, there has yet to be much progress in metabolisable protein/AA nutrition recommendation development over the last years.

1.4 Thesis Objectives

This doctoral thesis aimed to evaluate the potential role of MET as the first-limiting AA in growing Fleckvieh bulls. For this purpose, a comprehensive feeding trial was conducted. Both growth and slaughter performance, as well as meat quality parameters, were assessed. Moreover, metabolic pathways were analysed in liver and muscle tissues and blood serum. The feeding trial and its performance outcomes are described in detail in Inhuber, Windisch, Bächler, et al. (2021; Chapter 3). Details on the metabolic responses of the Fleckvieh bulls to dietary treatments are depicted in Inhuber, Windisch, Kleigrewe, et al. (2023; Chapter 4). The results were evaluated to further approach the IPC for practical beef cattle farming under central European feeding conditions, ultimately supporting more sustainable beef production chains.

Supplementation of performance-limiting AAs to diets low in CP (i.e., IPC) has been a common feeding practice in monogastric livestock nutrition for several years (van Milgen & Dourmad, 2015). Reducing dietary CP significantly improves nitrogen efficiency by reducing nitrogen excretion (especially urea or uric acid) into the environment. Supplementation of feed-grade AAs to these diets is inevitable to maintain animal performance and health (Cappelaere et al., 2021). However, knowledge of the limitation potentials of certain essential AAs and their metabolic functions in growing beef cattle still needs to be made available. The difficulty in applying the IPC to beef cattle stems from ruminal fermentation processes, which tremendously impair the precise quantification of the amount and composition of AAs reaching the small intestine for absorption (D'Mello, 2003). However, this knowledge would be essential for applying an ideal protein to beef cattle feed. Based on Richardson and Hatfield's (1978) ground research work, the first three limiting AAs for growing cattle might be MET, lysine and threonine. Only a few studies on these AAs were conducted in the last few decades; in summary, they did not yield conclusive solid results. However, a recent study (Cantalapiedra-Hijar et al., 2020) suggested that MET was first limiting in young fattening Charolais bulls (320 kg bodyweight). To our knowledge, this is the only study conducted under central European conditions with modern cattle genetics and feed.

Therefore, the first objective of this thesis was to investigate if MET is the first-limiting AA for growth in commonly used maize-based diets for growing Fleckvieh bulls. Since MET is not only crucial for protein synthesis but forms part of essential synthesis pathways (e.g., one-carbon cycle to synthesise antioxidant metabolites), the second objective of this thesis was to evaluate the metabolic response of growing Fleckvieh (German Simmental) bulls fed additional MET in reduced-protein diets.

For this study, 69 Fleckvieh bulls were allocated to three dietary treatments: a standard positive control (CON) adequate in CP (13.7%) and with 2.11 g MET/kg DM, a negative control (RED) reduced in CP (9.04%) and with 1.56 g MET/kg DM and a third diet (RED+MET) only differing in MET (2.56 g/kg DM) as compared to RED.[1] Additional MET in RED+MET was provided in a rumen-protected form. Differences in CP concentrations were achieved by replacing extracted rapeseed meal and urea with dried beet pulp. Metabolisable energy and other nutrients were equal between all three treatment groups. The CON diet met the current feeding recommendations (Daenicke et al., 1995) and reflected practical Fleckvieh bull feeding (bodyweight range: 350–600 kg).

First, we hypothesised that feeding diets with reduced CP levels compared to recommendations (Daenicke et al., 1995) should decrease performance due to protein (nitrogen) deficiency. If MET proves to be first-limiting for growth in a maize-based diet for Fleckvieh bulls during this growth range,

[1]The treatment name RED+MET in Chapter 2, as well as in the thesis manuscript, is denoted as RED+RPMET in Chapter 3 due to a review request.

depression of performance should be compensated by adding additional MET to the diet with reduced CP. The outcomes will elucidate the limitation potential of MET in maize-based diets for growing Fleckvieh bulls. This outcome is pivotal to approaching the IPC for beef cattle nutrition, tremendously supporting more efficient and sustainable beef production.

Second, we hypothesised that additional MET, if not used for muscle protein deposition, should push MET-related pathways in the metabolism of growing Fleckvieh bulls. Knowledge of the functional metabolic role of MET is crucial since productivity is not only based on performance but also on animal health and the proper functioning of the overall metabolism. Furthermore, animal health and welfare become increasingly important in the course of the holistic sustainability approach.

This project aimed to determine the growth, slaughter performance and metabolic responses of Fleckvieh bulls fed CP-reduced diets with supplemented rumen-protected MET. The experimental feeding period lasted for 105 days on average. Bulls were weighed at the start and end of the experiment – that is, immediately before leaving the stable and at regular intervals during the entire trial– to record growth performance. At slaughter, carcasses were evaluated. This should give information on the type of weight gain and whether dietary treatments affect meat quality. Liver, muscle, and blood serum samples underwent metabolic pathway analysis to assess the metabolic responses of the bulls to CP-reduced diets with or without additional MET.

The retrospective calculation of protein and AA supplies and requirements on a pre-cecal digestible level was a significant part of evaluating the performance trial results. Furthermore, metabolome analyses provided novel insights into the MET metabolism and prioritisation of Fleckvieh bulls under CP-reduced feeding conditions. They elucidated the prioritisation of MET in the metabolism of growing Fleckvieh bulls and, thus, suggest the incorporation of metabolic functions (besides growth) of AA into requirement calculations of (Fleckvieh; German Simmental) bulls to further enhance production efficiency and animal health. Therefore, the outcome of this doctoral project supplies new primary data that enable a more precise adaptation of the IPC for growing Fleckvieh bulls, supporting the industries' overall endeavours to minimise the environmental impact of beef cattle supply chains.

1.5 Thesis Outline

The aim of this doctoral research project was to evaluate the potential of MET as the first-limiting AA for growth in a CP-reduced diet fed to Fleckvieh bulls. Furthermore, the metabolic response of Fleckvieh bulls to additional MET in CP-reduced diets was elucidated. The comprehensive outcome delivers novel insights into the specific role of MET in growing Fleckvieh bulls. This knowledge is pivotal to developing more precise feeding recommendations and, ultimately, contributes to more efficient beef meat production chains. Therefore, a feeding trial was performed. Growth and slaughter parameters were assessed, and metabolome analyses were conducted. Chapter 2 describes the details of the materials and methods applied in this research project. Chapter 2.1 outlines the design of the feeding experiment. Chapter 2.2 elucidates the techniques applied for the analysis of feedstuff and blood AA and the metabolome analysis methods. Chapter 2.3 reveals details on statistical and bioinformatics analyses. Furthermore, calculations of protein supply and requirements are explained (Chapter 2.4).

The results of the experimental study are presented in two publications. The first publication (Chapter 3; Inhuber, Windisch, Bächler, et al., 2021) covers growth and slaughter performance results, the blood AA spectrum, and urea concentrations at slaughter. In more detail, the results comprise feed intake data, particularly pre-cecal digestible protein and AA (MET, lysine, and threonine) intake, and data on weight gain, dressing percentage and carcass and meat quality. A complete picture of protein evaluation regarding supply and requirement was drawn by calculating pre-cecal digestible MET, lysine and threonine requirements and intake.

The second publication (Chapter 4; Inhuber, Windisch, Kleigrewe, et al., 2023) elucidates the metabolic response of Fleckvieh bulls to CP-reduced diets with or without additional MET. In detail, volcano plots indicated the magnitude of significant changes in metabolite abundance in liver and muscle tissues and blood serum. Univariate analysis of reliably annotated metabolites revealed the metabolic prioritisation of MET under respective feeding conditions.

Chapter 5 contains a comprehensive discussion of the results of both publications. The combination of performance results and metabolic pathway analyses allows for a deeper understanding of the bulls' metabolism. This is pivotal to developing more precise feeding recommendations and, thus, to further approaching the IPC for future beef cattle nutrition.

Chapter 6 shows the synthesis of the experimental study outcomes and provides an outlook for further research. The key results of this doctoral project are put in a global context, and it is suggested that trial designs are required to obtain more details on the role of MET in the metabolism of Fleckvieh bulls. This information is crucial to reducing dietary protein concentrations without compromising performance. This nutritional strategy (IPC) will enable farmers to reduce beef production systems' environmental

impact and strongly support the whole industry's endeavours to reduce global warming and secure global food security.

2 GENERAL METHODOLOGY

This section details the materials and methods previously described in the two publications on this research project (Inhuber, Windisch, Bächler, et al., 2021; Inhuber, Windisch, Kleigrewe, et al., 2023). The targeted identification of performance-limiting AAs and their addition as feed supplements in a synthetic form allows for an overall reduction in dietary CP without compromising performance. Over the past decades, this nutritional strategy has been proven to be a successful feeding concept with reduced nitrogen losses to the environment in poultry and swine production (Cappelaere et al., 2021).

Therefore, this doctoral research project evaluated if MET is the first-limiting AA for growth in maize-based low CP diets for growing-finishing Fleckvieh bulls. Furthermore, the metabolic response of growing Fleckvieh bulls subjected to a CP-reduced diet with supplemental MET was assessed. However, the identification of performance-limiting AAs in beef cattle and dairy cows is impaired due to rumen fermentation: first, dietary CP is broken down by rumen microbiota, and second, the reaction products (i.e., AAs and peptides) are used for *de novo* protein synthesis (denoted as MP). This results in an incongruent AA pattern between dietary CP and MP. However, this AA pattern gap must be tackled to identify a specific AA's limitation potential and improve nitrogen efficiency.

The only way to determine a specific AA's (growth) limitation potential is to induce a deficiency in UCP, which comprises the fraction of endogenous secretions, RBP and MP. More than 50% of the absorbed AA in the small intestine is delivered by the duodenal MP flux. Therefore, it is the most critical AA fraction in the small intestine when determining the limitation potential of an AA. Second, the diet must deliver all other nutrients, including the (potentially) limiting AA in sufficient amounts. The AA in question has to be supplied either via infusion (abomasal or duodenal) or via the diet in a rumen-protected form to prevent it from being degraded by rumen microbiota. To prove the AA's role in being first limiting to growth, adding it to the diet must relieve the animal from protein deficit (i.e., stimulate growth performance). This means the limitation only becomes present if the quantitative requirement has not been previously met. However, this effect is only pronounced until a certain level at which the following AA, or another nutrient, becomes limiting (D'Mello, 2003; Storm & Ørskov, 1979). Several calculations and conversion factors modelling rumen transformation processes are necessary to determine the intestinal supply of pre-cecal digestible protein (Inhuber, Windisch, Bächler, et al., 2021).

Ruminant protein evaluation systems worldwide (Chapter 1.3) try to assess AA supply and requirements. Precise AA nutrition in dairy cows is far further developed than for growing cattle. In general, evaluation systems are calculation models that simulate the step-by-step transformation of dietary CP, the protein fraction that enters the rumen. After rumen fermentation, RBP, together with *de novo* synthesised protein (MP), leaves the rumen, and with a minor part derived from endogenous secretions, it represents the

duodenal flow of protein (denoted as UCP). It must be considered that only a particular part of UCP is true protein and that digestibility and metabolic utilisation factors for AAs vary widely. The latter two factors and their assessment are different between protein evaluation systems and are not known in detail yet. For instance, the 'American Cornell Net Carbohydrate and Protein System' (Higgs et al., 2015) denotes the absorbed AAs into the bloodstream (after transport through the intestinal epithelium) as 'pre-cecal digestible' according to the measurement techniques in monogastric AA determination. The current version of the German system (Daenicke et al., 1995) uses the UCP, which denotes the amount of protein flow to the duodenum for absorption. The reason for considering UCP instead of AA postabsorption is that both the true protein proportion of UCP and the digestibility factors of AAs are not well known yet. However, both systems and their conversion factors can be easily transferred into each other since constant factors are applied for the true protein proportion of UCP and AA digestibility factors. Currently, the German system for dairy cows is being revised. It will soon be based on AA postabsorption, as is in the American system (pre-cecal digestible), but these will be denoted as 'small intestine digestible' (SID). Since the 'SID system' has not been officially introduced yet and will only cover dairy cows, we will use the term 'pre-cecal digestible' in this doctoral thesis. However, we describe the same AA fraction.

Knowledge of limiting AA in beef cattle nutrition still needs to be improved. Methionine and lysine are potentially first limiting for growth (Klemesrud et al., 2000; Nimrick et al., 1970; Richardson & Hatfield, 1978; Storm & Ørskov, 1979; van Milgen & Dourmad, 2015; Wilkerson et al., 1993; Williams & Smith, 1974) but have been tested in sophisticated experimental setups. Moreover, these studies have only focused on the AAs' role as protein building blocks and not assessed further metabolic functions. Furthermore, studies applying practical feeding conditions with the aim of improving and adapting feeding recommendations are scarce.

Figure 2 provides an illustrational overview of the study design.

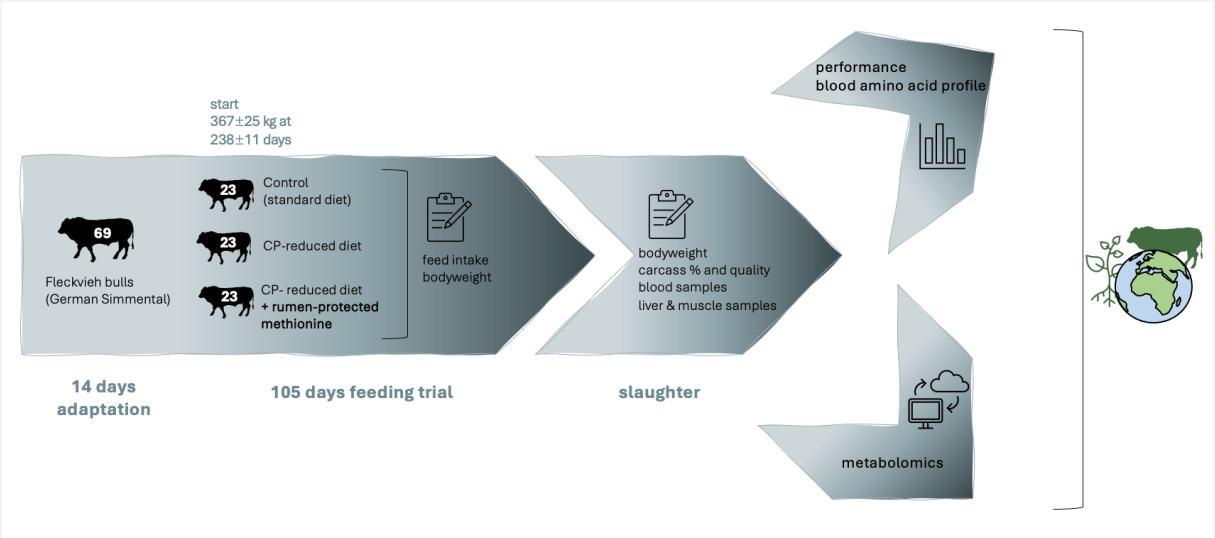

Figure 2: Overview of the experimental feeding study and subsequent data analysis.

Bulls were either subject to a standard dietary treatment or a CP-reduced feeding programme with or without additional rumen-protected MET. Growth performance was recorded throughout the whole time on feed to determine the role of MET as the first-limiting AA for growth. At slaughter, carcasses were evaluated. Blood samples underwent AA analyses, and together with muscle and liver tissues, they were subject to metabolomics analyses to assess the metabolic response of the bulls to additional MET in CP-reduced diets. The study outcomes provide a detailed insight into the specific role of MET for growing Fleckvieh bulls fed CP-reduced diets.

2.1 Feeding Experiment

The feeding experiment comprised three phases that ended with the slaughter of the animals. Details have been described in Inhuber, Windisch, Bächler, et al. (2021; Chapter 3) and Inhuber, Windisch, Kleigrewe, et al. (2023; Chapter 4). The research project was conducted at the Bavarian State Research Centre for Agriculture (LfL, Grub, Germany).

2.1.1 Animals

All animals were handled following the guidelines of the German law for animal protection of the German State and Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experiment included 69 Fleckvieh bulls from Bavaria, Southern Germany. All bulls were fed equal, standard diets, starting two weeks before the experiment. At day zero of the experiment, bulls were evenly assigned to three different feeding groups (n = 23/group) balanced for age, initial body weight and feed intake data (measured over two weeks before

the start of the experiment). They entered the experiment with an initial body weight of 367 ± 25 kg at 238 ± 11 days.

2.1.2 Dietary Treatments

Maize silage and ground premixed concentrate were the main diet components. The positive control diet (standard diet; CON) contained 13.7% CP, 15.7% UCP and 12.3 megajoules of metabolisable energy per kg DM. It was formulated according to the recommendations for nutrient and energy supply to growing Fleckvieh bulls in the bodyweight range of 350–600 kg (Daenicke et al., 1995). A deficit in CP characterised the negative control diet (RED; 9.04% CP) by removing rapeseed meal and urea from the CON diet and increasing the dried beet pulp concentration. Rumen-protected lysine (fat coated; LysiGEM, Kemin Industries, USA) was added to achieve the same dietary lysine concentration as CON. The third diet (RED+MET; RED+RPMET, respectively) was the same as RED, except for adding 1.6 g rumen-protected MET (Smartamine® M, Adisseo, France) per kg DM. The RED diet contained 9.04% CP, and the RED+MET diet had 9.08% CP. The experimental diets were kept constant throughout the entire study. Metabolisable energy concentration per kg DM was equal between all three diets. All diets were fed as a total mixed ration.

2.1.3 Feeding Periods

The experiment comprised three feeding periods. Period one lasted from day one until day 28, period two from day 29 until day 57 and period three from day 58 until slaughter. On average, the duration of the experiment was 105 days.

2.1.4 Slaughter

The bulls were slaughtered at the LfL Research Abattoir (Grub, Germany) according to the German law of animal protection of the German State and Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of the killing. Carcass evaluation (classification according to the EUROP system and quality) was performed following European standards (Council Regulation 1249/2008).

Bulls from all three feeding groups were slaughtered across all eight days. At all slaughter dates, they were weighed immediately before leaving the stable. Liver, muscle, and blood samples were taken at slaughter and immediately thrown into liquid nitrogen. They were then stored at −80°C until further analysis.

2.2 Analyses and Measurements

Analytical procedures were previously described in Inhuber, Windisch, Bächler, et al. (2021) and Inhuber, Windisch, Kleigrewe, et al. (2023).

2.2.1 Feedstuff

Wet chemistry analysis of all feedstuffs was performed at the LfL Department of Quality Assurance and Analytics (Grub, Germany) according to the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2012), if not indicated differently. The DM content of the total mixed ration was analysed twice per week and once per batch for the concentrate feed, according to VDLUFA (2012; Method 3.1).

The nutrient and AA composition of maize silage, concentrate feed and total mixed ration (VDLUFA, 2012; Method 8.1 for crude ash, Method 4.1.2 for CP, Method 7.1.1 for sugar, Method 6.5.1 for NDF after amylase treatment and ashing [$aNDF_{om}$]) were analysed in samples pooled over four weeks. Crude fat (Method 152-H) and starch (Method 152-L) analysis followed the methods of Commission Regulation (EC) No. 152/2009. Feed AA analysis, except for tryptophane, was conducted according to Commission Regulation (EG) 152/2009 App. III, F methods. Tryptophane analysis followed the Commission Regulation (EG) 152/2009 App. III, G method. The metabolisable energy content was estimated according to the Committee for Requirement Standards of the Society of Nutrition Physiology (2008) and Spiekers et al. (2013).

