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Determination of the protein value of feeds for ruminants

DISSERTATION

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“[...] a nutritionist conversant with the principles of protein metabolism in ruminants can if necessary turn his hand to non-ruminants. The reverse is a much more painful adaption!”¹

¹Ørskov, E.R., 1992. Protein Nutrition in Ruminants, 2nd ed. Academic Press Harcourt Brace Jovanovich, London, 175 pp.

Meinen Eltern

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List of abbreviations

with the exception for units defined by the international system of units (SI) and the international union of pure and applied chemistry (IUPAC).

0 h	Washout fraction at time point 0 h (<i>in situ</i>)
<i>a</i>	Soluble fraction (<i>in situ</i>)
<i>a+b</i>	Potential degradation (<i>in situ</i>)
AA	Amino acids
ADF _{om}	Acid detergent fibre expressed exclusive of residual ash
ADICP	Acid detergent insoluble crude protein
ADL	Acid detergent lignin
A _{max}	Maximum extent of bacterial colonization
aNDFom	Neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash
<i>b</i>	Insoluble, potentially degradable fraction (<i>in situ</i>)
<i>c</i>	Degradation rate (<i>in situ</i>)
CA	Crude ash
CDI	<i>In situ</i> disappearance corrected for small particle losses
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude protein (Nitrogen x 6.25)
DDGS	Dried distillers' grains with solubles
deg	Cumulative degradation at time <i>t</i>
DI	<i>In situ</i> disappearance
DM	Dry matter
DOM	Digestible organic matter
ED	Effective degradation <i>in situ</i>
ED _k CP	<i>In situ</i> effective ruminal degradability of crude protein at ruminal passage rate <i>k</i>
ED _k OM	<i>In situ</i> effective ruminal degradability of organic matter at ruminal passage rate <i>k</i>
EE	Ether extract
eHGT	Extended Hohenheim gas test
FOM	Fermentable organic matter
FOM _k	Fermented organic matter at passage rate <i>k</i>
FP	Fermentation products in silage
GP	Gas production
HGT	Hohenheim gas test
<i>k</i>	Ruminal passage rate (/h)
<i>L</i>	Lag phase (<i>in situ</i>)
LAB	Lactic acid bacteria
MA	Microbial attachment at time <i>t</i>
MCP	Microbial crude protein synthesized in the rumen
MCP _k	Effective microbial crude protein synthesized in the rumen at passage rate <i>k</i>
ME	Metabolisable energy

modHGT	Modified Hohenheim gas test
MP	Metabolisable protein
N	Nitrogen
NDFom	Neutral detergent fibre assayed without a heat stable amylase and expressed exclusive of residual ash
NDICP	Neutral detergent insoluble crude protein
NH ₃	Ammonia
NH ₃ -N	Ammonia-nitrogen
non-NH ₃ -N	Non-ammonia nitrogen
NPN	Non-protein-nitrogen
OM	Organic matter
PD	Purine derivate
prCP	Post-rumen crude protein
prCP _k <i>in situ</i>	Effective post-rumen crude protein at passage rate <i>k</i> determined <i>in situ</i>
prCP _k <i>in vitro</i>	Effective post-rumen crude protein at passage rate <i>k</i> determined <i>in vitro</i>
R	Rate of microbial colonization (/h)
R ²	Coefficient of determination
R ² _c	Conditional coefficient of determination
R ² _m	Marginal coefficient of determination
RMSE	Root mean square error
RNB	Ruminal nitrogen balance
RUCP	Rumen undegraded crude protein
RUCP _k	Effective rumen undegraded crude protein at passage rate <i>k</i>
RUSITEC	Rumen simulation technique
SCP	Soluble crude protein
SD	Standard deviation
SE	Standard error
SP	Small particles escaping from the polyester bags (<i>in situ</i>)
<i>t</i>	Time
uCP	Utilisable crude protein at the duodenum
VFA	Volatile fatty acids
WS	Water-soluble fraction (<i>in situ</i>)
WSC	Water-soluble carbohydrates

CHAPTER 1

Introduction

The impact of human activity on the environment has always been a central part of scientific research. Environmental protection has gained social and political importance in recent decades due to anthropogenic influences on climate, on water sources and on soils. Agriculture, especially animal husbandry, is often criticised for high nitrogen (N) emissions (i.e., ammonia, nitrate, and nitrous oxide) to the environment. Excess N which is not retained in animal tissues or animal products (e.g., milk) is excreted in urine and faeces contributing to environmental pollution (Hristov *et al.*, 2019; Hristov *et al.*, 2011). In dairy cattle production, increasing N use efficiency (i.e., proportion of milk N yield of total N intake) has become of growing interest for some time, in order to reduce N excretion and associated negative ecological impacts, and to reduce feeding costs. In addition to the knowledge of cows' nutrient requirement, a precise determination of feed protein value is a key prerequisite to maintain performance-adapted and nutrient-efficient feeding. In contrast to monogastric livestock, the ruminant digestive system is characterised by pregastric fermentation through symbiotic microorganisms (van Soest, 1994). This means, that feed consumed by the animal is first subjected to microbial degradation in the forestomachs. The symbiosis enables the host animal to utilize structural carbohydrates (cellulose, hemicelluloses, pectins) as an energy source, which could otherwise not be utilised. The end products of carbohydrate degradation are primarily volatile fatty acids (VFA; acetate, propionate, butyrate), which are absorbed via the rumen wall and are thus available to the animal's metabolism. Moreover, there is a significant protein or N turnover in the forestomachs, which can be divided into two distinct events occurring simultaneously: crude protein (CP; N × 6.25) degradation and microbial crude protein (MCP) synthesis (Bach *et al.*, 2005). Degradable CP is largely hydrolysed to amino acids (AA) and almost completely deaminated by proteolytic activity of microorganisms, mainly resulting in ammonia (NH₃) (Simon, 2020). Rumen-undegradable CP (RUCP) escapes fermentation and enters the duodenum undegraded. Microbial protein synthesis greatly depends on rumen available energy, which is mainly derived from fermentation of structural and non-structural carbohydrates, yielding VFA, and the process in turn produce microbial cells by incorporating AA and non-protein N (NPN) compounds (Ørskov, 1992). Ruminal fermentation occurs under continuous-culture conditions,

whereby continual microbial growth is maintained by ongoing nutrient supply and removal of fermentation end products and microbial biomass (Edwards *et al.*, 2008).

Fermentation in the forestomachs poses a challenge in feed evaluation, because concentration and composition of feed CP provide little information about the protein supply to the animal (Steingäß and Südekum, 2013). Enzymatic digestion of protein and absorption of AA by the animal take place in the small intestine, consequently, feed protein value is evaluated at an intestinal level. For this purpose, protein evaluation systems are used, which were normally based on either empirical or mechanistic models. In Germany, the determination of protein value is currently based on empirical equations derived from *in vivo* measurements on duodenal fistulated cows, including the variables metabolisable energy (**ME**) or digestible organic matter (**DOM**), CP and RUCP (GfE, 2001; Lebzien *et al.*, 1996). These equations account for the dependence of MCP yield on the supply of energy to the microbes, but it is not possible to consider other influencing factors. To account for additional influencing factors such as type of substrate fermented, ruminal microbiota or (fractional) degradation and passage rate of feed particles, mechanistic models are needed. In contrast to models based on experimental data, mechanistic models are much more complex, but might lead to a better estimation of the protein value (Dijkstra *et al.*, 2005; Dijkstra *et al.*, 1998). However, the high number of factors included therein might limit their routine use in feed evaluation (Krieg, 2017). Even if nomenclature, exact definitions, methods, and assumptions differ between protein evaluation systems, most of them are based on an estimation of RUCP and MCP supply. Herein, RUCP contents are commonly derived from *in situ* measurements, whereas MCP is predicted assuming a fixed (or variable) yield of MCP synthesised per unit of, for instance, fermentable organic matter (**FOM**), DOM or ME (GfE, 2001; Lebzien *et al.*, 1996), depending on the evaluation system.

The *in situ* technique is an internationally accepted method to determine ruminal CP degradation of feeds. It is based on an incubation of feed samples placed in polyester bags and incubated in the rumen of cannulated animals for various lengths of time. Despite its wide application, however, this method cannot be considered as an absolute reference, because there are some methodological limitations of varying importance depending on the feed material examined (Lebzien and Flachowsky, 2005; Michalet-Doreau and Ould-Bah, 1992; Ørskov *et al.*, 1980). Ruminant feeds vary widely in terms of their ruminal CP degradation (Universität Hohenheim - Dokumentationsstelle, 1997) and can roughly be divided into feeds with high (75-95%), medium (65-85%) and low (55-75%) degradability (GfE, 1986). Easily

degradable are e.g., CP from fresh or ensiled grass, whereas CP e.g., from soybean or rapeseed meal have low intraruminal degradability. Besides digestible CP, RUCP also contains some completely indigestible parts (Huhtanen, 2005). In the past, extensive research focussed on enhancing RUCP in feeds aiming on higher protein supply to the animal and subsequently increased performance. Physical or chemical feed treatments have been successfully applied to enhance RUCP content (van der Poel *et al.*, 2005). However, enhancing RUCP concentration in feed is not generally reflected in a milk production response as observed in meta-analyses (Ipharraguerre and Clark, 2005; Santos *et al.*, 1998). This can have several causes. For example, there is evidence that treated feeds could have reduced digestibility especially through heat damage (van Soest, 1994) and that RUCP supplements can have a negative impact on MCP synthesis (Ipharraguerre and Clark, 2005; Santos *et al.*, 1998). Presumably, in the past, RUCP was given a disproportionately high importance in the protein supply of the animals (Huhtanen and Hristov, 2009; Ipharraguerre and Clark, 2005; Santos *et al.*, 1998). Nevertheless, because of limited feed or energy intake capacity and the resulting limited MCP yield, RUCP supply to the animals, as well as its AA composition becomes more important with increasing milk yield (Flachowsky and Lebzien, 2006).

The MCP yield is derived from variables that reflect the amount of FOM. Besides the quantitative supply of FOM, microbial growth is influenced by a variety of factors such as FOM composition (van Duinkerken *et al.*, 2011) and rumen available N sources (Dijkstra *et al.*, 2013). Moreover, synchronous energy and N release in the rumen is important, as the potential of energy-dependent MCP synthesis is limited if there is a lack of N in the rumen (Huhtanen, 2005). In the case of N surplus relative to the microbial need, excess N is absorbed as NH_3 via the rumen wall, converted to urea in the liver and either excreted in urine or recycled (rumino-hepatic cycle). Recycling means that urea is returned into the rumen via saliva or diffusion through the rumen wall to be reused for the MCP synthesis (Simon, 2020). The ruminal N balance (**RNB**) characterizes the feed according to the ratio between the amount of N and energy available in the rumen. A positive RNB indicates, that there is more N available than can be used by the microorganisms. A negative RNB, on the other hand, describes an N deficiency for the microbes. Negative RNB can be balanced to some extent via the rumino-hepatic cycle (Hristov *et al.*, 2019), but strongly negative values can decrease dry matter intake, MCP synthesis, and milk yield as reviewed by Flachowsky and Lebzien (2006).

Outflow of MCP from the rumen significantly contributes to the protein supply to the cows by having a relatively constant AA ratio with high intestinal digestibility (Ørskov, 1992). In addition, MCP contains all essential AA in sufficient amounts, which means that the animals' AA requirements can also be met exclusively from NPN compounds (Ørskov, 1992; Steingäß and Südekum, 2013). However, only one fourth to one third of feed-N is transferred into milk i.e., N use efficiency averaged at 25-28% in lactating dairy cows as reported in a meta-analysis of large datasets of North American (736 diets) and North European (998 diets) milk production trials (Huhtanen and Hristov, 2009). This (seemingly) low N efficiency is caused, among other factors, by MCP contribution to the protein supply to the cows, because non-NH₃-N content in duodenal chyme contain about 27% non-AA-N (GfE, 2001), whereof a large part are nucleic acids from microbial origin. However, this should in no way diminish the benefits gained through fermentation.

Degradation of CP and RUCP content are analytically sufficiently represented by chemical methods or enzymatic approaches (Licitra *et al.*, 1998; Sniffen *et al.*, 1992) and their use is superior to the *in situ* technique in terms of effort, costs, and reproducibility. However, MCP yield can only be reliably represented by biological systems. The modified Hohenheim gas test (**modHGT**) offers a simple method for the combined determination of RUCP and MCP. This method is based on an incubation of feed samples in rumen-fluid buffer solution. Thus, possible interactions between CP degradation and MCP yield and other factors influencing MCP growth can be considered.

The sum of RUCP and MCP is referred to as utilisable CP at the duodenum (**uCP**) and used to describe the protein value of feeds and the requirement of dairy cows in the German protein evaluation system (GfE, 2001). But, as noted by Westreicher-Kristen *et al.* (2015) the term “utilisable” may lead to misunderstandings, as it describes the quantity of CP found at the duodenum, rather than the available quantity of CP to the metabolism. For this reason, the term post-rumen CP (**prCP**) is used instead of uCP within the present thesis. Post-rumen CP can be converted to the more common international value, metabolisable protein (**MP**), which is the total amount of digestible true protein (AA) available to the animal for metabolism (AFRC, 1993; NRC, 2001). The actual supply of AA to the animal depends on a variety of factors such as AA composition and AA absorption from prCP in the small intestine as well as utilization of absorbed AA for protein synthesis (Flachowsky and Lebzien, 2006). An assessment of these variables could not be made within the scope of this work.

Objectives of the thesis

An adequate protein supply to dairy cows is of central importance for ecological, economic, and animal welfare reasons. However, a direct measurement of the protein value of feeds or feed mixtures through routine application of *in vivo* investigations is not conceivable. Rather, protein value has to be estimated using protein evaluation systems based on empirical or mechanistic models. However, empirical equations are derived from experimental data, thus their applicability is restricted to the conditions during data collection (Dijkstra *et al.*, 2005). Mechanistic models are applicable to a broader range of data, but they are much more complex through consideration of a high number of factors which might limit their routine use in feed evaluation (Dijkstra *et al.*, 2005; Krieg, 2017). Furthermore, the basic principle of most evaluation systems are estimates of RUCP and MCP, but both estimations are subjected to various assumptions and different limitations. A reliable method suitable for routine testing is therefore imperative.

The overall aim of this work was to show the potential of the modHGT for the assessment of protein value of feeds for ruminants. For this purpose, two studies were conducted, within both of each, *in vitro* determined prCP values were compared to those obtained *in situ*. *In situ* values were calculated as the sum of RUCP and MCP, where a constant value of 181 g MCP/ kg FOM was used to estimate MCP yield (Lebzien and Voigt, 1999). In turn, FOM was derived from effective OM degradation determined *in situ*.

Study 1 (Chapter 2): *Effect of wilting and lactic acid bacteria inoculant on in situ and in vitro determined protein value of grass silages.*

In addition to the method comparison, the aim of the first study was also to investigate the effect of pre-ensiling treatments on RUCP and prCP content in grass silages. Therefore, grass silages were wilted to 8 different DM concentrations and ensiled with or without the use of lactic acid bacteria inoculum.

Study 2 (Chapter 3): *In situ and in vitro determination of the protein value of feeds for ruminants.*

In the second study, 9 feeds, 13 feed mixtures made thereof, and 12 feed mixtures provided by commercial dairy farms were examined, covering a broad range of feed components and chemical ingredients.

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CHAPTER 2

Effect of wilting and lactic acid bacteria inoculant on *in situ* and *in vitro* determined protein value of grass silages.¹

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¹ Besides minor formal changes, this version is identical to the publication.

2.1 Abstract

The objective of this study was to evaluate the effect of dry matter (**DM**) concentration and the use of lactic acid bacteria (**LAB**) inoculant on the protein value of grass silages. Grass samples containing 170 g DM/kg fresh material were wilted to different DM concentrations (310, 390, 420, 470, 530, 580, 600 g/kg fresh material) before ensiling. Half of the grass material at respective DM was treated with LAB, the other half remained untreated (control, **CON**). Ruminal degradation of organic matter (**OM**) and crude protein (**CP**) was determined using the *in situ* technique. For this, silage samples were incubated for 2, 4, 8, 16, 24, 48 and 72 h. Rumen undegraded CP (**RUCP**) was derived from *in situ* CP degradation. Microbial CP (**MCP**) synthesis was assumed to be 181 g per kg fermented OM. Post-ruminal CP (**prCP**) was defined as the sum of RUCP and MCP. Effective prCP *in situ* was calculated for passage rates (k) of 0.02, 0.04 and 0.06/h. In addition, the modified Hohenheim gas test (**modHGT**) was used to determine effective prCP *in vitro* for the same passage rates. The DM linearly increased effective prCP *in situ* in CON ($P<0.001$) and LAB ($P<0.001$). This was caused by both increased RUCP in the range of 580–600 g DM ($P<0.001$) and increased MCP ($P<0.001$) due to higher amount of water-soluble carbohydrates in the more intensively wilted silages. The use of LAB increased RUCP ($P<0.001$) but decreased the calculated MCP content ($P<0.001$). Therefore, these effects compensated each other leading to only slightly lower effective prCP *in situ* for LAB ($P<0.05$). The positive effect of DM on effective prCP *in situ* was confirmed *in vitro* ($P<0.001$) showing no effect of the inoculant. Based on both methods effective prCP increased to a small extent with 2.3 to 4.9 g/kg OM ($P<0.001$), when DM increases by 100 g/kg fresh material, depending on the assumed passage rate. Therefore, wilting and LAB addition to grass have the potential to improve protein value of grass silage. Both methods delivered similar estimates for effective prCP. But modHGT appeared to be superior to the *in situ* technique by being more sensitive, not requiring separate estimation of MCP and the assumption of a certain efficiency value, and by having lower operating expenses. The results of this study show the potential of the modHGT, however further studies are required to standardize and validate this technique and to evaluate the effect of wilting grass on animal performance.

Keywords: Forage conservation; Utilizable crude protein; Silage additive; Hohenheim gas test

2.2 Introduction

A precise determination of the protein value of feeds is crucial to meet the protein requirements in high yielding dairy cows, where both protein excess and protein deficit should be avoided for economic, environmental, and animal welfare reasons. The decisive factor here is the amount of post-rumen crude protein (**prCP**), which is referred to as utilisable crude protein at the duodenum (**uCP**, **nXP** in German) in the German protein evaluation system (GfE, 2001). It corresponds to the sum of microbial crude protein (**MCP**) synthesized in the rumen and rumen undegraded crude protein (**RUCP**), where MCP is quantitatively more important (Huhtanen, 2019; Tamminga, 1982). Due to the high proportion of grass silage in many diets, even a small enhancement of prCP in forages provides a significant contribution to meet the protein demand in ruminants. About 75% of crude protein (**CP**) in forage is degradable in the rumen (Merchen and Bourquin, 1994), of which 40–60% is present as non-protein nitrogen (**NPN**) in grass silages (Edmunds *et al.*, 2012b). Because MCP synthesis is predominantly energy-dependent, a great proportion of ammonia is absorbed in the rumen and excreted via urine, if – as the case in many forages – rapidly fermentable carbohydrates are not sufficiently available (Flachowsky and Lebzién, 2006; Tamminga, 1982). Therefore, N utilization in forages can be improved by either promoting MCP synthesis or by increasing the proportion of RUCP and consequently reducing N excretion. Literature indicates that wilting enhances the amount of fermentable organic matter available for MCP synthesis and MCP outflow of the rumen (Johansen *et al.*, 2017; Verbič *et al.*, 1999). Pre-wilting of forage also reduced the proportion of NPN (Hartinger *et al.*, 2019; Tamminga *et al.*, 1991) which has a positive effect on RUCP content (Aufrère *et al.*, 2003; Edmunds *et al.*, 2014; Johansen and Weisbjerg, 2016). Furthermore, inoculants containing lactic acid bacteria (**LAB**) reduces proteolysis during ensiling (Slottner and Bertilsson, 2006) and increases RUCP content (Kramer, 2011).

Many studies are available which determined different feed treatments affecting CP degradation and RUCP content, but little information is available about these effects on the extent of organic matter (**OM**) degradation, the main determinant for MCP yield. Therefore, a one-sided view regarding RUCP alone does not allow any conclusions about effects on prCP content. The standard procedure used in many protein evaluation systems is the determination of RUCP by the *in situ* method, allowing the description of nutrient degradation over time, but it shows distinct limits in estimating prCP content. In previous studies, the modified Hohenheim gas test (**modHGT**; Steingäß and Südekum, 2013) has been shown to provide reliable

figures for prCP content. A decisive advantage of this *in vitro* method is the measurement of the total amount of prCP instead of a separate determination of RUCP and MCP. This evades the problem that RUCP and MCP are not independent measures which questions additivity of both components (GfE, 2001). The aims of this study were (1) to investigate the effect of dry matter (DM) concentration and the use of an inoculant containing LAB on RUCP and prCP content in grass silages and (2) to compare prCP content measured *in vitro* with those obtained *in situ*. We hypothesized, that (1) wilting and LAB improves the protein value of grass silages, (2) both methods reflect the treatment effects and (3) the less laborious modHGT provides reliable estimates for prCP content in grass silages and can be used as an alternative to the *in situ* method.

2.3 Materials and methods

The studies reported here were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2014). *In situ* ruminal degradation experiment was performed at the Department of Animal Nutrition of the Hohenheim University, Stuttgart, Germany and was approved by the Regional Council Stuttgart of the Ministry of Rural Areas and Consumer Protection of the federal state Baden-Württemberg, Germany. *In vitro* incubation was carried out at the Institute of Animal Nutrition and Physiology of the Christian-Albrechts-Universität, Kiel, Germany, and the collection of rumen-fluid from fistulated heifers was approved by the Animal Welfare commission of the Ministry of Energy, Agriculture, Environment, and Rural Areas of the federal state of Schleswig-Holstein, Germany.

2.3.1 Grass Silages

Perennial ryegrass (*Lolium perenne*, second cut 2017, 31 d of regrowth) cultivated at the Chamber of Agriculture of Schleswig-Holstein (Futterkamp, Germany) was used for the experiment. After cutting, grass was wilted using floor ventilation with heated air (~40 °C) to obtain eight different DM concentrations (achieved values were: 170, 310, 390, 420, 470, 530, 580 and 600 g DM/kg fresh matter), where 170 g DM refers to fresh grass without any artificial drying. The treatments were denoted according to their DM concentration before ensiling as following: DM-g/kg fresh material, e.g., DM-170.

Total processing time after cutting took about 2 and 60 h for the first and the last silage, respectively. Herein, the artificial drying procedure – interrupted overnight – lasted between 1 and 12 h to obtain materials with 310 to 600 g DM/kg.

During interruption grass material was stored in a stockroom under atmospheric air (~14 °C). After wilting, the grass material was chopped (20-30 mm) using a laboratory forage chopper (Hege, Waldenburg, Germany). Unwilted and wilted grass was ensiled without (control, **CON**) or with an inoculant (BonsilagePlus, Co. Schau-mann, Germany; 1 g/t grass) containing a mixture of homo- and heterofermentative LAB. Subsequently, grass material was ensiled in quadruplicate in 1.5 L glass jars. Ensiled amount varied between 200 and 500 g DM per glass jar, depending on the DM concentration of the grass material. Glass jars were stored under controlled temperature of ~25 °C and lasted for 90 days. In total, 16 treatments were tested and are referred to the use of inoculant (without inoculant: CON, with inoculant: LAB) and to DM concentration (170, 310, 390, 420, 470, 530, 580, 600), e.g., LAB-170.

2.3.2 Chemical analysis

Silage samples were analysed for DM, ether extract (**EE**), water-soluble carbohydrates (**WSC**), neutral detergent fibre (assayed with a heat stable amylase and expressed exclusive of residual ash, **aNDFom**) and acid detergent fibre (expressed exclusive of residual ash, **ADFom**) using near-infrared reflectance spectroscopy. Fermentation pattern (lactic acid, acetic acid, propionic acid, propane-1,2-diol, ethanol, n-butyric acid, n-propanol) were analysed by high performance liquid chromatography. Silage pH and NH₃-N concentration were determined according to VDLUFA (2007; method 18.1) and VDLUFA (1995; method 3.2.6), respectively. These analyses were conducted by ISF Schaumann Forschung GmbH, Wahlstedt, Germany.

