Comparative studies of ruminal protein degradation using *in situ* and enzymatic *in vitro* methods

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Contents

I Abbreviations

CP	crude protein
DDGS	dried distillers' grains with solubles
degQ	degradation quotient
ED	effective ruminal crude protein degradation
GfE	Society of Nutrition Physiology
SGP	*Streptomyces griseus* protease
SGPM	*Streptomyces griseus* protease method
SGPM-L	*Streptomyces griseus* protease method according to Licitra et al. (1998)
SGPM-K	*Streptomyces griseus* protease method according to Krishnamoorthy et al. (1983)
SHU	spectrophotometric hemoglobin unit
TP	true protein

II Figures

General discussion

Supplements

III Tables

Background, General discussion

1. Introduction

An accurate protein evaluation of feedstuffs for ruminants is necessary to ensure that nitrogenous compounds are supplied in accordance with the specific nutritional requirements of the animal (Schwab and Broderick, 2017). In this context, the knowledge of rumen protein degradation kinetics has proven to be a key factor, as protein metabolism in the rumen has far-reaching consequences for the overall nitrogen balance of the animal (Danfær, 1994; Castillo et al., 2000).

Several methods can be used to determine the ruminal degradation of feed protein (Schwab and Broderick, 2017). The *in vivo* method using multi-fistulated cows is generally considered to be the real reference (Hvelplund and Weisbjerg, 2000). However, the complexity of the *in vivo* method and bias of the results caused by markers make it difficult to routinely determine ruminal crude protein (CP) degradation (Stern et al., 1994; Tamminga and Chen, 2000). The *in situ* method using rumen-fistulated cows is an accepted reference method and standardized by protocols (Hristov et al., 2019; GfE, 2022). However, the labor-intensive and time-consuming approach as well as limited controllable factors make it difficult to reliable estimate ruminal CP degradation using the *in situ* method (Nocek, 1988; Michalet-Doreau and Ould-Bah, 1992; Hristov et al., 2019). The German feed evaluation system refers to alternative or *in vitro* methods for estimating ruminal CP degradation (Society of Nutrition Physiology (GfE), 2023).

Approaches for the *in vitro* estimation of ruminal CP degradation that do not require animals appeared to be suitable for routine and reliable application and consider the 3 R's Principle (Replacement, Refinement, Reduction; Maestri, 2021). The *Streptomyces griseus* protease (SGP) is used in the *Streptomyces griseus* protease method (SGPM) according to Licitra et al. (1998) (SGPM-L) to estimate purely enzymatic ruminal CP degradation under standardized conditions. However, the protein in cereals, by-products of cereal processing, extraction meals and forages appears to be in complexes with fiber and/or starch (Assoumani et al., 1992; Abdelgadir et al., 1997; Rosenfelder et al., 2013; Jha et al., 2015; Ozturk et al., 2021). Within these feed- and feed-group-specific protein-carbohydrate complexes, the protein appeared to be less accessible to the SGP, resulting in reduced accuracy of the SGPM in estimating CP degradation compared to the *in situ* reference (Assoumani et al., 1992; Cone et al., 1996; Abdelgadir et al., 1997).

In summary, the accuracy of estimating ruminal CP degradation using the SGPM-L compared to *in situ* reference data might be reduced by feed-specific protein-carbohydrate complexes. The SGPM-L has never been applied to a wide range of feedstuffs to study the influence of protein-carbohydrate complexes on protein degradation by the SGP and validated to an *in situ* reference.

2. Background

2.1 Ruminal degradation of feed protein

The ingested feed is colonized and degraded by ruminal microorganisms. The result of the proteolytic activity of the microbes is the undegraded feed protein and the microbial protein that enter the duodenum (Bach et al., 2005; Kim and Lee, 2021). The ruminal protein degradation can be determined by using various methods. Selected methods are described below.

2.2 Determination of ruminal crude protein degradation

2.2.1 *In vivo* method

The estimation of CP degradation *in vivo* relies on ruminant with rumen and abomasal or duodenal fistulas. The *in vivo* method is regarded as the real reference, as the CP degradation is estimated simultaneously with the animal specific endogenous and microbial protein by different markers (Stern et al., 1994; Hristov et al., 2019). Therefore, the *in vivo* degradation data are used to evaluate, and assess other methods for the estimation of ruminal CP degradation (Krishnamoorthy et al., 1983). However, the *in vivo* method is subject to factors that severely limit its routine use (Vanzant et al., 1996; Hristov et al., 2019). Several markers are necessary to determine the digesta flow and to distinguish between degraded CP, microbial and endogenous protein (Hristov et al., 2019). It is assumed that the marker is homogeneously distributed in liquid and solid phases in the rumen and enter the small intestine via constant digesta flow. Inhomogeneous marker distribution and variations between animals are considered key reasons of unreliable estimation of microbial and endogenous protein, resulting in variable *in vivo* degradation data (Vanzant et al., 1996; Hristov et al., 2019). Finally, there is no standardized protocol that enables a standardized and thus reproducible experimental procedure. Alternative methods for estimating ruminal CP degradation are of important interest (Stern et al., 1994). For these reasons, the less intensive *in situ* method is used as standard for the estimation of ruminal CP degradation (Hvelplund and Weisbjerg, 2000). In the future, the possibility to carry out the *in vivo* method is not very promising, as animal protection laws make it difficult to obtain an approval for animal experiments (TSchG, 2022).

2.2.2 *In situ* method

The estimation of ruminal CP degradation using the *in situ* method (also known as the *in sacco*, nylon bag or polyester bag technique) is currently applied as the standard method for evaluating dietary protein in ruminants in several countries (Hristov et al., 2019). The methodology is briefly described, based on the protocol by Madsen and Hvelplund (1994). Nylon bags containing ground and precisely weighed feedstuff are exposed to the environment in the rumen for defined periods of time (2 - 96 h) in at least three ruminal-fistulated animals (cattle, sheep, goats). The 0 h incubation time is determined

by washing the nylon bags in the washing machine without spinning. During a lag time (*L*), rumen microbes colonize the nylon bag and after this time, microbial CP degradation begins. The ruminal microbes hydrolyze the protein in the cell matrix due to their diverse and adaptable enzymatic activities (Bach et al., 2005; Deusch et al., 2017). After a specified incubation period, the bags are removed from the rumen, rinsed under running water, dried and reweighed. The feedstuff residues are pooled and prepared for CP analysis (Nocek, 1988; Hvelplund and Weisbjerg, 2000; Hristov et al., 2019). The disappearance of CP at the specific incubation times, which is assumed as the degradation of CP, is summarized in an animal-specific degradation curve. By modeling the degradation curve, e.g., according to McDonald (1981), protein degradation parameters are estimated (*a*, immediately degradable/soluble fraction; *b*, potentially degradable fraction; *c*, degradation rate; *k*, assumed ruminal passage rate; *L*). These parameters are used to calculate the effective ruminal CP degradation (ED) (Wulf and Südekum, 2005).

The literature indicate a moderation relationship between *in vivo* and *in situ* CP degradation data. Gosselink et al. (2004) reported no significant relationship between *in vivo* and *in situ* CP degradation for 11 forages ($p > 0.1$). Vanzant et al. (1996) observed no significant differences between *in vivo* and *in situ* CP degradation due to bias in the *in vivo* CP degradation data. In general, The authors explained that the estimation of CP degradation in both methods is strongly influenced by individual animal effects and, for the *in vivo* method, by variations in CP degradation data caused by different methods of endogenous and microbial protein estimation (Vanzant et al., 1996; Gosselink et al., 2004).

The *in situ* degradation data are subject to feed-related, method-related and animal-related factors (Figure S1). The grinding intensity of the feedstuff influences the *in situ* CP degradation by alteration of its physical structure (Vanzant et al., 1998). The higher relative content of small soluble particles by intensive grinding increased the surface area of the feedstuff which promotes CP disappearance and microbial attack. In addition, if the feedstuff is too finely ground (< 0.6 mm), reduced degradability due to clumping has been observed (Weakley et al., 1983; Michalet-Doreau and Cerneau, 1991). Protocols recommend grinding levels of 2 mm (concentrated feed) and 3 mm (forages) (Vanzant et al., 1998; GfE, 2022). The pore size of the nylon bag influences the *in situ* CP degradation by permeability for food particles and microbes (Weakley et al., 1983; Nocek, 1985) (Figure S1). Other factors influencing *in situ* degradation are described in detail in the following literature sources: Weakley et al. (1983); Nocek (1985); Nocek (1988); Michalet-Doreau and Ould-Bah (1992) and Vanzant et al. (1998).

The following factors are considered relevant in the evaluation of *in situ* degradation data:

I) Correction of *in situ* degradation data for microbial nitrogen

During ruminal incubation in the *in situ* method, the feedstuff is colonized by microbes. The intensity of microbial colonization is related to the feedstuff and incubation time (Krawielitzki et al., 2006). The proportion of microbial nitrogen ranged between 5 and 85 % of the whole nitrogen contained in nylon bag for 48 h and 72 h of incubation (Alexandrov, 1998). The microbial colonization is positively correlated with the neutral detergent fiber content and negatively correlated with the CP content of the incubated feedstuff (Krawielitzki et al., 2006). The correction of microbial nitrogen attached to *in situ* feed residues is a key factor for estimating reproducible *in situ* degradation data, since uncorrected *in situ* CP degradation would be underestimated (Wanderley et al., 1993; Vanzant et al., 1998; Dixon and Chanchai, 2000). The correction of *in situ* CP degradation is generally recommended, especially for forages characterized by a high fiber and low protein content (Alexandrov, 1998). Parand and Spek (2021) developed equations for the estimation of microbial nitrogen contamination specific to incubation time and feedstuff based on CP and NDF content. These equations are explicitly recommended by the GfE for the correction of *in situ* CP degradation by microbial nitrogen, providing reliable estimates of *in situ* CP degradation (GfE, 2022).

II) Correction of *in situ* degradation data for small particles

A disadvantage of the *in situ* method is that the degradation of the soluble feedstuff fractions could not be determined (Nocek, 1985; Gierus et al., 2005). During ruminal incubation of the nylon bags and during rinsing of post-incubated nylon bags with water, parts of the feedstuff are rinsed out as a washout fraction. The washout fraction is calculated as the difference between the feedstuff nitrogen and the nitrogen remained in the 0 h-nylon bags after washing. This washout fraction can account for up to 74 % of the total feed nitrogen. It consisted of a soluble component and small insoluble particles, each with specific degradation kinetics (Gierus et al., 2005). It is assumed that the small particles are degraded in a similar way to feed residue in the nylon bag and it is therefore suggested by Hvelplund and Weisbjerg (2000) to correct the *in situ* ED for small particle losses. However, this data preparation step could lead to implausible *in situ* ED. The method for determining small particle losses itself appears to be inaccurate. The small particle fraction is estimated as the difference between the washout fraction and a water-soluble fraction determined by filtration (Hvelplund and Weisbjerg, 2000). This procedure combines two fractions, each determined by different dissolution methods (Vanzant et al., 1998; De Jonge et al., 2013; De Jonge et al., 2015). Therefore, it is recommended to carefully evaluate the *in situ* CP degradation data when correcting for small particles (Hvelplund and Weisbjerg, 2000; Gierus et al., 2005; GfE, 2022). De Jonge et al. (2013) suggested rinsing the nylon bags in a closed system in which a buffer solution (pH 6.2) is used instead of water. The simultaneous

determination of the soluble and insoluble feed fractions compensates for the limitations of the approach proposed by Hvelplund and Weisbjerg (2000) by standardized conditions in one approach.

III) Species and diet

Siddons and Paradine (1983) showed by the *in situ* CP degradation of six protein concentrates and one forage estimated in sheep and steers that the *in situ* CP degradation was up to 19 % higher in sheep than in steers (Figure S1). They suspect that the species effect on *in situ* CP degradation might be related to species-specific ruminal nitrogen metabolism. However, they concluded that when the nitrogen uptake of sheep and steers was considered in relation to absolute ruminal CP degradation, there were minor species-specific differences in ruminal CP degradation. Several studies also classify the effect of the animal species on the *in situ* CP degradation as negligible (Huntington and Givens, 1997; Nandra et al., 2000). Therefore, no explicit reference is made to an animal species in protocols of the *in situ* method (Madsen and Hvelplund, 1994; Vanzant et al., 1998; GfE, 2022). The ruminal microbiome itself appears to have a greater effect on ruminal CP degradation than the animal species (Henderson et al., 2015). The ruminal microbiome is primarily influenced by the administered diet (Henderson et al., 2015; Deusch et al., 2017). The global meta-analysis by Henderson et al. (2015) highlights dietary influences on the ruminal microbiome and thus on its function. It was shown that the ruminal microbiome and its enzymatic activities are diet-specific with a pronounced influence of the concentrate to forage ratio. For example, the *Fibrobacter* phylum is significantly more abundant in the rumen of ruminants fed a fiber-rich diet, whereas the *Prevotella* and *Succinivibrionaceae* phyla are more abundant in ruminants fed a concentrate-rich diet (Henderson et al., 2015). With reference to *in situ* estimated CP degradation, Weakley et al. (1983) observed decreased *in situ* CP protein degradation (24 h incubation time) of soybean meal by 7.5 %-points when concentrate mixture proportion (soybean meal and corn grain) of the diet was increased from 20 to 75 % in relation to alfalfa hay (Figure S1). Siddons and Paradine (1983) observed also decreased *in situ* CP degradation by maximal 36 %-points when barley was included in the administered diet at a proportion of 35 % (Figure S1). Therefore, the diet should include a broad spectrum of feedstuffs to ensure a wide diversity of the microbes and to reduce dietary influences on ruminal CP degradation estimated by the *in situ* method (Michalet-Doreau and Ould-Bah, 1992; Vanzant et al., 1998).

IV) Standardization of the method

Ring studies that included different institutions estimating *in situ* CP degradation of the same feedstuffs, indicated an unsatisfactory reproducibility of the *in situ* estimated degradation data by high variations among institutions (Madsen and Hvelplund, 1994; Wilkerson et al., 1995). The authors

attributed the variation in *in situ* CP degradation data to methodological factors such as the nylon bag characteristics, rinsing of incubated nylon bags and nitrogen analysis. In addition, Michalet-Doreau and Ould-Bah (1992) also suspect that the animal itself could attribute to variation in *in situ* CP degradation data. The microbial composition of the rumen as a diverse and dynamic system makes it difficult to reproducibly estimate *in situ* CP degradation under standardized conditions (Michalet-Doreau and Ould-Bah, 1992). The diet administered appears to be critical, as the CP degradability of a feedstuff cannot be applied equally to different feeding situations (Siddons and Paradine, 1983). It is recommend to use a standardized protocol for the estimation of *in situ* CP degradation with the aim of minimizing the number of factors and their interactions to improve reproducible estimation of *in situ* CP degradation (Vanzant et al., 1998; GfE, 2022). Although the *in situ* method is labor-intensive and cannot account for physiological processes, i.e., salivating the feedstuff and transport of the feedstuff through the rumen, *in situ* CP degradation serves as reference data (Hvelplund and Weisbjerg, 2000) and has been used for the validation of alternative methods estimating ruminal CP degradation (Michalet-Doreau and Ould-Bah, 1992). However, there is still a need for alternative methods to estimate ruminal CP degradation without using animals, as standardized conditions can be implemented for reproducible estimates.

2.2.3 *In vitro* method

Various *in vitro* approaches are available to determine ruminal CP degradation. An established standard *in vitro* method is the modified Hohenheim Gas Test developed by Raab et al. (1983). Since it is based on incubation of ruminal fluid batch cultures, it requires rumen cannulated donor animals. This is the same disadvantage that all manual or automatic batch culture or continuous incubation systems have. Ruminal fluid, diet of donor animals as well as incubation conditions (temperature control, agitation and anoxicity) are major sources of variation and bias (Getachew et al., 2002; Keim et al., 2017). Therefore, it seems to be necessary to apply methods which do not rely on cannulated animals and are potentially useful for routine analysis.

2.2.3.1 *Streptomyces griseus* protease method

Enzymatic approaches have the advantage of high sample throughput, good reproducibility and they do not require the use of animals (Hristov et al., 2019). However, the specificity and activity of commercial proteases may differ from the proteases in the rumen (Nocek, 1988). In addition, the specificity of proteases for only proteins appeared to be limiting, as the feed protein can be present in complex structures within the plant cell (Jha et al., 2015; Pedersen et al., 2015). Therefore, CP degradation by enzymatic approaches using only proteases may appear disadvantageous compared to the diverse enzymatic activities of the rumen.

Commercial proteases of bacterial, plant or fungal origin are used for the estimation of ruminal CP degradation (Table 1). The bacterial protease of *Streptomyces griseus* was frequently used to estimate ruminal CP degradation (Table 1). The SGP hydrolyzes proteins, oligo-, tri- and dipeptides non-specifically by a mixture of endo- and exopeptidases up to 90 % to free amino acids (Nomoto et al., 1960; Trop and Birk, 1970). Ficin, bromelain and papain protease used in other protease approaches (Table 1) are characterized by solely endo-protease activity and the *Aspergillus oryzea* protease by unknown activity (Luchini et al., 1996).

Krishnamoorthy et al. (1983) developed the SGPM (SGPM-K) by considering the conditions set by the rumen in terms of the pH of the buffer solution and the incubated sample quantity. The proteolytic activity of the rumen fluid of a donor cow was converted into a final SGP concentration. *In vivo* CP degradation data from 12 diets were used to validate this SGP concentration. This can be considered as a significant differentiating feature compared to enzymatic approaches including ficin, bromelain, papain or *Aspergillus oryzae* protease, where the protease dose was determined independently of the animal.

The correlations between the *in vitro* and *in situ* CP degradation data shown in Table 1 serve more as a tendency classification of the protease assays, since the *in vivo* and *in situ* degradation data are not corrected for the water-soluble protein (exception: De Boever et al. (1997)) nor for the microbial protein (exception: Gosselink et al. (2004) and Edmunds et al. (2012)). In the following, the factors influencing enzymatic CP degradation are presented with focus on the SGPM.

I) Use of biocides

The feedstuffs are naturally colonized to varying degrees with plant-own and environmentally associated microbes (Ikeda-Ohtsubo et al., 2018; Delitte et al., 2021; Bachmann et al., 2022). The incubation conditions of the approaches appeared favorable for microbial mass proliferation. Different antibiocides were applied in protease approaches to prevent microbial activity during *in vitro* incubation and to provide reliable estimation of *in vitro* ruminal CP degradation (Table 1). Sodium azide as it was used by Poos-Floyd et al. (1985) and Assoumani et al. (1992), is not recommended and should be replaced with agents that do not adversely affects protease activity. Devi et al. (2008) show that sodium azide concentration of 0.03 % (*w*/*v*; 5 mM) inhibited the activity of *Aspergillus niger* protease by reducing its activity by 30 %. Combined preparations of different antibiotics might be effective against bacteria of different categories (Cone et al., 2002; Cone et al., 2004; Gosselink et al., 2004) (Table 1). The combined preparation of penicillin-streptomycin appears to be suitable for this purpose. It is effective against gram-positive and gram-negative bacteria and has been used with trypsin in cell cultures for the hydrolysis of epithelial cells (You and Brody, 2012). For routine application, it should

be ensured that no or numerically small influence on CP degradation by the SGP are expected from the use of antibiotics.

II) Duration and timepoints of incubation

Krishnamoorthy et al. (1983) recommended an incubation time of 18 h for concentrates and 48 h for forages in the SGPM-K. However, the use of only one incubation time incorrectly assumes a linear progression of the enzymatic CP degradation. The enzymatic degradation curve corresponds more to the exponential curve ending in a degradation plateau (Figure S2) (Bisswanger, 2014). In addition, Licitra et al. (1999) reported a lag time of 1.9 h in the SGPM, which should also be considered. The incubation time series as used in the *in situ* method for at least 24 h and maximal 48 h appears to be suitable in the SGPM. Multiple incubation time points may take into account the lag time of the SGP, a degradation plateau of the protein, and the degradation kinetics of specific CP fractions. It would also allow reliable modelling of CP degradation data estimated by the SGPM (Ørskov and McDonald, 1979).

Another factor regarding the maximum incubation time is the activity of the SGP. It appears unclear whether the SGP exhibits measurable activity after 24 h incubation time. A maximum incubation time of 48 h was used in approaches using the SGP (Table 1).

III) pH of buffer solution

According to the manufacturer (Merck, KGaA, Darmstadt, Germany), the SGP is active in a pH range of 6.0 - 9.0 with an activity peak at pH 8.8. De Boever et al. (1997) concluded that a pH of 8.0 should be used in the incubation of the SGP to achieve optimal activity and thus, sufficient enzymatic CP hydrolysis. Furthermore, alkaline pH values also favor the dissolution of protein fractions of feed groups such as legume grains, cereal grains and silages, which has an additive effect on enzymatic CP degradation (Blethen et al., 1990; Licitra et al., 1998; De Jonge et al., 2009). However, Assoumani et al. (1992) suggest that rumen-similar pH should be applied in the SGPM-K to represent an *in vitro* protein solubility with physiological reference, although they observed reduced CP degradation by the SGP at pH 6.5 compared to pH 8.0. De Boever et al. (1997) detected with pH 6.7 in the SGPM according to Aufrere and Cartailler (1988) slightly lower correlation to *in situ* reference and, however, improved reproducibility of the results due to lower repeatability error compared to pH 8.0. The choice of the buffer solution pH is a central aspect of enzymatic approaches. The pH of the buffer solution determines whether optimal enzyme activity is achieved or whether physiological rumen pH conditions are incorporated, which may result in reduced enzyme activity.

IV) Dosage and concentration of *Streptomyces griseus* protease

According to the literature, the SGP dose can be divided into a feed-independent, i.e. constant dose according to Krishnamoorthy et al. (1983) and its modification according to Aufrere and Cartailler (1988) or a feed-specific dose according to Licitra et al. (1998). The latter is described in chapter 2.3.1. Krishnamoorthy et al. (1983) determined the proteolytic activity of rumen fluid with casein and converted it to an SGP concentration. The dose of 10 mL SGP solution per feedstuff (0.33 U/mL SGP solution that corresponds to 0.061 mg enzyme powder/mL buffer solution) (Table 1) was validated by *in vivo* CP degradation data of 12 diets. The correlation of r = 0.78 with the *in vivo* reference appeared to be sufficient, although it should be noted that the SGP was intentionally overdosed by Krishnamoorthy et al. (1983) to ensure full substrate saturation and thus achieve the highest possible CP degradation compared to *in vivo* CP degradation data. The SGP dose set by Krishnamoorthy et al. (1983) was validated once again on *in vivo* CP degradation data by Gosselink et al. (2004) using 11 different forages. They found no significant correlation between *in vivo* and *in vitro* CP degradation data ($p > 0.1$) (Gosselink et al., 2004).

Aufrere and Cartailler (1988) suggest an SGP concentration of 1 mg enzyme powder per 50 mL buffer solution (equivalent to 0.02 mg enzyme powder/mL buffer solution), According to their results, this SGP concentration resulted in smaller differences between *in vitro* and *in situ* CP degradation data. This validation by Aufrere and Cartailler (1988) should be viewed critically because the comparison of *in vitro* and *in situ* CP degradation data was limited to 24 h incubation time for both methods and the *in situ* CP degradation data was not corrected for microbial nitrogen.

V) Feed protein matrix

The degradation of feed protein is influenced by the its solubility and its embedding in complex structures within the plant cell (Shewry and Halford, 2002; Bach et al., 2005). The protein solubility is feed-specific and depends on the pH of the buffer solution (Shewry and Halford, 2002; Hedqvist and Udén, 2006). Protein solubility was found to be high in silages and legume grains and low in extraction meals and grain processing by-products (Hedqvist and Udén, 2006). Feed protein solubilization is enhanced by changing the pH to the alkaline range (Cone, 1993). Correlation analysis between protein solubility and *in situ* CP degradation revealed a weak relationship (r= 0.53 - 0.57) (Aufrère et al., 1991; Shannak et al., 2000). The protein of cereals, by-products of cereal processing, extraction meals and forages is complexed with carbohydrates, i.e., fiber and starch, which may act as a physical barrier to enzymatic hydrolysis (Assoumani et al., 1992; Abdelgadir et al., 1997; Rosenfelder et al., 2013; Ozturk et al., 2021). The protein in these feedstuff groups appeared to be less accessible to the SGP. This led to a decrease in accuracy when using the SGP to estimate CP degradation compared to the *in situ*

reference. (Assoumani et al., 1992; Cone et al., 1996; Abdelgadir et al., 1997). It has been reported in several studies that the addition of carbohydrases assist the protein hydrolysis by a protease, indicating the influence of protein-carbohydrate complexes on protein degradation. (Assoumani et al., 1992; Tománková and Kopečný, 1995; Abdelgadir et al., 1997; Pedersen et al., 2015; Ozturk et al., 2021). The influence of protein-starch complexes on CP degradation by the SGP was reported by Assoumani et al. (1992). Above the starch content of 230 g/kg dry matter, the CP degradation by the SGP should be supported by amylolytic enzymes to reduce the difference between *in situ* and *in vitro* CP degradation (Assoumani et al., 1992).

Table 1: Comparison of enzymatic tests using commercially proteases for the estimation of ruminal crude protein degradation

Reference	Enzymes	Biocide	Incubation	Feedstuffs	Enzyme dose	Correlation with *in situ* data
Assoumani et al. (1992)	a) *Streptomyces griseus* protease b) *Bacillus subtilis* protease	Sodium azide	a) pH 8.0 (borate-phosphate buffer) and pH 6.5 (sodium citrate buffer) b) pH 6.5 (sodium citrate buffer) at 39 °C; 4 h	2 protein concentrates; 3 cereals; 3 by-products	a) 10 mL protease solution (0.33 U[1]/mL) independent of feedstuff b) 0.2 mL to 50 mL buffer solution in total	a) pH 8.0: 0.78 (0.82)[2] pH 6.5: 0.95[2] b) 0.96[2]
a) Cone et al. (2002) b) Cone et al. (2004) c) Gosselink et al. (2004)	*Streptomyces griseus* protease	Tetracycline and nystatin	a, b) pH 8.0 (borate-phosphate buffer) at 40 °C; 0, 1, 6, 24 h c) pH 8.0 (borate-phosphate buffer) at 40 °C; 24 h	a) 9 protein concentrates; 11 by-products; 2 cereals; 3 legume grains; 1 cereal grain b) 22 forages c) 11 forages	1 mg enzyme powder per 50 mL buffer solution independent of feedstuff[3]	a) 0.74[4] b) 0.84[4] c) No relationship with *in vivo* and < 0.71 with *in situ* data
De Boever et al. (1997)	a) *Streptomyces griseus* protease b) *Ficus glabrate* protease	Not used	a,b) pH 6.7 (borate-phosphate buffer) at 40 °C; 1, 6, 24 h a) pH 8.0 (borate phosphate buffer) at 40 °C; 1, 6, 24 h	29 compound feeds; 2 protein concentrates; 8 forages; 2 by-products; 2 low-protein feed	a) 1 mg enzyme powder per 50 mL buffer solution[3] b) 5 mg enzyme powder per 50 mL buffer solution independent of feedstuff	a) pH 6.7: 0.86 pH 8.0: 0.88 pH 8.0: 0.78[5] b) 0.81

Table 1: Continuation

Reference	Enzymes	Biocide	Incubation	Feedstuffs	Enzyme dose	Correlation with *in situ* data
Edmunds et al. (2012)	*Streptomyces griseus* protease	Not used	pH 6.7 (borate-phosphate buffer) at 39 °C; 24 h	25 forages	Feed-specific depending on true protein concentration (0.58 U^1/mL at 24 U^1/g true protein	*k*2: 0.83 *k*4: 0.84 *k*6: 0.83
Krishnamoorthy et al. (1983)[6] (SGPM-K)	*Streptomyces griseus* protease	Not used	pH 8.0 (borate-phosphate buffer) at 39 °C; 0.25, 0.5, 1, 2, 4, 8, 12, 18, 24, 32, 40, 48 h	12 grain mixtures	10 mL protease solution (0.33 U^1/mL) independent of feedstuff	0.78[7]
Licitra et al. (1998)[6] (SGPM-L)	*Streptomyces griseus* protease	Not used	pH 6.7 – 6.8 (borate-phosphate buffer) at 39 °C; 18 h concentrates; 24 h by-products and 30 h forages	4 protein concentrates; 6 forages; 2 cereals; 3 by-products	Feed-specific depending on true protein concentration	Not determined
Poos-Floyd et al. (1985)	a) *Streptomyces griseus* protease b) *Ficus glabrata* ficin c) *Ananas comosus* bromelain d) *Corica papaya* papain e) *Aspergillus oryzae* protease	Sodium azide	pH 6.0 (potassium phosphate buffer) at 39 °C; 1, 4, 8, 24 h	6 protein concentrates; 3 by-products; 1 forage	0.5 SHU^8/mL enzyme solution independent of feedstuff	a) 0.55 [4,7] b) 0.82 [4,7] c) 0.68 [4,7] d) 0.71 [4,7] e) 0.95 [4,7]

Table 1: Continuation

Reference	Enzymes	Biocide	Incubation	Feedstuffs	Enzyme dose	Correlation with *in situ* data
Roe et al. (1991)	a) *Streptomyces griseus* protease b) *Ficus glabrate* ficin c) *Bacillus subtilis protease* + endo-ß-glucanase & α-amylase[9]	Not used	a) pH 7.8 – 8.0 (borate-phosphate buffer) b) pH 6.0 (phosphate buffer) c) pH 6.5 (sodium citrate buffer) at 39 °C; 0, 0.5, 1, 2, 4, 8, 12, 24, 48 h	2 protein concentrates; 2 cereal by-products; 2 legume grains	a) 10 mL protease solution (0.33 U/mL) b) 10 mL ficin solution (1.4 g ficin in 1 L of 200 mL phosphate buffer and 6.1 g cysteine hydrochloride) c) 0.2 mL neutrase enzyme use independent of feedstuff	a) 0.76 – 0.98 b) 0.68 – 0.94 c) 0.33 – 0.87
Tománková and Kopečný (1995)	*Ananas comosus* Bromelain	chloramphenicol	pH 7.2 (phosphate buffer) at 40 °C; 24 h	a) 22 protein concentrates; 18 protein concentrate mixtures b) 28 cereals c) 41 fresh forages d) 68 hay and silages e) 9 sugar beet leaf silages	0.06 mg bromelain per 1 mL buffer solution independent of feedstuff	a) 0.839 b) 0.423 (0.809)[10] c) 0.730 d) 0.741 e) 0.876

[1]: 1 unit is the amount of enzyme that hydrolyses casein to produce color with Folin-Ciocalteu reagent equivalent to 1 µmol tyrosine/min at pH 7.5 and 37 °C; [2]: 1 h pre-incubation with α-amylase and ß-glucanase at 55 °C; [3]: modified *Streptomyces griseus* protease dosage according to Aufrere and Cartailler (1988); [4]: correlation corresponds to 24 h incubation time; [5]: sample weight adjusted to 20 mg nitrogen; [6]: standardized protocol; [7]: Correlation based on *in vivo* reference; [8]: amount of enzyme releasing 1 µmole tyrosine/min at pH 6.0 and 39 °C from hemoglobin; [9]: according to Assoumani et al. (1992) [10]: co-incubation with α-amylase (0.5 mg/mL buffer solution).