2.2.2 Bodyweight and Feed Intake

Bodyweight was measured at day zero (bodyweight at start), at the end of periods one and two, and immediately before transportation to the slaughterhouse. Daily feed intake was recorded, and the bull's access to the feeding troughs was monitored (LfL Institute of Agricultural Engineering and Animal Husbandry; Wendl et al., 2001). Based on these measurements, body weight gain, average daily weight gain and feed conversion ratio were calculated. Supply of pre-cecal digestible protein and pre-cecal digestible AA (lysine, MET and threonine) were calculated by dietary contents multiplied by DM intake. Requirements of pre-cecal digestible protein were calculated by estimating the net requirements of the (ideal) protein while assuming a metabolic utilisation of pre-cecal digestible protein of 0.7 (Daenicke et al., 1995).

2.2.3 Blood AA and Urea

At exsanguination, blood serum samples were collected using VACUETTE tubes. They were handled according to instructions and stored at −20°C until further analysis. Blood AA analysis was performed at

the Bavarian Centre for Biomolecular Mass Spectrometry (BayBioMS), Freising, Germany. Method parameters were applied according to Hillmann and Hofmann (2016). Urea concentrations in the blood serum were analysed following the standardised recommended procedure of analysing urea and AA in physiological liquids (Biochrom, Cambridge, UK). Cystine and cysteine (Cys) concentrations were below the detection limit.

2.2.4 Metabolomics Analysis in Liver, Muscle and Blood Serum

Liver, muscle, and blood samples were thawed at 4°C. Subsequently, 10 mg of each liver and muscle tissue sample were transferred into 2 mL FastDNA Lysing Matrix A tubes that contained lysing matrix A for soft animal tissues (MP Biomedicals, Solon, OH, USA). Together with 1 mL of an extraction solvent (70% methanol, 30% water), samples were homogenised in the FastPrep-24™ Classic (MP Biomedicals, Irvine, CA, USA) system (30 sec, 5,500 rpm, 4°C, two times) and incubated on ice (30 sec after each spin). Subsequently, samples were centrifuged (10,000 g, 5 min at 4°C). The supernatants were then transferred to 1.5 mL autosampler vials.

Metabolomics analyses were done at BayBioMS, Technical University of Munich, Freising, Germany. The untargeted analysis was performed using a Nexera UHPLC system (Shimadzu), which was coupled to a Q-TOF mass spectrometer (TripleTOF 6600, AB Sciex, Toronto, Ont, Canada). It used the information-dependent acquisition (IDA) mode. A UPLC BEH Amide 2.1 × 100, 1.7 µm analytic column (Waters Corp., Milford, MA, USA) with a 400 µL/min flow rate was used to separate the biological samples. The mobile phase consisted of eluent A (5 mM ammonium acetate in water) and eluent B (5 mM ammonium acetate in acetonitrile/water [95/5 v/v]) being carried with the following elution gradient: 100% B from 0 to 1.5 min, 60% B at 8 min and 20% B at 10 min to 11.5 min and 100% B at 12 to 15 min. A volume of 10µL per sample was injected. The autosampler was cooled to 10°C. The column oven was heated to 40°C. After every 10 samples a quality control (QC) sample was run (analysed in randomised order). It consisted of a pool of all injected samples. Mass spectrometry settings in the positive mode were as follows: Gas 1 55, Gas 2 65, Curtain gas 35, temperature 500°C, ion spray voltage 5500, declustering potential 80. The mass range of the TOF MS and MS/MS scans was 50–2000 *m/z*. The collision energy was ramped from 15 to 55 V. Mass spectrometry settings in the negative mode were set as follows: Gas 1 55, Gas 2 65, Cur 35, temperature 500°C, ion spray voltage –4500, declustering potential –80. The mass range of the TOF MS and MS/MS scans was 50–2000 *m/z*. The collision energy was ramped from −15 to −55 V.

MS raw data files were converted to mzXML via 'msconvert' from ProteoWizard (Kessner et al., 2008). Data processing and feature identification was conducted with the bioconductor/R package xcms. This means, peaks were identified with the matched filter algorithm (full width at half maximum set to 7.5 seconds). The grouping of the peaks was based on the 'peak density' method (Smith et al., 2006). The

integrated area under the peaks represented feature abundance. Peak groups presented in most samples were used to adjust the retention time. The exact mass and MS2 fragmentation pattern of the measured features were compared to the records in the Human Metabolome Database (Wishart et al., 2018) and published MS/MS spectra compiled by MSDIAL (Tsugawa et al., 2015) to annotate possible metabolites to identified features (referring to MS1 and MS2, respectively).

In addition, an in-house annotation database was taken into consideration for feature annotation. Potential batch effects were controlled. They were removed according to QC samples. Features were categorised into four groups depending on the annotation quality: One means only the feature mass is matched with the metabolite candidate in the database and no MS2 information is used. Zero means the MS2 spectra matching suggests the annotation is likely incorrect. Two means a partial match of MS2 spectra (at least one peak match). Four means high similarity between the measured MS2 spectra and database MS2 spectra. A post-hoc test (Student–Newman–Keuls) of category-four metabolites was conducted to compare the metabolite abundance between the three feeding groups. The associated untargeted metabolomics data are available on MassIVE (repository number: MSV000092367).

2.3 Statistics

Statistical analysis of the first publication was performed using SAS 9.4 (SAS Institute, Cary, NC, USA). Zootechnical data of total time on feed and of each period (one, two and three) were analysed with a general linear mixed model (GLMM), with dietary treatment as a fixed effect and pen x treatment as a random effect. The residual variance was determined to be pen x treatment. The dietary treatment group means were tested using a Student–Newman–Keuls post-hoc test included in the GLMM. A nonparametric post-hoc test was applied in the case of 'EUROP' carcass classification and fat grade (Kruskal–Wallis H test). The $p\text{-value}_{GLMM}$ represents the statistical significance of the GLMM model in total. Linear contrasts, except for EUROP carcass classification and fat grade, were calculated to detect differences in response variables due to a reduction in CP (p-value CON vs RED and RED+MET) and, subsequently, due to the supplementation of rumen-protected MET (RED vs RED+MET). The standard error of the mean over the whole GLMM is indicated as SEM. Significant differences were declared at $p \leq 0.05$. Differences at $0.05 \leq p \leq 0.10$ were considered a trend.

Univariate statistical analysis of the second publication was performed using SAS 9.4 (SAS Institute, Cary, NC, USA). First, the Shapiro–Wilk test was used to test for normal distribution of the dataset. Treatment group means of significantly regulated metabolites, obtained from XCMS analyses, were analysed using a GLM with treatment as a fixed effect and a Student–Newman–Keuls post-hoc test, which was included in the GLM. The p-value of the GLM represents the statistical significance of the model. In contrast, the p-values of the linear contrasts I and II (Table 2) detect differences in response

variables due to a reduction in dietary CP (CON vs RED+RPMET) and the addition of rumen-protected MET (RED vs RED+RPMET). The SEM represents the standard error of the mean over the whole GLM. Statistical significance was declared at $p \leq 0.05$.

2.4 Calculation of Utilisable and Pre-Cecal Digestible Protein and Pre-Cecal AA Supply

As described in detail (Chapter 1.2), digestion and absorption of dietary CP in ruminants is a complex series of physiological processes. The calculation of pre-cecal digestible AAs comprises various steps described in this chapter. Figure 3 depicts the calculation scheme for pre-cecal digestible protein in growing cattle.

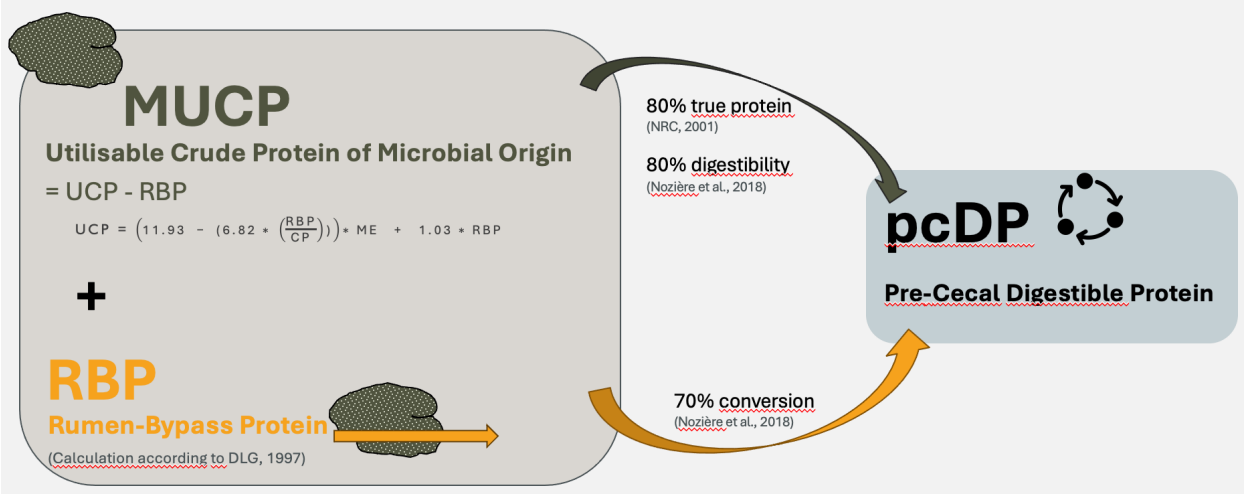

Figure 3: Calculation of pre-cecal digestible protein, derived from the contribution of utilisable crude protein of microbial origin and rumen-bypass protein.

The UCP concentration was calculated as follows:

11.93 − (6.82 × [RBP/CP (without urea)] x metabolisable energy + 1.03 x RBP (NRC, 2001).

RBP was calculated according to Spiekers et al. (2013). Dietary CP considered in this formula was derived from feed components, except for urea. We assumed that urea delivered 30% of CP as RBP. UCP of microbial origin (MUCP) was calculated by subtracting RBP from UCP.

Pre-cecal digestible protein describes the sum of the absorbed protein derived from the intestinal supply of microbial and rumen-bypass origin. The microbial contribution was calculated as follows: 80% were considered true protein (NRC, 2001), which was assumed to be 80% intestinal digestible ('PDI system' of L'Institut nationale de la recherche agronomique [INRAE]; Sauvant & Nozière, 2016). The contribution of rumen bypass to pre-cecal digestible protein was calculated by multiplication with 70%, which reflects the conversion factor suggested by INRA (Nozière et al., 2018).

According to the calculation of pre-cecal digestible protein, pre-cecal digestible MET, lysine and threonine include microbial and rumen-bypass origin contributions. Microbial contribution for MET was derived from 0.028 × 0.8 × MUCP × 0.8 for MET with 2.8% of MET in the true protein proportion of MUCP, 0.079 × 0.8 × MUCP × 0.8 for lysine with 7.9% of lysine in the true protein proportion of MUCP and 0.058 × 0.8 × MUCP × 0.8 for threonine with 5.8% of threonine in the true protein proportion of MUCP. The average AA concentrations in the true protein proportion of MUCP underlying the calculations were derived from the National Academies of Sciences, Engineering, and Medicine (2016) and NRC (2001), using data from Clark et al. (1992). Intestinal digestibility was 80%, as described above (Nozière et al., 2018).

The conversion factors of the proportion of RBP to dietary CP and from RBP to pre-cecal digestible protein (Nozière et al., 2018) were 0.3 and 0.7, respectively. These factors were multiplied by MET, lysine, and threonine dietary concentrations to obtain pre-cecal digestible MET, lysine and threonine concentrations.

In the case of added dietary rumen-protected MET or rumen-protected lysine, their contributions to pre-cecal digestible MET and pre-cecal digestible lysine were calculated by multiplication with the conversion factors for both rumen escape and intestinal digestibility: 0.8 and 0.85 for MET and lysine, respectively, and 0.99 and 0.95 for MET and lysine, respectively.

3 EFFECTS OF SUPPLEMENTING A CP-REDUCED DIET WITH RUMEN-PROTECTED METHIONINE ON FLECKVIEH BULL FATTENING

Author Contributions

The author contributions to the publication were as follows:

Vivienne Inhuber developed the concept of the publication and wrote the first draft of the manuscript. She led and conducted the review process of the publication, including all content and formal adaptations, as well as corrections to the calculation model. Additionally, she extensively reviewed the literature and suggested discussion points on the results obtained from this study (conceptualization). Data analysis and evaluation were performed by Vivienne Inhuber in collaboration with Wilhelm Windisch and Thomas Ettle. Both Wilhelm Windisch and Thomas Ettle were also involved in conceptualization and methodology, and they enhanced the quality of the research through their guidance and support. Hubert Spiekers reviewed the draft manuscript. Benedikt Bächler conducted amino acid analyses and assisted Vivienne Inhuber with amino acid data analysis.
Project administration and funding acquisition were organized by Thomas Ettle and Wilhelm Windisch.

Summary

The aim of this research project was to assess how supplementing a diet with reduced crude protein (CP) concentration with rumen-protected methionine affects the growth performance of Fleckvieh bulls. A total of 69 bulls (weighing 367 ± 25 kg) were evenly assigned to three feeding groups (n = 23 per group). The control (CON) diet contained 13.7% CP and 2.11 g of methionine per kg of diet (on a dry matter basis). This diet was considered the positive control. The reduced (RED) diet, serving as the negative control, along with the experimental RED+MET diet, both had reduced CP concentrations (9.04% CP). The RED+MET diet differed from the RED diet by having a higher concentration of methionine (2.54 g/kg DM vs. 1.56 g/kg DM, respectively) due to the addition of rumen-protected methionine. Rumen-protected lysine was added to both RED and RED+MET diets at 2.7 g/kg DM to ensure adequate lysine supply relative to total and metabolizable protein intake. Metabolizable energy (ME) and nutrient composition were similar for all three diets. The bulls were fed for an average of 105 days. Daily feed intake was recorded daily and individual body weights were recorded at the onset of the experiment, once a month during the experiment and directly before slaughter. Blood samples and carcass traits were assessed at slaughter.

Reducing dietary CP concentration led to decreased feed intake, and combined with lower CP concentration, daily intake of CP for RED and RED+MET diets was lower compared to CON ($P < 0.01$). Daily ME intake was also reduced in RED and RED+MET compared to CON ($P < 0.01$), resulting in reduced growth performance and carcass weights (both $P < 0.01$) in both RED and RED+MET compared

to CON. The supplementation of rumen-protected methionine led to increased serum methionine concentration in RED+MET ($P < 0.05$) compared to RED, but it did not affect growth performance, carcass traits, and other serum amino acid concentrations, except for lysine, which was reduced ($P < 0.01$) compared to CON and RED. In conclusion, bulls fed RED or RED+MET diets experienced a ruminal CP deficit and subsequently a deficit of pre-cecal digestible protein, but under the experimental conditions, methionine did not appear to be the first-limiting essential amino acid for growth.

See Appendix for full text.

4 EFFECT OF RUMEN-PROTECTED METHIONINE ON METABOLIC PROFILE OF LIVER, MUSCLE AND BLOOD SERUM SAMPLES OF GROWING GERMAN SIMMENTAL BULLS FED PROTEIN-REDUCED DIETS

Author Contributions

The author contributions to the publication were as follows:

Vivienne Inhuber led the conceptualization of the study in cooperation with Wilhelm Windisch and Thomas Ettle. She wrote the first draft of the manuscript, visualized the data and the content of the manuscript, and adapted the manuscript according to the official review process. Review and editing on the draft manuscript was done by Thomas Ettle, Wilhelm Windisch, Karin Kleigrewe, Chen Meng, and Julia Steinhoff Wagner. Vivienne Inhuber also led methodology development. In cooperation with Chen Meng, Benedikt Bächler, Michael Gigl, and Karin Kleigrewe, who developed the software, Vivienne Inhuber used the specific metabolomics analysis software. They altogether conducted validation of the metabolomics data. However, Vivienne Inhuber carried out formal analysis and led investigation. Resources were provided by Wilhelm Windisch, Julia Steinhoff-Wagner, and Thomas Ettle. Wilhelm Windisch provided supervision and Thomas Ettle oversaw project administration and carried out funding acquisition.

Summary

This study aimed to investigate the metabolic response of growing German Simmental bulls when fed low crude protein (CP) diets supplemented with rumen-protected methionine (RPMET). A total of 69 bulls, with an average age of 238 ± 11 days at the start and a body weight of 367 ± 25 kg, were divided into three dietary treatment groups (n = 23/group): Positive control (CON; 13.7% CP; 2.11 g methionine/kg DM), negative control deficient in CP (RED; 9.04% CP; 1.56 g methionine/kg DM), and a CP-deficient ration supplemented with RPMET (RED+RPMET; 9.04% CP; 2.54 g methionine/kg DM). Bulls were fed over 105 days on average. During slaughter, samples of liver, muscle, and blood serum were collected and subsequently subjected to metabolomics profiling using a UHPLC-QTOF-MS system. A total of 6,540 features were detected, with twenty metabolites in the liver, five in muscle, and thirty in blood serum showing significant differences ($p < 0.05$) due to dietary treatments. Six metabolites could be reliably annotated and were hence subjected to subsequent univariate analysis. The reduction in dietary CP had minimal effects on metabolite abundance in the target tissues of both RED and RED+RPMET bulls compared to CON bulls. The addition of RPMET altered the hepatic antioxidant status in RED+RPMET bulls compared to both RED and CON bulls. These results illustrate nutrient partitioning

in growing German Simmental bulls, where maintenance is the primary metabolic priority (homeostasis), followed by nutrient trafficking, which is directed towards specialized metabolic functions such as antioxidant pathways.

See Appendix for full text.

5 GENERAL DISCUSSION

This general discussion interprets the performance (Chapter 3; Inhuber, Windisch, Bächler, et al., 2021) and metabolic response (Chapter 4; Inhuber, Windisch, Kleigrewe, et al., 2023) of growing Fleckvieh bulls fed CP-reduced diets supplemented with rumen-protected MET. This research project aimed at determining the potential role of MET as the first-limiting AA for the growth of Fleckvieh bulls fed CP-reduced diets. Since MET is a protein building block for muscle protein and a functional AA, we examined the metabolic response of growing Fleckvieh bulls to a CP-reduced diet supplemented with rumen-protected MET. The overall objective of this experimental study was to generate the first quantitative insights to approach the IPC in beef cattle nutrition. The successful application of this nutritional strategy into practical beef cattle farming is crucial to enabling beef supply chains to become more efficient. It increases nitrogen utilisation efficiency and reduces beef production's environmental impact.

The IPC denotes a dietary protein whose AA pattern is as close as possible to animals' metabolic requirements. Its composition is designed so that all AAs are limiting to the same extent. This nutritional strategy hence minimises both the under- and oversupply of AAs – which would either lead to AA deficiency and, thus, a decrease of growth or to excess nitrogen excretion, which would place an additional burden on the environment (van Milgen & Dourmad, 2015). Pivotal steps in identifying performance-limiting AAs for both pigs and poultry resulted in the successful implementation and further optimisation of this concept during the past few decades (Lambert et al., 2023; Luise et al., 2021; Star et al., 2021). However, even though the concept has been proven successful, there are still many challenges to be solved (Selle et al., 2022).

