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Investigations on the effects of forage source and feed particle size on ruminal fermentation and microbial protein synthesis *in vitro*

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Abbreviations

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AA	amino acid
ADF	acid detergent fibre
CF	crude fibre
CP	crude protein
CV	coefficient of variation
DM	dry matter
e.g.	for example ('exempli gratia')
EMPS	efficiency of microbial protein synthesis
GS	grass silage
LAM	liquid-associated microbes
LAM-E	liquid-associated microbes in the effluent
LAM-F	liquid-associated microbes in the fermenter
LSMeans	least square means
ME	metabolisable energy
MS	maize silage
MSS	milling screen size
n	number of samples/ replicates
NDF	neutral detergent fibre
NSC	non-structural carbohydrates
OM	organic matter
r	correlation coefficient
RUSITEC	rumen simulation technique
SAM	solid-associated microbes
SCFA	short chain fatty acid
SD	standard deviation
SE	standard error
vs.	versus

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General introduction

Microbial protein and amino acid flow from the rumen

One important limiting factor for animal performance is the daily amount of absorbable amino acids (AA) in the small intestine. It is determined by the amount and the composition of the protein that enters the duodenum. In ruminants this protein is a mixture of microbial protein synthesised in the rumen, undegraded protein of dietary origin, and, to a much lesser extent, endogenous protein. The AA profile of microbial protein differs from that of feed protein. Therefore it is an important goal in protein evaluation systems to improve predictability of duodenal flow of microbial protein and rumen-undegradable feed protein (NRC 2001, Tamminga *et al.* 2007). Microbial protein typically supplies the majority of AA flowing to the small intestine. As the AA pattern of microbial protein is favourable to milk protein synthesis it is worthwhile to promote microbial protein synthesis due to feeding strategies (NRC 2001). Although microbial growth is linked with organic matter (OM) fermentation, there is no clear linear relationship between amount of ruminally fermented OM and daily flow of microbial protein. Moreover, a wide range in efficiency of microbial protein synthesis (EMPS) is reported in the literature (Clark *et al.* 1992, Figure I-1).

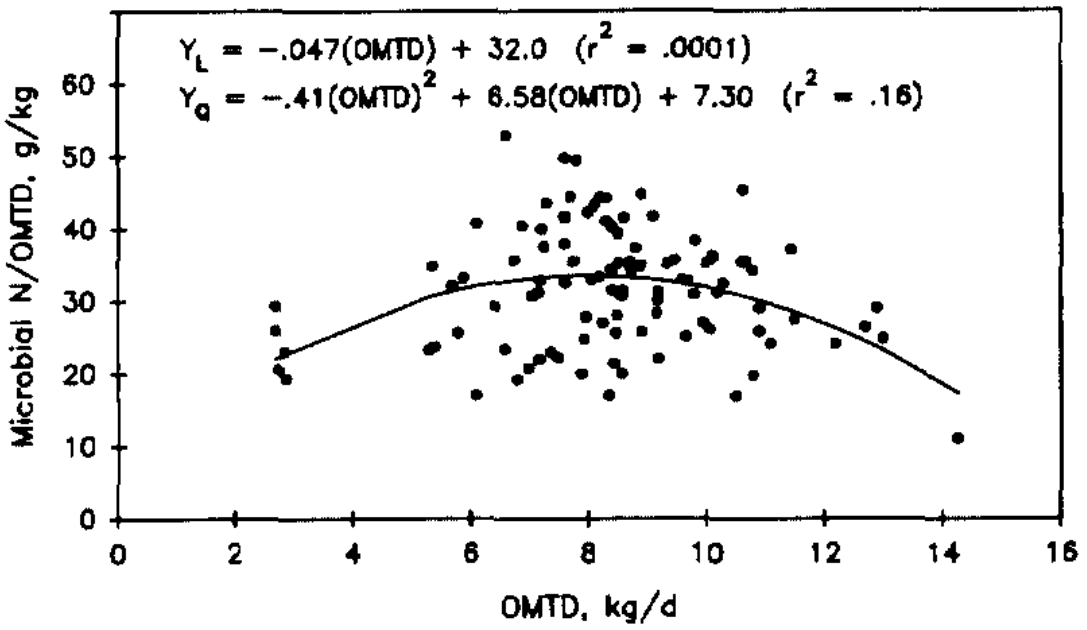

Figure I-1 Relationship between OM truly digested in the rumen (OMTD) and efficiency of microbial protein synthesis in dairy cows (Clark *et al.* 1992)

Values for EMPS are reported to range from 10 to 54 g microbial N/kg fermented OM in literature reviews (Stern and Hoover 1979, Lebzien and Voigt 1999, NRC 2001). Microbial growth and EMPS are significantly affected by feed intake and diet composition (Sniffen and Robinson 1987). Especially the amount and the source of carbohydrates and protein as well as their ruminal degradability were pointed out to be important dietary factors for microbial protein flow (Hoover and Stokes 1991). The content of crude protein (CP) and the inclusion rate of maize silage (MS) in 16 total mixed rations for dairy cows helped to explain part of the variation in EMPS (Boguhn *et al.* 2006b).

The mean AA profile of ruminal microbes as reviewed by Clark *et al.* (1992) is given in Table I-1. The effect of diet on the AA profile of microbial protein is discussed contrary, but large differences between results reported in the literature can be related to methodical aspects, particularly microbial isolation techniques (Clark *et al.* 1992). However, the use of different feedstuffs (Korhonen *et al.* 2002) and differences in the dietary fibre content (Rodríguez-Prado *et al.* 2004) seem to affect the AA profile of microbial mass. As indicated by the study of Boguhn *et al.* (2006a) the microbial AA profile seems to be affected by the proportion of MS and grass silage (GS) in total mixed rations.

Table I-1 Proportion of amino acids in microbial protein (Clark *et al.* 1992, n = 441)

Amino acid (AA)	Mean	Min	Max
		[g AA/ 100 g AA]	
Alanine	7.5	5.0	8.6
Arginine	5.1	3.8	6.8
Aspartic acid	12.2	10.9	13.5
Cystine	-	-	-
Glutamic acid	13.1	11.6	14.4
Glycine	5.8	5.0	7.6
Histidine	2.0	1.2	3.6
Isoleucine	5.7	4.6	6.7
Leucine	8.1	5.3	9.7
Lysine	7.9	4.9	9.5
Methionine	2.6	1.1	4.9
Phenylalanin	5.1	4.4	6.3
Proline	3.7	2.4	5.3
Serine	4.6	3.4	5.4
Threonine	5.8	5.0	7.8
Tyrosine	4.9	3.9	7.7
Valine	6.2	4.1	7.6

In order to determine the AA profile in microbial mass, microbes have to be isolated from the solid and liquid phase of digesta leaving the rumen. As shown by several researchers (Merry and McAllan 1983, Legay-Carmier and Bauchart 1989, Martín-Orúe *et al.* 1998) the chemical composition of microbial mass can differ between solid-associated microbes (SAM) and liquid-associated microbes (LAM). Moreover, differences in AA profile between SAM and LAM have to be expected (Martin *et al.* 1996, Volden *et al.* 1999). Therefore it is necessary to obtain both microbial fractions in terms of estimation of total microbial protein and AA flow.

Nutritional value of maize silage and grass silage

MS and GS are of significant importance in nutrition of indoor-kept dairy and beef cattle across Europe. In general MS is characterised by higher contents of OM, non-structural carbohydrates (NSC) and metabolisable energy (ME) than GS, whereas GS is known to be higher in CP and crude fibre than MS (DLG 1997). The differences in energy value, protein value and content of structural carbohydrates promote the complementary use of both forages in ruminant rations. Dependent on the nutrient requirements of animals and external conditions of production, the inclusion of the two forages in diets varies (Spiekers *et al.* 2009). Typically the partial replacement of GS by MS in the diet leads to an increase in dry matter intake (Phipps *et al.* 1995, O'Mara *et al.* 1998, Juniper *et al.* 2008), and hence an increased ME intake. However, a quadratic response in animal performance to a change in MS-to-GS ratio may occur, although feed intake increases linearly (O'Mara *et al.* 1998). Mixtures of GS and MS can result in a higher milk yield (O'Mara *et al.* 1998) and milk protein yield (Phipps *et al.* 1992) than feeding both silages as single forage source, indicating an improved AA supply to the animal when both forage sources are fed in mixture. As shown by Van Vuuren *et al.* (1999) the supply of maize starch to GS-based diets significantly improves the microbial protein flow to the duodenum in dairy cows. In general a higher microbial protein yield and EMPS for MS- than for GS-based diets is reported in the literature (Givens and Rulquin 2004, Owens *et al.* 2009). However, diets with a high proportion of MS need to be supplemented with protein sources, e.g. soybean meal, or mineral N in order to avoid a deficit in available N for microbial growth, and hence a reduction of nutrient fermentation (Hoover and Stokes 1991, GfE 2001, Spiekers *et al.* 2009).

The replacement of GS by MS in the diet is known to induce a decrease in ruminal pH value and can impair ruminal degradation of neutral detergent fibre (NDF) (Juniper *et al.* 2008,

Owens *et al.* 2009). Current feed evaluation systems assume that values for nutrient degradation of individual forages are additive in mixtures and do not consider possible interactions. If one feedstuff affects the degradability of another feedstuff in the diet, this kind of interaction can lead to positive or negative associative effects on fermentation characteristics in the rumen (Niderkorn and Baumont 2009). The extent of ruminal fermentation has a significant impact on the total tract digestibility of nutrients and the feedstuffs' nutritional value for the animal. Total tract digestibility of crude nutrients, measured in wether sheep, is the basis for calculations on ME content of single feedstuffs for ruminants in Germany (GfE 2001). Several studies have shown that the stepwise replacement of GS by MS can result in linear or quadratic responses in total tract digestibility (Moss *et al.* 1992, Browne *et al.* 2005, Vranić *et al.* 2008). However, it was not clear to what extent the respective observations were caused by differences in feed intake or composition of the supplemented concentrate feed, and in which way ruminal degradation of nutrients was affected by MS-to-GS ratio.

Investigation of ruminal fermentation *in vitro*

A wide range of methods to investigate ruminal fermentation has been developed in the past decades. The most widely applied way of measuring ruminal degradation of nutrients and microbial protein synthesis *in vivo* is the use of animals equipped with a cannula at the proximal duodenum. Because *in vivo* studies are expensive, labour intensive, time consuming, and subject to error, associated with the use of digesta flow markers and animals variation (Tamminga and Chen 2000), several alternative techniques have been developed to investigate ruminal fermentation with lower effort and under more constant conditions than *in vivo*. The most commonly used approaches are based on measurements conducted *in situ* or *in vitro* (Stern *et al.* 1997, López 2005). In order to unravel causal connections between dietary factors and the trait, it is useful to keep all other experimental conditions constant. Therefore the use of *in vitro* methods seems to be appropriate. *In vitro* systems usually work with rumen inocula taken from rumen-fistulated animals and can be classified into batch cultures and continuous cultures. A commonly used batch culture systems in German forage evaluation is the Hohenheim gas test, which is used to predict digestibility and ME content of feedstuffs (Menke and Steingass 1988, GfE 2008). Moreover, gas production techniques are useful tools to describe kinetics of fermentation, as development of gas production rate in

course of time is solely associated with fermentation of OM (Getachew *et al.* 1998). Several mathematical models were developed to describe the kinetics of gas production (Dhanoa *et al.* 2000). As proposed by Beuvink and Kogut (1993) a modified Gompertz function provides good results in modelling the curve of cumulative gas production from GS and MS.

In contrast to batch cultures, continuous culture systems are characterised by a regular addition of buffer solution and nutrients, and a continuous removal of fermentation products. This allows the establishment of steady-state conditions and thus a stable microbial population that is adapted to the incubated feedstuff and can be maintained for long periods of time (López 2005). The most commonly used continuous culture systems are the dual-flow system described by Hoover *et al.* (1976) and the rumen simulation technique (RUSITEC, Figure I-2) developed by Czerkawski and Breckenridge (1977).

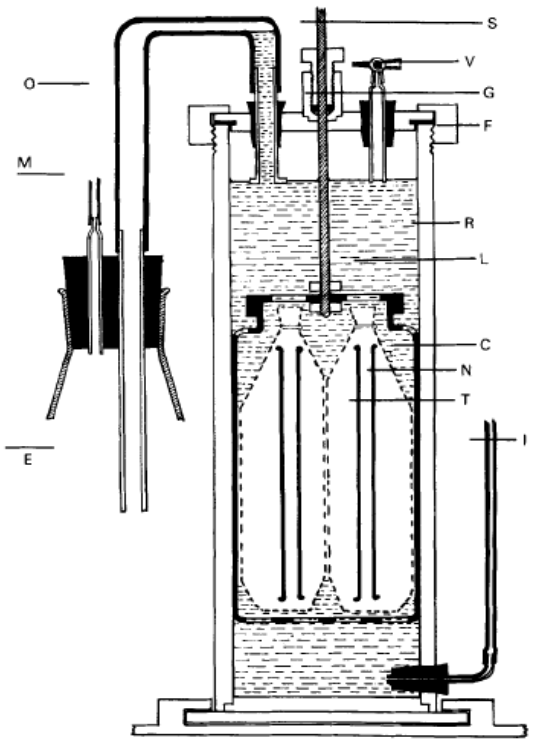

Figure I-2 Schematic diagram of a RUSITEC fermenter unit according to Czerkawski and Breckenridge (1977) with S = driving shaft, V = sampling valve, G = gland, F = flange, R = main reaction vessel, L = liquid phase (39°C); C = perforated food container, N = nylon bag, T = rigid tube, I = inlet of artificial saliva, O = outlet through overflow, M = outlet of fermentation gas, E= vessel for collection of effluent

In contrast to the dual-flow continuous culture system the RUSITEC system is fed only one time daily and nylon bags usually remain inside the fermenter for 48 hours. Therefore this technique is also called semi-continuous culture system. In most continuous flow studies a buffer solution gets infused continuously into the fermenter in order to regulate pH value. Continuous infusion also allows for the additional supplementation of soluble nutrients in order to simulate their ruminal availability in course of time. Moreover the rumeno-hepatic

circulation can be simulated by the infusion of mineral N, e.g. urea-N. As shown by Windschitl and Stern (1988) the infusion of urea led to improved fermentation of a maize-based diets in a continuous flow system, although total dietary CP content was 160 g/kg DM due to soybean meal inclusion into the diet, and therefore in the range of usual CP contents in ruminant rations. In this context the need for a high N supply in order to promote microbial protein synthesis from diets with high NSC contents was suggested by the continuous culture study of Stokes *et al.* (1991).

Variation of feed particle size *in vitro*

For most *in vitro* applications the feedstuffs have to be dried and ground before they get incubated. As sample size of incubated feed in general is small, in the first instance grinding is useful to generate homogenous feed samples. The second aspect is standardisation of *in vitro* procedures. A variation in particle size leads to a variation in growth conditions of several microbial groups and consequently in the extent of fermentation (Zhang *et al.* 2007). An increase in mean feed particle size can be induced by the increase in milling screen size (MSS) (Bossen *et al.* 2008, Figure I-3).

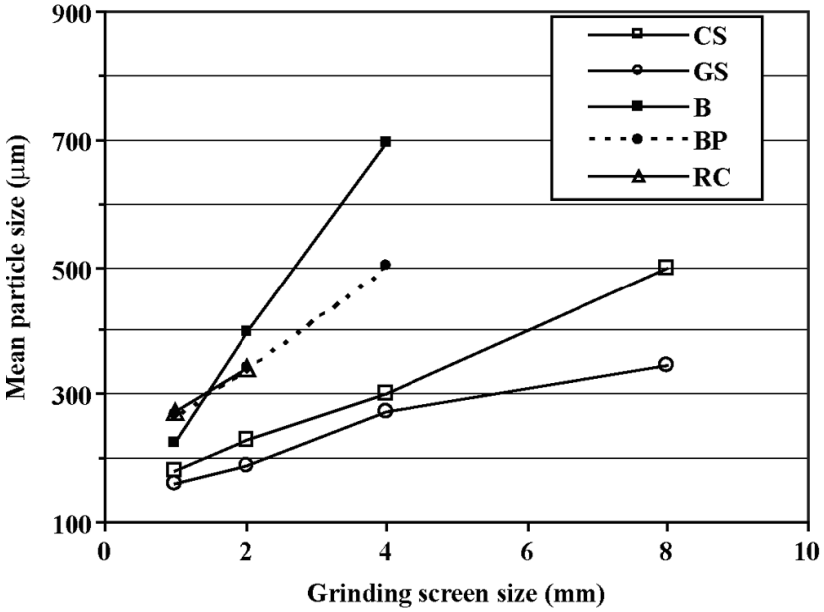

Figure I-3 Mean particle size from grinding barley (B), corn silage (CS), grass silage (GS), rapeseed cake (RC), and beet pulp (BP) through different screen sizes (Bossen *et al.* 2008)

Feedstuffs usually get milled through a 1-mm screen for use in the Hohenheim gas test in terms of feed evaluation (method 25.1, VDLUFA 2006). For batch cultures an increase in mean particle size by increasing the MSS is reported to impair cumulative gas production (Menke and Steingass 1988) and degradation of fibre fractions in forages (Robles *et al.* 1980, Bossen *et al.* 2008). The particle size of incubated feedstuffs has varied widely across continuous culture studies but attempts to investigate the consequences of this variation are rare. As a result of increasing MSS the difference in mean particle size becomes greater between feedstuffs (Figure I-3). Interactions between forage source and MSS on particle size distribution and mean particle size have been documented in the literature (Emanuele and Staples 1988, Michalet-Doreau and Cerneau 1991). An increase in mean particle size due to milling primarily relies on the increased size of particles originating from the stem fraction of forages (Kennedy and Doyle 1993). As leaf and stem fractions differ in their *in vitro* fermentation characteristics, this aspect has to be considered when interpreting results and when methods are used that benefit particle fractionation, for example nylon bag techniques (Udén 1992). As shown by Rodríguez-Prado *et al.* (2004) a variation in alfalfa hay stem particle size (1 vs. 3 mm screen size) affected the pattern of short chain fatty acids and ruminal N metabolism in continuous culture fermenters. However, effects of particle size on EMPS and AA profile were dependent on the considered microbial fraction, LAM or SAM, respectively. Because availability of nutrients and growth conditions of microbial groups seem to be closely related with the level of grinding (Michalet-Doreau and Cerneau 1991, Bowman and Firkins 1993, Zhang *et al.* 2007), the detection of differences in fermentation characteristics between forages might depend on the used MSS under *in vitro* conditions.

With particular relevance to the subject of this thesis the main conclusions from this introduction are:

- The simultaneous use of MS and GS may benefit microbial growth in the rumen to a higher extent than using only one forage source separately. However, systematic investigations in order to identify the MS-to-GS ratio that results in the highest microbial protein yield and EMPS are lacking.
- For investigations on the duodenal flow and AA profile of microbial protein the microbial mass in the solid and liquid phase of the digesta have to be considered.
- A deficit in dietary N may impair microbial fermentation of MS, and therefore N supply has to be considered in comparison studies investigating MS- and GS-based diets.

- Combining MS and GS in a diet may result in associative effects of both forage sources on ruminal fermentation characteristics and therefore in non-additivity of nutritional values of single forages in mixed diets.
- *In vitro* continuous culture systems are suitable experimental models for investigations on ruminal fermentation and microbial protein synthesis under more standardised conditions than *in vivo*.
- The reduction in feed particle size is an important aspect in standardisation of *in vitro* procedures; however information on the effect of variations in MSS on the results in continuous culture systems, particularly the RUSITEC system, is very rare in literature.

References

Beuvink JMW and Kogut J 1993. Modeling gas production kinetics of grass silages incubated with buffered ruminal fluid. Journal of Animal Science 71, 1041-1046.

Boguhn J, Kluth H and Rodehutscord M 2006a. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. Journal of Dairy Science 89, 1592-1603.

Boguhn J, Kluth H and Rodehutscord M 2006b. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. Journal of Dairy Science 89, 1580-1591.

Bossen D, Mertens DR and Weisbjerg MR 2008. Influence of fermentation methods on neutral detergent fiber degradation parameters. Journal of Dairy Science 91, 1464-1476.

Bowman JGP and Firkins JL 1993. Effects of forage species and particle size on bacterial cellulolytic activity and colonization in situ. Journal of Animal Science 71, 1623-1633.

Browne EM, Juniper DT, Bryant MJ and Beever DE 2005. Apparent digestibility and nitrogen utilisation of diets based on maize and grass silage fed to beef steers. Animal Feed Science and Technology 119, 55-68.

Clark JH, Klusmeyer TH and Cameron MR 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. Journal of Dairy Science 75, 2304-2323.

Czerkawski JW and Breckenridge G 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371-384.

Dhanoa MS, Lopez S, Dijkstra J, Davies DR, Sanderson R, Williams BA, Sileshi Z and France J 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: comparison of models. British Journal of Nutrition 83, 131-142.

DLG, Deutsche Landwirtschafts-Gesellschaft 1997. DLG-Futterwerttabellen Wiederkäuer. DLG-Verlag, Frankfurt am Main.

Emanuele SM and Staples CR 1988. Effect of Forage Particle Size on In Situ Digestion Kinetics. Journal of Dairy Science 71, 1947-1954.

Getachew G, Blümmel M, Makkar HPS and Becker K 1998. In vitro gas measuring techniques for assessment of nutritional quality of feeds: A review. Animal Feed Science and Technology 72, 261-281.

GfE, Gesellschaft für Ernährungsphysiologie 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG-Verlag, Frankfurt am Main, Germany.

GfE, Gesellschaft für Ernährungsphysiologie 2008. Mitteilungen des Ausschusses für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie: Neue Gleichungen zur Schätzung der Umsetzbaren Energie für Wiederkäuer von Gras- und Maisprodukten. Proceedings of the Society of Nutrition Physiology 17, 191-197.

Givens DI and Rulquin H 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Animal Feed Science and Technology 114, 1-18.

Hoover WH and Stokes SR 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield. Journal of Dairy Science 74, 3630-3644.

Hoover WH, Crooker BA and Sniffen CJ 1976. Effects of differential solid-liquid removal rates on protozoa numbers in continuous cultures of rumen contents. Journal of Animal Science 43, 528-534.

Juniper DT, Browne EM, Bryant MJ and Beever DE 2008. Digestion, rumen fermentation and circulating concentrations of insulin, growth hormone and IGF-1 in steers fed diets based on different proportions of maize silage and grass silage. Animal 2, 849-858.

Kennedy PM and Doyle PT 1993. Particle-size reduction by ruminants - Effects of cell wall composition and structure. In Forage cell wall structure and digestibility (eds. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 499-534, American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin, USA.

Korhonen M, Ahvenjärvi S, Vanhatalo A and Huhtanen P 2002. Supplementing barley or rapeseed meal to dairy cows fed grass-red clover silage: II. Amino acid profile of microbial fractions. Journal of Animal Science 80, 2188-2196.

Lebzien P and Voigt J 1999. Calculation of utilizable crude protein at the duodenum of cattle by two different approaches. Archives of Animal Nutrition 52, 363-369.

Legay-Carmier F and Bauchart D 1989. Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. British Journal of Nutrition 61, 725-740.

López S 2005. In vitro and in situ techniques for estimating digestibility. In Quantitative aspects of ruminant digestion and metabolism (eds. J Dijkstra, JM Forbes and J France), pp. 87-122, CABI Publishing, Wallingford, UK.

Martín-Orúe SM, Balcells J, Zakraoui F and Castrillo C 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure. Animal Feed Science and Technology 71, 269-282.

Martin C, Bernard L and Michalet-Doreau B 1996. Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. Journal of Animal Science 74, 1157-1163.

Menke KH and Steingass H 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development 28, 7-55.

Merry RJ and McAllan AB 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. British Journal of Nutrition 50, 701-709.

Michalet-Doreau B and Cerneau P 1991. Influence of foodstuff particle size on in situ degradation of nitrogen in the rumen. Animal Feed Science and Technology 35, 69-81.

Moss AR, Givens DI and Phipps RH 1992. Digestibility and energy value of combinations of forage mixtures. Animal Feed Science and Technology 39, 151-172.

Niderkorn V and Baumont R 2009. Associative effects between forages on feed intake and digestion in ruminants. Animal 3, 951-960.

NRC, National Research Council 2001. Protein and amino acids. In Nutrient Requirements of Dairy Cattle (ed. NRC), pp. 43-104, National Academic Press, Washington D.C.

O'Mara FP, Fitzgerald JJ, Murphy JJ and Rath M 1998. The effect on milk production of replacing grass silage with maize silage in the diet of dairy cows. Livestock Production Science 55, 79-87.