*k*2, 4, 6: assumed ruminal passage rate of 0.02, 0.04 and 0.06/h
SGPM-K: *Streptomyces griseus* protease method according to Krishnamoorthy et al. (1983); SGPM-L: *Streptomyces griseus* protease method according to Licitra et al. (1998); SHU: spectrophotometric hemoglobin unit; U: enzyme unit

2.3 *Streptomyces griseus* protease method according to Licitra et al. (1998)

2.3.1 Description and criticism

Licitra et al. (1998) modified the SGPM-K by adjusting the pH from 8.0 to 6.7 - 6.8 as the adoption provides incubation conditions similar to the rumen. The second modification concerns the SGP dose. The SGP solution of 10 mL (0.33 U/mL) in SGPM-K is applicated unspecific to the feedstuff (Table 1). This approach ignores any enzyme-substrate ratio which was initially defined by the SGP units to nitrogen concentration (Abdelgadir et al., 1997; De Boever et al., 1997). The substrate concentration can be a limiting factor in enzymatic reactions, as the substrate saturation allows an efficient rate of degradation by the enzyme (Bisswanger, 2014). The substrate-limiting factor in the SGPM was compensated by adjusting the sample weight to 15 mg or 20 mg nitrogen (Abdelgadir et al., 1997; De Boever et al., 1997; Mathis et al., 2001). However, the results of Abdelgadir et al. (1997) indicated that the *in situ* CP degradation was underestimated by this modified SGPM-K. This contrast to the results of a ring analysis in which *in situ* CP degradation tended to be overestimated by the modified SGPM-K (Mathis et al., 2001). De Boever et al. (1997) observed that considering an enzyme-to-substrate ratio by adjusting the sample weight appears to be successful in low protein feedstuffs as the underestimation of *in situ* CP degradation was reduced. However, these feedstuffs require impractical high sample weights to achieve the specific nitrogen amount of the defined substrate-enzyme ratio (Licitra et al., 1998).

To address this, the SGPM was modified by Licitra et al. (1998) by calculating the SGP dose according to the feed-specific true protein content (TP, CP minus A-fraction of the CP) according to the Cornell Net Carbohydrate and Protein System (Sniffen et al., 1992). The ratio of TP to SGP solution of the soybean meal used by Krishnamoorthy et al. (1983) is used as a calculation reference. In addition to this change, the incubation period in SGPM-L was arbitrarily set at 30 h for forages , 24 h for by-products and 18 h for concentrates (Licitra et al., 1998).

The CP degradation data estimated by the SGPM-L at 24 h incubation time has been validated by Edmunds et al. (2012) on *in situ* CP degradation data from forages. They observed a tendency of underestimated *in situ* CP degradation by the SGPM-L (Edmunds et al., 2012). Licitra et al. (1998) attributed lower enzymatic CP degradation in the SGPM-L to the feed-specific calculation of a lower SGP dose in the SGPM-L instead of using a constant 10 mL SGP solution in the SGPM-K. In addition, the previously described aspect of choosing only one incubation time may not provide reliable information on the degradation kinetics of the protein (Table 1). However, the accuracy of the SGPM-L may not be fully assessed by the results of Edmunds et al. (2012) which were limited to forages. More research is required on a wide range of feedstuffs incubated at multiple timepoints.

Bachmann et al. (2019) demonstrated successfully the sensitivity of the SGPM-L to feed treatments on ensiled + toasted pea grains. The sensitivity to treatment effects in feedstuffs is essential for the future

application as a feed evaluation method. Further studies on the sensitivity of the SGPM-L validated on *in situ* CP reference degradation data are necessary.

In summary, the SGPM-L protocol offers a promising approach to estimate purely enzymatic ruminal CP degradation under standardized conditions. The aspects of the *in situ* method related to the animal approach (diet effect, microbial nitrogen contamination and loss of small particles) are excluded and allow a comparatively high sample throughput (Hristov et al., 2019). The ring study by Mathis et al. (2001) comparing the *in situ* ED and the *in vitro* ED estimated by the SGPM based on the modifications of Abdelgadir et al. (1997) showed precisely estimated *in vitro* ED by their low standard errors. In addition, the SGPM-L has been included in purely enzymatic estimation of the intestinal CP digestibility for ruminants, considering new aspects of the protein evaluation for ruminants (Hippenstiel et al., 2015).

Some aspects of the SGPM-L protocol were ignored which might be relevant for reliable estimation of CP degradation. It seems unclear that no antibiotic solution was recommended in the SGPM-L protocol, although antibiotic solutions have been used in other protease approaches for preventing microbial growth (Table 1). The arbitrary choice of one single incubation time and the reduction of the incubation time to a maximum of 30 h do not allow a reliable modeling of *in vitro* CP degradation data for kinetic statements and may not ensure complete CP degradation by the SGP.

2.3.2 Extension of the *Streptomyces griseus* protease method according to Licitra et al. (1998)

Additional carbohydrases in the SGPM-K or in the SGPM according to Aufrere and Cartailler (1988) have only been investigated by Assoumani et al. (1992), Abdelgadir et al. (1997) and Cone et al. (1996). In general, the aim of additional carbohydrases was to assist the SGP in protein degradation of protein-carbohydrate complexes. For this purposes, approaches using the SGP or other proteases were supplemented with amylolytic or cellulolytic carbohydrases as separate incubation step prior to the SGP (pre-incubation) (Assoumani et al., 1992; Cone et al., 1996; Abdelgadir et al., 1997) or simultaneously with a protease (co-incubation) (Tománková and Kopečný, 1995; Pedersen et al., 2015; Karimi et al., 2018; Ozturk et al., 2021). Either solely enzyme (α-amylases, ß-glucanase, xylanase, cellulase in Abdelgadir et al., 1997; Assoumani et al., 1992; Pedersen et al., 2015) or enzyme mixtures of cell wall degrading enzymes (Driselase™ in Abdelgadir et al. (1997) , Viscozym® L in Cone et al. (1996) were used. Enzyme mixtures appear to be advantageous because multiple enzymatic specificities can dissolve different carbohydrates. The co-incubation approach appears to be more efficient than the pre-incubation because additional steps belonging to the pre-incubation, i.e., centrifugation, decanting, and pH adjustment can be avoided (Karimi et al., 2018). However, the co-incubation of protease and carbohydrase is decisively influenced by two points. First, co-incubation of different

enzymes represents a methodological compromise, as it cannot consider the optimal incubation conditions of both enzymes. The second point concerns the conflicting results of the literature. Saleh et al. (2004) assumed that the carbohydrase was degraded by the protease based on the reduced cellulase activity by approximately 75 % from co-incubation with a protease. This contrasts with the results of Tománková and Kopečný (1995), who successfully increased CP degradation in cereals by co-incubating bromelain protease and α-amylase (Table 1). However, the effect of co-incubating the SGP and carbohydrase under the conditions set by the SGPM-L on CP degradation has never been described in the literature. As demonstrated by Tománková and Kopečný (1995), the co-incubation of a protease and carbohydrase seems to be a promising approach to assist the hydrolysis of protein-carbohydrate complexes and reduce differences between *in situ* and *in vitro* CP degradation.

3. Scope of the thesis

The ruminal CP degradation can be estimated using several methods. The purely enzymatic approach using the protease from *Streptomyces griseus* proved to be suitable for future applications. However, protein-carbohydrate complexes that act as a physical barrier to enzymatic protein degradation are present in some feedstuffs. Within such a feed-specific matrix, the protein appeared to be less accessible and degradable to the SGP.

Our hypothesis was that specific characteristics (e.g. nutrient content, treatment) of individual feedstuffs or groups of feedstuffs lead to a differentiation with regard to the susceptibility of the feed protein to protease, and thus, to specific clustering.

For this purpose, the ED was estimated *in vitro* by the SGPM-L for 40 feedstuffs for which *in situ* reference degradation data were available. The difference between *in situ* and *in vitro* ED of a feedstuff was expressed as degradation quotient ($degQ = ED_{in\ vitro} - ED_{in\ situ} / ED_{in\ vitro}$). The grouping across the feedstuffs was examined using a cluster analysis. The clusters should provide information regarding the use of additional carbohydrases in the SGPM-L. The results are described in Paper I.

Feed-specific matrix effects can be reduced by pre-incubation or co-incubation with carbohydrases. Additional carbohydrases might improve the accuracy of the SGPM for estimating ruminal CP degradation. The carbohydrases have been applied either simultaneously with the protease as co-incubation or prior to the protease as pre-incubation. For methodological reasons, the co-incubation appears to be beneficial. However, the influence of the SGP on carbohydrase activity during co-incubation is not clear. In addition, the incubation conditions set by the SGPM (pH 6.75, 39 °C) have never been used in co-incubation approaches.

Our hypothesis was that during co-incubation, the SGP affects the α-amylase and cell wall degrading enzyme mixture Viscozym® L in the hydrolysis of carbohydrates by reducing the conversion of the substrates.

In order to test this hypothesis, the influence of the SGP on cellulose, pectin and starch degradation by carbohydrase (α-amylase) or carbohydrase mixture (Viscozym® L) was determined during co-incubation under conditions set by the SGPM-L (pH 6.75, 39 °C). The results are described in Paper II.

The moderate effect of the SGP on carbohydrase activity during co-incubation suggests the use of additional carbohydrases as a co-incubation approach with the SGP. The co-incubation approach appears to be appropriate to reduce the matrix effects and the differences to the *in situ* ED reference.

In addition, microbial activity during *in vitro* incubation might affect the accuracy of the SGPM. A penicillin-streptomycin solution seemed to suitable for incubation with the SGP.

The following two hypotheses were proposed:

(1) Additional penicillin-streptomycin solution, however, should have no or just a marginal effect on CP degradation.

(2) Additional carbohydrases assist the SGP to hydrolyze proteins from the matrix and reduce the differences to the *in situ* ED reference.

For this purpose, the ED of seven feedstuffs was estimated *in situ* using three rumen-fistulated cows and *in vitro* using the SGPM-L and SGPM-L with additional carbohydrase (α-amylase) or carbohydrase mixture (Viscozym® L). Each carbohydrase solution was added in four different doses as co-incubation to assist the SGP in hydrolyzation of protein-carbohydrate complexes. The influence of the penicillin-streptomycin solution on CP degradation by the SGP was investigated on seven feedstuffs. The results are described in Paper III.

4. Paper I

Feed Clusters According to In Situ and In Vitro Ruminal Crude Protein Degradation

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Feed Clusters According to In Situ and In Vitro Ruminal Crude Protein Degradation

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Simple Summary: The objective of the present study was to assess the suitability of an enzymatic laboratory method to estimate ruminal protein degradation. In situ data were used as reference. Appropriate in vitro methods are important to overcome methodological and ethical shortcomings, associated with the use of experimental animals. A cluster analysis was performed on the basis of differences between in vitro and in situ protein degradation. Among the 40 feedstuffs we tested, this difference was lowest in legume grains and highest in cereal by-products and barley. The feedstuffs clustered unspecific, not relatable to nutrient composition, origin or treatment. However, it was often obvious that additional carbohydrate-degrading enzymes should be used to assist the laboratory method, based solely on protease, to make it more conform with the in situ reference data.

Abstract: Effective degradation (ED) of crude protein (CP) was estimated in vitro at 0.02, 0.05 and 0.08 h^{-1} assumed ruminal passage rates for a total of 40 feedstuffs, for which in situ ED was available and used as reference degradation values. For this, the *Streptomyces griseus* protease test was used. The differences between in vitro CP degradation and the in situ CP degradation values were lowest in legume grains and highest in cereal by-products and barley. The differences between in situ and in vitro ED were expressed using a degradation quotient (degQ), where $degQ = (ED_{in\ vitro} - ED_{in\ situ})/ED_{in\ situ}$. Among the tested feedstuffs, eight specific clusters were identified according to degQ for the assumed passage rates. The feedstuffs clustered in an unspecific way, i.e., feedstuffs of different nutrient composition, origin or treatment did not necessarily group together. Formaldehyde–treated rapeseed meal, soybean meal, wheat, a treated lupin, sunflower meal and barley could not be assigned to any of the clusters. Groupwise degradation (range of degQ for assumed passage rates are given in brackets) was detected in grass silages (−0.17, −0.11), cereal by-products together with sugar beet pulp (−0.47, −0.35) and partly in legume grains (−0.14, 0.14). The clustering probably based on different specific nutrient composition and matrix effects that influence the solubility of feed protein and limit the performance of the protease. The matrix can be affected by treatment (chemically, thermally or mechanically), changing the chemical and physical structure of the protein within the plant. The *S. griseus* protease test had reliable sensitivity to reflect differences between native feedstuffs and treatments (thermally or chemically) that were found in situ. The in situ results, however, are mostly underestimated. The clustering results do not allow a clear conclusion on the groupwise or feed-specific use of carbohydrate-degrading enzymes as pre- or co-inoculants as part of the *S. griseus* protease test and need to be tested for its potential to make this test more conform with in situ data.

Keywords: concentrates; grass silages; ruminal crude protein degradation; in situ; in vitro; *Streptomyces griseus* protease

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1. Introduction

The estimation of ruminal crude protein (CP) degradation is an essential part of feed protein evaluation, with feed protein being a limited and expensive nutrient source. Therefore, sufficient quantification of ruminal CP degradation is of high interest in order to assess nitrogen utilization efficiency of ruminant livestock to meet precisely the animal's requirement. The degradation of CP which is measured in vivo has been considered as reference. However, this method is laborious, time-consuming and associated with errors due to variation among individual animals and use of markers [1–3]. Therefore, in situ determination of ruminal CP degradation is widely used as reference [4]. The use of in situ data is often critically discussed, especially in terms of repeatability of the results. There is, however, a reliable methodological protocol existing that ensures repeatability [5]. This protocol recommends, for example, the correction of in situ degradation data for the microbial nitrogen that is synthesized during the incubation of feeds in the rumen [6]. For future applications, it seems worthwhile to search for methods that do not rely on cannulated animals and are potentially useful for routine analysis [7]. A purely enzymatic in vitro method is the *Streptomyces griseus* protease test, which was developed by Krishnamoorthy et al. [8]. Protease from the bacterial species *S. griseus* has a broad activity spectrum and may hydrolyze proteins (i.e., oligopeptides) up to 90% [9,10]. Its high reactivity comes from endo- and exopeptidases, especially metalloendopeptidase activity [10,11]. Licitra et al. [10] indicated the ratio of protease to true protein (TP) concentration has influence on CP degradation and standardized the method to that effect. Moreover, incubation times were referenced to type of feed and feed characteristics [10,12]. Several studies have shown close agreement between CP degradation estimated in situ or using the *S. griseus* protease test both in concentrates and roughages [7,13–16]. Feed-specific degradation kinetics and effective degradation of protein (ED) at different passage rates have been barely described. A large part of feed protein is associated to carbohydrates, i.e., starch and fiber, as a kind of matrix that influences the degradation capability of proteases. Such matrix effects could be responsible for the inability of protease to degrade the entire feed protein [17–19].

We have evidence from a pilot study that the reliability of the *S. griseus* protease test estimating ED by the measure of in situ ED strongly depends on the incubated feedstuff and clusters may be defined that rely on feed or treatment characteristics [20]. From this, we conclude that the efficiency of the *S. griseus* protease may be influenced by matrix effects, which lead to protein degradation of feedstuffs clusters according to similar nutrient characteristics.

The objective of the present study was to assess the suitability of the *S. griseus* protease test for estimating ED of CP from 40 feedstuffs using the in situ test as a reference method.

Our hypothesis was that specific characteristics (e.g., nutrient content, treatment) of individual feedstuffs or groups of feedstuffs lead to a differentiation with regard to the susceptibility of the feed protein to protease, and thus, to specific clustering.

2. Materials and Methods

2.1. Feedstuffs and Treatments

A set of 40 different feedstuffs for which in situ CP degradation data were available has been used for the in vitro investigations to obtain a wide range of different feedstuffs. This set contained soybeans, soybean meal (SBM), sunflower meal (SFM), barley and wheat grains, wheat bran, corn gluten feed (CGF), sugar beet pulp (SBP) and dried distillers' grains with solubles (DDGS). In addition, some feedstuffs were subjected to treatment (Table 1). Lupin grains of cultivars *Boregine* and *Boruta* were tested, both native and treated. Additionally, six differently treated Rapeseed meals (RSM) were investigated indicated by letters a to d (Table 1). Two RSM, RSMc and RSMd, were tested native and treated as described in Table 1. With exception of the over-toasted RSM (RSMb), all further treated RSM (RSMa, RSMc and RSMd) were provided by industry, and specific treatment information was not available.

Table 1. Description of treatment procedures for lupin varieties and rapeseed meals.

Feedstuff	Treatment
Lupin *Boregine* native	Native
Lupin *Boregine* treated	Toasted at 115–120 °C for 1 min, conditioned for 30 min in cooling tower followed by cooling to 20 °C.
Lupin *Boruta* native	Native
Lupin *Boruta* treated	Moisture conditioned, short time toasted at 130 °C and drying to 940 g DM/kg.
RSMa expander-treated	Expanded (unknown conditions)
RSMb over-toasted	Toasted at 107 °C for 60 min under 450 kPa pressure [21]
RSMc native	Native
RSMc expander-treated	Expanded (unknown conditions)
RSMd native	Native
RSMd formaldehyde-treated	Formaldehyde-treated (unknown conditions)

DM: dry matter; RSM: rapeseed meal.

Three different cultivars of peas were investigated: *Hardy*, *Astronaute* and *Navarro*. As described by Rupp et al. [22], perennial ryegrass (*Lolium perenne*) was cut in 2017, wilted at 40 °C under temperature control and chopped to 20–30 mm particle size. A total of 16 grass silages were made from this material (90 d at 25 °C in glass jars), for which eight wilting stages were produced (I: 170 g dry matter (DM), II: 310 g DM, III: 390 g DM, IV: 420 g DM, V: 470 g DM, VI: 530 g DM, VII: 580 g DM and VIII: 600 g DM/kg) and ensiled with or without adding a mixture of homo- and heterofermentative lactic acid bacteria. Information on the ensiling process and silage quality parameters is given by Rupp et al. [22]. Nutrient concentrations of all feedstuffs are summarized in Table 2.

Table 2. Concentration of dry matter (DM, g/kg), proximate nutrients (g/kg DM) and soluble protein (SP, % of CP) of the feedstuffs.

Feedstuff	DM	CA	CP	TP	SP	AEE	aNDFom	ADFom	Starch
Barley	894	27	125	114	28	33	162	76	532
Wheat	857	19	140	126	32	31	86	36	544
Corn	894	17	83	76	18	43	84	30	705
Wheat bran	859	59	186	163	34	55	398	135	158
DDGS	863	64	312	252	21	81	338	198	28
CGF	867	84	169	87	55	36	343	95	159
Soybean	901	56	391	373	9	221	109	65	n.a.
SBM	893	71	504	465	11	26	111	66	18
SFM	910	78	318	294	36	30	402	307	17
RSMa et	790	79	358	340	20	45	321	225	38
RSMb ot	924	85	366	352	15	23	476	269	9
RSMc n	885	86	384	357	15	46	324	210	n.a.
RSMc et	900	86	383	358	15	48	321	207	n.a.
RSMd n	900	78	374	359	24	37	324	233	n.a.
RSMd ft	911	86	370	359	9	41	339	221	n.a.
Faba bean	897	39	279	233	55	23	173	129	358
Lupin *Boregine* n	917	37	298	289	75	68	264	235	298
Lupin *Boregine* t	929	38	320	300	32	75	252	212	320
Lupin *Boruta* n	900	37	319	311	64	67	249	213	319
Lupin *Boruta* t	925	38	328	311	36	67	265	201	328
Pea *Hardy*	902	32	219	206	68	19	115	83	451
Pea *Astronaute*	901	30	228	215	70	19	118	75	432
Pea *Navarro*	898	30	248	234	73	20	142	81	392
SBP	862	85	94	57	40	19	347	174	n.a.

Table 2. *Cont.*

Feedstuff	DM	CA	CP	TP	SP	AEE	aNDFom	ADFom	Starch
GS I	932	113	153	48	68	48	463	292	n.a.
GS I *	930	117	159	57	68	49	519	307	n.a.
GS II	926	102	153	50	66	40	498	293	n.a.
GS II *	917	105	156	56	65	46	503	309	n.a.
GS III	928	105	147	48	66	41	508	307	n.a.
GS III *	926	104	148	50	66	44	514	316	n.a.
GS IV	934	103	153	48	67	38	529	311	n.a.
GS IV *	937	107	155	50	66	42	520	315	n.a.
GS V	932	103	150	52	65	38	526	310	n.a.
GS V *	932	107	153	53	64	40	510	308	n.a.
GS VI	932	110	153	52	66	36	527	311	n.a.
GS VI *	929	110	153	51	64	38	518	312	n.a.
GS VII	926	108	146	55	65	34	523	307	n.a.
GS VII *	929	109	152	57	65	36	507	299	n.a.
GS VIII	927	111	151	58	65	33	526	309	n.a.
GS VIII *	932	110	149	60	65	34	514	301	n.a.

* Grass silage ensiled with bacterial inoculant; ADFom: acid detergent fiber expressed exclusive of residual ash; AEE: acid ether extract; aNDFom: neutral detergent fiber treated with amylase and expressed exclusive of residual ash; CA: crude ash; CGF: corn gluten feed; CP: crude protein; DDGS: dried distillers' grains with solubles; et: expander–treated; ft: formaldehyde–treated; GS: grass silage; n: native; n.a.: not analyzed; ot: over–toasted; RSM: rapeseed meal; SBM: soybean meal; SBP: sugar beet pulp; SFM: sunflower meal; t: treated; TP: true protein. SP was calculated according to Licitra et al. [23] and for GS according to Higgs et al. [24]. TP was calculated as CP—non-protein nitrogen according to Licitra et al. [23].

2.2. Origin of In Situ Data

Animal experiments were not part of this study because all in situ data originated from preliminary studies conducted under approval no. V319/14 TE. Ruminal CP degradation of concentrates was determined at the Institute of Animal Science, University of Hohenheim using a standardized assay [22,25–28]. In brief, feedstuffs were incubated in the rumen of rumen-fistulated Jersey cows and three cows were used for each feedstuff. Incubations were made in polyester bags (Ankom Technology, Macedon, New York, USA) with a pore size of 50 µm (30 µm for RSM) and internal dimensions of 5 × 10 cm, 10 × 20 cm and 11 × 22 cm for a time period of up to 72 h and in case of SFM and all pea cultivars for a time period of up to 48 h. A minimum of three bags was used of each point in time and cow and contents after incubation were pooled for chemical analysis. The in situ degradation data of RSMa, native and treated RSMc and RSMd were not published yet. In situ protein degradation was expressed as a percentage of CP at each specific incubation time.

2.3. In Vitro Incubations

The *S. griseus* protease test was performed according to Licitra et al. [10]. The feedstuffs were ground through 1 mm sieve size using a standard laboratory sample mill. Briefly, 0.5 g were weighed in 50 mL centrifuge tubes, filled with 40 mL of borate-phosphate buffer (12.20 g $NaH_2PO_4 \times H_2O$ + 8.91 g $Na_2B_4O_7 \times 10\ H_2O$/L with pH 6.7–6.8) and placed into a drying oven for 1 h at 39 °C as pre-incubation. The protease solution contained 0.58 U of nonspecific type XIV *S. griseus* protease (Sigma-Aldrich Chemie GmbH, Munich, Germany) per mL and was added after 1 h pre-incubation at a ratio of 24 U/g TP. The concentration of TP was calculated according to the Cornell Net Carbohydrate and Protein System (CNCPS) as CP minus non-protein nitrogen (fraction A) [23]. Samples of incubation time 0 h were taken immediately after pre-incubation without addition of protease solution. Subsequently, the feedstuffs were incubated in duplicate for 2, 4, 6, 8, 16 and 24 h, respectively. Afterwards, sample tubes were filtered through Whatman #41 filter circles and rinsed out with 150 mL distilled water each. The filters were air-dried overnight, and nitrogen was analyzed in the residues and blank filters using a FOSS Kjeltec^{TM} 8400 unit (Foss GmbH, Hamburg, Germany). This procedure was repeated a minimum of four and a maximum of six times to obtain at least four replicates for each feedstuff. Concentrations of rumen undegraded

protein (RUP) were determined according to Bachmann et al. [29] as follows (considering a sample weight of 0.5 g):

$$\text{RUP (g/kg DM)} = ((N_{residue} \times 6.25 \times 10)/(0.5 \times DM_{feed})) \times 10,$$

where $N_{resdiue}$ is the nitrogen measured in filter residues (mg) corrected by blank filters and DM_{feed} is the DM content of feedstuff (%). Degraded protein (% of CP) was considered the reciprocal of RUP at each specific incubation time.

2.4. Effective Protein Degradation

The following calculations were made using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). In a first step, the in situ CP degradation data of the tested substrates were reanalyzed by fitting CP degradation (as % of CP) measured after 0, 2, 4, 6, 8, 16, 24, 48 and, if applicable, for 72 h of incubation to the exponential equation provided by McDonald [30] and Steingass and Südekum [31] using the MODEL procedure of SAS 9.4. To describe CP degradation, the washout protein a, which instantly disappears at time $t = 0$, b, which is the protein potentially degradable in the rumen, and c, which is the degradation rate of fraction b, were estimated. The possible appearance of a discrete lag phase L, at which no ruminal degradation occurs, was considered using a broken-line approach. As long as $t \geq L$, CP degradation was fitted to the regression function, whereas if $t < L$, CP degradation was considered to be equal to a. The estimates of the lag phase were set to be greater or equal to zero; $a + b$ was restricted to be lower or equal 100%. Note that the slightly different methodological approach led to ED estimates which were somewhat different from those previously published [22,25–28] using partly the same feedstuffs as in the current study. In a second step, the in situ CP degradation data were corrected for the amount of microbial nitrogen present in the feed residues at each specific incubation time using the equations of Parand and Spek [6] as recommended by GfE [5]. For this, feedstuffs were grouped by roughages, concentrates and low protein concentrates (CP < 300 g/kg DM) and the specific equations provided by Parand and Spek [6] were applied. The potential contamination with microbial nitrogen over time is summarized in Table S1. In a third step, in situ CP degradation was estimated once with 72 h or 48 h maximal incubation time and once with maximal incubation time reduced to 24 h in accordance with maximal in vitro incubation time, and all estimations were repeated both without and with correction upon microbial nitrogen. The in situ dataset comprised three replicates per feed sample (i.e., three animals). The in vitro CP degradation was analyzed analogously with a maximal incubation time of 24 h. Within the in vitro dataset, outliers were identified using boxplots and eliminated. Outliers were defined as observations more far than three times of interquartile range. The in vitro dataset comprised six replicates (i.e., six runs) and a minimum of three replicates after elimination of outliers. Effective CP degradation, either in situ or in vitro, was calculated on the basis of the estimated parameters a, b, c, and L as described by Wulf and Südekum [32] for assumed ruminal passage rates of 0.02 (ED_2), 0.05 (ED_5) and 0.08 h^{-1} (ED_8). The whole investigation was conducted under the assumption that the degradation data obtained by the in situ method is the reference to which in vitro ED was compared. The SAS script used for all calculations can be obtained from the authors on request.

2.5. Chemical Analyses

Concentrations of DM, crude nutrients and detergent fiber fractions were analyzed according to the Association of German Agricultural Analytic and Research Institutes [33] using methods no. 3.1 (DM), 4.1.1 (CP), 5.1.1 B (AEE), 6.5.1 (aNDFom), 6.5.2 (ADFom) and 8.1 (CA). Neutral detergent fiber was determined after amylase treatment. Neutral detergent fiber and acid detergent fiber were expressed exclusive of residual ash. Starch was determined using the amyloglucosidase method (VDLUFA, 2012; method no. 7.2.5) similar to Grubješić et al. [25] and enzymatically according to Seifried et al. [34]. The CNCPS protein fraction A (non-protein nitrogen) was determined according to Licitra et al. [23]

and in grass silages according to Higgs et al. [24]. All measurements of nitrogen were performed using the Kjeldahl method.