Silage samples used for *in situ* and *in vitro* incubations as well as the residues in the polyester bags after fermentation were analysed for oven-DM (without correction for volatile compounds), crude ash (**CA**), and CP according to the official analytical methods in Germany (methods 3.1, 8.1 and 4.1.1, respectively; VDLUFA, 2007). Prior to analysis, silage samples were freeze-dried, pooled within treatment, and ground to pass through a 1-mm screen (Ultra centrifugal mill ZM 200, Retsch GmbH, Haan, Germany).

2.3.3 In situ procedure

Three ruminally cannulated and non-lactating Jersey-cows with an average body weight of 556 kg at the research station Meiereihof, Hohenheim University, Germany were used. The cows received hay and a mixed ration composed (in g/kg DM) of 350 maize silage, 350 grass silage, 250 hay, 30 barley straw and 20 of a mineral-vitamin mixture (including urea). Feed and water were offered for *ad libitum*

consumption. Mean DM intake was 2 kg hay and 9 kg mixed ration/d. For the *in situ* incubations, grass silage was pooled per treatment, freeze-dried, and ground to pass through a 2-mm sieve using a rotor mill (Brabender GmbH, Duisburg, Germany). Approximately 1.4 g on DM basis of each sample was placed into polyester bags (5 × 10 cm, 50 µm pore size, 14 mg DM sample/cm² polyester bag; ANKOM Technology Macedon, NY, USA). Bags were closed tightly with cable ties and fixed to an anchor weight. Prior to incubation, bags were soaked in warm tap water (~39 °C) for 5 min. Bags were introduced into the rumen immediately after morning feeding in the ventral sac. Four bags per treatment and time point (analytical replicates) were incubated in each cow (statistical replicates). After 2, 4, 8, 16, 24, 48 and 72 h polyester bags were removed from the rumen, immediately immersed into ice-cold water to minimize microbial activity, and rinsed in cold tap water to remove excess ruminal content. Additionally, four polyester bags per treatment and animal without incubation were used for determination of zero time (0 h) disappearance, the washout fraction at time point 0 h. All bags (including the 0 h) were washed together with cold water for 15 min in a washing machine (type WM14A160; Siemens GmbH, Munich, Germany) without centrifugation. Afterwards, bags were dried at 60 °C in an air forced oven for 24 h, weighted and stored until analysis. Two bags per treatment, time point, and cow were used for determining OM and CP degradation each.

Water-soluble (**WS**) fraction was estimated in quadruplicate by mixing samples (1 g fresh matter) in 50 ml warm water (37 °C) for 1 min before washed through a folded filter paper (No. 615 1/4, Ø: 150 mm, Macherey-Nagel, Düren, Germany). Water-insoluble fraction escaping as small particles (**SP**) from the polyester bags was estimated by subtracting WS fraction from 0 h washout fraction. The single values obtained for OM and CP disappearance (DI) were corrected (C) for loss of SP according to Hvelplund and Weisbjerg (2000) at each incubation time point as:

$$CDI = DI - SP \cdot (1 - ((DI - (SP + WS)) / (1 - (SP + WS)))) \quad (2-1)$$

where CDI, DI, SP and WS are given as fraction (g/g) of OM or CP, respectively.

The CDI of CP was corrected afterwards for microbial attachment (**MA**) according to Krawielitzki *et al.* (2006) as:

$$MA = A_{\max} \cdot (1 - e^{-R \cdot t}) \quad (2-2)$$

where MA is at time t (% of residue CP), t is incubation time in (h), A_{\max} is the maximum extent of bacterial colonization (% of residue CP) at $t \approx \infty$, and R is the rate of colonization (/h). Values for A_{\max} and R were calculated as:

$$A_{\max} (\% \text{ of residue CP}) = 50.6 + 0.48 \text{ NDF } (\% \text{ DM}) - 0.77 \text{ CP } (\% \text{ DM}) \quad (2-3)$$

$$R (/h) = 13.3 + 0.09 \text{ NDF } (\% \text{ DM}) - 0.35 \text{ CP } (\% \text{ DM}) \quad (2-4)$$

Instead of NDF, in this study, A_{\max} and R were calculated using aNDFom.

To represent OM and CP degradation over time, the exponential model of McDonald (1981) was adapted to CDI (g/kg), separately for every animal as:

$$\text{deg} = a + b \cdot (1 - e^{(-c \cdot (t-L))}) \quad (2-5)$$

where deg describes the cumulative degradation (g/kg) of OM or CP at time t , a represents the soluble fraction (g/kg), b the insoluble but potentially degradable fraction (g/kg), c the degradation rate of b (/h) and L the lag phase (h). Potential degradation is the sum of a and b fraction (asymptote of the curve) and is referred as $a+b$ fraction (g/kg), which was constrained to not exceed 1000 g.

Effective degradability of CP (**ED_kCP**; g/kg CP) and OM (**ED_kOM**; g/kg OM) was calculated for ruminal passage rates (k) of 0.02, 0.04 and 0.06/h (indicated as e.g., ED₂CP, ED₄CP and ED₆CP, respectively) according to McDonald (1981), but with the assumption that no degradation occurred during the lag phase (Wulf and Südekum, 2005):

$$ED_k = a + ((b \cdot c) / (c + k)) \cdot e^{-kL} \quad (2-6)$$

Effective RUCP (**RUCP_k**) was calculated for $k = 0.02, 0.04$ and $0.06/\text{h}$ (i.e., RUCP₂, RUCP₄, RUCP₆, respectively) as follows:

$$\text{RUCP}_k (\text{g/kg CP}) = 1000 - \text{ED}_k \text{CP} (\text{g/kg CP}) \quad (2-7)$$

$$\text{RUCP}_k (\text{g/kg OM}) = \text{RUCP}_k (\text{g/kg CP}) \cdot \text{CP} / \text{OM} \quad (2-8)$$

where CP and OM are given as a fraction of DM (g/g).

The amount of effective prCP *in situ* (**prCP_k in situ**; g/kg OM) was calculated by the sum of RUCP_k (g/kg OM) and effective MCP (**MCP_k**) synthesized in the rumen (g/kg OM) for the respective passage rates as:

$$\text{prCP}_k \text{ in situ} = \text{RUCP}_k + \text{MCP}_k \quad (2-9)$$

$$\text{MCP}_k = 0.181 \cdot (\text{ED}_k \text{OM} - (\text{lactate} + \text{propane-1,2-diol})) \quad (2-10)$$

where MCP_k was assumed to be 181 g per kg fermented OM (**FOM**; Lebzien and Voigt, 1999). The amount of FOM derived from *in situ* incubations was defined as ED_kOM minus the amounts of lactate and of propane-1,2-diol (g/kg OM), since lactate and propane-1,2-diol being themselves end products of microbial fermentation and therefore providing only a negligible amount of energy to rumen microbes (AFRC, 1993). The volatility of lactate and of propane-1,2-diol can be regarded as very small and therefore still present after freeze-drying, whereas all other fermentation acids and alcohols are completely volatile and no longer present in the incubated material.

2.3.4 *In vitro* procedure

The modHGT used in this study was performed as described in detail by Edmunds *et al.* (2012a). The procedure essentially corresponds to the Hohenheim gas test (**HGT**; Menke and Steingäß, 1988) with the modifications proposed by Steingäß and Südekum (2013) and is used for the *in vitro* estimation of prCP, which is the sum of RUCP and MCP. Rumen-fluid for incubation was taken from two rumen-cannulated German Black Pied heifers 1 h prior to the morning feeding. The heifers were fed a ration consisting of 3 kg of grass hay and 3 kg of concentrate divided into two equal meals (08:00 and 16:00 h). The concentrate contained (g/kg as fed) 232 rye, 210 rapeseed meal, 185 maize gluten feed, 150 milled barley grain, 120 rye bran, 45 beet pulp, 40 maize, and 11 calcium carbonate. Additionally, animals received 75 g of

mineral premix during morning feeding and water was offered for *ad libitum* consumption. The *in vitro* incubation was conducted in three runs in different days. Samples were weighed (200 ± 1 mg, on fresh matter basis) in duplicate and transferred into 100-mL glass syringes fitted with pistons (Haeberle Labortechnik GmbH & Co. KG, Lonsee-Ettlenschieß, Germany). Additionally, two replicates of standard feeds, and two blank syringes containing only rumen-fluid and no substrate for every run and incubation time were used. Samples were incubated for 8, 24 and 48 h. Standard feeds with defined gas production at 24 h of incubation (hay: 45.9 ml/200 mg DM, concentrate: 61.1 ml/200 mg DM) and defined prCP content at 8 and 48 h of incubation (232 and 97 g/kg DM, respectively) were provided by the Institute of Animal Science, Hohenheim University, Germany. Hay and concentrate standards were used for correcting gas production at 24 h of incubation. Post-rumen CP standard served as a control only, without correction of the measured prCP values. Blank syringes were used to correct for gas production after 24 h of incubation and NH₃-N content originating from rumen-fluid buffer solution after 8 and 48 h of incubation. After 8, 24 and 48 h gas volume was measured, and the syringes were immediately put on ice to suppress further microbial activity. Subsequently, NH₃-N content was determined by steam distillation as described by Westreicher-Kristen *et al.* (2015). The prCP content determined by the modHGT was calculated according to the following equation:

$$\text{prCP (g/kg OM)} = \quad (2-11)$$

$$(((\text{NH}_3\text{-N}_{\text{blank}} + \text{N}_{\text{sample}} - \text{NH}_3\text{-N}_{\text{sample}}) \times 6.25 \times 1000) / S) / \text{OM}$$

where NH₃-N_{blank} is the NH₃-N amount of the blanks after incubation (mg), N_{sample} is the N amount of the incubated sample (mg), NH₃-N_{sample} is the NH₃-N amount of the liquid contained in the syringes after incubation (mg) and S is the total amount of sample on DM basis (mg), OM refers to the OM content of the incubated sample (g/g DM).

The effective prCP *in vitro* (**prCP_k *in vitro***) was calculated through linear regression of prCP values at 8 h and 48 h of incubation against ln time (h) and calculating the function value for ln (50), ln (25) and ln (16.7) for each passage rate 0.02, 0.04 and 0.06/h.

Gas volume after 24 h of incubation was used to calculate amount of metabolizable energy (ME; GfE, 2008):

$$\text{ME (MJ/kg DM)} = \quad (2-12)$$

$$7.81 + 0.07559 \text{ GP24} - 0.00384 \text{ CA} + 0.00565 \text{ CP} + 0.01898 \text{ EE} - 0.00831 \text{ ADFom}.$$

where CA, CP, EE and ADFom are expressed as g/kg DM. GP24 is gas production at 24 h (ml/200 mg DM).

2.3.5 Statistical analysis

The *in situ* parameters (a , b , c , L) were estimated for each of the three animals via software program GraphPad Prism (version 8.00 for Windows, GraphPad Software Inc., San Diego, CA, USA). Linear mixed-effects models (Pinheiro and Bates, 2000) were used to compare target values using Software R (version 3.6.1, The R Foundation for Statistical Computing, Vienna, Austria). The statistical model included inoculant (categorical variable), DM concentration (DM, categorical variable) and their interaction as fixed effects and a random effect for animal (*in situ*) or run (*in vitro*). Comparison of the data was done using two-way analysis of variance to identify treatment effects. Additionally, multiple contrasts tests (Bretz *et al.*, 2010; Hothorn *et al.*, 2008) were used to test for a changepoint effect of DM concentration on response variables. Furthermore, linear mixed-effects models were used as described before, where DM was included as a continuous variable. Analysis of covariance was conducted to identify treatment effects. All reported values are least squares estimates (means, intercepts, slopes) and significance was declared at $P < 0.05$. Two coefficients of determination (R^2) are given, where marginal R^2 (R^2_m) describes the proportion of variance explained by the fixed factors (inoculant, DM) alone, and conditional R^2 (R^2_c) describes the proportion of variance explained by both the fixed and random factors.

2.4 Results

2.4.1 Chemical composition of the silages

Chemical composition and fermentation pattern results of the silage samples are shown in **Table 2-1**. Low contents of butyric acid (<3 g/kg DM) and $\text{NH}_3\text{-N}$ (<80 g/kg CP) indicate high quality of most silages. High proportion of acetic acid (71.1 g/kg DM) and propane-1,2-diol (42.3 g/kg DM) was found for LAB-170, which also was

the only treatment where meaningful contents of propionic acid (6.23 g/kg DM) and n-propanol (9.65 g/kg DM) could be detected.

The sum of fermentation acids and alcohols decreased with increasing DM ($P<0.001$) for both treatments (CON: 184 to 48.6 g/kg DM; LAB: 193 to 60.1 g/kg DM), with on average higher amounts of fermentation products for LAB (114 g/kg DM; $P<0.001$) compared to CON (89.7 g/kg DM). The concentration of WSC increased with increasing DM ($P<0.001$) for CON (from 9 to 66 g/kg DM) and LAB (from <0.1 to 48.8 g/kg DM). Consequently, pH value increased with DM and was higher for CON compared to LAB ($P<0.001$). Average $\text{NH}_3\text{-N}$ concentration was higher for CON (11.9 g/kg CP) compared to LAB (10.7 g/kg CP; $P<0.001$). Significant interactions between DM and inoculant were found for each parameter except for ethanol concentration. The interactions were not caused by a systematic effect of DM or inoculant, they occurred in some single treatments only.

Table 2-1: Chemical composition¹ and fermentation products² of grass silages pre-wilted to different dry matter (DM) concentrations without (control, CON) or treated with an inoculant containing homo- and heterofermentative lactic acid bacteria (LAB).

		DM concentration at ensiling (g/kg fresh material)								SEM	P-value		
		170	310	390	420	470	530	580	600		I	DM	I × DM
DM (g/kg fresh matter)	CON-	160	306	324	422	457	490	556	588	5.5	<0.001	<0.001	0.014
	LAB-	149	294	306	419	477	489	548	588				
CA ³ (g/kg DM)	CON-	113	102	105	103	103	110	108	111	0.5	<0.001	<0.001	0.001
	LAB-	117	105	104	107	107	110	109	110				
CP ³ (g/kg DM)	CON-	153	153	147	153	150	153	146	151	0.2	<0.001	<0.001	<0.001
	LAB-	159	156	148	155	153	153	152	149				
EE (g/kg DM)	CON-	47.7	40.0	41.0	38.3	38.3	36.0	33.5	32.8	0.62	<0.001	<0.001	<0.001
	LAB-	49.0	46.3	44.0	41.5	40.3	38.0	36.3	33.8				
WSC (g/kg DM)	CON-	9.00	31.5	31.7	29.8	34.5	40.8	55.5	66.0	1.76	<0.001	<0.001	<0.001
	LAB-	<0.1	<0.1	10.8	18.3	33.5	29.5	37.3	48.8				
aNDFom (g/kg DM)	CON-	463	498	508	529	526	527	523	526	2.6	0.356	<0.001	<0.001
	LAB-	519	503	514	520	510	518	507	514				
ADFom (g/kg DM)	CON-	292	293	307	311	310	311	307	309	1.5	0.001	<0.001	<0.001
	LAB-	307	309	316	315	308	312	299	301				
ME ⁴ (MJ/kg DM)	CON-	10.8	10.8	10.5	10.6	10.5	10.5	10.4	10.3	0.06	0.378	<0.001	<0.001
	LAB-	10.7	10.6	10.5	10.4	10.5	10.5	10.5	10.4				

SEM = standard error of the mean; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction; CA = crude ash; CP = crude protein; EE = ether extract; WSC = water-soluble carbohydrates; aNDFom = neutral detergent fibre (assayed with a heat stable amylase and expressed exclusive of residual ash); ADFom = acid detergent fibre (expressed exclusive of residual ash); ME = metabolisable energy.

¹analysed using near-infrared reflectance spectroscopy.

²fermentation acids and alcohols were determined using high performance liquid chromatography.

³determined according to VDLUFA (2007).

⁴determined according to GfE (2008) as: ME (MJ/kg DM) = 7.81 + 0.07559 GP24 – 0.00384 CA + 0.00565 CP + 0.01898 EE– 0.00831 ADFom, all fractions are expressed as g/kg DM, GP24 is gas production at 24 h (ml/200 mg DM).

Table 2-1: continued.

		DM concentration at ensiling (g/kg fresh material)								SEM	P-value		
		170	310	390	420	470	530	580	600		I	DM	I × DM
Lactic acid (g/kg DM)	CON-	129	73.8	78.6	55.7	55.8	53.5	41.6	33.0	2.55	<0.001	<0.001	<0.001
	LAB-	46.6	92.2	97.3	74.8	73.2	71.9	62.5	42.6				
Acetic acid (g/kg DM)	CON-	39.2	19.5	19.5	12.9	13.1	14.9	12.5	11.2	1.22	<0.001	<0.001	<0.001
	LAB-	71.1	33.0	25.1	18.2	14.1	16.0	12.5	12.4				
Propionic acid (g/kg DM)	CON-	0.06	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.111	<0.001	<0.001	<0.001
	LAB-	6.23	0.03	0.03	0.02	0.02	0.02	0.02	0.02				
Propane-1,2-diol (g/kg DM)	CON-	0.48	1.14	4.05	0.02	0.02	0.12	0.44	0.30	0.607	<0.001	<0.001	<0.001
	LAB-	42.3	22.2	13.8	4.98	1.39	1.11	0.48	1.63				
Ethanol (g/kg DM)	CON-	15.3	4.88	5.14	4.28	4.21	4.04	2.92	2.47	0.430	0.001	<0.001	0.179
	LAB-	17.3	5.34	4.89	4.42	4.00	3.97	2.45	3.06				
n-butyric acid (g/kg DM)	CON-	0.06	0.03	0.03	0.02	0.02	1.09	1.07	1.55	0.070	0.002	<0.001	<0.001
	LAB-	0.07	0.03	0.03	0.02	0.30	0.17	0.95	0.39				
n-propanol (g/kg DM)	CON-	0.06	0.03	0.03	0.02	0.02	0.02	0.02	0.02	0.030	<0.001	<0.001	<0.001
	LAB-	9.65	0.03	0.03	0.02	0.02	0.02	0.02	0.02				
NH ₃ -N ⁵ (g/kg CP)	CON-	12.3	10.1	10.5	10.9	10.2	14.0	14.5	12.9	0.307	<0.001	<0.001	<0.001
	LAB-	12.7	8.73	8.83	9.24	8.54	13.7	11.1	12.8				
pH value ⁶	CON-	3.87	4.12	4.11	4.30	4.36	4.54	4.82	5.01	0.022	<0.001	<0.001	<0.001
	LAB-	4.38	3.97	3.95	4.14	4.17	4.27	4.39	4.72				

SEM = standard error of the mean; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction.

⁵NH₃-N determined according to VDLUFA (1995).

⁶pH value determined according to VDLUFA (2007).

2.4.2 *In situ* OM and CP degradation and prCP concentration

The effect of silage DM and inoculant on *in situ* OM degradation parameters are given in **Table 2-2**. Average of *a* fraction was higher for CON ($P<0.001$) compared to LAB (295 and 277 g/kg OM, respectively) whereas *b* fraction was lower for CON ($P<0.001$) than LAB (576 and 595 g/kg OM, respectively). Interaction ($P<0.001$) was caused by a more pronounced deviation of *a* and *b* fraction between the treatments at lower DM compared to higher DM. There were no differences between CON and LAB for *a+b* values ($P = 0.92$), whereas a non-systematic effect of DM ($P<0.001$) was observed. The effective FOM¹ increased with DM concentration ($P<0.001$) and was higher in CON compared to LAB ($P<0.001$), whereas interaction ($P<0.001$) was not specific. Faster passage rates numerically reduced effective FOM.

For *a* and *b* values of CP (**Table 2-2**), changepoints (data not shown) existed between DM-530 and DM-580 ($P<0.001$): Fraction *a* averaged 648 and 627 g/kg CP within the DM range of 170 – 530 and decreased to 594 and 587 g/kg CP at the DM range of 580 – 600 for CON and LAB, respectively. Fraction *b* averaged 312 and 334 g/kg CP within the DM range of 170 – 530 and increased to 371 and 375 g/kg CP at the DM range of 580 – 600 for CON and LAB, respectively. These differences below and above this changepoint are also reflected by the content of effective RUCP. Depending on treatment and on assumed passage rate, effective RUCP was enhanced ($P<0.001$) by 3.7 to 13 g/kg CP at the DM range of 580 – 600 compared to DM range of 170 – 530. Average amounts of RUCP₂, RUCP₄, and RUCP₆ were 79, 110 and 137 g/kg CP for CON, and 82, 115 and 142 g/kg CP for LAB.

¹ Corresponds to ED₆OM determined *in situ* minus the amounts of lactate and propane-1,2-diol

Table 2-2: Effect of silage dry matter (DM) and inoculant¹ on estimated parameters describing the ruminal degradation *in situ*. Results of linear-mixed models and analyses of variance.

		DM concentration at ensiling (g/kg fresh material)								SEM	R ² _m	R ² _c	P-value		
		170	310	390	420	470	530	580	600				I	DM	I×DM
<i>In situ</i> ² parameters of organic matter (OM), g/kg OM															
<i>a</i>	CON-	301	326	286	297	269	276	301	304	4.1	0.86	0.91	<0.001	<0.001	<0.001
	LAB-	257	284	278	264	273	269	292	295						
<i>b</i>	CON-	589	549	578	572	588	597	580	556	5.4	0.81	0.86	<0.001	<0.001	<0.001
	LAB-	631	589	581	606	591	605	580	576						
<i>a+b</i>	CON-	890	876	864	869	857	873	881	860	4.0	0.64	0.71	0.921	<0.001	0.171
	LAB-	889	873	859	870	863	874	871	871						
<i>c</i> (/h)	CON-	0.087	0.084	0.079	0.074	0.080	0.076	0.076	0.085	0.006	0.15	0.84	0.172	<0.001	0.839
	LAB-	0.088	0.084	0.083	0.074	0.084	0.083	0.076	0.085						
<i>L</i> (h)	CON-	1.99	1.98	2.19	2.22	2.21	1.71	1.87	2.26	0.563	0.06	0.79	0.747	0.821	0.211
	LAB-	2.10	1.98	1.44	2.09	2.23	2.60	2.21	2.15						
FOM ₂	CON-	616	668	634	665	656	670	694	696	11.5	0.65	0.98	<0.001	<0.001	<0.001
	LAB-	649	613	609	632	644	649	660	691						
FOM ₄	CON-	529	586	545	574	565	578	604	612	15.8	0.51	0.97	<0.001	<0.001	<0.001
	LAB-	556	525	524	537	554	554	569	604						
FOM ₆	CON-	466	527	482	512	501	514	542	552	17.5	0.47	0.97	<0.001	<0.001	<0.001
	LAB-	488	463	463	471	490	487	506	542						

SEM = standard error of the mean; R² = coefficient of determination; R²_m = marginal R², R²_c = conditional R²; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction; *a* = soluble fraction; *b* = insoluble but potentially degradable fraction; *a+b* = potential degradation; *c* = degradation rate of *b*; *L* = Lag phase; FOM_k = fermented OM at passage rate *k* (*k* = 0.02, 0.04, 0.06/h), corresponds to effective degradation of OM at passage rate *k* determined *in situ* minus the amounts of lactate and propane-1,2-diol.

¹without (control, CON) or with inoculant containing homo- and heterofermentative lactic acid bacteria (LAB).

²calculated according to McDonald (1981).

Table 2-2: continued.