Rumen fermentation processes, however, impair the straightforward transfer of this concept to beef cattle nutrition. Rumen microbiota degrade dietary protein. These reaction products (i.e., peptides, AAs and dietary NPN compounds [e.g., urea]) are then utilised in *de novo* protein synthesis. This protein fraction, denoted as 'MP' with dietary RBP and endogenous secretions, serves as UCP to be absorbed in the small intestine. The AA pattern of the intestinal protein flow differs substantially from the dietary AA pattern. This incongruence is biologically set and cannot be changed significantly. However, this AA pattern mismatch can be minimised by identifying limiting AAs and supplementing those in a rumen-protected form to contribute to the UCP in the small intestine for absorption, thereby improving the pre-cecal digestible protein quality.

Therefore, this study mainly evaluated the role of MET as a potential first-limiting AA in reduced-AA diets for growing Fleckvieh bulls under practical feeding conditions. Previous scientific studies on MET in beef cattle nutrition imply that together with lysine and threonine, it may act as one of the first-limiting AAs for growth performance (Cantalapiedra-Hijar et al., 2020; Froidmont et al., 2002; Klemesrud et al., 2000;

Nimrick et al., 1970; Richardson & Hatfield, 1978; Wilkerson et al., 1993; Williams & Smith, 1974). Many of these studies, however, applied sophisticated scientific approaches to detect metabolic limitations. Additionally, the studies are quite old and are, therefore, unsuitable for deriving neither specific AA requirements of current beef cattle genotypes nor practical feeding recommendations. However, precise knowledge of AA requirements is pivotal to increasing the nitrogen efficiency of beef cattle supply chains and, ultimately, reducing the environmental impact.

Results of this beef cattle feeding trial demonstrated that reduced dietary AA decreased feed and overall nutrient intake (Chapter 3). Consequently, growth performance was depressed. Adding rumen-protected MET to a maize-based diet reduced in AAs did not relieve bulls from growth restriction. This implies that MET was not the first-limiting AA for growth performance under our practical feeding conditions. Metabolomics analyses (Chapter 4) in liver, muscle and blood serum revealed that reduction in dietary AA and supplemented rumen-protected MET had minimal effect on the bull's metabolism. However, additional MET was prioritised for the hepatic one-carbon metabolism, with a significant increase in hepatic antioxidant synthesis.

The clear metabolic prioritisation of nutrients – that is, homeostasis as the prevailing priority and homeorhesis as a secondary priority – may indicate that actual MET requirements of Fleckvieh bulls are higher than initially assumed. However, the experimental study did not allow for quantifying actual requirements. Future studies are needed to precisely assess metabolic requirements for specific physiological processes in the metabolism of growing Fleckvieh bulls. 'Metabotype' analyses (i.e., the combination of phenotype [performance] data and metabolome data) certainly contribute to a deeper understanding of the Fleckvieh bull physiology. This is critical knowledge for further developing precise feeding recommendations – that is, the IPC for beef cattle nutrition.

5.1 Evaluation of MET as First-Limiting AA for Growth Performance of Fleckvieh Bulls Fed Low AA Diets

To evaluate the potential role of MET as the first-limiting AA for the growth performance of Fleckvieh bulls, we established a respective scientific feeding model in which practical feeding conditions were still maintained (Chapter 3). Thus, conclusions derived from this study serve as crucial basic information to improve further the feed efficiency of growing Fleckvieh cattle in practical agricultural production, ultimately supporting the environmental sustainability of beef supply chains.

Establishing a suitable feeding model is pivotal; otherwise, the role of the AA in question cannot be precisely evaluated. The experimental model must meet two significant conditions: First, the protein supply to the animal must be deficient. In contrast, other nutrients and energy must be supplied to meet

or slightly exceed the requirements. Second, the AA in question must be added to this diet, and in case of being first limiting, it must relieve the bull from protein deficit and improve growth performance. However, this effect is only pronounced once another (nutritional) factor becomes limiting. Due to ruminal degradation of AAs, the targeted AA has to be supplied in a (rumen-) protected form or infused postruminally via abomasal or duodenal infusion (D'Mello, 2003; Storm & Ørskov, 1979).

This study used a feeding model with three dietary treatment groups: first, a control diet (CON), which met nutrient (13.7% AA; 15.4% UCP) and energy requirements (12.3 megajoules of metabolisable energy/kg DM) of growing Fleckvieh bulls at this growth stage (Daenicke et al., 1995). The second group was fed a diet deficient in AA (RED; 9.04% AA) but adequate in all other nutrients and metabolisable energy (12.2 MJ/kg DM). To prevent lysine from being limiting, it was added to this diet in a rumen-protected form (0.27% DM). Third, the experimental diet (RED+MET) was supplemented with rumen-protected MET (0.16% DM; 2.2 vs 3.2 g/kg DM, respectively). Since we used a practical feeding model instead of a sophisticated scientific model – that is, postruminal infusion (Hill et al., 1980) or specific diets (Richardson & Hatfield, 1978) – the study outcomes can be used for practical farming advice for current Fleckvieh breeds.

Detailed calculations on the intestinal supply of AAs relative to respective requirements are an inevitable condition to evaluate the limitation potential of AAs. In this study, we developed a calculation model (Chapter 2.4) to determine the amount of pre-cecal digestible protein (metabolisable protein) of CON, RED and RED+MET. Pre-cecal digestible protein supply was calculated as described in detail in Chapter 2.4. This calculation model, however, entails significant sources of variation. First, the amount of true protein in MUCP is uncertain. Generally, 80% true protein in MUCP is assumed (NRC, 2001). This value is based on Clark et al.'s (1992) analyses. However, Sok et al. (2017) strongly suggested a concentration of 82.4%. Second, differences are evident in not only the amount of true protein but also the AA pattern of MUCP between research approaches. Both Clark et al. (1992), which is used by the NRC (2001), and Le Hénaff (1991), used by INRA, only considered fluid-associated bacteria as the supplier of intestinal AAs and considered both sheep and cattle databases. Sok et al. (2017), however, used data only from cattle, including the AA composition of protozoa and accounting for 16.5% of true MP (Sylvester et al., 2005), and differentiated between fluid-associated (33.4% of true MP) and particle-associated bacteria (50.1% of true MP). Third, the digestibility factors for MP's and RBP's true protein vary. INRA (2018), for instance, applies 80% and 70%, respectively. On the contrary, Daenicke et al. (1995) applied 85% for both. Fourth, the utilisation efficiency of AAs may differ between growth stages, for example. Methionine utilisation efficiency, for instance, can vary between 14% and 66% (D'Mello, 2003). Therefore, our calculation model of pre-cecal digestible (metabolisable) protein and AA intake gives a good estimation but is uncertain.

Reduction in dietary AA led to a lower feed intake and a lower intake of pre-cecal digestible protein in the RED and RED+MET groups compared to the CON group. However, from a retrospective point of view, intake exceeded the requirement by 33% and 38% in the RED and RED+MET groups since only lower growth rates were realised. RED+MET bulls had a higher intake of pre-cecal digestible MET than RED bulls ($p < 0.01$). A daily pre-cecal digestible MET intake of 26.2 g accounted for 146% of their requirements but did not relieve them from growth restriction, although it was metabolically available. This was proven by increased MET concentrations in RED+MET bulls compared to RED bulls ($p < 0.01$).

Hence, our experimental setup matched the conditions to prove the limitation of MET as the first-limiting AA for growth. The results indicate that MET did not act as the first-limiting AA for growth in a maize-based diet under our practical feeding conditions. It may have also been that MET was first limiting for growth. However, phenotype effects did not become evident because the following AA was already limiting close to MET. High levels of maize silage in our diets could have prevented MET limitation since relatively high levels of MET characterise its AA composition.

This consolidated overview of the results clearly demonstrates the relevance of precise diet formulation. However, diet formulation calculations entail significant uncertainties in feedstuff's AA supply capacity and specific metabolic AA requirements. Approaching actual supply capacities and metabolic requirements is inevitable to increase nitrogen utilisation efficiency and decrease the environmental impact of beef cattle production.

5.2 Metabolomics as a Potential Tool to Develop Biomarkers for Growing Fleckvieh Bulls

Metabolomics is an 'omics' tool that quantifies a global set of metabolites within biological samples (e.g., liver and muscle tissues and blood serum). A particular cell type's 'metabolic snapshot' helps compare physiological phenotypes, including environmental effects (E. Gómez et al., 2020; Goldansaz et al., 2017). Over 50% of livestock metabolome studies have been conducted in bovine species (Goldansaz et al., 2017). Recently published studies have focused on the identification of biomarkers for desirable economic traits, such as feed efficiency (Artegoitia et al., 2022; Clemmons, Martino, et al., 2019; Clemmons, Mihelic, et al., 2017; Clemmons, Powers, et al., 2020; Connolly et al., 2019; Foroutan, Fitzsimmons, Mandal, Piri-Moghadam, et al., 2020; Novais et al., 2019), growth potential (Imaz et al., 2022) and dairy production challenges (Lisuzzo et al., 2022; López Radcenco et al., 2021; Rocchetti et al., 2022). However, very little is known about baseline values of essential metabolites in bovine tissues and fluids of healthy animals (Foroutan, Fitzsimmons, Mandal, Piri-Moghadam, et al., 2020). Minimal knowledge is a limiting factor for the correct ranking and placement of metabolomics results (Goldansaz

et al., 2017). Over the past decade, various metabolomics studies have been published, which contribute to filling the knowledge gap on bovine metabolomics to different extents.

Our study is the first to elucidate MET's functional role in the metabolism of growing Fleckvieh bulls under protein deficiency conditions. As described in Chapter 2.1.2, bulls were allotted to three treatments that represented, first, a practical standard diet (maize-based; CON) and second, a protein-deficient diet (RED), which in the third treatment was supplemented with MET (RED+MET) in a rumen-protected form to prevent it from microbial degradation. Reduction in dietary AA led to decreased growth performance, reflected by lower average daily weight gain in RED and RED+MET bulls compared to CON bulls (Chapter 3.5.4). The addition of rumen-protected MET did not relieve RED+MET bulls from growth restriction, which implies that MET was not first limiting for growth under our respective feeding conditions. It may have been that the next limiting AA was closely limiting after MET, thus hiding the growth-limiting potential of MET. Targeted AA analyses indicated that the addition of rumen-protected MET was successful. RED+MET bulls showed higher serum MET concentrations than RED and even CON bulls ($p < 0.05$).

Since we specifically analysed liver and muscle tissues and blood serum samples, our metabolomics results only allow us to draw conclusions based on defined target tissues. We chose blood samples since blood is generally helpful for assessing herd health and overall metabolic status and is widely used in animal nutrition studies. Liver samples were chosen since the liver is the critical node in metabolism. Additionally, we took muscle samples to evaluate their metabolic signature. For instance, J. Gómez et al. (2022) impressively showed a clear difference in beef muscle metabolic signature due to growth rate intensity. However, results cannot be compared easily, as they used a different feeding approach and Angus *x* Nellore crossbreed steers.

The reduction in dietary AA and the addition of rumen-protected MET had minimal effect on the metabolic signature of the liver and muscle cells and blood serum compared to the metabolic signature of standard-fed Fleckvieh bulls. This became evident in liver tissue, where out of 1,457 metabolites, 15 between CON and RED+MET and five between CON and RED+MET were significantly regulated ($p < 0.05$). In blood serum, out of 3,214, 29 metabolites between CON and RED+MET and one metabolite between RED and RED+MET were regulated ($p < 0.05$). We found 1,869 metabolites in muscle cells, from which five between CON and RED+MET were regulated ($p < 0.05$). We did not observe a significantly regulated metabolite between RED and RED+MET in muscle tissue. This means that out of 6,540 metabolites, only 55 were significantly regulated in our comparisons of CON vs RED+MET and RED vs RED+MET. Reliable annotation of significantly regulated metabolites was achieved for six metabolites – namely, hepatic carnosine, cystine, taurocholic acid, L-leucine/L-isoleucine/norleucine and Cys glutathione disulphide and pyrrolidone carboxylic acid in blood serum.

Reliable annotation in our data evaluation means a high similarity between measured MS2 spectra and database MS2 spectra. MS2 describes the fragmentation pattern of the precursor ion.

The fact that cellular metabolic signature did not differ extensively between CON, RED and RED+MET bulls in liver, muscle and blood serum implies that under reduced dietary protein conditions, bulls maintain nutrient utilisation for growth to maintain their well-being and the fundamental functioning of their metabolism. They set maintenance as the prevailing priority in nutrient utilisation. This concept of nutrient partitioning entails two main types of regulation: homeostasis and homeorhesis. Homeostasis aims to maintain a physiological equilibrium connecting all organs and tissues, ensuring they interact cooperatively. These processes sensitively work together and buffer external challenges (Baumgard et al., 2017; Piantoni & VandeHaar, 2023) on 'an acute moment-to-moment basis' (Baumgard et al., 2017). Homeorhesis, on the contrary, is defined as 'orchestrated changes for priorities of a physiological state', which originates from the regulation pattern of physiological processes during pregnancy and lactation, as well as growth (Bauman, 2000; Bauman et al., 1982; Bauman & Currie, 1980). Growth processes perfectly demonstrate that there are shifts in prioritising target tissues for nutrients that have been digested from feed. A higher order of endocrine regulation governs these nutrient flows. This hierarchical concept of nutrient partitioning sets the fulfilment of maintenance requirements as the prevailing priority (homeostasis) and then controls the targeted nutrient flow to recipient tissues (homeorhesis). With this background, the results of our metabolomics analyses exemplify nutrient partitioning in growing Fleckvieh bulls. Under conditions of a CP-reduced supply, metabolic maintenance functions were set as the top priority to the detriment of growth. Thus, there was hardly any change in single-cell metabolism between CON vs RED+MET and RED vs RED+MET bulls since the regulation of nutrient utilisation had presumably occurred on a higher endocrine organ level.

An interesting exception to this was the addition of rumen-protected MET (RED+MET) to the CP-reduced diet (RED): First, targeted AA analyses indicated changes in serum MET concentrations. RED+MET bulls had higher serum MET concentrations than both RED and CON bulls ($p < 0.01$), which proved the efficacy of the rumen-protected MET product. However, increased metabolic availability of MET in RED+MET bulls did not relieve them from growth restriction, which implied that MET was not the first-limiting AA for growth under our feeding conditions. However, it may also be that MET was first limiting for growth. Still, another AA was closely limiting growth and, thus, did not allow RED+MET bulls to increase growth performance (i.e., muscle protein synthesis).

Second, metabolomics analysis indicated that the addition of rumen-protected MET increased hepatic Cys glutathione disulphide (GSSG) synthesis in RED+MET bulls as compared to RED bulls and even exceeded the GSSG synthesis rate in CON bulls ($p < 0.01$). Glutathione disulphide is a significant component of cellular redox regulation. It scavenges reactive oxygen species to prevent cell damage

during oxidative stress. It is also synthesised via the transsulfuration pathway, as is taurine, and relies on MET. Various studies have investigated the positive effects of rumen-protected MET on (reproductive) performance and the antioxidant status of dairy cows (Batistel et al., 2018; Osorio, Trevisi, et al., 2014), but respective data on beef cattle are scarce. The addition of rumen-protected MET in RED+MET bulls also increased hepatic taurocholic acid concentrations compared to the CP-reduced RED group ($p < 0.05$) and reached comparable levels to the CON group. Taurine (aminoethane sulfonic acid) is a sulphur-containing beta-AA. Mammals mainly synthesise taurine in the liver (Jung et al., 2019). This synthesis consumes MET (transsulfuration pathway; Brosnan & Brosnan, 2006). The RED+MET bulls may have used additional MET to alter hepatic taurine metabolism, which finally increased taurocholic acid. The literature needs to provide more information on the specific role of taurocholic acid, particularly in beef cattle or generally in ruminants. However, Zhang et al. (2023) evaluated the effects of taurine on rumen fermentation in vitro to determine its usefulness as a potential feed additive in ruminants. Taurine inhibited ruminal fermentation (decreased DM degradability, volatile fatty acid and gas production) but increased MP synthesis. For instance, taurine has been shown to improve performance and antioxidant capacity in broiler chickens (Han et al., 2023). Another recent research (van Vliet et al., 2023) evaluated the effect of the production system (pasture finishing or pen finishing) on the metabolic profile of bison meat (*Bison bison*). Meat taurine concentrations were 1.5-fold higher in pen-finished bison meat than in pasture-finished bison meat. Of course, these studies cannot be used to explain our findings, but both groups of authors concluded that more research on taurine's role in beef cattle is needed.

Recapitulating the above, metabolomics analyses of our feeding trial allow for concluding on the metabolic prioritisation of MET in growing Fleckvieh bulls. Methionine is an AA that typically functions as a building block for proteins via peptide bonds (e.g., muscle protein synthesis) and plays a significant role in metabolism, which is not directly linked to protein synthesis. In this functional role, for instance, MET provides methyl groups in DNA methylation processes or acts as a precursor to functional metabolites in the body (e.g., GSSG). Its ability to increase the synthesis of critical components with antioxidant effects (such as glutathione, which serves as an immunometabolic status parameter in dairy cows) has been extensively demonstrated in dairy cow research (Osorio, Trevisi, et al., 2014).

Concluding the respective outcomes of our study, we may assume that MET did not have priority as a precursor to antioxidants. In this case, the nonsupplemented group (RED) would have displayed less growth intensity than the RED+MET group since MET would have been directly used as functional AA (i.e., as a precursor to antioxidant metabolites) and not as an AA for the synthesis of body muscle protein. It may have also been that MET was used for the immune system in RED bulls since they may have had a worse immune status than RED+MET bulls. Last, the additional MET in RED+MET bulls, which could not be used for muscle protein synthesis, was 'disposed of' via GSSG. However, we cannot conclude

whether this was a 'push or pull' effect on their metabolism. More studies are needed to further evaluate this finding.

5.3 Metabotype Analyses Allow for More Precise Diet Formulation Which Can Reduce the Environmental Impact of Beef Cattle Production

The metabotype – that is, the combined view of metabolome and phenotype (Fontanesi, 2016) – allows a comprehensive and detailed understanding of the physiological status of the animals. This will generate important information to derive more detailed beef cattle requirement data, allowing for more precise diet formulation. This holistic approach will enable farmers to minimise (nitrogen) environmental inputs while maintaining healthy beef cattle performance. Animal welfare also gets increasing attention from the public, not only because of being considered in the 'Sustainable Development Goals of the United Nations' (responsible consumption and production; UN General Assembly, 2015).