Owens D, McGee M, Boland T and O'Kiely P 2009. Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat. Journal of Animal Science 87, 658-668.

Phipps RH, Weller RF and Rook AJ 1992. Forage mixtures for dairy cows: The effect on dry matter intake and milk production of incorporating different proportions of maize silage into diets based on grass silages of differing energy value. Journal of Agricultural Science 118, 379-382.

Phipps RH, Sutton JD and Jones BA 1995. Forage mixtures for dairy cows: The effect on dry-matter intake and milk production of incorporating either fermented or urea-treated whole-crop wheat, brewers' grains, fodder beet or maize silage into diets based on grass silage. Animal Science 61, 491-496.

Robles AY, Belyea RL, Martz FA and Weiss MF 1980. Effect of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchardgrass forages. Journal of Animal Science 51, 783-790.

Rodríguez-Prado M, Calsamiglia S and Ferret A 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. Journal of Dairy Science 87, 1413-1424.

Sniffen CJ and Robinson PH 1987. Microbial growth and flow as influenced by dietary manipulations. Journal of Dairy Science 70, 425-441.

Spiekers H, Nußbaum H and Potthast V 2009. Erfolgreiche Milchviehfütterung. DLG-Verlag, Frankfurt am Main.

Stern MD and Hoover WH 1979. Methods for determining and factors affecting rumen microbial protein synthesis: A review. Journal of Animal Science 49, 1590-1603.

Stern MD, Bach A and Calsamiglia S 1997. Alternative techniques for measuring nutrient digestion in ruminants. Journal of Animal Science 75, 2256-2276.

Stokes SR, Hoover WH, Miller TK and Manski RP 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. Journal of Dairy Science 74, 860-870.

Tamminga S and Chen XB 2000. Animal-based techniques for the estimation of protein value of forages. In Forage Evaluation in Ruminant Nutrition (eds. DI Givens, E Owen, RFE Axford and HM Omed), pp. 215-232, CAB International, Wallingford, Oxon, UK.

Tamminga S, Brandsma GG, Dijkstra J, Van Duinkerken G, Van Vuuren AM and Blok MC 2007. Protein evaluation for ruminants: the DVE/OEB 2007 system. In CVB documentation report nr. 53 Centraal Veevoeder Bureau, CVB, Lelystad, Netherlands.

Udén P 1992. The influence of leaf and stem particle size in vitro and of sample size in sacco on neutral detergent fibre fermentation kinetics. Animal Feed Science and Technology 37, 85-97.

Van Vuuren AM, Klop A, Van Der Koelen CJ and De Visser H 1999. Starch and stage of maturity of grass silage: Site of digestion and intestinal nutrient supply in dairy cows. Journal of Dairy Science 82, 143-152.

VDLUFA, Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten 2006. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Volden H, Harstad OM and Mydland LT 1999. Amino acid content and profile of protozoal and bacterial fractions isolated from ruminal contents of lactating dairy cows fed diets differing in nitrogen supplementation. Acta Agriculturae Scandinavica, Section A, Animal Science 49, 245-250.

Vranić M, Knežević M, Bošnjak K, Leto J, Perčulija G and Matić I 2008. Effects of replacing grass silage harvested at two maturity stages with maize silage in the ration upon the intake, digestibility and N retention in wether sheep. Livestock Science 114, 84-92.

Windschitl PM and Stern MD 1988. Effects of urea supplementation of diets containing lignosulfonate-treated soybean meal on bacterial fermentation in continuous culture of ruminal contents. Journal of Animal Science 66, 2948-2958.

Zhang Y, Gao W and Meng Q 2007. Fermentation of plant cell walls by ruminal bacteria, protozoa and fungi and their interaction with fibre particle size. Archives of Animal Nutrition 61, 114-125.

Scope of the thesis

The first objective was to describe the effect of maize silage (MS)-to-grass silage (GS) ratio in the diet on ruminal fermentation characteristics and the synthesis, as well as amino acid (AA) profile, of microbial protein. Additionally the effect of inorganic N supply to MS with regard to the way of supplementation was investigated. The second objective was to study the effect of feed particle size, due to variation of milling screen size (MSS), on characteristics of fermentation and microbial protein synthesis, particularly in a semi-continuous flow system (RUSITEC), and to identify possible interactions between forage source and feed particle size.

In detail, the following questions are aimed to be answered in the three main chapters of this thesis:

1. In which way, linearly or quadratically, do *in vitro* ruminal fermentation characteristics and total tract digestibility coefficients change due to a stepwise replacement of MS by GS in the diet? (**Paper I**)
2. To what extent will *in vitro* fermentation characteristics change when feed particle size of MS- and GS-based diets is varied by MSS (1 vs. 4 mm)? (**Paper I**)
3. At which MS-to-GS ratio do the highest yield and efficiency of microbial protein synthesis in a RUSITEC occur, and what role does the MSS (1 vs. 4 mm) play? (**Paper II**)
4. How is the AA profile of microbial fractions in a RUSITEC affected by MS-to-GS ratio and feed particle size, and are there differences between microbes associated with the liquid and solid phase, respectively? (**Paper II**)
5. In which way will characteristics of fermentation and microbial protein synthesis differ between pure MS and GS in a RUSITEC, when MSS is 1, 4 or 9 mm? (**Paper III**)
6. How will the way of urea-N supply, continuously infused with buffer solution or given once daily in the nylon bag, affect fermentation characteristics of MS in a RUSITEC? (**Paper III**)

Paper I

Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation *in vitro*

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The effect of the forage source on ruminal fermentation in vitro *was investigated for fine (F) and coarse (C) milled diets, using a modified Hohenheim gas production test and a semi-continuous rumen simulation technique (Rusitec). It was hypothesised that the replacement of maize silage by grass silage might lead to associative effects and that interactions related to particle size variation could occur. Five diets with a maize silage to grass silage ratio of 100 : 0, 79 : 21, 52 : 48, 24 : 76 and 0 : 100 differed in their content of CP and carbohydrate fractions, as well as digestible crude nutrients, derived from a digestibility trial with wether sheep. For* in vitro *investigations, the diets were ground to pass a sieve of either 1 mm (F) or 4 mm (C) perforation. Cumulative gas production was recorded during 93 h of incubation and its capacity decreased with increasing proportion of grass silage in the diet. Across all diets, gas production was delayed in C treatments compared with F treatments. Degradation of crude nutrients and detergent fibre fractions was determined in a Rusitec system. Daily amounts of NH_3-N and short-chain fatty acids (SCFA) were measured in the effluent. Degradation of organic matter (OM) and fibre fractions, as well as amounts of NH_3-N, increased with stepwise replacement of maize silage by grass silage. Degradability of CP was unaffected by diet composition, as well as total SCFA production. In contrast to the results of the gas production test, degradation of OM and CP was higher in C than in F treatments, accompanied by higher amounts of NH_3-N and SCFA. Interactions of silage ratio and particle size were rare. It was concluded that the stepwise replacement of maize silage by grass silage might lead to a linear response of most fermentation characteristics* in vitro*. This linear effect was also supported by total tract digestibility data. However, further investigations with silages of variable quality seem to be necessary.*

Keywords: silage, particle size, associative effects, ruminal fermentation, *in vitro*

Implications

When grass silage and maize silage are incubated together but in different ratios *in vitro*, the associative effects on criteria of fermentation are unlikely to occur. Fermentation values for the individual silages are additive in these mixtures. However, the effect of variation in silage chemical composition remains to be investigated. The effects of feed particle size should be taken into account when comparing results of studies conducted with a Rusitec system. Further research investigating the effect of feed particle size, larger than particles produced by milling feeds with a 4-mm sieve, on degradation rates measured in rumen simulations is needed.

Introduction

The simultaneous use of maize silage and grass silage in ruminant nutrition is common practice in Europe, and intensity of feeding is dependent on environmental and economic aspects. Both forages differ in their contents of non-structural carbohydrates (NSC), generally higher in maize silages, and CP, mostly higher in grass silages. Current feed evaluation systems assume that nutritional values of individual forages are additive and do not consider possible interactions. However, one forage can affect the nutritional value of another forage in the diet (Niderkorn and Baumont, 2009). The incremental replacement of grass silage by maize silage has been reported to result in linear or quadratic responses of total tract digestibility (Browne *et al.*, 2005; Juniper *et al.*, 2008; Vranić *et al.*, 2008).

Published studies investigating the effect of a diets' maize silage to grass silage ratio on ruminal fermentation are limited in number. On the one hand, the adequate availability of fermentable carbohydrates and nitrogen has been intensively discussed to promote microbial fermentation in the rumen (Hoover and Stokes, 1991). On the other hand, high starch contents, as present in maize silage, have been shown

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to possibly lead to a depression in fibre degradation (Grant and Mertens, 1992). As recently demonstrated by Juniper *et al*. (2008), the inclusion of maize silage in replacement of grass silage in a diet (0, 330, 670 and 1000 g/kg forage) led to an increase in degradation of organic matter (OM) and a decrease in degradation of NDF in the rumen of steers. These observations were accompanied by an increase in feed intake and ruminal passage rate caused by the inclusion of maize silage, whereas the ruminal pH value decreased.

In vitro methods provide the opportunity to standardise the environment of ruminal fermentation, in general at lower costs than *in vivo* studies (Stern *et al*., 1997). One of the most commonly used *in vitro* methods is the Hohenheim gas production technique (Menke and Steingass, 1988), which is reported to detect interactions between forages in the early hours of incubation (Robinson *et al*., 2009). However, to consider the adaptation of the microbial community to the diet, *in vitro* continuous culture systems are suitable for measurements on nutrient degradation (Stern *et al*., 1997). To generate homogeneous samples, dry feedstuffs usually are milled before *in vitro* incubation. The particle size and shape of incubated feedstuffs affect the availability and degradability of nutrients (Michalet-Doreau and Cerneau, 1991; Bowman and Firkins, 1993). The particle size of incubated feedstuffs has varied widely across continuous culture studies in the literature, but attempts to research the consequences of this variation are rare. Inter-laboratory standardisation of *in vitro* set-ups is hard to achieve. However, the comparison of results from different laboratories is common practice in scientific publishing.

The first objective of this study was to investigate how the stepwise replacement of maize silage by grass silage affects gas production and nutrient degradation under *in vitro* conditions. The hypothesis was that one silage, maize silage and grass silage, respectively, might affect the fermentation and nutritional value of the other one when both are incubated together in several ratios. The effects on total tract digestibility were additionally studied with sheep. The second objective was to study to what extent the characteristics of *in vitro* fermentation change when particle size distribution is varied by fine and coarse milling.

Material and methods

Treatments

Five diets, equal in their proportion of forages (877 to 887 g/kg), soybean meal (102 to 111 g/kg) and a mineral and vitamin premix (11 to 12 g/kg), but different in their maize silage to grass silage ratio, were used. The maize silage and grass silage (fourth cut) were produced at a dairy cow farm near Halle upon Saale (Germany). The dry matter content was 350 g/kg (maize silage) and 380 g/kg (grass silage), and the pH values were 3.72 and 4.21, respectively. The concentrations of acetic acid, propionic acid, butyric acid and lactic acid were 25.1, 0.6, 0.3, 56.6 g/kg dry matter for maize silage and 6.2, 0.3, 0.3, 92.2 g/kg dry matter for grass silage. NH_3-N content was 116 mg/g total N in maize silage and 43 mg/g total N in grass silage. The starch concentration of the maize silage was 323 g/kg dry matter. The maize silage to grass silage ratios were 100 : 0, 79 : 21, 52 : 48, 24 : 76 and 0 : 100 (on dry matter basis) in diets M100, M79, M52, M24 and M0, respectively (Table 1). With the stepwise replacement of maize silage by grass silage, the concentrations of CP and fibre fractions in the diet increased, and the concentrations of OM, especially NSC, decreased. Before the *in vitro* incubations, the diets were dried at 65°C and ground by a cutting mill (Pulverisette 15, Fritsch GmbH, Idar-Oberstein, Germany). Particle size was varied by using two different milling sieves with either a 1- or 4-mm perforation, and treatments were classified as fine (F) and coarse (C), respectively. F treatments were characterised by a bulk density (g/100 ml) that was, on average, 45% higher than that of C treatments (Table 2). Differences in bulk density rely on differences in particle size distribution. The proportion of particles with a diameter lower than 1 mm was about 88%, 69% and 43% in F treatments and 57%, 44% and 30% in C treatments for diets M0, M24 and M52, respectively. Particle size distribution could not be measured for diets with a high proportion of maize silage (M100, M79) due to a high degree of particle agglomeration, which turned out to impair the suitability of the method of determination.

Experimental procedures

Total tract digestibility. For additional characterisation of diets before the *in vitro* assays, digestibility of crude nutrients was measured with four wether sheep per diet by a standard procedure (Gesellschaft für Ernährungsphysiologie, GfE, 1991) with methodological details as described by Boguhn *et al*. (2003). In brief, the daily rations were offered

Table 1 *Composition of experimental diets (g/kg DM)*

Diet[1]	M100	M79	M52	M24	M0
Ingredient composition					
Maize silage	887	697	457	215	–
Grass silage	–	188	427	664	877
Soybean meal	102	104	104	109	111
Mineral and vitamin premix[2]	11	11	12	12	12
Chemical composition					
DM (g/kg)[3]	936	933	934	926	942
OM	943	933	907	887	873
CP	130	153	178	204	231
EE	33	33	34	34	35
CF	177	195	199	209	222
NDF	338	352	381	406	410
ADF	203	213	224	223	239
NSC[4]	454	397	338	243	209

DM = dry matter; OM = organic matter; EE = ether extract; CF = Crude fibre; NSC = non-structural carbohydrates.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0)
[2]Ingredient/g: 180 mg Ca, 100 mg Na, 52 mg P, 20 mg Mg, 0.32 mg Zn, 0.064 mg Mn, 0.002 mg Se, 0.001 mg I, 0.001 mg Co, 24 IU vitamin A, 2.4 IU vitamin D_3, 0.024 mg vitamin E.
[3]Oven-dried at 65°C as used for *in vitro* incubation.
[4]NSC = 1000 − NDF − CP − EE − ash (Van Soest *et al*., 1991).

Table 2 *Bulk density of diets and classification of feed particles according to their diameter (%)*

Diet[1]	M100		M79		M52		M24		M0	
Particle size	F	C	F	C	F	C	F	C	F	C
Bulk density (g/100 ml)	33.7	26.5	33.4	26.0	35.5	22.6	35.3	22.6	37.4	23.5
Diameter (mm)										
<0.5	n.d.	n.d.	n.d.	n.d.	5	5	14	9	28	16
0.5 to 1.0	n.d.	n.d.	n.d.	n.d.	38	25	55	35	60	41
1.0 to 1.5	n.d.	n.d.	n.d.	n.d.	37	29	24	28	11	23
1.5 to 2.0	n.d.	n.d.	n.d.	n.d.	15	20	5	14	1	10
2.0 to 4.0	n.d.	n.d.	n.d.	n.d.	5	19	2	12	0.1	7
>4.0	n.d.	n.d.	n.d.	n.d.	0.1	3	0	2	0.1	3

F = fine; C = coarse; n.d. = non detectable because of agglomerated particles in maize silage.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0).

in two meals per day. The period of adaptation to the diet was at least 14 days. Feed intake ranged between 722 and 828 g dry matter for diets M0 and M100, respectively, and was adapted to the estimated maintenance metabolisable energy (ME) requirement. Wethers with a mean body weight of 67.1 kg were housed in single cages and were equipped with bags during collection of faeces for a period of 6 consecutive days. The bags were emptied once daily and a representative sample of faeces was stored at −20°C.

Gas production test. The intention was to describe the kinetics of gas production especially in the first hours of incubation as an indication of the extent of fermentation over time. Gas production was measured using the apparatus as described by Menke *et al*. (1979) and following the official method (25.1, Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA, 2006). At least eight replicates per treatment, distributed across four independent incubation periods, were used. Approximately 200 mg of the diets were weighed in glass syringes (150 ml). Rumen liquid was taken from three ruminally fistulated sheep (breed Schwarzköpfiges Fleischschaf) before the morning feeding, filtered through two layers of linen cloth and mixed. Animals had been fed grass hay for *ad libitum* intake, 250 g of a concentrate mix and 10 g of a mineral and vitamin mix daily. Rumen liquid and buffer medium (method 25.1, VDLUFA, 2006) were mixed at a ratio of 1 : 2 and dosed at a volume of 30 ml to each syringe. Gas production was recorded manually 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 17, 21, 25, 29, 33, 39, 45, 57, 69, 81 and 93 h after the incubation had been started. Venting was done four times in each incubation period at all syringes including the blanks. The correction factors for hay and concentrate as the two standard feedstuffs were not considered.

Rumen simulation. Degradation of crude nutrients and detergent fibre fractions was measured using a semi-continuous rumen simulation technique (Rusitec; Czerkawski and Breckenridge, 1977). Treatments were tested at four (F) and three (C) replications, respectively, and distributed to seven incubation periods, each lasted for 13 days. Five ruminally fistulated sheep, fed as described above, were used for obtaining liquid and solid samples of rumen content on the first day of incubation, immediately before the morning feeding. The whole procedure of sampling inocula, loading and running the Rusitec system, equipped with six fermenters having a capacity of 800 ml each, was carried out as described by Boguhn *et al*. (2006). Diets were weighed into nylon bags (pore size = 100 μm) at an amount of 15 g per bag. Incubation started with two nylon bags per fermenter, one contained experimental feed and the other was filled with solid samples of rumen content (~60 g). The latter was replaced by a second feed bag 24 h later. The buffer solution, following McDougall (1948), contained 0.7 mmol NH_4^+/l from $^{15}NH_4Cl$ (11.5% ^{15}N abundance) and was infused continuously at an average flow rate of 546 (s.d. = 29) ml/day. The amount of effluents, captured in cooled bottles (4°C), was measured daily. After 48 h of incubation, each bag was replaced by a new one, washed in 50 ml of buffer solution and squeezed moderately. The liquid was filled back into the respective fermenter. After 7 days of incubation, feed residues were collected for 6 consecutive days. In addition, 320 ml of daily effluents were centrifuged two times for 5 min at 2.000 × **g** and 4 °C to separate the feed particles that were washed out from the bags. After removing the supernatant with a pipette, the residuum was rinsed on a pre-weighed folded filter (MN 615¼ ø 150 mm, Macherey-Nagel GmbH & Co. KG, Düren, Germany). Bags and filters were dried at 65 °C for 24 h and weighed at room temperature after 24 h. Feed residues from the bags were pooled per fermenter and stored at room temperature until subsequent analyses. Thereafter, liquid effluents, free from feed particles, were centrifuged at 27.000 × **g** (15 min, 4°C), and 40 ml of the supernatant were sampled daily, pooled per fermenter and stored at −20°C for analysis of NH_3-N and short-chain fatty acids (SCFA). Microbes, associated with solids, were isolated from feed residues on the last day of incubation according to the method described by Carro and Miller (2002). The microbes were separated from the remaining fluid and further treated as described by Boguhn *et al*. (2006).

Analytical procedures

Bulk density of feed samples was measured by using a grain tester with a calibrated 250 ml cylinder (Louis Schopper, Leipzig, Germany) in three replicates per diet. Particle size distribution was detected by using a photo-optical particle analyser (HAVER-CPA 4, Haver & Boecker OHG, Oelde, Germany). The photo-optical method was used because sieving methods are less suitable for measuring feed samples that contain particles of elongated shapes, as in the case of grass silage. In brief, particles from 5 g of the experimental diets were individualised by an oscillating trough and dosed to fall down in front of a light source. The particles' silhouettes were detected by a charge-coupled device camera, which was connected to a control and output unit. The diameter of

the feed particles was calculated by the area of a circle, equating the area of the particles' silhouettes.

Crude nutrients were analysed following official methods (VDLUFA, 2006). Samples of feed, feed residues and freeze-dried faeces were ground to pass through a sieve of 1-mm pore size and analysed for dry matter and crude ash (method 3.1), CP (method 4.1.1), ether extract (EE, method 5.1.1) and crude fibre (CF, method 6.1.1). These samples, with the exception of faeces, were also analysed for contents of NDF, assayed with a thermally stable amylase, and ADF, both without residual ash (methods 6.5.1 and 6.5.2). Starch content in maize silage was determined via the polarimetric approach (method 7.2.1). NH_3-N was measured by steam distillation with sodium hydroxide (VAPODEST 4 titramatic, Gerhardt GmbH & Co. KG, Bonn, Germany) followed by end-point titration (titration unit S154, Schott, Hofheim, Germany). Concentrations of SCFA were analysed in pooled samples of each replicate, using a gas chromatograph (GC 14B, Shimadzu, Japan) equipped with a flame ionisation detector, and samples were treated with formic acid containing 4% of 2-methyl-valeric acid according to Geissler *et al.* (1976). Feedstuffs, feed residues and solid-associated microbes were analysed for N and ^{15}N in triplicate per sample using an elemental analyser (EuroEA, HEKAtech GmbH, Wegberg, Germany) combined with an isotope ratio mass spectrometer (Delta V advantage, Thermo Fisher Scientific, Bremen, Germany).

Calculations

Total tract digestibility of nutrients was calculated on the basis of quantitative data for feed intake and faeces and the analysed concentrations of crude nutrients as described by Boguhn *et al.* (2003). ME was calculated on the basis of digestible nutrients according to GfE (2001).

Gas production data were corrected for blank values (mean value of 3 syringes per incubation period, only rumen liquid-medium mixture) at each time of measurement. Considering the aspect that gas production primarily originates from OM fermentation and because diets differed in their OM content, the gas production data were standardised by expression per g of OM. To describe the course of cumulative gas production, the following modified Gompertz function according to Beuvink and Kogut (1993) was fitted to the data:

$$y = b \times \exp\left(-\frac{\mu_r}{D_r} \times \exp(-D_r \times t) - \frac{\mu_s}{D_s} \times \exp(-D_s \times t)\right) \quad (1)$$

with y = cumulative gas production at time t (ml/g OM); b = capacity of gas production (ml/g OM); μ_r = gas production rate of rapidly fermentable components (per h); D_r = fractional decay constants for μ_r; μ_s = gas production rate of slowly fermentable components (per h); D_s = fractional decay constants for μ_s; t = time after incubation started (h).

Model parameters were estimated for each syringe by using the software GraphPad Prism 5.01 for Windows (GraphPad Software Inc., La Jolla, San Diego, CA, USA). The rate of gas production was calculated by the first derivative of equation (1), and its maximal value (GP_{max}, ml/h) was reached at the point of inflection.

The extent of degradation of crude nutrients and detergent fibre fractions was calculated as the difference between the daily input and output of the respective nutrient in relation to its daily input. The amounts of NSC in feed and feed residues were calculated according to Van Soest *et al.* (1991). The daily amounts of N and OM in feed residues were corrected for the contribution of solid-associated microbes. Respective calculations, based on measurements of N and ^{15}N, followed Boguhn *et al.* (2006). CP from microbes was calculated as microbial N $\times$ 6.25.

Statistics

Data were statistically analysed using the MIXED procedure of the software package SAS for Windows (version 9.1.3, SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was performed for the two fixed effects of diet and particle size and their interaction. Incubation period was factored as a random effect into the statistical analysis. Variances were evaluated according to the method of Kenward and Roger (1997) for calculation of the degrees of freedom in mixed linear models. If significant interactions ($P < 0.05$) between the fixed effects were observed, the least square means of treatments F and C within a diet were additionally compared by *t*-test. Furthermore, the effect of replacing maize silage by grass silage was examined for linear and quadratic effects using the CONTRAST statement of SAS.

Results

Total tract digestibility in wether sheep

Diets were characterised by a linear increase in digestibility of OM, CP and CF with increasing proportion of grass silage ($P < 0.001$, Table 3). In contrast, the digestibility of EE decreased linearly with an increasing proportion of grass silage ($P < 0.001$). The content of ME was similar among all diets, with a mean value of 11.5 MJ/kg dry matter. Nevertheless, there was a slight linear decrease with increasing proportion of grass silage in the diet. No significant quadratic effects in response to changes in diet composition were observed for nutrient digestibility and ME content.