		DM concentration at ensiling (g/kg fresh material)								SEM	R ² _m	R ² _c	P-value		
		170	310	390	420	470	530	580	600				I	DM	I×DM
<i>In situ</i> ² parameters of crude protein (CP), g/kg CP															
<i>a</i>	CON-	650	632	653	667	645	643	589	598	2.7	0.97	0.97	<0.001	<0.001	<0.001
	LAB-	608	610	627	649	626	639	596	578						
<i>b</i>	CON-	309	325	306	296	316	319	374	367	3.3	0.96	0.96	<0.001	<0.001	<0.001
	LAB-	354	351	330	311	334	324	364	386						
<i>a+b</i>	CON-	960	957	959	963	962	963	962	965	1.9	0.30	0.73	0.273	0.001	0.109
	LAB-	961	961	957	959	960	963	960	963						
<i>c</i> (/h)	CON-	0.171	0.175	0.161	0.148	0.165	0.154	0.159	0.158	0.016	0.09	0.91	0.331	0.001	0.225
	LAB-	0.158	0.165	0.172	0.151	0.171	0.144	0.157	0.154						
<i>L</i> (h)	CON-	0.51	0.62	0.64	0.64	0.82	0.51	0.52	0.40	0.141	0.21	0.59	0.257	0.025	0.765
	LAB-	0.26	0.62	0.67	0.58	0.62	0.48	0.50	0.45						
RUCP ₂	CON-	76.2	80.4	78.9	75.8	77.7	77.4	83.2	79.7	2.45	0.28	0.92	<0.001	<0.001	0.056
	LAB-	81.5	81.6	81.9	80.5	78.9	80.1	84.9	84.6						
RUCP ₄	CON-	105	111	109	106	109	109	119	115	4.7	0.26	0.96	<0.001	<0.001	0.003
	LAB-	115	115	113	112	110	113	120	122						
RUCP ₆	CON-	129	136	134	131	135	134	149	144	6.3	0.28	0.97	<0.001	<0.001	0.001
	LAB-	142	143	139	137	136	140	149	153						

SEM = standard error of the mean; R² = coefficient of determination; R²_m = marginal R², R²_c = conditional R²; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction; *a* = soluble fraction; *b* = insoluble but potentially degradable fraction; *a+b* = potential degradation; *c* = degradation rate of *b*; *L* = Lag phase; RUCP_k = effective rumen undegraded CP at passage rate *k* (*k* = 0.02, 0.04, 0.06/h).

²calculated according to McDonald (1981).

The contents of MCP and RUCP required for calculating the effective prCP *in situ* and values of *in vitro* determined effective prCP are shown in **Table 2-3**. For most of these parameters' significant interaction of DM and inoculant were observed. To assist understanding and to quantify the effects of DM and inoculant linear regression equations are given in **Table 2-4**. Irrespective of assumed passage rate, MCP linearly increased ($P<0.001$) by 2.3 g/kg OM per 100 g DM increase, where CON had higher intercepts, reflecting the higher MCP for CON ($P<0.001$, **Table 2-3**). With increasing passage rate MCP numerically decreased. Similar linear effects of DM existed for effective prCP *in situ* ($P<0.001$) and are more pronounced (steeper slopes) at higher passage rates.

The *in vitro* estimated prCP₂, prCP₄ and prCP₆ increased linearly with increasing DM content ($P<0.001$). However, at lower passage rates intercepts numerically decreased, whereas slopes numerically increased, which reflected a higher sensitivity of the *in vitro* method at lower passage rates. No effect of inoculant on effective prCP *in vitro* could be detected.

The effects of animal and run on effective prCP values reveal the necessity of either using a respective standard feed to correct for these effects or of performing multiple runs in different days (*in vitro*) or multiple runs in several animals.

Table 2-3: Effect of silage dry matter (DM) and inoculant¹ on effective rumen undegradable crude protein, microbial crude protein, and effective post-rumen crude protein determined *in situ* and *in vitro* at passage rates (*k*) of 0.02 0.04 and 0.06/h. Results of linear-mixed models and analyses of variance.

		DM concentration at ensiling (g/kg fresh material)								SEM	R ² _m	R ² _c	P-value		
		170	310	390	420	470	530	580	600				I	DM	I×DM
Effective rumen undegradable crude protein (RUCP _k , g/kg OM) based on <i>in situ</i>															
RUCP ₂	CON-	13.1	13.7	13.0	12.9	13.0	13.3	13.6	13.5	0.42	0.34	0.93	<0.001	<0.001	0.006
	LAB-	14.7	14.2	13.5	14.0	13.5	13.8	14.5	14.2						
RUCP ₄	CON-	18.1	18.9	17.9	18.1	18.2	18.7	19.6	19.5	0.81	0.29	0.96	<0.001	<0.001	<0.001
	LAB-	20.7	20.1	18.7	19.4	18.9	19.5	20.5	20.5						
RUCP ₆	CON-	22.2	23.1	21.9	22.3	22.5	23.1	24.4	24.4	1.07	0.30	0.97	<0.001	<0.001	<0.001
	LAB-	25.6	25.0	23.0	23.8	23.3	24.1	25.5	25.6						
Microbial crude protein synthesised in the rumen (MCP _k ; g/kg OM) calculated based on <i>in situ</i> determined fermented organic matter															
MCP ₂	CON-	112	121	115	120	119	121	126	126	2.1	0.65	0.98	<0.001	<0.001	<0.001
	LAB-	118	111	110	114	117	118	120	125						
MCP ₄	CON-	96	106	99	104	102	105	109	111	2.9	0.51	0.97	<0.001	<0.001	<0.001
	LAB-	101	95	95	97	100	100	103	109						
MCP ₆	CON-	84	96	87	93	91	93	98	100	3.2	0.47	0.97	<0.001	<0.001	<0.001
	LAB-	88	84	84	85	89	88	92	98						

SEM = standard error of the mean; R² = coefficient of determination; R²_m = marginal R², R²_c = conditional R²; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction.

¹without (control, CON) or with inoculant containing homo- and heterofermentative lactic acid bacteria (LAB).

Table 2-3: continued.

		DM concentration at ensiling (g/kg fresh material)								SEM	R ² _m	R ² _c	P-value		
		170	310	390	420	470	530	580	600				I	DM	I×DM
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k ; g/kg OM)															
prCP ₂	CON-	125	135	128	133	132	135	139	140	1.7	0.73	0.97	<0.001	<0.001	<0.001
	LAB-	132	125	124	128	130	131	134	139						
prCP ₄	CON-	114	125	117	122	121	123	129	130	2.2	0.65	0.97	<0.001	<0.001	<0.001
	LAB-	121	115	114	117	119	120	124	130						
prCP ₆	CON-	107	119	109	115	113	116	122	124	2.4	0.65	0.96	<0.001	<0.001	<0.001
	LAB-	114	109	107	109	112	112	117	124						
Effective post-rumen crude protein determined <i>in vitro</i> using the modified Hohenheim gas test (prCP _k ; g/kg OM)															
prCP ₂	CON-	94	99	104	111	99	114	122	120	7.6	0.33	0.60	0.346	0.002	0.447
	LAB-	104	97	94	100	102	121	110	112						
prCP ₄	CON-	117	127	128	127	125	135	138	138	5.1	0.36	0.69	0.224	<0.001	0.156
	LAB-	126	121	117	124	127	137	133	131						
prCP ₆	CON-	131	143	142	136	140	146	147	149	3.9	0.41	0.75	0.150	<0.001	0.006
	LAB-	139	135	131	138	141	147	146	142						

SEM = standard error of the mean; R² = coefficient of determination; R²_m = marginal R², R²_c = conditional R²; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (categorical variable); I×DM = Interaction.

Table 2-4: Effect of dry matter (DM) content and inoculant¹ on microbial crude protein synthesised in the rumen and effective post-rumen crude protein at passage rates (*k*) of 0.02 0.04 and 0.06/h determined *in situ* and *in vitro*. Results of linear-mixed models and analyses of covariance.

		Intercept	SE _{Intercept}	P _{Intercept}	Slope	SE _{Slope}	P _{Slope}	R ² _m	R ² _c	P-value			
											I	DM	I×DM
Microbial crude protein synthesised in the rumen (MCP _k ; g/kg OM)													
MCP ₂	CON-	110	2.7	<0.001	0.023	0.004	<0.001	0.36	0.67	0.002	<0.001	nd ²	
	LAB-	106	2.7	<0.001									
MCP ₄	CON-	94	3.3	<0.001	0.023	0.004	<0.001	0.28	0.73	<0.001	<0.001	nd	
	LAB-	90	3.3	<0.001									
MCP ₆	CON-	83	3.6	<0.001	0.023	0.004	<0.001	0.26	0.75	<0.001	<0.001	nd	
	LAB-	78	3.6	<0.001									
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k ; g/kg OM)													
prCP ₂	CON-	123	2.5	<0.001	0.023	0.004	<0.001	0.35	0.57	0.020	<0.001	nd	
	LAB-	120	2.5	<0.001									
prCP ₄	CON-	112	3.0	<0.001	0.024	0.004	<0.001	0.30	0.59	0.034	<0.001	nd	
	LAB-	109	3.0	<0.001									
prCP ₆	CON-	105	3.2	<0.001	0.026	0.005	<0.001	0.29	0.58	0.044	<0.001	nd	
	LAB-	102	3.2	<0.001									
Effective post-rumen crude protein determined using the modified Hohenheim gas test (prCP _k ; g/kg OM)													
prCP ₂	CON-	85	7.1	<0.001	0.049	0.011	<0.001	0.20	0.49	nd	<0.001	nd	
	LAB-												
prCP ₄	CON-	112	4.9	<0.001	0.036	0.007	<0.001	0.22	0.58	nd	<0.001	nd	
	LAB-												
prCP ₆	CON-	128	4.0	<0.001	0.029	0.006	<0.001	0.22	0.58	nd	<0.001	nd	
	LAB-												

SE_{Intercept} = standard error of the intercept; SE_{Slope} = standard error of the slope; P_{Intercept} = probability of intercepts being different from zero; P_{Slope} = probability of slopes being different from zero; R² = coefficient of determination; R²_m = marginal R², R²_c = conditional R²; I = main effect of inoculant (categorical variable); DM = main effect of DM concentration (continuous variable); I×DM = Interaction.

¹without (control, CON) or with inoculant containing homo- and heterofermentative lactic acid bacteria (LAB).

²Not determined, variable was removed from the model since it was not significant.

2.5 Discussion

2.5.1 Effect of wilting

In general, high DM content results in an increased osmotic pressure which suppresses proteolytic activity of plant-based enzymes (McDonald *et al.*, 1991; Slottnér and Bertilsson, 2006). Therefore, soluble CP (fraction *a*) decreases, while insoluble but potentially degradable CP (fraction *b*) increases in wilted silages (Tamminga *et al.*, 1991; Verbič *et al.*, 1999). Indeed, a similar trend was observed in this study and an effect of DM on CP fractions *a* and *b* was reflected by a changepoint between the range of DM-170 to DM-530 and DM-580 to DM-600. The latter resulted in increased effective RUCP (g/kg CP) for all passage rates by showing the same DM changepoints, indicating reduced proteolysis in highly wilted silages. However, within the respective ranges no effect of DM was found for *a*, *b*, or effective RUCP. In accordance with this, Edmunds *et al.* (2014) have demonstrated that RUCP₃ increases through intensive wilting (DM-650), without any effect in less intensive wilted (DM-200, DM-350, DM-500) silages. However, DM effect observed by Edmunds *et al.* (2014) was more pronounced (46 to 61 g RUCP₃/kg CP) than in the present study (9 g RUCP₄/kg CP). Also, mean RUCP₄ was higher (160 ± 38 g/kg CP; Edmunds *et al.*, 2012b) as observed here (113 ± 5 g/kg CP). The differences between the studies might be explained by many factors, such as plant material, cut number, maturity or wilting conditions including processing time. Silages examined by Edmunds *et al.* (2012a) composed of meadow grass (approx. 0.80 grass, 0.10 legumes and 0.10 herbs; second harvest) with on average higher CP (189 g/kg DM) than in the present study (152 g/kg DM). Additionally, processing procedure took less time compared to our study. Highly wilted silage samples (DM-650) were prepared during 50 h (Edmunds *et al.*, 2014), whereas in this study about 10 h more were needed to produce samples with 600 g DM. The authors highlighted a positive effect of a shortened and more intensive wilting on effective RUCP and effective prCP *in vitro* (Edmunds *et al.*, 2014), which can also be assumed to be responsible for the deviation of values determined for effective RUCP and effective prCP and the strength of DM effect compared to our study. Possibly, a part of the deviations could be attributed to a missing or false correction for microbial contamination. It has been shown that the correction according to Krawielitzki *et al.* (2006) used here overestimates the maximum extent of bacterial colonisation (A_{\max}) of grass silage (Edmunds *et al.*, 2012b). Therefore, a slightly higher content of effective RUCP might be more realistic, although this does not fortify the effect of DM.

The wilting process reduced microbial fermentation of WSC during ensiling and consequently concentration of fermentation acids, which is consistent to the literature (Gordon *et al.*, 2000; Johansen *et al.*, 2017; Purwin *et al.*, 2009). Due to this positive effect of wilting on WSC content, effective FOM increased in by increasing DM concentration indifferent of passage rate. Because MCP is calculated based on effective FOM, its increase through wilting was mainly caused by the variation in the concentrations of fermentation acids and WSC. Johansen *et al.* (2017) studied the effect of pre-wilting of grass-clover silages on duodenal nutrient flow in lactating dairy cows. They reported a linear increase of duodenal flow of MCP by 3.2 g/kg DM, while DM of silages increases by 100 g. Also, Verbič *et al.* (1999) reported higher supply of microbial N in highly wilted silages (DM-520) compared to unwilted (DM-210) or moderately wilted silages (DM-430) calculated based on purine derivative excretion in sheep. In contrast, Merchen and Satter (1983) did not find significant differences in the MCP flow in cows fed alfalfa silages varying in a range of DM-290 to DM-660.

The effect of DM on effective prCP *in situ* was mainly caused by the extent of increased MCP and the proportion of MCP in prCP. The MCP accounted for 77% ($k = 0.06/h$) to 90% ($k = 0.02/h$) of effective prCP *in situ*. Moreover, while DM had only a small effect on effective RUCP (g/kg OM), the absolute MCP increased in average 9% ($k = 0.02/h$) and 15% ($k = 0.06/h$) with increasing the DM content. The latter resulted in 9 and 12% increases of effective prCP for the same passage rates. These effects on effective prCP *in situ* were confirmed by the linear increase of effective prCP *in vitro* with DM ($P < 0.001$; **Table 2-4**), where prCP₆ *in vitro* showed the best agreement (similar slopes of the linear regression equations). The positive effect of DM agrees with results of Bakken *et al.* (2017) who showed a negative relationship between the concentration of total acids and prCP₄ *in vitro*, showing that intensively wilted silages contain smaller amounts of fermentation acids and a higher amount of WSC. Similarly, Edmunds *et al.* (2014) observed elevated values of prCP₄ *in vitro* in grass silages pre-wilted to DM-650 (136 g/kg DM), whereas no effect of DM on prCP₄ *in vitro* occurred between DM-350 (128 g/kg DM) and DM-500 (130 g/kg DM). In contrast, wilting lucerne silages prior to ensiling (DM-250 and DM-350) had no effect on effective prCP *in vitro* at passage rates of 0.02 and 0.05/h and tended to be higher for DM-250 at passage rate 0.08/h (Hartinger *et al.*, 2019). The authors assumed that the missing effect on effective prCP *in vitro* was caused by a generally limited availability of WSC in the silages to reuse ammonia-N from CP degradation for MCP synthesis. Despite of the statistical significance of the effect of DM on

effective prCP values in this study, the biological relevance with 2.3 to 4.9 g/kg OM per 100 g DM increase is small.

2.5.2 Effect of inoculant

Compared to CON, soluble CP (fraction *a*) reduced and the slowly degradable CP (fraction *b*) increased in LAB, indicating reduced proteolysis, as also reflected by the lower NH₃-N content in LAB silages. This was probably due to a faster drop in pH value after ensiling and a lower final pH by using the inoculant. Consequently, pH-dependent plant enzymes and fermentation organisms were suppressed earlier (McDonald *et al.*, 1991; Muck *et al.*, 2018). Therefore, average effective RUCP enhanced for LAB by 3 g/kg CP at a passage rate of 0.02/h and by 5 g/kg CP at passage rate 0.04 and 0.06/h. Kramer (2011) reported a greater effect of 20-60 g RUCP/kg CP in first-cut grass silages varying in a range of DM-200 to DM-500. However, RUCP values were estimated using the Cornell Net Carbohydrate and Protein System (CNCPS), which is based on the chemical fractionation of CP and not on ruminal parameters from *in situ* incubations.

Due to the higher concentration of acids and lower WSC content in LAB, the amount of FOM in LAB was lower compared to CON, resulting also in lower calculated amount of MCP for LAB. However, higher RUCP (g/kg OM) in LAB compared to CON compensated to some extent for the calculated lower MCP yield. Nevertheless, effective prCP *in situ* was lower in LAB irrespective of passage rate. However, the decrease in effective prCP by using LAB cannot be generalised for silages. Moreover, although significant, the differences between CON and LAB were small in absolute terms and to a certain extent depending on the underlying assumptions, e.g., the efficiency of MCP synthesis. Additionally, results of the modHGT indicated no difference in effective prCP between CON and LAB for any chosen passage rate. Even if no increase in effective prCP content in grass silage could be shown by LAB in our study, the use of such inoculants is beneficial due to other well-known positive effects on silage quality which were confirmed by this study. The increase of butyric acid concentration in high wilted silages was suppressed by LAB, which might be even much more pronounced under practical conditions. Furthermore, LAB silages showed higher acetic acid concentration due to the combination of homo- and heterofermentative lactic acid bacteria, which resulted in a greater aerobic stability (data not shown).

2.5.3 Relationship between *in situ* and *in vitro* prCP values

The protein value of feeds for ruminants is mainly determined by their amounts of prCP. The flow of prCP to the duodenum can be measured *in vivo* with duodenal fistulated animals or estimated from results of ruminal *in situ* incubations as done in our study. Both procedures are more expensive, laborious, and time-consuming than *in vitro* measurements. Therefore, this study also aimed at evaluating the potential of the modHGT to show similar treatment effects as observed with the *in situ* method. Function values for effective prCP (**Table 2-4**) calculated for DM-350 were lower for *in vitro* than *in situ* at low passage rates, and *vice versa*. Effective prCP *in vitro* was lower with 28 g at $k = 0.02/\text{h}$ and higher with 26 g/kg OM at $k = 0.06/\text{h}$ than effective prCP *in situ*. Assuming a passage rate of $k = 0.04/\text{h}$, which seems to be realistic for forages (Sauvant and Nozière, 2016; Tamminga *et al.*, 2007), both methods resulted in similar function values for effective prCP (*in vitro*: 125 g; *in situ*: CON 121 g, LAB 118 g/kg OM). It should be emphasized that despite the distinctly different general conditions of both methods the prCP₄ values (**Table 2-3**) differed only by approximately 7 g/kg OM on average. As noted above, the microbial contamination might have been overestimated in this study, which would have led – if correctly estimated – to higher effective prCP *in situ*, furthermore, reducing the difference between the *in situ* and *in vitro* determined prCP₄. The differences in effective prCP values between methods were mainly caused by the assumed passage rate and supports previous observations (Edmunds *et al.*, 2012a; Westreicher-Kristen and Südekum, 2018). Edmunds *et al.* (2012a) examined several forages (i.a. white clover, lucerne, perennial ryegrass) differently conserved (unconserved, hay, silage and artificially dried). They reported lower values for effective prCP *in vitro* at $k = 0.02/\text{h}$ and higher values at $k = 0.06/\text{h}$ than effective prCP values determined according to GfE (2001), however a good agreement at $k = 0.04/\text{h}$ (Edmunds *et al.*, 2012a). Westreicher-Kristen and Südekum (2018) determined effective prCP of total-mixed rations composed of maize silage, red clover silage, lupine seeds, soybean meal and wheat, having a forage to concentrate ratio of 0.75:0.25. The authors reported higher values of effective prCP determined according to GfE (2001) for $k = 0.02/\text{h}$ and $k = 0.05/\text{h}$ compared to effective prCP *in vitro*, whereas minimal differences occurred at $k = 0.08/\text{h}$. To receive a more detailed comparison between both methods, effective prCP values obtained *in situ* were regressed against those estimated *in vitro* (data not shown). *In situ* prCP correlated with prCP *in vitro* at all rates of passage ($k = 0.02/\text{h}$: $P < 0.001$, $R^2 = 0.58$; $k = 0.04/\text{h}$: $P < 0.001$, $R^2 = 0.61$; $k = 0.06/\text{h}$: $P < 0.01$, $R^2 = 0.54$), in spite of the very near range of the values (**Table 2-3**). This relationship was most pronounced at $k = 0.04/\text{h}$ and can be described by the following equation: prCP₄ *in*

situ (g/kg OM) = $41.2 \pm 17.3 + 0.63 \pm 0.13 \times \text{prCP}_4 \text{ in vitro}$ (g/kg OM). In accordance with these observations, Edmunds *et al.* (2012a) reported for different forages increasing correlation coefficients with passage rate ($k = 0.02/\text{h}$: $P < 0.038$, $R^2 = 0.19$; $k = 0.04/\text{h}$: $P < 0.001$, $R^2 = 0.56$; $k = 0.06/\text{h}$: $P < 0.001$, $R^2 = 0.67$). Westreicher-Kristen and Südekum (2018) found no correlation at $k = 0.02/\text{h}$ ($R^2 = 0.72$, $P = 0.15$), but a close correlation at $k = 0.05/\text{h}$ ($R^2 = 0.94$, $P = 0.03$) and $k = 0.08/\text{h}$ ($R^2 = 0.95$; $P = 0.02$) for total-mixed rations.

Although the *in vitro* method does not realise the true situation in the rumen, it better reflects the differences in the availability of energy from fermented nutrients for microbial growth, as shown for the negative impact of higher RUCP on MCP (Ipharraguerre and Clark, 2005; Tamminga, 1982). Furthermore, it avoids the unsolved problem of the *in situ* method with regard to non-additivity of RUCP and MCP as outlined by GfE (2001). Since passage rate positively affects both the efficiency of MCP synthesis as well as RUCP, effective *prCP in vitro* might represent a more accurate measurement than using a constant efficiency for calculating of MCP.

2.5.4 Limitations of the *in situ* and *in vitro* methods

This chapter aims to address some of the main limitations of the *in situ* and *in vitro* method used in the present study. The *in situ* incubation technique is well suited to describe ruminal degradation of chemical constituents of feeds over time. Although this technique is widely used in several protein systems, some critical points still remain not satisfactory solved. The strength of *in situ* measurements are the exposure of feeds to ruminal conditions similar to those *in vivo* (Schwab *et al.*, 2003). Extensive research on forage evaluation was performed frequently focused on the assessment of factors reducing ruminal degradation of CP (Huhtanen, 2019; Huhtanen and Broderick, 2016). Although variations of RUCP concentration of forage detected by the *in situ* method are present, there is limited evidence that these differences are reflected in animal performance, which partly can be explained by inherent problems of the *in situ* method related to the soluble CP fraction and microbial contamination. The general assumption that the soluble fraction of CP is completely degraded in the rumen is evidently questionable, because there are indications that a considerable part of soluble non-ammonia N (free amino acids, peptides, and soluble proteins) reaches the small intestine undegraded (Ahvenjärvi *et al.*, 2018; Volden and Larsen, 2011). The soluble protein pool is reassigned to the outflow of the liquid phase of the rumen in the CNCPS (van Amburgh *et al.*, 2015) due to its high passage rate (Seo *et al.*, 2006; Volden and Larsen, 2011). Therefore, it can be assumed that RUCP values in our study were underestimated to a certain

extent. On the other hand, RUCP content might be overestimated, if microbial contamination of residues in the bags is not considered, especially relevant in fibre-rich feeds of low N content (Krawielitzki *et al.*, 2006; Tamminga *et al.*, 1991). According to the equation proposed by Krawielitzki *et al.* (2006), 14 to 64% of the residual CP consisted of microbial CP in the present study for incubation times of 2 to 72 h. This means that e.g., RUCP₄ ranged between 169-200 g/kg CP without and between 105-122 g/kg CP with correction for microbial CP contamination. However, it was proven that the mentioned equation overestimates the maximum extent of bacterial colonisation of grass silage (Edmunds *et al.*, 2012b) which probably leads to a slightly higher content of effective RUCP. The latter might partially explain some differences between *in vitro* and *in situ* results at certain passage rates.