Avoiding protein-rich feedstuff for beef cattle diets, such as soybean meal, impedes the appropriate design of a requirements-based feed ration. Nevertheless, reducing dietary AA – hence, at best without human-edible protein – is the most efficient measure to increase nitrogen utilisation efficiency. However, a decrease in performance will accompany this. Supplementing diets reduced in AA with rumen-protected AAs presents a promising strategy to counteract the compromise of performance (Cantalapiedra-Hijar et al., 2020; Zou et al., 2023). For growing cattle, MET, lysine and threonine have been considered most likely to be first limiting for growth (Inhuber, Windisch, Bächler, et al., 2021). Supplementation of rumen-protected AAs in beef cattle diets per se is feasible. Adding the correct dosage to the diets, a dependency on beef cattle's age and growth stage is still being questioned. However, requirement calculations of pre-cecal digestible AAs are based on numerous conversion factors and entail significant uncertainties (as discussed in Chapters 3.5.2 and 3.7). Until now, livestock's pre-cecal digestible AA requirement calculations were primarily based on their role as protein building blocks. This is correct, but the functional part of AAs should also be considered to ensure optimal animal growth and health.

Even though MET was not first limiting for growth in our study (Chapter 3), we observed a 'metabolic fingerprint' of this AA (Chapter 4) under our feeding conditions. This may imply that the MET requirement may have been higher than initially assumed since only growth response parameters were used in requirement calculations (Chapter 3.5.4). Accordingly, Chalvon-Demersay et al.'s (2021) review impressively demonstrated the potential of AA supplementation strategies based on not only the protein-building role of AAs but also the animals' AA requirements of specific AAs as precursors to energy and signalling molecules. The authors' example was drawn from a comprehensive examination of gut health parameters. Gut health is a significant issue in both broiler and pig production.

Beef cattle and both poultry and swine cannot be directly compared. However, there is research involving dairy cows clearly showing the potential of rumen-protected MET in low AA diets to not only maintain performance but also improve antioxidant status and, therefore, reduce nitrogen excretion (Batistel et al., 2018; Patton et al., 2014). Methionine, for instance, supports immune fitness via the reduction of proinflammatory cytokines and lymphocyte proliferation, as well as oxidative stress homeostasis via improving the synthesis of total glutathione and antioxidative enzymes and increasing the overall antioxidative capacity (Lopes et al., 2019; Osorio, Trevisi, et al., 2014). This is in accordance with our metabolomics analyses, which displayed the metabolic fingerprint of MET in antioxidative stress synthesis pathways (both taurine and Cys glutathione synthesis).

In conclusion, performance results clearly indicate the discrepancy between animals' metabolic requirements and nutrient supply capacity of respective diets. Metabolomics analyses allow animal scientists to obtain a more detailed and complete picture of animals' underlying physiological status, enabling a better understanding of the whole phenotype (Goldansaz et al., 2017).

6 CONCLUSION AND OUTLOOK

This research project evaluated the potential of MET as the first-limiting AA for the growth performance of Fleckvieh bulls fed CP-reduced diets. Moreover, we aimed to elucidate the bulls' metabolic response to additional MET in CP-reduced diets. Therefore, we conducted an experimental feeding study during which performance parameters were recorded. At slaughter, we evaluated carcasses and collected liver, muscle and blood samples to perform metabolome analyses.

The identification of performance-limiting AAs in growing beef cattle is impaired by ruminal transformation and MP synthesis. A crucial step to approaching the limitation potential of MET for growing Fleckvieh bulls was establishing a respective feeding model. Accordingly, three dietary treatments were used: a standard diet (reference diet according to current recommendations) and two diets reduced in CP (nitrogen). One of them was supplemented with MET. Methionine was supplemented in a rumen-protected form to secure its metabolic availability. A decrease in performance due to a reduction in dietary AA indicated (ruminal) nitrogen deficiency. Relief from growth restriction due to additional MET would have demonstrated its role as the first-limiting AA for growth. Hence, this feeding model allowed for displaying potential growth limitations due to MET deficiency. Retrospective calculations of nutrient supply and requirements on a pre-cecal digestible basis were pivotal to evaluating the bulls' actual performance and approaching the specific AA's limitation potential.

Performance results indicated a decrease in growth due to reduced dietary AA. Adding MET did not relieve the bulls from growth restriction, suggesting that MET was not first limiting for growth under these feeding conditions. It may have also been that another AA was limiting growth performance closely to MET and thus hid the actual limitation potential of MET.

Metabolomics analyses demonstrated that reduced dietary AA nearly unaffected cellular metabolism. This finding and phenotypic results on growth performance exemplify the body's nutrient partitioning ability. The animal sets its well-being and maintenance as a prevailing priority (homeostasis) at the expense of performance (homeorhesis). This hierarchical concept is controlled on a higher endocrine level, leaving single-cell metabolism nearly unaffected in bulls fed CP-reduced diets. However, adding rumen-protected MET significantly increased hepatic antioxidant synthesis (one-carbon metabolism).

In conclusion, this doctoral thesis demonstrated, for the first time, the decoupling of growth performance and specific metabolic processes in growing Fleckvieh bulls under a deficient dietary protein supply. Phenotypic results confirmed the successful implementation of our scientific model, and metabolomics analyses completed this picture to a certain extent. Furthermore, metabolomics analyses elucidated the metabolic prioritisation of MET for antioxidant synthesis under protein deficiency. However, our

experimental setup did not allow us to ascertain if the Fleckvieh bulls under protein deficiency exhibited a poorer immunometabolic status than those fed a standard diet.

For future studies, a new experimental set-up would be needed to evaluate if Fleckvieh bulls under protein deficit really exhibit a poorer immunometabolic status (pull for MET to synthesise antioxidants) or if the metabolism is only using antioxidant synthesis to discard additional MET (push). If the experiment's results display a pull for MET to alter antioxidant synthesis, (pre-cecal) digestible MET requirements would have to be adapted accordingly in respective calculation models to ensure optimal animal health and growth. Subsequently, other AAs known for their functional metabolic roles must be targeted in further (Fleckvieh) bull AA nutrition research. Additionally, it must be considered that healthy animals' baseline values are lacking. Thus, future studies should also focus on developing status parameters for different metabolic functions at different growth stages.

The comprehensive outcome of this doctoral project will tremendously contribute to successfully approaching the IPC for beef cattle nutrition. This, in turn, will enable farmers to increase the ecological and economic efficiency of beef meat production and reduce the incorporation of protein-rich, human-edible feed components. Combining this nutritional IPC with the utilisation of marginal areas with difficult agroecological conditions significantly contributes to minimising environmental impacts, feed-to-food competition and, finally, global food security.

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ACKNOWLEDGEMENTS

First, I would like to thank my doctoral supervisor, Prof Dr Wilhelm Windisch, for allowing me to do my doctorate at the Chair of Animal Nutrition, Technical University of Munich (TUM). In particular, I would like to thank you for your great patience, your never-ending support and your impressive expertise.

My special thanks go to the staff of the contributing institutes and facilities of TUM and the Bavarian State Research Centre for Agriculture (Grub). The conduction of this project was only possible with excellent cooperation between all institutions and the staff's expertise, support, and technical assistance. My special thanks go to Karin Kleigrewe and Chen Meng (BayBioMS, TUM); Monika Piller, Li Janovsky, Andrea Reichlmeir and Michael Gertitschke (Chair of Animal Nutrition, TUM); and Dr Manfred Schuster and the staff of the Department of Quality and Assurance and Analytics of the Bavarian State Research Center for Agriculture (LfL, Grub).

At this point, I would like to sincerely thank Marcel Bowens and the staff of the Research Abattoir (previously LfL, now Bayerische Staatsgüter, Grub, Germany) for their never-ending patience during slaughter and for supporting me in recording all data and collecting hundreds of tissue samples. I would have never been able to obtain such precise data in a conventional slaughterhouse. My special thanks also go to Peter Edelmann and Anton Obermeier for their continuous help in data collection and practical animal work. Your help is greatly appreciated.

I especially want to thank my bachelor's and master's students for their tremendous contribution to this research project. Thank you so much, Regina Fuchs, Wyona Hager, Lea Lorentz, Lisa Vilgertshofer, Sophia Wirth, Brett Boden and Chris Pend! Without you, I would have never been able to manage hundreds of samples!

I particularly thank Dr Thomas Ettle from the Institute for Animal Nutrition and Feed Management (LfL), who initiated the project 'Amino Acids in Ruminant Nutrition' and allowed me to do my doctorate research. In addition, I acknowledge the financial support from the Bavarian State Ministry of Food, Agriculture and Forestry (A/17/18).

Moreover, sincere thanks go to Aniela Honig and Maria Schlattl for their continuous support during the trial periods and subsequent data evaluation phase. Going through this all together means a lot!

Finally, I thank my family and my friends from the bottom of my heart for their helpful advice and their never-ending patience and support.

APPENDIX

This section comprises the two publications presented in Chapter 3 and 4 in original formats.

Animal 15 (2021) 100366

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

Effects of supplementing a CP-reduced diet with rumen-protected methionine on Fleckvieh bull fattening

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ARTICLE INFO

Article history:
Received 17 September 2020
Revised 6 August 2021
Accepted 13 August 2021
Available online 1 October 2021

Keywords:
Amino acids
Average daily gain
Beef cattle
Protein utilization
Requirements

ABSTRACT

The objective of this study was to evaluate the effect of supplementing a CP-reduced diet with rumen-protected methionine on growth performance of Fleckvieh bulls. A total of 69 bulls (367 ± 25 kg BW) were assigned to three feeding groups ($n = 23$ per group). The control (**CON**) diet contained 13.7% CP and 2.11 g methionine/kg diet (both DM basis) and was set as positive control. The diet reduced in CP (nitrogen) (**RED**) diet as negative control and the experimental RED + rumen-protected methionine (**MET**) diet were characterised by deficient CP concentrations (both 9.04% CP). The RED + MET diet differed from the RED diet in methionine concentration (2.54 g/kg DM vs. 1.56 g/kg DM, respectively) due to supplementation of rumen-protected methionine. Rumen-protected lysine was added to both RED and RED + MET at 2.7 g/kg DM to ensure a sufficient lysine supply relative to total and metabolisable protein intake. Metabolisable energy (**ME**) and nutrient composition were similar for CON, RED, and RED + MET. Bulls were fed for 105 days (**d**) on average. Individual feed intake was recorded daily; individual BW was recorded at the beginning of the experiment, once per month, and directly before slaughter. At slaughter, blood samples were collected and carcass traits were assessed. Reduction in dietary CP concentration reduced feed intake, and in combination with lower dietary CP concentration, daily intake of CP for RED and RED + MET was lower compared with CON ($P < 0.01$). Daily ME intake was reduced in RED and RED + MET compared with CON ($P < 0.01$). Consequently growth performance and carcass weights were reduced (both $P < 0.01$) in both RED and RED + MET compared with CON. Supplemental rumen-protected methionine was reflected in increased serum methionine concentration in RED + MET ($P < 0.05$) as compared to RED but it did not affect growth performance, carcass traits and serum amino acid (**AA**) concentrations, except for lysine which was reduced ($P < 0.01$) compared to CON and RED. In conclusion, bulls fed RED or RED + MET diets were exposed to a ruminal CP deficit and subsequently a deficit of prececal digestible protein, but methionine did not appear to be the first-limiting essential AA for growth under the respective experimental conditions.

Implications

Efficiency in meat production (i.e., poultry and swine) is particularly enhanced by supplementing single, limiting amino acids to protein-reduced diets to meet the animals' amino acid requirements more precisely. In a few studies, this concept was also successfully applied to bulls for fattening. In this study, a protein-reduced diet with limited supply of prececal digestible methionine depressed growth performance. Addition of rumen-protected methionine did not resolve depression of performance. Therefore, methionine did not seem to be the first-limiting amino acid under these feeding conditions. Hence, this strategy cannot be generally applied to beef cattle farmers to reduce nitrogen emissions.

Introduction

Dietary amino acid (**AA**) supplementation based on digestible or metabolisable AA requirements is common practice in conventional swine and poultry production (van Milgen and Dourmad, 2015). However, knowledge on limiting AA for growing cattle under different feeding conditions and diet formulations is still

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https://doi.org/10.1016/j.animal.2021.100366
1751-7311/

limited. Ruminal fermentation and microbial protein synthesis impede the quantification of the amount and composition of AA absorbed in the small intestine (Titgemeyer, 2003). Flux of microbial protein towards the duodenum combined with dietary protein escaping ruminal degradation is the main AA source in intestinal digesta (Chalupa, 1975).

Numerous studies have investigated the effects of rumen-protected AA on milk production and health in dairy cows. Broderick et al. (2008) demonstrated that supplementation of rumen-protected Met to cows consuming a CP-reduced diet (16.1% CP vs. 17.3% CP in a standard diet) reduced urinary N excretion, increased N efficiency measured as milk N/N intake, and improved milk fat content and milk yield. Several other studies found ameliorating effects on milk yield, milk composition, and N utilisation with rumen-protected methionine (Met), lysine (Lys) and histidine (His) supplementation (Kudrna et al., 2009; Lee et al., 2012; Arriola Apelo et al., 2014; Lee et al., 2015; Giallongo et al., 2015; Giallongo et al., 2016). Contrary to work in dairy cattle, limited research has been conducted to evaluate the effects of supplemental rumen-protected AA in growing beef cattle.

Foundational work conducted by Richardson and Hatfield (1978) indicated that the sequence of the three first-limiting AAs in growing cattle was Met, Lys, and threonine (Thr). Therefore, Met and Lys appear to be the most promising AA to investigate limitations in growing cattle. Hill et al. (1980) determined the effects of supplementing rumen-protected Met to growing Angus × Hereford steers. Steers (230 kg BW) were fed a diet adequate in Lys and sulphur. Lysine and varying amounts of Met were infused abomasally. Under their experimental conditions, Met did not appear to be the first-limiting AA for growth. In contrast, recent work by Cantalapiedra-Hijar et al. (2020) may indicate improvements in N metabolism of young fattening Charolais bulls (320 kg BW) when Met was supplemented in high-forage diets. In a 2 × 2 factorial design, a protein adequate (13.2% CP) and a high protein (16.2% CP) diet were either balanced with rumen-protected Met (supplemented at 2.6% of metabolisable protein) or unbalanced (rumen-protected Met supplemented at 2.0% of metabolisable protein). Average daily gain (**ADG**) was improved when diets were balanced for Met, with a larger improvement observed in the high CP diet. Teixeira et al. (2019) indicated that supplemental rumen-protected Arg and Lys did not improve performance, but Lys can increase lean meat yield in Angus × Simmental finishing steers.

The experiments mentioned above applied sophisticated experimental procedures to evaluate the limiting potential of Met and Lys, such as abomasal infusions via cannulas and unusual dietary compositions. Research investigating applications of Met and Lys under practical conditions, such as the supplementation of rumen-protected AA to common rations, is still lacking. Therefore, the objective of our study was to evaluate the relevance of rumen-protected Met as a putative first-limiting AA in dual-purpose Fleckvieh bulls for fattening under conditions of sufficient Lys supply under practical feeding and housing conditions.

Material and methods

Animals

A total of 69 growing-fattening Fleckvieh bulls (238 ± 11 days (**d**); 367 ± 25 kg initial BW) were evenly assigned to three dietary treatment groups: control diet (**CON**), diet reduced in CP (nitrogen) (**RED**) or CP-reduced diet with supplemental rumen-protected Met (RED + **MET**) (n = 23/treatment). The treatment groups were balanced for age, initial BW and feed intake, measured during two weeks prior to the start of the experiment. The bulls were kept in two pens per treatment equipped with fully slatted floors. Each of the two pens assigned to one treatment housed 11 and 12 bulls, respectively. Bulls in the same pen had access to all feeding troughs of their pen (six feeding troughs/pen) all the time. The bulls' access to the feeding trough was monitored during the whole experiment (LfL Institute for Agricultural Engineering and Animal Husbandry, Grub, Germany; Wendl et al., 2001). All diets were offered as total mixed ration. The total mixed rations were prepared and delivered once per d (0800) for *ad libitum* intake. Approximately 10% of feed refusals were targeted; refusals were removed daily before refilling the feeding trough. Refusals were not analysed for feed sorting.

Feeding groups, feed analyses, and feed evaluation

Experimental diets (Table 1) contained maize silage and ground premixed concentrate as major diet components. The CON diet contained 13.7% CP, 15.7% utilisable CP (**UCP**), and 12.3 MJ metabolisable energy (**ME**) per kg DM (Table 2) and met the recommendations for nutrient and energy supply to growing Fleckvieh bulls in the BW range of 350–600 kg (German Society for Nutrition Physiology, 1995). The second diet (RED) induced a deficit in CP (9.04% CP) by removing rapeseed meal and urea from the CON diet; proportion of dried beet pulp was increased to replace rapeseed meal and urea (Table 1). Rumen-protected Lys (fat coated; LysiGEM, Kemin Industries, USA) was added to achieve the same dietary Lys concentration as CON. LysiGEM contains 70% Lys–HCl with reported 85% rumen stability and 95% intestinal availability of bypass Lys. The third diet (RED + MET) was the same as RED, except for the addition of 1.6 g rumen-protected Met (Smartamine M®, Adisseo, France) per kg DM. Smartamine M ® consists of small beads that are physically protected by a pH-sensitive coating (Graulet et al., 2005). The product contains a minimum of 75% DL-Met with 80% rumen stability and 99% intestinal availability of rumen stable Met. The RED diet contained 9.04% CP and the RED + MET diet contained 9.08% CP. The experimental diets were kept constant throughout the entire study.

The DM content of the total mixed ration (Table 2) was analysed twice per week, and DM content of the concentrate feed was analysed once for every batch. Dry matter analysis was conducted in accordance with the Association of German Agricultural Analytic and Research Institutes (VDLUFA 2012, method 3.1). Nutrient composition of the maize silage, total mixed ration, and concentrate feed were analysed by wet chemistry analyses according to VDLUFA (2012; method 8.1 for crude ash, method 4.1.2 for CP, method 7.1.1 for sugar, method 6.5.1 for NDF after amylase treat-

Table 1
Ingredient composition of the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet fed to Fleckvieh bulls for fattening.

Item	Diet	
	CON	RED and RED + MET[1]
Ingredient (% DM)		
Maize silage	38.07	37.89
Corn, dry-rolled	25.35	25.01
Barley, dry-rolled	12.69	12.63
Rapeseed meal, extracted	8.88	–
Dried beet pulp	8.88	18.94
Barley straw	3.81	3.79
Urea	0.80	–
Mineral and Vitamin premix	0.72	0.72
Calcium carbonate	0.63	0.63
Salt	0.13	0.13
LysiGEM™	–	0.27
Smartamine M®[1]	–	0/0.16

[1] RED and RED + MET were equal in their ingredient composition except for supplemental rumen-protected methionine (0.16%, DM basis).

Table 2
Chemical and amino acid composition of the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet fed to Fleckvieh bulls for fattening.