Gas production

The modified Gompertz function was adjusted to the gas production data with a high goodness of fit ($R^2 \geqslant 0.99$). The capacity of gas production (b) decreased linearly with increasing proportions of grass silage in the diet for both particle size treatments (Table 4). Values averaged 338, 333, 321, 311 and 300 ml/g OM for diets M100, M79, M52, M24 and M0, respectively. Values were, on average, higher in F treatments than in C treatments ($P = 0.03$). Although differences between the two particle sizes became nominally lower with higher proportions of grass silage, no significant interaction was detected ($P = 0.33$). The gas production rate of rapidly fermentable components (μ_r) and its fractional

Table 3 *Total tract digestibility of nutrients (%) in wether sheep and ME (MJ/kg DM) of experimental diets (means and pooled s.e.)*

							Effect of diet[3]	
Diet[1]	M100	M79	M52	M24	M0	s.e.	Linear	Quadratic
OM	79	80	81	81	84	0.68	***	0.11
CP	75	77	79	81	84	1.48	***	0.43
EE	84	76	75	63	61	1.04	***	0.83
CF	66	73	76	82	86	0.53	***	0.55
ME[2]	11.6	11.6	11.5	11.3	11.5	0.07	*	0.12

ME = metabolisable energy; DM = dry matter; OM = organic matter; EE = ether extract; CF = Crude fibre.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0).
[2]Calculated on the basis of digestible nutrients according to GfE (2001).
[3]Linear and quadratic effects in response to changes in maize silage to grass silage ratio.
$*P < 0.05$; $***P < 0.001$.

decay constant (D_f) were affected by diet and significantly higher at particle size F than at C across all diets. Interactions of diet and particle size were found for the gas production rate of slowly fermentable substrate (μ_s). Although μ_s was higher at particle size C than F for diets M52, M79 and M100, it was not significantly affected by particle size in diets M24 and M0. Maximal gas production rate (GP_{max}), on average, was highest for M100 (19 ml/h) and lowest for M24 and M0 (17 ml/h). GP_{max} was higher in F treatments than in C treatments for all diets. The point of GP_{max} was reached after approximately 4.3 h for diets M100, M79 and M52, but was detected 0.6 and 1.1 h later for diets M24 and M0, respectively. For all diets, the occurrence of GP_{max} was significantly later in C treatments compared with F treatments, at least 0.6 h for diet M100, and at most 1.4 h for diet M0. These differences between F and C treatments might explain the observed interactions for GP_{max} and its time of occurrence. In Figure 1, the development of gas production rates within 93 h of incubation is shown for these two diets. A delay of gas production in C treatments compared with F treatments is obvious for both diets. As shown in Table 4, significant quadratic effects were detected for the parameters μ_r, D_r, μ_s and D_s, as well as for the time of occurrence of GP_{max}. In contrast, the capacity of gas production (b) and GP_{max} only showed a significant linear response.

Rumen simulation

Degradation of OM, CF, NDF and ADF during 48 h of incubation in the Rusitec system was significantly affected by diet (Table 5). The stepwise replacement of maize silage by grass silage from diet M100 to diet M0 led to a linear increase in degradation of OM from about 0.40 to 0.46. This was accompanied by a linear increase in degradation of fibre fractions and NSC with increasing proportion of grass silage in the diet. Apparently unaffected by the diet, CP was degraded to an extent of 0.57. The degradation of OM was, on average, 2 percentage points higher in C treatments than in F treatments. This observation seems to result from the

Table 4 *Estimated parameters of the modified Gompertz function (equation (1)) and maximal gas production rates (means and pooled s.e.)*

															Effect of diet[4]					
	M100		M79		M52		M24		M0			*P*-values (ANOVA)			Linear		Quadratic			
Diet[1]																				
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet × PS	F	C	F	C		
b (ml/g OM)[2]	342	334	335	330	323	318	311	311	299	300	2.917	***	*	0.33	***	***	0.47	0.29		
μ_r (per h)[2]	0.82	0.60	0.72	0.54	0.73	0.49	0.63	0.45	0.70	0.50	0.017	***	***	0.24	***	***	**	***		
D_r^2	0.30	0.26	0.30	0.24	0.31	0.25	0.27	0.21	0.26	0.20	0.007	***	***	0.47	***	***	*	0.06		
μ_s (per h)[2]	0.039[b]	0.052[a]	0.044[b]	0.052[a]	0.048[b]	0.062[a]	0.048	0.045	0.035	0.029	0.003	***	*	**	0.74	***	***	***		
D_s^2	0.049	0.052	0.051	0.053	0.057	0.058	0.058	0.053	0.053[a]	0.046[b]	0.002	***	0.41	*	*	0.09	**	***		
GP_{max} (ml/h)[3]	22[a]	17[b]	20[a]	16[b]	21[a]	15[b]	18[a]	15[b]	19[a]	15[b]	0.250	***	***	***	***	***	0.20	0.08		
Time GP_{max} (h)	4.0[b]	4.6[a]	3.9[b]	4.8[a]	3.7[b]	4.7[a]	4.4[b]	5.4[a]	4.7[b]	6.1[a]	0.134	***	***	**	***	***	***	**		

PS = particle size; F = fine; C = coarse.
[1]Maize silage to grass silage ratio of 100:0 (M100), 79:21 (M79), 52:48 (M52), 24:76 (M24) and 0:100 (M0).
[2]Abbreviations according to equation (1).
[3]Maximal gas production rate.
[4]Linear and quadratic effects in response to changes in maize silage to grass silage ratio.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[a,b]In case of significant interaction: different superscripts indicate significant differences between particle sizes F and C within diets (*t*-test, $P < 0.05$).

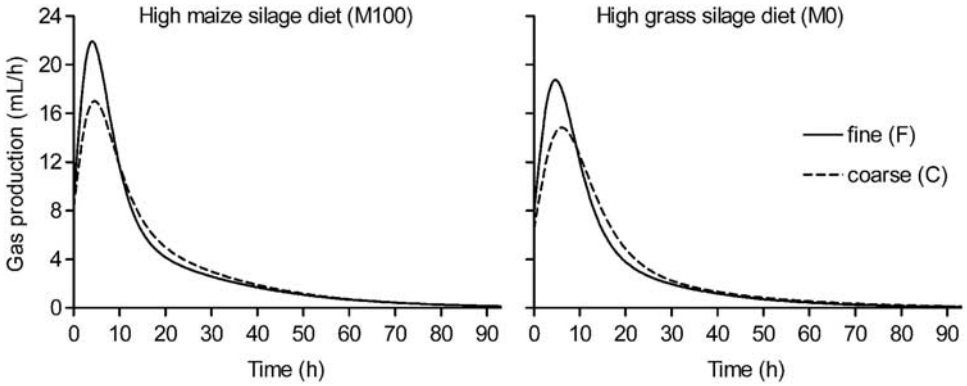

Figure 1 Development of gas production rates from 1 g organic matter of diet M100 and diet M0 at particle sizes F and C during 93 h of incubation.

Table 5 *Degradation of crude nutrients, detergent fibre fractions and non-structural carbohydrates during 48-h incubation in the Rusitec (%), daily outflow of NH_3-N (mg/day) and SCFA (mmol/day) measured in the effluents (means and pooled s.e.)*

															Effect of diet[4]			
Diet[1]	M100		M79		M52		M24		M0			*P*-values (ANOVA)			Linear		Quadratic	
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet x PS	F	C	F	C
OM_{corr}[2]	39.0	41.1	39.8	42.2	40.6	43.6	43.0	44.5	45.1	47.2	1.64	***	***	0.87	***	*	**	0.72
CP_{corr}[2]	54.3	57.9	56.0	60.8	52.6	58.0	56.8	58.2	56.3	57.3	1.88	0.53	***	0.25	0.28	0.41	0.19	0.54
CF	9.0	−0.6	15.1	12.2	12.4	8.1	15.2	13.9	13.3	17.2	2.94	***	*	0.07	0.18	**	0.23	0.51
NDF	4.7	2.5	10.3	9.4	13.7[a]	7.7[b]	24.3	25.5	23.3[b]	26.3[a]	2.32	***	0.99	*	***	***	0.92	0.37
ADF	−1.5	0.1	2.9	1.4	13.1	13.7	7.1	5.5	18.1	24.4	2.56	***	0.91	0.59	***	***	0.67	0.10
NSC	61.1	67.0	61.8	66.7	66.6	78.6	67.9	70.2	81.8	85.4	2.71	***	**	0.44	***	*	**	0.45
NH_3-N	51	54	77	83	81	96	111	128	117	135	3.31	***	***	0.23	***	***	0.32	0.10
Total SCFA	36.2	38.9	35.5	40.6	33.4	40.1	31.8	39.1	30.3	38.8	1.52	0.11	***	0.29	***	0.80	0.98	0.58
Acetate	15.2	17.6	15.1	18.5	15.7	18.8	15.0	18.8	14.4	19.5	0.92	0.83	***	0.44	0.35	0.24	0.33	0.87
Propionate	13.5	12.6	12.5	11.9	11.0	11.9	9.6	10.8	7.3	9.6	0.64	***	0.24	0.11	***	*	0.27	0.54
Iso-butyrate	0.21	0.27	0.27	0.38	0.29	0.44	0.43	0.57	0.52	0.61	0.02	***	***	0.13	***	***	***	0.57
Butyrate	3.39	4.00	3.49	4.78	3.03	4.72	3.51	4.63	5.38	5.74	0.24	***	***	0.09	***	**	***	0.60
Iso-valerate	1.94	2.39	1.30	1.81	1.50	1.97	0.89	1.30	1.14	1.55	0.13	***	***	0.93	***	***	0.25	0.06
Valerate	1.96	1.98	2.83	3.21	1.94	2.26	2.34	2.94	1.55	1.74	0.19	**	**	0.66	*	0.22	0.10	**
C2 : C3[3]	1.12	1.41	1.21	1.57	1.44	1.59	1.55	1.76	1.97	2.03	0.07	***	***	0.25	***	**	*	0.31

PS = particle size; F = fine; C = coarse; CF = Crude fibre; NSC = non-structural carbohydrates; SCFA = short-chain fatty acids.
[1]Maize silage to grass silage ratio of 100:0 (M100), 79:21 (M79), 52:48 (M52), 24:76 (M24) and 0:100 (M0).
[2]Corrected for contribution of solid-associated microbes.
[3]Acetate-to-propionate ratio.
[4]Linear and quadratic effects in response to changes in maize silage to grass silage ratio.
$*P < 0.05$; $**P < 0.01$; $***P < 0.001$.
[a,b]In case of significant interaction: different superscripts indicate significant differences between particle sizes F and C within diets (*t*-test, $P < 0.05$).

significantly higher degradation of the CP and NSC fractions in C treatments compared with F treatments, with 0.58 *v.* 0.55 and 0.74 *v.* 0.68, respectively. The effect of milling on degradation of fibre fractions was less clear. Coarse milling seemed to have a negative effect compared with fine milling at high maize silage proportions, whereas the opposite seems to be the case at high grass silage proportions. The trend for an interaction for CF degradation was also supported by the linear effect determined for C treatment but not for F treatment.

Higher proportions of grass silage in the diet resulted in a linearly increasing amount of NH_3-N in the daily effluents from Rusitec, with, on average, 52 mg/day for M100 and 126 mg/day for M0 (Table 5). The daily outflow of NH_3-N was higher in C treatments than in F treatments, with a minimum difference of about 3 mg and a difference of maximally 18 mg for M100 and M0, respectively. No main effect of diet on the amount of SCFA was detected by ANOVA ($P = 0.11$). Nevertheless, the test for linear effects showed a decrease in SCFA production with increasing proportion of grass silage within F treatments (Table 5). The daily outflow of the individual SCFA was significantly affected by diet, with the exception of acetate. With a higher proportion of grass silage in the diet, the daily amounts of iso-butyrate and butyrate

increased, and those of propionate and iso-valerate decreased linearly. The response in iso-butyrate and butyrate also showed a quadratic effect, but only within the F treatments. Compared with F treatments, C treatments had a higher production of total and individual SCFA, except for propionate. Consequently, the acetate-to-propionate ratio was significantly affected by maize silage to grass silage ratio and particle size. Molar proportions of acetate, iso-butyrate and butyrate increased from diet M100 to diet M0, whereas the proportion of propionate and iso-valerate decreased (data not shown). Compared to fine milled diets, C treatments had an increasing effect on the molar proportions of acetate, iso-butyrate and butyrate but led to reductions in the proportion of propionate.

Discussion

Effect of maize silage to grass silage ratio

It was hypothesised that maize silage and grass silage might affect each other in their characteristics of fermentation when both are incubated together *in vitro* at several ratios. In general, this hypothesis could not be confirmed. Although some quadratic effects on parameters of the estimated functions were detected, the combination of maize silage and grass silage (M79, M52 and M24) showed no associative effect on the capacity of gas production (Figure 2). The differences between diet M100 and diet M0 are in agreement with the results of García-Rodriguez *et al.* (2005), who found a mean difference of 24% in the cumulative gas production between maize silages and grass silages after 96 h of incubation. Some associative effects of forage mixtures, compared with single feedstuff incubation, were reported by Robinson *et al.* (2009), but only for the first 8 h of incubation. This could be confirmed by the present results when performing some calculations with the estimated parameters (equation (1)). During the first 4 h of incubation, cumulative gas production was the highest for both F and C treatments of diet M52, but after 8 h the ranking of diets was equal to the capacity of gas production and did not change anymore (quadratic effect at 2 h: $P < 0.001$, 4 h: $P = 0.01$ and 8 h: $P = 0.32$). Consequently, the presence of associative effects depends on the duration of incubation.

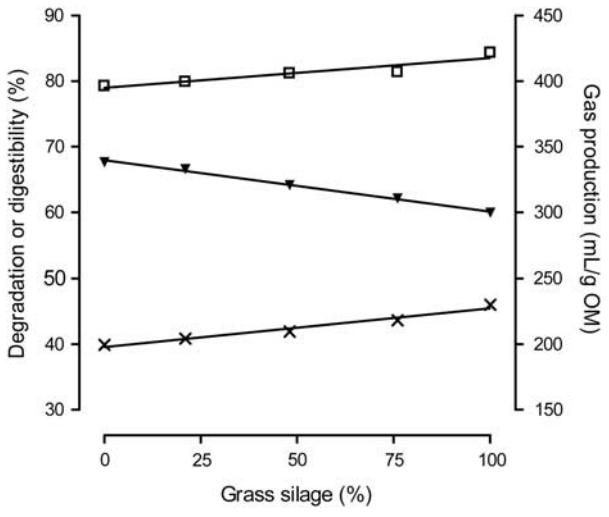

Figure 2 Linear responses of organic matter (OM) digestibility in wether sheep (□; $y = 0.045x + 78.98$; $R^2 = 0.68$), OM degradation in the Rusitec (×; $y = 0.059x + 39.56$; $R^2 = 0.33$) and capacity of gas production (▼; $y = -0.394x + 339.8$; $R^2 = 0.70$) to the replacement of maize silage by grass silage in the forage portion of the diet.

Increasing the proportion of grass silage in the diet resulted in an increase in degradation of OM (Figure 2). However, the amount of apparently degraded carbohydrates (NDF + NSC, without consideration of microbial carbohydrates) was higher in high maize silage diets than in high grass silage diets (4.2 *v.* 3.8 g in diets M100 and M0, respectively). This was confirmed by the measured amounts of total SCFA, which averaged 37, 38, 36, 35 and 34 mmol/day for diets M100, M79, M52, M24 and M0, respectively. These results are in agreement with the quantitative gas production data, and *in vitro* gas production arises to a greater extent from NSC fermentation than CP fermentation (Menke and Steingass, 1988). The amount of degraded CP increased with increasing proportion of grass silage (1.0 *v.* 1.8 g in diets M100 and M0, respectively), which contributed to the degradability of OM in the Rusitec. However, the degradability of CP was not affected by diet, probably because the solubility of CP in grass silage and maize silage is similar (Givens and Rulquin, 2004). In addition, differences in the availability of N sources between diets were indicated by changes in iso-acid production (Griswold *et al.*, 2003).

Differences in fibre degradation between diets, incubated in the Rusitec system, were in accordance with the acetate-to-propionate ratio, increasing from high maize silage to high grass silage diets. However, the general level of fibre degradation was very low and close to zero for ADF for diet M100. Microbial enzyme activity and not the fibre itself limits the rate of fibre degradation (Wallace *et al.*, 2001). High dietary contents of NSC, such as maize starch, are often associated with a decrease in ruminal pH value and consequently a reduction in cellulolytic activity, but this effect is marginal when the pH value does not fall below 6.2 (Huhtanen *et al.*, 2006). *In vitro* conditions allow the pH value to be maintained at a constant level, which in this study was around 6.6 immediately before daily feeding. A negative effect of maize starch addition on the potential extent of NDF digestion (Grant and Mertens, 1992) and lag time of fibre digestion (Mertens and Loften, 1980) was observed, although the pH was maintained at 6.8 *in vitro*. In conclusion, the presence of starch might have had a negative effect on microbial fibrolytic activity in the present Rusitec study. However, the increase in fibre degradation with inclusion of grass silage in the diet is in accordance with the digestible CF determined in wether sheep (Table 3), indicating a higher content of fermentable fibre from grass silage. Linear responses in the total tract digestibility (Table 3 and Figure 2) of nutrients are also reported by Browne *et al.* (2005) and Juniper *et al.* (2008) when the ratio of maize silage to grass silage was changed. However, the occurrence of associative effects is dependent on the quality of grass

silage (Vranić *et al*., 2008). The quality of grass silage can greatly vary between single batches and the digestibility of OM is positively related ($P < 0.001$) to CP concentration (Yan and Agnew, 2004). Contrary results from studies concerning maize silage to grass silage ratio may arise because of the high variations in the chemical composition of silages. Therefore, the results of this study do not allow for conclusions on these types of diet *per se*. The content of CP and fibre fractions in forages, as well as their ruminal fermentation characteristics, especially seems to play an important role in the occurrence of associative effects (Niderkorn and Baumont, 2009).

Effect of particle size

Our second objective was to identify the effect of milling on the characteristics of fermentation *in vitro.* The use of milling sieves with either a 1- or 4-mm perforation showed apparently contrary results between the two *in vitro* methods used. Gas production was higher in F treatments than in C treatments, but fermentation benefited in the Rusitec system when diets were milled at 4 mm than at 1 mm perforation.

Particle size distribution differs between feedstuffs also when the same sieve is used (Michalet-Doreau and Cerneau, 1991; Bossen *et al*., 2008). Owing to the agglomeration of fine particles in high maize silage diets (M100 and M79), particle size distribution could not be measured by photo-optical particle analysis in this study. As shown by Bossen *et al*. (2008), who used a sieve shaker particle separator system, the mean particle size was higher when maize silage and grass silage were milled at 4-mm than at 1-mm perforation. The mean particle size was similar between both silages, which is also in accordance with the measurements in bulk density of this study. In routine use of the gas production test, feedstuffs are milled at 1-mm perforation (Menke and Steingass, 1988). As expected, C treatments showed a decreasing effect on cumulative gas production and gas production rate during the early stages of fermentation compared with F treatments (Menke and Steingass, 1988). Nutrient degradation was not measured in the present gas production study. However, other batch culture studies showed that the degradation of NDF can be reduced by an increase in the mean particle size, with this effect being dependent on the kind of incubated forage (Robles *et al*., 1980; Bossen *et al*. 2008). Furthermore, differences in the rate of NDF degradation are much higher between different forages than within the different batches of the same forage (Robles *et al*., 1980).

As known from *in situ* studies, particle losses through the pores of nylon bags increase with the fineness of milling (Michalet-Doreau and Cerneau, 1991). Surprisingly, the disappearance of OM and CP was higher at the coarse milled treatments in this Rusitec study. The increased degradation of OM was accompanied by an increase in the production of total SCFA and in the acetate-to-propionate ratio, indicating an increase in fibrolytic activity. However, the effect of the particle size on fibre degradation was inconsistent in this study. The large feed particles generated because of mechanical crushing rather originated from the stem fraction than from the leaf fraction (Kennedy and Doyle, 1993). Rodríguez-Prado *et al*. (2004) used dual-flow continuous culture fermenters and varied the particle size of the stem fraction from alfalfa hay in the diet. According to the present study, no significant differences in the degradation of NDF were found. An increased production of acetate, as well as a higher acetate-to-propionate ratio at a large particle size ($\geqslant$3 mm) compared with a small particle size ($\leqslant$1 mm), was also described by Rodríguez-Prado *et al*. (2004). It can be assumed that large particles benefit cellulolytic microorganisms, which is the reason why the acetate proportion was increased in this study. Zhang *et al*. (2007) evaluated the contribution of various ruminal microbial groups (bacteria, protozoa, fungi) to the fermentation of cell walls extracted from corn stover with two different particle sizes, coarse and fine. Gas production and degradability after 72 h was always higher for fine milled samples in the presence of bacteria (alone and in mixtures with fungi or protozoa), but was higher for coarse ground samples in the presence of fungi alone. Akin (1993) suggested that increased particle size may benefit the penetration of particles by fungi and consequently the degradation of fibre by respective bacteria. In accordance with the results of Bowman and Firkins (1993), interactions between forage source and feed particle size were rare in this study. Some interactions were found for the parameters of gas production; however, particle size did not affect the general conclusions on the effect of diet at all. Interactions found for the Rusitec study concerning fibre degradation and SCFA production and the kind of effect in response to replacing maize silage by grass silage seemed to be dependent on feed particle size for a few of these *in vitro* characteristics.

Conclusions

A stepwise replacement of maize silage by grass silage induces an increase in degradability of fibre and OM in a rumen simulation. The associative effects of both forages were marginal for the used feed evaluation methods. However, results might be dependent on the chemical composition of the used silages.

Milling silage-based diets at the 4-mm sieve size seem to benefit fermentation processes in the Rusitec system, compared with fine milling at the 1-mm sieve size. A negative effect of coarse milling on fermentation in the Hohenheim gas production test was confirmed. All these observations underline the importance of using standardised milling sieves in feed evaluation methods. The effect of the particle size should be investigated further to make it easier to compare studies using rumen simulations.

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References

Akin DE 1993. Perspectives of cell wall biodegradation. In Forage cell wall structure and digestibility (ed. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 73–82. American Society of Agronomy Inc., Crop Science Society of America, Inc., Soil Science Society of America Inc., Madison, WI, USA.

Beuvink JMW and Kogut J 1993. Modeling gas production kinetics of grass silages incubated with buffered ruminal fluid. Journal of Animal Science 71, 1041–1046.

Boguhn J, Kluth H and Rodehutscord M 2006. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. Journal of Dairy Science 89, 1580–1591.

Boguhn J, Kluth H, Steinhöfel O, Peterhänsel M and Rodehutscord M 2003. Nutrient digestibility and prediction of metabolizable energy in total mixed rations for ruminants. Archives of Animal Nutrition 57, 253–266.

Bossen D, Mertens DR and Weisbjerg MR 2008. Influence of fermentation methods on neutral detergent fiber degradation parameters. Journal of Dairy Science 91, 1464–1476.

Bowman JGP and Firkins JL 1993. Effects of forage species and particle size on bacterial cellulolytic activity and colonization in situ. Journal of Animal Science 71, 1623–1633.

Browne EM, Juniper DT, Bryant MJ and Beever DE 2005. Apparent digestibility and nitrogen utilisation of diets based on maize and grass silage fed to beef steers. Animal Feed Science and Technology 119, 55–68.

Carro MD and Miller EL 2002. Comparison of microbial markers (^{15}N and purine bases) and bacterial isolates for the estimation of rumen microbial protein synthesis. Animal Science 75, 315–321.

Czerkawski JW and Breckenridge G 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371–384.

García-Rodriguez A, Mandaluniz N, Flores G and Oregui LM 2005. A gas production technique as a tool to predict organic matter digestibility of grass and maize silage. Animal Feed Science and Technology 123–124, 267–276.

Geissler C, Hoffmann M and Hickel B 1976. Ein Beitrag zur gaschromatographischen Bestimmung flüchtiger Fettsäuren. Archiv für Tierernährung 26, 123–129.

Gesellschaft für Ernährungsphysiologie 1991. Leitlinien für die Bestimmung der Verdaulichkeit von Rohnährstoffen an Wiederkäuern. Journal of Animal Physiology and Animal Nutrition 65, 229–234.

Gesellschaft für Ernährungsphysiologie 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG-Verlag, Frankfurt am Main, Germany.

Givens DI and Rulquin H 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Animal Feed Science and Technology 114, 1–18.

Grant RJ and Mertens DR 1992. Influence of buffer pH and raw corn starch addition on in vitro fiber digestion kinetics. Journal of Dairy Science 75, 2762–2768.

Griswold KE, Apgar GA, Bouton J and Firkins JL 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. Journal of Animal Science 81, 329–336.

Hoover WH and Stokes SR 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield. Journal of Dairy Science 74, 3630–3644.