The prerequisite for estimating MCP synthesis is the precise information about the amount of energy available to the rumen microbes. A correction for substances which escape the bag, however, are not used as energy source for microbial growth (AFRC, 1993; Tamminga *et al.*, 2007; Vérité and Peyraud, 1989), e.g., fermentation acids, seems to be necessary when calculating MCP from ED_kOM determined *in situ*. In our study, ED_kOM was corrected for lactate and propane-1,2-diol, other fermentation products during ensiling were negligible and volatile during drying. Assuming a factor of 181 g MCP/kg FOM generated plausible values in the present study. However, the absolute value must be critically discussed, and the assumption of a constant efficiency of MCP yield is even more questionable. Rather, the efficiency of MCP yield depends on many factors such as the fractional rumen passage rates of the liquid, solid, concentrate and forage fraction (Thomas, 2004), the feed components as well as their fractional degradation rates (Tamminga *et al.*, 2007), DM intake (Volden, 2011), and the type of degraded substrates (van Duinkerken *et al.*, 2011). Among these factors passage rate plays a prominent role, since it affects microbial efficiency by affecting the energy requirement for maintenance of the microbes (Hackmann and Firkins, 2015). Therefore, this simplification by omitting different fractional passage rates of the liquid and solid phase is one of the main limitations. On the other hand, the missing outflow is a substantial disadvantage of closed *in vitro*-systems in which both, nutrient supply and removal of degradation products do not occur, affecting the activity of the microbial population (Gidlund *et al.*, 2018; Westreicher-Kristen and Südekum, 2018). Additionally, at long incubation times (low passage rates) effective prCP *in vitro* decreases due to progressive feed CP degradation and lysis of microbes (Steingaß and Südekum, 2013). Therefore, short *in vitro* incubation times (corresponding to passage rates at high feed intakes) result in

more reliable estimates of *in vivo* MCP synthesis (Westreicher-Kristen and Südekum, 2018).

It is an obvious limit of the present study, that two indirect methods were compared against each other, since as mentioned above the *in situ* method cannot be considered as an absolute reference. However, it can serve as a guide due to its widespread use (GfE, 2001; NRC, 2001; Thomas, 2004; van Duinkerken *et al.*, 2011), whereas its high effort prevents routine application. The present study was designed to evaluate the effects of wilting and inoculant application on RUCP, MCP and prCP. Although the study does not give protein values of grass silages directly applicable for ration formulation, it provides a valid indication about the effect of wilting and inoculant administration on the protein value of grass silage for dairy cows.

In vivo experiments using animals fitted with an abomasal or a duodenal cannula (Huhtanen, 2005) might be seen as the most appropriate method to generate true feed protein values. Besides the need for surgically prepared animals, digesta flow and microbial markers are required to measure microbial protein synthesis (Huhtanen, 2005), which are regarded as potential sources of several errors (Hristov *et al.*, 2019). The ultimate method to define the protein value of a diet is the measurement of the response in milk protein yield of lactating animals (Huhtanen and Broderick, 2016). However due to the enormous effort, such trials are not suitable for routine evaluation. Furthermore, since various factors affect protein yield, it is difficult to formulate diets where protein supply is the first-limiting factor. Nevertheless, in future a small number of such studies should be performed to calibrate *in vitro* and *in situ* measurements.

Conclusions

The positive effect of wilting on RUCP concentration of grass silages as reported in the literature was confirmed by our results, although the effects were less pronounced in our study. Despite statistically significant the effect of wilting on effective prCP was small in absolute terms and can be considered as biologically not meaningful. Inoculant containing LAB increased RUCP content to a small extent, whereas effective prCP remained unaffected. Although representing real fermentation conditions in the rumen less accurately, the modHGT shows some significant advantages over the *in situ* method – besides better suitability for routine analysis – which are the potential to better reflect the incomplete additivity of RUCP and MCP and avoiding the problematic simplification of assuming a constant efficiency of MCP synthesis.

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CHAPTER 3

***In situ* and *in vitro* determination of the protein value of feeds for ruminants¹**

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¹ Besides minor formal changes, this version is identical to the publication.

3.1 Abstract

The objective of this study was to estimate the amount of post-rumen crude protein (**prCP**), a precursor to metabolisable protein for ruminants, of feeds and feed mixtures using an *in situ* and *in vitro* method, and to contrast the results of both approaches. For this, 34 samples were examined: 9 feeds, 13 feed mixtures made thereof, and 12 feed mixtures provided by commercial dairy farms. As a minor aspect, additivity of protein values from feeds was evaluated by comparing measured and calculated values of feed mixtures. Effective prCP was calculated for assumed rumen passage rates (k) of 0.05/h (**prCP_s**) and 0.08/h (**prCP₈**) by *in situ* measurements of rumen undegraded CP and fermented organic matter (**FOM**) assuming an efficiency of 181 g microbial CP/kg FOM. Additionally, effective prCP *in vitro* was estimated using the modified Hohenheim gas test (**modHGT**) through incubation in rumen-fluid buffer solution for 8 h and 24 h followed by ammonia distillation. *In vitro* estimations were highly correlated with *in situ* values for both passage rates ($k = 0.05/\text{h}$: $P < 0.001$, $R^2 = 0.68$; $k = 0.08/\text{h}$: $P < 0.001$, $R^2 = 0.76$). The *in vitro* method yielded higher values for effective prCP than the *in situ* approach with 29 g/kg OM ($k = 0.05/\text{h}$) and 37 g/kg OM ($k = 0.08/\text{h}$) on average for all samples. Small positive associative effects – reflecting non-additivity – were found, averaging at 2.2 g prCP₈/kg OM ($p < 0.05$) for the *in situ* and 10.7 g prCP₈/kg OM ($P < 0.001$) for the *in vitro* approach. Due to the need of an assumption of a certain value for microbial yield *in situ*, effective prCP might be more accurately estimated *in vitro*, accounting for nutrient-specific efficiencies as well as interactions between carbohydrate and protein degradation by rumen microbes. Furthermore, the modHGT highlighted associative effects more pronounced and seems suitable as a routine method due to the comparably low effort and high sample throughput. The potential of the modHGT to determine the protein value of feeds could be demonstrated by our study.

Keywords: associative effects; compound feeds; ruminants; rumen undegradable crude protein; microbial crude protein yield

3.2 Introduction

Protein requirement of dairy cows should be precisely met by feeding, to avoid negative effects on health and performance, and to minimize excess N and consequently environmental pollution (Dijkstra *et al.*, 2013; Hristov *et al.*, 2019). In this context, one of the key variables in feed evaluation is the content of post-rumen crude protein (**prCP**) corresponding to the sum of rumen undegraded CP (**RUCP**) and microbial CP synthesized in the rumen (**MCP**), which is referred to as utilisable crude protein at the duodenum (**uCP**, **nXP** in German) in the German protein evaluation system (GfE, 2001).

The *in situ* technique is a frequently used method to estimate RUCP, but high effort and limited sample throughput prevent its routine application. However, MCP yield has to be estimated from variables describing the amount of energy available for microbial growth (Edmunds *et al.*, 2012). This estimate differs between protein evaluation systems, for example, MCP is calculated as a function of digestible organic matter (**DOM**; NRC, 2001) or using DOM corrected for substances that provide little or no energy to rumen microbes, such as fermentation products in silages, ether extract (**EE**), RUCP (Vérité and Peyraud 1989) and bypass starch (Tamminga *et al.*, 1994). Furthermore, microbial efficiency depends on a variety of factors such as feed fractions (soluble, degradable, non-degradable), properties of the feeds (forage vs. concentrate) and kind of fermented nutrients (van Duinkerken *et al.*, 2011). Thus, the estimate of MCP yield is variable, associated with various assumptions and consequently limited in its accuracy, which is especially crucial, since MCP is quantitatively more important than RUCP (Steingäß and Südekum 2013). Furthermore, Voigt and Piatkowski (1987) reported an interaction between measurements of RUCP and MCP, thus a more accurate assessment of the protein value might be received by a measurement of total prCP. The modified Hohenheim gas test (**modHGT**, Steingäß and Südekum, 2013) offers a simple method to estimate prCP from ammonia N concentration of samples incubated in rumen-fluid buffer solution and bypasses the need to estimate RUCP (Südekum and Böttger 2019). Gidlund *et al.* (2018) described a close relationship between *in vitro* determined prCP and omasal flow of CP in lactating dairy cows, while previous studies confirmed the reliability of the modHGT (Edmunds *et al.*, 2012; Westreicher-Kristen *et al.*, 2015) in comparison to reference values calculated based on GfE (2001).

In vitro methods provide an alternative to *in vivo* or *in situ* studies, which are criticized for animal welfare reasons, and simplify standardization while giving the possibility for routine application.

Moreover, the occurrence of associative effects on *in vitro* prCP in feed mixtures was demonstrated by Zhao *et al.* (2005) and the above-mentioned multiple influences on MCP synthesis such as nutrient specific efficiencies might also be considered *in vitro*.

The aim of this study was to validate the suitability of the modHGT in estimating prCP *in vitro* from different feeds and feed mixtures, where prCP estimated using *in situ* RUCP and estimates of MCP (based on *in situ* measurements of fermented organic matter, **FOM**) served as reference. It was hypothesised that the modHGT yields in prCP values like those received by the *in situ* method and additionally shows advantages regarding practicability and sensitivity to influences on MCP synthesis and interactions between OM and CP degradation.

3.3 Materials and methods

The studies reported here were conducted in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2014). The *in situ* ruminal degradation experiment was performed at the Department of Animal Nutrition of the Hohenheim University, Stuttgart and was approved by the Regional Council Stuttgart of the Ministry of Rural Areas and Consumer Protection of the federal state Baden-Württemberg, Germany. The *in vitro* incubation was carried out at the Institute of Animal Nutrition and Physiology of the Christian-Albrechts-Universität, Kiel and the collection of rumen-fluid from fistulated heifers was approved by the Animal Welfare commission of the Ministry of Energy, Agriculture, Environment, and Rural Areas of the federal state of Schleswig-Holstein, Germany.

3.3.1 Samples

Nine feeds : grass silage (freeze-dried), maize silage (freeze-dried), grass hay, wheat straw, corn, dried distillers' grains with solubles (**DDGS**), rapeseed meal, soybean meal, and sunflower meal were used for lab scale production of 13 feed mixtures (500 g DM each, **Table 3-1**) added with mineral mixture and subsequently ground to pass through a 4 mm screen. The formulation aimed to cover a large variety of feed mixtures and a range of about 120 to 210 g CP/kg DM. Feed mixtures were numbered from 1 to 13 based on increasing CP concentration. Additionally, 12 feed mixtures provided by commercial dairy farms (freeze-dried) covering a broad range of different diets were used, which were numbered from 1 to 12 according to their CP concentration and are hereinafter referred to as "commercial feed mixtures".

Table 3-1: Ingredient composition of the 13 feed mixtures prepared on lab scale (g/kg DM).

Feed component	Feed mixtures (No.)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Grass silage	91	-	151	264	152	-	252	181	152	250	182	423	152
Maize silage	413	248	295	147	296	-	245	410	345	244	413	177	344
Grass hay	86	168	142	83	143	584	-	-	-	-	-	-	-
Wheat straw	-	70	-	-	-	-	-	-	-	-	-	-	-
Corn	299	402	323	396	285	173	273	272	250	345	120	75	179
DDGS	96	-	-	95	-	-	215	-	-	-	-	311	-
Rapeseed meal	-	97	-	-	-	227	-	-	-	-	271	-	309
Soybean meal	-	-	74	-	-	-	-	122	-	146	-	-	-
Sunflower meal	-	-	-	-	110	-	-	-	238	-	-	-	-
Mineral mixture	15	15	15	15	15	15	15	15	15	15	15	15	15

DDGS = dried distillers' grains with solubles.

Chemical composition of the 9 feeds and 25 feed mixtures used for *in situ* and *in vitro* incubation is given in **Table 3-2**. All samples were analysed for DM, crude ash (CA) and CP according to the official analytical methods in Germany (methods 3.1, 8.1 and 4.1.1, respectively; VDLUFA, 2007). Organic matter was calculated as 1000 – CA, where CA is in g/kg DM. Feeds and feed mixtures were analysed according to VDLUFA (2007) for neutral detergent fibre (assayed without a heat stable amylase and expressed exclusive of residual ash, **NDFom**; method 6.5.1), acid detergent fibre (expressed exclusive of residual ash, **ADFom**; method 6.5.2), acid detergent lignin (**ADL**; method 6.5.3) and sugar (method 7.1.1). Starch was quantified after enzymatic hydrolysis and subsequent oxidation by glucose oxidase as described by Brandt *et al.* (1987). Prior to analysis, samples were ground to pass through a 1.0-mm screen (Retsch centrifugal mill ZM1, Retsch GmbH, Haan, Germany) for DM, CA, CP, and sugar, through a 2.0-mm screen for NDFom, ADFom and ADL, and through a 0.2-mm screen for analysis of starch. NDFom (assayed with a heat stable amylase; **aNDFom**), ADFom, starch and sugar contents of commercial feed mixtures were determined by Landwirtschaftliche Kommunikations- und Servicegesellschaft mbH, Lichtenwalde, Germany, using near-infrared reflectance spectroscopy.

Table 3-2: Chemical composition and digestibility of organic matter (DOM) of feeds and feed mixtures.

Chemical composition (g/kg DM)	CA	CP	NDFom	ADFom	ADL	Starch	Sugar	DOM ¹
Feeds								
Grass silage	94	173	476	322	42	0	9	72.6
Maize silage	35	75	505	266	31	299	0	68.2
Grass hay	62	91	636	338	61	0	133	56.2
Wheat straw	65	38	810	528	63	0	0	54.8
Corn	22	118	153	42	16	628	26	87.7
DDGS	50	290	370	151	68	74	83	72.6
Rapeseed meal	79	368	300	208	82	0	92	78.4
Soybean meal	63	525	128	71	6	38	101	89.5
Sunflower meal	67	383	335	254	67	0	77	72.1
Mineral mixture	911	3	13	0	5	0	0	–
Feed mixtures produced on lab scale								
No.1	54	122	414	205	27	357	29	75.6
No.2	54	128	425	218	33	373	10	74.6
No.3	59	141	429	196	22	316	37	76.8
No.4	60	141	378	184	28	308	28	77.2
No.5	55	146	388	217	29	279	44	74.9
No.6	64	163	517	268	51	163	115	65.0
No.7	62	165	392	183	39	279	23	75.7
No.8	64	178	372	192	22	316	12	76.9
No.9	62	185	352	222	36	288	21	72.0
No.10	61	192	352	196	17	280	30	79.7
No.11	70	193	442	239	46	196	15	72.1
No.12	73	196	452	236	54	126	34	73.9
No.13	68	211	385	229	45	235	38	77.0
Commercial feed mixtures provided by dairy farms								
No.1	64	144	418	229	-	270	27	74.4
No. 2	70	152	456	240	-	186	32	74.1
No.3	54	154	366	204	-	296	25	76.9
No.4	77	154	441	233	-	211	30	75.1
No. 5	66	155	410	227	-	244	36	73.0
No.6	65	157	407	227	-	266	34	76.7
No.7	67	158	395	217	-	247	35	75.3
No. 8	77	162	463	252	-	161	39	77.0
No. 9	85	163	420	252	-	142	24	74.8
No.10	66	167	367	206	-	248	39	76.2
No.11	76	177	397	226	-	214	39	75.6
No.12	73	186	373	210	-	249	40	77.9

DM = dry matter; CA = crude ash; CP = crude protein; NDFom = neutral detergent fibre assayed with (commercial feed mixtures) or without (feeds and feed mixtures) a heat stable amylase exclusive of residual ash; ADFom = acid detergent fibre exclusive of residual ash; ADL = acid detergent lignin; DDGS = dried distillers' grains with solubles; - = not determined.

¹calculated according to Menke and Steingäß (1988) as: $DOM(\%) = 14.88 + 0.8893 \cdot GP24 + 0.0448 \cdot CP$ (g/kg DM) $+ 0.0651 \cdot CA$ (g/kg DM), where GP24 is corrected gas production after 24 h of incubation (ml/200 mg).

3.3.2 *In situ* procedure

The *in situ* procedure was conducted using four ruminally cannulated and lactating Jersey-cows with an average body weight of 546 kg and an average milk yield of 18 kg/d. The cows received a mixed ration composed (in kg/kg DM) of 0.25 concentrate mixture (0.27 barley, maize, and faba beans each, and 0.20 peas), 0.24 maize silage, 0.24 grass silage, 0.17 meadow hay, 0.05 rapeseed meal, 0.02 barley straw and 0.02 of a mineral mixture. Feed and water were offered for *ad libitum* consumption. Mean DM intake was 15 kg/d. For *in situ* incubations, feed and feed mixtures were ground to pass through a 2 mm sieve (mill: Brabender GmbH, Duisburg, Germany) and about 1.4 g on DM basis of each sample was placed into polyester bags (5 × 10 cm, 50 µm pore size, 14 mg DM sample/cm² polyester bag; ANKOM Technology Macedon, NY, USA). Bags were fixed to an anchor weight (600 g) and introduced into the rumen after morning feeding in the ventral sac. Immediately before rumen incubation, bags were soaked in warm tap water (≈39 °C) for 5 min. Six bags per sample and time point were assigned to the four cows (i.e., one or two repetitions each per cow and time point) and incubated for 8, 24 and 48 h. Polyester bags were removed from the rumen after each corresponding incubation time, subsequently immersed in ice-water to stop microbial activity. Additionally, six polyester bags per sample without incubation were used for the determination of the bag content at the initial 0 h time point. All bags (including 0 h bags) were thorough and carefully washed by hand in cold tap water until the water was no longer turbid. Afterwards, bags were freeze-dried, weighted, and stored until analysis of CA and CP (steam distillation).

Water-soluble (**WS**) fraction was measured in quadruplicate by mixing samples (1 g fresh matter) in 50 ml water (37 °C) for 1 min before filtration through filter paper (No. 615 1/4, Ø: 150 mm, Macherey-Nagel, Germany). Water-insoluble fraction escaping from the polyester bags as small particles (**SP**) was calculated by subtracting WS fraction from 0 h washout fraction.

Out of the 6 bag residuals per feedstuff and time point, 3 bags each were used for the determination of CA and CP concentration, respectively. It was assumed that the CA concentration in the 3 bags analysed did not differ from the CA concentration in the 3 bags used for the N determination and *vice versa*. Since variation of N concentration between the 3 residues did account for a negligible small proportion of the variation in CP degradability within feedstuff and time point, mean CP concentration was used for calculating CP degradation for all 6 bags. This implicates

that variation of CP degradability values between bags are nearly completely caused by differences in DM degradability.

Therefore, 6 values of OM and the CP disappearance (**DI**) per feedstuff and time point were obtained and corrected (**C**) for loss of SP according to Hvelplund and Weisbjerg (2000) as:

$$CDI = DI - SP \cdot (1 - ((DI - (SP + WS)) / (1 - (SP + WS)))) \quad (3-1)$$

where CDI, DI, SP and WS are given as fraction (g/g) of OM or CP, respectively.

Subsequently, CDI of CP was corrected for microbial attachment (**MA**) according to Krawielitzki *et al.* (2006) with:

$$MA = A_{\max} \cdot (1 - e^{-R \cdot t}) \quad (3-2)$$

where MA is at time t (% of residue CP), t is incubation time in (h), A_{\max} is the maximum extent of bacterial colonization (% of residue CP) at $t \approx \infty$, and R is the rate of colonization (/h). Values for A_{\max} and R were calculated as:

$$A_{\max} (\% \text{ of residue CP}) = 50.6 + 0.48 \text{ NDF } (\% \text{ DM}) - 0.77 \text{ CP } (\% \text{ DM}) \quad (3-3)$$

$$R (/h) = 13.3 + 0.09 \text{ NDF } (\% \text{ DM}) - 0.35 \text{ CP } (\% \text{ DM}) \quad (3-4)$$

In this study, instead of NDF, A_{\max} and R were calculated using NDFom for feeds and feed mixtures, and aNDFom for commercial feed mixtures.

Ruminal degradation of OM and CP depending on the time was calculated adapting the exponential model suggested by Ørskov and McDonald (1979) to CDI (g/kg) using the four observations at time points t ($t = 0, 8, 24, 48$ h) for every animal ($n = 4$):

$$\text{deg} = a + b \cdot (1 - e^{-c \cdot t}) \quad (3-5)$$

where deg describes the cumulative degradation (g/kg) of OM or CP at time t (h), a represents the soluble fraction (g/kg), b the insoluble but potentially degradable fraction (g/kg) and c the degradation rate of b (/h). Potential degradation is the sum of a and b fraction (asymptote of the curve) and is referred as $a+b$ fraction (g/kg), which was constrained to not exceed 1000 g. Lag phase could not be considered due to few incubation time points.

Effective degradability of CP (g/kg CP) and OM (g/kg OM) was calculated according to Ørskov and McDonald (1979) for ruminal passage rates (k) of 0.05/h (ED₅) and 0.08/h (ED₈) indicated as e.g., ED₅CP, ED₈CP, ED₅OM and ED₈OM.

$$ED_k = a + ((b \cdot c) / (c + k)) \quad (3-6)$$

Effective RUCP _{k} was calculated for the same passage rates (i.e., RUCP₅, RUCP₈, respectively) as follows:

$$RUCP_k \text{ (g/kg CP)} = 1000 - ED_k \text{CP (g/kg CP)} \quad (3-7)$$

$$RUCP_k \text{ (g/kg OM)} = RUCP_k \text{ (g/kg CP)} \cdot \text{CP} / \text{OM} \quad (3-8)$$

where CP and OM are given as a fraction of DM (g/g).

The amount of effective prCP *in situ* (**prCP _{k} *in situ***; g/kg OM) was calculated by the sum of RUCP _{k} (g/kg OM) and MCP _{k} synthesized in the rumen (g/kg OM) for the respective passage rates:

$$\text{prCP}_k \text{ in situ} = RUCP_k + MCP_k \quad (3-9)$$

$$MCP_k = 0.181 \cdot ED_k \text{OM} \quad (3-10)$$

where MCP _{k} was assumed to be 181 g per kg effective FOM (Lebzien and Voigt, 1999). The amount of effective FOM derived from *in situ* incubations and corresponds to ED _{k} OM of the respective passage rate (g/kg OM) in this study.

3.3.3 *In vitro* estimation of post-rumen crude protein

The *in vitro* estimation of effective prCP was conducted using the modHGT described by Steingäß and Südekum (2013), measuring ammonia production from feed incubated in a rumen-fluid buffer solution. The procedure is described in detail by Westreicher-Kristen *et al.* (2017). Briefly, two rumen-cannulated German Black Pied heifers served as donor animals, where rumen-fluid was taken 1 h prior to the morning feeding. The ration provided to the heifers consisted of (kg DM) 2.6 grass hay and 2.7 of concentrate divided into two equal meals (08:00 and 16:00 h). Composition (g/kg as fed) of the concentrate feed was: 232 rye, 210 rapeseed meal, 185 maize gluten feed, 150 milled barley grain, 120 rye bran, 45 beet pulp, 40 maize and 11 calcium carbonate. In addition, heifers received 75 g of mineral premix during

morning feeding and had *ad libitum* access to water. Ground samples (mill: ZM1, Retsch GmbH, Haan, Germany; sieve size 1 mm) were weighed (200 ± 1 mg, on fresh matter basis) and transferred into 100-mL graduated glass syringes (Haeberle Labortechnik GmbH & Co. KG, Lonsee-Ettlenschieß, Germany) closed airproof with vaseline-greased pistons. Additionally, each run included three blank syringes (containing only rumen-fluid buffer solution and no substrate) and three syringes with a standard sample of known prCP content (prCP-standard). Target values of prCP-standard were 232 and 183 g/kg DM for 8 and 24 h of incubation, respectively. The prCP-standard was provided by the Institute of Animal Science, Hohenheim University, Germany, and served as a control for undisturbed conditions. Two replicates in three runs were carried out for each sample and incubation time (8 and 24 h). At the end of each incubation time, gas volume was measured, and the syringes were immediately put on ice to minimize further microbial activity. Subsequently, $\text{NH}_3\text{-N}$ content was determined by steam distillation as described by Westreicher-Kristen *et al.* (2017).