Item	Diet		
	CON	RED	RED + MET
DM (g/kg)	539	537	531
Analysed Composition (% of DM, if not indicated differently)			
Ash	5.3	5.0	4.9
$aNDF_{om}$	28	29	29
Starch and Sugar	40	42	42
Crude Fat	3.0	2.9	2.9
Nitrogen (g/kg DM)	22	15	15
Amino Acids (g/kg DM)			
Lys	4.8	4.9	4.8
Met	2.1	1.6	2.5
Cys	2.1	1.4	1.2
Thr	4.7	3.6	3.4
Trp	1.1	0.8	0.7
Ile	3.9	3.5	3.1
Leu	9.6	8.3	7.8
Val	5.4	4.8	4.4
Ala	7.0	6.0	5.7
Arg	5.0	3.4	3.1
Asx	8.5	6.7	6.
Glx	20	16	15
Gly	5.4	4.1	3.9
His	3.2	2.4	2.2
Phe	4.5	3.90	3.6
Pro	8.2	6.8	6.3
Ser	5.5	4.0	3.7
Tyr	3.3	2.8	2.6
Calculated parameters (g/kg DM, if not indicated differently)			
CP[1], total	137	90	90
from urea	23	–	–
Utilisable CP[2]	157	149	149
Prececal Digestible Protein	103	97	99
Prececal Digestible Met	2.3	2.2	3.2
Prececal Digestible Lys	6.3	7.8	7.8
Prececal Digestible Thr	4.7	4.6	4.6
Metabolisable Energy[3] (MJ/kg DM)	12.3	12.2	12.2

Abbreviation: MJ = Megajoule.
[1] CP = N × 6.25.
[2] Calculation of utilisable CP according to German Society of Nutrition Physiology (1995) excluding CP from urea.
[3] Estimation of metabolisable energy concentration according to German Society of Nutrition Physiology (2008) and DLG (2011).

ment and ashing ($aNDF_{om}$)) in samples pooled over four weeks. Crude fat (method 152-H) and starch (method 152-L) were determined according to the methods of Commission Regulation (EC) No. 152/2009. Feed AA, except tryptophane (Trp), was analysed according to methods of Commission Regulation ((EG) 152/2009 App. III, F). Tryptophane analysis was conducted according to the method of Commission Regulation (EG) 152/2009 App. III, G. Utilisable CP concentration was calculated as follows: $(11.93 - (6.82 \times (RBP/CP_{(without\ urea)}))) * ME + 1.03 \times RBP$ (German Society of Nutrition Physiology, 2001) with RBP presenting rumen bypass protein (**RBP**) according to German Agricultural Society (DLG, 1997). In this formula, we used dietary CP that originated from feed components except urea. This component of dietary CP was considered to provide 30% of CP as RBP. Utilisable CP of microbial origin (**MUCP**) was calculated by subtracting RBP from total UCP. Prececal digestible (**pcD**) protein was calculated as the sum of absorbed protein from both MUCP and RBP. Microbial contribution to pcD protein was calculated as follows: 80% of MUCP was considered to be true protein according to National Research Council (NRC, 2001), which was assumed to be 80% intestinal digestible according to the 'PDI system' of the Institute National de la Recherche Agronomique (Sauvant and Nozière, 2016; Institut National de la Recherche Agronomique, 2018). Contribution of RPB to pcD protein was calculated by RBP × 0.7, reflecting the conversion factor of the 'PDI system' of the Institut National de la Recherche Agronomique (2018). Accordingly, pcDMet, pcDLys and pcDThr concentrations comprise the respective sum of pcDMet, pcDLys and pcDThr from both the MUCP and RBP. Respectively, contributions from MUCP were calculated as follows: $(0.028 \times 0.8 \times MUCP \times 0.8)$ for Met with 2.8% of Met in the true protein proportion of MUCP, $(0.079 \times 0.8 \times MUCP \times 0.8)$ for Lys with 7.9% of Lys in the true protein proportion of MUCP and $(0.058 \times 0.8 \times MUCP \times 0.8)$ for Thr with 5.8% of Thr in the true protein proportion of MUCP. Average AA concentrations in the true protein proportion of MUCP were obtained from the National Research Council (NRC, 2001; 2016) that uses data from Clark et al. (1992). Intestinal digestibility was set to 0.8 as indicated above (Institut National de la Recherche Agronomique, 2018). Accordingly, contributions of RBP to pcDMet, pcDLys and pcDThr were calculcated as respective dietary concentrations of Met, Lys, and Thr multiplied 0.3 and 0.7 with the latter factors representing the proportion of RBP to dietary CP and the conversion factor from RBP to pcD protein (Institut National de la Recherche Agronomique, 2018). In case of added dietary rumen-protected Met or rumen-protected Lys, their contributions to pcDMet and pcDLys were calculated by multiplying with 0.8 and 0.85 for rumen escape and 0.99 and 0.95, for digestibility, respectively, as stated above in the product description.

Slaughter, blood sampling and analyses

The experiment was divided into three periods. Period one lasted from d 1 until d 28, period two from d 29 until d 57. Period three lasted from d 58 until slaughter. Bulls were slaughtered across eight d and bulls from all three treatments were slaughtered on each date. The average duration of the experiment was 105 d.

On d of slaughter, bulls were transported from the stable to the research abattoir at 0600 h. Duration of transport was not longer than five minutes. Bulls were always weighed immediately before leaving the stable and refilling the feeding troughs and when entering the slaughterhouse; bulls were not fasted before slaughter. Carcasses were classified (EUROP with E = excellent, U = very good, R = good, O = Fair and P = poor) and carcass quality was determined following European Standards (Council Regulation No. 1249/2008).

Blood samples were collected using vacuette tubes (VACUETTE TUBE 4 ml CAT Serum Clot Activator, Greiner Bio-One International GmbH, Kremsmünster, Austria) during exsanguination. Tubes were inverted, centrifuged (2 000*g* × 10 min at room temperature) and then stored at −20 °C for further analysis.

The AAs in the blood serum were analysed at the Bavarian Centre for Biomolecular Mass Spectrometry (BayBioMS, Freising, Germany) using stable isotope dilution analysis and LC-ESI-MS/MS (MRM) measurement after the extraction of AA from the serum with a mixture of methanol/water 70/30 (v/v). Fifteen isotope-labelled standards were used to quantify 19 proteinogenic AA. Method parameters were applied according to Hillmann and Hofmann (2016).

Urea concentrations in the blood serum were analysed using a BioChrom30 Amino Acid Analyser following the standardised recommended procedure of analysing urea and AA in physiological liquids (Biochrom Ltd., Cambridge, UK). Cystine and cysteine (Cys) concentrations were below detection limit and therefore omitted from statistical analyses.

BW measurements and calculations

The BW of the bulls was recorded using an electronic scale directly before beginning of the study (BW at start), at the end of

period one and period two, and immediately prior to transportation to the slaughterhouse (BW at slaughter).

Calculations were conducted for the three distinct periods (periods one, two, three) as well as for the entire time on feed. Average daily gain (g/d) total was calulcated as (BW at slaughter (kg) − BW at start (kg))/total (d) × 1 000. Accordingly, ADG during periods one, two and three was calculated as the ratio of BW gain (kg) during the period and the time being the number of d in the period (i.e., between two measurements). Daily total DM, nutrient and ME intake were calculated as the ratio of total DM, nutrient, and ME intake and total d on feed and were conducted accordingly for periods one, two and three. Feed conversion ratio of each bull was calculated as the ratio of total DM intake/total weight gain and calculated accordingly for periods one, two, three, and total.

Supply of pcD protein, pcDMet, pcDLys, and pcDThr was calculated by dietary contents multiplied with DM intake in the respective periods of time. Requirements of pcD protein of animals were calculated by estimating the net requirements of (ideal) protein and assuming a metabolic utilisation of pcD protein of 0.7 according to German Society of Nutrition Physiology (1995). Net requirements comprised estimates of maintenance requirements (i.e., urinary, faecal, and surface losses derived by DM intake and mean BW according to German Society of Nutrition Physiology (1995)) and net protein requirements for growth that were assumed to account for 20% of ADG (Honig et al., 2020). Supply of pcD protein, pcDMet, pcDLys, and pcDThr was expressed as percentage of respective requirements.

Statistical analyses

Statistical analysis was performed using SAS (SAS 9.4, SAS Institute, Cary, NC, USA). Zootechnical data of total time on feed and of each period (1, 2, 3) were analysed with a general linear mixed model (**GLMM**) with dietary treatment as fixed effect and pen × treatment as random effect. Residual variance was determined to be pen × treatment. Dietary treatment group means were tested using a Student-Newman-Keuls posthoc test included in the GLMM. In the case of EUROP carcass classification and fat grade, a non-parametric posthoc test was applied (Kruskal-Wallis H Test).

The $P\text{-value}_{GLMM}$ represents the statistical significance of the GLMM model in total. Linear contrasts, except for EUROP carcass classification and fat grade, were calculated to detect differences in response variables due to CP reduction (*P*-value CON vs. RED and RED + MET) and subsequently, due to the supplementation of rumen-protected Met (RED vs. RED + MET). The standard error of the mean over the whole GLMM is indicated as SEM.

Significant differences were declared at $P \leq 0.05$. Differences at $0.05 \leq P \leq 0.10$ were considered a trend.

Results

DM, nutrient, and metabolisable energy intake

Table 3 presents the average DM, ME, nutrient, and pcDAA intake during experimental periods one to three and over the entire feeding period, referred to as 'total'. The reduction in CP decreased DM intake ($P < 0.01$) in both RED and RED + MET (8.49 and 8.27 kg/d, respectively) compared to CON (9.43 kg/d) during the whole time on feed. Consequently, ME and nutrient intake were also lower ($P < 0.01$) in RED and RED + MET compared to CON. In total, pcD protein intake of CON bulls accounted for 968 g/d, which represented 134% of their daily requirement. In comparison, RED and RED + MET bulls had lesser ($P < 0.01$) supply of pcD protein (both 822 g/d) meeting their requirements (calculated retrospectively on the base of actual performance) at 133 and 138%, respectively. Intake of pcDLys accounted for 59.7, 66.0 and 64.2 g/d in CON, RED and RED + MET, respective intake of pcDThr accounted for 44.1, 38.9 and 37.8 g/d. In CON and RED bulls, intake of pcDMet (21.2 and 18.8 g/d, respectively) matched their requirements at 98 and 101%, respectively. RED + MET bulls had a greater ($P < 0.01$) daily pcDMet intake (26.2 g) relative to CON and RED, which accounted for 146% of their daily requirement.

Serum amino acids and urea concentrations

The reduction in dietary CP concentration depressed serum concentrations (Table 4) of almost all essential AA, with Lys, Met, Thr, and the sum EAA being statistically different (linear contrasts of both RED and RED + MET vs. CON; $P < 0.05$). Furthermore, Ala and Asx were reduced ($P < 0.01$), while serum levels of Tyr increased ($P < 0.05$) relative to RED and RED + MET vs CON. Serum urea decreased from 2 081 to ∼702 mol/L on average in CP deficient bulls ($P < 0.01$). Within CP deficient treatments, addition of rumen-protected Met (group RED + MET) increased serum Met ($P < 0.01$) and reduced Lys (linear contrast between RED and RED + MET; $P = 0.02$).

Growth performance

The initial BW was 366 kg in CON and RED + MET bulls and 368 kg in RED bulls (Table 5). BW at slaughter was lower ($P < 0.01$) in both RED and RED + MET groups (499 and 498 kg) compared to CON bulls (532 kg). Additionally, higher dietary CP concentration increased ($P < 0.01$) ADG in CON (1 579 g/d) as compared to RED (1 256 g/d). On average, RED + MET bulls gained 1 199 g/d. Due to reduction in dietary CP concentration, total feed conversion ratio (**FCR**) of RED and RED + MET bulls was impaired (7.07 and 7.15, respectively) compared to FCR of CON bulls (6.07; $P < 0.01$).

Carcass traits

Reduced dietary CP concentration (RED and RED + MET) decreased both carcass weight (Table 6, 298 kg for CON vs. 274 kg for average of RED and RED + MET, $P < 0.01$) and dressing percentage (56.9% for CON vs. 55.6% for average of RED and RED + MET, $P < 0.01$) relative to CON; the addition of rumen-protected Met had no effect on either parameter (RED vs. RED + MET, $P \geq 0.33$). Carcass quality was not affected by dietary CP reduction or Met supplementation ($P = 0.2$). Fat grade of CON bulls was found to be 2.34. Compared to CON, RED + MET bulls had a lower fat grade (2.34 vs. 2.04) while that of RED bulls was intermediate (2.13; $P = 0.3$). There were no differences between treatments for pH after 1 and 24 h or in the kidney fat proportion.

Discussion

Studies evaluating metabolic AA requirements of ruminants must consider microbial degradation of dietary protein and de novo microbial protein synthesis in the rumen, as they alter the AA composition of protein reaching the small intestine (Titgemeyer, 2003). The dominant proportion of the duodenal AA flow is of microbial origin (Clark et al., 1992). Apart from that, ruminal bypass protein and endogenous secretions add to the total duodenal flow of AA (Richardson and Hatfield, 1978). The relevant protein supply to the animal, however, is the AA absorbed from duodenal digesta into the bloodstream. These are assessed differently by various protein evaluation systems - such as 'metabolisa-

Table 3
Daily nutrient intake of the bulls fed the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet.

	Diet				*P*-value		
	CON	RED	RED + MET	SEM	GLMM[1] (CON vs. RED vs. RED + MET)	Lin. Contrast I[2] (CON vs. RED/RED + MET)	Lin. Contrast II[2] (RED vs. RED + MET)
Period 1							
DM (kg)	8.27	7.56	7.01	0.47	0.19	<0.01	0.03
Metabolisable Energy (MJ)	102	92	86	5.77	0.17	<0.01	0.02
CP (g)	1 133[a]	683[b]	637[b]	49.0	0.02	<0.01	0.03
Prececal Digestible Protein (g)	850	732	696	46.3	0.09	<0.01	0.03
% requirement	*137*	*136*	*156*				
Prececal Digestible Met (g)	18.6[ab]	16.7[a]	22.2[b]	1.2	0.04	0.13	<0.01
% requirement	*100*	*103*	*166*				
Prececal Digestible Lys (g)	52.4	58.7	54.4	3.5	0.32	<0.01	0.02
% requirement	*106*	*136*	*153*				
Prececal Digestible Thr (g)	38.7	34.6	32.0	2.17	0.15	<0.01	0.02
% requirement	*120*	*124*	*139*				
Period 2							
DM (kg)	9.14	8.6	8.31	0.54	0.21	0.01	0.29
Metabolisable Energy (MJ)	113	105	101	6.66	0.17	<0.01	0.28
CP (g)	1 251[a]	777[b]	754[b]	55.8	<0.01	<0.01	0.41
Prececal Digestible Protein (g)	938	833	825	53.4	0.07	<0.01	0.29
% requirement	*128*	*129*	*131*				
Prececal Digestible Met (g)	20.6[a]	19.0[a]	26.3[b]	1.39	<0.01	<0.01	<0.01
% requirement	*94*	*98*	*139*				
Prececal Digestible Lys (g)	57.8	66.8	64.5	4.07	0.08	<0.01	0.25
% requirement	*99*	*130*	*128*				
Prececal Digestible Thr (g)	42.8	39.4	38.0	2.50	0.13	<0.01	0.26
% requirement	*112*	*118*	*116*				
Period 3							
DM (kg)	10.24	9.1	9.22	0.60	0.08	<0.01	0.7
Metabolisable Energy (MJ)	126	111	113	7.37	0.06	<0.01	0.72
CP (g)	1 402[a]	823[b]	837[b]	65.0	<0.01	<0.01	0.66
Prececal Digestible Protein (g)	1 051[a]	881[b]	916[b]	59.5	0.03	<0.01	0.7
% requirement	*130*	*132*	*133*				
Prececal Digestible Met (g)	23.0[a]	20.1[b]	29.2[c]	1.54	<0.01	0.02	<0.01
% requirement	*95*	*100*	*141*				
Prececal Digestible Lys (g)	64.8	70.7	71.5	4.42	0.11	<0.01	0.71
% requirement	*100*	*132*	*130*				
Prececal Digestible Thr (g)	47.9	41.7	42.1	2.78	0.05	<0.01	0.75
% requirement	*114*	*120*	*118*				
Total duration of the experiment							
DM (kg)	9.43	8.49	8.27	0.52	0.07	<0.01	0.43
Metabolisable Energy (MJ)	116	104	101	6.42	0.06	<0.01	0.42
CP (g)	1 292[a]	767[b]	751[b]	54	<0.01	<0.01	0.58
Prececal Digestible Protein (g)	968[a]	822[b]	822[b]	0.02	0.03	<0.01	0.43
% requirement	*134*	*133*	*138*				
Prececal Digestible Met (g)	21.2[a]	18.8[b]	26.2[c]	1.35	<0.01	0.04	<0.01
% requirement	*98*	*101*	*146*				
Prececal Digestible Lys (g)	59.7	66.0	64.2	3.91	0.13	<0.01	0.39
% requirement	*103*	*134*	*134*				
Prececal Digestible Thr (g)	44.1	38.9	37.8	2.41	0.05	<0.01	0.39
% requirement	*117*	*121*	*122*				

Abbreviation: GLMM = general linear mixed model, SNK = Student-Newman-Keuls posthoc test.
[1] *P*-values of the SNK in the GLMM.
[2] *P*-values of the linear contrasts of CON vs. RED and RED + MET or RED vs. RED + MET.
[a,b,c] Values within a row with different superscripts differ significantly in the SNK at $P < 0.05$.

ble protein' according to the National Research Council (2001) and the Cornell Net Carbohydrate System (Lapierre et al., 2018), as 'intestinal digestible protein' (DVE) according to the Dutch 'DVE/OEB system' (Tamminga et al., 1994; 2007; van Duinkerken et al., 2011), as 'protein digestible in the intestine' (PDI) according to the French 'PDI system' (Institut National de la Recherche Agronomique, 2018), or as 'AA absorbed in the small intestine' (AAT_n) according to the Scandinavian 'NorFor system' (2011). In our study, we refer to pcD according to the system used in monogastrics (German Society of Nutrition Physiology, 2008).

Within protein supply, the AA Met and Lys are likely to be most limiting to growing animals (Nimrick et al., 1970; Williams and Smith 1974; Richardson and Hatfield, 1978, Storm and Ørskov 1984; Wilkerson et al., 1993, Klemesrud et al., 2000; van Milgen and Dourmad, 2015). The first condition to prove the limitation of an EAA is a deficient protein supply while all other nutrients and ME are supplied in sufficient amounts. Secondly, the AA in question must be added to the protein-deficient diet and must relieve protein deficit (e.g., stimulation of growth). In the case of ruminants, the AA must be supplied postruminally, either via abomasal or duodenal infusion, or by a rumen-protected AA added to the diet. However, the positive effect of this AA stops once the next critical AA starts to become limiting (Storm and Ørskov, 1984; Titgemeyer, 2003).

This study was conducted to determine whether Met limits growth performance of fattening Fleckvieh bulls. Dietary concentrations of ME and other nutrients were adequate in all treatments. Regarding ME, bulls with approx. 450 kg BW have a daily ME

Table 4
Concentrations of amino acids and urea in blood serum from bulls fed the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet.