Huhtanen P, Ahvenjärvi S, Weisbjerg MR and Nørgaard P 2006. Digestion and passage of fibre in ruminants. In Ruminant physiology – digestion, metabolism and impact of nutrition on gene expression, immunology and stress (ed. K Sejrsen, T Hvelplund and MO Nielsen), pp. 87–135. Wageningen Academic Publishers, Wageningen, The Netherlands.

Juniper DT, Browne EM, Bryant MJ and Beever DE 2008. Digestion, rumen fermentation and circulating concentrations of insulin, growth hormone and IGF-1 in steers fed diets based on different proportions of maize silage and grass silage. Animal 2, 849–858.

Kennedy PM and Doyle PT 1993. Particle-size reduction by ruminants – effects of cell wall composition and structure. In Forage cell wall structure and digestibility (ed. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 499–534. American Society of Agronomy Inc., Crop Science Society of America Inc., Soil Science Society of America Inc., Madison, WI, USA.

Kenward MG and Roger JH 1997. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53, 983–997.

McDougall EI 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochemical Journal 43, 99–109.

Menke KH and Steingass H 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Animal Research and Development 28, 7–55.

Menke KH, Raab L, Salewski A, Steingass H, Fritz D and Schneider W 1979. The estimation of the digestibility and metabolizable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. Journal of Agricultural Science 93, 217–222.

Mertens DR and Loften JR 1980. The effect of starch on forage fiber digestion kinetics in vitro. Journal of Dairy Science 63, 1437–1446.

Michalet-Doreau B and Cerneau P 1991. Influence of foodstuff particle size on in situ degradation of nitrogen in the rumen. Animal Feed Science and Technology 35, 69–81.

Niderkorn V and Baumont R 2009. Associative effects between forages on feed intake and digestion in ruminants. Animal 3, 951–960.

Robinson PH, Getachew G and Cone JW 2009. Evaluation of the extent of associative effects of two groups of four feeds using an *in vitro* gas production procedure. Animal Feed Science and Technology 150, 9–17.

Robles AY, Belyea RL, Martz FA and Weiss MF 1980. Effect of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchardgrass forages. Journal of Animal Science 51, 783–790.

Rodríguez-Prado M, Calsamiglia S and Ferret A 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. Journal of Dairy Science 87, 1413–1424.

Stern MD, Bach A and Calsamiglia S 1997. Alternative techniques for measuring nutrient digestion in ruminants. Journal of Animal Science 75, 2256–2276.

Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) 2006. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Vranić M, Knežević M, Bošnjak K, Leto J, Perčulija G and Matić I 2008. Effects of replacing grass silage harvested at two maturity stages with maize silage in the ration upon the intake, digestibility and N retention in wether sheep. Livestock Science 114, 84–92.

Wallace RJ, Wallace SJ, McKain N, Nsereko VL and Hartnell GF 2001. Influence of supplementary fibrolytic enzymes on the fermentation of corn and grass silages by mixed ruminal microorganisms in vitro. Journal of Animal Science 79, 1905–1916.

Yan T and Agnew RE 2004. Prediction of nutritive values in grass silages: I. Nutrient digestibility and energy concentrations using nutrient compositions and fermentation characteristics. Journal of Animal Science 82, 1367–1379.

Zhang Y, Gao W and Meng Q 2007. Fermentation of plant cell walls by ruminal bacteria, protozoa and fungi and their interaction with fibre particle size. Archives of Animal Nutrition 61, 114–125.

Paper II

Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation

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Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation

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The objective was to investigate the effect of variation in forage source and feed particle size of a diet, including interactions, on the amount and the composition of microbial crude protein (CP) in a semi-continuous culture system (Rusitec). Different microbial CP fractions were compared. Five diets with mean forage proportion of 0.88 and different maize silage to grass silage ratios (100 : 0, 79 : 21, 52 : 48, 24 : 76 and 0 : 100) were used. Diets were ground through sieves with a pore size of either 1 or 4 mm, matching the particle size of fine (F) and coarse (C), respectively. Diets were characterised by increasing concentrations of CP and fibre fractions, and decreasing concentrations of starch with ascending inclusion rates of grass silage. Microbial mass was isolated from feed residues after incubation from the liquid phase of the fermenter and from the liquid effluent. The amount of synthesised microbial CP was determined on the basis of ^{15}N balance. It increased quite linearly by the stepwise replacement of maize silage by grass silage, and was higher in C treatments compared to F treatments. Efficiency of microbial CP synthesis (EMPS) was improved from 29 to 43 mg microbial N/g degraded organic matter (OM) by increasing the proportion of grass silage in the diet, but was unaffected by particle size. The N content as well as the profiles of amino acids of the three microbial fractions was affected by diet composition and particle size. The ratio of solid- to liquid-associated microbes was affected by diet composition and feed particle size. The amount and EMPS seemed to be improved by degradation of OM from grass silage and an increasing availability of N. Moreover, the results of this study indicated a shift in the composition of the microbial community caused by variation in forage composition and feed particle size.

Keywords: silage, particle size, microbial protein, amino acid profile, Rusitec

Implications

This study suggests that grass silage promotes rumen microbial growth more than maize silage when both forages are incubated under the same conditions in a rumen simulation technique (Rusitec). The changes in composition and contribution of microbial fractions give indications to a shift in microbial community as a result of variation of silage type and feed particle size. A standardisation in the milling of feedstuffs would improve comparability of Rusitec studies. Further research in identifying the accountability of dietary factors, for example, the amount and type of crude protein, on microbial protein synthesis in continuous culture systems is needed.

Introduction

Microbial protein leaving the rumen is the most important source of amino acids (AA) to meet the requirements for maintenance and performance in ruminants. The amount and efficiency of microbial CP synthesis (EMPS) depends on the availability of energy and N in favour of the microbial growth (Clark *et al.*, 1992; Stern *et al.*, 1994). Variable proportions of feedstuffs in the diet alter the availability of carbohydrates and CP to microbial protein synthesis. As shown by Boguhn *et al.* (2006a and 2006b), maize silage and grass silage in total mixed rations seemed to affect the EMPS, as well as the microbial AA profile *in vitro* (rumen simulation technique (Rusitec)). Both forages differ in their nutrient composition. Grass forages typically contain no starch, and maize silages are known for lower concentrations of CP and fibre fractions than grass silages. Therefore, it is assumed that a combination of both forages could promote microbial CP synthesis in terms of synchronisation of available energy and N.

The use of rumen simulations allows the quantification of daily microbial N yield with less effort and under more constant conditions than *in vivo* (Stern *et al.*, 1994). The feedstuffs are usually reduced in particle size by milling or cutting

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before incubation. Those changes in physical characteristics also affect the availability of nutrients, both in the amount and in the course over time (Michalet-Doreau and Ould-Bah, 1992). A variation in feed particle size (1 *v.* 3 mm sieve size) affected ruminal N metabolism in continuous culture fermenters, but the effects depended on the microbial fraction that was analysed (Rodríguez-Prado *et al.*, 2004). These authors supported the concept that the origin of the microbial samples is very important for the estimation of the microbial AA amount. Differences in the AA profile between and within the microbial fractions isolated from the solid and the liquid phases of a Rusitec were found when the availability of nutrients was varied (Boguhn *et al.*, 2006b). The effect of feed particle size as an additional dietary factor on the synthesis of microbial CP and AA profile of microbial fractions in the Rusitec has not been clarified yet.

The first objective of this study was to identify the maize silage to grass silage ratio at which the maximum amount and EMPS occurs, and in which way the variation in particle size plays a role in a Rusitec system. We hypothesised that a combination of maize silage and grass silage benefits microbial N yield and EMPS to a higher extent than using either forage source separately. Furthermore, it should be clarified to what extent microbial fractions differ in their AA profile and their contribution to AA yield in response to variations in forage source and particle size distribution in the diet. As both dietary factors influence the availability of nutrients, interactions between diet and particle size on microbial CP synthesis might occur.

Material and methods

Treatments

Maize silage and grass silage used in this study originated from a dairy cow farm near Halle upon Saale, Germany. The dry matter content was 350 g/kg (maize silage) and 380 g/kg (grass silage), and the pH values 3.72 and 4.21, respectively. The concentrations of acetic acid, propionic acid, butyric acid and lactic acid were 25.1, 0.6, 0.3 and 56.6 g/kg dry matter for maize silage and 6.2, 0.3, 0.3 and 92.2 g/kg dry matter for grass silage. Ammonia-N content was 116 mg/g total N in maize silage and 43 mg/g total N in grass silage. Five diets were composed to match a forage proportion of about 0.88 in the diet (Table 1). The diets, namely M100, M79, M52, M24 and M0 were characterised by maize silage to grass silage ratios of 100 : 0, 79 : 21, 52 : 48, 24 : 76 and 0 : 100, respectively. Soyabean meal was included at a proportion of approximately 0.11 across all diets in order to reach a minimum CP concentration of 130 g/kg dry matter. The stepwise replacement of maize silage by grass silage from diet M100 to diet M0 was accompanied by an increase in CP concentration of up to 231 g/kg dry matter in diet M0, and by an increase in fibre fractions (crude fibre, NDF and ADF). The maize silage contained 323 g starch per kg dry matter, and a change in dietary maize starch content proportional to the inclusion rate of maize silage was assumed. Differences in starch content were also reflected by the calculated fraction of non-structural carbohydrates (NSC; Table 1). In order to realise a variation in particle size distribution, the oven-dried diets (65°C) were ground through sieves with a pore size of 1 or 4 mm (cutting mill, Pulverisette 15, Fritsch GmbH, Idar-Oberstein, Germany). Hence, each diet was proofed at fine (F) and coarse (C) particle size.

Table 1 *Composition of experimental diets (g/kg DM)*

Diet	M100	M79	M52	M24	M0
Ingredient composition					
Maize silage	887	697	457	215	–
Grass silage	–	188	427	664	877
Soyabean meal	102	104	104	109	111
Mineral and vitamin premix	11	11	12	12	12
Chemical composition					
DM (g/kg)	936	933	934	926	942
OM	943	933	907	887	873
CP	130	153	178	204	231
EE	33	33	34	34	35
CF	177	195	199	209	222
NDF	338	352	381	406	410
ADF	203	213	224	223	239
NSC[1]	454	397	338	243	209

DM = dry matter; OM = organic matter; EE = ether extract; CF = crude fibre; NSC = non-structural carbohydrates.
[1]NSC = 1000 − NDF − CP − EE − ash (Van Soest *et al.*, 1991).

Rumen simulation

The *in vitro* procedure, using a semi-continuous Rusitec (Czerkawski and Breckenridge, 1977) is described in more detail in a companion study (Hildebrand *et al.*, 2010) and in general followed the procedures as described by Boguhn *et al.* (2006a). In brief, six fermenters, with a volume of 800 ml each, were used per period. One incubation period lasted for 13 days. Randomised across seven periods, F and C treatments were tested in four and three replications per diet, respectively. The ruminal inoculum was obtained from five wether sheeps that were fed hay *ad libitum* and 250 g of a concentrate feed per day. Each fermenter contained two nylon bags of 100 μm pore size, which were filled with 15 g of the respective diet. Every 24 h, one bag was replaced by a new one, and therefore each bag was incubated for 48 h. The continuously infused buffer solution (546 ml/day, s.d. = 29) contained 0.7 mmol ammonium per litre from $^{15}NH_4Cl$ (115 μg ^{15}N/mg N) to label the N pool and quantify the microbial protein synthesis. Liquid effluents were collected in cooled bottles (4°C).

Sampling procedure

The total amount of effluent was quantified daily from days 7 to 13, assuming that the plateau of ^{15}N enrichment in bacteria and thus a steady state was reached within 6 days after starting the ^{15}N infusion (Boguhn *et al.*, 2006a). A daily subsample of 320 ml effluent was centrifuged twice for 5 min at 2000 × **g** and at 4°C to separate feed particles.

Liquid-associated microbes in the effluent (LAM-E) were obtained by differential centrifugation as described by Boguhn *et al.* (2006a). Briefly, particle-free effluents were centrifuged three times at 27 000 × **g** and at 4°C for 15 min. After the first centrifugation step, 40 ml of supernatant was taken for all 7 days and pooled per fermenter for storage at −20°C. Subsequent to the first and second centrifugations, the microbial pellet was re-suspended in saline solution (9 g NaCl/l). After the third centrifugation step, the pellet was conveyed into plastic tubes for storage.

Feed residues in nylon bags were taken daily from days 7 to 12, washed in 50 ml of buffer solution, squeezed moderately, dried at 65°C, weighed and pooled per fermenter. On the last day of incubation, the two bags per fermenter were evacuated to isolate solid-associated microbes (SAM) according to the method described by Carro and Miller (2002). The microbes were separated from the remaining fluid and further treated as described by Boguhn *et al.* (2006a). In brief, the bags were squeezed moderately and incubated in a saline solution of methylcellulose (1 g methylcellulose + 9 g NaCl/l distilled water), about 30 min at 39°C and thereafter about 6 h at 4°C. After washing and removing the bags, the remaining suspension was centrifuged stepwise, as described for the LAM-E.

The entire liquid content of the fermenter vessel at the end of the incubation period, including the suspension of the washing step from feed residues was used to obtain a further microbial fraction, the LAM in the fermenter (LAM-F). The procedure of obtaining the LAM-F was equal to isolating the LAM-E fraction as described before.

Microbial isolates of fractions LAM-E, SAM and LAM-F were frozen at −20°C immediately after the last centrifugation step. After freeze-drying, all microbial pellets were ground by mortar and pestle and daily samples of the LAM-E fraction were pooled per fermenter.

Analyses

Crude nutrients, fibre fractions and starch in feed and feed residues were analysed according to the official methods in Germany (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA, 2006), and specifications as described in the companion study (Hildebrand *et al.*, 2010). Supernatants obtained during LAM-E isolation were analysed for NH_3-N (Hildebrand *et al.*, 2010). Finely ground samples of $^{15}NH_4Cl$, feed, feed residues, freeze-dried supernatants and microbial isolates were filled into tin capsules and delivered to an elemental analyser (EuroEA, HEKAtech GmbH, Wegberg, Germany) combined with an isotope ratio mass spectrometer (Delta V advantage, Thermo Fisher Scientific, Bremen, Germany). Concentrations of N and ^{15}N were determined in triplicate per sample. AA were analysed in 100 mg of the freeze-dried microbial mass of SAM, LAM-F and LAM-E. Analysis followed standard procedures (method 4.11.1; VDLUFA, 2006) with laboratory details as described by Rodehutscord *et al.* (2004). In brief, after a performic acid oxidation step, samples were hydrolysed in 6 *N* HCl. Norleucine was used as an internal standard. Tryptophan, histidine and tyrosine were not determined. AA were separated and detected by an AA analyser (Biochrom 30, Biochrom Ltd, Cambridge, UK), using various citrate buffer solutions and ninhydrin. Absorbance was determined at 570 nm, with the exception of proline, which was quantified from absorbance at 440 nm. AA analysis was run in duplicate.

Calculations

The daily amount of microbial N originating from the LAM-E fraction ($N_{LAM\text{-}E}$, mg/day), was calculated as:

$$N_{LAM\text{-}E} = \frac{^{15}N_{in} - {}^{15}N_{out}}{^{15}N_{LAM\text{-}E}} \quad (1)$$

with $^{15}N_{in}$ (μg/day) defined as the sum of daily input of ^{15}N via buffer solution and feed. The daily output of ^{15}N ($^{15}N_{out}$, μg/day) was calculated by summarising the amounts of ^{15}N in NH_3-N and feed residues, including SAM. The daily input of ^{15}N was assumed to be equal to the daily ^{15}N output. N losses by evaporation of ammonia were assumed to be negligibly low. The difference between $^{15}N_{in}$ and $^{15}N_{out}$ was attributed to the LAM-E fraction and therefore related to the proportion of ^{15}N in $N_{LAM\text{-}E}$ ($^{15}N_{LAM\text{-}E}$, μg/mg).

Feed residues were defined as the sum of SAM and undegraded feed. The daily amount of N originating from the SAM fraction (N_{SAM}, mg/day) was determined as:

$$N_{SAM} = N_{FR} \times \frac{^{15}N_{FR} - {}^{15}N_{Diet}}{^{15}N_{SAM} - 3.66} \quad (2)$$

with the amount of N in feed residues (N_{FR}, mg/day), the proportion of ^{15}N in N_{FR} ($^{15}N_{FR}$, μg/mg), the proportion of ^{15}N in dietary N ($^{15}N_{Diet}$, μg/mg), the proportion of ^{15}N in N_{SAM} ($^{15}N_{SAM}$, μg/mg) and the assumed natural abundance of ^{15}N in unlabelled N_{SAM} (3.66 μg/mg).

For the calculation of EMPS, the daily outflow of microbial N, as the sum of N_{SAM} and $N_{LAM\text{-}E}$ was related to the amount of degraded organic matter (OM). The degradation of OM was calculated as the difference between daily input and output of OM in relation to its daily input. The amount of OM in feed residues was corrected for the contribution of SAM, according to Boguhn *et al.* (2006a). The efficiency of microbial N utilisation was calculated as the ratio of daily microbial N and available N, determined as the sum of degraded dietary N and N from buffer solution (Bach *et al.*, 2005).

The concentrations of individual AA were expressed as percentage of analysed AA. The proportion of analysed amino acid N (AA-N) in the total N of the microbial fractions was calculated by consideration of the molar proportion of N in individual AA.

Statistics

Data were analysed using the MIXED procedure of the software package SAS for windows (version 9.1.3, SAS Institute, Cary, NC, USA). Analysis of variance was performed for the fixed effects of diet and particle size and their interaction. The effect of period was factored as random into

Table 2 *N in microbial isolates, proportion of ^{15}N and AA-N (means and pooled s.e.)*

Diet[1]	M100		M79		M52		M24		M0			*P*-values		
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet × PS
N (mg/g freeze-dried matter)														
SAM	79^{B}	82^{B}	81^{B}	81^{C}	85^{C}	88^{B}	85^{C}	81^{C}	90^{C}	90^{B}	1.6	***	0.95	0.14
LAM-F	86^{A}	86^{B}	87^{A}	88^{B}	88^{B}	91^{B}	91^{B}	92^{B}	95^{B}	94^{AB}	1.5	***	0.61	0.75
LAM-E	91^{A}	94^{A}	88^{bA}	92^{aA}	95^{A}	98^{A}	94^{A}	96^{A}	100^{aA}	98^{bA}	1.1	***	0.11	*
^{15}N (μg/mg N)														
SAM	5.23^{C}	5.64^{C}	4.55^{C}	4.78^{C}	4.50^{C}	4.80^{C}	4.14^{C}	4.30^{C}	4.20^{C}	4.38^{C}	0.06	***	***	0.12
LAM-F	6.10^{B}	6.17^{B}	5.12^{B}	5.20^{B}	5.28^{B}	5.26^{B}	4.75^{B}	4.58^{B}	4.87^{B}	4.71^{B}	0.07	***	**	0.10
LAM-E	7.18^{A}	6.81^{A}	6.15^{A}	5.75^{A}	6.26^{A}	5.56^{A}	5.41^{A}	5.08^{A}	5.53^{A}	5.04^{A}	0.12	***	***	0.23
AA-N (mg/g N)[2]														
SAM	656^{B}	662	667	665	663	653	669	680^{A}	668^{A}	655	6.0	0.50	0.85	0.23
LAM-F	656^{B}	667	655	669	665	660	652	658^{B}	644^{B}	648	7.5	0.08	0.17	0.59
LAM-E	679^{A}	668	681	680	675	657	669	658^{B}	660^{AB}	661	4.5	***	***	0.09

AA-N = amino acids-N; PS = particle size; F = fine; C = coarse; SAM = solid-associated microbes from feed residues; LAM-F = liquid-associated microbes from fermenter; LAM-E = liquid-associated microbes from effluent.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0).
[2]On the basis of the 15 analysed AA.
[a,b]In case of significant interaction between diet and PS: different superscripts indicate significant differences between particle size F and C within diets (Student's *t*-test, $P < 0.05$).
[A,B,C]Different superscripts indicate significant differences between microbial fractions within treatments (Student's *t*-test, $P < 0.05$).
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

statistical analysis. Degrees of freedom were adjusted by the method of Kenward and Roger (1997). In case of significant interactions ($P < 0.05$) between diet and particle size, the least square means of treatments F and C within a diet were compared by Student's *t*-test. Measurements on chemical composition were compared between microbial fractions within each treatment by Student's *t*-test.

Results

Composition of microbial fractions

The three microbial fractions SAM, LAM-F and LAM-E differed in their content of N ($P < 0.01$) that averaged 84, 90 and 94 mg/g freeze-dried matter, respectively (Table 2). The LAM-E contained significantly more N than the SAM. The diet significantly affected the N concentration, which improved with increasing inclusion of grass silage in the diet. Particle size had no effect on the N concentration of the microbial fractions, but there was an interaction between diet and particle size for the LAM-E isolates. The proportion of ^{15}N in microbial N differed between microbial fractions ($P < 0.01$) by mean values of 4.6, 5.2 and 5.9 μg/mg N for the SAM, LAM-F and LAM-E, respectively. The ^{15}N ratio in total N decreased in order of LAM-E, LAM-F and SAM in all treatments. The incorporation of ^{15}N changed with the maize silage to grass silage ratio and was highest in diet M100 and lowest in diets M24 and M0 across all microbial fractions. ^{15}N content was also affected by particle size in all microbial fractions. For SAM, it was higher in C treatments than in F treatments. In contrast, ^{15}N in the LAM-E fraction was higher for particle size F compared to particle size C.

The concentration of AA-N in microbial N averaged 664, 657 and 669 mg/g N for SAM, LAM-F and LAM-E fractions, respectively (Table 2). The proportion of AA-N was significantly affected by diet and particle size only in the LAM-E fraction. AA-N was highest for diet M79 (680 mg/g N) and lowest for diet M0 (660 mg/g N), as well as higher in F treatments than in C treatments. Significant differences between microbial fractions in AA-N were detected only for three of the 10 treatments.