2.6. Statistical Analysis

Statistical analysis was performed using SAS 9.4. The effects of a correction of in situ CP degradation for percentages of microbial nitrogen contained in the feed residues and a reduction of maximal incubation time (t = 72 h/48 h or t = 24 h) on ED_2, ED_5 and ED_8 were tested using the NPAR1WAY procedure and Kruskal–Wallis test. Differences between in situ and in vitro estimates of ED_2, ED_5 or ED_8 for both 24 and 72 h maximal incubation times (in situ) were tested using pooled t-test or the Satterthwaite approximation of the t-test if applicable according to folded F-test. In cases where Gaussian distribution of the studentized residuals was not given, we used the Wilcoxon rank sum test with the NPAR1WAY procedure. Differences between in situ and in vitro estimates of ED_2, ED_5 or ED_8 in native or treated feed samples were tested by pooled t-test. Homogeneity of sample variances and Gaussian distribution of the studentized residuals were confirmed. For all tests, statistical significance was given with $p < 0.05$. To compare effective CP degradation between the two different methodologies (in situ vs. in vitro), differences between in situ and in vitro ED_2, ED_5 and ED_8 were additionally expressed as a degradation quotient (degQ). The degQ was calculated as follows:

$$\text{degQ} = (ED_{\text{in vitro}} - ED_{\text{in situ}})/ED_{\text{in situ}}.$$

Clustering was examined by single linkage method separately for every feedstuff and for assumed ruminal passage rates of 0.02 h^{-1}, 0.05 h^{-1} and 0.08 h^{-1} including degQ or including only the concentrations of crude nutrients, detergent fibers and starch. Missing data of starch concentration in feedstuffs were provided by DLG [35]. Grass silages, SBP, native and treated RSMc and RSMd did not contain starch and soybeans contained 58 g starch/kg DM. A dendrogram was created showing the clusters.

3. Results

The estimated amount of microbial nitrogen present in feed residues is listed in Table S1. Microbial nitrogen was highest in grass silages (55.8–58.5% of total nitrogen) and lowest in the native lupin *Boregine* (5.1% of total nitrogen) after 72 h incubation time. The effects of a correction for microbial nitrogen contamination and a reduction of maximal incubation time (72 h or 48 h to 24 h) on in situ ED_2, ED_5 and ED_8 are shown in Table S2 and Figure S1. Irrespective of the incubation time (72 h or 48 h or 24 h), correction for microbial nitrogen elevated in situ ED which reached significance in wheat grain with up to 2% points, in RSMa (expander-treated) and in RSMb (over-toasted) with up to 5% points and in all grass silages with up to 6% points ($p < 0.05$). The reduction of incubation time to maximal 24 h had mostly no or merely a small effect on in situ ED; only in RSMd (formaldehyde-treated and nitrogen-uncorrected), ED_2 was reduced by a maximal of 11% points. However, reduction of incubation time from 72 h or 48 h to 24 h affected ED of protein and comparison to in vitro ED (Table S4) far less than the correction for microbial nitrogen contamination (Table S2). On that basis, in situ CP degradation over maximal 72 h corrected for microbial nitrogen contaminations was used as reference for comparison of in vitro results reported in the following (Table 3).

Reliable estimation of ED in vitro was not possible in case of faba beans and corn due to implausible estimates of CP degradation parameters (Table S3). Effective CP degradation was mainly underestimated using the *S. griseus* protease test (by maximal 48% points; $p < 0.05$; Table 3), which is shown by negative quotients (Table 4). Only in the treated lupins *Boregine* and *Boruta*, the pea *Navarro* and the SFM at a passage rate of 0.08 h^{-1} and in the native lupins *Boregine* and *Boruta* and SBM at 0.05 and 0.08 h^{-1} passage rates, ED was higher in vitro than in situ (Table 3). Regardless of ruminal passage rate, the largest differences between in situ and in vitro CP degradation were found in barley grains and industrial by-products (DDGS, CGF, wheat bran and SBP). In these feedstuffs, a, b

or *c* were underestimated up to 53% points by the in vitro method ($p < 0.05$). Significant differences between in situ and in vitro estimates were also found in oilseeds following fat extraction and other processing processes ($p < 0.05$). In legume grains (with exception of faba beans), in situ and in vitro estimates agreed well, although some differences were significant ($p < 0.05$).

Table 3. Comparison of in situ (72 h incubation time) and in vitro (24 h incubation time) estimates of effective CP degradation (ED, % of CP) at 0.02 (ED_2), 0.05 (ED_5) and 0.08 h^{-1} (ED_8) assumed ruminal passage rate.

Feedstuff	ED_2		ED_5		ED_8	
	In Situ	In Vitro	In Situ	In Vitro	In Situ	In Vitro
Barley	91 ^{aA}	43 ^{bB}	87 ^{aA}	42 ^{bB}	83 ^{aA}	40 ^{bB}
Wheat	92 ^{aA}	73 ^{bB}	84 ^{aA}	70 ^{bB}	78 ^{aA}	67 ^{bB}
Wheat bran	91 ^{aA}	58 ^{bA}	87 ^{aA}	56 ^{bA}	83 ^{aA}	54 ^{bA}
DDGS	86 ^{aA}	55 ^{bB}	82 ^{aA}	50 ^{bB}	79 ^{aA}	46 ^{bB}
Corn gluten feed	92 ^{aA}	51 ^{bA}	89 ^{aA}	49 ^{bA}	87 ^{aA}	48 ^{bA}
Soybeans	92 ^{aA}	70 ^{bB}	84 ^{aA}	65 ^{bB}	79 ^{aA}	61 ^{bB}
SBM	84 ^{aA}	80 ^{bB}	68 ^{aA}	75 ^{bB}	56 ^{aA}	71 ^{bB}
SFM	89 ^{aA}	81 ^{bB}	81 ^{aA}	77 ^{bB}	74 ^{aA}	75 ^{aA}
RSMa et	81 ^{aA}	67 ^{bB}	71 ^{aA}	60 ^{bB}	63 ^{aA}	55 ^{bB}
RSMb ot	70 ^{aA}	58 ^{bB}	58 ^{aA}	52 ^{bB}	50 ^{A}	47 ^{B}
RSMc n	85 ^{aA}	71 ^{bA}	72 ^{aA}	59 ^{bA}	63 ^{aA}	52 ^{bA}
RSMc et	88 ^{A}	72 ^{A}	81 ^{A}	62 ^{A}	76 ^{aA}	55 ^{bA}
RSMd n	86 ^{aA}	72 ^{bA}	77 ^{aA}	64 ^{bA}	70 ^{aA}	58 ^{bA}
RSMd ft	57 ^{A}	62 ^{A}	45 ^{aA}	41 ^{aA}	38 ^{A}	32 ^{A}
Lupin *Boregine* n	92 ^{aA}	88 ^{bB}	82 ^{aA}	87 ^{bB}	75 ^{aA}	86 ^{bB}
Lupin *Boregine* t	89 ^{aA}	76 ^{bB}	77 ^{aA}	73 ^{bA}	69 ^{aA}	70 ^{aA}
Lupin *Boruta* n	92 ^{aA}	87 ^{bB}	83 ^{aA}	86 ^{aA}	77 ^{aA}	84 ^{bB}
Lupin *Boruta* t	87 ^{aA}	77 ^{bB}	74 ^{aA}	74 ^{aA}	65 ^{aA}	72 ^{bB}
Pea *Hardy*	93 ^{aA}	80 ^{bB}	85 ^{aA}	79 ^{bB}	79 ^{aA}	78 ^{aA}
Pea *Astronaute*	92 ^{aA}	81 ^{bA}	84 ^{aA}	81 ^{aA}	77 ^{aA}	80 ^{aA}
Pea *Navarro*	92 ^{A}	82 ^{A}	83 ^{aA}	81 ^{aA}	76 ^{aA}	80 ^{bA}
Sugar beet pulp	89 ^{aA}	47 ^{bA}	77 ^{aA}	42 ^{bA}	69 ^{aA}	39 ^{bA}
Grass Silage I	94 ^{aA}	78 ^{aA}	91 ^{aA}	77 ^{bA}	89 ^{aA}	76 ^{bA}
Grass Silage I *	94 ^{aA}	77 ^{bA}	91 ^{aA}	75 ^{bA}	88 ^{aA}	74 ^{bA}
Grass Silage II	93 ^{aA}	79 ^{bA}	90 ^{aA}	77 ^{bA}	88 ^{aA}	76 ^{bA}
Grass Silage II *	93 ^{aA}	77 ^{bA}	89 ^{aA}	75 ^{bA}	86 ^{aA}	74 ^{bA}
Grass Silage III	93 ^{aA}	78 ^{bA}	89 ^{aA}	76 ^{bA}	87 ^{aA}	75 ^{bA}
Grass Silage III *	93 ^{aA}	78 ^{bA}	89 ^{aA}	76 ^{bA}	87 ^{aA}	74 ^{bA}
Grass Silage IV	93 ^{aA}	79 ^{bA}	89 ^{aA}	78 ^{bA}	87 ^{aA}	77 ^{bA}
Grass Silage IV *	92 ^{aA}	79 ^{bA}	89 ^{aA}	77 ^{bA}	86 ^{aA}	76 ^{bA}
Grass Silage V	92 ^{aA}	78 ^{bA}	88 ^{aA}	76 ^{bA}	86 ^{aA}	75 ^{bA}
Grass Silage V *	92 ^{A}	77 ^{A}	88 ^{aA}	75 ^{bA}	86 ^{aA}	74 ^{bA}
Grass Silage VI	93 ^{aA}	79 ^{bA}	89 ^{aA}	77 ^{bA}	86 ^{aA}	76 ^{bA}
Grass Silage VI *	93 ^{aA}	79 ^{bA}	89 ^{aA}	77 ^{bA}	86 ^{aA}	75 ^{bA}
Grass Silage VII	92 ^{aA}	77 ^{bA}	87 ^{aA}	75 ^{bA}	84 ^{aA}	73 ^{bA}
Grass Silage VII *	92 ^{aA}	78 ^{bA}	88 ^{aA}	75 ^{bA}	85 ^{aA}	73 ^{bA}
Grass Silage VIII	92 ^{aA}	77 ^{bA}	87 ^{aA}	75 ^{bA}	84 ^{A}	73 ^{A}
Grass Silage VIII *	92 ^{aA}	77 ^{bA}	87 ^{aA}	74 ^{bA}	83 ^{aA}	72 ^{bA}
Range of SD	0.07–16.38	0.29–3.68	0.31–9.22	0.24–3.16	0.16–5.8	0.17–3.70

* Grass silage ensiled with bacterial inoculant; ^{ab} different lowercase superscripts mark significant differences with *t*-test between in situ and in vitro ED ($p < 0.05$); ^{AB} different uppercase superscripts mark significant differences with Wilcoxon rank sum test between in situ and in vitro ED ($p < 0.05$); CP: crude protein; DDGS: dried distillers' grains with solubles; et: expander–treated; ft: formaldehyde–treated; n: native; ot: over–toasted; RSM: rapeseed meal; SBM: soybean meal; SFM: sunflower meal; t: treated. The in situ CP degradation data were corrected for the amount of microbial nitrogen present in the feed residues at each specific incubation time using the equations of Parand and Spek [6].

Table 4. Degradation quotient (degQ) at 0.02 h^{-1}, 0.05 h^{-1} and 0.08 h^{-1} assumed ruminal passage rates.

Feedstuffs	**0.02 h^{-1}**	**0.05 h^{-1}**	**0.08 h^{-1}**
Barley	−0.52	−0.52	−0.51
Wheat	−0.21	−0.16	−0.13
Wheat bran	−0.37	−0.36	−0.35
DDGS	−0.36	−0.39	−0.41
CGF	−0.44	−0.45	−0.45
Soybeans	−0.24	−0.23	−0.23
SBM	−0.05	0.11	0.27
SFM	−0.09	−0.04	0.01
RSMa et	−0.17	−0.15	−0.13
RSMb ot	−0.17	−0.11	−0.05
RSMc n	−0.16	−0.17	−0.18
RSMc et	−0.18	−0.23	−0.27
RSMd n	−0.16	−0.17	−0.17
RSMd ft	0.09	−0.08	−0.14
Lupin *Boregine* n	−0.04	0.06	0.14
Lupin *Boregine* t	−0.14	−0.06	0.01
Lupin *Boruta* n	−0.05	0.03	0.10
Lupin *Boruta* t	−0.11	0.00	0.11
Pea *Hardy*	−0.14	−0.07	−0.01
Pea *Astronaute*	−0.12	−0.04	0.03
Pea *Navarro*	−0.11	−0.02	0.05
Sugar beet pulp	−0.47	−0.46	−0.43
GS I	−0.17	−0.15	−0.14
GS I *	−0.18	−0.17	−0.16
GS II	−0.15	−0.14	−0.13
GS II *	−0.17	−0.16	−0.15
GS III	−0.16	−0.15	−0.13
GS III *	−0.16	−0.15	−0.14
GS IV	−0.15	−0.13	−0.11
GS IV *	−0.14	−0.13	−0.12
GS V	−0.15	−0.14	−0.12
GS V *	−0.17	−0.15	−0.14
GS VI	−0.15	−0.13	−0.12
GS VI *	−0.15	−0.14	−0.12
GS VII	−0.16	−0.14	−0.13
GS VII *	−0.16	−0.15	−0.13
GS VIII	−0.17	−0.15	−0.13
GS VIII *	−0.17	−0.15	−0.14

* Grass silage ensiled with bacterial inoculant; CGF: corn gluten feed; DDGS: dried distillers' grains with solubles; et: expander–treated; ft: formaldehyde–treated; n: native; ot: over–toasted; t: treated; RSM: rapeseed meal; SBM: soybean meal; SFM: sunflower meal.

The calculated degQ for the assumed passage rates showed that lupins and pea grains had nearly degQ of 0, whereas by-products and barley grain had the lowest degQ of less than −0.50 (Table 4).

The cluster analysis including crude nutrient, detergent fiber and starch concentrations of the feedstuffs clearly showed eight clusters (cluster 1: native and treated variants of RSMc and RSMd; cluster 2: treated variants of lupins *Boregine* and *Boruta*; cluster 3: over-toasted RSM (RSMb) and SFM; cluster 4: expander-treated RSM (RSMa) and DDGS; cluster 5: all grass silages; cluster 6: wheat bran and CGF; cluster 7: pea varieties *Astronaute* and *Hardy*; cluster 8: wheat and barley). Soybeans, SBM, native variants of lupins *Boregine* and *Boruta*, SBP and the pea *Navarro* were arranged outside of any cluster (Figure S2).

Separate inclusion of degQ at 0.02 h^{-1}, 0.05 h^{-1}, 0.08 h^{-1} and all degQ together revealed that 37 clusters appeared (Figure 1, Table S5). The grass silages, native variants of RSMc and RSMd, SBP, CGF, wheat bran and DDGS clustered together irrespective of passage rate. The lupins were combined with the peas in varying cluster combinations. The other RSM, SFM and wheat clustered diffusely in varying combinations with other

feedstuffs. Some feedstuffs, however, were not attributed to any cluster: for degQ at 0.02 h^{-1}, formaldehyde-treated RSM (RSMd), SFM and barley; for degQ at 0.05 h^{-1}, SBM and barley, for degQ at 0.08 h^{-1}, SBM, over-toasted RSM (RSMb), wheat bran, the native lupin *Boregine* and barley; and for all degQ together, formaldehyde-treated RSM (RSMd), SBM, over-toasted RSM (RSMb), treated lupin *Boruta* and barley (Figures 1 and 2).

In Figure 3, all feedstuffs were arranged in the same order as in Figure 2. A total of eight clusters were identified and were delimited by dashed lines (cluster 1: soybeans and expander treated RSM (RSMc); cluster 2: native variants of RSMc and RSMd; cluster 3: all grass silages and expander-treated RSM (RSMa); cluster 4: pea varieties *Navarro* and *Astronaute*; cluster 5: pea variety *Hardy* and treated variant of lupin *Boregine*; cluster 6: native variants of lupin *Boruta* and *Boregine*; cluster 7: wheat bran and DDGS; cluster 8: SBP and CGF) (Figure 2, Table S5).

Within the in situ dataset, significant differences in ED were found between native and treated feedstuffs in most of the comparisons (Figure S3). These differences between native and treated feedstuffs were likewise obtained using the *S. griseus* protease test (Figure 4).

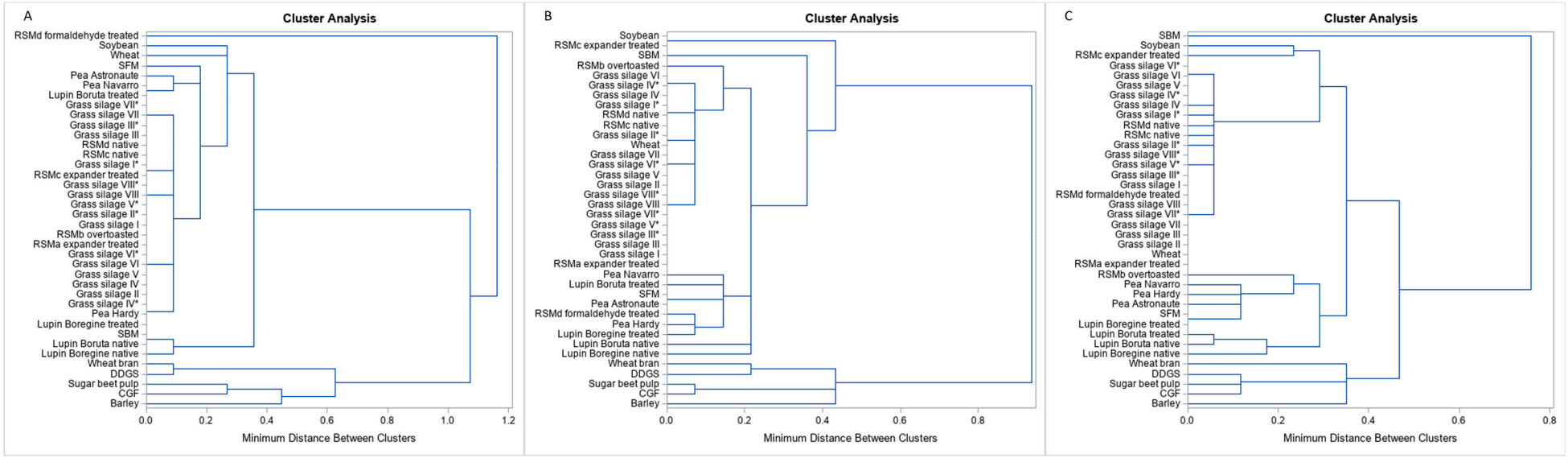

Figure 1. Cluster analysis according to degQ at 0.02 h^{-1} (**A**), 0.05 h^{-1} (**B**) and 0.08 h^{-1} (**C**) assumed ruminal passage rate. * GS ensiled with bacterial inoculant; CGF: corn gluten feed; DDGS: dried distillers' grains with solubles; GS: grass silage; RSM: rapeseed meal; SBM: soybean meal; SBP: sugar beet pulp; SFM: sunflower meal.

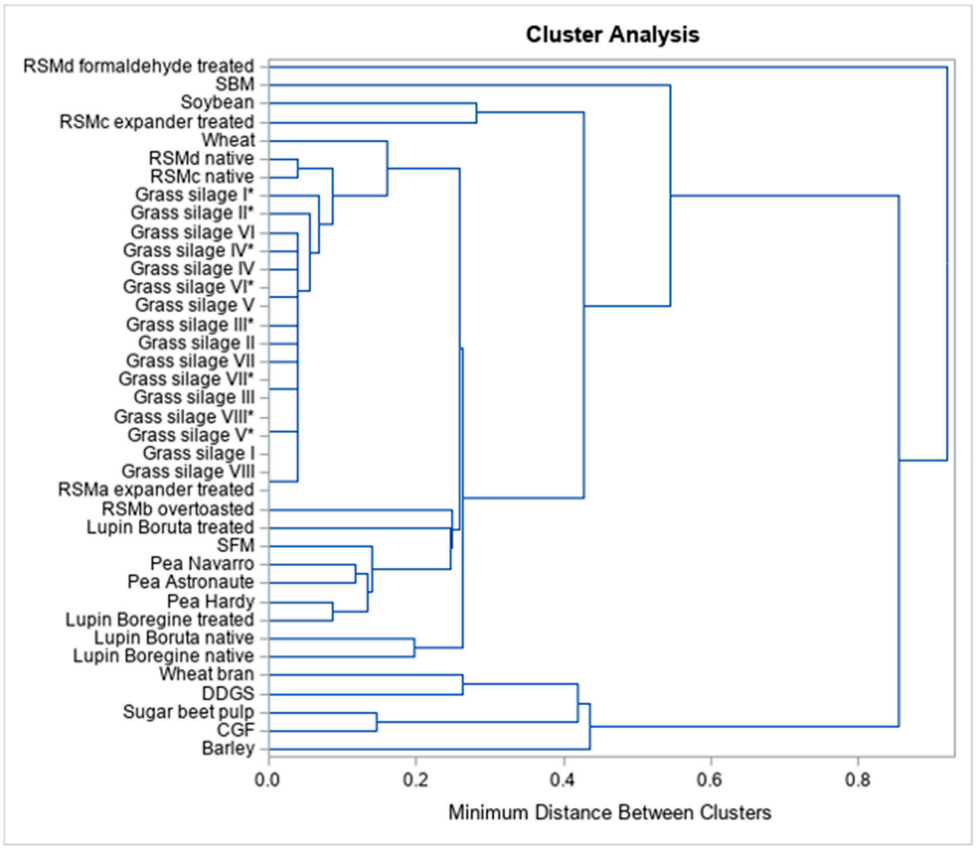

Figure 2. Cluster analysis including all degQ. * GS ensiled with bacterial inoculant; CGF: corn gluten feed; DDGS: dried distillers' grains with solubles; GS: grass silage; RSM: rapeseed meal; SBM: soybean meal; SBP: sugar beet pulp; SFM: sunflower meal.

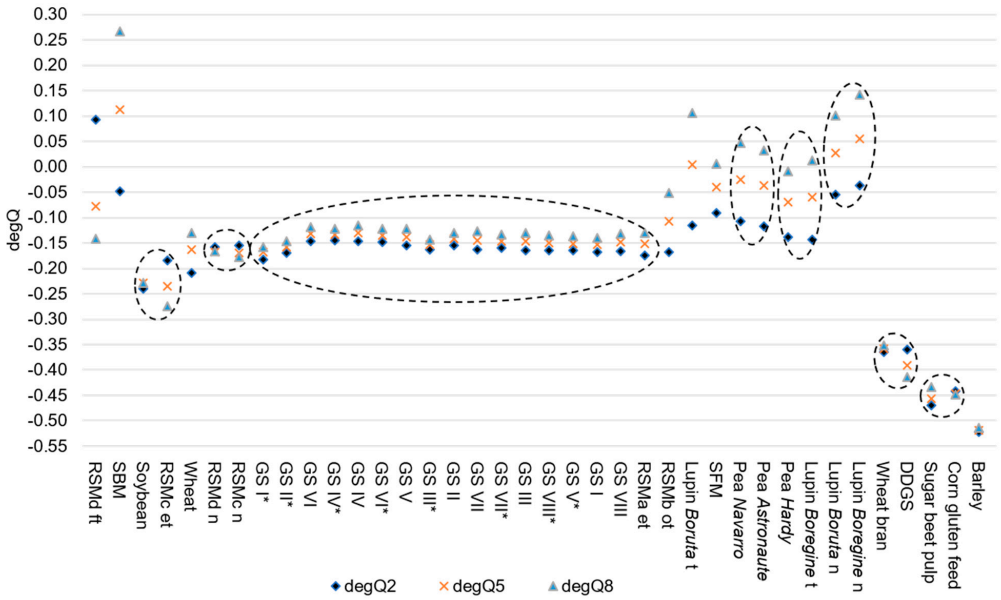

Figure 3. Clusters based on included degQ at 0.02 h^{-1}, 0.05 h^{-1} and 0.08 h^{-1} assumed ruminal passage rate. * GS ensiled with bacterial inoculant; DDGS: dried distillers' grains with solubles; et: expander–treated ft: formaldehyde–treated; GS: grass silage; n: native; ot: over–toasted; RSM: rapeseed meal; SBM: soybean meal; SFM: sunflower meal; t: treated. Dashed line indicates a cluster.

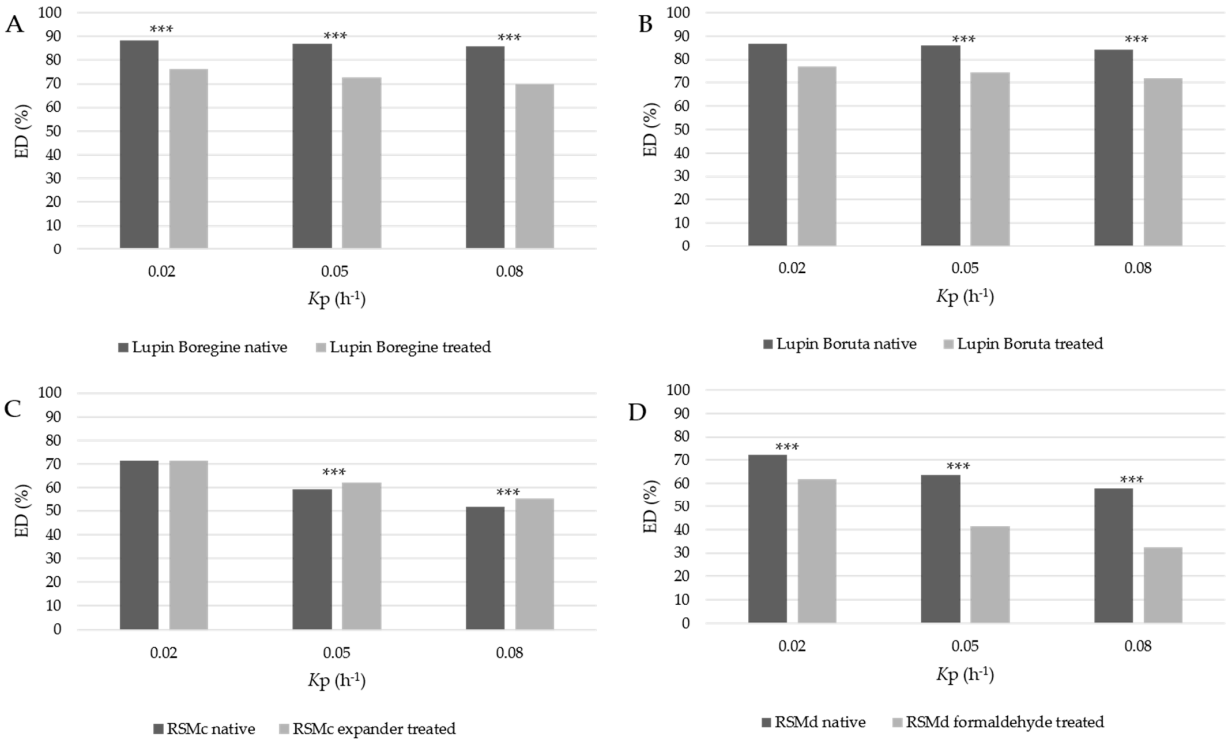

Figure 4. Effective crude protein degradation (ED) at 0.02 h^{-1}, 0.05 h^{-1} and 0.08 h^{-1} ruminal passage rate estimated in vitro in native and treated feedstuffs: lupin *Boregine* (**A**), lupin *Boruta* (**B**), RSMc (**C**) and RSMd (**D**) (24 h incubation time). *** Asterisks indicate significant differences ($p < 0.001$); *Kp*: assumed ruminal passage rate; lupin *Boregine* treated: toasted at 115–120 °C for 1 min, conditioned for 30 min in a cooling tower followed by cooling to 20 °C; lupin Boruta treated: moisture conditioning, short time toasting at 130 °C and drying to 940 g DM/kg; RSM: rapeseed meal.

4. Discussion

Protease from *S. griseus* has widely been used for estimation of ruminal CP degradation [7,13,16,36]. As Edmunds et al. [7] described, comparison among studies is difficult, because either enzyme concentration or incubation conditions differ. The standardized protocol of Licitra et al. [10] is, therefore, a basis on which the *S. griseus* protease test can be performed under defined conditions. In accordance with Cone et al. [36] and Cone et al. [37], in this study, we measured degradation of CP at 0, 2, 4, 6, 8, 16 and 24 h, which allowed displaying specific degradation kinetics and to compare them with in situ results. Although degradation values from in vivo studies are considered to be the best possible reference, they are almost not available. The results of the *S. griseus* protease test were, therefore, compared to in situ degradation values as the best available reference. It should be noted that the in situ method is associated with relevant uncertainties (i.e., microbial attachment and particle losses), which is why the results are subjected to variability and bias [38]. These limitations highlight the potential of in vitro methods in terms of standardizable, reproducible and inexpensive methods for estimating ruminal protein degradation.

As a first step, we examined the impact of nitrogen from increasing adherence of microbial biomass to the feed residues during in situ incubation [6,39], and secondly of the reduction of the incubation time on in situ predictions of ED_2, ED_5 and ED_8. Microbial nitrogen adhering to in situ feed residues was estimated according to Parand and Spek [6]. As shown previously [6,39], microbial nitrogen contamination of feed residues during ruminal in situ incubation is substantially lower in concentrates than in roughages. We calculated a maximal contamination with microbial nitrogen of 58% of total nitrogen in feed residues after 72 h of ruminal in situ incubation with lower values for concentrates (5–45% of total nitrogen) and higher values for grass silages (55%–58% of total nitrogen) as the only forage source in the current study. The correction for microbial nitrogen contamination

elevated the estimated ED_2, ED_5 and ED_8 in all tested feedstuffs. The correction for microbial nitrogen resulted in greater differences between in situ and in vitro estimated ED especially in grass silages and SBP and is, therefore, deemed to be relevant in at least some of the concentrates, and especially in roughages.

For routine applications, a simple and timely affordable in vitro test for the determination of CP degradation of feeds is required. Using *S. griseus* protease, incubation times of maximal 30 h are thought to be reliable for concentrates, by-products from food processing and forages [10]. Although in our re-calculation of in situ data, the reduction of incubation time in situ from 72 h or 48 h to 24 h had just a small effect on ED_2, ED_5 and ED_8 (Table S2), we followed the assumption of Steingass and Südekum [31] and recommendations by GfE [5] that the incubation time of at least 48 h is necessary for the reliable estimation of ED.