Calculation of prCP *in vitro* using modHGT was done according to the following equation:

$$\text{prCP in vitro (g/kg OM)} = \frac{((\text{NH}_3\text{-N}_{\text{blank}} + \text{N}_{\text{sample}} - \text{NH}_3\text{-N}_{\text{sample}}) \cdot 6.25 \cdot 1000) / S}{\text{OM}} \quad (3-11)$$

where $\text{NH}_3\text{-N}_{\text{blank}}$ is the $\text{NH}_3\text{-N}$ amount of the blanks after incubation (mg), N_{sample} is the N amount of the incubated sample (mg), $\text{NH}_3\text{-N}_{\text{sample}}$ is the $\text{NH}_3\text{-N}$ amount of the liquid contained in the syringes after incubation (mg) and S is the total amount of sample on DM basis (mg), OM refers to the OM content of the incubated sample (g/g DM).

Effective prCP *in vitro* (g/kg OM) was calculated for $k = 0.05$ and $k = 0.08/\text{h}$ through linear regression of prCP values after 8 and 24 h of incubation against \ln of time (h) to the values for $\ln(20)$ and $\ln(12.5)$, respectively.

3.3.4 Associative effects on protein value

Additivity of protein values of feeds was tested by comparing the measured values of the feed mixtures (No. 1–13) with the sum of the respective proportions of protein value from feeds used (calculated feed mixtures). Differences in means between measured and calculated feed mixtures were computed for effective RUCP, effective MCP, effective prCP *in situ* and effective prCP *in vitro*.

3.3.5 Statistical analysis

The statistical model used included the sample as a fixed effect and a random effect for animal (*in situ*) or run (*in vitro*), respectively. Linear regressions between effective prCP determined *in situ* and *in vitro* were performed and characterized by the coefficient of determination (R^2) and the root mean square error (RMSE). Comparison of the mean of feed mixtures ($n = 13$) and the mean of summed feed mixtures ($n = 13$) was done using paired t-test. Significant differences for *in situ* values or effective prCP *in vitro*, respectively, were considered as associative effects and expressed in the respective unit (g/kg OM or g/kg CP). All reported values are least squares estimates (means, intercepts, slopes) and significance was declared at $P < 0.05$. Calculations were done using Software R (version 3.6.1, The R Foundation for Statistical Computing, Vienna, Austria).

3.4 Results

3.4.1 *In situ* and *in vitro* measurements

Feeds

An overview of estimated *in situ* and *in vitro* values of feeds is given in **Table 3-3**. Coefficients of the exponential model (a , b , c) do not reflect the feed properties because of the limited number of incubation time points, rather they are presented for calculating effective degradability. Effective FOM varied from 303 to 774 g/kg OM ($k = 0.05/\text{h}$) and from 237 to 705 g/kg OM ($k = 0.08/\text{h}$), where grass hay, maize silage and wheat straw represented the lower third, DDGS, soybean meal and sunflower meal the upper third.

Effective RUCP was lowest in sunflower meal (189 g/kg CP, $k = 0.05/\text{h}$) and grass silage (240 g/kg CP, $k = 0.08/\text{h}$) and highest in wheat straw (448 and 534 g/kg CP, for $k = 0.05$ and $k = 0.08/\text{h}$, respectively).

Effective MCP was lowest in wheat straw and highest in soybean meal at both passage rates. Effective RUCP was low (< 65 g/kg OM) in grass silage, grass hay, maize silage, and wheat straw and higher (> 140 g/kg OM) in rapeseed meal and soybean meal. *In situ* prCP varied between 73 and 306 g/kg OM ($k = 0.05/\text{h}$) and 64 to 346 g/kg OM ($k = 0.08/\text{h}$) with lowest and highest values for wheat straw and soybean meal, respectively. *In vitro* prCP varied in a smaller range from 95 to 266 g/kg OM ($k = 0.05/\text{h}$) and 75 to 297 g/kg OM ($k = 0.08/\text{h}$), with lowest and highest values for wheat straw and DDGS, respectively.

Feed mixtures

In situ and *in vitro* values of feed mixtures and commercial feed mixtures are given in **Table 3-4**. Effective FOM varied between 434 and 606 g/kg OM for $k = 0.05/\text{h}$ and between 381 and 541 g/kg OM for $k = 0.08/\text{h}$, with mean values of 540 and 472 g/kg OM, respectively.

Soluble CP fraction a was 255 g/kg CP on average, showing a huge margin from 36 to 357 g/kg CP. Potentially degradable CP fraction $a+b$ varied from 859 to 958 g/kg CP. The proportion of effective RUCP averaged at 287 ($k = 0.05/\text{h}$) and 355 g/kg CP ($k = 0.08/\text{h}$) ranging from 231 to 394 g/kg CP and from 290 to 483 g/kg CP, respectively.

Effective MCP was 98 ($k = 0.05/\text{h}$) and 85 g/kg OM ($k = 0.08/\text{h}$) on average. Effective RUCP varied in the range of 39.5 to 68.8 g/kg OM ($k = 0.05/\text{h}$) and of 48.0 to 84.2 g/kg OM ($k = 0.08/\text{h}$). Therefore, effective prCP_5 *in situ* averaged at 148 g/kg OM and varied between 133 and 169 g/kg OM. A slightly lower mean value of 147 g/kg OM was found for prCP_8 *in situ*. The *in vitro* method ranked effective prCP values similarly to *in situ*, but they were generally higher with an average of 184 and 192 g/kg OM for prCP_5 and prCP_8 *in vitro*.

Table 3-3: *In situ* and *in vitro* values of feeds.

	Feed									SEM ¹	SD ¹	
	GS	MS	Hay	Straw	Corn	DDGS	RSM	SBM	SFM		ran	res
<i>In situ</i> ² degradation of organic matter (OM; g/kg OM)												
<i>a</i>	325	164	247	81	96	337	234	293	225	6.3	3.2	12.2
<i>b</i>	457	536	389	782	877	442	591	670	518	18.6	<0.01	37.2
<i>a+b</i>	782	699	635	862	973	779	825	963	744	19.2	<0.01	38.3
<i>c</i>	0.081	0.069	0.029	0.021	0.063	0.108	0.087	0.129	0.245	0.008	<0.01	0.015
FOM ₅	607	470	383	303	581	629	608	774	655	7.7	5.4	14.3
FOM ₈	554	407	345	237	479	581	541	705	615	8.3	5.8	15.6
<i>In situ</i> ² degradation of crude protein (CP; g/kg CP)												
<i>a</i>	548	629	189	0	85	122	84	103	50	5.3	0.6	10.6
<i>b</i>	354	222	628	798	862	770	868	874	901	5.3	0.7	16.8
<i>a+b</i>	902	852	817	798	948	892	953	977	951	7.7	<0.001	15.4
<i>c</i>	0.119	0.044	0.108	0.113	0.073	0.116	0.089	0.111	0.272	0.006	<0.01	0.011
RUCP ₅	203	275	382	448	405	344	359	295	189	4.7	3.1	8.9
RUCP ₈	240	299	451	534	505	426	458	390	254	5.6	3.5	10.6
Microbial crude protein (MCP _k ; g/kg OM) calculated based on FOM _k <i>in situ</i>												
MCP ₅	110	85	69	55	105	114	110	140	119	13.9	9.9	25.9
MCP ₈	100	74	63	43	87	105	98	128	111	15.1	10.5	28.2
Effective rumen undegraded crude protein (RUCP _k ; g/kg OM) based on <i>in situ</i> measurement												
RUCP ₅	38.6	21.4	37.2	18.1	48.6	105	144	166	77.5	1.40	0.61	2.73
RUCP ₈	45.8	23.2	43.8	21.5	60.7	130	183	219	104	1.62	0.68	3.18
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k <i>in situ</i> ; g/kg OM)												
prCP ₅	148	106	107	73	154	219	254	306	196	1.1	<0.001	2.2
prCP ₈	146	97	106	64	147	235	281	346	216	1.2	<0.001	2.4
Effective post-rumen crude protein determined <i>in vitro</i> by modified Hohenheim gas test (prCP _k <i>in vitro</i> ; g/kg OM)												
prCP ₅	158	148	136	95	192	266	225	244	171	2.5	0.5	4.2
prCP ₈	164	144	136	75	209	297	281	281	196	2.8	1.6	4.5
Difference between prCP _k <i>in situ</i> and prCP _k <i>in vitro</i> ; g/kg OM												
prCP ₅	-10	-42	-29	-22	-38	-47	29	62	25	-	-	-
prCP ₈	-18	-47	-30	-11	-62	-62	0	65	20	-	-	-

GS = grass silage; MS = maize silage; Hay = grass hay; Straw = wheat straw; DDGS = dried distillers' grains with solubles; RSM = rapeseed meal; SBM = soybean meal; SFM = sunflower meal; SEM = standard error of the mean; SD = standard deviation; ran = SD of the random factor (animal, run); res = SD of the residuals; *a* = soluble fraction; *b* = insoluble but potentially degradable fraction; *a+b* = potential degradation; *c* = degradation rate of *b*; FOM_k, fermented OM at passage rate *k* (*k* = 0.05, 0.08/h); RUCP_k = rumen undegraded CP at passage rate *k*; - = not determined.

¹calculated including all samples (feed, feed mixtures and commercial feed mixtures),

²calculated according to Ørskov and McDonald (1979).

Table 3-4: *In situ* and *in vitro* values of feed mixtures.

	Feed mixtures produced on lab scale (No.)													SEM ¹	SD ¹	
	1	2	3	4	5	6	7	8	9	10	11	12	13		ran	res
<i>In situ</i> ² degradation of OM (g/kg OM)																
<i>a</i>	168	106	172	195	185	204	220	151	172	192	193	286	199	6.3	3.2	12.2
<i>b</i>	607	695	623	624	629	455	612	719	599	682	591	488	604	18.6	<0.01	37.2
<i>a+b</i>	775	801	795	820	814	659	832	870	771	874	784	773	803	19.2	<0.01	38.3
<i>c</i>	0.072	0.054	0.078	0.081	0.05	0.052	0.07	0.055	0.11	0.078	0.068	0.092	0.077	0.008	<0.01	0.015
FOM ₅	526	465	549	579	494	434	574	522	583	606	533	596	565	7.7	5.4	14.3
FOM ₈	455	384	477	508	423	381	503	439	518	527	464	541	495	8.3	5.82	15.6
<i>In situ</i> ² degradation of CP (g/kg CP)																
<i>a</i>	282	117	265	287	245	36	260	192	176	227	226	286	229	5.3	0.6	10.6
<i>b</i>	604	757	658	618	642	823	646	712	736	731	692	591	700	5.3	0.7	16.8
<i>a+b</i>	886	874	923	906	887	859	906	904	912	958	918	877	928	7.7	<0.001	15.4
<i>c</i>	0.106	0.11	0.115	0.105	0.123	0.113	0.106	0.113	0.191	0.106	0.103	0.132	0.109	0.006	<0.01	0.011
RUCP ₅	307	364	277	295	299	394	302	315	241	277	308	287	292	4.7	3.1	8.9
RUCP ₈	373	446	348	363	366	483	373	392	305	357	384	348	369	5.6	3.5	10.6
Microbial crude protein (MCP _k ; g/kg OM) calculated based on FOM _k <i>in situ</i>																
MCP ₅	95	84	100	105	90	79	104	94	106	110	97	108	102	13.9	9.9	25.9
MCP ₈	82	70	86	92	77	69	91	80	94	95	84	98	90	15.1	10.5	28.2
Effective rumen undegradable crude protein (RUCP _k , g/kg OM) based on <i>in situ</i> measurement																
RUCP ₅	39.5	49.3	41.5	44.3	46.1	68.8	53.2	59.9	47.4	56.7	64	60.7	66.1	1.4	0.61	2.73
RUCP ₈	48	60.4	52.1	54.5	56.5	84.2	65.7	74.5	60.1	73.1	79.9	73.5	83.3	1.6	0.68	3.18
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k <i>in situ</i>), g/kg OM																
prCP ₅	135	133	141	149	136	147	157	154	153	166	161	169	169	1.1	<0.001	2.2
prCP ₈	130	130	139	146	133	153	157	154	154	169	164	171	173	1.2	<0.001	2.4
Effective post-rumen crude protein determined <i>in vitro</i> by modified Hohenheim gas test (prCP _k <i>in vitro</i>), g/kg OM																
prCP ₅	180	176	181	191	176	192	198	182	167	194	192	201	209	1.9	0.6	4.7
prCP ₈	183	182	186	204	179	201	210	192	182	201	206	214	227	2.6	1.7	5.9
Difference between prCP _k <i>in situ</i> and prCP _k <i>in vitro</i> ; g/kg OM																
prCP ₅	-45	-43	-40	-42	-40	-45	-41	-28	-14	-28	-31	-32	-40	-	-	-
prCP ₈	-53	-52	-47	-58	-46	-48	-53	-38	-28	-32	-42	-43	-54	-	-	-

SEM = standard error of the mean; SD = standard deviation; ran = SD of the random factor (animal, run); res = SD of the residuals; *a* = soluble fraction; *b* = insoluble but potentially degradable fraction; *a+b* = potential degradation; *c* = degradation rate of *b*; FOM_k, fermented OM at passage rate *k* (*k* = 0.05, 0.08/h); RUCP_k = rumen undegraded CP at passage rate *k*; - = not determined; ¹calculated including all samples (feed, feed mixtures and commercial feed mixtures), ²calculated according to Ørskov and McDonald (1979).

Table 3-4: continued.

	Commercial feed mixtures provided by dairy farms (No.)												SEM ¹		SD ¹	
	1	2	3	4	5	6	7	8	9	10	11	12	ran.	res.		
<i>In situ</i> ² degradation of OM (g/kg OM)																
<i>a</i>	173	213	195	208	173	176	177	221	224	218	200	208	6.3	3.2	12.2	
<i>b</i>	606	553	593	579	542	593	567	592	579	539	572	581	18.6	<0.01	37.2	
<i>a+b</i>	779	767	788	787	714	769	744	813	803	758	772	789	19.2	<0.01	38.3	
<i>c</i>	0.057	0.069	0.079	0.061	0.084	0.066	0.077	0.075	0.089	0.077	0.081	0.083	0.008	<0.01	0.015	
FOM ₅	493	535	557	511	510	513	519	576	592	545	554	567	7.7	5.4	14.3	
FOM ₈	423	470	489	446	448	444	454	508	526	482	488	501	8.3	5.82	15.6	
<i>In situ</i> ² degradation of CP (g/kg CP)																
<i>a</i>	290	331	302	357	274	218	239	317	285	298	342	295	5.3	0.6	10.6	
<i>b</i>	613	563	593	527	621	675	665	591	633	580	573	622	5.3	0.7	16.8	
<i>a+b</i>	902	894	895	884	895	893	905	908	918	878	916	917	7.7	<0.001	15.4	
<i>c</i>	0.084	0.114	0.139	0.147	0.145	0.117	0.128	0.139	0.153	0.13	0.125	0.161	0.006	<0.01	0.011	
RUCP ₅	327	278	262	251	264	309	282	248	238	283	249	231	4.7	3.1	8.9	
RUCP ₈	398	339	322	303	326	382	351	308	299	343	308	290	5.6	3.5	10.6	
Microbial crude protein (MCP _k ; g/kg OM) calculated based on FOM _k <i>in situ</i>																
MCP ₅	89	97	101	93	92	93	94	104	107	99	100	103	13.9	9.9	25.9	
MCP ₈	77	85	89	81	81	80	82	92	95	87	88	91	15.1	10.5	28.2	
Effective rumen undegradable crude protein (RUCP _k , g/kg OM) based on <i>in situ</i> measurement																
RUCP ₅	50.4	45.4	42.6	41.9	43.8	52	47.8	43.7	42.5	50.7	47.7	46.3	1.4	0.61	2.73	
RUCP ₈	61.2	55.3	52.3	50.6	54.1	64.2	59.5	54.2	53.5	61.5	59.2	58.1	1.62	0.68	3.18	
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k <i>in situ</i>), g/kg OM																
prCP ₅	140	142	144	135	136	145	142	148	150	149	148	149	1.1	<0.001	2.2	
prCP ₈	138	140	141	131	135	145	142	146	149	149	148	149	1.2	<0.001	2.4	
Effective post-rumen crude protein determined <i>in vitro</i> by modified Hohenheim gas test (prCP _k <i>in vitro</i>), g/kg OM																
prCP ₅	175	181	177	180	175	184	182	186	184	184	177	184	1.9	0.6	4.7	
prCP ₈	179	182	182	184	181	191	190	192	191	192	183	193	2.6	1.7	5.9	
Difference between prCP _k <i>in situ</i> and prCP _k <i>in vitro</i> ; g/kg OM																
prCP ₅	-35	-39	-33	-45	-39	-39	-40	-38	-34	-35	-29	-35	-	-	-	
prCP ₈	-41	-42	-41	-53	-46	-46	-48	-46	-42	-43	-35	-44	-	-	-	

SEM = standard error of the mean; SD = standard deviation; ran = SD of the random factor (animal, run); res = SD of the residuals; *a* = soluble fraction; *b* = insoluble but potentially degradable fraction; *a+b* = potential degradation; *c* = degradation rate of *b*; FOM_k, fermented OM at passage rate *k* (*k* = 0.05, 0.08/h); RUCP_k = rumen undegraded CP at passage rate *k*; - = not determined. ¹calculated including all samples (feed, feed mixtures and commercial feed mixtures); ²calculated according to Ørskov and McDonald (1979).

3.4.2 Relationship between *in situ* and *in vitro* values of prCP

The differences between *in situ* and *in vitro* values (prCP *in situ* minus prCP *in vitro*) ranged between -47 to 62 g/kg OM ($k = 0.05/\text{h}$) and from -62 to 65 g/kg OM ($k = 0.08/\text{h}$; Table 3-3, Table 3-4). The largest differences in feeds were observed for soybean meal with 62 g prCP₅ and 65 g prCP₈/kg OM. The largest differences in feed mixtures were observed for no. 1, no. 6, and no. 4 (commercial feed mixture) with -45 g prCP₅/kg OM each, and for no. 4 (commercial feed mixture) with -58 g prCP₈/kg OM. On average of all samples, the modHGT tended to yield higher values of prCP₅ and prCP₈ by 29 and 37 g/kg OM, respectively. Nevertheless, close relationships between *in vitro* and *in situ* determined prCP ($n = 34$) were found at $k = 0.05/\text{h}$ ($R^2 = 0.68$, $P < 0.001$) and $k = 0.08/\text{h}$ ($R^2 = 0.76$, $P < 0.001$). The regression equation for $k = 0.08/\text{h}$ is: $\text{prCP}_8 \text{ in situ} = -58.1 \pm 21.7 + 1.108 \pm 0.11 \text{ prCP}_8 \text{ in vitro}$ (Figure 3-1; solid line), and the slope is not significantly different from 1. The relationship between *in vitro* and *in situ* prCP for feed mixtures and commercial feed mixtures ($n = 25$) was also significant at $k = 0.05/\text{h}$ ($R^2 = 0.57$, $P < 0.001$) and $k = 0.08/\text{h}$ ($R^2 = 0.70$, $P < 0.001$).

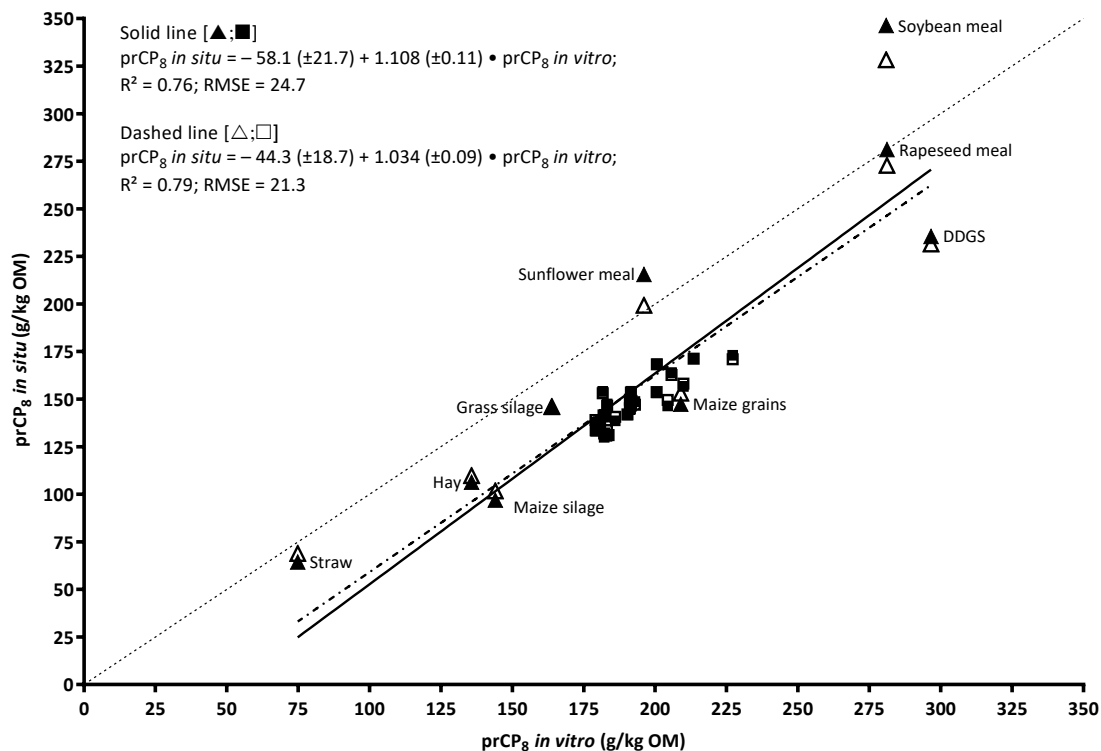


Figure 3-1: Relationship between *in vitro* and *in situ* determined post-rumen crude protein of feeds [\blacktriangle , \triangle] ($n = 9$) and feed mixtures [\blacksquare , \square] ($n = 25$) at passage rate of $k = 0.08/\text{h}$ (prCP₈). The dotted line represents the angle bisector. Contents of prCP₈ *in situ* were calculated as the sum of rumen undegraded crude protein (RUCP) and microbial crude protein (MCP) using either one value of 181 g MCP yield per kg fermented organic matter (FOM; filled symbols) or two different values of 208 g MCP/kg crude protein-free FOM and 104 g MCP/kg fermented crude protein (clear symbols).

3.4.3 Associative effects on protein value

A comparison of measured and calculated feed mixtures for effective RUCP, effective MCP, effective prCP *in situ* and effective prCP *in vitro* is given in **Table 3-5**. Additivity is given for all *in situ* values except for RUCP₈ and prCP₈, where measured values were higher ($P < 0.05$) compared to calculated values but only by 3.3 and 2.2 g/kg OM, respectively. However, effective prCP *in vitro* was higher ($P < 0.001$) with 12.6 ($k = 0.05/\text{h}$) and 10.7 g/kg OM ($k = 0.08/\text{h}$) for measured than calculated feed mixtures, which indicates that modHGT reflected positive associative effects.

Table 3-5: Comparison of measured protein values of feed mixtures produced on lab scale ($n = 13$) and protein value of feed mixtures calculated based on protein value from feeds multiplied by their respective proportion in the feed mixture at passage rates of $k = 0.05/\text{h}$ and $0.08/\text{h}$.

		mean	SD	range		difference		P-value
				min	max	mean	SD	
Effective rumen undegraded crude protein (RUCP _k ; g/kg CP) based on <i>in situ</i> measurement								
RUCP ₅	measured	305	38.7	241	394	-7.1	20.1	0.227
	calculated	312	35.6	241	373			
RUCP ₈	measured	377	44.8	305	483	-5.4	22.0	0.389
	calculated	383	43.3	301	461			
Microbial crude protein synthesized in the rumen (MCP _k ; g/kg OM) ¹								
MCP ₅	measured	97.9	9.41	78.6	110	-0.74	4.4	0.558
	calculated	98.6	5.97	85.0	106			
MCP ₈	measured	85.2	9.41	69.1	98.0	-1.1	4.8	0.440
	calculated	86.3	5.85	74.8	95.8			
Effective rumen undegraded crude protein (RUCP _k ; g/kg OM) based on <i>in situ</i> measurement								
RUCP ₅	measured	53.7	9.74	39.5	68.8	2.2	3.8	0.063
	calculated	51.5	8.58	40.3	66.4			
RUCP ₈	measured	66.6	12.27	48.0	84.2	3.3	4.4	<0.05
	calculated	63.3	10.89	48.7	82.5			
Effective post-rumen crude protein determined <i>in situ</i> (prCP _k <i>in situ</i> ; g/kg OM)								
prCP ₅	measured	152	12.7	133	169	1.4	3.1	0.118
	calculated	150	10.6	135	166			
prCP ₈	measured	152	15.2	130	173	2.2	3.6	<0.05
	calculated	150	12.9	131	170			
Effective post-rumen crude protein determined <i>in vitro</i> (prCP _k <i>in vitro</i> ; g/kg OM) ²								
prCP ₅	measured	188	11.7	167	209	12.6	7.4	<0.001
	calculated	175	9.0	163	193			
prCP ₈	measured	197	14.8	179	227	10.7	6.3	<0.001
	calculated	187	11.1	170	206			

SD = standard deviation

¹calculated based on effective fermented organic matter determined *in situ*

²modified Hohenheim gas test

3.5 Discussion

3.5.1 Principles and limits of the methods

The pool of samples used in this study included different forages, concentrates, and protein rich feedstuffs, as well as their mixtures, covering a wide range of contents of CP (38-525 g/kg DM), NDFom (128-810 g/kg DM) and starch (0-628 g/kg DM), which therefore provided a suitable data set for comparison between the *in situ* and *in vitro* approach conducted herein.