The proportion of individual AA analysed in the SAM, LAM-F and LAM-E fractions are presented in Table 3. Generally, changing the grass silage proportion changed the proportion of individual AA within the microbial mass. Maize silage to grass silage ratio affected the proportion of alanine, arginine, aspartic acid, cystine, glutamic acid, leucine and methionine in the SAM fraction; the proportion of alanine, lysine, methionine, phenylalanine and proline in the LAM-F fraction; and the proportion of alanine, arginine, leucine, lysine, methionine, phenylalanine and threonine in the LAM-E fraction significantly. Higher proportions of grass silage in the diet led to higher contents of arginine and phenylalanine, whereas those of lysine and threonine decreased within the respective microbial fractions. However, the change in AA profile in response to diet variations differed between microbial fractions. Interactions between diet and microbial fraction were observed. For example, the contents of leucine and methionine increased within the SAM fraction at higher dietary proportions of maize silage, but decreased within the LAM-E fraction. A significant effect of particle size on AA contents was detected mostly for the LAM-E fraction. Concentrations of arginine, leucine and phenylalanine increased in F treatments compared to C treatments. In contrast, the proportions of cystine, leucine and methionine were higher in particle size C than in F. Interactions of diet and particle size on the proportion of AA only occurred once for cystine, proline and serine. For all AA, a significant effect ($P < 0.05$) of microbial fraction on the relative AA proportion was found

Table 3 *Proportion of AA in the fractions of solid- and liquid-associated microbes (% of analysed AA; means and pooled s.e.)*

Diet[1]	M100		M79		M52		M24		M0			*P*-values		
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet × PS
Alanine														
SAM	8.53^{B}	8.75^{B}	8.16^{B}	8.35^{B}	8.30^{B}	8.64	8.49^{B}	8.45^{B}	8.74	8.69	0.12	*	0.37	0.17
LAM-F	9.14^{A}	9.51^{A}	8.91^{A}	9.02^{A}	9.07^{A}	8.90	8.83^{A}	8.93^{A}	9.01	8.74	0.11	**	0.78	0.07
LAM-E	8.94^{A}	9.06^{B}	9.14^{A}	9.11^{A}	8.93^{A}	8.79	9.04^{A}	9.03^{A}	8.87	8.74	0.07	*	0.64	0.50
Arginine														
SAM	5.64	5.64	5.23	5.26	5.76^{AB}	5.78^{A}	5.54	5.58	5.79^{AB}	5.78	0.14	*	0.48	0.95
LAM-F	5.41	5.31	5.38	5.28	5.42^{B}	5.37^{B}	5.37	5.32	5.32^{B}	5.54	0.10	0.94	0.34	0.32
LAM-E	5.77	5.59	5.67	5.61	6.01^{A}	5.76^{A}	5.81	5.71	6.02^{A}	5.95	0.10	*	*	0.76
Aspartic acid														
SAM	12.1^{B}	12.1	13.1^{AB}	13.1	12.4	12.3	13.2^{A}	13.2^{A}	12.5	12.3	0.12	**	0.34	0.15
LAM-F	12.6^{A}	12.3	13.4^{A}	13.3	12.7	12.4	13.3^{A}	13.3^{A}	12.7	12.4	0.13	0.07	0.38	0.95
LAM-E	12.5^{A}	12.4	13.0^{B}	13.1	12.5	12.4	12.9^{B}	12.9^{B}	12.5	12.3	0.12	0.36	0.37	0.92
Cystine														
SAM	1.14^{A}	1.08^{B}	1.10^{B}	1.16^{A}	1.04^{B}	0.96^{bC}	1.01^{B}	1.01^{B}	1.00^{B}	0.98^{C}	0.02	***	0.21	*
LAM-F	1.21^{A}	1.25^{A}	1.22^{A}	1.23^{A}	1.19^{A}	1.23^{A}	1.23^{A}	1.22^{A}	1.23^{A}	1.28^{A}	0.03	0.41	0.09	0.66
LAM-E	1.02^{B}	1.06^{B}	1.00^{C}	1.05^{B}	0.99^{B}	1.12^{B}	1.04^{B}	1.05^{B}	1.02^{B}	1.12^{B}	0.03	0.59	**	0.23
Glutamic acid														
SAM	14.9^{A}	14.8^{A}	15.2^{A}	15.0^{A}	15.2^{A}	15.4^{A}	15.6^{A}	15.2^{A}	15.2^{A}	15.2^{A}	0.14	*	0.61	0.24
LAM-F	14.5^{B}	14.6^{A}	14.4^{B}	14.4^{B}	14.4^{B}	14.6^{B}	14.7^{B}	14.8^{B}	14.5^{B}	14.7^{B}	0.12	0.08	*	0.78
LAM-E	14.0^{C}	14.3^{B}	13.7^{C}	13.8^{C}	14.0^{B}	14.2^{B}	13.7^{C}	13.8^{C}	13.9^{C}	14.2^{C}	0.08	0.36	***	0.28
Glycine														
SAM	5.87	5.84	5.84^{B}	5.87^{B}	5.80	5.83	5.87^{B}	5.90^{B}	5.77	5.81	0.06	0.60	0.77	0.94
LAM-F	5.95	5.95	5.89^{B}	5.89^{B}	5.94	5.88	5.90^{B}	5.88^{B}	6.06	5.99	0.06	0.08	0.07	0.69
LAM-E	6.01	5.92	6.18^{A}	6.16^{A}	6.00	5.97	6.22^{A}	6.21^{A}	6.07	5.98	0.05	0.24	0.82	0.95
Isoleucine														
SAM	5.64	5.50	5.55^{A}	5.53	5.63	5.70	5.40^{AB}	5.53	5.55	5.67	0.08	0.54	0.86	0.47
LAM-F	5.56	5.68	5.25^{B}	5.38	5.53	5.49	5.29^{B}	5.35	5.48	5.78	0.08	0.21	0.46	0.07
LAM-E	5.64	5.72	5.51^{A}	5.64	5.53	5.73	5.59^{A}	5.68	5.62	5.81	0.08	0.48	*	0.83
Leucine														
SAM	8.63^{A}	8.65	8.37^{B}	8.49^{A}	8.60^{A}	8.58	8.08^{B}	8.18^{A}	8.30^{B}	8.41	0.10	**	0.47	0.91
LAM-F	8.03^{B}	8.11	7.73^{A}	7.69^{C}	8.02^{B}	8.30	7.81^{C}	7.73^{B}	8.15^{B}	8.19	0.09	0.16	0.78	0.10
LAM-E	8.39^{A}	8.35	8.15^{B}	8.03^{B}	8.50^{A}	8.43	8.31^{A}	8.25^{A}	8.68^{A}	8.51	0.06	**	**	0.77
Lysine														
SAM	8.22^{B}	8.29	8.24^{B}	8.08^{C}	8.34	8.34	8.21^{B}	8.04^{B}	8.22^{A}	8.20	0.08	0.36	0.14	0.56
LAM-F	8.61^{A}	8.52	8.73^{A}	8.64^{A}	8.50	8.40	8.56^{A}	8.46^{A}	8.26^{A}	8.18	0.07	***	0.14	1.00
LAM-E	8.32^{AB}	8.26	8.39^{B}	8.37^{B}	8.12	8.20	8.13^{B}	8.12^{B}	7.72^{B}	7.95	0.07	***	0.43	0.53

Table 3 *Continued*

Diet[1]	M100		M79		M52		M24		M0			*P*-values		
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet × PS
Methionine														
SAM	2.73	2.70	2.63^{B}	2.71^{B}	2.56	2.54	2.57^{B}	2.52^{B}	2.64^{B}	2.63	0.05	**	0.57	0.70
LAM-F	2.80	2.80	2.86^{A}	2.90^{A}	2.72	2.54	2.83^{A}	2.89^{A}	2.90^{A}	2.85	0.06	**	0.81	0.20
LAM-E	2.68	2.72	2.73^{B}	2.83^{AB}	2.73	2.82	2.83^{A}	2.88^{A}	2.83^{A}	2.87	0.03	**	*	0.80
Phenylalanine														
SAM	5.43	5.39^{A}	5.36^{A}	5.39^{A}	5.41	5.39	5.34	5.48^{A}	5.40	5.36	0.06	0.96	0.67	0.44
LAM-F	5.15	5.20^{AB}	5.18^{B}	5.09^{B}	5.21	5.16	5.35	5.17^{B}	5.30	5.33	0.07	*	0.29	0.23
LAM-E	5.16	5.07^{B}	5.30^{A}	5.16^{AB}	5.19	5.12	5.39	5.23^{B}	5.22	5.08	0.06	*	***	0.08
Proline														
SAM	4.51	4.43^{A}	4.11^{A}	3.85^{A}	4.31	4.08^{B}	3.99^{A}	3.99	4.29	4.46	0.13	0.33	0.38	0.69
LAM-F	4.07	3.95^{B}	3.56^{B}	3.56^{B}	4.13^{b}	5.24^{aA}	3.63^{B}	3.63	4.06	4.11	0.17	**	0.26	*
LAM-E	4.33	4.46^{A}	3.65^{B}	3.71^{AB}	4.38	4.5 7^{AB}	3.57^{B}	3.86	4.30	4.44	0.13	0.49	0.97	0.13
Serine														
SAM	4.55	4.40	5.27^{A}	5.33^{A}	4.64^{a}	4.19^{b}	5.23^{A}	5.32^{A}	4.58	4.33	0.12	0.25	0.43	*
LAM-F	4.43	4.25	5.09^{A}	5.08^{B}	4.52	4.08	5.06^{B}	5.08^{B}	4.56	4.35	0.11	0.15	0.68	0.16
LAM-E	4.41	4.30	4.79^{B}	4.71^{C}	4.42	4.26	4.79^{C}	4.68^{C}	4.47	4.29	0.08	0.85	0.67	0.81
Threonine														
SAM	5.59^{C}	5.70^{B}	5.94^{B}	6.02^{B}	5.63^{B}	5.45^{B}	5.77^{B}	5.89^{B}	5.53^{C}	5.45^{B}	0.08	0.11	0.59	0.50
LAM-F	5.90^{B}	5.75^{B}	6.22^{A}	6.25^{A}	6.02^{A}	5.78^{A}	6.08^{A}	6.12^{A}	5.88^{B}	5.78^{A}	0.09	0.24	0.78	0.53
LAM-E	6.15^{A}	6.07^{A}	6.28^{A}	6.22^{A}	6.09^{A}	5.96^{A}	6.19^{A}	6.09^{A}	6.08^{A}	5.92^{A}	0.05	***	0.10	0.48
Valine														
SAM	6.53	6.72	5.83^{C}	5.81^{B}	6.43	6.76	5.69^{C}	5.73^{C}	6.41	6.73	0.11	0.17	0.93	0.39
LAM-F	6.71	6.79	6.18^{B}	6.26^{A}	6.63	6.68	6.11^{B}	6.13^{B}	6.56	6.77	0.10	0.11	0.43	0.49
LAM-E	6.71	6.71	6.49^{A}	6.47^{A}	6.67	6.72	6.49^{A}	6.49^{A}	6.77	6.81	0.09	0.46	0.22	0.99

AA = amino acids; PS = particle size; F = fine; C = coarse; SAM = solid-associated microbes from feed residues; LAM-F = liquid-associated microbes from fermenter; LAM-E = liquid-associated microbes from effluent.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0).
[a,b]In case of significant interaction between diet and PS: different superscripts indicate significant differences between particle size F and C within diets (Student's *t*-test, $P < 0.05$).
[A,B,C]Different superscripts indicate significant differences between microbial fractions within treatments (Student's *t*-test, $P < 0.05$).
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4 *Outflow of microbial N and microbial AA from the Rusitec (means and pooled s.e.)*

Diet[1]	M100		M79		M52		M24		M0			*P*-values		
PS	F	C	F	C	F	C	F	C	F	C	s.e.	Diet	PS	Diet × PS
Microbial N														
mg/day	144	156	170	180	181	200	210	212	237	240	7.12	***	***	0.07
mg/day with SAM	38.9	41.4	40.8	35.1	55.3	46.2	59.3[a]	40.5[b]	87.4[a]	68.7[b]	3.38	***	**	*
mg/day with LAM-E	105	114	129	145	126	153	151	171	150	172	6.51	***	***	0.35
SAM to LAM-E ratio	0.37	0.36	0.31	0.24	0.45	0.30	0.40	0.24	0.59	0.41	0.03	***	***	0.12
g/g available N	0.90	0.92	0.87	0.85	0.86	0.86	0.81	0.79	0.80	0.80	0.01	***	0.88	0.79
mg/g degraded OM[2]	28.3	29.3	32.8	32.8	35.5	36.5	39.8	38.7	43.4	42.4	0.95	***	0.39	0.31
Microbial AA[3]														
mg/day with SAM	189	204	203	174	272	224	294[a]	205[b]	433[a]	333[b]	17.2	***	**	*
mg/day with LAM-E	528	565	649	728	624	744	744	832	728	836	30.7	***	***	0.45

AA = amino acids; Rusitec = rumen simulation technique; PS = particle size; F = fine; C = coarse; SAM = solid-associated microbes from feed residues; LAM-E = liquid-associated microbes from effluent; OM = organic matter.
[1]Maize silage to grass silage ratio of 100 : 0 (M100), 79 : 21 (M79), 52 : 48 (M52), 24 : 76 (M24) and 0 : 100 (M0).
[2]Values for degraded OM were taken from Hildebrand *et al.* (2010).
[3]Sum of the 15 analysed AA.
[a,b]In case of significant interaction between diet and PS: different superscripts indicate significant differences between particle size F and C within diets (Student's *t*-test, $P < 0.05$).
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

for some of the treatments, furthermore indicating interactions of particle size and microbial fraction (Table 3).

Microbial N and AA yield

The daily output of microbial N from the SAM and LAM-E was significantly increased by the replacement of maize silage by grass silage in the diet (Table 4). An interaction of diet and particle size was detected for the SAM fraction, in which F treatments resulted in a higher amount of daily microbial N compared to C treatments in diets M24 and M0. In contrast, microbial N from LAM-E was higher for C treatments than for F treatments across all diets. Consequently, the SAM to LAM-E ratio was higher at particle size F than at particle size C on average (0.42 *v.* 0.31). Differences between F and C treatments became greater as the proportion of grass silage increased, but no significant interaction was detected ($P = 0.12$). The efficiency of microbial N utilisation (mg microbial N/mg available N) and EMPS (mg microbial N/g degraded OM) were not affected by particle size. The use of available N for microbial CP synthesis was significantly increased by the inclusion of maize silage in the diet and averaged 91% and 80% in diets M100 and M0, respectively. In contrast, EMPS significantly improved by the rising proportion of grass silage and increased by about 14 mg/g from diets M100 to M0.

The daily outflow of all individual AA and total AA as a sum of the SAM and LAM-E fraction was significantly affected by diet and particle size, and no interaction between these two dietary factors was detected (data not shown). The daily amount of all AA increased with ascending proportion of grass silage in the diet. Similar to microbial N the daily microbial AA yield from the SAM fraction was characterised by an interaction of diet and particle size, whereas the amount of total AA originating from the LAM-E fraction was improved by an increasing inclusion rate of grass silage and coarse milling compared to fine milling (Table 4).

Discussion

Amount and efficiency of microbial CP synthesis

It was hypothesised that an optimal ratio of available energy and N by changing the maize silage to grass silage ratio in a diet maximises the microbial CP synthesis. These results show that daily microbial N as well as EMPS increased with the stepwise replacement of maize silage by grass silage in the diet. These results are in contrast to *in vivo* studies, which showed that the inclusion of maize silage in the diet promotes the postruminal supply of microbial protein, compared to grass silage-based diets (Givens and Rulquin, 2004). Effects observed under *in vitro* conditions can be masked or other limiting factors might occur *in vivo.* Constant daily feeding and feed retention time as well as limited fluctuations in pH value in the Rusitec system compared to the intact rumen might impair the direct comparison of these results with *in vivo* findings. As shown by Hildebrand *et al.* (2010), the degradation of OM increased slightly when replacing maize silage by grass silage, and a high correlation between degraded OM and the microbial CP synthesis was reported for the Rusitec system by Boguhn *et al.* (2006a). However, a wide range in EMPS was documented (29 to 43 mg microbial N/g degraded OM). Large differences in EMPS are also known from other *in vitro* studies dealing with changes in the amount and type of carbohydrates and N (Stokes *et al.*, 1991; Bach *et al.*, 1999; Boguhn *et al.*, 2006a).

Similar amounts of total short-chain fatty acids indicated that the amount of fermented carbohydrates was similar between diets of different maize silage to grass silage ratio (Hildebrand *et al.*, 2010). The amount of ATP derived from

fermentation of carbohydrates is assumed to be similar between structural and non-structural polysaccharides (Russell and Strobel, 2005; Tamminga *et al.*, 2007). However, the use of energy could have been limited by the availability of other nutrients. Diets in this study were characterised by an increase in available N with a rising proportion of grass silage in the diet. As the ratio of dietary NSC to degradable CP decreased, the EMPS increased in continuous culture (Stokes *et al.*, 1991). This observation is in accordance with these results, in which the ratio of NSC to degraded CP decreased from 6.4 to 1.6 from diets M100 to M0. These findings emphasise the importance of adequate amounts of available N when availability of energy is not limited (Bach *et al.*, 2005). Furthermore, an increase in NH_3-N concentration in the liquid phase is generally associated with a decrease in efficiency of N utilisation (g microbial N/g available N) in continuous culture systems (Bach *et al.*, 2005). The current results are similar to the findings of Bach *et al.* (1999). They found that the efficiency of N utilisation was increased by supplementation of cracked maize to lush pasture compared to pure lush pasture, whereas EMPS was lower with maize addition.

The findings of Satter and Slyter (1974) suggested an available CP equivalent of 120 g/kg diet for maximal microbial growth in the liquid phase *in vitro*. In this study, available CP equivalent (degraded CP + N from buffer solution) was 74, 91, 100, 119 and 134 g/kg for diets M100, M79, M52, M24 and M0, respectively. Therefore, a deficit in available N is possible for the diets with high proportions of maize silage. Moreover, the N source might have played a major role in this study. Microbial growth can be enhanced by addition of AA and peptides, especially under *in vitro* conditions (Carro and Miller, 1999; Demeyer and Fievez, 2004; Bach *et al.*, 2005). The supply of AA originating from silages more than doubled from diets M100 to M0 (0.77 *v.* 1.82 g AA/day). However, it is difficult to estimate the available AA and the direct use for the microbial protein synthesis, so that a discussion of this aspect can only be hypothetical.

It has been demonstrated in this study that daily microbial N was higher in C treatments compared to F treatments. The degradation of OM and CP, as well as the daily amounts of NH_3-N and short-chain fatty acids in the effluent were higher in coarse-milled than in fine-milled treatments (Hildebrand *et al.*, 2010). An improved microbial protein synthesis in response to increased availability of energy and N is probable. But, in contrast to the continuous culture experiment of Rodríguez-Prado *et al.* (2004), in this study there was no significant effect of feed particle size on EMPS.

Composition of microbial fractions

A wide range in chemical composition of ruminal bacteria has been reported in the literature, but techniques and location of bacterial isolations have differed among experiments (Clark *et al.*, 1992; Martin *et al.*, 1996; Yang *et al.*, 2001). Therefore, microbial mass was isolated from three compartments of the Rusitec system in this study to investigate effects of dietary forage composition and particle size. The contents of N and ^{15}N in the LAM-F fraction were intermediate between those of the two other fractions across all diets, indicating that this fraction represents a mixture of SAM and LAM-E. However, the proportions of AA were not intermediate at all. The higher proportion of ^{15}N in the LAM-E compared to the SAM is in accordance with most studies using ^{15}N as microbial marker (Rodríguez *et al.*, 2000; Yang *et al.*, 2001; Carro and Miller, 2002) and indicated differences in the N source used (Atasoglu *et al.*, 1999).

Dietary influences on the N content of ruminal bacteria are rarely identified (Hvelplund, 1986; Philipczyk *et al.*, 1996; Yang *et al.*, 2001). As pointed out by Bach *et al.* (2005), bacterial chemical composition is affected by the availability of energy and N as well as by the availability of AA (Wang *et al.*, 2008). High proportions of concentrate in the diet (Ranilla and Carro, 2003) and supplementation of maize starch to grass pasture (Bach *et al.*, 1999) were reported to reduce the N content in the microbial mass. In this study, N contents of microbial fractions were lowest in maize silage- and highest in grass silage-based diets. Changes in the availability of nutrients, as described between treatments F and C (Hildebrand *et al.*, 2010), did not affect N contents of the microbial fractions. However, in contrast to LAM-E, ^{15}N enrichment in the SAM fraction was higher in C treatments than in F treatments. It is supposable that SAM in C treatments had easier access to the ^{15}N pool of the liquid phase because of lower bulk density, and consequently, a better flushing of feedbags compared to F treatments. Differences in the effect of feed particle size on microbial N metabolism between SAM and LAM were also reported by Rodríguez-Prado *et al.* (2004). In accordance with Molina-Alcaide *et al.* (2009) interactions between microbial fraction and diet on AA profile could be detected in this study. This fact underlines the hypothesis that the origin of microbial sample might affect the results of continuous culture studies when particle size or diet varies. Diet composition has been reported to have only small effects on microbial AA profile (Martin *et al.*, 1996; Volden and Harstad, 1998; Yang *et al.*, 2001). Although the proportion of several AA was significantly affected by the maize silage to grass silage ratio in this study, the nominal differences in individual AA were not of biological importance and in the range of AA profiles reported *in vivo* (Clark *et al.*, 1992). In accordance with Rodríguez-Prado *et al.* (2004), the effect of particle size on microbial AA profile was low. However, similar AA profiles do not prove the fact that the microbial community was similar across treatments. A companion study using different molecular techniques showed that the community structure of the bacterial group of *Bacteroides–Prevotella* was significantly influenced by both maize silage to grass silage ratio and feed particle size (Witzig *et al.*, 2010). The relative abundance of *Prevotella bryantii* increased with increasing proportion of grass silage in the diet and the authors attributed this to the changes in dietary CP.

Dietary factors influencing SAM to LAM-E ratio might be the forage to concentrate ratio (Ranilla and Carro, 2003;

Gómez *et al*., 2005, Vlaeminck *et al*., 2006) and the availability of N (Carro and Miller, 1999; Kajikawa *et al*., 2007). As the SAM may have greater requirements for NH_3-N than the LAM fraction (Carro and Miller, 1999), higher amounts of NH_3-N in the course of grass silage inclusion might have promoted the synthesis of SAM and led to an increase in the SAM to LAM-E ratio. Furthermore, the higher SAM to LAM-E ratio in F treatments compared to C treatments indicates a higher microbial colonisation of small particles than large particles (Yang *et al*., 2001). However, it cannot be guaranteed that the detached SAM fraction was representative for all particle associated microbes (Martín-Orúe *et al*., 1998; Ranilla and Carro, 2003). An underestimation of the SAM fraction is likely, because the firmly adherent, not detached microbes may be lower in ^{15}N enrichment than the loosely attached microbes. The contribution of SAM to total microbial N was, on average, 27% and lower than commonly reported *in vivo* (Craig *et al*., 1987; Vlaeminck *et al*., 2006). However, the ratio of solid to liquid phase was lower in the used rumen simulation than in an intact rumen and therefore the proportion of SAM in total microbial CP might have been lower. In accordance, the mass of LAM was reported to be higher than the mass of SAM in other *in vitro* studies (Carro and Miller, 1999; Gómez *et al*., 2005; Kajikawa *et al*., 2007).

Conclusions

This study showed that the amount of available nutrients to favour microbial CP synthesis and EMPS is most optimised when grass silage is used instead of maize silage. Grinding forage-based diets through a sieve with a pore size of 4 mm leads to an increase in daily microbial N, but not in EMPS compared to using a 1-mm milling sieve. Diet and feed particle size affect the AA profile of microbial fractions, and interactions of both factors might occur. Nevertheless, biological significance of the changed AA profile is negligible. In addition, interactions between dietary factors and microbial fractions are assumed. Differences in the amount and composition of microbial protein might indicate that specific microbial communities are promoted.

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References

Atasoglu C, Valdes C, Newbold CJ and Wallace RJ 1999. Influence of peptides and amino acids on fermentation rate and de novo synthesis of amino acids by mixed micro-organisms from the sheep rumen. British Journal of Nutrition 81, 307–314.

Bach A, Calsamiglia S and Stern MD 2005. Nitrogen metabolism in the rumen. Journal of Dairy Science 88, E9–E21.

Bach A, Yoon IK, Stern MD, Jung HG and Chester-Jones H 1999. Effects of type of carbohydrate supplementation to lush pasture on microbial fermentation in continuous culture. Journal of Dairy Science 82, 153–160.

Boguhn J, Kluth H and Rodehutscord M 2006a. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. Journal of Dairy Science 89, 1580–1591.

Boguhn J, Kluth H and Rodehutscord M 2006b. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. Journal of Dairy Science 89, 1592–1603.

Carro MD and Miller EL 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semi-continuous culture system (Rusitec). British Journal of Nutrition 82, 149–157.

Carro MD and Miller EL 2002. Comparison of microbial markers (^{15}N and purine bases) and bacterial isolates for the estimation of rumen microbial protein synthesis. Animal Science 75, 315–321.

Clark JH, Klusmeyer TH and Cameron MR 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. Journal of Dairy Science 75, 2304–2323.

Craig WM, Broderick GA and Ricker DB 1987. Quantitation of microorganisms associated with the particulate phase of ruminal ingesta. Journal of Nutrition 117, 56–62.

Czerkawski JW and Breckenridge G 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371–384.

Demeyer D and Fievez V 2004. Is the synthesis of rumen bacterial protein limited by the availability of pre-formed amino acids and/or peptides? British Journal of Nutrition 91, 175–176.

Givens DI and Rulquin H 2004. Utilisation by ruminants of nitrogen compounds in silage-based diets. Animal Feed Science and Technology 114, 1–18.

Gómez JA, Tejido ML and Carro MD 2005. Influence of disodium malate on microbial growth and fermentation in rumen-simulation technique fermenters receiving medium- and high-concentrate diets. British Journal of Nutrition 93, 479–484.

Hildebrand B, Boguhn J and Rodehutscord M 2010. Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation *in vitro*. Animal 5, 528–536.

Hvelplund T 1986. The influence of diet on nitrogen and amino acid content of mixed rumen bacteria. Acta Agriculturae Scandinavica 36, 325–331.

Kajikawa H, Tajima K, Mitsumori M and Takenaka A 2007. Effects of amino nitrogen on fermentation parameters by mixed ruminal microbes when energy or nitrogen is limited. Animal Science Journal 78, 121–128.

Kenward MG and Roger JH 1997. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53, 983–997.

Martin C, Bernard L and Michalet-Doreau B 1996. Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. Journal of Animal Science 74, 1157–1163.

Martín-Orúe SM, Balcells J, Zakraoui F and Castrillo C 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure. Animal Feed Science and Technology 71, 269–282.

Michalet-Doreau B and Ould-Bah MY 1992. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: a review. Animal Feed Science and Technology 40, 57–86.