The rumen is colonized by a diverse commensal microbiota consisting of bacteria, protozoa, and anaerobic fungi [40]. Among bacteria, the most intensively studied group of rumen microbes, *Prevotella* was the most dominant genus found in ruminal fluid [41,42]. They are closely associated with protein and carbohydrate degradation [42,43] and may also act cellulolytic or synergistically with other cellulolytic microorganisms [42,44]. Rate and extent of CP degradation largely depend on the proteolytic activity of ruminal microflora and feed protein composition [45], but also amylolytic and cellulolytic activities of the microbes support ruminal degradation of proteins [17,18,45,46]. This might cause the gap between in situ estimates and those obtained using *S. griseus* protease as a sole agent.

Degradation of feed protein is mainly influenced by its solubility. In grass silages and legume grains, soluble protein was highest, whereas it was lowest in RSM and soybeans (Table 2). The proportion of washout protein (*a*) (in situ) or the protein soluble in borate-phosphate buffer (in vitro) plays an essential role especially in legume grains (with exception of faba beans). Hedqvist and Uden [47] determined the highest proportion of buffer soluble nitrogen (B1) in pea grains, lupin grains and grass silages. The protein solubility is influenced by the native protein composition, i.e., the distribution of prolamins, glutelins, albumins and globulins [48,49]. Most important, however, is the localization of proteins. The plant protein is structurally enclosed in the matrix composed of cellulose, hemicellulose and pectin or associated to starch and have to be dissolved prior to efficient CP degradation [45]. The cereal by-products (wheat bran, CGF and DDGS) are enriched by plant cell wall constituents (aleurone and pericarp). These parts of the grain comprise cell wall associated proteins [50,51]. More than 50% of total protein of wheat bran, CGF and DDGS is associated to fiber and is, therefore, less accessible to proteases [50,52]. These feedstuffs and SBP had the lowest degQ compared to the oilseed by-products (RSM, SFM and SBM). Pedersen et al. [19] found protein solubilization in DDGS to be increased by up to 31% following the addition of xylanase. The combination of xylanases and protease had the greatest potential to degrade non-starch polysaccharides, such as arabinoxylan, and release nutrients from DDGS [19]. The fiber-protein matrix can be influenced by heating and chemical treatment during food/feed processing. The large number of treatment options, i.e., the combination of time, temperature, use of water and reducing substances, results in a wide range of differently processed feedstuffs [53,54]. As a result of processing, the protein as a component of the matrix is structurally and chemically altered [54–57]. During heating and chemical treatment, the resistance to proteolysis might increase by denaturation of protein and/or formation of Maillard reaction products [54,58–61]. Effective CP degradation of by-products was considerably underestimated in vitro compared to the in situ results. The reduced in vitro ED can be attributed to separation processing, which leads to products enriched in cell wall-associated proteins (wheat bran, DDGS and CGF) or to physical or chemical post production treatments, as in DDGS, RSM, SFM, SBM and SBP.

Proteins localized in grains (cereal grains and legume grains) are associated to starch and this may influence protein solubility by interactions between protein and starch. These proteins surround or encapsulate starch granules and are a physical barrier to starch digestion [62]. In corn, in situ starch degradation was negatively correlated with the CP concentration of the grain [34]. In the endosperm, embedding of starch granules occurs in

highly variable spherical structures which depend on source, genotype and environmental conditions. This affects the vulnerability to enzymes [63–65]. Assoumani et al. [18] reported increased CP degradation through *S. griseus* protease with the addition of amylase and ß-glucanase in feeds with more than 23% starch on DM basis (corn, wheat and barley). They attributed the smaller differences between in situ and in vitro CP degradation to improved accessibility or vulnerability of protease to the protein matrix. Literature data revealed that toasting may decrease degradation of protein in lupins [26,58,59]. Bachmann et al. [66] showed, on the basis of scanning electron micrographs, that in pea grains, heat treatment led to an alteration of the protein matrix. Then, heat treatment limits proteolysis of the matrix surrounding starch granules. This is probably an effect of Maillard reactions or the inactivation of trypsin inhibitor activity [67,68].

Full fat soybeans, as another example, are characterized by large differences between in situ and in vitro ED estimates as well. Guillamón et al. [69] reported that soybeans contain high levels of trypsin inhibitors, which can inhibit protease activity. Another reason could be the high concentration of AEE influencing the activity of protease.

In forages, it was found that the *S. griseus* protease test had good accuracy with estimating ruminal CP degradation probably due to a high protein solubility [7,15,37]. In grass silages, specifically, proteolysis during ensiling increased the proportion of non-protein nitrogen, whereby protein is released from the fiber matrix [7]. Despite their high solubility, the grass silages in the present study had markedly lower in vitro ED compared to in situ ED. Abdelgadir et al. [17] used fibrolytic enzymes prior to the incubation with protease, which reduced differences between in situ and in vitro degradation of CP from alfalfa and meadow hay. The grass silages mostly had a degQ between −0.1 and −0.2, which was very uniform both among variants and among ED_2, ED_5 and ED_8. Prospectively, this makes mathematical correction of in vitro estimates possible and does not necessarily require modification of the test.

Our hypothesis was that specific characteristics (i.e., nutrient content or treatment) of individual feedstuffs or groups of feedstuffs lead to feed clusters with regard to the susceptibility of the CP to protease. On the basis of the selected feeds, clusters could be identified with regards to the degQ at 0.02, 0.05 and 0.08 h^{-1} assumed ruminal passage rates. The inclusion of all degQ resulted in eight clusters (Figure 3). Other feedstuffs could not be assigned to any cluster. Some clusters were characterized by similar nutrient compositions of included feedstuffs (clusters 2, 4 and 6), others by partial (cluster 1) or very clear differences (clusters 3, 5, 7 and 8). Such differences can be attributed to treatment effects on feed protein. The clustering of degQ clearly shows diffuse assignment of untreated with treated feedstuffs in common clusters (clusters 1, 3 and 5) or individually outside of any cluster (SBM, SFM and treated lupin *Boruta*) (Figure 3). Especially, the aggressive treatments of RSM could contribute to separate arrangement of over-toasted RSM (RSMb) and formaldehyde-treated RSM (RSMd). This contrasts with the cereal by-products and SBP, which were also subjected to heat and pressure treatments, but clustered together regardless of the assumed ruminal passage rate. Cereal grains (barley and wheat) were allocated differently although they have similar nutrient composition (Figure S2). Matrix effects and treatments of feedstuffs seem to have a decisive influence on in vitro CP degradation as well and determine whether and to what extent protease can work. Thus, matrix effects determine the feed-specific difference between in situ and in vitro CP degradation.

Clustering was tried to identify groups of feedstuffs that should be tested with specific additional enzymes. In general, most of the feedstuffs clustered diffusely of origin and treatment, resulting in clusters that were not characterized by feedstuffs with uniform nutrient composition. The feedstuffs which were arranged outside of any cluster were not characterized by uniform nutrient composition. This makes it difficult to derive clear specific recommendations for type and quantity of the additions of carbohydrate-degrading enzymes to improve the vulnerability of the feed protein in the *S. griseus* protease test. Groupwise degradation occurred in grass silages, cereal based-byproducts together with

SBP and partly in legume grains. The addition of fiber- or cell wall-degrading enzymes seems appropriate for the above-mentioned feedstuffs with exception of legume grains, to minimize differences to in situ CP degradation. The legume grains clustered, but the low degQ showed good agreement between in situ and in vitro ED (Figure 3). However, in the case of faba beans and corn, the addition of starch-degrading enzymes appears to be necessary to enable the estimation of effective CP degradation.

An objective of the present study was to assess the sensitivity of the *S. griseus* protease test displaying feed-specific treatment effects, because this is an essential requirement for feed evaluation purposes. As our results have shown, thermic, chemical and expander treatments were well discerned from the untreated materials. The differences of in situ ED (Figure S3) were in principle reflected by ED estimated in vitro (Figure 4). Thus, our results clearly confirmed that the sensitivity of the *S. griseus* protease test is reliable for evaluating specific treatment effects.

5. Conclusions

Results of the current study revealed that in situ CP degradation was mainly underestimated using the *S. griseus* protease test, probably due to associations of protein to carbohydrates. Feed characteristics such as nutrient composition or treatment did not fully explain the clustering of feedstuffs we observed with regard to differences between in situ and in vitro CP degradation. The clustering results do not allow a clear conclusion on the groupwise or feed-specific use of carbohydrate-degrading enzymes. The addition of amylolytic and/or fibrolytic enzymes or multi-enzyme mixtures as pre- or coincubation agents in the *S. griseus* protease test seems to be required in some cases to support starch associated and fiber-bound protein degradation. The *S. griseus* protease test displays effects of nutrient composition and treatment and could, therefore, become a reliable tool in routine feed evaluation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani13020224/s1, Figure S1: Effect of the correction for microbial nitrogen contained in the degradation residues and reduction of maximal incubation time on in situ effective crude protein degradation (ED) at 0.02 h^{-1} (ED_2), 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8) assumed ruminal passage rate; Figure S2: Cluster analysis including crude nutrients, detergent fibers and starch of used feedstuffs; Figure S3: Effective crude protein degradation (ED) at 0.02 h^{-1}, 0.05 h^{-1} and 0.08 h^{-1} ruminal passage rate estimated in situ in native and treated feedstuffs: lupin *Boregine* (A), lupin *Boruta* (B), RSMc (C) and RSMd (D) (72 h incubation time); Table S1: Estimated microbial nitrogen in the residues of in situ crude protein degradation at the various incubation times; Table S2: Estimated parameters of ruminal in situ crude protein degradation at 72 h and 24 h incubation time considering the effect of the correction for microbial nitrogen contained in the degradation residues; Table S3: Estimated parameters of ruminal in vitro crude protein degradation at 24 h incubation time; Table S4: Comparison of in situ and in vitro estimates of effective crude protein degradation (ED) at 0.02 h^{-1} (ED_2), 0.05 h^{-1} (ED_5) and 0.08 h^{-1} (ED_8) assumed ruminal passage rate and 24 h incubation time; Table S5: Parameter describing clusters.

Author Contributions: P.O. conducted the *S. griseus* protease test and wrote the original draft of the manuscript. M.B. conceptualized the study, curated and prepared the data, performed statistical analysis and reviewed the manuscript. M.W.-D. performed statistical analysis. N.T. and M.R. provided feedstuffs, in situ data and supporting information. N.T. was involved in data preparation and analysis. C.R. and A.S. provided feedstuffs, in situ data and supporting information. J.M.G. provided analyses of starch concentrations. A.Z. conceptualized and supervised the study. All authors contributed to reviewing and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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5. Paper II

The Impact of *Streptomyces griseus* Protease Reserved for Protein Evaluation of Ruminant Feed on Carbohydrase Activity during Co-Incubation

Okon, P., Liebscher, S., Simon, A. H., Wensch-Dorendorf, M., Bachmann, M., Bordusa, F., Zeyner, A.

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Article

The Impact of *Streptomyces griseus* Protease Reserved for Protein Evaluation of Ruminant Feed on Carbohydrase Activity during Co-Incubation

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Simple Summary: Ruminal feed protein degradation can be estimated in vitro using a protease. Plant proteins associated with carbohydrates might, however, not be completely hydrolyzed by the protease alone. For this reason, carbohydrase is additionally required in the test. The co-incubation of carbohydrase and protease appears to be a suitable methodological approach. It is not clear, however, whether this approach would be feasible as the carbohydrase could be inhibited by the protease. The objective of the present study was thus to investigate the effect of co-incubation of carbohydrase (α-amylase, a cell wall-degrading enzyme mixture) and protease on the hydrolysis of carbohydrates. The co-incubation of protease tended to reduce the hydrolysis of carbohydrates by carbohydrase.

Abstract: For protein evaluation of feedstuffs for ruminants, the *Streptomyces griseus* protease test provides a solely enzymatic method for estimating ruminal protein degradation. Since plant proteins are often structured in carbohydrate complexes, the use of carbohydrase during the test might improve its accuracy. It is advisable to co-incubate protease and carbohydrase, risking that the carbohydrase activity is reduced under the influence of the protease. The present study was conducted to investigate this impact by using α-amylase or the multi-enzyme complex Viscozym® L as carbohydrase. The detection of active protease was determined fluorescence photometrically using internally quenched fluorogenic substrates (IQFS). Cellulose, pectin, and starch degradation were determined spectrophotometrically using 3,5-dinitro salicylic acid as a colorimetric agent. The *Streptomyces griseus* protease mixture proved to be active for the selected IQFS immediately after the start of measurements ($p < 0.05$). Starch hydrolysis catalyzed by α-amylase or Viscozym® L, respectively, was decreased by co-incubation with protease mixture by maximal 3% or 37%, respectively, at 5 h incubation time ($p > 0.05$). Pectin and cellulose hydrolysis catalyzed by Viscozym® L, respectively, was not significantly influenced by co-incubation with a protease mixture ($p > 0.05$). Although a decrease in carbohydrase activity during co-incubation with *Streptomyces griseus* protease occurred, it was only numerical and might be counteracted by an adapted carbohydrase activity.

Keywords: *Streptomyces griseus* protease test; ruminal protein degradation; co-incubation; in vitro

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1. Introduction

Ruminal protein degradation is an important parameter for assessing the protein quality of feed for ruminants. Various approaches can be used to determine ruminal feed protein degradation. Alternative methods not based on the use of animals appear to be valuable due to their standardized and routine application. An example of an alternative method for determining ruminal protein degradation is the standardized *Streptomyces griseus* protease test. The method is based on the incubation of a feedstuff at different time

points with the *S. griseus* protease mixture consisting of endo- and exopeptidases [1]. The advantage of the *S. griseus* protease mixture lies in its diverse enzymatic composition, which ensures extensive protein degradation [2]. The *S. griseus* protease test was used at pH 6.75 and 39 °C on a broad spectrum of feedstuffs in comparison to in situ degradation data. However, the accuracy of the *S. griseus* protease test is limited by feed-specific complexes consisting of proteins and carbohydrates such as fiber and starch [3]. Such complexes cannot be hydrolyzed by proteases [4–6], which is why additional carbohydrases with fiber and/or starch-hydrolyzing activities are probably necessary in *S. griseus* protease test. The commercial enzyme preparations Viscozym® L and Termamyl® 2X (α-amylase) were used in pre-incubation with a supporting effect on feed protein degradation by *S. griseus* protease [7]. Viscozym® L contains several cell wall-degrading enzymes such as cellulases, hemicellulases, pectinase and ß-glucanase and degrades starch and cellulose to measurable sugar monomers [8].

Methodologically speaking, carbohydrases and proteases could be incubated either separately in two successive steps as pre-incubation [4,5,7] or together as co-incubation [9]. However, the pre-incubation process requires additional incubation steps, including centrifugation and pH adjustment, for optimal enzymatic activity [4,5]. The co-incubation offers the potential to reduce effort and time and improve method handling. However, contradictory results have been reported regarding the co-incubation of proteases and carbohydrases, and it appears possible that added carbohydrases can be degraded or inactivated [10,11].

The aim of the study was to investigate the effects of the co-incubation of the *S. griseus* protease and carbohydrase (α-amylase/Viscozym® L) on the release of reducing sugars from different carbohydrates at different incubation times. If the co-incubation of carbohydrase and *S. griseus* protease appears to be possible, the addition of carbohydrase might be a suitable approach to increase the accuracy of the *S. griseus* protease test by reducing the protein-carbohydrate complexes.

We hypothesized that during co-incubation, the *S. griseus* protease mixture affects the carbohydrase Viscozym® L and α-amylase in the hydrolysis of carbohydrates by reducing the conversion.

2. Materials and Methods

The study was divided into a preliminary and a main experiment. The preliminary experiment was conducted:

1. to determine whether the *S. griseus* protease mixture is active at 39 °C and pH 6.75, measuring the hydrolysis of two peptides through an increased fluorescence signal (fluorescence assay) and,
2. to investigate the relationship between increasing doses of *S. griseus* protease mixture on the release of reducing sugars due to the degradation of starch by α-amylase (absorbance assay).

The main experiment aimed to investigate the effect of *S. griseus* protease on different carbohydrates during co-incubation with carbohydrase, based on the results of the preliminary experiment. In general, Viscozym® L and *Streptomyces griseus* protease are both mixtures of enzymes, making it difficult to detect activity with just one substrate. Therefore, different substrates are incubated with the respective enzyme.

2.1. Enzymes and Buffer Solution

The following enzyme preparations were used after the first opening: Viscozym® L (V2010, Merck KGaA, Darmstadt, Germany) and α-amylase (Termamyl® 2X, Univar Solutions, Essen, Germany). According to the manufacturer, Viscozym® L contains several cell wall-degrading enzymes such as arabanase, cellulase, hemicellulase, pectinase and ß-glucanase. The manufacturer specifies the activity of Viscozym® L as ≥100 fungal ß-glucanase units per g at a concentration of 1.10–1.30 g/mL. One fungal ß-glucanase unit is

the enzyme required to hydrolyze barley β-glucan to reducing carbohydrates at standard conditions (pH 5.0, 30 °C, 30 min reaction time) at 1 µmol glucose per min [12].

The activity of α-amylase is given as 240 kilo novo units alpha-amylase with a concentration of 1.25 g/mL. One kilo novo unit is the amount of enzyme that hydrolyzes 4870 mg of starch per hour under standard conditions (pH 5.6, 37 °C and 0.3 mM Ca^{2+}) [13].

The *S. griseus* protease stock solution contained 0.58 U/mL [14] of nonspecific proteases (Merck KGaA, Darmstadt, Germany). This concentration was used for all reactions. One unit is defined as the amount of enzyme that hydrolyzes casein, producing a color equivalent by the Folin–Ciocalteu reagent to 1.0 µmol (181 µg) of tyrosine per min at pH 7.5 and 37 °C.

The buffer solution was prepared as a borate-phosphate buffer (23 mM NaH_2PO_4 and 88 mM $Na_2B_4O_7$ with pH 6.75) [1]. The borate-phosphate buffer was used in all experiments.

2.2. Substrates

For protease activity test, two internally quenched fluorogenic substrates (IQFS) peptides [15] of the general sequence Abz-Ala-Ala-Xaa-Phe-Ala-Ala-Lys-(Dnp) (Abz: 2-amino benzoic acid; Ala: alanine; Dnp: 2,4-dinitrophenol; Phe: phenylalanine; Lys: lysine; IQFS 1: Xaa = Alanine; IQFS 2: Xaa = Arginine) were used. The N-terminal 2-Abz functionality serves as a fluorescence donor. 2,4-dinitrophenol located at the N-terminal end of the lysine peptide acts as a fluorescence acceptor, resulting in a quenched fluorescence signal of the intact substrate. Any proteolytic event within the peptide goes along with a respective fluorescence signal (wavelength (λ) of fluorescence excitation (λex) = 320 nm, wavelength (λ) of fluorescence emission (λem) = 420 nm), which was measured in a fluorescence plate reader (Greiner Bio-One GmbH, Frickenhausen, Germany). Peptides were built up manually onto 2-chlorotrytil resin via standard fluorenylmethoxycarbonyl chemistry [16,17].

The following polysaccharides were used as substrates for carbohydrase: cellulose (R200 Vitacel J. Rettenmaier & Söhne GmbH + Co KG, Rosenberg, Germany), starch (CAS-No: 9005-25-8, Merck KGaA, Darmstadt, Germany) and pectin (agroPect Instant; agro Food Solution GmbH, Werder (Havel), Germany). Pectin was dissolved under continuous stirring with a magnet stirrer, cellulose and starch each through manually shaking in a borate-phosphate buffer. Cellulose, pectin and starch were dissolved to obtain a concentration of 20 mg/mL (*w*/*v*) [18]. Pectin was completely entirely dissolved, and cellulose and starch formed an emulsion in the buffer solution. All substrates were freshly prepared.

2.3. Fluorescence Assay

Fluorescence measurements were conducted in a 96-well flat-bottom fluorescence plate (Greiner Bio-One GmbH, Frickenhausen, Germany) at 39 °C. IQFS peptides were dissolved in borate-phosphate buffer (pH 6.75) in a concentration of 100 µM in the presence and absence of pectin (20 mg/mL *w*/*v*) [18], respectively. A total of 100 µL of respective IQFS solution were pipetted into the 96-well microplate and 2.5 µL *S. griseus* protease solution were added. The increasing fluorescence measurement started immediately after the protease solution was added using a NOVOstar microplate reader (BMG LABTECH, Ortenberg, Germany). Fluorescence (λex = 320 nm, λem = 420 nm) was measured every min over an incubation period of 60 min. All measurements were conducted with three replicates of each variant consisting of IQFS in buffer solution (variant 1), IQFS in buffer solution with *S. griseus* protease solution (variant 2), IQFS in buffer-pectin solution (variant 3), IQFS in buffer-pectin-solution with *S. griseus* protease solution (variant 4).

2.4. Absorbance Assay

The main measurement was conducted in a flat-bottom 96-well plate using 3,5-dinitro salicylic acid (3,5 DNS) as a colorimetric agent as described previously [18,19] with slight modifications. Briefly, the carbohydrase substrate (cellulose/ pectin/ starch) was dissolved in the borate-phosphate buffer. Then, 10 µL of enzyme preparation as provided by the manufacturer (α-amylase or Viscozym® L) was added to 1 mL of substrate suspension in

safe-lock Eppendorf tubes. Immediately afterwards, a freshly prepared *S. griseus* protease solution (0.58 U/mL) was added. For the preliminary experiments, 5 (dose 1), 25 (dose 2) and 50 µL (dose 3) of *S. griseus* protease solution were added to the carbohydrase-substrate mixture. For the main experiments, only 25 µL of *S. griseus* protease solution were used. Reaction mixtures were incubated at 39 °C at 900 rpm in an Eppendorf Thermomixer C (Eppendorf, Hamburg, Germany). Incubation times of 0, 3, and 5 h were used in the preliminary experiments and 0, 1, 2, 3 and 5 h in the main ones. The incubation times were based on the conditions set by the *S. griseus* protease test [1,3]. At these time points, 12 µL of the reaction mixture was mixed with 24 µL 3,5-DNS. The mixture was heated at 95 °C for 5 min in HBT 130-2 block thermostat (Haep Labor Consult Bio Tech, Bovenden, Germany) and cooled down in an ice bath for up to 1 min. Then, 160 µL bi-distilled water were added and subsequently centrifuged for 10 s at 11,000× *g*. A total of 180 µL of the final mixture were pipetted into a 96 flat-bottom microwell plate (Greiner Bio-One GmbH, Frickenhausen, Germany). The 3-nitrogroup of 3,5-DNS is reduced by the aldehyde function of the monomeric sugars forming 3-amino, 5-nitrosalicylic acid resulting in an intensive color change. The absorbance was measured at λ = 540 nm using a Tecan Sunrise absorbance microplate reader (Tecan Trading AG, Männedorf, Switzerland). The absorbance values were corrected by blanks containing specific enzyme and substrate-buffer solution. In order to convert absorbance units into product concentrations, calibration curves using glucose in the respective carbohydrate-buffer solutions were created. Glucose was dissolved in duplicates at 0, 0.25, 0.5, 1, and 2 mg/mL in borate-phosphate buffer (pH 6.75) containing cellulose, pectin, and starch solution at 20 mg/mL *(w/v)* [18]. All measurements were conducted with three replicates of each variant consisting of carbohydrase-substrate with Viscozym® L or α-amylase (variant 1) and carbohydrase-substrate with Viscozym® L or α-amylase and, in each case, *S. griseus* protease solution (variant 2).

2.5. Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Least squares means of fluorescence units (FU) and reducing sugar concentrations were estimated using the MIXED procedure and the model given below, separately for each variant and each incubation time. The Gaussian distribution of studentized residuals was confirmed using the UNIVARIATE procedure. For the analysis of FU and reducing sugar concentrations in the dose–response relationship, repeated measures and residual effects were considered specific for treatment *j*. For the analysis of reducing sugar concentrations of the main experiment, homogenous residual variances were considered.

The used mixed model was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + r_{jk} + e_{ijk},$$

where Y_{ijk} is FU, or reducing sugar concentration (mg/mL),

μ is the general mean,

α_i is the fixed effect of time level i (i = 0, . . ., 60 min in the fluorescence experiment, i = 0, 3, 5 h in the dose–response experiment and i = 0, . . .,5 h in the main experiment),

β_j is the fixed effect of variant j (j = 1, . . ., 4 in the fluorescence experiment, j = 1, . . ., 4, where 1 = 0 µL of *S. griseus* protease solution, 2 = dose 1, 3 = dose 2, 4 = dose 3 in the dose–response experiment and j = 1, 2 in the main experiment),

$(\alpha\beta)_{ij}$ is the interaction of level i for α and level j for β,

r_{jk} is the random effect of repetition k (1, . . ., 3) within level j of treatment β with r_{jk}~N (0, $\sigma^2 r_j$) and

e_{ijk} is the random residual effect with e_{ijk}~N (0, $\sigma^2 e_j$). Differences among least squares means with $p < 0.05$ were considered to be significant.

3. Results

Calibration curves using glucose in the respective carbohydrate-buffer solutions (0, 0.25, 0.5, 1.0, and 2.0 mg/mL) yielded regression slopes with a coefficient of determination of $R^2 = 0.973$, $R^2 = 0.993$ and $R^2 = 0.975$ in cellulose, pectin and starch solution, respectively.

3.1. Preliminary Experiments

Incubation of IQFS peptides with *S. griseus* protease solution increased fluorescent units immediately after the start of the incubation ($p < 0.05$). The pectin in the buffer-substrate solution itself significantly increased fluorescence units after 3 and 6 min in IQFS 1 and IQFS 2, respectively ($p < 0.05$) (Table 1).

Table 1. Means of fluorescent units from internally quenched fluorogenic substrate hydrolyzed by *Streptomyces griseus* protease mixture with and without pectin measured for the first 10 min of incubation.

	IQFS 1 +		*p*-Value	IQFS 1 +		*p*-Value	IQFS 2 +		*p*-Value	IQFS 2 +		*p*-Value
Time (min)	**Buffer**	**Buffer + Protease**	**Time × Variant**	**Pectin**	**Pectin + Protease**	**Time × Variant**	**Buffer**	**Buffer + Protease**	**Time × Variant**	**Pectin**	**Pectin + Protease**	**Time × Variant**
0	12.2 ^{b}	14.3 ^{a}	<0.0001	12.6 ^{a}	13.3 ^{a}	0.4883	12.9 ^{b}	16.7 ^{a}	0.0005	14.7 ^{a}	13.6 ^{a}	0.3769
1	12.2 ^{b}	18.4 ^{a}	<0.0001	12.6 ^{a}	13.8 ^{a}	0.2105	12.9 ^{b}	21.1 ^{a}	<0.0001	14.8 ^{a}	14.0 ^{a}	0.5406
2	12.2 ^{b}	22.4 ^{a}	<0.0001	12.8 ^{a}	14.6 ^{a}	0.0689	12.8 ^{b}	25.8 ^{a}	<0.0001	15.1 ^{a}	15.1 ^{a}	0.9771
3	12.2 ^{b}	26.0 ^{a}	<0.0001	12.7 ^{b}	15.5 ^{a}	0.0040	12.8 ^{b}	30.0 ^{a}	<0.0001	14.9 ^{a}	15.6 ^{a}	0.5933
4	12.1 ^{b}	29.5 ^{a}	<0.0001	12.5 ^{b}	16.2 ^{a}	0.0001	12.8 ^{b}	33.4 ^{a}	<0.0001	14.7 ^{a}	16.3 ^{a}	0.2260
5	12.1 ^{b}	32.6 ^{a}	<0.0001	12.7 ^{b}	17.2 ^{a}	<0.0001	12.7 ^{b}	37.6 ^{a}	<0.0001	14.7 ^{a}	17.3 ^{a}	0.0640
6	12.1 ^{b}	35.8 ^{a}	<0.0001	12.8 ^{b}	17.9 ^{a}	<0.0001	12.8 ^{b}	41.0 ^{a}	<0.0001	14.8 ^{b}	18.4 ^{a}	0.0096
7	12.2 ^{b}	38.1 ^{a}	<0.0001	12.6 ^{b}	19.2 ^{a}	<0.0001	12.8 ^{b}	43.4 ^{a}	<0.0001	14.6 ^{b}	19.7 ^{a}	0.0003
8	12.2 ^{b}	40.9 ^{a}	<0.0001	12.5 ^{b}	20.4 ^{a}	<0.0001	12.8 ^{b}	45.5 ^{a}	<0.0001	14.6 ^{b}	21.5 ^{a}	<0.0001
9	12.3 ^{b}	42.2 ^{a}	<0.0001	12.6 ^{b}	21.6 ^{a}	<0.0001	12.8 ^{b}	47.8 ^{a}	<0.0001	14.7 ^{b}	23.0 ^{a}	<0.0001
10	12.2 ^{b}	44.7 ^{a}	<0.0001	12.5 ^{b}	22.8 ^{a}	<0.0001	12.7 ^{b}	49.9 ^{a}	<0.0001	14.6 ^{b}	25.1 ^{a}	<0.0001
SD	0.1–0.2	0.0–1.2	-	0.2–0.5	0.5–2.3	-	0.8–1.2	0.5–1.5	-	0.1–0.3	1.1–2.8	-

$^{a, b}$ different lowercase superscripts indicate significant differences within heptapeptide between buffer/protease or pectin/protease variants ($p < 0.05$); IQFS: internally quenched fluorogenic substrate of the general structure: 2-amino benzoic acid-alanine-alanine-Xaa-phenylalanine-alanine-alanine-lysine-2,4-dinitrophenol, where IQFS 1: Xaa = alanine; IQFS 2: Xaa = arginine; SD: standard deviation. All values are given fluorescent units $\times 10^3$. Fluorescence was measured in a fluorescence plate reader (λex = 320 nm, λem = 420 nm). Pectin was dissolved at 20 mg/mL, *S. griseus* protease mixture at 0.58 U/mL, and heptapeptides at 100 µM in borate-phosphate buffer at pH 6.75. A total of 2.5 µL of *S. griseus* protease solution was used.

Over the entire incubation period, maximum fluorescence was reached after 16 min for IQFS 1 and 13 min for IQFS 2 (Figure 1). For the pectin-containing buffer, the maximum fluorescence was detected after 36 min for IQFS 1 and after 40 min for IQFS 2 (Figure 2).