	Diet				*P*-value		
	CON	RED	RED + MET	SEM	GLMM[1] (CON vs. RED vs. RED + MET)	Lin. Contrast I[2] (CON vs. RED/RED + MET)	Lin. Contrast II[2] (RED vs. RED + MET)
Amino Acid [μmol/L]							
Lys	90.8[a]	69.4[b]	45.6[c]	4.1	<0.01	<0.01	0.02
Meth	33.5[a]	35.0[a]	43.4[b]	1.6	<0.01	<0.01	<0.01
Cys[3]	n.q.	n.q.	n.q.				
Thr	120[a]	100[b]	97.1[b]	5.15	0.12	<0.05	0.70
Trp	61.3[a]	54.8[ab]	53.1[b]	2.55	0.15	0.08	0.46
Ile + Leu	251	220	227	11.7	0.25	0.1	0.81
Val	260	226	236	10.8	0.25	0.12	0.49
His	95.6	83.9	82.9	4.75	0.276	0.11	0.83
Phe	65.4	69.6	61.6	3.65	0.42	0.67	0.20
Essential Amino Acids	978[a]	859[b]	847[b]	36.9	0.08	0.03	0.80
Ala	221[a]	181[b]	168[b]	13.3	<0.01	<0.01	0.93
Arg	187	179	174	8.80	0.80	0.52	0.91
Asx	124[a]	105[b]	97.1[b]	6.75	<0.01	<0.01	0.09
Glx	481	487	479	24.3	0.99	0.93	0.93
Pro	87.0[a]	90.8[a]	77.2[b]	3.70	0.18	0.60	0.09
Ser	88.3[a]	103.5[b]	95.5[ab]	4.45	0.23	0.11	0.45
Tyr	122	139	141	7.80	0.11	0.04	0.73
Non-Essential Amino Acids	1 309	1 284	1 231	270	0.66	0.40	0.79
Sum of Amino Acids	2 188	2 037	1 970	57.5	0.37	0.17	0.86
Urea	2 081[a]	658[b]	747[b]	94.9	<0.001	<0.001	0.39

Abbreviation: GLMM = general linear mixed model, SNK = Student-Newman-Keuls posthoc test.
[1] *P*-values of the SNK in the GLMM.
[2] *P*-values of the linear contrasts of CON vs. RED and RED + MET or RED vs. RED + MET.
[3] Cys could not be analysed because quantities were below the detection limit.
[a,b,c] Values within a row with different superscripts differ significantly in the SNK at $P < 0.05$.

Table 5
Growth performances of the bulls fed the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet.

	Diet				*P*-value		
	CON	RED	RED + MET	SEM	GLMM[1] (CON vs. RED vs. RED + MET)	Lin. Contrast I[2] (CON vs. RED/RED + MET)	Lin. Contrast II[2] (RED vs. RED + MET)
Period 1							
BW at start[3] (kg)	366	368	366	14.8	0.65	0.87	0.8
Mean BW (kg)	385	383	377	14.1	0.18	0.46	0.43
Average daily gain (g)	1 290	1 023	805	233	0.12	<0.01	0.07
Feed Conversion Ratio	7.53	8.98	9.62	2.8	0.05	0.15	0.67
Period 2							
Mean BW (kg)	425	416	406	14.7	0.08	0.03	0.21
Average daily gain (g)	1 606	1 339	1 316	228	0.24	<0.01	0.92
Feed Conversion Ratio	6.21	7.13	6.68	1.2	0.76	0.21	0.39
Period 3							
Mean BW (kg)	490	468	462	21.0	0.06	0.01	0.6
Average daily gain (g)	1 791[A]	1 379[B]	1 446[B]	192	<0.01	<0.01	0.51
Feed Conversion Ratio	5.85	6.99	6.7	1.0	0.21	0.02	0.59
Total duration of the experiment							
Mean BW (kg)	449	434	432	19.5	0.15	0.07	0.86
BW at slaughter[4] (kg)	532	499	498	34.1	0.17	0.03	0.93
Average daily gain (g)	1 580[a]	1 256[b]	1 199[b]	0.15	0.01	<0.01	0.46
Feed Conversion Ratio	6.07	7.07	7.15	0.8	0.18	<0.01	0.85

Abbreviation: GLMM = general linear mixed model, SNK = Student-Newman-Keuls posthoc test.
[1] *P*-values of the SNK in the GLMM.
[2] *P*-values of the linear contrasts of CON vs. RED and RED + MET or RED vs. RED + MET.
[3] BW at start indicates BW immediately before start of the experiment.
[4] BW at slaughter indicates BW immediately before transportation to the slaughterhouse.
[A,B] Values within a row with different superscripts differ significantly in the SNK at $P < 0.01$.
[a,b] Values within a row with different superscripts differ significantly in the SNK at $P < 0.05$.

requirement of 96 MJ for ADG of 1 600 g (German Society of Nutrition Physiology, 1995). Mean energy intake was 116, 104, and 101 MJ ME per d in CON, RED, and RED + MET, respectively. In the CON diet, pcDMet, pcDLys, and pcDThr concentrations per 100 g pcDP (2.25 g, 6.33 g, and 4.68 g, respectively; Table 2) were below the recommendations given by Wilkerson et al. (1993) (per 100 g metabolisable protein: 3.0 g, 8.0 g, 5.2 g, respectively). Despite the low dietary AA concentrations, due to high DM intake, daily intake of this pcDAA was near or above the requirement (Table 3).

Table 6
Carcass traits of the bulls fed the control (CON), CP-deficient (RED) and RED supplemented with rumen-protected Methionine (RED + MET) diet.

Items	Diet			SEM	*P*-value		
	CON	RED	RED + MET		GLMM[1] (CON vs. RED vs. RED + MET)	Lin. Contrast I[2] (CON vs. RED/RED + MET)	Lin. Contrast II[2] (RED vs. RED + MET)
Carcass weight[4] (kg)	298	275	272	20.6	0.09	<0.01	0.72
Dressing percentage (%)	56.7	55.7	55.3	0.72	0.11	<0.01	0.33
Kidney fat/carcass weight (%)	2.66	2.68	2.59	0.39	0.94	0.79	0.67
pH1[5]	6.91	6.86	6.89	0.05	0.22	0.10	0.21
pH24[5]	5.47	5.46	5.46	0.04	0.13	0.81	0.83
EUROP[6]	2.65	2.96	2.87		0.20[3]	0.09[3]	0.64[3]
Fat grade[7]	2.34	2.13	2.04		0.02[3]	0.08[3]	0.30[3]

Abbreviation: GLMM = general linear mixed model, SNK = Student-Newman-Keuls posthoc test.
[1] *P*-values of the SNK in the GLMM.
[2] *P*-values of the linear contrasts of CON vs. RED and RED + MET or RED vs. RED + MET.
[3] *P*-values of the Kruskal-Wallis H Test between diet group means.
[4] Weight (kg) of both warm carcass-halves.
[5] Measured 1 and 24 h after slaughter.
[6] EUROP carcass classification (E = 1 = best. U = 2. R = 3. O = 4. P = 5 = poorest). https://www.agriculture.gov.ie/farmingsectors/beef/eubeefcarcaseclassificationscheme/.
[7] Degree of fat is denoted by the numbers 1. 2. 3. 4. and 5 in order of increasing fatness.

RED and RED + MET groups were exposed to a CP deficit in order to reduce microbial protein synthesis and limit protein supply. Thus, DM intake was depressed compared to the CON group as a consequence of low dietary CP concentrations, which reduced pcDP supply and hence limited growth. However, for lower realised growth rates of RED and RED + MET, their true pcDP intake was above the actual requirement – from a retrospective point of view (i.e., RED and RED + MET bulls met the pdDP requirement for their actual rates of growth). In fact, their pcDP intake (822 g/d of both) should have been adequate even for CON growth rates, as 100% pcDP requirement of CON intake accounted for 722 g pcDP/d. Retrospectively, pcDP, pcDLys, and pcDThr intake rates exceeded calculated requirements, while pcDMet supply matched the retrospective requirement in RED bulls (101% of requirement, Table 3). In RED + MET bulls, pcDMet supply was improved compared to RED (142% of requirement; $P < 0.01$, Table 3). Therefore, addition of rumen-protected Met to the RED + MET bulls should stimulate growth, provided Met was the first-limiting AA in our study.

Calculations on protein requirement and supply, however, entail major uncertainties. For example, ideal AA composition of metabolisable protein derived by Wilkerson et al. (1993) was based on data of British type Hereford × Angus crossbred steers with initial BW of 253 kg and ADG of 0.49 kg/d. Hence, these bulls were in an earlier life stage and had considerably lesser ADG. The first uncertainty in the calculation of protein supply is the amount of true protein in MUCP. It is generally assumed to contain 80% true protein (NRC, 2001). Sok et al. (2017), however, proposed 82.4% due to differing AA composition of particle-associated bacteria, fluid-associated bacteria, and protozoa. Secondly, estimates on the AA composition of MUCP true protein differ between protein evaluation systems due to varying methodological approaches. Clark et al. (1992), which is widely used by NRC (2001), took into account data from 18 studies, from which seven were performed in sheep. The Institut National de la Recherche Agronomique (Rulquin et al., 1998) uses the AA composition of fluid-associated bacteria (Le Hènaff, 1991) representing average values of 66 studies in both sheep and cattle. Thirdly, estimates for digestibility of true protein from both sources, the MUCP and the RBP, are different. The Institut National de la Recherche Agronomique (2018) uses 80 and 70%, respectively, whereas German Society of Nutrition Physiology (1995) indicates 85% for both. The fourth uncertainty is AA utilisation efficiency in metabolism. Growing ruminants primarily utilise absorbed AA for muscle tissue accretion, but additional losses due to oxidative processes also take place to a significant extent. Methionine utilisation efficiency, for instance, can range from 14 to 66% (Titgemeyer, 2003).

In conclusion, the calculation of AA supply through the diets and of the AA requirements of the bulls as done in our study suggests only rough estimates of effective AA supply among treatment groups.

Different Met supplies of the CON, RED, and RED + MET diets were evident in respective blood serum Met concentrations. In particular, RED + MET showed greater serum Met than RED bulls, which proves the efficacy of the added Met source to deliver absorbable Met. Hence, our experimental setup matched in principle the preconditions to test the hypothesis that Met might be the first-limiting AA for the growth performance of Fleckvieh bulls. The fact that serum Met concentration was not reduced in RED compared to CON despite the lower Met intake may have resulted from lower ADG in the RED group which, retrospectively, entailed a lower requirement of pcDMet. Interestingly, blood Lys concentration was significantly lower in RED + MET compared to RED. This may suggest that Lys could have been the first-limiting AA for growth, but not Met. However, theoretical calculations of pcDLys supply show only a mere difference between RED and RED + MET. Hence, it can be assumed that circulating Lys was apparently more utilised for protein synthesis in the RED + MET group compared to RED, but lack of response in growth suggests that it was not translated into pronounced muscle tissue accretion in favour of other body proteins (i.e., organs, functional proteins, etc.). This hypothesis is supported by the fact that supply of pcDLys was sufficiently high and hence did not limit muscle tissue accretion of bulls in our study. Another point that may be considered is the efficacy of the rumen-protection of the Lys product. We did not determine rumen stability of the product in this study, but this has been done by Francia et al. (2020). They indicate ruminal degradation of 23.2% and intestinal digestibility of 87.3% for the Lys product.

Several studies have identified Met as one of the three first-limiting AAs (Richardson and Hatfield, 1978; Greenwood and Titgemeyer, 2000; Froidmont et al., 2001; Cantalapiedra-Hijar et al., 2020). In contrast, Hill et al. (1980) did not find Met as a first-limiting AA. In addition, Ettle et al. (1999) did not consistently elicit the growth-enhancing effects of rumen-protected Met to Fleckvieh bulls for fattening in the live weight range of 100–200 kg. These findings contradict most other studies that applied artificial experimental conditions, but they are in accordance with our results. As the diets in our study and that of Hill et al. (1980) were primarily based on maize silage, this particular feedstuff might have additionally affected experimental outcomes because the AA profile of maize is characterised by comparably high levels of Met. Another point to be considered is the stage of the develop-

ment of the growing bulls. In comparison with the abovementioned studies with beef cattle, our bulls grew to greater live weights, suggesting that the dietary deficit of pcDP was less pronounced. This might have additionally reduced the potential of rumen-protected AA added to the diet to be first-limiting to growth.

In our study, a reduction in dietary CP in RED and RED + MET reduced the fat cover compared to CON. This finding seems to contradict numerous studies with growing monogastric livestock that showed increased body fat accretion when the protein supply fell below requirements (e.g. Kerr et al., 1995). Indeed, quantitative limitations in EAAs force the metabolism to redirect the utilisation of the relative excess of non-limiting AA from protein synthesis towards fat accretion, provided that overall consumption of DM and the corresponding dietary energy remains unchanged. However, in our study, the intake of DM and ME was significantly reduced in RED and RED + MET bulls, which seemed to prevent the expected rise in body fat accretion due to an insufficient supply of dietary AA.

When comparing RED with RED + MET bulls, the addition of rumen-protected Met did not affect fat deposition. This finding additionally supports that Met was not the first-limiting AA for growth, because the relief of such a limitation should have stimulated protein synthesis at the expense of fat deposition as has been widely shown in other studies with monogastric animals (e.g., Kerr et al., 1995; Ettle et al., 2003).

In summary, our data suggest the presence of pcDP deficiency in RED and RED + MET groups due to dietary CP restriction. Furthermore, we succeeded in supplementing RED + MET bulls with rumen-protected Met, which is strongly supported by serum Met concentrations. However, the growth and slaughter performance results did not elicit a significant effect of supplemental Met. Therefore, we assume that under our experimental conditions, Met was not the first growth-limiting AA for Fleckvieh bulls of 350–500 kg BW fed a maize silage-based ration. It may also be possible that another EAA became first-limiting closely behind Met. This could not have been Lys and probably also not Thr, provided that the estimates of pcDLys and pcDThr supply status reflected actual experimental conditions and that rumen-protection of rumen-protected Lys was sufficient. For consecutive studies under practical feeding conditions, we suggest the use of younger bulls, which grow more intensively and have relatively higher rates of protein deposition and, consequently, greater AA requirements.

Ethics approval

The study was conducted at the Bavarian State Research Centre for Agriculture in Grub (LfL, Germany). The experimental procedures followed the guidelines of the German law for animal protection of the German State and Directive 2010/63/EU of the European Parliament and of the Council of September 22nd 2010 on the protection of animals used for scientific purposes. The bulls were slaughtered at the LfL research abattoir (Grub, Germany) according to the German law of animal protection of the German State and Council Regulation (EC) No. 1099/2009 of September 24th 2009 on the protection of animals at the time of killing.

Data and model availability

Data analysis was performed with SAS (SAS 9.4, SAS Institute, Cary, NC, USA). None of the data were deposited in an official repository. The data that support the study findings are available upon reasonable request.

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Declaration of interest

None.

Acknowledgements

Slaughterhouse personnel is greatly appreciated for their help collecting data and samples during slaughter. Thanks to the stable personnel for their daily animal welfare care and their help weighing the bulls. The authors highly appreciate the help of Mrs. Madeline Sue Grant.

Financial support statement

This study received funding from the Bavarian State Ministry for Food, Agriculture and Forestry. The project “Amino Acids in Ruminants” is registered by the number A/17/18.

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Effect of Rumen-Protected Methionine on Metabolic Profile of Liver, Muscle and Blood Serum Samples of Growing German Simmental Bulls Fed Protein-Reduced Diets

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Abstract: This study aimed to determine the metabolic response of growing German Simmental bulls fed rations low in crude protein (CP) supplemented with rumen-protected methionine (RPMET). In total, 69 bulls (on average 238 ± 11 days of age at start and 367 ± 25 kg of bodyweight) were assigned to three dietary treatments (n = 23/group): Positive control (CON; 13.7% CP; 2.11 g methionine/kg DM), negative control deficient in CP (RED; 9.04% CP; 1.56 g methionine/kg DM) and crude protein-deficient ration supplemented with RPMET (RED+RPMET; 9.04% CP; 2.54 g methionine/kg DM). At slaughter, samples of liver, muscle and blood serum were taken and underwent subsequent metabolomics profiling using a UHPLC-QTOF-MS system. A total of 6540 features could be detected. Twenty metabolites in the liver, five metabolites in muscle and thirty metabolites in blood serum were affected ($p < 0.05$) due to dietary treatments. In total, six metabolites could be reliably annotated and were thus subjected to subsequent univariate analysis. Reduction in dietary CP had minimal effect on metabolite abundance in target tissues of both RED and RED+RPMET bulls as compared to CON bulls. The addition of RPMET altered the hepatic anti-oxidant status in RED+RPMET bulls compared to both RED and CON bulls. Results exemplify nutrient partitioning in growing German Simmental bulls: bulls set maintenance as the prevailing metabolic priority (homeostasis) and nutrient trafficking as the second priority, which was directed toward special metabolic functions, such as anti-oxidant pathways.

Keywords: ruminants; amino acids; anti-oxidants; nutrient partitioning; metabolomics; ideal protein concept

Citation: Inhuber, V.; Windisch, W.; Kleigrewe, K.; Meng, C.; Bächler, B.; Gigl, M.; Steinhoff-Wagner, J.; Ettle, T. Effect of Rumen-Protected Methionine on Metabolic Profile of Liver, Muscle and Blood Serum Samples of Growing German Simmental Bulls Fed Protein-Reduced Diets. *Metabolites* **2023**, *13*, 946. https://doi.org/10.3390/metabo13080946

Academic Editor: Baichuan Deng

Received: 17 July 2023
Revised: 8 August 2023
Accepted: 10 August 2023
Published: 15 August 2023

1. Introduction

Beef supply chains have gained significant attention in the global discussions on livestock production. This public opinion is mainly derived from the fact that beef cattle have lower feed-to-food transformation efficiency as compared to both poultry and swine [1], meanwhile excluding other specific attributes of beef cattle to explain their importance for a bio-circular economy [2,3]. However, in recent decades, nutritional strategies that have focused on increasing the efficiency of livestock have been well established, including the 'ideal protein concept' in monogastric nutrition. The 'ideal protein' denotes a dietary protein that does not under- or oversupply intestinal digestible amino acids [4] and thereby ensures an efficient feed-to-food protein turnover. This concept allows for a reduction in dietary crude protein due to the supplementation of single amino acids. The successful application of this concept, however, requires precise knowledge on the metabolic role and requirements of single performance-limiting amino acids [5].

This nutritional concept cannot be simply transferred to beef and dairy nutrition, since the fore stomach system synthesizes its own protein in the rumen (microbial protein). Thus, there is an incongruence between ingested and absorbed amino acid patterns, which impedes the determination of performance-limiting amino acids [6].

Nevertheless, numerous studies imply that lysine and methionine might be the first-limiting amino acids in growing ruminants [6], more specifically when microbial protein derived from the rumen is the major supplier of amino acids at the small intestine [7].

Methionine is a sulfurized amino acid, which enters the one-carbon metabolism in its active form S-adenosylmethionine. As such, it donates its methyl group to an acceptor, thereby producing S-adenosylhomocysteine, which can be then hydrolyzed to homocysteine. This trans-methylation process, which is restricted to the liver, kidney, intestine and pancreas, is the first step of numerous subsequent metabolic pathways. It is followed by the trans-sulfuration pathway, which converts homocysteine into cysteine. Cysteine, in turn, serves as the intermediate metabolite for glutathione synthesis, an important antioxidant, or is oxidized into cystine, for instance. Other metabolic fates of cysteine are, e.g., the synthesis of taurine. Taurine, in turn, has several metabolic functions, such as antioxidant activity [8–10].