The *in situ* technique is an established method to measure particularly ruminal degradation of feeds over time and is often considered as a reference method to estimate CP degradation (Böttger *et al.* 2019), thus it enables a comparison of different feeds in relation to RUCP content. However, a measurement of MCP yield *in situ* is unfeasible, for this, *in vivo* approaches are needed using duodenally cannulated animals. Due to the difficulty and cost of *in vivo* measurements, MCP yield is usually estimated from variables representing the amount of energy available in the rumen, seen as the main factor responsible for microbial growth (Sauvant and Nozière, 2016). In feed evaluation systems, MCP synthesis is estimated from total tract DOM by NRC (2001), DOM discounting for digestible but non fermented fractions by INRA (2018) or by summation of rumen degraded fractions determined *in situ* by the NorFor-system (Volden, 2011). In the present study, MCP yield was assumed to be 181 g/kg FOM according to Lebzien and Voigt (1999). These authors determined FOM *in vivo* as the difference between OM intake and OM reaching the duodenum minus microbial OM. In our opinion, FOM determined *in situ* as reference seems to be a reliable basis and superior to DOM. The predictive value of FOM measured *in situ* might be improved by correcting for nutrients that do not provide energy for MCP synthesis, such as EE or fermentation products in silages. However, in the present study MCP was calculated using FOM *in situ* without further corrections, because the figure given by Lebzien and Voigt (1999) is related to uncorrected FOM of mixed rations. Thus, discounting fermentation acids and EE from FOM in our data set would have required respective information, which are not provided for the diets in their study. Indeed, the greatest uncertainties in evaluation of protein value of feeds concern the estimate of MCP (Sauvant and Nozière, 2016), which is quantitatively more important than RUCP (Steingäß and Südekum, 2013) and accounted for 62% on average of effective prCP *in situ* in feed mixtures of the present study. The fact of non-additivity of RUCP and MCP from independent measurements (Voigt and Piatkowski, 1987) was considered in our study by *in situ*

measurement of both, RUCP and FOM. The use of a constant efficiency of MCP yield (181 g/kg FOM) results in a decrease of prCP *in situ* with increasing passage rates without consideration of possible changes in efficiency of MCP yield (Krieg *et al.*, 2018). Rather, the efficiency of MCP synthesis differs between liquid, forage and concentrate fractions (Thomas, 2004), and also varies between fermented substrates (starch, NDF, proteins and other carbohydrates; Volden, 2011). On the basis of a meta-analysis, Sauvant and Nozière (2016) confirmed the substrate specific microbial growth, which is already included in the Nordic (NorFor; Volden, 2011) and the Dutch (DVE/OEB; van Duinkerken *et al.*, 2011) protein evaluation systems with lower efficiencies for proteins than for carbohydrates. The DVE/OEB2010 system (van Duinkerken *et al.*, 2011) is based on the yield of ATP from fermented substrates into VFA, with 27.3 mmol/g polysaccharides (structural polysaccharides, starch), 23.9 mmol/g mono-, di- and oligosaccharides, and only 13.7 mmol/g CP. These nutrient-specific efficiencies could also have affected differences between *in situ* and *in vitro* results in our study.

Therefore, based on the assumption, that the efficiency is twice as high in CP-free FOM than in degraded CP, prCP₈ *in situ* was recalculated assuming an efficiency of MCP yield of 104 g/kg degraded CP and 208 g/kg CP-free FOM derived from the values 181 g MCP/kg FOM and 25.6% degraded CP in FOM (mean value of all samples in our study assumed to be near to that in the study of Lebzien and Voigt, 1999). The relationship between *in vitro* and *in situ* prCP₈ values calculated assuming uniform efficiency (filled symbols) or substrate-specific efficiencies for degraded CP and FOM-CP free (clear symbols), respectively, is given in **Figure 3-1**. This differentiation notably affected prCP₈ *in situ* values of solvent-extracted oilseed meals (sunflower meal, soybean meal, rapeseed meal), since about 40 to 50% of their total FOM composed of fermented CP, approaching the respective values to those obtained *in vitro*. Consequently, assuming a substrate-specific efficiency improved the relationship between both methods ($R^2 = 0.79$, RMSE = 21.3 vs. $R^2 = 0.76$, RMSE = 24.7). Although, this recalculation does not yet provide the true values for prCP₈ *in situ*, it obviously indicated the insufficient assumption of a constant efficiency for MCP synthesis *in situ* and the possible improvement through the *in vitro* estimate. Additionally, slightly higher values of prCP₈ *in vitro* for measured feed mixtures than for calculated feed mixtures indicated positive associative effects, which were less pronounced *in situ*. This in turn would suggest a variable yield of MCP depending on the composition of the FOM, further highlighted by generally lower prCP₈ *in situ* compared to prCP₈ *in vitro*. The modHGT might therefore be more sensitive to reflect differences in the efficiency of microbial CP yield.

Using FOM determined *in situ* to calculate prCP *in situ* considers the ruminal degradation of OM depending on the passage rate (Krieg *et al.*, 2018), however, a uniform passage through the rumen of all OM components cannot be assumed. Rather, fractional passage rates are discussed, which determine both the extent of degradation of the respective substrate and the efficiency of MCP yield (Sauvant and Nozière, 2016). Fractional passage rates largely depends on DM intake (NRC, 2001; Sauvant and Nozière, 2016) and are further differentiated into liquid, concentrate and forage particles (INRA, 2018; Thomas, 2004; Volden, 2011) as well as nature of nutrients (CP, starch, NDF, non starch polysaccharides; van Duinkerken *et al.*, 2011). For the methods used in this study a uniform retention time, which corresponds to a uniform passage rate, is realized, since samples retain in polyester bags (*in situ*) and syringes (*in vitro*) without any outflow. On the other hand, small particles and the soluble fraction can escape from polyester bags without being fermented (Ahvenjärvi *et al.*, 2018), which may lead to an underestimation of RUCP, especially in samples with a higher proportion of soluble CP. In the modHGT system removal of the fermentation products cannot occur, which seems to be a disadvantage of this method especially regarding MCP yield. Indeed, lysis of MCP might be higher *in vitro* than in the rumen *in vivo*, whereas calculated MCP from FOM *in situ* neglects any lysis or supposes lysis identical to the study of Lebzién and Voigt (1999). Therefore, prCP *in vitro* limited to incubation times of 8 and 24 h without consideration of 48 h measurements seems to be necessary to avoid an extensive lysis of MCP already synthesized.

Conclusions

This study shows that both methods provide realistic estimates of the protein value of feeds, when assuming passage rates of 0.05/h and 0.08/h. Despite of their completely different approach a notable accordance between *in situ* and *in vitro* determined prCP values exists. Minor associative effects occur for *in situ* and *in vitro* determined prCP which were more pronounced by the modHGT indicating a higher sensitivity to reflect associative effects, but they were not of practical relevance in both cases. The modHGT can be seen as a sound alternative approach to *in situ*, while reducing effort and providing a routine method. Further studies are needed to validate and to calibrate *in vitro* data with *in vivo* observations.

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CHAPTER 4

General discussion

4.1 Methods for evaluating the protein value of diets for ruminants

This chapter does not aim to give a comprehensive overview of all methods used for the estimation of protein value of ruminant feeds, which have been refined continuously, but still having different types of methodological limitations (Gidlund *et al.*, 2018). The presentation of different protein evaluation systems and their comparison will also not be addressed here. Rather, the focus is on a discussion of some basic methods available for analysing feed protein value to show the suitability of different methods for mapping prCP.

4.1.1 *In vivo* techniques

In vivo techniques serve as references in protein evaluation and are used to validate other methodological approaches, as they provide results received at physiological conditions and thus represents the “true” protein value most accurately. Measurements basically required are intake and excretion (urine, faecal) of nitrogen (N), duodenal flow and proportion of non-ammonia nitrogen (**non-NH₃-N**) from microbial origin in the duodenal chyme. For this, surgical prepared animals fitted with abomasal or duodenal cannulas are needed, where flow samples can be obtained either through total collection or spot sampling (Rohr *et al.*, 1984). Estimation of digesta flow and microbial crude protein (**MCP**) necessitates the application of reliable marker substances, often used are chromium oxide or the isotope ¹⁵N, respectively (van Soest, 1994; Zhao and Cao, 2004). Alternatively, rumen outflow can be determined through the less invasive omasal sampling technique (Huhtanen *et al.*, 1997), where samples are taken from omasum through rumen cannula. Advantageously, there is no contamination through abomasal secretions and studies of digesta flow are precise and demonstrates good repeatability, but efforts will not be lowered because the measurement requires the use of multiple markers (Ahvenjärvi *et al.*, 2000; Broderick *et al.*, 2010). In addition, omasal sampling technique is not prospected to be able to generate a sufficient dataset for calibration of less elaborate methods (Steingäß and Südekum, 2013). Due to the time-consuming, laborious, and expensive *in vivo* measurements (Hristov *et al.*, 2019; Westreicher-Kristen *et al.*, 2015), only small numbers of samples can be analysed. Hence, this technique is not suitable for routine application (Gidlund *et al.*, 2018). It should be taken into account that *in vivo*

values are also only estimates, whose underlying measured values are subject to numerous uncertainties, which can therefore lead to misjudgements of the protein value (Steingäß and Südekum, 2013). *In vivo* methods are limited in accuracy mainly due the reliability of the digesta markers and variation among animals (Stern *et al.*, 1997). For instance, the markers used must be non-absorbable (unless that's the purpose), closely associated with the material it is to mark, and must be homogeneously mixed with the digesta (Faichney, 2005). Furthermore, knowledge about the amount of endogenous N is needed (Flachowsky and Lebzien, 2006), commonly derived from a fixed value related to chyme DM flow.

To overcome the problems of animal welfare and limitations due to the use of markers, methods based on indicator substances were developed. These substances occur under natural conditions at least partly in animal excreta, thereby allowing for non-invasive sampling even under on-farm conditions (Dickhoefer *et al.*, 2015). Degradation of nucleic acids or of adenine and guanine, respectively, forms purine derivatives (**PD**; hypoxanthine, xanthine, uric acid, allantoin), which are predominantly excreted in urine. The urinary PD originate from absorbed purines of exogenous sources (feed, MCP) as well as endogenous contribution of PD from body tissue (Tas and Susenbeth, 2007). The proportion of nucleic acids absorbed from feed is variable but would not contribute significantly to PD excretion in urine (Kehraus *et al.*, 2006), whereas the excretion of endogenous PD is described as a constant per kg metabolic body weight (Chen and Ørskov, 2003). Thus, PD excreted via urine in ruminants mainly originate from absorbed purines from microbial origin. Hence, microbial N flow to the duodenum can be estimated by quantifying urinary PD excretion (Tas and Susenbeth, 2007). First, duodenal absorption of purines from microbial origin is estimated from PD excretion in the urine considering endogenous contribution. Next, absorption of purines is used to calculate total duodenal microbial N flow taking account of the digestibility of microbial purines, the N content of purines and the ratio of purine-N to total-N in mixed rumen microbes (Chen and Ørskov, 2003). Besides being non-invasive and suitable to be applied in experimental studies, PD method is advantageous over the invasive *in vivo* methods as PD analysis in urine is rapid and cheap. However, using the urinary PD method, the following variables have to be assumed to be constant: the ratio of purine-N to total-N in microbial mass, the true intestinal digestibility of purine nucleosides, and recovery rate of absorbed exogenous purines in urine.

In contrast to the approaches described above, where MCP yield is estimated, the *in situ* technique is usually used to determine RUCP (Zhao and Cao, 2004). Although the *in situ* method is normally not counted as an *in vivo* method, it is also included

in this section, as the samples are incubated directly in the rumen by introducing feed samples placed in polyester bags into the ventral sack. After a certain incubation time, bags are removed, and the remaining nutrient mass is measured. An exponential model is applied to the data, which allows the calculation of effective degradability for different passage rates (Ørskov and McDonald, 1979). This procedure has been widely used and a huge data basis is available, because *in situ* RUCP values are part of feed evaluation systems for example in northern countries (Volden, 2011), the Netherlands (Tamminga *et al.*, 2007; van Duinkerken *et al.*, 2011), the United States (NRC, 2001), and also in Germany (GfE, 2001). However, several problems of the *in situ* technique regarding RUCP evaluation are still present (Borrick and Cochran, 2000; Michalet-Doreau and Ould-Bah, 1992) and the reproducibility was not satisfactory in interlaboratory tests mainly due to insufficient standardisation (i.a., grinding, drying, bag material, bag pore size, sample size to bag surface ratio, washing procedure) (Madsen and Hvelplund, 1994). Furthermore, a high sample throughput or routine application of the *in situ* method is not possible, which fortified the need to estimate protein values by laboratory methods (Böttger *et al.*, 2019b).

4.1.2 *In vitro* techniques

To determine the protein value *in vitro* a variety of methods have been developed, but unfortunately there are only few comparisons with *in vivo* data (Hristov *et al.*, 2019). Generally, these *in vitro* methods are based on incubation of feed samples in a medium reflecting rumen condition. Most of these aim at measurement of CP degradation, whereas only a few methods can estimate MCP yield or the total amount of prCP. Furthermore, “it is important to point out that *in vitro* techniques may provide satisfactory accuracy for comparative purposes, but rarely absolute values that are comparable to *in vivo* data (Hristov *et al.*, 2019)”.

4.1.3 Rumen-fluid based *in vitro* methods

One of the pioneering work in rumen-fluid based methods was described by Tilley and Terry (1963) using rumen-fluid from cannulated sheep. This method based on two main stages starting with an incubation of a small feed sample in rumen-fluid buffer-solution under anaerobic conditions, followed by a second stage to remove insoluble protein (RUCP and MCP) through incubation in a pepsin-HCl-solution. The method was proposed to be suitable for routine evaluation of *in vitro* DM digestibility, due to simplicity, reproducibility, and the opportunity to evaluate many samples within a single experiment.

Czerkawski and Breckenridge (1977) developed a semi-continuous rumen simulation technique (**RUSITEC**) aiming to reproduce the physiological processes in the rumen as closely as possible. For this, feed samples enclosed in polyester bags are introduced in perforated fermenter vessels and incubated in fermentation chambers in a rumen-fluid buffer solution, while fermentation conditions (e.g., pH value, temperature, flow rate, feed quantity, feed composition) are maintained continuously, or adapted to research questions accordingly (Boguhn, 2004). This enables to preserve microbial population under ‘natural conditions’ over a longer period of time of up to 34 days in the study of Czerkawski and Breckenridge (1977). Basically, the feed residues in the bags, the liquid and gaseous overflow, and the fermenter liquid are available for sampling, which enables a variety of different analyses (e.g., degradation of crude nutrients and detergent fibres, liquid-associated microbes, microbial efficiency, methane production). Compared to closed *in vitro* methods one of the most important advantages of this system is the continuous removal of fermentation products, i.e. volatile fatty acids (**VFA**) and ammonia (**NH₃**), which potentially inhibit fermentation in batch culture systems (Brandao *et al.*, 2020). Much research has been done to further improve this system, but the representation of different passage rates between the liquid and solid phases as well as the absorption of degradation products by the rumen wall cannot yet be depicted with sufficient accuracy (Boguhn, 2004). The daily exchange of the bags needed leads to a brief entry of air into the system, which can have a negative effect on certain microorganisms. Further, a strikingly low OM or neutral-detergent fibre degradation of 44 and 47% in maize silage and grass silage, respectively, was recorded by Zuber (2017). Thus, microbial yield derived by RUSITEC studies might not be correctly estimated. The low standardization of the method regarding polyester bag pore sizes, feed particle lengths, or buffer infusion rates limits repeatability and reproducibility of the results. Due to the considerable time and personnel expenditure involved in maintaining the system, RUSITEC cannot be seen as a suitable routine method and should therefore be regarded as a system for basic research and at best as a screening method.

Zhao and Lebzien (2000) developed a technique, which is based on the first stage of the two-stage technique according to Tilley and Terry (1963). Feed samples and blanks are incubated for 24 h in glass tubes containing a rumen-fluid buffer solution. Subsequently, the samples are filtered, and N is determined in both, the liquid, and the solid parts. Post-rumen CP is further calculated from N measurements of the samples and the residues corrected for the blanks (only rumen fluid buffer solution without substrate). *In vitro* prCP was significantly correlated to *in vivo* data (Lebzien

et al., 1996; Zhao and Lebzien, 2000) and the incubation technique was slightly modified for the estimation of utilizable AA (Zhao and Lebzien, 2002).

Similarly, the Hohenheim gas test (**HGT**) as well as its extension (**eHGT**) or modification (**modHGT**), are based on incubation of feed samples in a rumen-fluid buffer solution, with minor differences between each approach in terms of incubation times and composition of the incubation medium. In contrast to the RUSITEC, these approaches are static systems, which neither allow nutrient supply nor removal of fermentation products. The HGT (Menke and Steingäß, 1988) acts as a standard method in Germany to estimate DOM and ME in ruminant feeds, for which gas production (**GP**) after 24 h of incubation and chemical composition of the feeds are used (GfE, 2001). The eHGT (Raab *et al.*, 1983) is based on an incubation of the feed samples with and without addition of carbohydrate mixture and the subsequent recording of GP and $\text{NH}_3\text{-N}$ content (steam distillation) in the liquid of the incubated feed sample and the respective blanks. *In vitro* degradable N is estimated through linear regression between $\text{NH}_3\text{-N}$ and GP. The theoretical point of zero GP (intercept) represents the amount of $\text{NH}_3\text{-N}$, which would be released due to CP degradation only, as no energy is available for MCP synthesis. *In vitro* degradable N is thus calculated by the difference between the intercept of the regression and $\text{NH}_3\text{-N}$ of the blank samples related to the total N in the incubated feedstuff (Raab *et al.*, 1983). This method is also described in detail by Wild *et al.* (2019) and Böttger *et al.* (2019b) and a modification was published by Karlsson *et al.* (2009). This modification included some methodological changes like sampling at different times from the same flask, as well as a pre-incubation of the rumen-fluid with carbohydrates to increase the microbial activity on the one hand and to reduce $\text{NH}_3\text{-N}$ background values on the other hand. By allowing samples to be taken from the liquid phase within the same flask during incubation, the method is more practical and consistent than the use of glass syringes within the eHGT (Karlsson *et al.*, 2009). In addition, higher sampling frequency opens the possibility to estimate kinetic parameters of CP degradation. But comparison to *in situ* data seemed to be problematic and ranking of the feed samples differed between *in situ* and *in vitro* estimated effective protein degradation in the study Karlsson *et al.* (2009), where the loss of SP out of the bag and escape of soluble N from rumen degradation was mentioned as most probable reasons. The buffer solution used for incubation of feeds in the modHGT (Steingäß *et al.*, 2001; Steingäß and Südekum, 2013) contains additional N compared to the HGT to ensure unlimited microbial fermentation. In contrast to the method of Zhao and Lebzien (2000) where prCP is determined as precipitant in the incubation residue, the modHGT estimates the prCP as the difference between $\text{NH}_3\text{-N}$

N of the incubated sample and total N of the *in vitro* batch (Westreicher-Kristen *et al.*, 2015). Principals have been described in detail e.g. by Edmunds *et al.* (2012a), Böttger and Südekum (2017a, 2017b) and in our studies (**Chapter 2, Chapter 3**) This method also shows potential to represent effective prCP for a certain passage rate providing suitable protein values at different feeding levels (Südekum and Böttger, 2019; Westreicher-Kristen *et al.*, 2015). Gidlund *et al.* (2018) studied the relationship between *in vitro* determined prCP and omasal flow of CP in lactating dairy cows and found a close relationship ($R^2 = 0.87$) between the data.

In vitro methods for determination of prCP are suitable for routine analysis, because they are fast, simple, and of low costs (with exception for the cost of keeping fistulated animals) (Zhao and Cao, 2004).

4.1.4 *In vitro* methods without rumen-fluid

The suitability of *in vitro* methods described above is limited by the need of rumen-cannulated animals, which may not be available in all laboratories. Alternatively, animal-independent methods are required (Westreicher-Kristen *et al.*, 2015). Such methods can provide information about CP degradation and could therefore be an alternative to the *in situ* method. However, they are not able to give any information about MCP yield.

The simulation of feed CP degradation by the use of enzymes commercially available aims for results similar to those found *in vivo* (Broderick and Cochran, 2000). Basically, the pure protein content of the feed sample is first determined by protein precipitation and true protein remaining in an enzymatic solution is used to estimate CP degradation (Steingäß and Südekum, 2013). In a review, Stern *et al.* (1997) illustrated the use of various proteases of bacterial (*Streptomyces griseus*), plant (*papain*, *ficin*, *bromelain*) or fungal (*Aspergillus oryzae*) origin in their ability to predict ruminal CP degradation compared to *in situ* measurements. Herein, some aspects affecting the results exist, such as enzyme concentration, enzymatic activity, pH value, and the presence of reducing factors or starch. One year later, Licitra *et al.* (1998) published a standardized regulation using a *Streptomyces griseus* protease. In addition, an extensive study was conducted by Gallo *et al.* (2018) to identify a suitable combination of enzyme concentration and incubation time. By now, many studies conducted using *Streptomyces griseus* have been published, also recently (Bachmann *et al.*, 2020b; Bachmann *et al.*, 2020a; Böttger *et al.*, 2019a), but the main criticism of enzymatic approaches is that the rumen spectrum of proteolytic activity cannot be represented with enough precision (Hristov *et al.*, 2019). However, the

great advantage of this procedure is, that it can be easily standardised, and a high sample throughput can be implemented with good repeatability and reproducibility. Thus results may be sufficiently accurate for comparative purposes and to evaluate treatment effects on RUCP (Hristov *et al.*, 2019).