The increasing dose of *S. griseus* protease solution (5, 25 and 50 µL) resulted in different responses regarding the time-dependent release of reducing sugars from starch degraded by α-amylase (control) (Figure 2 and Table S1). The 5 µL protease solution dose had no significant effect on reducing sugar concentrations at incubation times of 3 h and 5 h, respectively ($p > 0.05$). At a dose of 25 µL of protease solution, a significant decrease in reducing sugar concentrations by 37% was observed at an incubation time of 5 h ($p < 0.05$). The dose of 50 µL protease solution led to a significant decrease in reducing sugar concentrations at 3 h (40%) and 5 h (33%), respectively, compared to the control ($p < 0.05$) (Figure 1, Table S1).

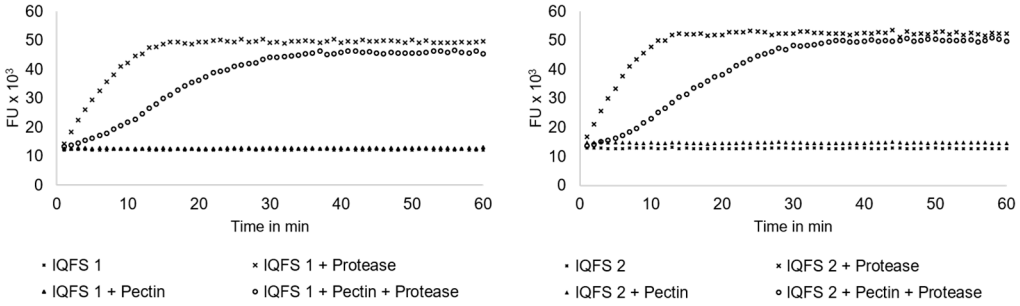

Figure 1. Means of fluorescent units from internally quenched fluorogenic substrate hydrolyzed by *Streptomyces griseus* protease mixture with and without pectin during 60 min of incubation. IQFS: internally quenched fluorogenic substrate of the general structure: 2-amino benzoic acid-alanine-alanine-Xaa-phenylalanine-alanine-alanine-lysine-2,4-dinitrophenol, where IQFS 1: Xaa = alanine; IQFS 2: Xaa = arginine. All values are given in fluorescent units (FU). Fluorescence was measured in a fluorescence plate reader (λex = 320 nm, λem = 420 nm). Pectin was dissolved at 20 mg/mL, *S. griseus* protease mixture at 0.58 U/mL and heptapeptides at 100 µM in borate-phosphate buffer at pH 6.75. A total of 2.5 µL of *S. griseus* protease solution was used.

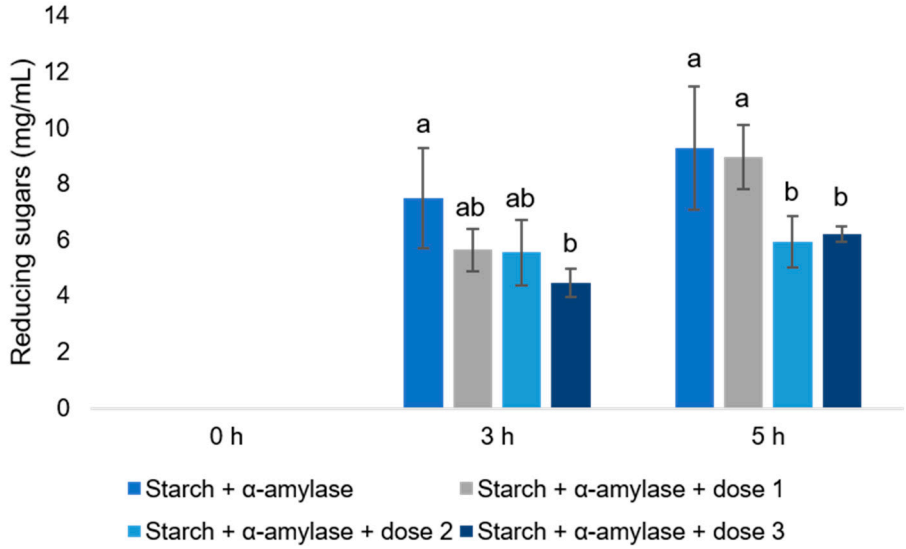

Figure 2. Means with standard deviation of reducing sugars from starch degraded by α-amylase with increased doses of *Streptomyces griseus* protease solution. a, b: different letters indicate significant differences within incubation time point between *S. griseus* protease doses ($p < 0.05$); dose 1: 5 µL protease solution; dose 2: 25 µL protease solution; dose 3: 50 µL protease solution Starch was dissolved at 20 mg/mL and *S. griseus* protease mixture at 0.58 U/mL in borate-phosphate-buffer at pH 6.75. Error bars show the standard deviation.

Based on these results, the dose of 25 µL protease solution seems to be appropriate to investigate any impact of *S. griseus* protease on the release of reducing sugars by α-amylase and Viscozym® L from complex carbohydrates during a 5 h co-incubation. It avoids over- or under-dosing of the *S. griseus* protease and reduces the release of reducing sugars by α-amylase by a similar amount compared to the 50 µL protease dose.

3.2. Main Experiments

The effect of co-incubated *S. griseus* protease and carbohydrase (α-amylase/Viscozym® L) on the release of reducing sugars was investigated using cellulose, pectin and starch as substrates during a 5 h co-incubation (Tables 2–5; Figure S1). The coefficient of determination of carbohydrate degradation by carbohydrase at different incubation times is shown

in Table S2. The coefficient serves as a parameter for the evaluation of the results, as an evaluation can only be carried out in the linear range between the incubation times [20].

Table 2. Means of reducing sugar concentration (mg/mL) from pectin degraded by Viscozym® L in presence of *Streptomyces griseus* protease mixture during 5 h of incubation.

	Reducing Sugar Concentration			*p*-Value
Time (h)	**Pectin**	**Pectin + Viscozym L**	**Pectin + Viscozym L + Protease**	**Time × Variant**
0	0.3	0 [a]	0 [a]	10.000
1	0	2.6 [a]	2.5 [a]	0.9889
2	0	4.6 [a]	4.1 [a]	0.1636
3	0	4.7 [b]	5.4 [a]	0.0304
5	0	5.9 [a]	5.8 [a]	0.6590
Range of SD	0–0.1	0–0.5	0–0.7	-

[a, b] different lowercase superscripts indicate significant differences for an incubation time point ($p < 0.05$); SD: standard deviation. Pectin was dissolved at 20 mg/mL and *S. griseus* protease mixture at 0.58 U/mL in borate-phosphate buffer at pH 6.75. A total of 10 µL of Viscozym® L as provided by the manufacturer and 25 µL of *S. griseus* protease solution was used in the experiment.

Table 3. Means of reducing sugar concentration (mg/mL) from starch degraded by α-amylase in presence of *Streptomyces griseus* protease mixture during 5 h of incubation.

	Reducing Sugar Concentration			*p*-Value
Time (h)	**Starch**	**Starch + α-Amylase**	**Starch + α-Amylase + Protease**	**Time × Variant**
0	0.1	0 [a]	0 [a]	10.000
1	0.1	4.2 [a]	4.0 [a]	0.6507
2	0.1	6.2 [a]	5.9 [a]	0.4366
3	0.1	7.0 [a]	7.1 [a]	0.9124
5	0.1	9.2 [a]	8.9 [a]	0.5111
Range of SD	0	0–0.6	0–1.3	-

[a, b] different lowercase superscripts indicate significant differences for an incubation time point ($p < 0.05$); SD: standard deviation. Starch was dissolved at 20 mg/mL and *S. griseus* protease mixture at 0.58 U/mL in borate-phosphate buffer at pH 6.75. A total of 10 µL of α-amylase as provided by the manufacturer and 25 µL of *S. griseus* protease solution was used in the experiment.

Table 4. Means of reducing sugar concentration (mg/mL) from starch degraded by Viscozym® L in presence of *Streptomyces griseus* protease mixture during 5 h of incubation.

	Reducing Sugar Concentration			*p*-Value
Time (h)	**Starch**	**Starch + Viscozym L**	**Starch + Viscozym L + Protease**	**Time × Variant**
0	0.2	0 [a]	0 [a]	10.000
1	0.1	0.9 [a]	0.3 [a]	0.3150
2	0.2	1.2 [a]	0.9 [a]	0.5643
3	0.1	1.3 [a]	1.1 [a]	0.6843
5	0.1	2.7 [a]	1.7 [a]	0.0680
Range of SD	0	0–1.1	0–0.9	-

[a, b] different lowercase superscripts indicate significant differences for an incubation time point ($p < 0.05$); SD: standard deviation. Starch was dissolved at 20 mg/mL and *S. griseus* protease mixture at 0.58 U/mL in borate-phosphate buffer at pH 6.75. A total of 10 µL of Viscozym® L as provided by the manufacturer and 25 µL of *S. griseus* protease solution was used in the experiment.

Table 5. Means of reducing sugar concentration (mg/mL) from cellulose degraded by Viscozym® L in presence of *Streptomyces griseus* protease mixture during 5 h of incubation.

	Reducing Sugar Concentration			*p*-Value
Time (h)	**Cellulose**	**Cellulose + Viscozym L**	**Cellulose + Viscozym L + Protease**	**Time × Variant**
0	0	0.1 [a]	0.6 [a]	0.3379
1	0.1	0.8 [a]	0.5 [a]	0.5868
2	0	0.9 [a]	0.8 [a]	0.9552
3	0.1	1.3 [a]	1.2 [a]	0.7714
5	0	1.7 [a]	1.6 [a]	0.9009
Range of SD	0–0.1	0.1–1.2	0.4–1.1	-

[a, b] different lowercase superscripts indicate significant differences for an incubation time point ($p < 0.05$); SD: standard deviation. Cellulose was dissolved at 20 mg/mL and *S. griseus* protease mixture at 0.58 U/mL in borate-phosphate buffer at pH 6.75. A total of 10 µL of Viscozym® L as provided by the manufacturer and 25 µL of *S. griseus* protease solution were used in the experiment.

The co-incubation of Viscozym® L and *S. griseus* protease resulted in a significant increase in reducing sugar concentrations by 15% with an incubation time of 3 h and with pectin as substrate ($p < 0.05$) (Table 2 and Figure S1).

The co-incubation of α-amylase and protease resulted in a decrease in reducing sugar concentrations by 3% at 5 h incubation time with starch as substrate (Table 3 and Figure S1).

The co-incubation of Viscozym® L resulted in a decrease in reducing sugar concentrations by 37% with 5 h of incubation time and starch as substrate (Table 4 and Figure S1).

The differences resulting from cellulose degradation between Viscozym® L and co-incubation of both enzymes were low ($p > 0.05$) (Table 5 and Figure S1).

4. Discussion

The aim of the study was to investigate the effects of the co-incubation of the *S. griseus* protease and carbohydrase on the release of reducing sugars from different carbohydrates under the conditions set by the *S. griseus* protease test at selected incubation times [1].

The coefficient of determination of linear regression was added to give information on whether degradation occurred within the linear range. Results outside of the linear range should be carefully interpreted [20].

We decided to use the commercial enzyme preparations α-amylase (synonym: Termamyl® 2X) and Viscozym® L, as they had already been used as pre-incubation agents in a study in which ruminal protein degradation was determined using *S. griseus* protease [7]. Viscozym® L appears to be advantageous as a co-incubation agent in the *S. griseus* protease test, as the enzyme mixture provides multiple cell wall-degrading enzymes for extensive hydrolysis of complexes of protein and fiber/starch.

To our knowledge, the exact enzyme composition of Viscozym® L is actually not known. Uncontrollable interactions within the *S. griseus* protease and Viscozym® L mixtures or between protease and α-amylase or Viscozym® L could have contributed to high standard deviations. A certain inhomogeneity of the substrate-buffer solution, an emulsion of cellulose and starch, was probably another source of bias. Additionally, sucrose was added to the carbohydrase preparations according to the manufacturer's information. As a non-reducing substance, sucrose could interact with 3,5-DNS during incubation by enzymatic degradation or dissolution in buffer, resulting in increased absorbance units.

According to the manufacturer, the *S. griseus* protease mixture has its highest activity at pH 8.8, α-amylase between pH 6–7/>80 °C, and Viscozym® L at pH 3.5–5.5/ 55 °C [12]. The incubation conditions set for the standardized *S. griseus* protease test (39 °C and pH 6.75) do not correspond to the optimal conditions of either the α-amylase, Viscozym® L or the *S. griseus* protease mixture. However, the release of reducing sugars by carbohydrases was determined for different carbohydrates.

The *S. griseus* protease was proved to be active immediately after adding to IQFS in borate-phosphate buffer at pH 6.75. A lag time in increasing fluorescence units was

observed with pectin as an additional supplement in the buffer solution (Table 1, Figure 1). Pectin is a viscous component that might have influenced *S. griseus* protease by decelerating the degradation of the peptides (IQFS). It can be assumed that pectin forms a gel-like emulsion with peptides [21] and this resulted in decelerating degradation through *S. griseus* protease.

The relationship between increasing *S. griseus* protease mixture doses and the release of reducing sugars by α-amylase showed that the reducing sugar concentrations declined in relation to an increasing dosage of protease solution. The results revealed that the *S. griseus* protease dose of 25 µL (0.58 U/mL) appears to be useful for further experiments in this study.

The final results regarding the co-incubation of carbohydrases (α-amylase/Viscozym® L) and *S. griseus* protease revealed that reducing sugar concentrations decreased up to 37% after 5 h of incubation compared to an incubation without protease using starch as substrate. To give a practical example, literature data showed an apparent influence of *Aspergillus saitoi* protease in co-incubation with cellulase, pectinase, xylanase or glucanase as dry matter and crude protein digestibility of maize and a soybean-maize mixture decreased. They suspect that the protease degrades the carbohydrases [10,11]. However, it may be difficult to determine carbohydrase level after co-incubation because of the secondary effects of the enzyme protein during incubation. These are the denaturation and sticking of the enzyme protein to the wall of the reaction vessel, which would lead to incorrect determination of the carbohydrase level [20].

The co-incubation of Viscozym® L and *S. griseus* protease with pectin and cellulose as substrates showed no significant influence.

However, it is difficult to interpret the results, knowing that Viscozym® L and *S. griseus* protease preparation are enzyme mixtures with unknown enzyme compositions, which makes it quite difficult to determine the enzyme activity. Each enzyme in these mixtures has its own substrate specificity and affinity. If one substrate's release of reducing sugars was reduced in the co-incubation of carbohydrase and *S. griseus* protease, the reduction is not necessarily transferable to other substrates. It remains uncertain whether the activity of each enzyme in the enzyme mixture is equally reduced. In the case of *S. griseus* protease mixture, it remains unclear how high the affinity of each single protease to the carbohydrase as a potential substrate is. It remains unclear whether a single enzyme or all enzymes cause the observed influence of the *S. griseus* protease mixture on the carbohydrase. Based on these results, influence could be observed, which presumably becomes more effective at an incubation time of 5 h. An additional reason for the moderate influence of *S. griseus* protease could be related to autolysis. According to the manufacturer, the *S. griseus* protease mixture contains a serine protease known for autolysis loop [22]. Over the incubation period of 5 h, the hydrolysis might inactivate some of the proteases within the mixture, resulting in lower proteolytic potential.

5. Conclusions

Despite predominantly not being significant, the co-incubation of α-amylase or a cellulolytic enzyme mixture with *Streptomyces griseus* protease decreased the release of reducing sugars from different carbohydrates. From a methodical point of view, co-incubation of protease and carbohydrase during the *Streptomyces griseus* protease test still appears promising, and further studies should be intended to investigate which carbohydrase activities are required to counteract the disturbing protease effect on carbohydrases.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14131931/s1, Table S1 Means with standard deviation (SD) of reducing sugars from starch degraded by α-amylase with increased dosage of *Streptomyces griseus* protease solution; Table S2: Coefficient of determination (R^2) of substrates degraded by α-amylase or Viscozym® L in presence of *Streptomyces griseus* protease mixture during 5 h of incubation; Figure S1: Means of reducing sugar concentrations from substrates degraded by Viscozym® L or α-amylase in presence of *Streptomyces griseus* protease during 5 h of incubation.

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

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6. Paper III

Ruminal Crude Protein Degradation Determined in Sacco and by Co-Incubation of *Streptomyces griseus* Protease and Carbohydrases

Okon, P., Wensch-Dorendorf, M., Bachmann, M., von Soosten, D., Ulrich, M., Greef, J.M., Dänicke, S., Zeyner, A.

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Ruminal Crude Protein Degradation Determined in Sacco and by Co-Incubation of *Streptomyces griseus* Protease and Carbohydrases

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Simple Summary: Ruminal protein degradation of seven feedstuffs was estimated in sacco and in vitro by using a protease assay. In sacco protein degradation data were used as reference. The accuracy of the protease assay is reduced by two methodological aspects. During in vitro incubation, microbial activity induced by microbial colonization of the feedstuff may increase, requiring antibiotic solutions in protease assays. The protease alone cannot sufficiently hydrolyze protein–carbohydrate complexes. Therefore, a carbohydrase of fiber- or starch-hydrolyzing activity was added to the protease assay as a simultaneous incubation. The antibiotic solution reduced protein degradation by protease. The antibiotic solution is recommended to prevent microbial activity and improve standardization of the protease assay. Differences between in sacco and in vitro degradation data were not essentially reduced by additional carbohydrases. Unfavorable incubation conditions and the inhibitory effect of protease on the carbohydrase activity during simultaneous incubation may be responsible for the insufficient hydrolysis of protein–carbohydrate complexes by the carbohydrases. It does not seem promising to incubate protease and carbohydrase simultaneously.

Abstract: The objectives of the study were to examine the effect of an antibiotic solution applied in the *Streptomyces griseus* protease method (SGPM) and the effect of carbohydrases in SGPM on the effective crude protein (CP) degradation (ED) with reference to in sacco ED. For this purpose, the ruminal CP degradation of rapeseed meal, dried distillers' grains with solubles, wheat grain, corn grain, corn silage, grass silage and partial crop field pea silage was determined in sacco using three rumen-fistulated dairy cows and in vitro using SGPM. The impact of the antibiotic solution on CP degradation by *S. griseus* protease was investigated by supplementing SGPM with Penicillin–Streptomycin solution to reduce microbial mass proliferation during incubation. The carbohydrase α-amylase or Viscozym® L (cell wall-degrading enzyme mixture) was added to the SGPM at four different doses simultaneously as a co-incubation to improve feed protein accessibility. For most feedstuffs, ED was lower when the antibiotic solution was used in SGPM ($p < 0.05$). The use of an antibiotic solution in the SGPM is recommended to standardize the SGPM. The in sacco ED values were significantly underestimated by the SGPM and by the SGPM with co-incubated carbohydrase ($p < 0.05$). Co-incubation of *S. griseus* protease and carbohydrase was not successful in reducing the differences to the in sacco CP degradation.

Keywords: protein evaluation; in vitro method; α-amylase; Viscozym® L; simultaneous incubation; antibiotic solution

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1. Introduction

In ruminants, the key to efficient use of feed protein is to know its rate of ruminal protein degradation [1]. Various approaches to determine the ruminal crude protein (CP) degradation rely on the use of animals or laboratory methods. The real reference for measuring ruminal CP degradation is the in vivo method as ruminal CP degradation, microbial contamination and endogenous proteins are measured in the same animal [2]. The complexity of the in vivo method and the bias of the results caused by markers make it difficult to routinely determine ruminal CP degradation [1,3]. An accepted reference method is the in sacco determination of CP degradation using rumen-fistulated cows [4]. This method is standardized, but time-consuming and labor-intensive. Furthermore, factors such as diet, animal species and the microbial contamination of incubated feeds generate large variation in the measured data [1,5,6]. Alternative methods for estimating ruminal CP degradation that do not include animals, such as the purely enzymatic *Streptomyces griseus* protease method (SGPM) according to Licitra et al. [7], appear to be promising approaches. Challenges in terms of time, cost, ethics and logistics are reduced and, thus, the standardization of these in vitro methods seems to be more achievable. However, the accuracy of the SGPM may be limited by two methodological shortcomings.

In a previous study [8], we used SGPM in 40 feedstuffs for which in sacco degradation data were available. The in sacco CP degradation data were underestimated by SGPM in most of the feedstuffs. This was probably related to the feed-specific matrices of proteins and carbohydrates. Within such a matrix, the protein is bonded and less accessible to the *Streptomyces griseus* protease (SGP). The addition of a carbohydrase with amylolytic and fibrolytic activity to the SGPM appears to be a suitable approach to better hydrolyze the protein–carbohydrate matrices [8]. The carbohydrases Termamyl 2X® (α-amylase) and Viscozym® L (a mixture of cell wall-degrading enzymes such as cellulases, hemicellulases, pectinase and ß-glucanase) were used to improve the degradation of CP by SGP as a pre-incubation step [9]. However, pre-incubation requires additional pH adjustment, centrifugation, decantation and rinsing, which are potential sources of error and are time-consuming [10]. Recently published data have shown that the co-incubation of a carbohydrase (α-amylase/Viscozym® L) and SGP is possible at incubation conditions set by the SGPM (pH 6.75, 39 °C), although these are not optimal for each of the enzymes [11]. However, adverse effect of the SGP were also evident as SGP seemed to reduce the activity of α-amylase and Viscozym® L [11]. The effect of SGP during co-incubation on α-amylase/Viscozym® L might be compensated for by increasing the doses of the carbohydrases. Further investigations are necessary to prove this hypothesis with reference to in sacco CP degradation data.

The second point concerns the increase in microbial activity during the co-incubation of SGP and carbohydrase favored by the release of degradable nutrients [12]. Because of this, the use of antibiotics is required to reduce microbial activity, other than that suggested in the original SGPM protocol [7]. The combined preparation of Penicillin–Streptomycin appears to be suitable for this purpose, as it is effective against gram-positive and gram-negative bacteria and has been used with trypsin in cell cultures [13]. The use of a Penicillin–Streptomycin solution in SGPM should be investigated first to ensure that there are no adverse effects on CP degradation by SGP.

We hypothesized that an additional Penicillin–Streptomycin solution should have no effect or just a marginal effect on CP degradation, as it has been used with trypsin.

Additional carbohydrases assist the SGP to hydrolyze proteins from the matrix and reduce the difference to the in sacco ED reference.

The objectives of this study were to investigate the impact of a Penicillin–Streptomycin solution applied to the SGPM and the effect of α-amylase or Viscozym® L on effective CP degradation (ED) in the SGPM with reference to in sacco ED.

2. Materials and Methods

2.1. Feedstuffs

The selection of feedstuffs for this study was on the basis of the results of a previous study in which the ED was estimated in sacco and by using SGPM [8]. The differences between in sacco and SGPM-estimated ED were clustered. One feedstuff was selected from each cluster, representing all feedstuffs of the respective cluster in terms of nutrient composition and treatment. In total, the following seven feedstuffs were used: rapeseed meal, wheat grains, dried distillers' grains with solubles (DDGS), corn grains, corn silage, grass silage and partial crop field pea silage (PCFPS).

The rapeseed meal was provided by Raiffeisen Waren GmbH (Kassel, Germany). Wheat grains and DDGS were provided by producers who want to remain anonymous. The wheat represented the major component of DDGS for which the producer did not provide us any information. The corn variety LG 30.258 was grown and harvested as grain in November 2021 on the experimental fields near the Institute of Animal Nutrition of the Friedrich-Loeffler-Institut (FLI) in Braunschweig (Germany).

The corn silage (corn variety LG 30.258) and grass silage plant materials were grown and harvested on adjacent fields of the FLI in Braunschweig (Germany). At the time of harvest, the corn plants ranged in Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und Chemische Industrie growth stages between 82 and 85 [14]. The corn plant material was harvested as a whole crop, chopped to a particle length of 8–10 mm and ensiled in silo stock in September 2021. The grass plant material was harvested as a second cut in June 2020, chopped to a particle length of 20–30 mm and ensiled in silo stock and opened in April 2021. The pea variety *Astronaute* (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany) was sown in April/May 2020 near the Saxon State Farm for Teaching and Research Köllitsch and harvested in July 2020 by direct cutting of the plants at an approximate height of 25 cm. Subsequently, the material was ensiled in round bales which were opened in the period from November 2020 to January 2021. Aliquot samples of the round bales were merged into one bulk sample. Pea harvesting and processing are described in detail by Okon et al. [15]. All silages were ensiled without silage additives.

2.2. In Sacco Procedure

The in sacco experiment was conducted in compliance with German animal protection laws and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (approval no. 33.19-42502-04-17/2577), in consultation with an independent ethics committee.

The in sacco experiment was conducted in 2021 and 2022 according to Wroblewitz et al. [16] at the FLI experimental station in Braunschweig (Germany) using three lactating German Holstein dairy cows fitted with permanent cannulas in the dorsal rumen. The average body weight was 691 ± 56 kg in 2021 and 632 ± 84 kg in 2022; note that different cows were used in 2022. The days in milk of the cows ranged between 179 and 284 (4th–6th lactation) in 2021 and between 123 and 203 (2nd–5th lactation) in 2022. The average milk yields were 30 ± 9 kg and 34 ± 7 kg, respectively. The cows had free access to tap water and were fed *ad libitum*. The partial mixed ration (PMR) was formulated according to the Society of Nutrition Physiology recommendations [17] and consisted of a dry matter (DM) basis of 50% corn and 50% grass silage in 2021 and of 57% corn, 29% grass silage and 14% concentrate (33% wheat, 29% dried sugar beet pulp, 16% rapeseed meal, 18% soybean meal, 2.5% minerals, 1.5% soybean oil and 1.3% urea) in 2022. The average fresh matter intake was 35 ± 7 kg in 2021 and 37 ± 6 kg in 2022. Additionally, the cows had access to an automatic concentrate feeder, which provided a maximum of 4 ± 2 kg/d in 2021 and 8 ± 2 kg/d in 2022.

The feedstuffs for ruminal incubation were ground through a 3 mm sieve (Retsch ZM 100, Haan, Germany), and amounts of 4 g were weighed into pre-washed and dried nylon bags (100 × 200 mm; pore size: 50 ± 10 µm; Ankom Technology, New York, NY, USA),

closed with a cable tie and fixed to a cast iron ring (542 g) with an additional weight (913 g). Before incubation, the bags were placed in warm tap water for at least one minute. The nylon bags were incubated according to Paine et al. [18] as a complete exchange. Each incubation time point was incubated separately by removing all bags from the rumen at the end of the incubation period and placed into ice water to stop microbial activity. The time series comprised seven separate incubation time points (2, 4, 8, 16, 24, 48 and 72 h). Subsequently after ruminal incubation, the bags were rinsed with cold tap water to remove rumen digesta, washed in a washing machine (Gorenje, WA 1042, Velenje, Slovenia) without spinning for 20 min with cold tap water and dried at 60 °C for 24 h. After cooling down in an exicator, the bags were weighed and all the residues of one animal were pooled into one bulk sample. The nylon bags of incubation time 0 h were washed in the washing machine and prepared for analysis as described above without ruminal incubation. Then, the pooled samples were analyzed for DM and CP concentration.

2.3. In Vitro Procedure

The SGPM was conducted according to Licitra et al. [7]. The feedstuffs were ground to pass through a 1 mm sieve size using a standard laboratory sample mill. Briefly, duplicates of 0.5 g were weighed in 50 mL centrifuge tubes and filled with 40 mL of borate–phosphate buffer (12.20 g $NaH_2PO_4 \times H_2O$ + 8.91 g $Na_2B_4O_7 \times 10\ H_2O$/L with pH 6.75).

The effect of an antibiotic solution in SGPM on CP degradation was tested by adding 0.5 mL of Penicillin–Streptomycin solution (10,000 units/mL Penicillin; 10,000 µg/ mL Streptomycin by Thermo Fisher Scientific, Massachusetts, USA). Afterwards, the tubes were placed into a drying oven for 1 h at 39 °C as pre-incubation. After pre-incubation, the SGP solution was added. The SGP solution contained 0.58 U of nonspecific type XIV SGP (Merck KGaA, Darmstadt, Germany) per mL at a ratio of 24 U/g true protein (TP) [19]. The concentration of TP in the samples was calculated according to the Cornell Net Carbohydrate and Protein System (CNCPS) as CP minus non-protein nitrogen (fraction A) [20]. Samples of incubation time 0 h were taken immediately after pre-incubation without the addition of an enzyme solution. Subsequently, the feedstuffs were incubated for 2, 4, 8, 24 and 48 h, respectively. Afterwards, sample tubes were filtered through Whatman #41 filter circles and rinsed out with 100 mL bi-distilled water each. The filters were air-dried overnight, and nitrogen was analyzed in the residues and in blank filters using a FOSS Kjeltec^{TM} 8400 unit (Foss GmbH, Hamburg, Germany).

The effect of carbohydrases in SGPM on CP degradation was investigated by adding fresh α-amylase (Termamyl® 2X, Univar Solutions, Essen, Germany) or Viscozym® L (V2010, Merck KGaA, Darmstadt, Germany) in duplicates, as provided by the manufacturer, after the pre-incubation step. Enzyme specifications and doses are described in Table 1. Subsequently, the SGP solution was added as described above and the SGPM procedure was continued as described above. Figure 1 shows the methodological approach of the co-incubation.

The correction of the co-incubation data for the enzymatical protein of carbohydrase was performed by means of using three runs of blank samples. For this approach, duplicates of falcon tubes containing buffer solution, antibiotic solution and the four aforementioned α-amylase or Viscozym® L doses were incubated for 2, 4, 8, 24 and 48 h and the residual nitrogen was determined as described above.

Table 1. Enzyme density and enzyme activity of α-amylase and Viscozym® L.