Protecting dietary methionine from ruminal degradation increases its metabolic availability in ruminants [11]. This has recently been demonstrated in a study [6], where growing German Simmental bulls received either a control diet (CON) or a diet low in crude protein (RED) supplemented with rumen-protected methionine (RPMET; RED+RPMET; 0.16% of DM Smartamine M®, Adisseo, France). Reduction in dietary crude protein led to a decrease ($p < 0.05$) in growth performance over the whole experiment in both RED and RED+RPMET as compared to the CON group. The addition of RPMET did not relieve the RED+RPMET bulls from growth limitations, suggesting that under those feeding conditions, methionine was not firstly limiting for growth. However, the supplementation of RPMET led to an increase ($p < 0.01$) in serum methionine concentrations in RED+RPMET as compared to RED bulls (43.4 vs. 35.0 µmol/L) and hence, increased its metabolic availability [6]. Based on this background, the objective of this study was to evaluate the metabolic response of growing German Simmental bulls to diets low in crude protein with supplemented RPMET.

2. Materials and Methods

2.1. Feeding Experiment

The feeding experiment was conducted at the Bavarian State Research Center for Agriculture (LfL, Grub, Germany). All experimental procedures followed the guidelines of the German law under the German State and Directive 2010/63/EU of the Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Slaughter of the animals was conducted in accordance with the German law of animal protection of the German State and Council Regulation (EC) No. 1099/2009 of 24 September 2009 on the protection of animals at the time of killing.

2.1.1. Animals and Dietary Treatments

A feeding trial was conducted to harvest liver and muscle tissue and serum samples at the slaughter of the bulls.

In brief, a total of 69 growing fattening German Simmental bulls (on average 238 ± 11 days of age at beginning of the experiment; 367 ± 25 kg initial bodyweight) were allotted to a control group (CON) and two dietary treatment groups ($n = 23$/group). Bulls were weighed at the beginning and immediately before slaughter as well as in regular intervals during the experimental period. The CON ration provided adequate metabolizable energy (ME; 12.3 MJ/kg total mixed ration), crude protein (13.7%) and utilizable crude protein (15.7%), referring to the current national recommendations of German Simmental bull feeding [12]. Both treatment groups (RED and RED+RPMET) contained less crude protein (both 9.04%) and utilizable crude protein (both 14.9%), but were supplemented with rumen-protected lysine to reach CON diet levels. The diets RED and RED+RPMET only differed in their

concentration of digestible methionine (2.2 g/kg dry matter vs. 3.2 g/kg dry matter, respectively) due to supplementation of RPMET (0.16% of dry matter; Smartamine M®, Adisseo SAS, Antony, France). The feeding trial lasted for 105 days on average.

Details on the feeding experiment were previously described [6].

2.1.2. Slaughter and Sample Collection

At the end of the feeding experiment, bulls were slaughtered over a time period of eight d and bulls from all three treatments were slaughtered on each date. At slaughter, liver (3 cm^2 of the left lobe of the liver) and muscle (*Musculus masseter*) tissue samples of each bull were removed and directly frozen in a liquid nitrogen container to minimize metabolic degradation. Samples were stored in 1.5 mL cryotubes at −80 °C until assayed. Additionally, blood samples were collected in Vacuette tubes (VACUETTE TUBE 4 mL CAT Serum Clot Activator, Greiner Bio-One International GmbH, Kremsmünster, Austria) during exsanguination. Vacuette tubes were inverted and centrifuged (2000× *g* 10 min, at 4 °C) and the serum samples were transferred to 1.5 mL cryotubes and then stored at −80 °C until further analysis.

2.2. Sample Preparation and Metabolomics Analysis

Muscle, liver and serum samples were thawed at 4 °C. Subsequently, 10 mg of each tissue (muscle and liver) sample were transferred into 2 mL FastDNA Lysing Matrix A tubes containing lysing matrix A for soft animal tissues (MP Biomedicals, Solon, OH, USA). After adding 1 mL of an extraction solvent (70% methanol, 30% water), samples were homogenized using FastPrep-24TM Classic (MP Biomedicals, Irvine, CA, USA; 30 s, 5500 RPMET, 4 °C, 2 times) and incubated on ice (30 sec after each spin). Samples were then centrifuged (10,000× *g*, 5 min at 4 °C) and the clear supernatants were transferred to 1.5 mL glass autosampler vials. Blood serum samples (200 µL) were diluted in the same extraction solvent used for tissue samples (1 mL).

Untargeted metabolomics analyses were carried out at the Bavarian Center for Biomolecular Mass Spectrometry (BayBioMS, Technical University of Munich, Freising, Germany). The untargeted analysis was performed using a Nexera UHPLC system (Shimadzu), which was coupled to a Q-TOF mass spectrometer (TripleTOF 6600, AB Sciex, Toronto, Ont., Canada) using the information-dependent acquisition (IDA) mode. A UPLC BEH Amide 2.1 × 100, 1.7 µm analytic column (Waters Corp., Milford, MA, USA) with a 400 µL/min flow rate was used for separation of the biological samples. The mobile phase consisted of eluent A (5 mM ammonium acetate in water) and eluent B (5 mM ammonium acetate in acetonitrile/water (95/5, *v*/*v*)) and was performed with the following elution gradient: 100% B from 0 to 1.5 min, 60% B at 8 min and 20% B at 10 min to 11.5 min and 100% B at 12 to 15 min. A volume of 10 µL per sample was injected. The autosampler was cooled to 10 °C and the column oven was heated to 40 °C. A quality control (QC) sample was run after every ten samples (analyzed in randomized order). It consisted of a pool of all injected samples. MS settings in the positive mode were as follows: gas 1, 55; gas 2, 65; curtain gas, 35; temperature, 500 °C; ion spray voltage, 5500; declustering potential, 80. The mass range of the TOF MS and MS/MS scans were 50–2000 m/z. The collision energy was ramped from 15 to 55 V. MS settings in the negative mode were set as follows: gas 1, 55; gas 2, 65; cur, 35; temperature, 500 °C; ion spray voltage, −4500; declustering potential, −80. The mass range of the TOF MS and MS/MS scans was 50–2000 m/z. The collision energy was ramped from −15 to −55 V.

2.3. Statistical Data Analysis and Bioinformatics

MS raw data files were converted to mzXML via the "msconvert" from ProteoWizard [13]. Data processing and feature identification were conducted with the Bioconductor/R package XCMS [14]. In detail, peaks were identified with the matched filter algorithm (full width at half maximum set to 7.5 s). Grouping of the peaks was based on the "peak density" method [14]. Feature abundance was represented by the integrated area under the

peaks. Peak groups presented in most samples were used to adjust the retention time. The MS1 (exact mass of the precursor ion) and MS2 (fragmentation pattern of precursor ion) of the measured features were compared to the records in the Human Metabolome Database (HMDB) [15] and published MS2 spectra compiled by MSDIAL [16] to annotate possible metabolites for the identification of features (referring to MS1 and MS2, respectively). Additionally, in-house annotation database was taken into consideration for feature annotation. Potential batch effects were controlled and removed according to QC samples. Features were categorized into four categories depending on the quality of annotation: One means only the feature mass is matched with the metabolite candidate in the database, and no MS2 information is used. Zero means the MS2 spectra matching suggests the annotation is likely incorrect. Two means the partial match of MS2 spectra (at least one peak match). Four means high similarity between measured MS2 spectra and database MS2 spectra. The associated untargeted metabolomics data are made available on the MassIVE repository under the ID MassIVE MSV000092367: https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp, last accessed on 16 July 2023.

Univariate analysis was performed using SAS (SAS 9.4, SAS Institute, Cary, NC, USA). Treatment group means of significantly regulated metabolites, which were obtained from XCMS analyses, were tested via normal distribution (Shapiro–Wilk's test) and then analyzed using a general linear model (GLM) with treatment as fixed effect and a Student–Newman–Keuls (SNK) post-hoc test according to the SAS user's guide [17]. The p-value of the GLM represents the statistical significance of the GLM model, whereas the p-values of the linear contrasts I and II indicate differences in response variables due to reduction in dietary crude protein and addition of RPMET (CON vs. RED+RPMET) and due to the addition of RPMET (RED vs. RED+RPMET). The SEM represents the standard error of the mean over the whole GLM. Statistical significance was declared at $p \leq 0.05$.

3. Results

3.1. Zootechnical Results

Zootechnical results on the feeding experiment were previously described [6]. In brief, dietary treatments had a significant effect on performance parameters. Bulls from both the RED and RED+RPMET groups had a lower ($p < 0.05$) dry matter intake (8.49 kg/day and 8.27 kg/day, respectively) as compared to the standard group (9.43 kg/day in CON) over the whole duration of the experiment. This led to a reduction ($p < 0.01$) in digestible protein, metabolizable energy and nutrient intake in both RED and RED+RPMET groups as compared to the CON group. Consequently, growth rates were lower in RED and RED+RPMET bulls ($p < 0.01$) than in CON bulls. Considering the lower growth rates in both RED and RED+RPMET in a retrospective view, digestible protein supply in both RED and RED+RPMET met their requirements at 133 and 138%, respectively. Due to the specific addition of RPMET in RED+RPMET, these bulls had a higher ($p < 0.01$) intake of pre-cecal digestible methionine (26.2 g/day) as compared to RED bulls (18.8 g/day). Again, considering the lower growth rates in a retrospective view, methionine intake matched their daily requirements at 146% and 101%, respectively.

3.2. Relative Quantification and Identification of Compounds in Liver and Muscle Tissue and Blood Serum Samples

In total, 930 and 527 features were identified in positive (P) and negative (N) ionization modes, respectively, in liver tissue. A total of 1122 and 747 features were identified in P and N ionization modes, respectively, in muscle tissue, and 1835 and 1379 features were identified in blood serum samples, respectively (Table 1).

Figures 1–3 visualize liver, muscle and blood serum metabolites that were detected during metabolomics analysis. Violet areas in the volcano plots display significantly regulated metabolites. The significance level was set at 0.05 for the false discovery rate (FDR) of Benjamini–Hochberg (BH)-corrected p-value. Features depicted in 'green' show a match with MS2 spectra, whereas features in 'violet' lack a respective match. Each figure

shows four volcano plots. In all figures (Figures 1–3), volcano plots 1 show the comparison between CON and RED+RPMET and volcano plots 2 visualize the comparison between RED and RED+RPMET, in P (a) and N (b) detection (ionization) mode each.

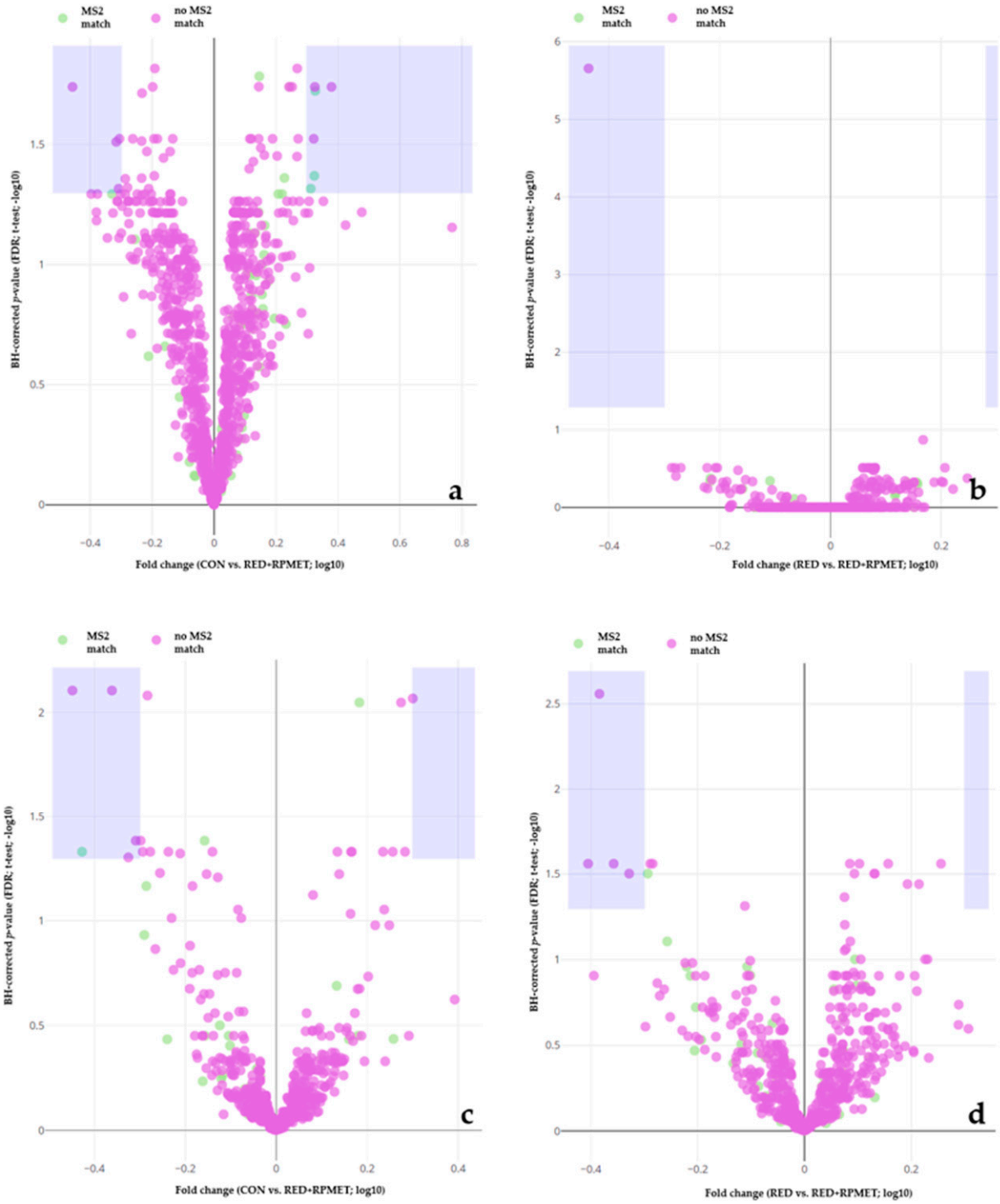

Figure 1. Volcano plots of liver metabolites. The comparison of CON vs. RED+RPMET is depicted in either the P detection mode (**a**) or the N detection mode (**c**) and the comparison of RED vs. RED+RPMET in either the P detection mode (**b**) or the N detection mode (**d**). Y-axis depicts Benjamini–Hochberg (BH)-adjusted *p*-value's false discovery rate (FDR). X-axis depicts the fold change between the treatment groups with CON = control diet according to requirements, RED = reduced in crude protein and RED+RPMET = reduced in crude protein + addition of RPMET (0.16% DM).

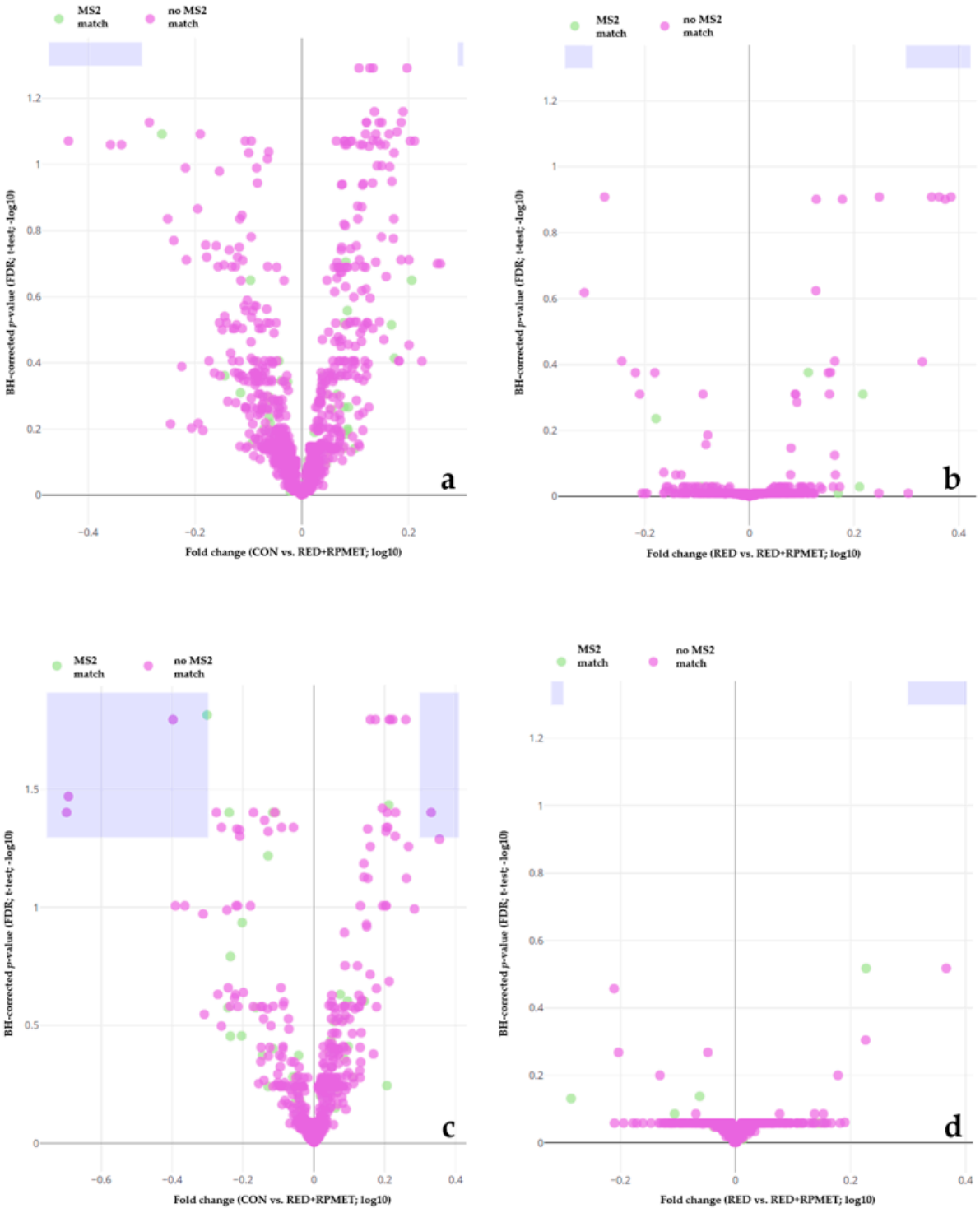

Figure 2. Volcano plots of muscle metabolites. The comparison of CON vs. RED+RPMET is depicted in either the P detection mode (**a**) or the N detection mode (**c**) and the comparison of RED vs. RED+RPMET in either the P detection mode (**b**) or the N detection mode (**d**). Y-axis depicts Benjamini–Hochberg (BH)-adjusted *p*-value's false discovery rate (FDR). X-axis depicts the fold change between the treatment groups with CON = control diet according to requirements, RED = reduced in crude protein and RED+RPMET = reduced in crude protein + addition of RPMET (0.16% DM).