Molina-Alcaide E, Moumen A, Martín-García I and Carro MD 2009. Comparison of bacterial pellets and microbial markers for the estimation of the microbial nitrogen and amino acids flows from single flow continuous culture fermenters fed diets containing two-stage olive cake. Journal of Animal Physiology and Animal Nutrition 93, 527–537.

Philipczyk I, Südekum KH, Ahrens F and Stangassinger M 1996. Bestimmung des Anteils bakterieller Biomasse im Pansen von Ochsen mit Diaminopimelinsäure als Marker: Einfluß des Erntezeitpunktes von Futterpflanzen, des Probenahmezeitpunktes und der Methode zur Isolierung der Bakterienfraktion (Quantification of bacterial biomass in the rumen of steers with diaminopimelic acid as marker: effects of forage stage of maturity, time of sampling and method of isolating the bacterial fraction). Journal of Animal Physiology and Animal Nutrition 75, 105–119.

Ranilla MJ and Carro MD 2003. Diet and procedures used to detach particle-associated microbes from ruminal digesta influence chemical composition of

microbes and estimation of microbial growth in Rusitec fermenters. Journal of Animal Science 81, 537–544.

Rodehutscord M, Kapocius M, Timmler R and Dieckmann A 2004. Linear regression approach to study amino acid digestibility in broiler chickens. British Poultry Science 45, 85–92.

Rodríguez CA, González J, Alvir MR, Repetto JL, Centeno C and Lamrani F 2000. Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. British Journal of Nutrition 84, 369–376.

Rodríguez-Prado M, Calsamiglia S and Ferret A 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. Journal of Dairy Science 87, 1413–1424.

Russell J.B. and Strobel H.J. 2005. Microbial energetics. In Quantitative aspects of ruminant digestion and metabolism (ed. J Dijkstra, JM Forbes and J France), pp. 229–261. CABI Publishing, Wallingford, UK.

Satter LD and Slyter LL 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. British Journal of Nutrition 32, 199–208.

Stern MD, Varga GA, Clark JH, Firkins JL, Huber JT and Palmquist DL 1994. Evaluation of chemical and physical properties of feeds that affect protein metabolism in the rumen. Journal of Dairy Science 77, 2762–2786.

Stokes SR, Hoover WH, Miller TK and Manski RP 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. Journal of Dairy Science 74, 860–870.

Tamminga S, Brandsma GG, Dijkstra J, van Duinkerken G, van Vuuren AM and Blok MC 2007. Protein evaluation for ruminants: the DVE/OEB 2007-System. CVB documentation report no. 53. Centraal Veevoeder Bureau, Lelystad, Netherlands.

Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583–3597.

Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA) 2006. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Vlaeminck B, Fievez V, Demeyer D and Dewhurst RJ 2006. Effect of forage : ‘concentrate ratio on fatty acid composition of rumen bacteria isolated from ruminal and duodenal digesta. Journal of Dairy Science 89, 2668–2678.

Volden H and Harstad OM 1998. Amino acid composition of bacteria harvested from the rumen of dairy cows fed three diets differing in protein content and rumen protein degradability at two levels of intake. Acta Agriculturae Scandinavica, Section A – Animal Science 48, 210–215.

Wang M-Z, Wang H-R, Cao H-C, Li G-X and Zhang J 2008. Effects of limiting amino acids on rumen fermentation and microbial community *in vitro*. Agricultural Sciences in China 7, 1524–1531.

Witzig M, Boguhn J, Kleinsteuber S, Fetzer I and Rodehutscord M 2010. Effect of the corn silage to grass silage ratio and feed particle size of diets for ruminants on the ruminal *Bacteroides-Prevotella* community *in vitro*. Anaerobe 16, 412–419.

Yang WZ, Beauchemin KA and Rode LM 2001. Effect of dietary factors on distribution and chemical composition of liquid- or solid-associated bacterial populations in the rumen of dairy cows. Journal of Animal Science 79, 2736–2746.

Paper III

Investigations on the effect of forage source, grinding, and urea supplementation on ruminal fermentation and microbial protein synthesis in a semi-continuous rumen simulation system

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Abstract

The objective of the present study was to compare the effect of maize silage and grass silage on microbial fermentation and protein flow in a semi-continuous rumen simulation system (Rusitec) when milling screen size (MSS) during grinding was varied. Oven-dried silages were milled through screens of 1, 4 or 9 mm pore size and incubated for 48 h in a Rusitec system. Furthermore, the effect of N supplementation to maize silage (MSS: 4 mm) was investigated and single dose *vs.* continuous infusion of urea-N were compared. Degradation of organic matter (OM), crude protein (CP), fibre fractions and non-structural carbohydrates (NSC) as well as short-chain fatty acid production differed significantly between forage sources. Urea-N supplementation improved the degradation of NSC, but not that of fibre fractions in maize silage. The way of urea supply had only marginal effects on fermentation characteristics. An increase in MSS, and consequently in mean feed particle size, led to an improvement in the degradation of OM, CP and NSC, but efficiency of microbial net protein synthesis (EMPS; mg microbial N flow/g degraded OM)and microbial amino acid profile were less affected. EMPS was higher in grass silage than in maize silage and was improved by urea-N supplementation in maize silage. This study indicates that fermentation of NSC as well as EMPS during incubation of maize silage was limited by availability of NH_3-N. Furthermore, an increase in MSS above 1 mm seems to improve fermentation of silages in the Rusitec system.

Keywords

Silage, particle size, urea, fermentation, microbial protein, Rusitec

1. Introduction

For *in vitro* procedures used to investigate the effect of feedstuffs on ruminal fermentation, the feedstuffs are usually ground before incubation. Changes in feed particle size distribution are known to influence growth conditions of several microbial groups and the extent of fermentation (Zhang et al. 2007). As shown in batch culture studies, an increase in milling screen size (MSS) tends to induce a decrease in gas production (Menke and Steingass 1988) and a reduction in the degradation of fibre fractions in forages (Robles et al. 1980, Bossen et al. 2008). However, information on the effect of MSS variation in forage evaluation by continuous flow rumen simulation systems is scarce (Rodríguez-Prado et al. 2004); moreover, no standardisation in grinding exists between laboratories. As indicated by previous studies (Hildebrand et al. 2011a, 2011b), using a MSS of 4 mm, compared to 1 mm, seems to benefit fermentation of maize silage- and grass silage-based diets as well as microbial N flow in a semi-continuous rumen simulation system (Rusitec). As a result of a further increase in MSS, the difference in mean particle size becomes greater between forages (Bossen et al. 2008). Hence, interactions of forage source and MSS on fermentation characteristics might occur. Identifying those kinds of interactions would provide further information on the comparability of studies using rumen simulation systems. In order to avoid interactions of forage with other dietary components (Niderkorn and Baumont 2009) and thus to allow a better understanding of direct forage effects, forages need to be incubated purely. The most important forage sources in ruminant feeding across Europe, particularly for indoor keeping of dairy cows, are maize silage and grass silage. Therefore, the first objective of this study was to compare the effect of pure maize silage and grass silage on fermentation characteristics and microbial protein synthesis in a Rusitec when MSS was varied.

Maize silage and grass silage differ in their nutrient composition. Because of lacking rumino-hepatic circulation *in vitro*, a deficit in available N might impair fermentation of maize silage in comparison with grass silage (Stokes et al. 1991, Hildebrand et al. 2011a, 2011b). As shown by Windschitl and Stern (1988) and Griswold et al. (2003), the supplementation of urea-N could lead to an increase in microbial N flow and the degradation of carbohydrate fractions. In this context, the availability of urea-N over time can play a role (Cherdthong and Wanapat 2010). Hence, the second objective of this study was to investigate the effect of urea supplementation to maize silage, either supplied continuously via buffer solution or once daily together with feed.

2. Materials & methods

2.1 Feedstuffs

A batch of maize silage and a batch of grass silage (4th cut) were used in two *in vitro* experiments conducted at Martin-Luther-Universität Halle-Wittenberg and at Universität Hohenheim, respectively. Both forages were obtained from a dairy cow farm near Halle upon Saale (Germany) and typically differed in fermentation characteristics as well as nutrient composition and amino acid (AA) contents (Table 1). Silages were oven-dried at 65°C and the subsequent dry matter (DM) content was approximately 95 %. Thereafter, the silages were milled through screens of 1, 4 or 9 mm pore size (cutting mill, Pulverisette 15, Fritsch GmbH, Idar-Oberstein, Germany), inducing differences in bulk density (Table 1).

2.2 Experiment 1

In the first experiment, maize silage and grass silage, ground at 1 or 4 mm-MSS each, were filled into nylon bags (pore size = 100 μm) to an amount of 15 g prior to incubation in a Rusitec (Czerkawski and Breckenridge 1977) that comprised 6 fermenters. The four treatments were tested in at least 3 replicates distributed across 6 incubation periods. One period lasted 13 days and the whole procedure of incubation and sampling was done as described by Hildebrand et al. (2011b). The inoculum was obtained from the rumen of at least two fistulated wether sheep before the morning feeding. Animals were fed on hay to satiation and 250 g of a concentrate feed per day. Two nylon bags were incubated at 39°C in a fermenter of 800 ml volume and replaced daily by a new one after 48 h of incubation. A buffer solution, following McDougall (1948) and containing 0.7 mmol NH_4^+/l from $^{15}NH_4Cl$ (11.5% ^{15}N abundance per analysis), was infused continuously with an average flow rate of 549 ml/day. Effluents were captured in cooled bottles (4°C) and volume was measured daily. Daily subsamples of 320 ml were taken from day 7 to day 13 and conveyed to differential centrifugation. In order to separate feed particles, samples were centrifuged twice at 2,000 × *g* and 4°C for 5 min. The residue was rinsed on a folded filter, dried at 65°C and weighed. The feed particle-free effluent was centrifuged at 27,000 × *g* and 4°C for 15 min, obtaining a pellet of liquid-associated microbes (LAM). The supernatant was evacuated and stored at -20°C for subsequent analysis of short-chain fatty acids (SCFA), NH_3-N and ^{15}N. The LAM pellets were further treated as described by Boguhn et al. (2006b) and pooled per fermenter after freeze drying. Daily samples of feed residues in the nylon bags (days 7-12) were washed in 50 ml of buffer solution, squeezed moderately, dried at 65°C for 24 h, weighed and pooled per

fermenter. Solid-associated microbes (SAM) were isolated from feed residues on the last day of incubation. After squeezing, the bags were incubated in a saline solution of methylcellulose as described by Boguhn et al. (2006b). After removing of the feed bags, the samples of SAM were obtained by differential centrifugation from the remaining suspension as detailed above for the LAM fraction.

2.3 Experiment 2

Maize silage and grass silage of 4 and 9 mm MSS were incubated by using the same procedure and apparatus as described for Experiment 1. In addition, the effect of N supply was to be investigated. Therefore, the maize silage, milled at 4 mm-MSS, was supplemented with urea in order to achieve at least the same amount of N supply as with the grass silage. Urea was either weighed into the bags (283 mg urea-N/d) or added to the buffer solution (on average 288 mg urea-N/d). The experiment comprised at least 3 replicates per treatment, distributed across 4 incubation periods. The flow rate of the buffer solution was on average 563 ml/d. Sampling and further treatment of effluents and feed residues as well as isolation of LAM and SAM were done as described for Experiment 1.

2.4 Analyses

Bulk density of ground feeds was determined by using a grain tester with a calibrated cylinder (Louis Schopper, Leipzig, Germany). Samples of feedstuffs and feed residues were ground to pass a 0.5-mm screen and were analysed for crude ash (method 3.1, Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA 2006), crude protein (CP, method 4.1.1, VDLUFA 2006), ether extract (method 5.1.1, VDLUFA 2006), crude fibre (CF, method 6.1.1, VDLUFA 2006), and starch (method 7.2.1., VDLUFA 2006). Neutral detergent fibre, assayed with a heat-stable amylase (aNDFom), and acid detergent fibre (ADFom) were analysed according to VDLUFA (2006), methods 6.5.1 and 6.5.2, respectively, and were expressed exclusive of residual ash. Analysis of AA in silages and freeze-dried microbial pellets followed standard procedures (method 4.11.1, VDLUFA 2006) with laboratory details as described by Rodehutscord et al. (2004), using an amino acid analyser (Biochrom 30, Biochrom Ltd, Cambridge, UK). Concentrations of SCFA in particle-free effluents were measured by gas chromatography according to Geissler et al. (1976), using 2-methylvaleric acid as internal standard. NH_3-N was determined by steam distillation with sodium hydroxide followed by end-point titration. Samples of feedstuffs, feed residues,

$^{15}NH_4Cl$, freeze-dried microbial pellets and supernatant were ground finely and analysed for ^{15}N and N, using an isotope mass spectrometer (Delta V advantage, Thermo Fisher Scientific, Bremen, Germany) coupled with an elemental analyser (EuroEA, HEKAtech GmbH, Wegberg, Germany).

2.5 Calculations and statistics

Degradation of nutrients was calculated by daily input of feed and daily output of feed residues. Daily weight of feed residues was complemented by the weight of feed particles separated from the effluent. The amounts of non-structural carbohydrates (NSC) were calculated from DM minus aNDFom, CP, ether extract and crude ash (Van Soest et al. 1991). The supplemented urea was assumed to solubilise completely and therefore not considered in calculations of the degradation of OM and CP. The daily output of microbial OM and CP attached to feed residues was estimated as described by Boguhn et al. (2006a), using ^{15}N and N measurements. CP from microbes was calculated as microbial N × 6.25. The amount of microbial N in the daily effluent was calculated on the basis of a ^{15}N balance as described in detail by Hildebrand et al. (2011a). The amounts of microbial AA were calculated by the daily amount of microbial N and the concentration of analysed AA (g/g microbial N) for the SAM and the LAM, respectively. The proportion of AA-N in the total microbial N was calculated by consideration of the molar proportion of N in individual AA.

Data were statistically analysed by using the MIXED procedure of the software package SAS for Windows (version 9.1.3, SAS Institute, Cary, NC, USA). A two-factorial ANOVA was done for the two fixed effects silage and MSS. In case of significant interactions ($P<0.05$), the treatments were additionally compared by t-test. The effect of urea supply was tested separately by a one-factorial ANOVA of the 4 mm-maize silage treatments. The option KENWARDROGER was included in the model statement and the experimental period was considered as random effect.

3. Results

3.1 Experiment 1

The degradation of OM, CF, aNDFom, ADFom and NSC after 48 h was significantly higher in grass silage than in maize silage, but no significant effects of MSS were detected (Table 2). In contrast, the degradation of CP, on average, was higher in maize silage than in grass silage (74.3 vs. 69.5 %), and 4 mm-MSS led to a higher degradation of CP than 1 mm-MSS. Because

of differences in dietary CP content, the total amount of degraded CP was higher in grass silage than in maize silage. This was accompanied by a higher ammonia-N concentration in the effluent of grass silage than of maize silage (P<0.001), but no significant effect of MSS was detected.

Several interactions of silage and MSS became obvious in SCFA production. An effect of silage on acetate and butyrate production was only detected with 4 mm-MSS, whereas propionate was only affected by silage with 1 mm-MSS. Acetate, propionate and total SCFA production were higher with 4 mm-MSS than with 1 mm-MSS for grass silage, but there was no effect of MSS in maize silage. In contrast, the production of butyrate was higher with 4 mm-MSS than with 1 mm-MSS in maize silage, but no effect of MSS was detected for grass silage. Production of iso-valerate and valerate in general was higher in maize silage than in grass silage, whereas the production of iso-butyrate was higher at incubation of grass silage. Significant main effects of MSS were found for iso-butyrate and valerate. A large discrepancy was found for the efficiency of microbial net protein synthesis (EMPS), which averaged 27 and 46 mg microbial N flow per g of degraded OM for maize silage and grass silage, respectively. EMPS was higher with 4 mm-MSS than with 1 mm-MSS. The ratio of SAM-N to LAM-N was higher in grass silage than in maize silage (0.35 vs. 0.20) and was higher with 1 mm-MSS than with 4 mm-MSS (0.34 vs. 0.22).

The AA pattern of microbial protein, calculated from the sum of daily AA originating from the SAM and LAM fraction, was significantly affected by the kind of forage and, to a lesser extent, by MSS (Table 3). Concentrations of arginine, glycine, isoleucine, methionine and phenylalanine in microbial protein were higher after incubation of grass silage, whereas the proportions of lysine and threonine were significantly higher after maize silage incubation. The proportions of arginine and threonine were significantly higher with 4 mm-MSS than with 1 mm-MSS. Further differences between 1 and 4 mm-MSS (P<0.10) were detected for the proportion of cystine, glycine, isoleucine and serine.

3.2 Experiment 2

Degradation of nutrient fractions was higher in grass silage than in maize silage, with exception of CP (Table 4). Addition of urea-N to maize silage led to an increase in degradation of OM, primarily caused by an improved degradation of NSC and starch. The 9 mm-MSS benefited the fermentation of OM, CP and NSC, compared to the 4 mm-MSS. Interactions between silage and MSS were detected for fibre fractions. Degradation of CF,

aNDFom and ADFom was similar between both MSS in maize silage, but higher with 9 mm-MSS than with 4 mm-MSS in grass silage. Degradation of starch was only measured for maize silage and was higher for 9 mm-MSS than for 4 mm-MSS (P=0.08). Ammonia-N in the daily effluents was highest in urea treatments, and higher in grass silage than in maize silage without N supplementation. No significant effect of MSS on ammonia-N was detected. Total SCFA production averaged 34.6 and 40.9 mmol/d for maize silage and grass silage, respectively (Table 4). This difference was primarily caused by an increase in acetate production, which averaged 12.7 and 20.5 mmol/d for maize silage and grass silage, respectively. There was also found a significant effect of silage on iso-butyrate, iso-valerate and valerate. An addition of urea to maize silage led to an increase in the production of total SCFA, acetate, propionate and iso-valerate. Supplementing urea to maize silage via buffer solution resulted in a higher production of acetate and iso-valerate than supplementing urea to the feeding bag. A higher production of total SCFA, acetate, iso-butyrate and iso-valerate was measured with 9 mm-MSS than with 4 mm-MSS, whereas valerate was unaffected by MSS. Significant interactions of silage and MSS were detected for propionate and butyrate. Daily microbial N as well as EMPS were highest in grass silage and were significantly increased by urea supplementation to maize silage. However, the way of urea supply did not affect microbial N production. Furthermore, the amount of microbial N was higher with 9 mm-MSS than with 4 mm-MSS, but no effect of MSS on EMPS was detected. The ratio of SAM-N to LAM-N was higher in grass silage than in maize silage (0.29 vs. 0.23), but no significant effects of MSS and urea supply were detected.

4. Discussion

4.1 Milling screen size

The results of the present study indicate that coarse milling of silage by a cutting mill promotes microbial fermentation in a Rusitec more than fine milling. The degradation of CP and NSC was improved by an increase in MSS, as also indicated by higher SCFA production at coarse milling compared to finer milling for both types of silage. The increase in production of valerate and iso-valerate by coarse milling supports the measurements of increased CP degradation. Results of Experiment 1 are in accordance with the study of Hildebrand et al. (2011b), indicating a higher fermentation of silage-based diets in a Rusitec with 4 mm-MSS than with 1 mm-MSS. Unlike general expectation, the increase in MSS to 9 mm led to a further increase in degradation of OM, NSC and CP, as well as in SCFA

production in both types of silage, and an increase in degradation of fibre fractions in grass silage. Significant differences in fermentation characteristics between 4- to 9-mm treatments were found more often for grass silage than for maize silage; this observation might be attributed to the small difference in bulk density of maize silage (Table 1).

The increase in MSS seemed to have improved growth conditions of some microbial groups. Particle size distribution of the coarse milled treatments was more similar to that in the native rumen and might therefore have offered a more adequate environment for the microbial community than homogenously fine milled plant tissues. Fine milling of feedstuffs is reported to benefit fermentation by bacteria rather than coarse milling (Zhang et al. 2007). As shown by Rodríguez-Prado et al. (2004), the daily bacterial N flow was increased by fine milling in continuous culture fermenters; however, the digestibility of nutrients was not affected. This is in contrast to the present results, indicating an increase in bacterial mass with an increase in MSS. This increase in bacterial N flow might be explained by the increase in the degradation of CP (Stokes et al. 1991). Furthermore, the increased CP degradation by coarse milling might have affected the use of different N sources and thus the composition of bacterial mass, as indicated by small changes in the AA profile (Table 3). Differences in ^{15}N incorporation between bacterial fractions indicated a preferred use of ^{15}N from the liquid phase of coarse milled treatments, and an effect of better flushing of the feed bag was discussed (Hildebrand et al. 2011a). Moreover, the increase in the SAM to LAM ratio by fine milling (Table 2) suggested a change in the composition of bacterial mass. As shown in studies of Witzig et al. (2010a, 2010b), the composition of LAM was affected by milling treatments. A variation in MSS influenced the bacterial community structure of the ruminal *Firmicutes* and the *Bacteroides-Prevotella* group in a Rusitec, when maize silage- and grass silage-based diets were incubated.

Although the number of protozoa in a Rusitec usually decreases extensively during incubation, this microbial group might have played a role in the present study (Czerkawski and Breckenridge 1977, Carro et al. 1995). As shown by Hoover et al. (1976), fine milling reduced the number of protozoa and the SCFA production in continuous culture. As reported by Zhang et al. (2007), an increase in mean particle size seems to benefit fungi and a mixture of fungi and protozoa in the absence of bacteria. Especially high-fibre diets and the availability of vascular stem tissues as well as large feed particles seem to promote growth conditions of fungi (Bauchop 1989, Akin 1993). As fungi seem to play an important role in fibre digestion (Bauchop 1989), this would indicate an expected increase in the degradation

of fibre fractions with increasing MSS. However, the present results do not support this hypothesis consistently, as the degradation of fibre fractions was improved significantly only in grass silage due to the increase in MSS from 4 to 9 mm.

4.2 Forage source and urea supply

A significant effect of forage source was found for nearly all fermentation characteristics in both experiments. The degradation of OM, fibre fractions and NSC as well as microbial N flow was higher in grass silage than in maize silage. This confirms results by Hildebrand et al. (2011a, 2011b), who used the same types of silage but incubated them together with soybean meal. Total SCFA production was higher in grass silage than in maize silage in Experiment 2 and can be attributed to the higher difference in the amount of degraded fibre between forages than in Experiment 1 (data not shown). Based on the results of Hildebrand et al. (2011b), a lower degradation of CP in grass silage than in maize silage was not expected. Consequently, this observation indicates that the degradability of CP originating from maize silage was higher at incubation of pure silage than at incubation together with an additional protein source or that the CP degradability of soybean meal was affected by forage source in the study of Hildebrand et al. (2011b).

The release of NH_3-N increases nearly proportionally with urea supplementation in the Rusitec system (Czerkawski and Breckenridge 1982). It was hypothesised that this increase in NH_3-N might improve microbial N flow and carbohydrate fermentation in maize silage. As also reported in previous studies (Windschitl and Stern 1988, Griswold et al. 2003), an increase in degradation rate was observed for NSC when urea was supplemented, accompanied by an increase in total SCFA production, particularly in acetate production. These results suggest that the fermentation of NSC in maize silage was limited by the availability of NH_3-N. In accordance with Griswold et al. (2003) and Windschitl and Stern (1988), the proportion of valerate was higher when no urea was added ($P<0.05$, data not shown), indicating differences in the use of the N source by microbes. Contrary to the results of Windschitl and Stern (1988) and Griswold et al. (2003), urea supply did not improve fibre degradation, although the increase in NH_3-N was expected to benefit structural carbohydrate degrading microbes (Russell et al. 1992). This lack in the degradation of fibre fractions might result from of a deficit in adequate N sources. Several studies indicated that N forms other than NH_3-N are needed for maximum fibre digestion. The degradation of NSC was highest with urea supplementation and the degradation of ADF and NDF highest with peptide

supplementation in the study of Griswold et al. (1996). An improved degradation of fibre fractions in the Rusitec system was also found by Carro and Miller (1999) when AA or protein were supplemented instead of NH_3-N. However, replacing urea N with peptide N by more than 10% led to a decrease in the degradation of OM, NDF and ADF in the continuous culture study of Jones et al. (1998), indicating a negative effect of high peptide supply on fibre digesting microbes using NH_3-N. Another reason for the apparent inhibition of fibrolytic activity in maize silage treatments might have been the availability of starch (Grant and Mertens 1992) and the accumulation of fermentation products (Joblin and Naylor 1993). The latter might also explain the overall low level of fibre degradation and the low acetate-to-propionate ratio in both experiments.