The CP fractionation (Licitra *et al.*, 1996) is based on an analytical determination of CP, soluble CP (**SCP**), neutral detergent insoluble CP (**NDICP**), and acid detergent insoluble CP (**ADICP**) of feeds. Subsequently, multiple linear regressions are used to derive an overall RUCP value for the total CP in the feed (Kirchhof *et al.*, 2010; Kirchhof, 2007; Shannak *et al.*, 2000). From the analytical fractions (CP, SCP, ADICP, NDICP), five subfractions of CP are estimated related to the rate of rumen degradation in the American Cornell Net Carbohydrate and Protein System (**CNCPS**; Sniffen *et al.*, 1992): NPN (**A**), rapidly (**B1**), moderately (**B2**) and slowly degradable (**B3**) true protein, and CP bound to ADF considered neither as rumen degradable nor digestible in the intestine (**C**). By contrast to the method of CP fractionation, an undegraded fraction is assigned to each individual CP fraction in the CNCPS system (Sniffen *et al.*, 1992). Prediction of *in situ* RUCP from chemical fractionation of feed CP according to CNCPS was proven for concentrates (Shannak *et al.*, 2000), dried distillers grains with solubles (**DDGS**) (Westreicher-Kristen *et al.*, 2012) and forages (Kirchhof *et al.*, 2010). However, Böttger *et al.* (2019b) point out the importance of the quality of the prediction equations and the need for feed-specific equations, as differences can also occur within different types of feedstuff. The equations proposed by Shannak *et al.* (2000) were validated for concentrates, but are unsuitable for roughages (Edmunds *et al.*, 2012b; Kirchhof *et al.*, 2010; Kirchhof, 2007), for DDGS (Westreicher-Kristen *et al.*, 2012), or rapeseed meal (Böttger *et al.*, 2019b). In a comparison of RUCP estimates by laboratory methods, CP fractionation yielded generally higher values compared to an enzymatic approach (*Streptomyces griseus*) or eHGT, which were partly higher than the theoretical maximum (Böttger *et al.*, 2019b). However, besides differed absolute RUCP values (g/kg CP) the ranking of the samples was comparable among methods (Böttger *et al.*, 2019b). Zhao and Cao (2004) found a significant multiple regression relationship ($R^2 = 0.90$; $P < 0.0001$) between *in vitro* prCP (determined according to Zhao and Lebzien, 2000) and CNCPS CP fractions in feeds and suggested their use as a possible alternative to estimate prCP of ruminant feeds, if donor animals are not available. Chemical analysis were more complicated than rumen-fluid based *in vitro* techniques, but could be easily performed in most laboratories (Zhao and Cao, 2004).

4.2 Detailed comparison of the *in situ* method and the modHFT

This chapter focusses on a detailed discussion and comparison of the *in situ* and *in vitro* method – applicated within the present thesis– with respect to their suitability for prCP estimation.

4.2.1 *In situ* method

The protein value of ruminant feeds is usually assessed through the application of different protein evaluation systems. Comparability of the literature data is hampered by the fact that systems consider different influencing factors and are based on various assumptions. In particular, this refers to the estimation of MCP yield. However, there is broad agreement between different systems on the use of RUCP values derived from *in situ* measurements (GfE, 2001; NRC, 2001; Thomas, 2004; van Duinkerken *et al.*, 2011). Due to the widespread use, these values can act as a guide, but they could not be seen as an absolute reference, because many factors influence the accuracy of the measurement of *in situ* degradation. Some of them are listed by the work of Lebzien and Flachowsky (2005) such as a uniform grinding of the feedstuffs and the resulting change in degradability compared to the original material, an unrepresentative microbial activity within the bags compared to the activity in the rumen (Huhtanen *et al.*, 1998), small particles and water-soluble fraction escaping ruminal fermentation (Hvelplund and Weisbjerg, 2000), or microbial contamination of the bag residues (Krawielitzki *et al.*, 2006). The last two aspects are frequently mentioned and important for the present work. Both will be briefly addressed in the following.

With the *in situ* method, degradation of feed components is estimated through incubation of feeds placed in polyester bags in the rumen of cannulated animals for various length of time. Herein it is assumed, that each component can be separated into a non-washout and a washout fraction, whereas non-washout fraction consists of potentially degradable and non-degradable parts. Washout fraction includes the components that have leaked from the bags due to water solubility (**WS**) or small particle (**SP**) size (Tamminga *et al.*, 2007). Commonly, degradation profile of feeds is described by first-order kinetics model according to Ørskov and McDonald (1979) or the model including the lag phase (McDonald, 1981). In these equations, washout fraction is regarded as soluble and immediately degraded which, however, is not correct. So, Hvelplund and Weisbjerg (2000) proposed to correct degradation data for SP losses, by transferring the degradation kinetics from samples incubated in the bags to SP, which was realized in the present studies accordingly. However, SP

and WS have the potential to escape the rumen undegraded via the liquid phase (Ahvenjärvi *et al.*, 2018; Volden and Larsen, 2011). In this context, Ahvenjärvi *et al.* (2018) reported a 12.5% escape of soluble non-NH₃-N (free AA, peptides, and soluble proteins) in grass silages. Consequently, RUCP content may be underestimated by 11-12 g RUCP/kg CP for grass silages evaluated in **Chapter 2** containing about 60-65% soluble CP (in CP; *in situ* fraction *a*). However, NH₃-N content of the grass silages after freeze-drying was unknown and it cannot be assumed that *in situ* fraction *a* of the grass silages was entirely composed of non-NH₃-N. The volatility of NH₃-N during the drying process depends on the pH value and is reported to be 32% for silages with a pH value between 4-4.5 (Weißbach and Kuhla, 1995), which applies to about two thirds of the silages evaluated in our study. On the other hand, NH₃-N content of the silages was about 11 g/kg CP, corresponding to only about 2% of the soluble fraction. Thus, the actual absolute value of escape fraction cannot be given at this point, the figures only intended to show the meaning of the escape of soluble N compounds.

The principle of the *in situ* method is that microorganisms entering the porous bags and subsequently colonize the feed samples for its degradation in a similar way than in the rumen (Hvelplund and Weisbjerg, 2000). Washing procedure after incubation aims at removing rumen content, degraded substrates, and microbes. However, some microbes will be still attached to feed residuals ('microbial contamination') and accounted as undegraded feed fraction (Hvelplund and Weisbjerg, 2000). Since microbial mass contains 512 g CP, 167 g EE, 51 g starch, and 270 g residual fraction (cell wall) per kg OM (Volden, 2011), microbial attachment can lead to an underestimation of ruminal CP degradation, in particular, whereas the effect on DM degradation will be of minor importance (Hvelplund and Weisbjerg, 2000). Krawielitzki *et al.* (2006) published an estimation formula to correct the residuals for microbial contamination. The variables A_{\max} (maximum contamination) and R (rate of contamination) required for this can be calculated from CP and NDF content of the samples. This correction was applied to all samples of the present work. However, Edmunds *et al.* (2012b) showed that A_{\max} of grass silages was overestimated through the equation proposed by Krawielitzki *et al.* (2006) by 75 (± 31) mg microbial CP/g residue CP compared to an analytical measurement of A_{\max} . A correction for microbial contamination may therefore be more accurately if A_{\max} is determined analytically.

Several studies indicated, that the importance of RUCP is overrated (Beever and Cottrill, 1994; Ipharraguerre and Clark, 2005; Santos *et al.*, 1998). Consequently, a

precise determination of MCP would be of greater importance for the assessment of the protein value. To estimate MCP synthesis, information on the amount of FOM is needed, which provides the energy for microbial synthesis. A variety of equations exist, whereby FOM is estimated based on DOM, or other easily available parameters (Huhtanen, 2005). The main problem using DOM is that variable proportions of disappeared substances are fermented in the rumen (Huhtanen, 2005). Thus, in some protein evaluation systems, DOM is corrected for substances that provide little or no energy to the microbes such as EE, fermentation products (FP) in silages, RUCP or bypass starch (INRA, 2018; Tamminga *et al.*, 1994). Others determine FOM by summation of rumen degraded fractions determined *in situ* (Thomas, 2004; Volden, 2011). Within the present work, it was assumed that FOM was precisely given by effective OM degradation determined *in situ* (EDOM), although part of the OM washout-fraction might also have escaped fermentation. Correction for substances that have leaked from the bag but do not provide energy may allow further improvement. This includes FP or EE, whose consideration may be particularly important if these ingredients are present in high amounts. Grass silages (**Chapter 2**) originating from the same material were used and tested for treatment effects of 'DM concentration before ensiling' and 'addition of lactic acid bacteria (LAB) inoculant'. Both treatments primarily affected fermentation pattern of the silages. In general, there was a more intensive fermentation in both, restrictively wilted and LAB treated silages, resulting in a higher content of FP compared to more intensively wilted and/or untreated silages. Thus, differences between the samples mainly occur through amount of FOM. Main contributors to FP are lactic acid (LA) and VFA, but depending on component and drying conditions variable amounts of FP are lost during the drying process (Tamminga *et al.*, 2007). Porter and Murray (2001) described an almost complete evaporation of alcohols, whereas VFA evaporated to 55-90% and LA to 10-40%. In the DVE/OEB2007 system, 100%, 50% and 8% evaporation is assumed for alcohols, VFA and LA, respectively (Tamminga *et al.*, 2007). In our study (**Chapter 2**) the evaporation of LA and of propane-1,2-diol was tested separately by adding the respective FP to grass hay and subsequent freeze-drying. This resulted in a weight change of treated hay samples and showed that major amounts (>85%) of both substances were still present on the feed after the drying procedure. Therefore, FOM in grass silages was derived from EDOM corrected for the amount of LA and of propane-1,2-diol, as both being themselves the end products of microbial fermentation in the silo and were expected to provide no energy to rumen microbes. The inaccurate assumption of complete non-volatility of LA and of propane-1,2-diol was, in our opinion, justified by the fact that a small

proportion of the VFA might have still been present on the feed material, which was accounted as completely volatile.

Concentration of EE decreased with increased DM concentration in grass-clover silages evaluated by Johansen *et al.* (2017). The same effect was also seen for the grass silages evaluated in our study (**Chapter 2**). If additionally discounting EE from EDOM to calculate FOM, the positive effect of wilting on prCP might be slightly more pronounced. However, the change in EE might be related to oxidation of unsaturated fatty acids during wilting (Johansen *et al.*, 2017; van Ranst *et al.*, 2009) and occur due to analytical inaccuracy rather than an actual change in the amount of non-fermentable substances. Thus, no correction for EE was made within the present work. The FOM from feeds and feed mixtures (**Chapter 3**) was derived from EDOM without further discounts for FP und EE, because these differences were considered as marginal and should not have affected the ranking of the feeds.

It is conceivable that feeds/feed components are fermented at different rates and to different extents, which further complicates a precise estimation of FOM. According to the Dutch DVE/OEB2007 system (Tamminga *et al.*, 2007), OM in ruminant feeds is separated into CP, starch, sugars (mono- and disaccharides), glucose oligosaccharides, EE, NDF, FP, and residual non-starch polysaccharides (pectins, arabans, xy-lans and beta-glucans). Theoretically, all these ingredients would have to be determined in the bags before and after incubation to give precise information about the extent of disappeared or fermented nutrients. Due to the enormous number of additional bags required, it is not possible to determine the degradation of individual ingredients. With two replicates per sample at each incubation time (about 7 time points) in each animal (minimum 3 animals) about 42 bags would be needed per additional nutrient fraction. However, Olaisen *et al.* (2003) have found that incubation times could be narrowed down further, as degradation was estimated with sufficient accuracy by two or three incubation times, reducing the number of polyester bags by 58 to 78% in their study. In addition, near-infrared spectroscopy offers the possibility to further limit the sample size (Krieg *et al.*, 2018) or to keep it at the previous level. Total analysis of the ingredients using near-infrared spectroscopy is conceivable, but requires precise validation, which will also involve an enormous effort.

The most critical point in our *in situ* approach is not the estimation of FOM, but the calculated MCP therefrom. Data on the efficiency of MCP yield and its variation is available in the literature. For example, the NRC (2001) declares a variation of 12-45 g microbial N/kg FOM or 75-338 g MCP/kg FOM, respectively, within a summarised literature data set. In an early version of the Dutch DVE/OEB system a fixed amount

of 150 g MCP/ kg FOM was assumed (Tamminga *et al.*, 1994) and the value of 181 g MCP/ kg FOM used in our studies is given with a range of 69-266 g/kg FOM (Lebzien and Voigt, 1999). Despite the wide variation in the efficiency of MCP yield, it is not so much the absolute value rather than the assumption of a constant value of MCP/kg FOM that is to be questioned. By assuming a constant value of MCP yield, *in situ* prCP content may decrease with an increase in passage rate (Krieg *et al.*, 2018). However, this is not to be assumed, since in general, increasing passage rate has a positive effect on both RUCP content and MCP yield. Faster passage rates reduces CP degradation in the rumen on the one hand, and increases the efficiency of MCP yield on the other (Sauvant and Nozière, 2016). The increased MCP yield in turn is largely attributed to the reduction of energy demand for maintenance requirement of the microbes, and reduced lysis in the rumen of MCP already synthesised (Hackmann and Firkins, 2015). Although the measurement of FOM *in situ* considers the ruminal degradation of OM in relation to (uniform) passage rate (Krieg *et al.*, 2018), the neglect of the fractional passage rate of liquid, solid, concentrate and forage fractions (Thomas, 2004) represents a further limitation of the *in situ* method. Moreover, fermented substances deliver variable amounts of energy for MCP synthesis. The yield of ATP from fermented substrates into VFA is 27.3 mmol/g polysaccharides (structural polysaccharides, starch), 23.9 mmol/g mono-, di- and oligosaccharides, and only 13.7 mmol/g CP (van Duinkerken *et al.*, 2011). Although VFA do not yield ATP for microbial growth, LA is known to provide a small amount of energy for MCP synthesis of about 20% of those supplied from carbohydrates (Volden, 2011). Thus, the complete discount of the amount of LA from EDOM in grass silages (**Chapter 2**) could have provided slightly underestimated values of FOM. An even more accurate assessment of MCP synthesis would therefore be possible, if nutrient-specific efficiencies could be applied. An attempt was made in **Chapter 3** where a lower efficiency for rumen-degradable CP and a higher one for FOM-CP free was applied to estimate MCP yield. It was concluded that this could lead to a more accurate assessment of the prCP, especially for high-protein feeds.

Prospectively, it could be expected, that *in vivo* experiments (including *in situ* measurements) will no longer be carried out in Europe for animal welfare reasons and routine testing in animals is therefore not conceivable.

4.2.2 Modified Hohenheim gas test (modHGT)

The key problem with prCP determined by the *in situ* method is that on the one hand, RUCP measurement shows some inaccuracies, and on the other hand, that the estimation of MCP is subjected to various assumptions. Consequently, a simple

addition of both variables might lead to an accumulation of errors (Lebzien *et al.*, 1996). Some of the mentioned problems of the *in situ* approach as SP loss, escape of soluble N, or microbial contamination, are removed by an *in vitro* batch system, additionally microbial access to the feed is not limited through pore size of the polyester bags needed for *in situ* incubations (Gidlund *et al.*, 2018).

Recent studies (**Table 4-1**) have used the modHGT, which provides a direct estimate of combined RUCP and MCP to characterise prCP *in vitro* of various feeds. A few of these studies compared *in vitro* determined prCP to reference values calculated based on equations proposed by GfE (2001). Only Krieg *et al.* (2018) compared *in vitro* determined prCP to reference values calculated according to Lebzien and Voigt (1999) as done in our studies. Therefore, the comparability of the results concerning the relationship between *in vitro* and *in situ* data of the studies conducted within this work with those described in the literature may be limited. Irrespective of this, two general findings can be highlighted in a consistent manner: (1) closer relationships between *in vitro* prCP and reference¹ values at higher compared to lower passage rates and (2) higher values for prCP *in vitro* compared to reference values at high passage rates and *vice versa*.

Difficulties concerning the interpretation of modHGT measurements mainly arise, regarding the choice of an adequate incubation time or passage rate, respectively. In addition, as with the *in situ* approach, fractional passage rates cannot be considered which might influence the results (Edmunds *et al.*, 2012a). However, the consistent findings of the studies about closer relationships between *in vitro* and reference values at higher compared to lower passage rates (**Table 4-1**) support the assumption of Westreicher-Kristen and Südekum (2018) about a more precise reflection of *in vivo* MCP synthesis at short incubation times (corresponding to passage rates at high feeding level). Closer relationships due to higher passage rates were found in feeds and feed mixtures composed thereof in our study ($k = 0.05/\text{h}$, $R^2 = 0.68$; $k = 0.08/\text{h}$, $R^2 = 0.76$; **Chapter 3**), in feed mixtures based on maize silage, red clover silage, lupin seed, soybean meal, and wheat grain (Westreicher-Kristen and Südekum, 2018), in DDGS (Westreicher-Kristen *et al.*, 2015), and in forages (Edmunds *et al.*, 2012a). However, the relationship between *in vitro* and *in situ* prCP values was not improved from $k = 0.04$ ($R^2 = 0.61$) to $0.06/\text{h}$ ($R^2 = 0.54$) in grass silages (**Chapter 2**). Moreover, the modHGT yielded higher prCP values compared to

¹ The term "reference value" refers to prCP values used as a reference and compared to *in vitro* prCP values within the respective studies (**Table 4-1**). In most cases, these reference values were determined using an equation proposed by GfE (2001). Within our studies (**Chapter 2**, **Chapter 3**) and the study of Krieg *et al.* (2018) reference values were calculated according to Lebzien and Voigt (1999).

reference values (prCP reference minus prCP *in vitro*) at high passage rates and *vice versa* (**Table 4-1**) for forages (Edmunds *et al.*, 2012a), DDGS (Westreicher-Kristen *et al.*, 2015), and grains (Krieg *et al.*, 2018). Similar observations were made for grass silages (**Chapter 2**) and feed mixtures (**Chapter 3**) evaluated within our studies.

Although the relationships described in the literature “indicates that the modHGT displays accuracy in ranking feeds according to protein value (Edmunds *et al.*, 2012a)”, the authors classify the coefficient of determination as moderate for forages (Edmunds *et al.*, 2012a) and DDGS (Westreicher-Kristen *et al.*, 2015). This was attributed to both, possible limitations in determining the reference values with the GfE (2001) equation, and some characteristics of the modHGT which are supposed as limitations (Westreicher-Kristen *et al.*, 2015). The authors point out that the only factor that changes in GfE equation, if different passage rates are applied, is effective RUCP. This value, in turn, is determined using the *in situ* method, which is subjected to a high level of variation (**Chapter 4.2.1**). Since CP fractions are not considered in the GfE equation, Westreicher-Kristen *et al.* (2015) concluded that reference values of prCP in DDGS may be underestimated, as differences between *in vitro* and reference values were significantly correlated to CP fraction A (CNCPS, Sniffen *et al.*, 1992). Thus, a possible explanation for the differences between the methods could be the combined effect of enough available energy and a high amount of NPN (CP Fraction A) in DDGS promoting MCP growth and consequently leads to higher prCP *in vitro* compared to reference values calculated based on GfE (Westreicher-Kristen *et al.*, 2015). In addition, it should be noted that the *in vivo* method used to derive the regression equations proposed by GfE (2001) is also subject of errors (Edmunds *et al.*, 2012a). For these reasons, Edmunds *et al.* (2012a) concluded that a more accurate representation of prCP at the chosen passage rate is probably given by the modHGT compared to the GfE equation.

Limitations of the modHGT are related to the closed system where neither nutrient supply nor outflow of fermentation products and N recycling can be considered (Edmunds *et al.*, 2012a; Gidlund *et al.*, 2018; Westreicher-Kristen and Südekum, 2018). As a result, increased incubation time leads to decreased prCP due to progressive feed CP degradation and lysis of MCP already synthesized (Steingäß and Südekum, 2013; Walker *et al.*, 2005). Closer relationships between the methods at higher passage rates might therefore mainly be a result of neglectable lysis. Any effect of N recycling, however, would be minimal and of minor importance, as the system has been developed so that N is not a limiting factor (Edmunds *et al.*, 2012a).

Table 4-1: Literature overview of studies in which post-rumen crude protein (prCP) in feeds was determined using the modified Hohenheim gas test.

Authors	Feed	Incubation time (h) ¹	Reference value ²	Passage rate (/h)	R ² [†]	Difference ³ (g/kg DM)
Edmunds <i>et al.</i> (2012a)	forages (<i>n</i> = 23) (fresh, silage, hay, artificially dried)	8, 48	GfE (2001) Eq. 9	0.02	0.19	27 (±11)
				0.04	0.56	−2 (±10)
				0.06	0.67	−18 (±11)
Hamacher <i>et al.</i> (2014)	legumes, herbs, grass	8, 48	- ⁴	0.04	-	-
				0.06	-	-
				0.08	-	-
Westreicher-Kristen <i>et al.</i> (2015)	DDGS ⁶ (<i>n</i> = 13)	8, 24, 48	GfE (2001) Eq. 9, 10	0.02	ns ⁵	7 (±33)
				0.05	0.54	−36 (±25)
				0.08	0.70	−56 (±25)
Bakken <i>et al.</i> (2017)	grass–clover silages)	8, 48	-	0.04	-	-
Böttger and Südekum (2017a)	DDGS ⁶	8, 24	GfE (2001) Eq. 9, 10	0.05		-
Böttger and Südekum (2017b)				0.08		-
Vaga <i>et al.</i> (2017)	field beans, sweet blue lupins, peas	8, 24, 48	-	0.06	-	-
Westreicher-Kristen <i>et al.</i> (2017) Westreicher-Kristen and Südekum(2018)	total-mixed rations (<i>n</i> = 4)	8, 48	GfE (2001) Eq. 9	0.02	ns	58 (±6)
				0.05	0.94	17 (±3)
				0.08	0.95	−2 (±1)
Krieg <i>et al.</i> (2018)	barley, rye, triticale	8, 24, 48	GfE (2001) Eq. 9	0.05	-	1
				0.08	-	−22
			Lebzien and Voigt (1999)	0.05	-	−4
				0.08	-	−27
Böttger <i>et al.</i> (2019a)	grass silages, grass hay	8, 48	-	0.05	-	-
Böttger <i>et al.</i> (2019b)	rapeseed meal	8, 24	-	0.05	-	-

¹incubation time after which NH₃-N was measured to calculate prCP *in vitro*

²reference values calculated according to the given literature reference

[†]coefficient of determination; for the regression equation between *in vitro* prCP and the respective reference values of prCP for the passage rates adjoining on the left column

³difference between the reference values (calculated according to the given studies) and prCP *in vitro* (prCP reference minus prCP *in vitro*)

⁴not specified

⁵not significant

⁶dried distillers' grain's with solubles

Table 4-1: continued.

Authors	Feed	Incubation time (h) ¹	Reference value ²	Passage rate (/h)	R ² [†]	Difference ³ (g/kg DM)
Hartinger <i>et al.</i> (2019)	lucerne silages	8, 48	- ⁴	0.02	-	-
				0.05	-	-
				0.08	-	-
Ramin <i>et al.</i> (2019)	grass silages, seaweeds	8, 16, 24, 30	-	0.06	-	-
Wild <i>et al.</i> (2019)	microalgae	8, 48	-	0.02	-	-
				0.05	-	-
				0.08	-	-
Bachmann <i>et al.</i> (2020a)	field peas, field beans (native, ensiled, toasted)	8, 24	-	0.02	-	-
				0.04	-	-
				0.06	-	-
				0.08	-	-
				0.12	-	-
Grubješić <i>et al.</i> (2020)	concentrate feeds concentrate mixtures)	8, 24	-	0.05	-	-
				0.08	-	-
Krieg <i>et al.</i> (2020)	durum wheat	8, 24	-	0.08	-	-

¹incubation time after which NH₃-N was measured to calculate prCP *in vitro*

²reference values calculated according to the given literature reference

[†]coefficient of determination; for the regression equation between *in vitro* prCP and the respective reference values of prCP for the passage rates adjoining on the left column

³difference between the reference values (calculated according to the given studies) and prCP *in vitro* (prCP reference minus prCP *in vitro*)

⁴not specified

⁵not significant

Concerning the problematic assumption of a nutrient-unspecific MCP yield as well as the uncertainties in FOM estimation, the modHGT shows advantages over the *in situ* method. For example, Bakken *et al.* (2017) assessed the effect of wilting rate and the use of silage additives (formic acid, LAB) on *in vitro* protein value in silages from two cuts (first, second) with different proportions of grass or clover species. Differences in prCP due to herbage composition or wilting rate could not be detected within their study. This apparent insensitivity of the modHGT was attributed by the authors to possible factors limiting MCP synthesis, such as a lack of CP or energy in the silages of the first and second cut, respectively. The efficient use of CP for MCP synthesis due to enough available energy in the first cut and the surplus of CP in the second cut may have led to equal prCP *in vitro* in both (Bakken *et al.*, 2017). Nevertheless, the modHGT revealed an effect of silage additive within the cuts with higher prCP values for restrictively fermented silages (formic acid) than intensively fermented silages (LAB). For grass silages examined in **Chapter 2**, no significant differences in prCP *in vitro* between the control group and the LAB treatment was found. Similarly, Bakken *et al.* (2017) did not find any significant differences between the control group and the LAB treatment, but only between LAB and FA. Restrictively fermented grass silages were ranked above intensively fermented silages in terms of prCP *in vitro* also in **Chapter 2**, but in this case the fermentation pattern was mainly determined by DM content of the samples, whereby DM content between samples did not differ in the study of Bakken *et al.* (2017). Restrictive fermentation due to formic acid treatment or higher DM content might lead to a more intensive MCP synthesis due to a higher amount of fermentable energy and a higher proportion of free AA and peptides in the NPN fraction (Dijkstra *et al.*, 2013). If the amount of prCP₄ *in vitro* determined for grass silages ($n = 16$, **Chapter 2**) is regressed against the sum (g/kg OM) of LA and propane-1-diol, a negative relation was found ($R^2 = 0.69$). This relation may help to reinforce the need to correct EDOM for these fermentation products to calculate FOM or MCP yield (**Chapter 2**), respectively, but there is not any correction needed using modHGT.