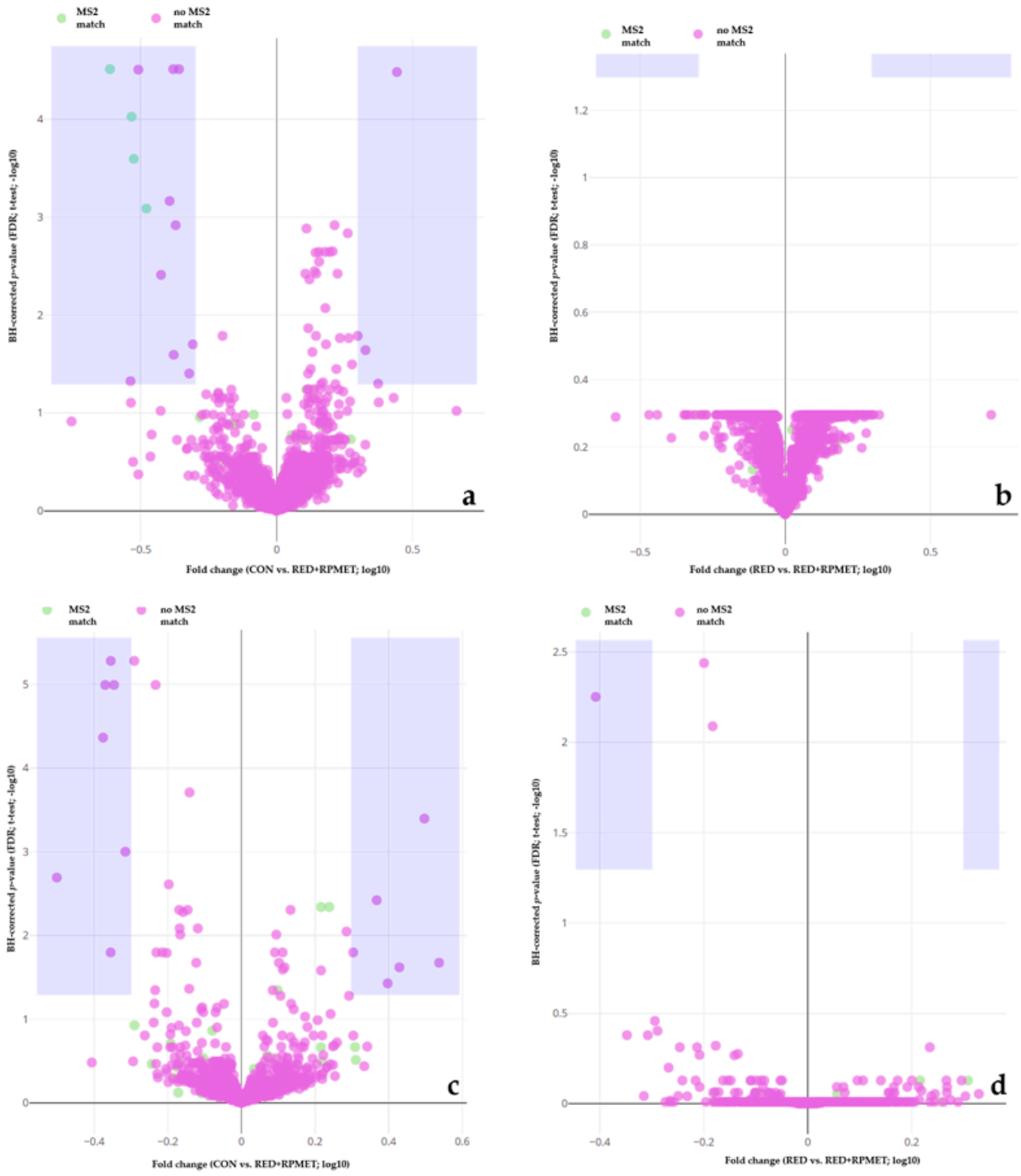

Figure 3. Volcano plots of blood serum metabolites. The comparison of CON vs. RED+RPMET is depicted in either the P detection mode (**a**) or the N detection mode (**c**) and the comparison of RED vs. RED+RPMET in either the P detection mode (**b**) or the N detection mode (**d**). Y-axis depicts Benjamini–Hochberg (BH)-adjusted *p*-value's false discovery rate (FDR). X-axis depicts the fold change between the treatment groups with CON = control diet according to requirements, RED = reduced in crude protein and RED+RPMET = reduced in crude protein + addition of RPMET (0.16% DM).

Overall, there were 20 significantly regulated metabolites in liver tissue, from which 15 were significantly different between CON and RED+RPMET (5 detected in N mode and 10 detected in P mode) and five between RED and RED+RPMET (four detected in N mode and one detected in P mode), respectively (Figure 1).

In muscle tissue, five metabolites were found to be statistically different between CON and RED+RPMET (all detected in N mode) (Figure 2).

In total, 30 serum metabolites were significantly regulated, 29 between CON and RED+RPMET (13 detected in N mode and 16 detected in P mode) and one between RED and RED+RPMET (detected in N mode).

Table 1. Number of features in liver and muscle tissue and blood serum samples of German Simmental bulls for fattening in positive (P) and negative (N) detection modes.

Sample Type	Detection Mode	Number of Features
Liver	P	930
	N	527
Muscle	P	1122
	N	747
Blood serum	P	1835
	N	1379
Total		6540

3.3. Univariate Analysis of Annotated Metabolites

Table 2 shows the results of the univariate analysis of significantly regulated metabolites that could be annotated reliably. This means that the MS2 spectra of these metabolites showed a high similarity with the MS2 spectra of the databases used for analysis (compare Section 2.3). Reduction in dietary crude protein (CON vs. RED) led to a decrease ($p < 0.01$) in hepatic carnosine (5.09 vs. 4.79), cystine (4.65 vs. 4.18) and taurocholic acid (4.69 vs. 4.31) and blood serum pyrrolidonecarboxylic acid (4.61 in CON vs. 3.97 in RED) concentrations. Additionally, the MS1 and MS2 spectra indicated the presence of L-leucine/L-isoleucine/norleucine with ($p < 0.01$) between CON and RED (4.37 vs. 4.13). Based on the retention time and the fragmentation pattern, we cannot differentiate between these amino acids and therefore combine them. The addition of RPMET (RED+RPMET) did not affect metabolite concentrations as compared to RED, with the exception of taurocholic acid (4.53 vs. 4.31, *p* Lin. Contrast II = 0.01) and cysteine glutathione disulfide (4.96 in RED+RPMET vs. 4.63 in RED; *p* Lin. Contrast II < 0.01).

Table 2. Concentration of annotated metabolites in liver and muscle tissue and blood serum samples of growing German Simmental bulls.

						Dietary Treatment				*p*-Value		
						Mean				GLM [4]	Linear Contrast I [5]	Linear Contrast II [6]
Tissue	Mode [1]	Annotation	Prec m/z [2]	Retention Time	Quality [3]	CON	RED	RED+ RPMET	SEM	CON vs. RED vs. RED+ RPMET	CON vs. RED+ RPMET	RED vs. RED+ RPMET
Liver	P	Carnosine	227.11	462.55	4	5.09 [a]	4.79 [b]	4.85 [b]	0.11	<0.01	<0.01	0.31
Liver	P	Cystine	241.03	492	4	4.65 [a]	4.18 [b]	4.32 [b]	0.20	<0.01	<0.01	0.21
Liver	P	Taurocholic acid	516.3	291.72	4	4.69 [a]	4.31 [b]	4.53 [a]	0.15	<0.01	<0.01	0.01
Liver	P	L-leucine/L-isoleucine/ norleucine	132.1	318.27	4	4.37 [a]	4.13 [b]	4.05 [b]	0.15	<0.01	<0.01	0.32
Liver	N	Cysteine glutathione disulfide	425.08	512.25	4	4.79 [b]	4.63 [c]	4.96 [a]	0.15	<0.01	0.99	<0.01
Blood serum	N	Pyrrolidone-carboxylic acid	128.04	119.04	4	4.61 [a]	3.97 [b]	4.11 [b]	0.19	<0.01	<0.01	0.14

[1] Mode describes detection mode with P = positive mode and N = negative mode. [2] m/z of precursor. [3] Quality denotes the quality of annotation as described in Section 2.3. [4] GLM is the general linear model with a Student–Newman–Keuls' post-hoc test to detect group mean differences. [5] *p*-Values of the linear contrast analysis of CON vs. RED+RPMET. [6] *p*-Values of the linear contrast analysis of RED vs. RED+RPMET. [a,b,c] Values within a row with different superscripts were statistically different in the SNK. Statistical significance was declared at $p < 0.05$.

4. Discussion

The transformation efficiency of human non-edible biomass into protein food derived from ruminants is relatively low compared to other species such as poultry and swine [18]. However, ruminants are exclusively able to utilize the feed and by-products of either very small or no value for human food as well as non-protein nitrogen to produce high-quality human food, such as milk and beef. Both swine and poultry are not able to digest such raw materials, but in general, they are more efficient in utilizing dietary nutrients. Moreover, their diets can be balanced more precisely. In terms of protein, the 'ideal protein concept' allows for the reduction in dietary protein concentration, because the supplementation of individual limiting amino acids then secures both an adequate amount and pattern of amino acids supplied to the intestinal tract [4]. Knowledge on limiting amino acids in ruminants, and especially in beef cattle nutrition under practical feeding conditions, is still scarce. This is mainly due to the difficulty to quantify the amount and pattern of amino acids reaching the small intestine for absorption. Rumen-protected amino acids present an opportunity to overcome the challenge of ruminal degradation. However, to identify a limitation of a certain amino acid, the supplementation of itself to a diet deficient in this amino acid should relieve the animal from the deficiency. Therefore, this results in an increase in growth performance [19]. Numerous studies imply that methionine and lysine may be the most promising amino acids for growth limitation [6]. Therefore, we conducted a feeding trial (published in [6]) to evaluate the role of methionine as a first-limiting amino acid in diets low in crude protein for growing German Simmental bulls. Methionine, however, is not only known as a protein building block via peptide bonds, but also as a functional amino acid that acts as a precursor to numerous metabolites. As such, it plays a pivotal role in epigenetics via DNA methylation [20] and forms a key part in the one-carbon metabolism in its biologically active form of S-adenosyl-methionine. Via the trans-sulfuration pathway and the intermediate metabolite cysteine, it is involved in the synthesis of the antioxidants glutathione and taurine [21].

Hence, the objective of this study was to evaluate the effect of RPMET in reduced crude protein diets on metabolic pathways in growing German Simmental bulls under practical feeding conditions.

As to our knowledge, this is the first study evaluating the metabolic response of German Simmental beef cattle that were fed diets reduced in crude protein supplemented with RPMET. Until 2023, there were 153 publications on metabolomics approaches in research with bovine species available [22]. Recently published studies predominantly aimed to identify biomarkers for desirable economic traits, such as feed efficiency [23–30], growth potential [31] and dairy production [32–34]. Metabolomics studies on the effect of supplementing RPMET to dairy/beef diets are limited. Among those few studies, a substantial part focuses on the metabolic programming effect of feeding RPMET to the parental generation. Palombo et al. [35], for example, evaluated the effect of feeding RPMET to late-gestation Holstein dairy cows on metabolic changes in neonatal calves. They found that due to maternal methionine supply neonatal calves experienced beneficial effects on their antioxidant status. The supplementation of RPMET to peripartal dairy cows also led to an improved antioxidant status [21,36,37]. Alfaro et al. [38] conducted a nutrigenetics study in beef cattle. They evaluated the effect of RPMET supplementation on the preconditioning of beef heifers experiencing long-duration transportation stress. They concluded that heifers receiving RPMET had a better-controlled oxidant–antioxidant balance in skeletal muscle.

Metabolomics is an 'omics' tool to quantify a global set of metabolites within biological samples. Hence, this analysis tool delivers the entirety of metabolic downstream products derived from genomic, transcriptomic and proteomic processes [39]. These datasets reflect the physiological status of cells, and hence enable us to elucidate changes in a biological system induced by different factors, such as environment, diseases and nutrition [22,40,41]. Metabolomics data in combination with phenotype results therefore allow for a more

precise understanding of the physiology of the animals [42]. Therefore, we discuss our metabolomics results in context with the performance results of our study [6].

In our previous study, bulls were allotted to three different treatment groups: a standard group (CON), which was designed to meet the requirements for German Simmental bulls for fattening at this stage of growth [12] and a diet reduced in crude protein (RED) with supplemental RPMET (RED+RPMET). The latter ones only differed in their digestible methionine concentration (2.2 vs. 3.2 g/kg DM, respectively). As published earlier [6], reduction in dietary crude protein (RED) led to a significant decrease in dry matter intake and hence, metabolizable energy and pre-cecal digestible protein intake as compared to the CON group. The addition of RPMET did not recover feed intake and, therefore, feed and nutrient intake were comparable with the non-supplemented RED group. Consequently, both the bulls from RED and RED+RPMET groups had a lower ($p < 0.05$) growth performance than CON bulls. Metabolomics analyses on a cellular level in muscle, blood and liver tissue revealed that there was hardly any difference in metabolite abundance between CON and both RED and RED+RPMET bulls. This exemplifies the hierarchical concept of nutrient partitioning. The animals set their well-being and maintenance as a prevailing priority of nutrient trafficking (homeostasis) at the expense of (growth) performance (homeorhesis). The lower feed intake in both RED and RED+RPMET led to a lower nutrient availability for metabolism, and hence, bulls grew only to that extent which could be realized with the difference between the total intake of nutrients and energy, and the requirements for maintenance. This concept is controlled at a higher endocrine organ level [43,44] and therefore may have not been visible on a single-cell level as presented via metabolome analysis.

Considering the actually realized growth rates of both RED and RED+RPMET, the intake of pre-cecal digestible methionine met 101% of RED and 146% of RED+RPMET requirements, respectively. Both RED and RED+RPMET had equal ($p > 0.05$) dry matter and hence, energy and protein intake, which were, from a retrospective point of view, sufficient for their actually realized growth rates. Therefore, we could exclude both energy and protein intake as limiting factors for growth response in the RED+RPMET group. If methionine had been the first-limiting amino acid for growth, dietary protein quality (i.e., amino acid pattern) would have been improved. This means that more amino acids would have been utilized for protein deposition and this would have then resulted in a decrease in fat deposition in RED+RPMET compared to RED. Respective results can be found in [6].

However, additional methionine in the RED+RPMET group did not relieve bulls from growth limitation, but altered hepatic anti-oxidant pathways. This may imply that bulls of the RED group used metabolically available methionine for growth as the highest priority, supporting our assumption of nutrient partitioning and the decoupling of homeostasis and homeorhesis.

Additional methionine in the RED+RPMET group was directed toward anti-oxidant pathways, represented by an increase ($p < 0.01$) in cysteine glutathione disulfide and taurocholic acid, the conjugate of cholic acid and taurine, an important cellular antioxidant defender. The supplementation of RPMET (RED+RPMET) increased ($p = 0.01$) hepatic taurocholic acid concentrations as compared to the non-supplemented group (RED; 4.53 vs. 4.31, respectively) and reached a comparable level to the CON group (4.69). Taurocholic acid, a bile acid, is the conjugate of cholic acid and taurine. Taurine synthesis needs methionine [45] via cysteine (trans-sulfuration pathway) in a three-step enzymatic process. The main biological effects of taurine comprise antioxidant activity by inhibiting mitochondrial reactive oxygen species generation, glucose homeostasis by interfering the insulin-signaling pathway and osmoregulation due to counteracting hyperglycemia-induced osmotic imbalance.

Hepatic cystine was lower ($p < 0.01$) in both CP-deficient groups (RED and RED+RPMET) as compared to the standard diet (CON). Interestingly, the addition of RPMET (RED+RPMET) failed to increase hepatic cystine concentrations. This would have been reasonable, since cystine is the oxidized form of cysteine, which is directly synthesized from methionine in

the trans-sulfuration pathway [8]. It may be that first, other metabolic pathways had higher demands for methionine/methyl groups, or second, that an increase in cystine could not be displayed in that 'snapshot' of the metabolism, since it had already been used for other syntheses (e.g., cystine–glutamate antiporter), or third, that it was reduced back to cysteine and as such, used for the synthesis of subsequent metabolites. Glutathione synthesis, for instance, is restricted by the availability of cysteine. Especially in hepatocytes, the trans-sulfuration pathway provides half of the cysteine required for glutathione synthesis, even if cysteine is present in physiological concentrations [46]. Interestingly, the addition of RPMET increased ($p < 0.01$) the abundance of hepatic cysteine glutathione disulfide (4.96 in RED+RPMET vs. 4.63 in RED) and even exceeded the abundance in the standard group (4.79 in CON).

In conclusion, our results show that growing German Simmental bulls face a dietary protein reduction with nutrient partitioning by setting maintenance and physiological equilibrium as the first priority in nutrient trafficking to ensure that all organs and tissues are interacting correctly. Additional methionine, which was not first limiting for growth under our feeding conditions [6], was directed toward the hepatic synthesis of important anti-oxidant metabolites, such as cysteine glutathione disulfide and taurocholic acid.

Since our study was the first to elucidate the comprehensive metabolic role of methionine under such feeding conditions, further studies are required to sharpen the role of methionine in growing beef cattle. Further research should evaluate the 'metabotype', i.e., the combination of phenotype and metabolome [42], which offers a promising strategy to determine the comprehensive role of amino acids in the metabolism of growing beef cattle.

Knowledge on amino acid partitioning is particularly important to approach the 'ideal protein concept' for growing beef cattle. This is pivotal to increase the production efficiency of cattle genetic resources, thereby to drive further bio-economic circularity [2] and improve public health and environmental resilience [3].

Author Contributions: Conceptualization, T.E., W.W. and V.I.; methodology, V.I.; software, C.M., B.B., M.G., V.I. and K.K.; validation, C.M., K.K., B.B., M.G. and V.I.; formal analysis, V.I.; investigation, V.I.; resources, W.W., J.S.-W. and T.E.; data curation, V.I., C.M., K.K., M.G. and B.B.; writing—original draft preparation, V.I.; writing—review and editing, T.E., W.W., K.K., C.M. and J.S.-W.; visualization, V.I.; supervision, W.W.; project administration, T.E.; funding acquisition, T.E. All authors have read and agreed to the published version of the manuscript.

Funding: This study received specific funding from the Bavarian State Ministry for Food, Agriculture and Forestry. The research project 'Amino Acids in Ruminants' is registered by the number A/17/18.

Institutional Review Board Statement: The feeding experiment was conducted at the Bavarian State Research Center for Agriculture (LfL, Grub, Germany). All experimental procedures followed the guidelines of the German law under the German State and Directive 2010/63/EU of the Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Slaughter of the animals was conducted in accordance with the German law of animal protection of the German State and Council Regulation (EC) No. 1099/2009 of 24 September 2009 on the protection of animals at the time of killing.

Informed Consent Statement: Not applicable.

Data Availability Statement: We confirm that all data supporting our conclusions are available in the article and accessible in MassIVE MSV000092367.

Acknowledgments: We gratefully acknowledge the support of stable personnel of the Bavarian State Research Center (Grub, Germany) for daily animal care and slaughterhouse personnel of the Bavarian State Research Center (Grub, Germany) for sample taking and data collection during slaughter process.

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

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