	α-Amylase	Viscozym® L
Enzyme activity	240 KNU/g [1]	≥100 FBGU/g [2]
Enzyme density	1.25 g/mL [3]	1.2 g/mL [4]
Enzyme activity per dose	0.1 mL~30 KNU 0.2 mL~60 KNU 0.4 mL~120 KNU 0.8 mL~240 KNU	0.188 mL~22.56 FBGU 0.375 mL~45 FBGU 0.750 mL~90 FBGU 1.5 mL~180 FBGU
Reference	Cone et al. [9]	Ansharullah et al. [21]

[1] One kilo novo unit (KNU) is the amount of enzyme that hydrolyzes 4870 mg of starch per hour under standard conditions (pH 5.6, 37 °C and 0.3 mM Ca^{2+}) [22]; [2] one fungal ß-glucanase unit (FBGU) is the enzyme amount required to hydrolyze barley β-glucan to measurable carbohydrates at standard conditions (pH 5.0, 30 °C, 30 min reaction time) at 1 µmol glucose per min [23]; [3] according to the manufacturer (Univar Solutions, Essen, Germany); [4] according to the manufacturer (Merck KGaA, Darmstadt, Germany).

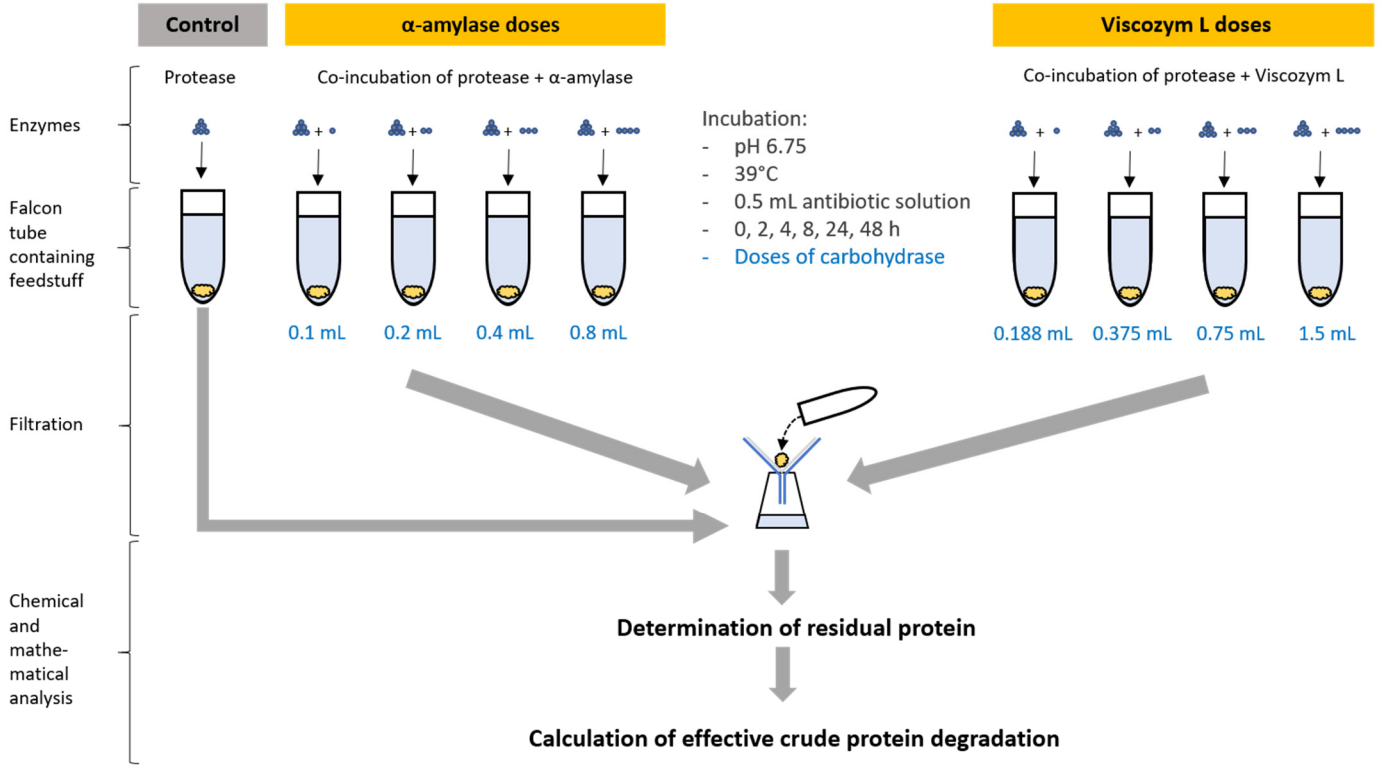

Figure 1. Co-incubation scheme consisting of control variant (*Streptomyces griseus* protease alone) and α-amylase variant and Viscozym® L variant each including four carbohydrase doses.

Concentrations of undegraded protein after SGP incubation (UP) were calculated as follows (considering a sample weight of 0.5 g): UP (g/kg DM) = (($N_{residue}$ × 6.25 × 10)/(0.5 × DM_{feed})) × 10, where $N_{resdiue}$ is the nitrogen measured in filter residues (mg) corrected by blank filters and the nitrogen of α-amylase or Viscozym® L, and DM_{feed} is the DM concentration of the feedstuff (%). Degraded protein (% of CP) was considered to be the reciprocal of UP at each specific incubation time [24].

In total, the first part of the study consisted of two variants including SGPM with Penicillin–Streptomycin solution and SGPM without Penicillin–Streptomycin solution. Degradation data from the SGPM without added antibiotics were determined prior to the co-incubation approach. The second part of the study comprised 10 variants including CP degradation in sacco, by SGP, by co-incubation of SGP and four α-amylase doses, and by

co-incubation of SGP and four Viscozym® L doses. The ED estimates of SGPM without co-incubating carbohydrase (α-amylase/Viscozym® L) were used as the control.

2.4. Effective Protein Degradation

The following calculations were made using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). In a first step, the in sacco CP degradation data were corrected for the amount of microbial nitrogen present in the feed residues at each specific incubation time [25]. In a second step, the in sacco CP degradation data of the tested feedstuffs were analyzed by fitting CP degradation (as % of CP) measured after 0, 2, 4, 8, 16, 24, 48 and 72 h of incubation to the exponential Equation (1) provided by McDonald [26] using the MODEL procedure:

$$DEG = a + b(1 - e^{-c(t - L)}) \quad (1)$$

where DEG is the disappearance at time t, a is the washout protein instantly disappearing at time $t = 0$, b is the protein potentially degradable in the rumen and c is the degradation rate of fraction b. The incubation time of corn grain is limited to 24 h incubation as the feedstuff residues almost completely disappeared at 48 h incubation. The possible appearance of a discrete lag phase L, at which no ruminal degradation occurs, was considered using a broken-line approach. As long as $t \geq L$, CP degradation was fitted to the regression function, whereas if $t < L$, CP degradation was considered to be equal to a. The estimates of the lag phase were set to be greater than or equal to zero; $a + b$ was restricted to be lower than or equal to 100%. The in sacco data set comprised three replicates per feed sample (i.e., three animals).

The in vitro CP degradation was analyzed analogously by the exponential equation provided by McDonald [26] using the MODEL procedure. The in vitro degradation data were corrected for blanks containing specific carbohydrase and antibiotic solutions. Within the in vitro data set, outliers were identified using boxplots and eliminated. Outliers were defined as observations greater than three times the interquartile range. The in vitro data set comprised four replicates (i.e., four runs).

The ED values estimated in sacco and by SGPM were calculated on the basis of the estimated parameters a, b, c and L as described by Wulf and Südekum [27] for assumed ruminal passage rates of 0.02 (ED_2), 0.05 (ED_5) and 0.08 h^{-1} (ED_8).

2.5. Microscopy

Wheat samples co-incubated with SGP and α-amylase/Viscozym® L, respectively, for a period of 48 h were filtered and air-dried. The feed residue was scraped off the filter and carefully crushed with a spatula within a falcon tube. A small sample amount was stained with 2.5%-Lugol's iodine (0.25 iodine, 1 g potassium iodide in 1000 mL water) on a microscope slide. After an exposure time of 30 s, a cover slip was placed on the sample and the Lugol's iodine solution was removed using a paper towel and remoistened using bi-distilled water. The sample was then examined microscopically using an inverted microscope with light as the source (Nikon, Eclipse Ts2, Tokio, Japan) for staining behavior and cell wall conditions at 20× magnification. Microscopy images were produced by a Nikon camera (DS-Fi3, Tokio, Japan) and processed by Nikon imaging software (Element, Software 5.21.01, Tokio, Japan). Microscopy images were used for the descriptive evaluation of the α-amylase and Viscozym® L effects on the enzymatic starch hydrolysis and cell wall conditions.

2.6. Chemical Analysis

Concentrations of DM, crude nutrients and detergent fibers were analyzed according to the Association of German Agricultural Analytic and Research Institutes [28] using methods no. 3.1 (DM), no. 4.1.1 (CP kjehldahl), 4.1.2 (CP dumas), 5.1.1 (acid ether extract), 6.1.1 (crude fiber), 6.5.1 (neutral detergent fiber after amylase pre-treatment exclusive of residual ash), 6.5.2 (acid detergent fiber exclusive of residual ash), 7.2.5 (starch) and 8.1 (crude ash), respectively. Starch was determined using the amyloglucosidase method (7.2.5).

Short chain fatty acids produced during fermentation of the silages were determined after aqueous extraction by gas chromatography using a Shimadzu GC2010 (Shimadzu Corp., Kyoto, Japan) fitted with a flame ionization detector described in Okon et al. [15]. Ammonia (NH_3-N) was determined according to the method of Conway and Byrne [29].

Lactic acid concentrations were determined by a high-performance liquid chromatography device using a Shimadzu LC-20 HPLC fitted with a photo-diode array detector (Shimadzu Corp., Kyoto, Japan), with a 300 mm × 7.8 mm Rezex ROA-Organic Acid H^+ separation column and a Carbo-H 4 × 3 mm Security Guard cartridge (Phenomenex Ltd., Aschaffenburg, Germany). Silage extracts were mixed 1:1 (v/v) with 0.016 N sulfuric acid and frozen at −20 °C. Then, thawed extracts were centrifuged 10 min at 14,000 rpm and 20 °C. An amount of 1 mL of the supernatant was mixed with 0.016 N sulfuric acid 1:2 (v/v) to achieve a total volume of 2 mL. The samples were filtered and injected onto the chromatograph with a volume of 20 µL. The oven temperature was set to 45 °C. An amount of 0.016 N sulfuric acid was used as eluent at 0.6 mL/min isocratic flow. Lactic acid was detected at 210 nm. An amount of 1 g L-(+)-lactic acid in 200 mL 0.016 N sulfuric acid was used as standard stock solution. An 8-point external calibration in a range of 0.2 to 4 g/L was applied.

2.7. Statistical Analysis

Statistical analysis was performed using SAS 9.4. Outliers of the in sacco and in vitro estimated ED values were identified by studentized residuals greater than three according to the 3σ rule using PROC UNIVARIATE. Finally, least squares means were estimated for ED at an assumed ruminal passage rate of 2% (ED_2), 5% (ED_5) and 8% (ED_8) per hour separately for each feed using the MIXED procedure and the following model for both hypotheses:

$$Y_{ij} = \mu + \alpha_i + e_{ij} \quad (2)$$

where Y_{ij} is ED_2, ED_5 and ED_8; μ is the general mean; α_i is the fixed effect of the variant (i = 1, 2, where 1 = SGPM with Penicillin–Streptomycin solution, 2 = SGPM without Penicillin–Streptomycin solution and i = 1, . . ., 10, where 1 = the in sacco estimation of CP degradation, 2 = the CP degradation estimated by SGPM, 3 = the CP degradation estimated by the co-incubation of SGP and 0.1 mL α-amylase solution, 4 = the CP degradation estimated by the co-incubation of SGP and 0.2 mL α-amylase solution, 5 = the CP degradation estimated by the co-incubation of SGP and 0.4 mL α-amylase solution, 6 = the CP degradation estimated by the co-incubation of SGP and 0.8 mL α-amylase solution, 7 = the CP degradation estimated by the co-incubation of SGP and 0.188 mL Viscozym® L solution, 8 = the CP degradation estimated by the co-incubation of SGP and 0.375 mL Viscozym® L solution, 9 = the CP degradation estimated by the co-incubation of SGP and 0.750 mL Viscozym® L solution, 10 = the CP degradation estimated by the co-incubation of SGP and 1.5 mL Viscozym® L solution); and e_{ij} is the random residual effect with e_{ij}~N(0,$\sigma^2 e_i$) or e_{ij}~N(0,$\sigma^2 e$). Homogeneous or heterogeneous residual variances were considered according to the likelihood ratio test for the analysis of ED between treatments. Differences between the least squares means with $p < 0.05$ were considered to be significant with the Tukey–Kramer adjustment because the data set is characterized by an unequal number of replications in the treatments. The studentized residuals were confirmed to have Gaussian distribution using the UNIVARIATE procedure.

3. Results

The analyzed concentrations of crude nutrients, detergent fibers and starch are presented in Table 2.

All silages were characterized by a low pH and distinct lactic acid concentrations. The concentration of n-butyric acid and NH_3-N was on a low level and therefore negligible. The concentrations of i-butyric, valeric and caproic acid were below the limit of detection (Table 3).

The ED estimates of all tested feedstuffs were significantly lower when the antibiotic solution was used in SGPM ($p < 0.05$) (Table 3). Regardless of the ruminal passage rate, the largest differences were observed in corn silage (5%-points) by the decreased degradation rate (Tables 4 and S1).

Table 2. Concentrations of crude nutrients, detergent fibers and starch (g/kg DM).

Feedstuff	DM	CA	CP	AEE	CF	aNDFom	ADFom	Starch
Rapeseed meal	880	75	374	38	156	249	227	n.a.
DDGS	901	47	321	79	75	293	120	n.a.
Wheat grain	984	19	140	24	25	145	34	743
Corn grain	876	14	93	56	27	119	13	783
Grass silage	377	103	151	41	289	500	329	n.a.
PCFPS	610	69	153	22	220	308	274	201
Corn silage	328	38	74	32	188	398	217	443

AEE: acid ether extract; aNDFom: neutral detergent fiber treated with amylase and expressed exclusive of residual ash; ADFom: acid detergent fiber expressed exclusive of residual ash; CA: crude ash; CF: crude fiber; CP: crude protein; DDGS: dried distillers' grains with solubles; DM: dry matter; n.a.: not analyzed; PCFPS: partial crop field pea silage.

Table 3. Ensiling characteristics of the silages.

Feedstuff	DM	pH	Lactic Acid	Acetic Acid	n-Butyric Acid	NH_3-N
Grass silage	377	4.35	266.8 (14.1)	21.4 (0.0)	1.5 (0.0)	1.8 (0.0)
PCFPS	610	4.38	91.6 (3.5)	13.7 (0.1)	n.d.	1.0 (0.0)
Corn silage	328	3.80	90.9 (1.3)	17.7 (0.3)	n.d.	1.5 (0.0)

DM: dry matter; n.d.: below limit of detection; PCFPS: partial crop field pea silage; DM is given as g /kg; lactic acid, acetic acid, n-butyric acid is given as g/kg DM. NH_3-N is given as %/CP. Standard deviation is given in brackets.

Table 4. Least squares means of effective crude protein degradation (ED, % of CP) at 0.02 (ED_2), 0.05 (ED_5) and 0.08 h^{-1} (ED_8) assumed ruminal passage rates determined by *Streptomyces griseus* protease method with (+) and without antibiotic solution (−).

	ED_2			ED_5			ED_8		
Feedstuff	**−**	**+**	***p*-Value**	**−**	**+**	***p*-Value**	**−**	**+**	***p*-Value**
Rapeseed meal	70 ^{a}	69 ^{a}	0.1030	62 ^{a}	60 ^{b}	<0.001	56 ^{a}	53 ^{b}	<0.001
DDGS	61 ^{a}	61 ^{a}	0.6266	55 ^{a}	55 ^{a}	0.0913	51 ^{a}	50 ^{b}	0.0299
Wheat grains	74 ^{a}	71 ^{b}	<0.001	71 ^{a}	67 ^{b}	<0.001	67 ^{a}	64 ^{b}	<0.001
Corn grains	28 ^{a}	25 ^{b}	0.0016	27 ^{a}	24 ^{b}	<0.001	27 ^{a}	23 ^{b}	<0.001
Grass silage	73 ^{a}	70 ^{b}	<0.001	71 ^{a}	68 ^{b}	<0.001	70 ^{a}	66 ^{b}	<0.001
PCFPS	80 ^{a}	80 ^{a}	0.5976	79 ^{a}	78 ^{b}	0.0422	78 ^{a}	77 ^{b}	0.0412
Corn silage	70 ^{a}	65 ^{b}	<0.001	69 ^{a}	64 ^{b}	<0.001	68 ^{a}	63 ^{b}	<0.001
Range of SE	0.18–0.42			0.16–0.31			0.07–0.32		

^{a,b} different lower-case letters indicate significant differences between ED determined with or without antibiotic solution ($p < 0.05$); DDGS: dried distillers' grains with solubles; PCFPS: partial crop field pea silage; SE: standard error. The antibiotic solution consisted of 10,000 units/mL Penicillin and 10,000 µg/mL Streptomycin.

The in sacco and in vitro estimates of ED are summarized in Table 5. The in sacco ED values were significantly underestimated by SGPM and by SGPM with co-incubated carbohydrase (α-amylase/Viscozym® L) by maximal 60%-points ($p < 0.05$).

Table 5. Least squares means of effective crude protein degradation (ED, % of CP) determined in sacco and by co-incubation of *Streptomyces griseus* protease and α-amylase/ Viscozym® L (in vitro) at 0.02 (ED_2), 0.05 (ED_5) and 0.08 h^{-1} (ED_8) assumed ruminal passage rates.

Feedstuff		In Sacco	SGP Solo	SGP + α-A_1	SGP + α-A_2	SGP + α-A_3	SGP + α-A_4	SGP + V_1	SGP + V_2	SGP + V_3	SGP + V_4	Range of SE
Rapeseed meal	ED_2	85 ^{A}	69 ^{Bb}	70 ^{Bb}	69 ^{Bb}	69 ^{Bb}	69 ^{Bb}	71 ^{Ba}	71 ^{Ba}	72 ^{Ba}	72 ^{Ba}	0.12–0.84
	ED_5	78 ^{A}	60 ^{Bb}	60 ^{Bb}	59 ^{Bb}	59 ^{Bb}	59 ^{Bb}	62 ^{Ba}	61 ^{Ba}	63 ^{Ba}	62 ^{Ba}	0.28–0.33
	ED_8	72 ^{A}	53 ^{Bb}	54 ^{Bb}	53 ^{Bb}	53 ^{Bb}	52 ^{Bb}	55 ^{Ba}	54 ^{Bb}	56 ^{Ba}	55 ^{Ba}	0.13–1.16
DDGS	ED_2	93 ^{A}	61 ^{Bb}	61 ^{Bb}	61 ^{Bb}	61 ^{Bb}	61 ^{Bb}	62 ^{Ba}	61 ^{Bb}	61 ^{Bb}	59 ^{Bb}	0.08–0.55
	ED_5	91 ^{A}	55 ^{Bb}	55 ^{Bb}	55 ^{Bb}	55 ^{Bb}	55 ^{Bb}	56 ^{Ba}	56 ^{Ba}	55 ^{Bb}	54 ^{Bb}	0.16–0.19
	ED_8	89 ^{A}	50 ^{Bb}	50 ^{Bb}	51 ^{Ba}	50 ^{Bb}	51 ^{Ba}	51 ^{Ba}	52 ^{Ba}	51 ^{Bb}	50 ^{Bb}	0.15–0.17
Wheat grain	ED_2	94 ^{A}	71 ^{Bb}	73 ^{Ba}	74 ^{Ba}	74 ^{Ba}	77 ^{Ba}	76 ^{Ba}	78 ^{Ba}	79 ^{Ba}	82 ^{Ba}	0.16–0.70
	ED_5	89 ^{A}	67 ^{Bb}	69 ^{Ba}	70 ^{Ba}	70 ^{Ba}	72 ^{Ba}	72 ^{Ba}	74 ^{Ba}	74 ^{Ba}	78 ^{Ba}	0.31–0.36
	ED_8	84 ^{A}	64 ^{Bb}	66 ^{Ba}	67 ^{Ba}	67 ^{Ba}	68 ^{Ba}	68 ^{Ba}	70 ^{Ba}	71 ^{Ba}	75 ^{Ba}	0.30–0.34
Corn grain	ED_2	85 ^{A}	25 ^{Bb}	27 ^{Bb}	27 ^{Bb}	27 ^{Ba}	28 ^{Ba}	27 ^{Bb}	25 ^{Bb}	25 ^{Bb}	25 ^{Bb}	0.52–0.60
	ED_5	74 ^{A}	24 ^{Bb}	26 ^{Bb}	26 ^{Ba}	26 ^{Ba}	26 ^{Ba}	25 ^{Ba}	25 ^{Ba}	25 ^{Bb}	25 ^{Ba}	0.10–3.32
	ED_8	67 ^{A}	23 ^{Bb}	25 ^{Ba}	25 ^{Ba}	25 ^{Ba}	26 ^{Ba}	25 ^{Ba}	25 ^{Bb}	25 ^{Bb}	26 ^{Ba}	0.06–3.99
Grass silage	ED_2	91 ^{A}	70 ^{Bb}	70 ^{Bb}	70 ^{Bb}	69 ^{Bb}	69 ^{Ba}	72 ^{Ba}	72 ^{Ba}	72 ^{Ba}	73 ^{Ba}	0.23–0.26
	ED_5	86 ^{A}	68 ^{Bb}	68 ^{Bb}	68 ^{Bb}	67 ^{Bb}	67 ^{Bb}	69 ^{Ba}	69 ^{Ba}	69 ^{Ba}	68 ^{Bb}	0.20–0.23
	ED_8	83 ^{A}	66 ^{Bb}	66 ^{Bb}	66 ^{Bb}	66 ^{Bb}	66 ^{Bb}	67 ^{Ba}	67 ^{Ba}	67 ^{Ba}	66 ^{Bb}	0.21–0.24
Partial crop field pea silage	ED_2	90 ^{A}	80 ^{Bb}	80 ^{Bb}	80 ^{Bb}	80 ^{Bb}	80 ^{Bb}	83 ^{Ba}	83 ^{Ba}	83 ^{Ba}	83 ^{Ba}	0.25–0.29
	ED_5	87 ^{A}	78 ^{Bb}	79 ^{Bb}	79 ^{Bb}	78 ^{Bb}	79 ^{Bb}	81 ^{Ba}	81 ^{Ba}	81 ^{Ba}	82 ^{Ba}	0.16–0.19
	ED_8	84 ^{A}	77 ^{Bb}	77 ^{Bb}	77 ^{Bb}	77 ^{Bb}	78 ^{Bb}	79 ^{Ba}	80 ^{Ba}	80 ^{Ba}	80 ^{Ba}	0.18–0.21
Corn silage	ED_2	92 ^{A}	65 ^{Bb}	68 ^{Ba}	67 ^{Bb}	67 ^{Bb}	68 ^{Ba}	69 ^{Ba}	69 ^{Ba}	69 ^{Ba}	71 ^{Ba}	0.38–0.44
	ED_5	89 ^{A}	64 ^{Bb}	66 ^{Ba}	66 ^{Ba}	66 ^{Ba}	67 ^{Ba}	67 ^{Ba}	67 ^{Ba}	67 ^{Ba}	66 ^{Ba}	0.26–0.30
	ED_8	86 ^{A}	63 ^{Bb}	65 ^{Ba}	65 ^{Ba}	65 ^{Ba}	66 ^{Ba}	66 ^{Ba}	65 ^{Ba}	66 ^{Ba}	65 ^{Ba}	0.25–0.28

^{A,B} different upper-case letters indicate significant differences between ED values estimated in sacco and in vitro, respectively ($p < 0.05$). ^{a,b} different lower-case letters indicate significant differences between ED values estimated by SGP solo and by SGP + α-A_n/ SGP + V_n ($p < 0.05$). α-A: α-amylase; CP: crude protein; DDGS: dried distillers' grains with solubles; SGP: *Streptomyces griseus* protease; SE: standard error; V: Viscozym® L; SGP + α-A_1: co-incubation of SGP and 0.1 mL α-amylase; SGP + α-A_2: co-incubation of SGP and 0.2 mL α-amylase; SGP + α-A_3: co-incubation of SGP and 0.4 mL α-amylase; SGP + α-A_4: co-incubation of SGP and 0.8 mL α-amylase; SGP + V_1: co-incubation of SGP and 0.188 mL Viscozym® L; SGP + V_2: co-incubation of SGP and 0.375 mL Viscozym® L; SGP + V_3: co-incubation of SGP and 0.750 mL Viscozym® L; SGP + V_4: co-incubation of SGP and 1.5 mL Viscozym® L. The in sacco degradation data were corrected for microbial nitrogen according to Parand and Spek [25]. The degradation data estimated by enzymatic co-incubation were corrected for the enzymatic protein of α-amylase/ Viscozym® L.

When α-amylase and SGP were co-incubated, significantly higher ED was determined in grains of wheat (4–6%-points) and corn (2–3%-points) and in corn silage (3%-points) compared to the control variant (SGPM) ($p < 0.05$).

When Viscozym® L and SGP were co-incubated, higher ED was determined in rapeseed meal (3%-points), wheat grain (11%-points), grass silage (3%-points), PCFPS (3–4%-points) and corn silage (3–6%-points) ($p < 0.05$). The ED of the other feedstuffs was little changed or not affected by the additional carbohydrase compared to the control variant (Table 5). An increase in ED by SGPM with additional carbohydrase was associated with reduced lag time and an increased degradation rate (Tables S2 and S3).

Microscopic images of wheat residues incubated with SGP and α-amylase or Viscozym® L, respectively, for 48 h, showed different staining behaviors depending on the carbohydrase (Figures 2 and 3). Co-incubating SGP and α-amylase resulted in decreased staining intensity as fewer stained starch granules were present. When SGP and Viscozym® L were co-incubated, however, the violet staining was consistently preserved. In every case, the cell wall appeared to stay intact.

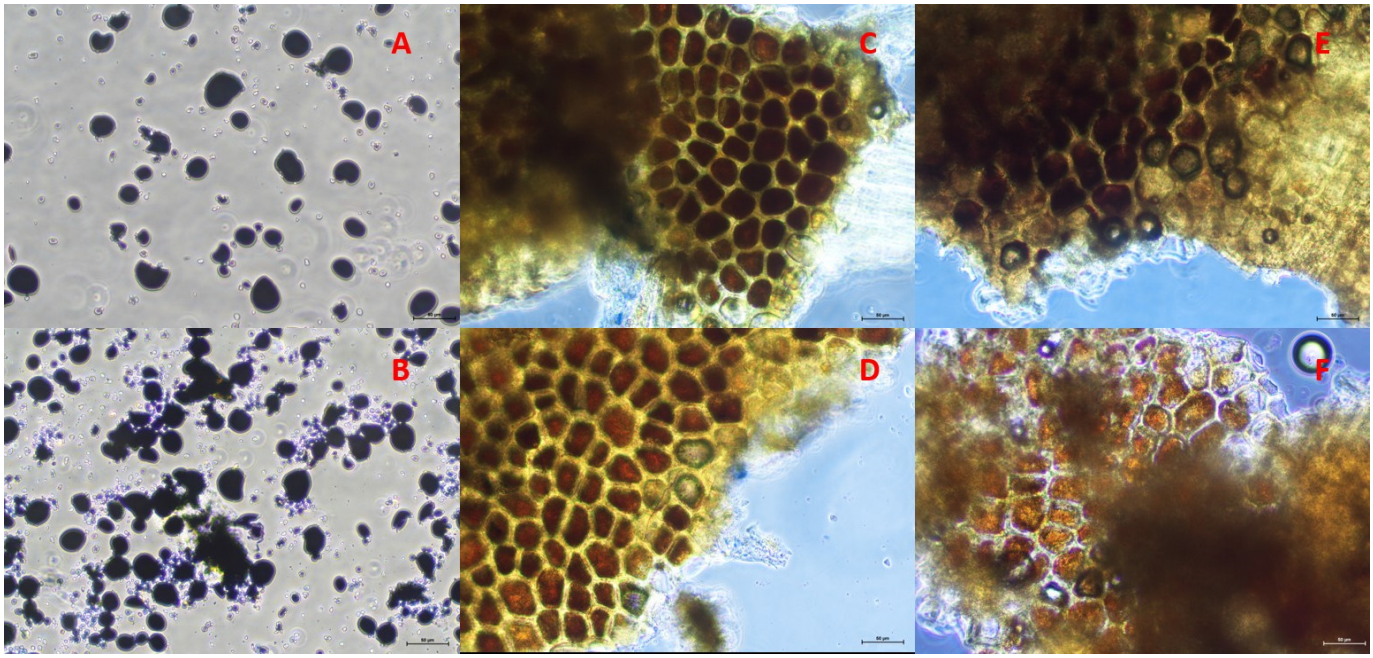

Figure 2. Microscopy images of stained wheat residues with Lugol's iodine solution after 48 h incubation without (**A**) or with *Streptomyces griseus* protease and α-amylase (**B**–**F**). (**A**): Wheat incubated in borate–phosphate buffer for 48 h; (**B**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease for 48 h; (**C**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.1 mL α-amylase for 48 h; (**D**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.2 mL α-amylase for 48 h; (**E**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.4 mL α-amylase for 48 h; (**F**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.8 mL α-amylase for 48 h. Scale = 50 µm.