Moreover, both methods seem to be more reliable for the evaluation of mixed rations compared to individual components. On the one hand, GfE equations were based on *in vivo* experiments, which were carried out using mixed rations (Westreich-Kristen and Südekum, 2018). On the other hand, the modHGT might deliver more reliable prCP values due to the consideration of possible interactions between feeds as well as various effects on MCP synthesis (Gidlund *et al.*, 2018). For instance, MCP synthesis may not be fully detected in protein feeds like soybean meal (**Chapter 3**) if incubated without other dietary components, because MCP growth may be

restricted due to excess of degradable CP relative to the supply of FOM (Vaga *et al.*, 2017).

Furthermore, the modHGT shows additional advantages like a high sample throughput and a comparatively low effort. It also offers the possibility to combine it with the original HGT to determine ME and DOM (Steingäß and Südekum, 2013) or extend it to determine both, RUCP and prCP within the same procedure (eHGT). Therefore, it can overcome few methodological imprecisions of modern protein evaluation systems (Gidlund *et al.*, 2018) and shows great potential for routine application in the future. Moreover, associative effects were recorded using the modHGT in feed mixtures at $k = 0.08/\text{h}$ (**Chapter 3**) accounts for 10.7 g prCP/kg OM (equals 7.6 g prCP/kg DM) which seems of minor relevance. However, assuming a dry matter intake of 22 kg/d it results in a potential difference of 167 g prCP/d which corresponds to the requirement for about 2 kg milk (GfE, 2001). It should be noted that our figure only corresponds to the average value from the comparison of 13 rations. It is quite conceivable that associative effects are more pronounced, if the 13 rations were evaluated on individual basis or if rations were composed using other feed combinations. The modHGT therefore shows the potential to illustrate associative effects in feed mixtures and may allow for a more precise formulation of diets and consequently help to reduced feed cost and diminish environmental pollution, whereby the latter issue will become increasingly important worldwide. Further technical development described by Karlsson *et al.* (2009) enables frequent sampling from the same incubation vessels can still improve the applicability of the *in vitro* method (Vaga *et al.*, 2017). To ensure standardisation of the method, a prCP standard is often carried along and used to correct the measured prCP values. In our study, but also in the study of Westreicher-Kristen *et al.* (2015) these standards were included, but only served as an internal control. However, Westreicher-Kristen *et al.* (2015) emphasises that such a correction is useful to reduce variations between different laboratories. Edmunds *et al.* (2012a) suggests possible improvement by the use of a forage based prCP standard to correct forage prCP data rather than the actually used concentrate based prCP standard. Bakken *et al.* (2017) additionally suggests a preincubation of carbohydrates in the rumen-fluid as described by Lorenz *et al.* (2011) to normalise NH_3 concentration in the incubation medium.

Unquestionably, one of the main criticisms on rumen-fluid based *in vitro* methods is the need for donor animals. It can be assumed that there will be further restrictions in the use of experimental animals, so research should focus on the development of an alternative to rumen-fluid inoculum. Literature described the

possibility of the use of fresh as well as frozen and thawed rumen-fluid from slaughtered animals to predict *in vitro* degradation of feeds (Chaudhry and Mohamed, 2012). Furthermore, the potential use of faecal inoculum for the *in vitro* gas production technique (Aiple *et al.*, 1992; Hughes *et al.*, 2012; Zicarelli *et al.*, 2011) and *in vitro* estimation of prCP (Zhao and Chen, 2004) was studied. Whatever future methods will look like, biological approaches will be necessary, because MCP synthesis cannot be achieved by purely chemical methods.

4.2.3 Consistency of methods

In order to demonstrate the suitability of the modHGT and its wide range of application, *in situ* values of the feeds and diets presented in **Chapter 2** and **Chapter 3** were regressed against the respective *in vitro* values (**Figure 4-1**). To ensure a uniform presentation, values of the silage samples were plotted at an assumed passage rate of 0.08/h, although data was presented only at $k = 0.02, 0.04$ and $0.06/\text{h}$ in **Chapter 2**. Besides being rather unusual to calculate effective prCP from $k = 0.08/\text{h}$ in forages, it was considered adequate for comparing both methods for demonstration purposes. No further adjustment of the data was made. Accordingly, the presented *in situ* data of the grass silages (**Chapter 2**) and of the feeds and mixed feeds (**Chapter 3**) were calculated from FOM derived from EDOM corrected for the proportion of FP (LA and propane-1,2-diol) or assuming a substrate-specific yield of MCP from degraded CP (104 g MCP/kg) and FOM-CP free (208 g MCP/kg), respectively. Further, data of effective prCP *in vitro* were derived from the incubation times of 8 and 48 h for grass silages (**Chapter 2**) or 8 and 24 h for feeds and feed mixtures (**Chapter 3**).

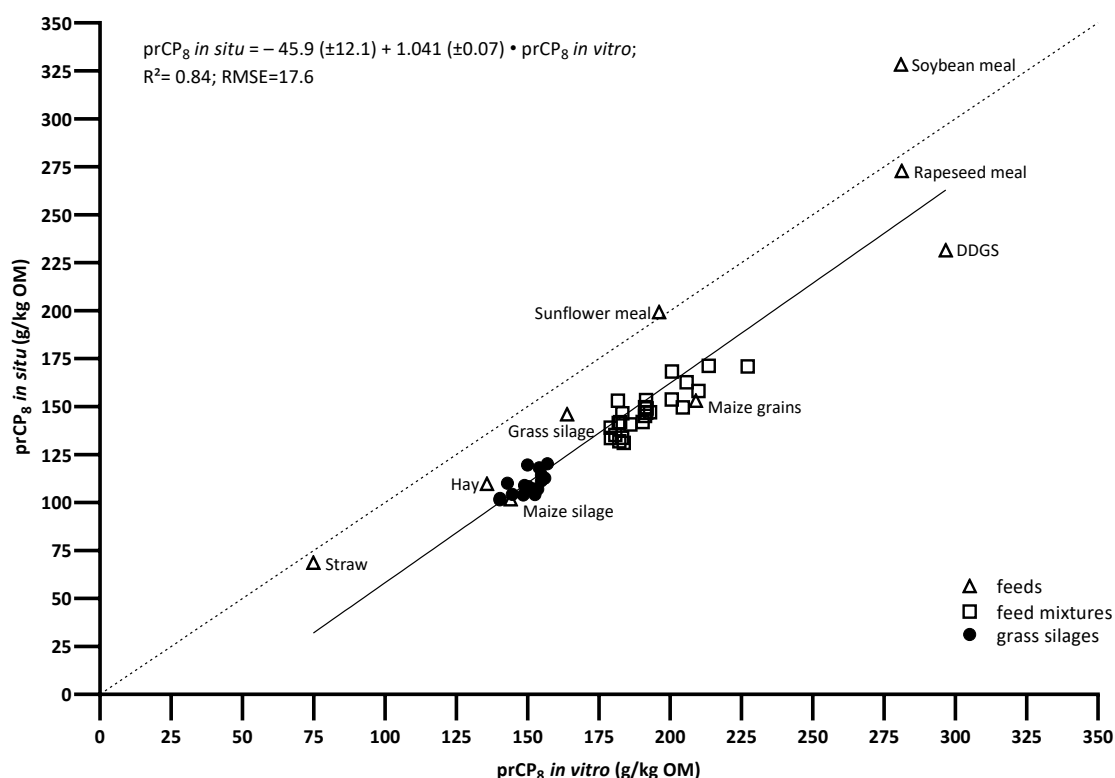


Figure 4-1: Relationship between *in vitro* and *in situ* determined post-rumen CP (prCP) of feeds [Δ], feed mixtures [□] and grass silages [●] at passage rate of $k = 0.08/\text{h}$. Data was adapted from **Chapter 3** and **Chapter 2**, respectively. The dotted line represents the angle bisector.

To simplify readability, the relationships described in the following have been numbered (No. 1-3) and listed in **Table 4-2**. Compared to the relation between *in situ* and *in vitro* values described for feeds and feed mixtures in **Chapter 3** (**Figure 3-1**) – again listed in **Table 4-2** (No. 1) – the inclusion of silage samples (No. 2) reduced standard error of the slope (not significant different from 1) and intercept as well as RSME, while improved R². The mean difference between absolute *in situ* and *in vitro* values of all samples was -39 (±17.5) g prCP₈/kg OM.

The graph shows a consistent assessment for roughage and feed mixtures i.e., the relation between both methods do not apply exclusively to a specific feed group. However, the agreement was less in some feeds of extreme nutrient composition, such as straw or soybean meal. No statement can be made which method provides the biologically more correct value. If soybean meal and straw were removed before analysis (**Table 4-2**; No. 3), regression equation remained similar, but standard error of the slope and intercept was smaller and RMSE was reduced to 11.5.

Table 4-2: Results of linear regression analysis between *in situ* and *in vitro* post-rumen crude protein (prCP) adopted from **Chapter 2** and **Chapter 3** presented at a passage rate of $k = 0.08/h$.

		prCP ₈ (g/kg OM)	Diff.	Intercept	SE _{Intercept}	Slope	SE _{Slope}	RMSE	R ²	P-value
(1) $n = 34$	<i>in situ</i>	156 (±45.6)	-38 (±21.0)	-44.3	18.7	1.034	0.09	21.3	0.79	<0.001
	<i>in vitro</i>	194 (±39.2)								
(2) $n = 50$	<i>in situ</i>	141 (±43.5)	-39 (±17.5)	-45.9	12.1	1.041	0.07	17.6	0.84	<0.001
	<i>in vitro</i>	180 (±38.3)								
(3) $n = 48$	<i>in situ</i>	139 (±33.3)	-41 (±11.5)	-32.7	9.4	0.954	0.05	11.5	0.88	<0.001
	<i>in vitro</i>	180 (±32.8)								

prCP₈ (g/kg OM) = mean value and standard deviation of prCP₈; Diff. = differences in mean and standard deviation; SE_{Intercept} = standard error of the intercept, SE_{Slope} = standard error of the slope; RMSE = root mean squared error; R² = coefficient of determination.

(1) corresponds to the equation given in **Figure 3-1**, including feeds and mixed feeds (**Chapter 3**) assuming an efficiency of microbial crude protein (MCP) yield of 208 g/kg CP free fermented organic matter (FOM) and 104 g MCP/kg fermented CP;

(2) data includes those of equation (1) plus silage samples (**Chapter 2**);

(3) equals equation (2) with exclusion of straw and soybean meal data.

The main problem of all *in vitro* methods is the lack of a reliable reference. But even if *in vivo* measurements in duodenal/abomasal cannulated animals are seen as the 'gold standard' (Edmunds *et al.*, 2012a), they have some inaccuracies as described here. Therefore, the question about which method reflects the biologically 'true protein value' best, remains still open to discussion. For these reasons, the modHGT should not be misjudged as a possible method in feed evaluation (Westreicher-Kristen *et al.*, 2015). The underlying data confirms the conclusions of previous studies regarding a reliable estimation of the prCP content by means of the modHGT, in particular for short incubation times reflecting passage rates at high feeding levels. The ultimate method to define the protein value of a diet or to show possible changes through diets and feed treatments is a production trial (Huhtanen and Broderick, 2016). However, production parameters have not been used to validate protein evaluation systems till now, although such data can be measured more precisely than *in vivo* protein degradation and MCP yield (Huhtanen, 2005). Before routine use in practice, further standardization is needed and interlaboratory tests are required. It might be helpful to perform a limited number of *in vivo* trials covering the range of commonly used diets for calibration of the *in vitro* values, to allow those to be used in diet formulation and for the calculation of the protein supply to the animals. On a small number of production trials, *in vitro* methods should be calibrated to achieve a relative scale of absolute values.

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CHAPTER 5

Summary

An important goal in dairy cattle feeding is to reduce nutrient excretion for ecological as well as economic reasons. In addition to knowledge of the animals' energy and protein requirements, precise information on the energy and protein values of the feedstuffs used is needed, to ensure adequate feeding of high-yielding dairy cows while minimising nutrient excretion. In the current German protein evaluation system, protein requirement of the animals and protein value of the feedstuffs are characterised by the amount of utilisable crude protein at the duodenum (**uCP**), which corresponds to the sum of rumen-undegraded crude protein (**RUCP**), and microbial crude protein (**MCP**) synthesised in the rumen. The term post-rumen crude protein (**prCP**) is used instead of uCP in the following and throughout this work. Considering the amino acid concentration and its intestinal digestibility, prCP can be converted into the internationally more common value metabolisable protein (**MP**).

Ruminal CP degradation, or RUCP content, respectively, is often determined using the *in situ* method, which can be regarded as a reference method due to its wide application. Animal-independent alternatives to determine CP degradation are given by enzymatic and chemical approaches. In contrast, a measurement of MCP yield can only be realised with considerable experimental effort in duodenally fistulated animals. Since MCP synthesis is primarily an energy-dependent process, MCP yield is derived from variables representing the amount of fermentable organic matter (**FOM**) in the rumen. However, the extent of MCP yield depends not only on the amount of FOM, but among other factors, on its nutrient composition, rumen available N sources and dry matter intake of the animal.

A promising *in vitro* method for measuring the content of prCP - the modified Hohenheim gas test (**modHGT**) - was evaluated in this work on the basis of two studies with regards to its suitability for determining the protein value. This method is based on incubation of feed samples in a rumen-fluid buffer solution and is sensitive to influences on MCP yield. To assess the modHGT method, prCP values determined *in vitro* and *in situ* were compared assuming different passages of the feed from the rumen to the small intestine (effective prCP). *In situ* prCP values were calculated from the sum of RUCP and MCP, assuming a formation of 181 g MCP/kg

FOM. The FOM was in turn derived from effective OM degradation determined *in situ*.

In addition to the method comparison, in the first study, the effects of wilting grass before ensiling, as well as the use of lactic acid bacteria (**LAB**) on the protein value of grass silage were tested *in vitro* and *in situ*. The sample material comprised a total of 16 grass silages, which differed in dry matter content before ensiling (170, 310, 390, 420, 470, 530, 580, 600 g/kg) and by addition of LAB (with, without). Both treatments primarily influenced the fermentation pattern of the silages. Compared to restrictively wilted or LAB treated grass silages, intensively wilted or untreated grass silages showed a lower fermentation acid concentration and a higher concentration of water-soluble carbohydrates, and *vice versa*. The results of the *in situ* and *in vitro* approach showed a positive linear effect of dry matter concentration on prCP content. The application of LAB increased RUCP, but decreased the calculated MCP, resulting in a lower *in situ* prCP content compared to untreated silages. *In vitro*, no effect of LAB treatment could be shown, indicating a complete compensation between increased RUCP content and decreased MCP yield. The *in vitro* method probably reacted more sensitively to treatment differences that primarily influence MCP yield, which could not be represented with sufficient accuracy due to the non-specific calculation of MCP *in situ*. Since all samples were prepared from the same material, effective prCP contents differed between the different grass silages only in a range of approx. 15-30 g/kg OM. Nevertheless, significant relationships between *in situ* and *in vitro* determined effective prCP values could be shown at all passage rates (0.02, 0.04, 0.06/h), which confirms the suitability of the modHGT for prCP analysis.

In the second study, a total of 9 feeds, 13 feed mixtures made thereof, and 12 feed mixtures provided by commercial dairy farms were examined. The feeds used in this trial covered a wide range of different chemical ingredients and were therefore suitable for a method comparison. Significant relationships between the effective prCP values determined *in situ* and *in vitro* could be shown at a passage rate of 0.05/h ($R^2 = 0.68$) and 0.08/h ($R^2 = 0.76$). The already problematic assumption of a constant efficiency of MCP yield of 181 g/kg FOM appeared to be insufficient for the underlying data set, as it could be assumed that the composition of FOM differed significantly between feeds. Subdividing FOM into fermented CP and FOM-CP free and subsequent recalculation of MCP (or prCP) assuming an efficiency of 104 g MCP/kg fermented CP and 208 g MCP/kg FOM-CP free, respectively, improved the relationship between both methods ($R^2 = 0.79$).

Despite the completely different methodological approach, the results of both studies showed a notable agreement between prCP values determined *in situ* and *in vitro*. The results suggested a more precise estimation of prCP values by the modHGT compared to the *in situ* method and indicated a sensitive reaction of the modHGT with regard to changes in MCP yield.

Zusammenfassung

Ein wichtiges Ziel in der Milchviehfütterung ist es, Nährstoffausscheidungen aus ökologischen, aber auch aus ökonomischen Aspekten zu reduzieren. Um eine adäquate Fütterung hochleistender Milchkühe bei Minimierung der Nährstoffausscheidung zu gewährleisten sind, neben der Kenntnis des Energie- und Proteinbedarfs der Tiere, präzise Angaben zum Energie- und Proteinlieferungsvermögen der eingesetzten Futtermittel erforderlich. Der Proteinbedarf der Tiere, sowie das Proteinlieferungsvermögen der Futtermittel werden im aktuellen deutschen Proteinbewertungssystem durch die Größe nutzbares Rohprotein am Duodenum (**nXP**) charakterisiert, welche der Summe aus pansenstabilem Rohprotein (**UDXP**) und im Pansen mikrobiell synthetisiertem Rohprotein (**MXP**) entspricht. Unter Berücksichtigung der Aminosäurenkonzentration und dessen intestinaler Verdaulichkeit kann das nXP in die international gebräuchlichere Kenngröße metabolisierbares Protein (**MP**) umgerechnet werden.

Ein anerkanntes Verfahren zur Bestimmung des Proteinabbaus im Pansen, respektive des UDXP, ist die *in situ*-Methode, welche aufgrund ihrer verbreiteten Anwendung als Referenz angesehen werden kann. Tierunabhängige Alternativen zur UDXP-Bestimmung bieten enzymatische und chemische Verfahren. Eine Messung des MXP hingegen kann nur unter erheblichem experimentellem Aufwand an duodenal fistulierten Tieren realisiert werden. Da die MXP-Bildung vor allem ein energieabhängiger Prozess ist, wird diese Größe daher von Variablen abgeleitet, welche die Menge an fermentierbarer organischer Masse (**FOM**) im Pansen repräsentieren. Der Umfang der MXP-Bildung hängt jedoch nicht nur von der Menge an FOM ab, sondern unter anderem auch von dessen Nährstoffzusammensetzung, der im Pansen zur Verfügung stehenden N-Quellen und der Trockenmasseaufnahme.

Eine vielversprechende *in vitro*-Methode zur Messung des Gehalts an nXP – der modifizierte Hohenheimer Futterwerttest (**modHGT**) – wurde im Rahmen dieser Arbeit anhand von zwei Studien hinsichtlich seiner Eignung zur Bestimmung des Proteinwerts geprüft. Diese Methode basiert auf einer Inkubation von Futtermittelproben in einer Pansensaft-Pufferlösung und reagiert empfindlich gegenüber Einflüssen auf die MXP-Bildung. Zur Beurteilung des modHGT erfolgte eine Gegenüberstellung von *in vitro* und *in situ* ermittelten nXP-Gehalten bei Annahme unterschiedlicher Passageraten des Futters vom Pansen in den Dünndarm (**effektives nXP**). Der *in situ* nXP-Wert wurde anhand der Summe von RUCP und MCP kalkuliert, wobei

eine Bildung von 181 g MXP/kg FOM unterstellt wurde. Die FOM wurde wiederum aus dem *in situ* ermittelten effektiven OM-Abbau abgeleitet.

In der ersten Studie wurde neben dem Methodenvergleich der Effekt des Anwelkens von Gras vor der Silierung, sowie der Einsatz von Milchsäurebakterien (**MSB**) auf den Proteinwert von Grassilagen *in vitro* und *in situ* untersucht. Das Probenmaterial umfasste insgesamt 16 Grassilagen, die sich durch den Trockenmassegehalt vor der Silierung (170, 310, 390, 420, 470, 530, 580, 600 g/kg) sowie den Zusatz von MSB (mit, ohne) unterschieden. Beide Behandlungen beeinflussten in erster Linie das Fermentationsmuster der Silagen. Im Vergleich zu restriktiv angewelkten bzw. mit MSB versetzten Grassilagen, wiesen intensiv angewelkte bzw. unbehandelte Grassilagen eine geringere Gärssäurekonzentration und eine höhere Konzentration an wasserlöslichen Kohlenhydraten auf, und umgekehrt. Die Ergebnisse des *in situ* und *in vitro* Ansatzes zeigten einen positiven linearen Effekt der Trockenmassekonzentration auf den nXP-Gehalt. Der Einsatz von MSB führte zu einer Erhöhung des UDXP, verringerte jedoch das kalkulierte MXP und bedingte dadurch einen geringeren *in situ* nXP-Gehalt gegenüber unbehandelten Silagen. *In vitro* konnte kein Effekt der MSB-Behandlung gezeigt werden, was auf eine vollständige Kompensation zwischen erhöhtem UDXP und verringerter MXP-Bildung hinweist. Die *in vitro* Methode reagierte vermutlich sensibler auf die Behandlungsunterschiede, die in erster Linie die MXP-Bildung beeinflussen, welche aufgrund der unspezifischen Kalkulation des MXP *in situ* nicht ausreichend genau dargestellt werden konnten. Da alle Proben aus demselben Ausgangsmaterial hergestellt wurden, unterschieden sich die effektiven nXP Gehalte zwischen den verschiedenen Grassilagen lediglich in einem Bereich von ca. 15-30 g/kg OM. Dennoch konnte zu allen unterstellten Passageraten (0.02, 0.04, 0.06/h) signifikante Beziehungen zwischen den *in situ* und *in vitro* bestimmten nXP-Werten aufgezeigt werden, was die Eignung des modHGT zur nXP Analyse bekräftigt.

Bei der zweiten Studie standen insgesamt 9 Einzelfuttermittel, 13 daraus hergestellte Rationen, sowie 12 von landwirtschaftlichen Betrieben bereitgestellte Rationen zur Verfügung. Die in diesem Versuch verwendeten Futtermittel deckten eine breite Variation verschiedener Inhaltsstoffe ab und waren daher für einen Methodenvergleich geeignet. Signifikante Beziehungen zwischen den *in situ* und *in vitro* bestimmten nXP-Werten konnten sowohl bei einer Passagerate von 0.05/h ($R^2 = 0.68$), als auch bei 0.08/h ($R^2 = 0.76$) aufgezeigt werden. Die ohnehin problematische Unterstellung einer konstanten Effizienz der MXP Synthese von 181 g/kg FOM erschien für den zugrundeliegenden Datensatz als unzureichend, da davon

auszugehen war, dass sich die Zusammensetzung FOM deutlich zwischen den Futtermitteln unterschied. Eine Unterteilung der FOM in fermentiertes XP und FOM-XP-frei und die anschließende Neuberechnung des MXP (bzw. nXP) unter der Annahme einer Effizienz von 104 g MXP/kg fermentiertes XP bzw. 208 g MXP/kg FOM-XP frei, verbesserte die Beziehung zwischen den beiden Methoden ($R^2 = 0.79$).

Trotz des völlig unterschiedlichen Methodenansatzes haben die Ergebnisse beider Studien eine auffallend gute Übereinstimmung zwischen den *in situ* und *in vitro* ermittelten nXP-Gehalten gezeigt. Die Ergebnisse deuten eine im Vergleich zur *in situ*-Methode präzisere Einschätzung des nXP-Gehalts durch den modHGT an und weisen auf eine sensitive Reaktion des modHGT hinsichtlich Veränderungen der MXP-Bildung hin.

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