In accordance with other researchers (Czerkawski and Breckenridge 1982, Windschitl and Stern 1988, Griswold et al. 2003), the supply of urea led to an increase in microbial N flow and EMPS. The amount of available N (>32 g available N/kg DM, calculated as sum of N originating from buffer solution and degraded dietary CP) was assumed to be sufficient in both urea treatments as levels of available N above 24 g/kg DM were reported to no further increase bacterial N flow (Satter and Slyter 1974, Windschitl and Stern 1988). *In vivo* even lower levels of degradable dietary N (17 g/kg DM) for maximal microbial N flow and EMPS were reported by Boucher et al. (2007) when a maize silage-based diet was supplemented with urea at several levels. However, results of *in vitro* and *in vivo* studies have to be compared carefully, as rumino-hepatic circulation cannot be simulated completely in continuous culture.

The amount of available N was highest in urea treated maize silage, but the microbial N flow and EMPS were highest in grass silage. This observation supports the hypothesis that other factors than available N *per se* are responsible for an improvement in microbial protein synthesis in the Rusitec system. In accordance with the continuous culture studies of Bach et al. (1999) and Castillejos et al. (2005), the diet with a high NSC concentration led to a lower EMPS than a diet high in detergent fibre fractions and CP. Stokes et al. (1991) reported an increase in microbial N flow and EMPS induced by a decrease in the ratio of dietary NSC to degradable CP. This ratio was 5.5, 2.0 and 1.2 for maize silage, urea-supplemented maize silage and grass silage, respectively, and thus might partly explain the observed differences in microbial N flow. As shown by Windschitl and Stern (1988), a stepwise increase in available N from 11 to 23 g/kg DM due to urea supply led to a quadratic effect on the daily bacterial N flow and to a linear increase in EMPS. Maybe the supply of protein-N instead of

urea-N to maize silage would have led to a further increase in microbial protein flow and degradation of starch (Meng et al. 2000) as well as in EMPS (Carro and Miller 1999). However, no significant difference between urea-N and protein-N in regard to microbial N flow and EMPS were reported by Griswold et al. (1996).

Two ways of urea-N administration were compared in the present study. No significant differences between single dose and continuous infusion of urea were found in the measured characteristics of fermentation with the exception of the acetate-to-propionate ratio. These results indicate that the effect of the time pattern of urea-N supply on the fermentation of maize silage was negligible at the present level of available N. Effects of the way of urea administration might occur at lower N levels; however, no differences in DM digestibility, SCFA production and microbial protein flow between urea supply via bag or buffer solution up to 7 mg urea-N/g DM were found in the Rusitec study of Czerkawski and Breckenridge (1982). As concluded by Henning et al. (1991), the time pattern of energy supply is more important than the time pattern of N supply for maximising microbial growth *in vitro*.

5. Conclusions

The present study suggests that an increase in mean feed particle size of oven-dried silages, by increasing the MSS, improves the simulation of several fermentation characteristics in a Rusitec system. However, possible underlying mechanisms still have to be clarified. The degradation of OM as well as the efficiency of utilising the available nutrients for microbial protein synthesis is higher in grass silage than in maize silage in the Rusitec system. The fermentation of NSC in maize silage and the respective microbial N flow is impaired when availability of NH_3-N is limited. The way of urea supplementation, singly dosed or continuously infused, has only marginal effects on fermentation characteristics. Further research on the optimal level of NH_3-N and the identification of limiting factors on fermentation characteristics in the Rusitec system is needed.

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References

Akin DE. 1993. Forage cell wall structure and digestibility. Madison, Wisconsin, USA: American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc. Chapter 3, Perspectives of cell wall biodegradation; 73-82.

Bach A, Yoon IK, Stern MD, Jung HG, Chester-Jones H. 1999. Effects of type of carbohydrate supplementation to lush pasture on microbial fermentation in continuous culture. J Dairy Sci. 82:153-160.

Bauchop T. 1989. Biology of gut anaerobic fungi. Biosystems. 23:53-64.

Boguhn J, Kluth H, Rodehutscord M. 2006a. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. J Dairy Sci. 89:1580-1591.

Boguhn J, Kluth H, Rodehutscord M. 2006b. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. J Dairy Sci. 89:1592-1603.

Bossen D, Mertens DR, Weisbjerg MR. 2008. Influence of fermentation methods on neutral detergent fiber degradation parameters. J Dairy Sci. 91:1464-1476.

Boucher SE, Ordway RS, Whitehouse NL, Lundy FP, Kononoff PJ, Schwab CG. 2007. Effect of incremental urea supplementation of a conventional corn silage-based diet on ruminal ammonia concentration and synthesis of microbial protein. J Dairy Sci. 90:5619-5633.

Carro MD, Lebzien P, Rohr K. 1995. Effects of pore size of nylon bags and dilution rate on fermentation parameters in a semi-continuous artificial rumen. Small Ruminant Res. 15:113-119.

Carro MD, Miller EL. 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semi-continuous culture system (RUSITEC). Br J Nutr. 82:149-157.

Castillejos L, Calsamiglia S, Ferret A, Losa R. 2005. Effects of a specific blend of essential oil compounds and the type of diet on rumen microbial fermentation and nutrient flow from a continuous culture system. Anim Feed Sci Technol. 119:29-41.

Cherdthong A, Wanapat M. 2010. Development of urea products as rumen slow-release feed for ruminant production: A review. Aust J Basic Appl Sci. 4:2232-2241.

Czerkawski JW, Breckenridge G. 1977. Design and development of a long-term rumen simulation technique (Rusitec). Br J Nutr. 38:371-384.

Czerkawski JW, Breckenridge G. 1982. Distribution and changes in urease (EC 3.5.1.5) activity in Rumen Simulation Technique (Rusitec). Br J Nutr. 47:331-348.

Geissler C, Hoffmann M, Hickel B. 1976. Ein Beitrag zur gaschromatographischen Bestimmung flüchtiger Fettsäuren. Arch Tierernaehrg. 26:123-129.

Grant RJ, Mertens DR. 1992. Influence of buffer pH and raw corn starch addition on in vitro fiber digestion kinetics. J Dairy Sci. 75:2762-2768.

Griswold KE, Hoover WH, Miller TK, Thayne WV. 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. J Anim Sci. 74:483-491.

Griswold KE, Apgar GA, Bouton J, Firkins JL. 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. J Anim Sci. 81:329-336.

Henning PH, Steyn DG, Meissner HH. 1991. The effect of energy and nitrogen supply pattern on rumen bacterial growth in vitro. Anim Prod. 53:165-175.

Hildebrand B, Boguhn J, Rodehutscord M. 2011a. Effect of maize silage to grass silage ratio and feed particle size on protein synthesis and amino acid profile in different microbial fractions in a semi-continuous rumen simulation. Animal. 5:537-546.

Hildebrand B, Boguhn J, Rodehutscord M. 2011b. Effect of maize silage to grass silage ratio and feed particle size on ruminal fermentation *in vitro*. Animal. 5:528-536.

Hoover WH, Knowlton PH, Stern MD, Sniffen CJ. 1976. Effects of differential solid-liquid removal rates on fermentation parameters in continuous cultures of rumen contents. J Anim Sci. 43:535-542.

Joblin KN, Naylor GE. 1993. Inhibition of the rumen anaerobic fungus Neocallimastix frontalis by fermentation products. Lett Appl Microbiol. 16:254-256.

Jones DF, Hoover WH, Miller Webster TK. 1998. Effects of concentrations of peptides on microbial metabolism in continuous culture. J Anim Sci. 76:611-616.

McDougall EI. 1948. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem J. 43:99-109.

Meng QX, Xia ZG, Kerley MS. 2000. The requirement of ruminal degradable protein for non-structural carbohydrate-fermenting microbes and its reaction with dilution rate in continuous culture. Asian-Australas J Anim Sci. 13:1399-1406.

Menke KH, Steingass H. 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim Res Dev. 28:7-55.

Niderkorn V, Baumont R. 2009. Associative effects between forages on feed intake and digestion in ruminants. Animal. 3:951-960.

Robles AY, Belyea RL, Martz FA, Weiss MF. 1980. Effect of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchardgrass forages. J Anim Sci. 51:783-790.

Rodehutscord M, Kapocius M, Timmler R, Dieckmann A. 2004. Linear regression approach to study amino acid digestibility in broiler chickens. Br Poult Sci. 45:85-92.

Rodríguez-Prado M, Calsamiglia S, Ferret A. 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. J Dairy Sci. 87:1413-1424.

Russell JB, O'Connor JD, Fox DG, Van Soest PJ, Sniffen CJ. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J Anim Sci. 70:3551-3561.

Satter LD, Slyter LL. 1974. Effect of ammonia concentration on rumen microbial protein production in vitro. Br J Nutr. 32:199-208.

Stokes SR, Hoover WH, Miller TK, Manski RP. 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. J Dairy Sci. 74:860-870.

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci. 74:3583-3597.

Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten, VDLUFA. 2006. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III Die chemische Untersuchung von Futtermitteln. Darmstadt, Germany: VDLUFA-Verlag.

Windschitl PM, Stern MD. 1988. Effects of urea supplementation of diets containing lignosulfonate-treated soybean meal on bacterial fermentation in continuous culture of ruminal contents. J Anim Sci. 66:2948-2958.

Witzig M, Boguhn J, Kleinsteuber S, Fetzer I, Rodehutscord M. 2010a. Effect of the corn silage to grass silage ratio and feed particle size of diets for ruminants on the ruminal *Bacteroides-Prevotella* community *in vitro*. Anaerobe. 16:412-419.

Witzig M, Boguhn J, Kleinsteuber S, Fetzer I, Rodehutscord M. 2010b. Influence of the maize silage to grass silage ratio and feed particle size of rations for ruminants on the community structure of ruminal Firmicutes in vitro. J Appl Microbiol. 109:1998-2010.

Zhang Y, Gao W, Meng Q. 2007. Fermentation of plant cell walls by ruminal bacteria, protozoa and fungi and their interaction with fibre particle size. Arch Anim Nutr. 61:114-125.

Table 1 Fermentation characteristics, nutrient composition, amino acid contents and bulk density of maize silage and grass silage

	Maize silage	Grass silage
Fresh forage characteristics		
Dry matter (DM, g/kg)	350	380
pH	3.7	4.2
Acetic acid (g/kg DM)	25.1	6.2
Propionic acid (g/kg DM)	0.6	0.3
Butyric acid (g/kg DM)	0.3	0.3
Lactic acid (g/kg DM)	56.6	92.2
Ammonia-N (mg/g total N)	116	43
Nutrient composition (g/kg DM)		
Organic matter (OM)	952	874
Crude protein (CP)	93	203
Ether extract (EE)	36	37
Crude fibre (CF)	183	233
Neutral detergent fibre (aNDFom)	398	447
Acid detergent fibre (ADFom)	228	299
Non-structural carbohydrates (NSC)	426	188
Starch	323	n.a.[#]
Amino acids (g/kg DM)		
Alanine	10.0	13.8
Arginine	1.0	9.0
Aspartic acid	3.6	18.3
Cystine	0.6	1.4
Glutamic acid	9.3	17.0
Glycine	3.6	9.3
Isoleucine	3.2	7.6
Leucine	8.8	15.0
Lysine	1.3	9.5
Methionine	1.5	3.4
Phenylalanine	3.3	9.2
Proline	6.3	11.0
Serine	2.8	7.2
Threonine	3.2	8.3
Valine	4.5	10.3
Bulk density (g/100 ml)		
1 mm[†]	27.0	33.8
4 mm[†]	22.6	19.4
9 mm[†]	20.8	14.0

Notes: [#]n.a., Not analysed; [†]Milling screen size

Table 2 Experiment 1: Degradation of nutrients after 48 h of incubation, ammonia-N and short chain fatty acids (SCFA) in the effluent and microbial N flow; LSMeans and pooled standard error (SE), n ≥ 3

Silage	Maize silage		Grass silage		SE	*P* values		
MSS[#]	1 mm	4 mm	1 mm	4 mm		Silage	MSS	Silage × MSS
Degradation (%)								
OM[†]	38.6	40.4	44.5	46.3	1.40	**	0.29	0.99
CP[†]	70.7	78.3	67.2	72.1	1.43	**	**	0.30
CF	9.1	7.9	18.1	13.5	2.06	**	0.25	0.28
aNDFom	8.9	7.4	21.0	18.6	1.53	***	0.31	0.69
ADFom	1.6	-2.3	19.4	11.6	2.77	***	0.09	0.41
NSC	59.4	62.3	76.5	84.0	3.02	***	0.12	0.46
Ammonia-N (mg/d)	49	52	154	158	4.56	***	0.41	0.85
SCFA (mmol/d)								
Total	35.4^{bc}	38.8^{ab}	31.9^{c}	41.0^{a}	1.88	0.57	*	*
Acetate	14.6^{b}	15.2^{b}	15.1^{b}	19.9^{a}	1.20	**	0.05	*
Propionate	10.4^{a}	10.1^{a}	7.3^{b}	10.5^{a}	0.72	0.10	0.07	*
Iso-butyrate	0.3	0.4	0.6	0.7	0.03	***	**	0.37
Butyrate	6.4^{ab}	7.3^{a}	6.9^{ab}	6.1^{b}	0.39	0.26	0.97	*
Iso-valerate	1.9	2.5	0.9	1.4	0.25	**	0.10	0.67
Valerate	2.2	2.8	1.3	1.9	0.20	**	*	0.76
Acetate:propionate	1.4	1.5	2.1	1.9	0.13	**	0.49	0.28
Microbial N								
mg/d	135	155	248	268	6.41	***	*	0.95
mg/g degraded OM	26.3	28.3	45.2	47.2	0.76	***	*	0.98
SAM:LAM[‡⊥]	0.25	0.16	0.42	0.29	0.02	***	***	0.16

Notes: [#]MSS, Milling screen size; [†]Corrected for contribution of SAM; [‡]SAM, Solid-associated microbes; [⊥]LAM, Liquid-associated microbes; [abc] indicate significant differences between treatments, when an interaction between silage and MSS was detected; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 3 Experiment 1: Proportion of amino acids (% of analysed amino acids) and amino acid-N (% of microbial N) in microbial protein[#]; LSMeans and pooled standard error (SE)

Silage	Maize silage		Grass silage		SE	*P* values		
MSS[†]	1 mm	4 mm	1 mm	4 mm		Silage	MSS	Silage × MSS
Alanine	9.19	9.19	9.09	9.18	0.13	0.57	0.82	0.65
Arginine	5.49[d]	5.73[b]	5.63[c]	6.01[a]	0.24	***	***	*
Aspartic acid	12.67	12.70	12.73	12.63	0.19	0.96	0.79	0.47
Cystine	1.20	1.06	1.12	0.98	0.06	0.08	0.05	0.95
Glutamic acid	14.28	14.00	14.07	14.03	0.16	0.33	0.30	0.22
Glycine	5.92	6.07	6.12	6.19	0.07	**	0.08	0.26
Isoleucine	5.62	5.58	5.84	5.61	0.08	*	0.09	0.07
Leucine	8.18	8.23	8.29	8.39	0.11	0.08	0.51	0.69
Lysine	8.40	8.24	8.13	8.00	0.11	**	0.13	0.68
Methionine	2.77	2.76	2.91	2.88	0.05	**	0.59	0.84
Phenylalanine	5.14	5.21	5.34	5.32	0.05	**	0.62	0.21
Proline	3.97	3.82	3.71	3.53	0.22	0.18	0.54	0.94
Serine	4.69	4.81	4.67	4.80	0.11	0.57	0.06	0.83
Threonine	6.09	6.22	6.00	6.06	0.06	**	*	0.15
Valine	6.34	6.39	6.33	6.37	0.06	0.64	0.42	0.91
Amino acid-N	68.2	69.1	66.7	66.3	0.85	*	0.77	0.48

Notes: [†]MSS, milling screen size; [#] sum of solid- and liquid-associated microbes; [abcd] indicate significant differences between the treatments, when an interaction between silage and MSS was detected; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 4 Experiment 2: Degradation of nutrients after 48 h of incubation, ammonia-N and short chain fatty acids (SCFA) in the effluent and microbial N flow; LSMeans and pooled standard error (SE), n ≥ 3

Silage	Maize silage				Grass silage		SE	*P* values			
MSS[#]	4 mm	4 mm + urea (bag)	4 mm + urea (buffer)	9 mm	4 mm	9 mm		Urea supply	Silage	MSS	Silage × MSS
Degradation (%)											
OM[†]	41.6^{B}	45.7^{A}	46.7^{A}	42.7	49.9	53.4	1.12	*	***	**	0.07
CP[†]	78.0	80.2	82.6	82.7	72.0	74.6	1.52	0.25	***	**	0.28
CF	3.8^{c}	5.6	4.9	2.3^{c}	18.4^{b}	23.9^{a}	1.08	0.51	***	0.09	**
aNDFom	13.1^{c}	13.6	14.7	12.0^{c}	27.9^{b}	31.7^{a}	0.89	0.50	***	0.09	**
ADFom	3.5^{c}	2.4	3.5	2.5^{c}	19.6^{b}	24.6^{a}	1.18	0.71	***	0.09	*
NSC	59.9^{B}	66.6^{A}	67.6^{A}	62.0	76.1	81.3	1.55	*	***	**	0.18
Starch	63.9^{B}	73.5^{A}	73.9^{A}	67.9	n.a.[§]	n.a.	1.78	*		0.08	
Ammonia-N (mg/d)	52^{B}	276^{A}	292^{A}	52	154	153	5.00	***	***	0.71	0.43
SCFA (mmol/d)											
Total	32.0^{B}	37.5^{A}	39.1^{A}	37.2	37.8	43.9	0.81	**	***	***	0.54
Acetate	11.2^{B}	13.8^{A}	15.1^{A}	14.2	18.7	22.4	0.37	**	***	***	0.35
Propionate	10.1^{Bb}	12.3^{A}	12.1^{A}	11.3^{b}	10.4^{b}	13.8^{a}	0.38	**	**	***	*
Iso-butyrate	0.38	0.38	0.41	0.42	0.64	0.67	0.01	0.16	***	***	0.34
Butyrate	5.9^{b}	6.4	6.5	6.3^{a}	5.2^{c}	3.8^{d}	0.40	0.68	***	**	***
Iso-valerate	2.0^{B}	2.5^{A}	2.6^{A}	2.7	1.2	1.6	0.10	*	***	***	0.08
Valerate	2.4^{A}	2.1^{B}	2.3^{A}	2.3	1.7	1.7	0.11	*	***	0.10	0.09
Acetate:propionate	1.10^{Bd}	1.13^{B}	1.25^{A}	1.26^{c}	1.79^{a}	1.62^{b}	0.04	**	***	0.85	**
Microbial N											
mg/d	145^{B}	220^{A}	218^{A}	157	253	267	6.20	***	***	**	0.71
mg/g degraded OM	25.9^{B}	35.6^{A}	34.5^{A}	27.3	40.9	40.3	0.78	***	***	0.45	0.07
SAM:LAM[‡⊥]	0.22	0.19	0.19	0.24	0.31	0.27	0.02	0.33	**	0.57	0.07

Notes: [#]MSS, Milling screen size; [†]Corrected for contribution of SAM; [‡]SAM, Solid-associated microbes; [⊥]LAM, Liquid-associated microbes; [§]n.a., Not analysed; ^{AB} indicate significant differences between the 4 mm maize silage treatments; ^{abcd} indicate significant differences between treatments, when interactions between silage and MSS were detected; * P<0.05; ** P<0.01; *** P<0.001

General discussion

Feed evaluation by established methods

The use of maize silage (MS) and grass silage (GS) in ruminant rations is common practice in Germany and other European countries. In the present studies (Paper I-III) only one batch of MS and GS was used in order to allow comparison between studies. It is well-known that silage quality can highly differ between batches and in general a higher variation in chemical composition and nutritional value is reported for GS than for MS, due to a higher variation in the date of harvest and of the botanical diversity (DLG 1997). Furthermore the average chemical composition of silages can highly differ between years of harvest for the same location. Average values of chemical composition in silages produced in Germany in the same year of harvest are presented in Table D-1.

Table D-1 Composition of maize silages and grass silages (without first cut) produced in Germany in 2005; Mean values (Min – Max)

	n	DM g/kg	OM	CP	CF	NDF	ADF	Starch	ME MJ/kg DM
			g/kg DM						
Maize silage									
Own study	1	350	952	93	183	398	228	323	11.0 [1)]
Steinhöfel & Krieg (2006)	801	317	959	80	189	-	-	322	11.0
Leberl (2006)	-	321 (249-435)	963 (896-974)	80 (63-93)	196 (145-257)	390 (298-492)	216 (158-286)	307 (142-449)	10.9 (10.1-11.7)
Dunker (2006)	-	354	962 (898-980)	84 (61-117)	190 (132-250)			327 (89-467)	11.0
Spiekers *et al.* (2006)	3126	328	960	83	204	-	-	-	10.7
Target values [2)]		280-350	≥955	≤90	170-200			≥300	≥10.8
Grass silage									
Own study	1	380	874	203	233	447	299	-	9.8 [2)]
Steinhöfel & Krieg (2006)	628	389	899	157	258	-	-	-	9.7
Leberl (2006)	-	403 (204-755)	889 (773-927)	158 (104-228)	239 (154-305)	466 (347-587)	307 (240-378)	-	9.9 (8.3-10.8)
Dunker (2006)	-	440	897 (731-936)	172 (88-239)	261 (189-342)	-	-	-	9.9
Spiekers *et al.* (2006)	3781	371	891	172	240	-	-	-	9.9
Target values [4)]		300-400	≥900	≤170	220-250	-	-	-	≥10.0

n = number of samples; DM = dry matter; OM = organic matter; CP = crude protein; CF = crude fibre; NDF = neutral detergent fibre; ADF = acid detergent fibre; ME = metabolisable energy;
[1)] calculated according to GfE (1998) by using content of CF and ash; [2)] according to Landwirtschaftskammer Nordrhein-Westfalen (2005); [3)] calculated according to GfE (2008) by using gas production data and content of ash, CP, ether extract and ADF; [4)] according to Pries (2004)

Chemical composition and metabolisable energy (ME) content of MS and GS, used in the own study, was within the range of reports on the silage quality of the year 2005 in Germany (Table-D1). However, it is noticeable that the ash and crude protein (CP) content of the used silage batches were higher than average values, as well as the general target values. An effect of location and fertilisation management can be assumed. Moreover, cutting at a young stage of maturity of the grass might have contributed to the high CP content, as also indicated by the slightly lower content of crude fibre (CF) compared to average values (Table D-1). It was not the primary intention in the present study to use silage batches, which represent the average chemical composition of MS and GS in Germany, but the MS and the GS should significantly differ in their chemical composition in regard to protein value, ME values and fibre fractions. These requirements were met by the current silage batches. Nevertheless, the question arises, to what extent are the results and conclusions from the current studies transferable to other silage batches?

A high variation in silage quality is known to induce a high variation in total tract digestibility coefficients and *in vitro* gas production data. Wide ranges in total tract digestibility of organic matter (OM) from 0.57 to 0.79 and from 0.54 to 0.84 are reported for MS (Givens *et al.* 1995, De Boever *et al.* 1997, Ferret *et al.* 1997) and GS (Givens *et al.* 1989, De Boever *et al.* 1996, Nousiainen *et al.* 2003, Yan and Agnew 2004a), respectively, when silages were fed near to maintenance feeding level. Total tract digestibility of OM in MS is known to be negatively correlated with the content of CF, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin, and positively correlated with the proportion of OM and starch (Givens *et al.* 1995, De Boever *et al.* 1997). Negative correlation coefficients between OM digestibility and content of fibre fractions are also reported for GS, whereas increasing contents of ether extract and soluble CP seem to benefit OM digestibility in GS (De Boever *et al.* 1996, Nousiainen *et al.* 2003, Yan and Agnew 2004a).

Although cumulative gas production in general is higher for MS than for GS, a wide range is reported for both forages (García-Rodriguez *et al.* 2005). The extent of gas production is known to be positively correlated with the proportion of non-fibre carbohydrates in ruminant feeds, whereas negative correlations with the content of dietary fibre fractions and CP were identified (Getachew *et al.* 2004). Accordingly, the amount of degradable starch in MS (Chai *et al.* 2004, De Boever *et al.* 2005), and the amount of CP in grass (Cone *et al.* 1998) were shown to affect gas production. Moreover, for both forages an increase in ADF content was reported to impair the amount of produced gas (García-Rodriguez *et al.* 2005). Because

of high variation in nutrient composition of silages it can not be generally assumed that the stepwise replacement of MS by GS leads to an increase in total tract digestibility of OM or a decrease in cumulative gas production *in vitro*, as shown for the silage batches used in the present study (Paper I).