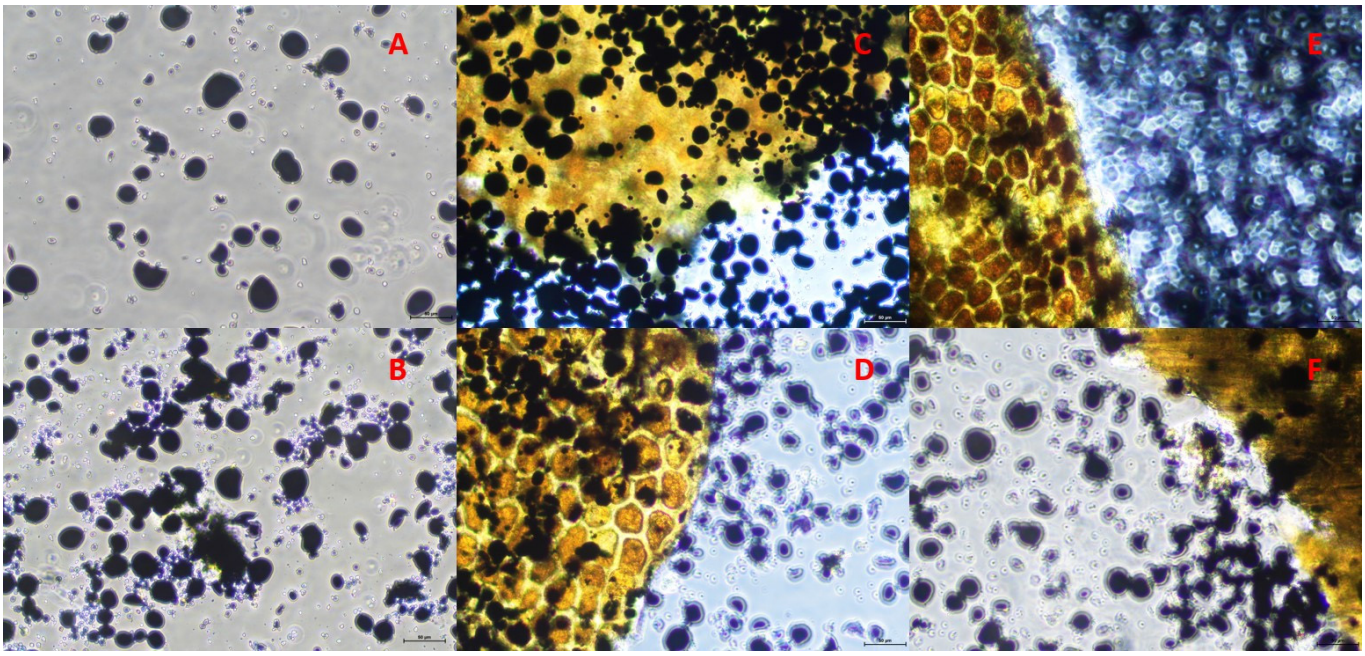

Figure 3. Microscopy images of stained wheat residues with Lugol's iodine solution after 48 h incubation without (**A**) or with *Streptomyces griseus* protease and Viscozym® L (**B**–**F**). (**A**): Wheat incubated in borate–phosphate buffer for 48 h; (**B**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease for 48 h; (**C**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.188 mL Viscozym® L for 48 h; (**D**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.375 mL Viscozym® L for 48 h; (**E**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 0.750 mL Viscozym® L for 48 h; (**F**): Wheat incubated in borate–phosphate buffer with *Streptomyces griseus* protease + 1.5 mL Viscozym® L for 48 h. Scale = 50 µm.

4. Discussion

The nutrient concentrations of the feedstuffs correspond to the literature data [30–35]. The silage fermentation parameters are similar to the literature data, whereby the lactic acid concentrations are higher than those reported in the literature [30,34,35].

The objectives of this study were to investigate the effect of an antibiotic solution in the SGPM and the impact of α-amylase or Viscozym® L co-incubated in the SGPM on ED with reference to in sacco ED.

The naturally occurring microbes in the feed favor microbial activity during in vitro incubation [36,37]. However, the accuracy of the SGPM is limited by two aspects regarding plant microbes. Similar to rumen microbes, plant-associated microbes could catabolize plant nutrients, i.e., carbohydrates and proteins, for their anabolism during incubation. With respect to the SGP, the plant microbiome could act synergistically with the protease in degrading the CP to a limited extent. The plant microbiome is specific to genotype, plant organ and environmental factors (soil, plant disease, fertilization) [36,37]. The inconsistent microbial composition of incubated plant material could lead to the strongly sample-specific microbial degradation of CP to an unknown extent. The second aspect contributing to the bias of CP degradation data by SGP is microbial mass proliferation during incubation. In particular, the release of fermentable substrates during the co-incubation of SGP and carbohydrase emphasize this effect [12]. Therefore, the use of an antibiotic solution is suggested, enabling uniform incubation conditions [38–41]. The SGPM protocol of Licitra et al. [7], however, did not recommend the use of an antibiotic solution. The results indicated significantly lower ED estimates with the use of antibiotics. The Penicillin–Streptomycin solution we applied is used in cell cultures to inhibit transpeptidases, which occur exclusively in bacteria [42]. However, the review by Blumberg and Strominger [42] showed that different

Streptomyces strains secrete enzymes with transpeptidase specificities. It remains unclear whether this specificity also occurs within the SGP mixture, which would explain the reduced ED when using the Penicillin–Streptomycin solution. Nevertheless, preparations that combine different antibiotics appear to be a recommendable tool preventing microbial activity during in vitro incubation and, for reasons of standardization, ensuring reliable estimations of ruminal CP degradation with the SGPM by providing uniform incubation conditions for different feedstuffs.

From a methodological point of view, several aspects can influence in sacco CP degradation estimates. These include microbial attachment, particle losses and animal-related factors; as such, they make reproducible estimates difficult [1]. The SGPM [7] as an alternative method compensates for such limitations by estimating the reproducible ruminal CP degradation under standardized conditions and without the use of animals. However, it was assumed that the feed-specific complexes of protein, starch and fiber act as a physical barrier hindering SGP to access and sufficiently hydrolyze the protein. Therefore, it was proposed to add carbohydrases as co-incubation agents [8,11].

The in sacco ED estimates were used as a reference to evaluate the estimates of ED by SGPM in terms of estimation accuracy. The in sacco ED values of tested feedstuffs were in the range given in the literature [31,33,34]. The in sacco ED values of corn silage and DDGS are slightly higher than those reported in the literature [31,33]. The DDGS is influenced by factors related to its production process (i.e., grinding, heat, drying and pressure) [43] and the corn silage by factors related to growing conditions (i.e., fertilization, maturity, weather conditions) [44]. Consequently, these factors could contribute to the sample-specific degradation properties differing from those in the literature. The ED values estimated by SGPM without carbohydrase were in the range of those in the published literature [8].

The estimates of the in sacco ED were underestimated both by SGPM and by SGPM with carbohydrase (α-amylase/Viscozym® L). Successful co-incubation regarding an improvement in the enzymatic CP degradation was observed with the bromelain protease and α-amylase in cereals [41]. The authors reported an increased enzymatic protein degradation by approximately 8% with the co-incubation of bromelain protease and amylase [41]. However, compared to the SGP mixture of endo- and exopeptidases [45], the bromelain protease is specified by solely endo-protease activity which might be limiting for an efficient CP hydrolysis [46]. Our results revealed slightly increased ED for specific feedstuffs when SGP was co-incubated with α-amylase or Viscozym® L, compared to the control. In wheat grain, an increase of maximal 6 and 11%-points in ED by co-incubating SGP with α-amylase and Viscozym® L was observed, respectively. This effect probably resulted from the synergistic actions of the protease and the carbohydrase affecting the protein matrix that embeds the starch granules and the protein that is enclosed in the starch granules [10,47]. The microscopy images of co-incubated SGP and α-amylase showed discolored cell structures and, macroscopically, no visible wheat starch remained at the bottom of the falcon tubes after 48 h incubation. We concluded that most of the starch was degraded (Figures 2 and S1). Viscozym® L, however, obviously did not degrade the wheat starch during co-incubation (Figures 3 and S2). Co-incubation of SGP and α-amylase seemed to work quite well, but it remains unclear why this was only evident in the wheat grains. Successful co-incubation of protease and carbohydrase was also reported by other studies [10,41,48,49].

Generally, it can be assumed that the carbohydrases did not sufficiently dissolve the feed-specific complexes of proteins and carbohydrates, despite their high doses applied in the SGPM. In particular, the effect of the Viscozym® L on ED estimates was minimal in rapeseed meal, DDGS and the silages, although these feedstuffs are mainly composed of cell wall components such as cellulose, xylose, arabinose and pectin, which are all targets of Viscozym® L [50]. With regard to the α-amylase, its effect was absent in corn grain and in PCFPS, which contained 78 and 20% starch, respectively. The differences between the ED estimated in sacco and that estimated by SGPM with carbohydrase might also be related to the incubation conditions. Enzymatic degradation reactions are generally dependent

on a number of factors including the enzyme concentration, incubation time, temperature and pH of the buffer solution [10,51]. The incubation conditions set by the SGPM (39 °C, pH 6.75; [7]) were not the optimum for Viscozym® L and α-amylase. The optimal conditions of Viscozym® L are 44–55 °C in pH 5.0 [23,52] and, according to the manufacturer, for α-amylase are >80 °C in pH 6–7. Therefore, a total of four doses per carbohydrase were used (Table 1). All doses exceeded those recommended by the manufacturer or reported in the literature [9,21], to ensure sufficient enzyme activity and to compensate for inhibitory influences on the conversion of carbohydrate–protein complexes (i.e., unfavorable incubation conditions and the disturbing effect of SGP). The incubation time of 48 h is sufficient for the enzymatic conversion of the substrates contained in the feedstuff since the relevant enzymatic reactions take place in the first hours of incubation [10]. In an experiment, Karimi et al. [10] observed that the degradation of the protein and starch of barley bran mainly occurred within the first three hours considering the optimal temperature range for the protease (Alcalase®) and α-amylase (Termamyl®). Following this initial phase, higher enzyme activities or longer incubation times did not result in the increased extraction of starch [10]. Another important factor limiting sufficient carbohydrase activity is the assumption that the carbohydrases themselves might act as substrates for the protease during co-incubation [53,54]. Recently published data confirmed the inhibitory effects of SGP on α-amylase or Viscozym® L during co-incubation [11].

5. Conclusions

The use of an antibiotic solution slightly reduced in vitro CP degradation. It is recommended to prevent microbial activity and improve the standardization of in vitro estimates. The co-incubation of SGP and carbohydrase did not sufficiently reduce the differences between in sacco and in vitro CP degradation. It seems therefore not to be a promising approach. It is assumed that the incubation conditions and enzyme interactions lead to insufficient activity of the carbohydrases. The pre-incubation of carbohydrase prior to the SGPM appears to be more promising as the carbohydrase requirements for optimal incubation conditions can be implemented.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14202982/s1, Table S1: Means of estimated parameters of in vitro crude protein degradation determined by *Streptomyces griseus* protease method with (+) and without antibiotic solution (−); Table S2: Means of estimated parameters of protein degradation determined in sacco and by co-incubation of *Streptomyces griseus* protease and α-amylase; Table S3: Means of estimated parameters of protein degradation determined in sacco and by co-incubation of *Streptomyces griseus* protease and Viscozym® L; Figure S1: Falcon tubes containing wheat incubated 48 h with *Streptomyces griseus* protease and co-incubated with *Streptomyces griseus* protease and α-amylase; Figure S2: Falcon tubes containing wheat incubated 48 h with *Streptomyces griseus* protease and co-incubated with *Streptomyces griseus* protease and Viscozym® L.

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7. General Discussion

7.1 Estimation of ruminal crude protein degradation using the *in situ* method

The lack of *in vivo* CP degradation data requires reference methods. The ruminal CP degradation data estimated by the *in situ* method are used as a reference for evaluating alternative methods estimating ruminal CP degradation (Michalet-Doreau and Ould-Bah, 1992). As already explained, the *in situ* CP degradation is subjected to numerous influences including methodologically and animal related factors (Weakley et al., 1983; Michalet-Doreau and Ould-Bah, 1992; Hristov et al., 2019) (Figure S1). These factors affect the reliable estimation of ruminal CP degradation and thus its function as reference data. It is therefore highly recommended to carry out the *in situ* method and estimate *in situ* ruminal CP degradation according to established protocols (Madsen and Hvelplund, 1994; Vanzant et al., 1998; GfE, 2022). The use of a protocol for the *in situ* method allows the standardized estimation of reproducible CP degradation data. An essential aspect of reliable *in situ* estimates is the correction for microbial nitrogen (Krawielitzki et al., 2006). Therefore, according to the protocol of the GfE (2022), it is advisable to correct the *in situ* CP degradation data for the microbial nitrogen estimated according to Parand and Spek (2021). The results of Paper I clearly show that the correction for microbial nitrogen provide more accurately estimated ED by avoiding their underestimation, especially in the case of forages. Although the animal-specific estimation of the microbial protein, as in the *in vivo* method, would be ideal, the equations of Parand and Spek (2021) provide an suitable tool for routinely correction of *in situ* CP degradation data.

7.2 Estimation of ruminal crude protein degradation using the *Streptomyces griseus* protease method

Aspects relating to the feedstuff

The feed protein degradation is mainly defined by its solubility and interaction with other nutrients of the plant cell (Bach et al., 2005). In Paper I, slight tendencies between the protein solubility and the degradation quotients (degQ = $ED_{in\ vitro}$ - $ED_{in\ situ}$/$ED_{in\ situ}$) of individual feedstuffs and feedstuff groups seemed to be visible. The protein solubility of native legume grains (peas, lupins) was high and the degQ was close to 0. Their high buffer soluble protein might have an additive effect on CP degradation by the SGP. The low protein solubilities of the by-products (wheat bran, dried distillers' grains with solubles (DDGS), corn gluten feed), sugar beet pulp and barley are in accordance with their low degQ. However, the extraction meals (sunflower-, soybean-, formaldehyde-treated- and over-toasted rapeseed meal) contrasted this tendency as they were characterized by a low protein solubility and degQ close to 0. In addition, no *in vitro* ED could be estimated for field beans and corn grains (Paper I). Consequently, feed-specific complexes of protein and nutrients, i.e., carbohydrates, might lead to

differences between *in situ* and *in vitro* ED. Depending on the localization of the protein in embryo, endosperm, cell wall/aleurone layer (Shewry and Halford, 2002), the protein appeared to be sterically structured with oil, fiber, and starch, which may affect the accessibility of the protein to enzymatic hydrolysis (Assoumani et al., 1992; McAllister et al., 1993; Abdelgadir et al., 1997; Doiron et al., 2009). The treatment of a feedstuff can pronounce the influence of the protein-carbohydrate complexes on enzymatic CP degradation by formation of Maillard reaction products (Hofmann et al., 2020). This led to the hypothesis that specific characteristics (e.g. nutrient composition, treatment) of individual feedstuffs or groups of feedstuffs lead to a differentiation with regard to the susceptibility of the feed protein to protease, and thus, to specific clustering (Paper I, hypothesis).

The influence of oil-protein complexes on the accessibility of the protein was suspected in flaxseed (Doiron et al., 2009). The ruminal protein degradability and the protein solubility increased in the extruded flaxseed. It was assumed that oil-protein complexes were dissolved by the extrusion. As a result, the protein was more accessible for ruminal degradation (Mustafa et al., 2003; Doiron et al., 2009). With reference to the native soybean described in Paper I, this could contribute to its moderate CP degradation in the SGPM-L. However, it remains unclear to what extent the inhibitory influence of the oil-protein complexes on protein accessibility in extraction meals is maintained. With reference to Paper I, the soybean and an expander-treated rapeseed meal clustered together (Paper I).

In the plant cell and in the plant cell wall, the protein is in complex structures with carbohydrates, which may reduce access and degradation of the protein by a protease (Abdelgadir et al., 1997; Pedersen et al., 2015; Ozturk et al., 2021). The non-starch polysaccharides (arabinose, xylose, arabinoxylans and pectin-like substances) are known to form gel-like aggregates within the plant cell, and thus, represent a physical barrier to the protein (Rosenfelder et al., 2013; Jakobsen et al., 2015). This influence of pectin was demonstrated by dissolving it with the SGP and a heptapeptide in the borate-phosphate buffer solution. During 60 min incubation, the degradation and rate of degradation of two different heptapeptides were reduced by the pectin-containing buffer (Paper II). The structural relationship between plant cell wall/fiber and protein has been shown several times in the literature. Pedersen et al. (2015) showed in wheat and corn DGGS that protein as well as pentosans were released by the protease. A synergistic effect on the dissolution of protein and pentosan was observed with the combined use of protease and xylanase (Pedersen et al., 2015). The influence of protein-carbohydrate complexes on CP degradation by the SGP might particularly pronounced in feedstuffs that have been concentrated with cell wall material during their production process, i.e., DDGS, corn gluten meal, wheat bran and extraction meals (Jayanegara et al., 2016). With reference to these feedstuffs in Paper I, the degQ of these feedstuffs clustered diffusely, regardless of nutrient composition or treatment (Paper I).

According to Assoumani et al. (1992), protein-starch complexes affect CP degradation by the SGP by through reduction of protein accessibility. For feedstuffs containing more than 230 g starch/kg dry matter, they reported that CP degradation by the SGP was enhanced by additional pre-incubated mixture of α-amylase and ß-glucanase. The effect of protein-starch complexes was also confirmed by Tománková and Kopečný (1995). In cereals, they reported increased CP degradation by co-incubating the protease (bromelain protease secreted by *Ananas comosus*) with an α-amylase. With reference to the degQ of starch-containing feedstuffs, they cluster diffusely regardless of origin and treatment (Paper I).

Finally, high levels of feed-specific nutrients such as acid ether extract, non-starch polysaccharides, and starch, as well as treatment effects, appeared to differentially affect CP degradation by the SGP. This confirms the first part of hypothesis (Paper I). However, the diffuse clustering of degQ across different feedstuffs regardless of origin, nutrient composition and treatment lead to rejection of the second part of hypothesis (Paper I).

Aspects relating to the enzymes

Enzymatic reactions are generally dependent on incubation conditions, including enzyme and substrate concentrations, pH of the buffer solution, incubation temperature, incubation time, and, in the case of co-incubation, enzyme interactions (Bisswanger, 2014; Karimi et al., 2018).

The CP degradation by the SGP is decisively influenced by the concentration of the SGP stock solution (Licitra et al., 1998; Licitra et al., 1999). Licitra et al. (1999) estimated CP degradation in the SGPM-L at SPG concentrations of 0.33 - 33 U/mL and validated the *in vitro* CP degradation against *in situ* CP reference. It was found that the SGP concentration between 1.0 - 2.4 U/mL is recommended to estimate *in vitro* CP degradation similar to *in situ* reference. With reference to the SPG stock solution of 0.58 U/mL used in Paper I and III, this might explain, in addition to the other factors, i.e., the protein-carbohydrate complexes and incubation conditions, why the *in situ* CP degradation was underestimated by the SGPM-L. If the SGP concentration recommended by Licitra et al. (1999) were applied to the feedstuffs of Paper I, the already overestimated ED of legume grains and extraction meals by the SGPM-L would be even more pronounced. The SGP dose recommended by Licitra et al. (1999) applied to the feedstuffs in Paper I and Paper III, which were underestimated by the SGPM-L, may reduce the difference to the *in situ* ED. Finally, as shown by the results of Paper I, although the SGP is feed-specific applicated, over- and underestimation of *in situ* CP degradation data cannot be avoided with the SGPM-L, confirming the already mentioned adverse aspect of one single SGP concentration.

The incubation conditions set by the SGPM-L do not correspond to the recommended incubation conditions of the SGP, α-amylase and a mixture of cell wall degrading enzymes (Viscozym® L) (Table 2), which contributes to insufficient enzymatic substrate hydrolysis.

Table 2: Recommended incubation conditions for the α-amylase and Viscozym® L in comparison to the conditions set by the *Streptomyces griseus* protease method according to Licitra et al. (1998)

	SGP	**α-amylase**	**Viscozym® L**	**SGPM-L**
Origin	*Streptomyces griseus*	Unknown	*Aspergillus sp.*	-
Composition	Unknown enzyme mixture consisting of endo- and exopeptidase	Single enzyme	Unknown enzyme mixture consisting of ß-glucanase, pectinase, cellulase, arabanase, xylanase	-
pH	8.8	6 - 7	4.6, 5.0	6.7 – 6.8
Temperature	37 °C	> 80 °C	44; 55 °C	39 °C
Reference	Manufacturer[1], Trop and Birk (1970), Bisswanger (2014)	Manufacturer[2]	Manufacturer[1] Liu et al. (2008), Rosset et al., (2012)	Licitra et al. (1998)

[1] Merck, KGaA, Darmstadt, Germany; [2] Univar Solutions, Essen, Germany; SGP: *Streptomyces griseus* protease; SGPM: *Streptomyces griseus* protease method; SGPM-L: *Streptomyces griseus* protease method according to Licitra et al. (1998)

Licitra et al. (1998) indicate a limiting effect of pH 6.7 on CP degradation by the SGP compared to pH 8.0. At pH 8.0, CP degradation by the SGP was up to 30 %-points higher for specific feedstuffs, indicating reserves in the CP degradation by the alkaline SGP (Licitra et al., 1998). The incubation temperature of 39 °C set by the SGPM-L might limit the hydrolytic potential of the carbohydrases in relation to the recommended incubation temperature of the carbohydrases (Table 2). However, adjusting the pH value of the buffer solution (pH 6.75) and the incubation temperature set by the SGPM-L to the recommendations of the carbohydrases (Table 2) would exclude rumen physiological aspects of the SGPM-L, might denature the SGP during co-incubation, and might contribute to less degradable feed protein by its denaturation (Hofmann et al., 2020). Nevertheless, the results of Paper II demonstrated that the SGP, α-amylase and Viscozym® L hydrolyze the substrates under SPGM conditions. However, no quantitative classification of the carbohydrase activities is possible due to a lack of literature data.

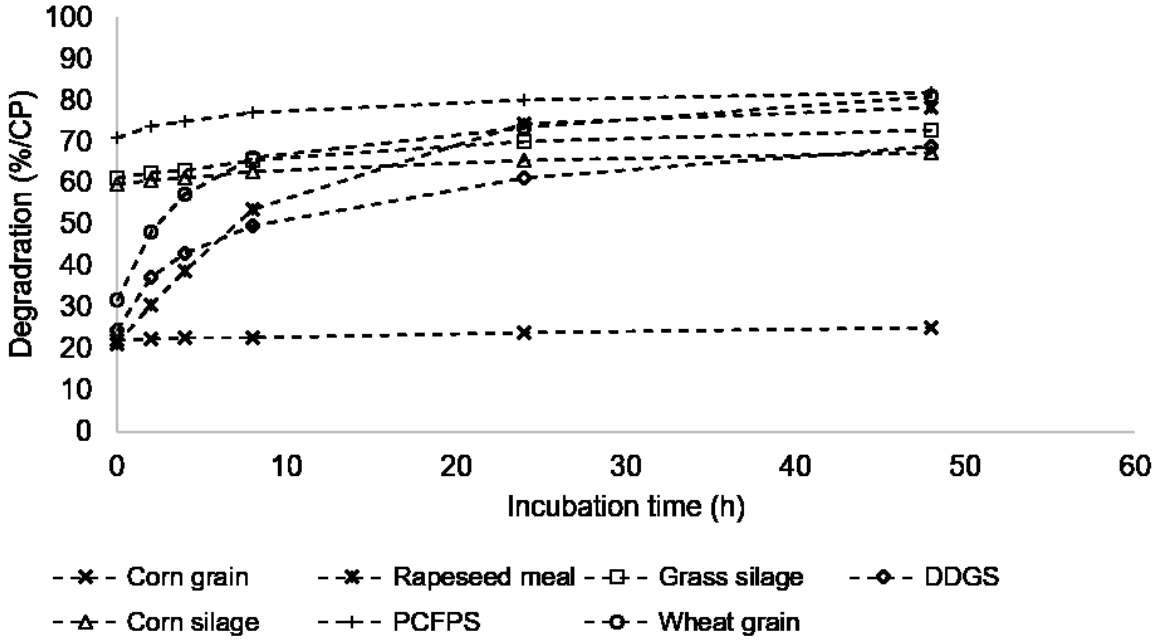
Additional carbohydrase in the SGPM-L appeared to be necessary to assist the SGP in hydrolyzing protein complexed with fiber and starch (Assoumani et al., 1992; Cone et al., 1996; Abdelgadir et al., 1997). The results in the literature regarding an effect of the protease during co-incubation on carbohydrase are contradictory, as either no effect was observed (Treimo et al., 2009; Pedersen et al.,

2015; Ozturk et al., 2021) or the carbohydrase activity was reduced by approximately 75 % by the protease (Saleh et al., 2004). However, the SGP was never part of the investigations and the incubation conditions in literature did not correspond to those of the SGPM-L. Paper II was intended to investigate the effect of the SGP on a carbohydrase (α-amylase) or carbohydrase mixture activity (Viscozym® L) during co-incubation under the conditions set by the SGPM-L. Our hypothesis was that the SGP affect the carbohydrase mixture Viscozym® L and the α-amylase in the hydrolysis of carbohydrates during co-incubation by reducing the conversion of the substrates (Paper II, hypothesis). The results confirm the hypothesis of Paper II that under the incubation conditions set by the SGPM-L, the α-amylase or Viscozym® L activity was reduced by 3 % or 37 %, respectively, during co-incubation with the SGP compared to the control variant without the SGP. Therefore, it can be assumed that SGP has an inhibitory effect on carbohydrase activity during co-incubation. In this context, it remains unclear whether the carbohydrase activity is influenced by a single protease or more proteases of the SGP mixture. Furthermore, it is also not clear whether one or more of the enzymes in the Viscozym® L mixture were inhibited by the SGP during co-incubation.

According to the Michaelis-Menten kinetics (Figure S2), the enzymatic substrate conversion can be limited by the concentration of the substrate and also by the concentration of the enzyme (Bisswanger, 2014). Carbohydrase substrates (starch and non-starch polysaccharides) are sufficiently available in the feedstuff. According to the Viscozym® L manufacturer Novozymes (Bagsværd, Denmark), its recommended dose per 1000 kg of plant material is 100 - 250 mL. For a 0.5 g sample weight, the recommended dose corresponds to 0.5 - 1.25 x 10^{-4} mL. This recommended dose was clearly exceeded in Paper III. In the case of the α-amylase, no dose is recommended by the manufacturer. It was hypothesized that additional carbohydrases assist the SGP to hydrolyze proteins from the matrix and reduce the differences to the *in situ* ED reference (Paper III, hypothesis 2). Although carbohydrases were overdosed, and substrates were available, the feed-specific protein-carbohydrate complexes might not be sufficiently hydrolyzed. It can be assumed that the unfavorable incubation conditions set by the SGPM-L appeared as the decisive aspect for the insufficient hydrolysis of the protein-carbohydrate complexes. The differences between the CP degradation estimated by the SGPM-L and the *in situ* reference can therefore not be reduced, which leads to the rejection of hypothesis 2 of Paper III.

The incubation time defines the time frame for enzymatic reactions. As indicated by Karimi et al. (2018), relevant enzymatic reactions take place in the first hours of incubation. The duration of CP degradation by the SGP depends on the stability or activity of the SGP during incubation. However, it does not seem to be clear whether the SGP is still active after 24 h or 48 h incubation time, as the maximal recommended incubation time in the SGPM-L was lowered to 30 h by Licitra et al. (1998) (Table 1). The SGP activity depends on concentration and availability of a substrate. The protease is

active as long as a substrate for the protease is present. The degradation curves of rapeseed meal, DDGS and wheat showing that the SGP appears to be active up to the incubation time of 48 h (Figure 1). The degradation curves of grass and partial crop field pea silage has only a moderate slope, indicating less accessible protein (Abdelgadir et al., 1997). The protein characteristics of corn grain and corn silage - low CP content and rich in poorly degradable di-sulfide bonds (Mahadevan et al., 1980) - seem to explain why either no CP degradation could be determined (Paper I) or why it was low (Paper III) (Figure 1). If no substrate is available for the protease, the proteases deactivates themselves. This autolysis has been observed with serine proteases (Jiang et al., 2021). According to the manufacturer, the SGP mixture contains serine proteases. Conclusively, contrary to the SGPM-L protocol (Table 1), an incubation time of 48 h in the SGPM-L is recommended to provide sufficient time for CP degradation by the SGP and thus, for accurate statements on CP degradation kinetics.



CP: crude protein; DDGS: dried distillers' grains with solubles; PCFPS: partial crop field pea silage
Figure 1: Means of crude protein degradation by *Streptomyces griseus* protease during 48 h incubation (Paper III)
The *Streptomyces griseus* protease solution contained 0.58 U/mL and the dose of *Streptomyces griseus* protease solution was feed-specific applicated according to Licitra et al. (1998) at a ratio of 24 U/g true protein.

Aspects relating to methodology

In general, some methodological aspects of the SGPM-K and SGPM-L are worth discussing. Compared to the other protease approaches for the estimation of ruminal CP degradation (Table 1), the uniqueness of the SGPM-K lies in its SGP concentration determined from the proteolytic activity of

rumen fluid and its validation on *in vivo* CP degradation data. However, the specification of the final SGP dose (10 mL SGP solution at 0.33 U/mL) appears to be incorrect. The determination of the SGP concentration and the resulting SGP dose is based on the *in vitro* incubation of azo-casein with rumen fluid from a cow fed with only one diet. The enzymatic composition of the rumen is related to the administered diet and differs between intra-ruminal components (solid and liquid phase, rumen fluid) (Falconer and Wallace, 1998; Deusch et al., 2017; Bowen et al., 2018). In conclusion, the SGP dose by Krishnamoorthy et al. (1983) is rumen-fluid-, diet-, animal- and substrate-specific. This SGP dose was validated by Krishnamoorthy et al. (1983) using incorrect *in vivo* degradation data. The *in vivo* CP degradation data was not corrected for the protozoal and endogenous protein resulted in underestimated *in vivo* CP degradation (Krishnamoorthy et al., 1983).

The SGP dose in the SGPM-L is calculated according to the feed-specific TP content. The advantage of this approach is that a specific enzyme-substrate ratio is maintained, ensuring that the substrate is not a limiting factor for the enzymatic degradation (Licitra et al., 1998; Bisswanger, 2014). However, the SGP dose according to Licitra et al. (1998) compared to the constant feed-unspecific application according to Krishnamoorthy et al. (1983) appears to have a limiting effect on CP degradation in certain feedstuffs. The CP degradation using the SGP dose according to Krishnamoorthy et al. (1983) was for most feedstuffs higher than the SGP dose according to Licitra et al. (1998). The differences of maximal 24 %-points were characteristically pronounced in feedstuffs with a low TP content, for which a low SGP dosage was calculated according to Licitra et al. (1998). Consequently, this modification of the SGPM-L may contribute to a limited extent to the insufficient CP degradation by the SGP of feedstuffs used in this study with low TP concentration (Paper I, III).

The carbohydrase α-amylase ("Termamyl® 2X") and the carbohydrase mixture Viscozym® L were already used in pre-incubation by Cone et al. (1996) to assist ruminal CP degradation by the SGP. From a methodological point of view, however, the use of carbohydrases as pre-incubation is associated with additional incubation steps including centrifugation, decantation and pH adjustment (Assoumani et al., 1992). The additional effort could be associated with bias and reduce sample throughput. The methodological approach of co-incubating the SGP and an α-amylase or Viscozym® L, respectively, was methodologically verified by the experiments of Paper II. The co-incubation improves handling in the SGPM-L by reducing effort and time (Karimi et al., 2018). However, the purely methodological advantages of co-incubating the SGP and α-amylase or Viscozym® L are not confirmed by its results on CP degradation, which is discussed in the corresponding chapter.

The incubation conditions set by the SGPM-L favor microbial activity during incubation. This could reduce the accuracy of the SGPM-L due to microbial mass proliferation and, similar to rumen microbes, due to the limited extent of protein and carbohydrate degradation by microbes for their own metabolism (Gustaw et al., 2021). The SGPM-L protocol does not recommend the use of an antibiotic

solution, although antibiotic preparations have been used in other approaches using the SGP to estimate ruminal CP degradation (Table 1). The carbohydrase might increase the microbial activity during incubation by releasing degradable nutrients (Okon et al., 2022). The penicillin-streptomycin solution seemed to be appropriate, as it has already been used in cell cultures together with trypsin and is equally effective against gram-positive and gram-negative bacteria (You and Brody, 2012). The hypothesis was that additional penicillin-streptomycin solution should have no or just a marginal effect on CP degradation in SGPM-L (Paper III, hypothesis 1). When the penicillin-streptomycin solution was added to the SGPM-L, the CP degradation was systematically reduced by maximal 5 %-points compared to CP degradation by the SGPM-L without antibiotic solution, confirming hypothesis 1 of Paper III. This observation has not been documented in any literature. Therefore, it is unclear why adding penicillin-streptomycin solution reduces CP degradation by the SGP. However, the use of the antibiotic solution is recommended to reduce microbial growth and microbial protein degradation. Additionally, CP estimation using the SGPM-L supplemented with antibiotic solution improve standardization of the *in vitro* estimates.

In general, the SGPM-L as a purely enzymatic *in vitro* method, offers the potential to estimate ruminal CP degradation reproducibly and under standardized conditions without the use of animals. Another important feature of the SGPM-L is its sensitivity to display feed-specific treatments which was confirmed and validated by comparing thermal, chemical and expander-treated feedstuffs with the *in situ* reference (Paper I). The modular structure of the SGPM enables further application possibilities, as it is integrated for the *in vitro* estimation of intestinal protein digestibility in ruminants (Hippenstiel et al., 2015). There are inconsistencies regarding the choice of established SGPM-L or SGPM-K. While Edmunds et al. (2012) used the SGPM-L protocol, other research groups (Cone et al., 2002; Cone et al., 2004; Gosselink et al., 2004) have used the SGPM protocol according to Aufrere and Cartailler (1988), which is based on the studies of Krishnamoorthy et al. (1983). From a methodological point of view, the SGPM appears to be undynamic, as a single SGP concentration may not adequately reflect different proteolytic activities in the rumen and varying *in situ* reference data (Licitra et al., 1999). Another important aspect of the SGPM is its dependence on the manufacturer of the SGP. The SGPM can only be conducted as long as the SGP is available on the market.

8. Conclusion

The present study shows that the *in situ* CP degradation is systematic but differently pronounced underestimated by the SGPM-L. The differences between *in situ* and *in vitro* CP degradation data are less attributable to methodological shortcomings of the SGPM-L, but rather by the complex structures of the feed protein to carbohydrates. Within such protein-carbohydrate complexes, the protein appeared to be less accessible and degradable to the SGP. The diffuse clustering of the feedstuffs could not be explained by groupwise or feed-specific characteristics, i.e., origin, nutrient composition and treatment. It was not possible to draw any conclusions about the groupwise or feed-specific use of a carbohydrase in the SGPM-L. However, additional carbohydrases are required in the SGPM-L for the hydrolysis of protein-carbohydrate complexes. The co-incubation approach under conditions set by the SGPM-L was verified in a model approach by exclusive co-incubation of the SGP and an α-amylase or a mixture of cell wall-degrading enzymes with carbohydrates. However, the carbohydrase activity was reduced by the SGP during co-incubation. It has been shown on different feedstuffs that the co-incubation of the SGP and α-amylase or mixture of cell wall degrading enzymes is not a promising approach to reduce the differences between *in situ* and *in vitro* CP degradation data. It is assumed that the inhibitory influence of the SGP and unfavorable incubation conditions for the carbohydrases lead to insufficient hydrolysis of the protein-carbohydrate complexes. The methodological approach of pre-incubating a carbohydrase prior to the SGPM-L appears to be more promising, as enzyme-specific incubation conditions can be considered. The addition of antibiotic solution to the SGPM is recommended for the reduction of microbial activity and the standardized estimation of CP degradation. Overall, the SGPM-L does not appear to be sufficiently adaptable to *in situ* CP reference data, as the *in situ* reference are over- and underestimated despite feed-specific protease dosing.

9. Summary

An essential part of the evaluation of feedstuffs for ruminants is the knowledge of the degradation of protein in the rumen. The ruminal crude protein (CP) degradation can be determined *in vivo* and estimated by *in situ* and *in vitro* methods. However, the complexity of the *in vivo* method using multi-fistulated cows makes the routine estimation of ruminal CP degradation difficult. The *in situ* method using rumen-fistulated cows is an accepted reference method and standardized by protocols. However, the labor-intensive and time-consuming methodology and limited controllable factors (microbial colonization of the feed residues, feed particle losses, animal-related factors) make it difficult to estimate ruminal CP degradation using the *in situ* method. The *in vitro* estimation of ruminal CP degradation using the *Streptomyces griseus* protease method (SGPM) seems to be advantageous, because of its purely enzymatic approach under standardized incubation conditions. However, the accuracy of the SGPM compared to *in situ* reference data might be impaired by two methodological shortcomings. The feed protein that is complexed with starch and fiber, appeared to be less degradable by the *Streptomyces griseus* protease (SGP). The addition of an α-amylase or a mixture of cell wall degrading enzymes (Viscozym® L: ß-glucanase, cellulase, pectinase, arabanase, xylanase) in the SGPM implemented as a pre-incubation step supported the CP degradation by increasing the protein accessibility to the SGP within these protein-carbohydrate complexes. However, the simultaneous incubation of the SGP and carbohydrase as co-incubation seems to be a more promising approach for reasons of efficiency. In this context, it is unclear whether the carbohydrase activity is affected by the SGP during co-incubation. The second point concerns microbial activity during the co-incubation of the SGP and carbohydrase caused by natural feed-specific microbes. Similar to the rumen microbes, plant-associated microbes are able to catabolize plant proteins, but to a different extent. Without the addition of an antibiotic solution during co-incubation, the accuracy of the SGPM could be reduced due to microbial activity. According to the literature, a penicillin-streptomycin solution seemed to be appropriate.

The hypothesis was that specific characteristics (e.g., nutrient content, treatment) of individual feedstuffs or groups of feedstuffs lead to a differentiation with regard to the susceptibility of the feed protein to protease, and thus, to grouping of feedstuffs in specific clusters. The clusters were used for the decision of additional carbohydrase in the SGPM (Paper I). It was assumed that during co-incubation the SGP affects the α-amylase and Viscozym® L in the hydrolysis of carbohydrates by reducing their conversion into measurable sugars (Paper II). Following the results of Paper II, it was hypothesized that additional carbohydrases assist the SGP to hydrolyze proteins from the matrices and reduce the difference to the *in situ* reference. Additionally, we hypothesized that an additional

penicillin-streptomycin solution should have no effect or just a marginal effect on CP degradation (Paper III).

The effective CP degradation (ED) was estimated by the SGPM for 40 feedstuffs (8 extraction meals, 3 cereal grains species, 9 legume grains, 4 by-products, 16 grass silages) for which *in situ* reference CP degradation data were available. The *in situ* CP degradation data were corrected for microbial nitrogen estimated *via* equations from the literature. The SGP solution (0.58 U/mL) was feed-specific added. The samples were incubated for 2, 4, 6, 8, 16 and 24 h at pH 6.75 and 39 °C. The differences between *in situ* and *in vitro* ED of a feedstuff were expressed as degradation quotient (degQ = $ED_{in\ vitro}$ - $ED_{in\ situ}$/$ED_{in\ situ}$). The grouping of degQ across the feedstuffs was examined using a cluster analysis (Paper I). The influence of the SGP on α-amylase or Viscozym® L during co-incubation was investigated by measuring carbohydrase activity on cellulose, pectin and starch under SGPM conditions (pH 6.75, 39 °C) within 5 h incubation (Paper II). The ED of rapeseed meal, dried distillers' grains with solubles, wheat grains, corn grains, corn silage, grass silage and partial crop field pea silage was estimated *in situ* using three rumen-fistulated dairy cows and *in vitro* using the SGPM and the SGPM with additional carbohydrase. The *in situ* ruminal CP degradation data were corrected for microbial nitrogen estimated *via* equations from the literature. Methodologically validated by Paper II, the α-amylase or Viscozym® L solution (as in Paper II) was added in four different doses, respectively, followed by the SGP solution as a co-incubation. The samples were incubated for 2, 4, 8, 24 and 48 h at pH 6.75 and 39 °C. In addition, the influence of a penicillin-streptomycin solution in SGPM on the ED was investigated (Paper III).

The *in situ* ED were systematically and differently pronounced underestimated by the SGPM by maximal 48 %-points ($p < 0.05$). The differences between *in situ* and *in vitro* ED were lowest in legume grains and highest in by-products and barley, indicating the need for additional carbohydrases in the SGPM to support CP degradation. The diffuse clustering of the degQ across different feedstuffs does not allow clear conclusions about the use of carbohydrase in the SGPM, as the feedstuffs were grouped independently of origin, nutrient composition and treatment (Paper I). Although predominantly not significant, the α-amylase and Viscozym® L activity was reduced up to maximal 3 % and 37 %, respectively during 5 h co-incubation with the SGP (Paper II). The *in situ* ED were underestimated by the SGPM up to 60 %-points and by the SGPM with additional α-amylase and Viscozym® L up to 58 %-points and 60 %-points, respectively ($p < 0.05$). The penicillin-streptomycin solution in the SGPM reduced the ED by a maximum of 5 %-points ($p < 0.05$).

In conclusion, the *in situ* CP degradation data were underestimated by the SGPM which might be related to feed-specific protein-carbohydrate complexes. During co-incubation, the unfavorable incubation conditions set by the SGPM and the inhibitory effect of the SGP on carbohydrase activity might lead to insufficient hydrolysis of the protein-carbohydrate complexes. The co-incubation of the SGP and carbohydrase seems not to be a promising approach. The pre-incubation appears to be more

promising, as the recommended incubation conditions of a carbohydrase can be integrated. The addition of an antibiotic solution in the SGPM is recommended provide uniform incubation conditions for a standardized estimation of ED by the SGPM.

All in all, the methodological shortcoming of the SGPM by its purely proteolytic specificity required the need for carbohydrases to improve its accuracy. Further research is necessary for the validation of the pre-incubation approach in the SGPM with *in situ* reference data.

10. Zusammenfassung

Kenntnisse zum ruminalen Proteinabbau sind ein wesentlicher Bestandteil der Bewertung von Futtermitteln für Wiederkäuer. Der ruminale Abbau des Rohproteins (CP) kann *in vivo* bestimmt, und mit *in situ*- und *in vitro*-Methoden geschätzt werden. Die komplexe Durchführung der *in vivo*-Methode, bei der Kühe mit mehreren Fisteln zum Einsatz kommen, erschwert die routinemäßige Bestimmung des ruminalen CP-Abbaus. Die *in situ*-Methode, bei der pansenfistulierte Kühe verwendet werden, ist als Referenzmethode zur Schätzung des ruminalen CP-Abbaus anerkannt und anhand von Protokollen standardisiert. Die zeit- und arbeitsaufwendige Versuchsdurchführung und die nur bedingt kontrollierbaren Faktoren (mikrobielle Besiedlung der Futtermittelresiduen, Partikelverluste des Futtermittels und tierbezogene Aspekte) erschweren die Schätzung des ruminalen CP-Abbaus mit der *in situ*-Methode. Die *in vitro*-Schätzung des ruminalen CP-Abbaus mit der *Streptomyces griseus* Protease-Methode (SGPM) scheint, aufgrund des reinen enzymatischen Versuchsansatzes unter standardisierten Bedingungen, vorteilhafter zu sein. Die Genauigkeit der SGPM im Vergleich zu *in situ*-Referenzdaten ist jedoch vermutlich durch zwei methodische Mängel beeinträchtigt. Das Protein, welches in Futtermitteln in komplexen Strukturen mit Faser und Stärke vorliegt, scheint für die *Streptomyces griseus* Protease (SGP) schwer abbaubar zu sein. Der Zusatz einer α-Amylase oder einer Mixtur aus zellwandspaltenden Enzymen (Viscozym® L: ß-Glukanase, Cellulase, Pektinase, Arabanase, Xylanase) zur SGPM als Vor-Inkubationsschritt unterstützt den CP-Abbau, indem die Zugänglichkeit des Proteins innerhalb der Protein-Kohlenhydrat-Matrizen für die SGP erhöht wird. Die simultane Inkubation der SGP und Carbohydrase als Co-Inkubation scheint aus Gründen der Effizienz erfolgsversprechender zu sein. In diesem Zusammenhang ist jedoch unklar, ob die Carbohydrase-Aktivität während der Co-Inkubation durch die SGP beeinträchtigt wird. Der zweite Punkt betrifft die mikrobielle Aktivität während der Co-Inkubation von SGP und Carbohydrase, die durch futtermittelspezifische Mikroben bedingt ist. Die futtermittelassoziierten Mikroben sind in der Lage, ähnlich den Mikroben im Pansen, Pflanzenproteine zu verstoffwechseln. Dies geschieht in unterschiedlichem Ausmaß. Ohne den Zusatz einer Antibiotikalösung zur Co-Inkubation würde die Genauigkeit der SGPM infolge mikrobieller Aktivität beeinträchtigt sein. Nach den Angaben in der Literatur scheint eine Penicillin-Streptomycin-Lösung einsatzwürdig zu sein.

Die Hypothese war, dass futtermittelspezifische oder futtermittelgruppenspezifische Eigenschaften (Nährstoffzusammensetzung und Behandlung) zu einer Differenzierung aufgrund der Abbaubarkeit des Proteins gegenüber der Protease und damit zur Gruppierung von Futtermitteln in spezifischen Clustern führt. Die Cluster wurden für die Entscheidung über zusätzliche Carbohydrasen in der SGPM verwendet (Paper I). Es wurde angenommen, dass die SGP während der Co-Inkubation den Kohlenhydratabbau, gemessen anhand des umgesetzten Zuckers, durch α-Amylase und Viscozym® L

beeinträchtigt wird (Paper II). Nach den Ergebnissen von Paper II wurde angenommen, dass zusätzliche Carbohydrasen die SGP im Abbau des Matrix-Proteins unterstützen und die Differenzen zur *in situ*-Referenz verringern. Des Weiteren wurde angenommen, dass der Zusatz einer Penicillin-Streptomycin-Lösung keinen oder nur einen geringen Einfluss auf den CP-Abbau ausübt (Paper III).

Der effektive CP-Abbau (ED) wurde mittels SGPM von 40 Futtermitteln geschätzt (8 Extraktionsschrote, 3 Getreidearten, 9 Leguminosenkörner, 4 Nebenprodukte und 16 Grassilagen). Zu diesen Futtermitteln waren *in situ*-Referenzdaten des CP-Abbaus vorhanden. Die SGP-Lösung (0,58 U/ml) wurde futtermittelspezifisch appliziert. Die Proben wurden für 2, 4, 6, 8, 16 und 24 h bei einem pH-Wert von 6,75 und 39 °C inkubiert. Die *in situ*-Abbaudaten des CP wurden um den mikrobiellen Stickstoff korrigiert, der anhand von Gleichungen aus der Literatur geschätzt wurde. Die Differenz zwischen dem *in situ*- und *in vitro*-ED eines Futtermittels wurde als Abbauquotient ($degQ = ED_{in\ vitro} - ED_{in\ situ}/ED_{in\ situ}$) ausgedrückt. Die Gruppierung der degQ der Futtermittel wurde mittels einer Clusteranalyse untersucht (Paper I). Der Einfluss der SGP auf die α-Amylase und das Viscozym® L während der Co-Inkubation wurde anhand der Carbohydrase-Aktivität an den Substraten Cellulose, Pektin und Stärke unter den Bedingungen des SGPM (pH 6,75; 39 °C) untersucht (Paper II). Der ED von Rapsextraktionsschrot, Trockenschlempe, Körnerweizen, Körnermais, Maissilage, Grassilage und Erbsensilage wurde mittels der *in situ*-Methode an drei pansenfistulierten Milchkühen bestimmt. Die *in situ*-Abbaudaten des CP wurden um den mikrobiellen Stickstoff korrigiert, der anhand von Gleichungen aus der Literatur geschätzt wurde. Die *in vitro*-ED-Schätzung wurde mittels SGPM und SGPM mit zusätzlicher Carbohydrase, abgesichert durch Paper II, vorgenommen. Dazu wurde die α-Amylase- oder Viscozym® L-Lösung (wie in Paper II) in vier verschiedenen Dosierungen hinzugegeben. Unmittelbar danach folgte die Zugabe der SGP-Lösung als Co-Inkubation. Die Proben wurden für 2, 4, 8, 24 und 48 h bei einem pH-Wert von 6,75 und 39 °C inkubiert. Des Weiteren wurde der Einfluss einer Penicillin-Streptomycin-Lösung in der SGPM auf den ED untersucht (Paper III).

Der *in situ*-ED wurde mit der SGPM systematisch und unterschiedlich stark um maximal 48 %-Punkte unterschätzt ($p < 0,05$). Die Differenzen zwischen *in situ*- und *in vitro*-ED waren bei den Körnerleguminosen am geringsten und bei den Nebenprodukten und Gerste am größten, weshalb zusätzliche Carbohydrasen in der SGPM zur Unterstützung des CP-Abbaus erforderlich sind. Die diffuse Gruppierung der degQ nach der Herkunft, Nährstoffzusammensetzung und Behandlung der Futtermittel lässt keine eindeutige Schlussfolgerung zur Verwendung von Carbohydrasen in der SGPM zu (Paper I). Die α-Amylase-Aktivität wurde um maximal 3 % und die Viscozym® L-Aktivität um maximal 37 % reduziert, was überwiegend nicht signifikant war (Paper II). Der *in situ*-ED wurde durch die SGPM um bis zu 60 %-Punkte und durch die SGPM mit zusätzlicher α-Amylase und Viscozym® L um bis zu 58 bzw. 60 %-Punkte unterschätzt ($p < 0,05$). Der Zusatz der Penicillin-Streptomycin-Lösung in der SGPM reduzierte den ED maximal um 5 %-Punkte ($p < 0,05$).

Zusammenfassend wurden die *in situ*-Abbaudaten des CP durch die SGPM unterschätzt, was auf futtermittelspezifische Protein-Kohlenhydrat-Komplexe zurückgeführt werden könnte. Vermutlich führen die ungünstigen Inkubationsbedingungen, vorgegeben durch die SGPM, und der inhibitorische Effekt der SGP während der Co-Inkubation dazu, dass die Protein-Kohlenhydrat-Komplexe unzureichend hydrolisiert werden. Die Co-Inkubation von SGP und einer Carbohydrase erscheint nicht erfolgsversprechend. Die Vor-Inkubation erscheint dagegen aussichtsreicher, da die empfohlenen Inkubationsbedingungen der Carbohydrase berücksichtigt werden können. Der Zusatz einer Antibiotikalösung ist empfehlenswert, um einheitliche Inkubationsbedingungen für eine standardisierte Schätzung des ED mit der SGPM zu ermöglichen.

Insgesamt erfordert der methodische Mangel der SGPM durch deren reine proteolytische Spezifität den Einsatz von Carbohydrasen, um die Genauigkeit der SGPM zu verbessern. Weitere Untersuchungen sind erforderlich, um den Vor-Inkubationsansatz in der SGPM an *in situ*-Referenzdaten zu validieren.

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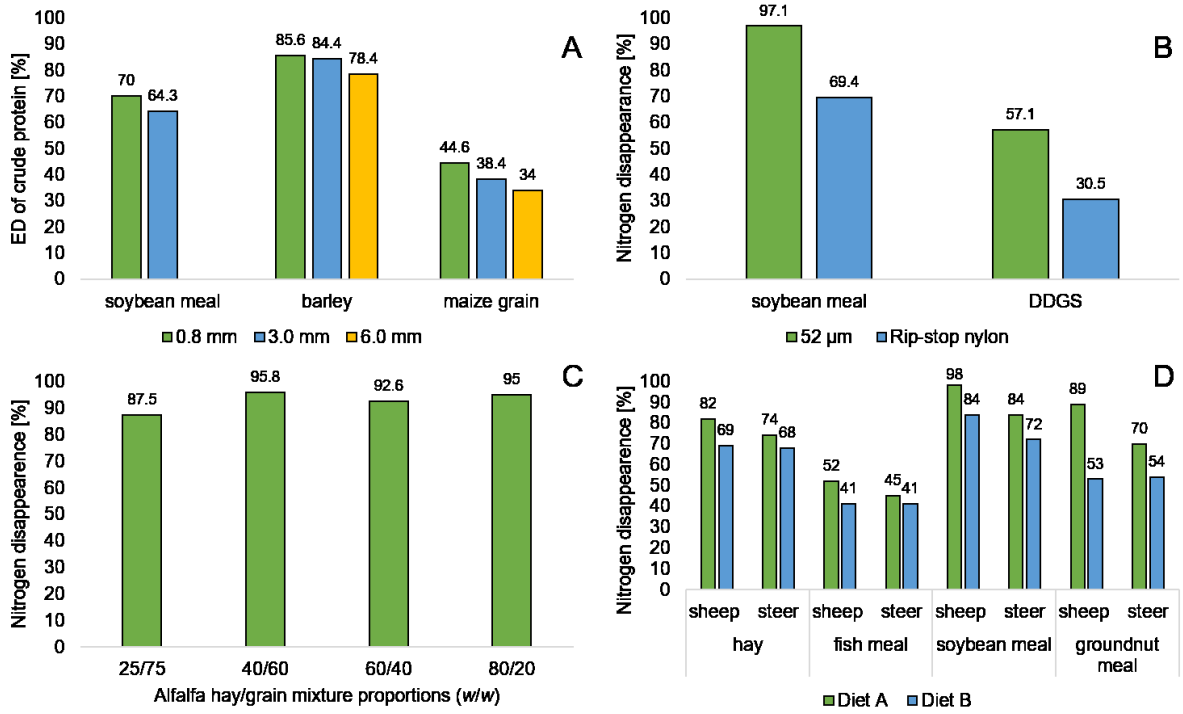
12. Supplements



DDGS: dried distillers' grains with solubles; ED: effective degradation

A) Michalet-Doreau and Cerneau, (1991): Effective crude protein degradation estimated according to Ørskov and McDonald, (1979) with assumed ruminal passage rate of 0.06 h^{-1}; grinding intensity of soybean meal was 2.5 mm instead of 3 mm

B) Weakley et al. (1983): Rip-stop nylon had no visible pores according to the authors; 24 h incubation time

C) Weakley et al. (1983): 24 h incubation of soybean meal in cows fed diet with increasing alfalfa hay and decreasing grain mixture (corn grain and soybean meal)

D) Siddons and Paradine (1983): Diet A consisted of dried grass (*Lolium perenne*) and Diet B consisted of 65 % dried grass (*Lolium perenne*) and 35 % barley

Figure S 1: Influence of grinding intensity (A), nylon bag porosity (B), diet (C) and diet + species (D) on crude protein degradation and nitrogen disappearance estimated by the *in situ* method

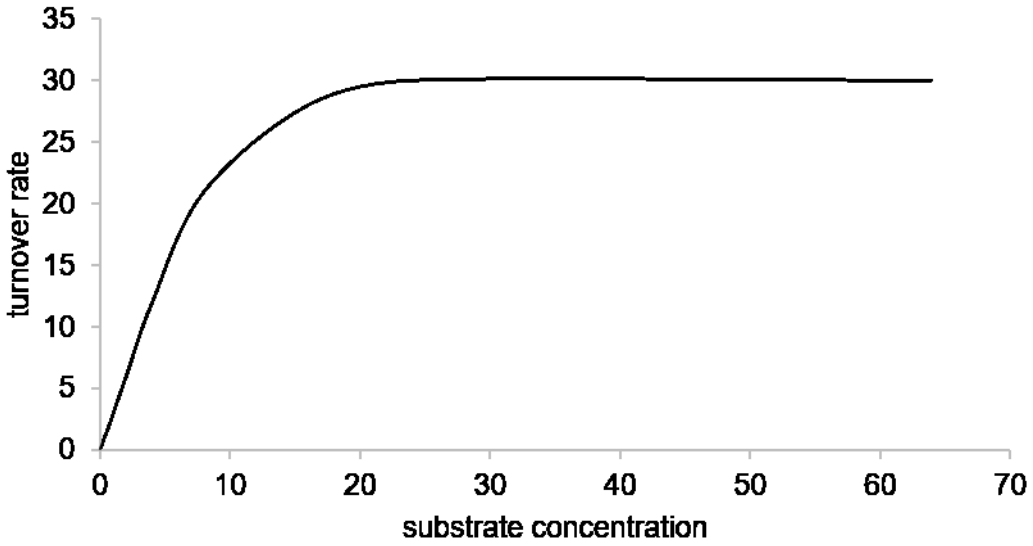


Figure S 2: Michaelis-Menten kinetics of enzymatic degradation with substrate concentration-dependent conversion rate

13. Appendix

Publications (peer reviewed)

Bachmann, M., Kuhnitzsch, C., Okon, P., Martens, S.D., Greef, J.M., Steinhöfel, O., Zeyner, A., 2019. Ruminal In Vitro Protein Degradation and Apparent Digestibility of Energy and Nutrients in Sheep Fed Native or Ensiled + Toasted Pea (*Pisum sativum*) Grains, in animals, 9 (7), 401., available at https://doi.org/10.3390/ani9070401.

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Conference contributions

Okon, P., Bachmann, M., Titze, N., Rodehutscord, M., Zeyner, A., 2021. Determination of ruminal protein degradation of concentrate feeds in situ and in vitro, in Proceedings of the 75th Society of Nutrition Physiology, 30, p. 132. Conference held digital, Germany, 16 – 18 March 2021.

Okon, P., Bachmann, M., Blunk, C., Kuhnitzsch, C., Martens, S., Steinhöfel, O., Zeyner, A., 2022. Effects of ensiling and thermic treatment on protein solubility and in vitro fermentation of faba bean partial crops, in Proceedings of the 76th Society of Nutrition Physiology, 31, p. 96. Conference held digital, Germany, 8 – 10 March 2022.

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kohlenhydratspaltenden Enzymen, in 133. VDLUFA KONGRESS |Kurzfassungen der Beiträge, p. 84. Conference held in Halle (Saale), Germany, 13 – 16 September 2022.

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Okon, P., Bachmann, M., Wensch-Dorendorf, M., Titze, N., Rodehutscord, M., Rupp, C., Susenbeth. A., Zeyner, A., 2023. Does clustering of degradation data help to identify necessary modifications of the *Streptomyces griseus* protease test?, in Proceedings of the 77th Society of Nutrition Physiology, 32, p. 35. Conference held in Göttingen, Germany, 7 – 9 March 2023.

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Kluth, H., Bachmann, M., Okon, P., Abraham, U., Zeyner, A., 2023. Zur Wirkung von Schälen und Toasten auf die praecaecale Aminosäurenverdaulichkeit von Erbsen und Lupinen beim Broiler, in 16. Tagung Schweine- und Geflügelernährung, 16, p. 48 – 50, Conference held in Lutherstadt Wittenberg, Germany, 14 – 16 November 2023.

Kluth, H., Bachmann, M., Okon, P., Abraham, U., Zeyner, A., 2023. Untersuchungen zur Verdaulichkeit von Aminosäuren aus Körnerleguminosen unter Berücksichtigung von Vermahlen, Schälen und Toasten sowie Alter und Geschlecht beim Broiler, in Leguminosentag Ost, Conference held in Dummerstorf, Germany, 5 – 6 December 2023.

Okon, P., Liebscher, S., Simon, A.H., Wensch-Dorendorf, M., Bachmann, M., Bordusa, F., Zeyner, A., Spectrophotometric investigation of the co-incubation of *Streptomyces griseus* protease and carbohydrase on the release of reducing sugars, in Proceedings of the 78th Society of Nutrition Physiology, 33, p. 53. Conference held in Göttingen, Germany, 5 – 7 March 2024.

Okon P., Bachmann, M., Wensch-Dorendorf, M., von Soosten, D., Meyer U., Dänicke, S., Zeyner, A., Bestimmung des ruminalen Futterproteinabbaus *in sacco* und mittels Co-Inkubation von *Streptomyces griseus* Protease + Carbohydrase, in 135. VDLUFA KONGRESS |Kurzfassungen der Beiträge, p 60. Conference held in Karlsruhe, Germany, 24 – 27 September 2024.

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Halle (Saale), January 2025

Eidesstaatliche Erklärung / *Declaration under oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Datum / *Date* Paul Okon

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