Diets (M100, M79, M52, M24 and M0) in the present study (Paper I + II) were characterised by a stepwise replacement of MS with GS in the forage portion, averaging 0.88 in the complete diets. Such high forage proportions are not common in diets for high yielding dairy cows (Spiekers *et al.* 2009). But it was aimed to identify possible associative effects of both forages in regard to the nutritional value of forage mixtures. In order to assure certain differences in chemical composition between the five diets, a high proportion of forage was realised in diet formulation, and all dietary factors were kept constant, with exception of MS-to-GS ratio. Consequently, diets were not balanced for energy- and protein value. To provide a certain minimum of 120 g CP per kg dry matter (DM) in diet M100, as advised for total tract digestibility studies (GfE 1991), all five diets contained about 106 g soybean meal per kg DM. Calculated energy values, according to the ME contents of single feedstuffs, were 11.2, 10.9, 10.6, 10.4 and 10.1 MJ ME/kg DM for diet M100, M79, M52, M24 and M0, respectively. However, ME values measured in wether sheep (Paper I) were slightly higher than the calculated values, and similar between treatments (on average 11.5 MJ ME/kg DM). The generally higher level of ME values, measured in wether sheep, may be attributed to a higher ME content of soybean meal than the assumed one (13.8 MJ/kg DM, DLG 1997). As the difference in ME between diet M100 and M0 was smaller for the *in vivo* data (11.6 vs. 11.5 MJ ME/kg DM) than for the predicted ME values, it can be hypothesised that the ME content of GS was actually higher than assumed on the basis of chemical composition and *in vitro* gas production. On the other hand, it cannot be excluded that the estimation of ME in the high GS-diet (M0) by measurements in wether sheep might have been slightly overestimated because of the high CP content of GS (GfE 1991), or that interactions between forage source and soybean meal on digestibility occurred *in vivo*. The latter aspect was not discussed in the present papers, because investigations in the present study focussed on possible associative effects of MS and GS, and not on associative effects of soybean meal and silage.

Comparing gas production data from incubation of pure silages (Hildebrand, unpublished work) and silages with soybean meal inclusion (diet M100 and M0, Paper I) showed a nominal increase in total gas production for GS, but not for MS after 93 hours of incubation (Figure D-1). This observation might indicate an interaction of forage source and supply of

soybean meal. On the one hand the measured values primarily can be attributed to the fact, that the profile of cumulative gas production from soybean meal is more similar to that of MS than to that of GS (Hildebrand, unpublished work).

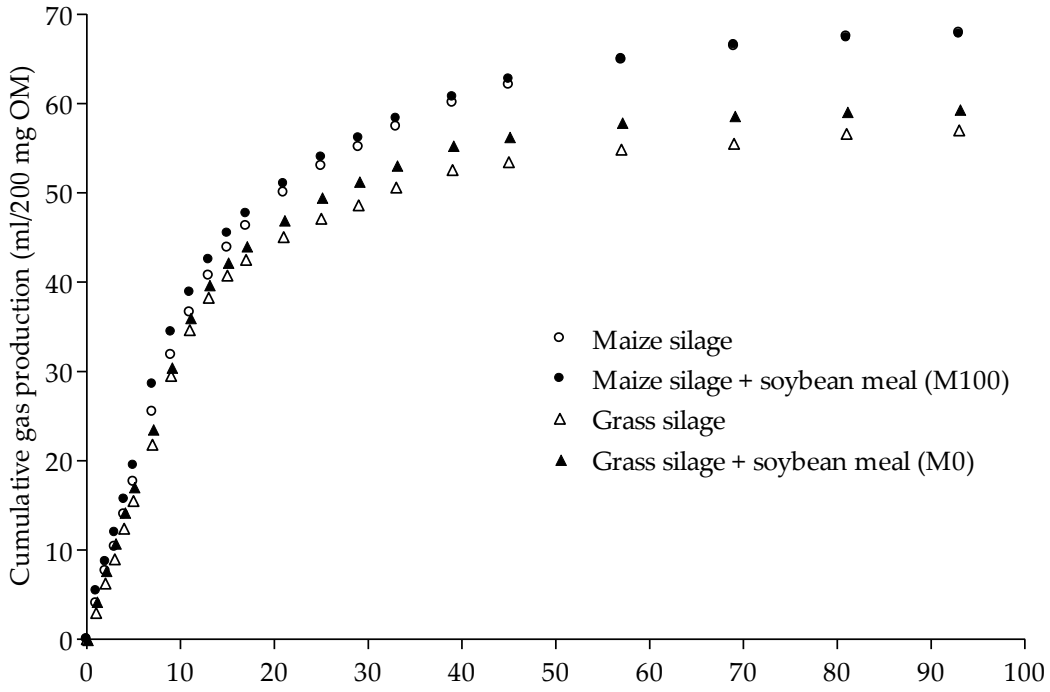

Figure D-1 Effect of soybean meal inclusion to maize silage and grass silage on cumulative gas production during 93 hours of incubation (1 mm screen size; means; n ≥ 8)

On the other hand, associative effects of soybean meal and silage on gas production cannot be excluded when both single feedstuffs are incubated together (Robinson *et al.* 2009). The theoretical gas production profile of feed mixtures can be calculated from data of single feedstuffs. The actual measured values of cumulative gas production were higher than the expected values for diet M100 and M0 in the present study (Table D-2). These data confirm reports by other researchers (Robinson *et al.* 2009, Arhab *et al.* 2010), that associative effects primarily occur in the first 8 hours of incubation. Cumulative gas production of expected and measured values was similar for the MS-based diet (M100) at the end of incubation (93 hours), but was nominally higher for the GS-based diet (M0) and by this giving indication for interactions of forage source and soybean meal inclusion.

Table D-2 Difference between expected values of cumulative gas production (ml/200 mg OM) and actual measured values in diet M100 and M0 (1 mm treatments)

	Time after incubation started (hours)						
	2	4	8	12	24	48	93
M100							
expected [1)]	8.3	14.7	29.0	38.8	52.4	62.7	67.5
measured	+ 5.4%	+ 7.0%	+ 8.8%	+ 4.8%	+ 1.6 %	+ 1.1%	+ 0.3%
M0							
expected [1)]	7.1	13.4	26.4	36.9	47.4	54.7	57.8
measured	+ 8.9%	+ 6.7%	+ 2.6%	+ 2.8%	+ 3.2%	+ 3.7%	+ 2.7%

[1)] calculated from gas production of single feedstuffs and the respective proportions in diet M100 and M0

The mixed diets were investigated by using different milling screen sizes (MSS) in the modified Hohenheim gas production test. It was shown that an increase in MSS impaired the general level of cumulative gas production and delayed the occurrence of maximal gas production rate (Paper I). These results confirmed other reports, that an increase in mean particle size, irrespective of forage composition, leads to a reduction in the extent and the rate of fermentation in batch culture systems (Menke and Steingass 1988, Ferreira and Mertens 2005, Bossen *et al.* 2008).

Nutrient degradation in the rumen simulation technique (RUSITEC)

Organic matter

In the present RUSITEC studies it was shown that degradation of OM was higher in GS-based diets than in MS-based diets (Paper I), and similar results were shown by incubation of pure silages (Paper III). For discussion of OM degradation it has to be considered that composition of OM highly varied between MS and GS (Table D-1), and the contribution of the individual nutrient fractions has to be discussed separately (Figure D-2). As continuous culture systems are not practical for routine forage evaluation, information on the effect of silage quality on ruminal fermentation characteristics of MS and GS in continuous culture is rare in literature. The incubation of several batches of MS and GS via nylon bags in the rumen showed a high variation in degradability of DM for both forage sources (Von Keyserlingk *et al.* 1996). As ruminal and total tract digestibility of fibre fractions are closely related, correlations between total tract digestibility of OM and content of fibre fractions as described previously, can be assumed to be similar for ruminal OM digestibility of a forage

source. Accordingly, negative correlations between ruminal DM degradation and the content of detergent fibre fractions were reported for GS (Yan and Agnew 2004b, Jančík *et al.* 2009) and MS (Ferret *et al.* 1997, De Boever *et al.* 2005) *in situ*. Moreover high proportions of soluble CP in GS contribute to OM degradation (Von Keyserlingk *et al.* 1996, Yan and Agnew 2004b). Besides forage source, the degradation of OM was affected by MSS and was improved by increasing MSS and bulk volume of feed samples (Figure D-2).

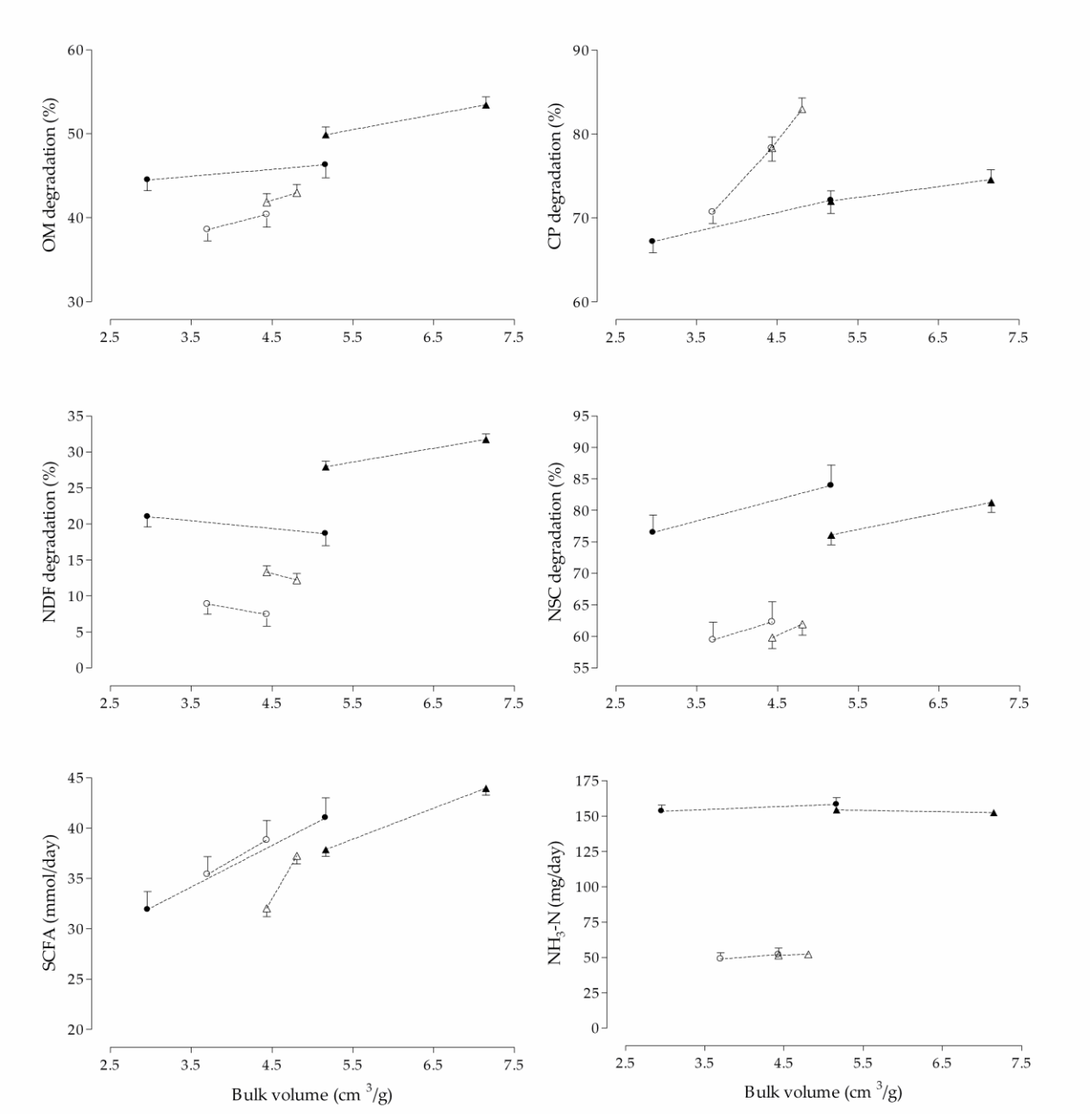

Figure D-2 Effect of bulk volume on ruminal fermentation characteristics of maize silage (○Δ) and grass silage (●▲) in the RUSITEC system measured in two laboratories (Halle/S. ○●; Hohenheim Δ▲) (LSMeans, SE, $n \geq 3$; Paper III)

This was observed for mixed diets (Paper I) and pure silages (Paper III). In accordance with the present study, nominally, but not significantly, higher values of OM degradation in continuous culture fermenters were reported for coarse milled (3 mm MSS) than for fine milled (1 mm MSS) alfalfa hay stems by Rodríguez-Prado *et al.* (2004). Variations in feed particle size were shown to benefit degradation of DM by the main microbial groups (bacteria, protozoa, fungi) in different ways *in vitro* (Zhang *et al.* 2007). Particularly anaerobe fungi were discussed to have an invasive growth form that should allow them to effectively invade and degrade large plant particles (Orpin 1974, Joblin *et al.* 1989, Akin 1993). However, no investigations on the presence of protozoa and fungi were performed in the present RUSITEC study, and therefore a discussion on the effect of these microorganisms on the measured fermentation characteristics can only be hypothetical. Continuous flow culture systems are particularly suitable to investigate bacterial growth (Stern *et al.* 1997). However, growth conditions of protozoa were shown to be disadvantageous in the RUSITEC system (Carro *et al.* 1995, Martínez *et al.* 2010b) and the dual flow system (Mansfield *et al.* 1995) compared to the rumen. Information on the role of anaerobe fungi in the RUSITEC system are rare in the literature. Several anaerobe fungi strains (*Neocallimastix, Piromyces,* and *Caecomyces*) were shown to be viable in RUSITEC fermenters (Kostyukovsky *et al.* 1995) and similar numbers of fungal cells were measured in a dual-continuous flow system and *in vivo* by Mansfield *et al.* (1995). Therefore it is likely that anaerobe fungi also played a role in OM degradation in the present study.

The OM degradation was corrected for microbial contribution of OM in the feed residues according to Boguhn *et al.* (2006b). For this correction a constant ash content of 12% in the solid-associated microbes (SAM) was assumed. Moreover, the contribution of carbohydrates and lipids of bacterial origin was neglected. Bacterial contents of ash were shown to vary between dietary treatments (Legay-Carmier and Bauchart 1989), and therefore possible differences in the ash content of the SAM between MS- and GS-treatments were not considered in the own study. Contents of total lipids in the DM of SAM were reported to range from 17 to 26% (Merry and McAllan 1983, Legay-Carmier and Bauchart 1989, Rodríguez *et al.* 2000) and an average proportion of 5% total carbohydrates was reported by Merry and McAllan (1983). Consequently, the degradation of dietary OM might have been underestimated in the present study.

Crude protein

Because of a high CP content in the presently used GS (203 g/kg DM), a high proportion of rumen degradable CP can be assumed (Hoffman *et al.* 1999, Yan and Agnew 2004b). However, no extra analyses on the soluble and degradable protein fractions in silages (Licitra *et al.* 1996) were performed in the present studies. A mean value of 0.82 for *in situ* CP degradability in GS was reported by Hoffman *et al.* (1999) and Yan and Agnew (2004b). In the German feed evaluation system mean values of 0.80-0.85 and 0.75 are assumed for potential ruminal degradability of CP (calculated from the given values of undegradable protein) in GS and MS, respectively (DLG 1997). Degradation of CP, measured in the RUSITEC within 48 hours (Paper III, experiment 1) averaged 0.69 and 0.74 for GS and MS, respectively (Figure D-2). Similar values (0.63 vs. 0.73) were reported for effective CP degradability in GS and MS *in situ* (Von Keyserlingk *et al.* 1996). As CP content was more than two times higher in GS than in MS, the higher amounts of degraded CP in GS than in MS contributed to degradation of OM in mixed diets (Paper I). Accordingly the amount of NH_3 in the effluent was higher in GS than in MS (Figure D-2) and the respective mixed diets.

The CP degradation of diets M100 and M0 averaged 56% and 57%, respectively (Paper I). However, degradation of CP in pure MS and GS averaged 74% and 69%, respectively (Paper III, experiment 1). As discussed in Paper III an interaction of soybean meal inclusion and forage source on CP degradation might have occurred. This hypothesis was supported by some further investigations, not published in the present papers. Soybean meal was incubated as pure feedstuff in the RUSITEC system, and degradability of CP averaged 57% (SD = 2.4; n = 5; Hildebrand, unpublished work). As CP content of MS, GS and soybean meal was 9.3%, 20.3% and 44.1%, respectively, CP degradation rates of 68% and 66% for diet M100 and M0, respectively, were expected in theory, and thus indicating a negative associative effect of soybean meal and silage on CP degradation in mixed diets. It can be assumed that microbial community in fermenters that were fed only with soybean meal differed from that fed high-forage diets, and therefore non-additivity of fermentation characteristics of single feedstuffs was not surprising. It was hypothesised in the present studies (Paper I-III) that microbial community was affected by MSS, and a benefiting effect of increasing feed particle size on growth conditions of anaerobe fungi was discussed (Akin 1993). Particularly the degradation of dietary CP was improved by increasing MSS (Paper I + III) and proteolytic activity of ruminal fungi, belonging to the genus *Neocallimastix* and *Piromyces*, was reported by Wallace and Joblin (1985) and Asao *et al.* (1993).

Fibre fractions

Fibre degradation was highly affected by forage source (Paper I + III), and was higher in GS-based than in MS-based diets. This observation was in accordance with the total tract digestibility of CF (r = 0.84) measured in wether sheep (Paper I). However, the general level of fibre degradation in MS- and GS-based diets was much lower in the RUSITEC studies, and near to zero for ADF degradation in MS-based diets. Degradation of NDF in MS- and GS-based diets was below 15% and 32%, respectively, and therefore significantly lower than values reported *in situ* and *in vivo* (Van Vuuren *et al.* 1999, De Boever *et al.* 2002, Jensen *et al.* 2005, Juniper *et al.* 2008, Owens *et al.* 2009). A low degradation of NDF (27%) in the RUSITEC system was also reported by Carro *et al.* (2009), but no differences between a high and a low forage diet were detected. Accordingly a lower proportion of cellulolytic bacteria in total bacteria was reported for the RUSITEC system compared to measurements in the rumen (Martínez *et al.* 2010b). A methodical aspect that might have contributed to the low level of fibre degradation is procedure of daily feeding. In order to change the nylon bags the vessel had to be opened, and a negative effect of oxygen input on anaerobe bacteria can not be excluded in the RUSITEC system (Gizzi *et al.* 1998).

The low level of degradation in fibre fractions was discussed in Paper I, however no clear responsible factors could be pointed out. A deficit in adequate N sources was discussed, but NH_3-N was shown to have not limited fibre degradation in MS (Paper III). The results of Atasoglu *et al.* (2001) indicated that cellulolytic bacteria incorporate pre-formed AA and that AA stimulate the growth of these bacteria. Accordingly a higher degradation of fibre fractions was shown when non-ammonia-N sources were supplemented instead of NH_3-N (Griswold *et al.* 1996, Carro and Miller 1999).

As discussed in Paper III the accumulation of fermentation products might have impaired the fibrolytic activity, and hence degradation of fibre fractions. A decrease in concentration of fermentation products in the RUSITEC fermenters can be achieved by an increase in dilution rate of buffer solution (Czerkawski and Breckenridge 1977). The dilution rate of the present Rusitec studies was similar between experiments and averaged 0.68 (daily flow/fermenter volume). Carro *et al.* (1995) compared dilution rates of 0.55 and 0.84 in a RUSITEC system, however degradation of dietary NDF was unaffected. In contrast an increase in dilution rate from 0.91 to 1.30 resulted in an increase in degradation of DM, NDF and ADF, as well as in short chain fatty acid (SCFA) production and xylanase activity in the RUSITEC study of Martínez *et al.* (2009). In contrast to the study of Carro *et al.* (1995) buffer

solutions differed in concentrations of salt in order to maintain similar pH values at 6.55 in the study of Martínez *et al.* (2009). Hoover *et al.* (1984) compared different dilution rates in continuous culture fermenters at different pH values. Degradation of nutrients was rather affected by pH value than by dilution rate, and pH of 6.5 was found to be optimum for fibre and OM degradation. PH values in the own study ranged between 6.5 and 6.8, and increased with increasing proportion of GS in the diet diet. However, pH was only measured once daily before feeding and fluctuations in pH during 24 h were not detected. Values can be assumed to decrease immediately after feeding, and the difference to the pH value measured before feeding was reported to average 0.13 during the first 9 h after feeding when a high concentrate diet was fed (Martínez *et al.* 2009).

As discussed above the growth conditions of anaerobe fungi might have been improved by increasing MSS and mean feed particle size. This microbial group is known to be involved in degradation of fibre fractions. As reviewed by Bauchop (1989) large populations of anaerobic fungi colonise plant fragments from high forage diets and actively ferment cellulose, resulting in an increase of acetate production. Consequently the acetate-to-propionate ratio increased when anaerobe fungi were inoculated with hay in the RUSITEC system (Kostyukovsky *et al.* 1995). An increase in acetate production and acetate-to-propionate ratio with increasing MSS was shown in the present studies (Paper I + III), however no significant increase in degradation of fibre fractions was detected, with exception of increasing MSS from 4 to 9 mm in GS (Paper III). Many rumen fungi are able to produce cellulases and xylanases, which enables them to degrade the major structural polysaccharides in plant cell walls, however they do not metabolise lignin (Wubah *et al.* 1993). The results from Paper III indicated that increased acetate production with increasing MSS rather was a result of fermentation of non-structural carbohydrates (NSC) than of fibre fractions.

Non-structural carbohydrates

The degradation of NSC was higher in GS than in MS (Figure D-2), but the total amount of degraded NSC was higher in MS-based diets than in GS-based diets and contributed mostly to the degraded OM in the MS-based diets. The fraction of NSC was not analysed directly, but calculated from OM minus the sum of CP, NDF and ether extract. Thus values for NSC degradation are directly affected by measurements of the other nutrient fractions, and therefore have to be discussed carefully. However, close relations between the degradation of NSC in MS and the respective degradation of starch were shown (Paper III, experiment 2).

It was demonstrated that degradability of starch depended on the presence of NH_3-N (Paper III), and this was in accordance with the results of Windschitl and Stern (1988) and Griswold *et al.* (2003). Nevertheless, degradation of starch in MS did not increase above 0.74 when ammonia-N was available in excess, and thus was lower than values reported *in situ* and *in vivo* (Fernandez *et al.* 2004, Jensen *et al.* 2005).

Furthermore, the degradation of starch in MS, as well as degradation of the NSC fraction in MS and GS (Figure D-2) and the respective mixed diets (Paper I) was shown to be improved by an increase in mean feed particle size. Accordingly the amounts of total SCFA increased with increasing MSS (Figure D-2). These results indicate that the access to the NSC fraction for microbial fermentation was limited at fine milling. Once again a possible effect of ruminal fungi can be hypothesised. Fungal penetration results in a weakening of plant stem tissue, and therefore improves the access for nutrient degradation by bacteria (Akin and Borneman 1990). Moreover, it was shown that fungi (*Neocallimastix frontalis*) are able to utilise not only cellulose, but also hemicelluloses and starch for growth (Orpin and Letcher 1979).

Microbial protein flow

The microbial N yield as well as efficiency of microbial protein synthesis (EMPS) in the present studies was higher at incubation of GS-based diets than at MS-based diets (Paper II + III). The effect of inclusion of MS to GS-based diets depends on the composition of the total diet. Particularly high proportions of MS in mixed rations increase the need for supplementing protein feedstuffs in order to comply ruminal N balance (Spiekers *et al.* 2009). The supplemented amount of soybean meal (11%) was not sufficient to promote nitrogen requirements for maximal microbial growth in MS-based diets in the own study. Higher proportions of protein supply seem to be necessary for maximal microbial growth in MS-based diets. Accordingly, an increase in the inclusion rate of soybean meal in a maize-based diet (105 vs. 149 g CP/kg DM) increased EMPS in continuous culture (Legleiter *et al.* 2005). As shown by Windschitl and Stern (1988) the infusion of urea led to increased microbial N flow at incubation of a maize-based diets in a continuous flow system, although total dietary CP already counted 160 g/kg DM due to soybean meal inclusion in the basal diet. As pointed out in Papers II and III, the amount of degraded CP and supplemented mineral N, and hence the amount of available N seems to determine microbial protein synthesis in the used RUSITEC

system. A plot of available N against microbial N flow from data sets of all own experiments (Figure D-3) underlines this hypothesis.

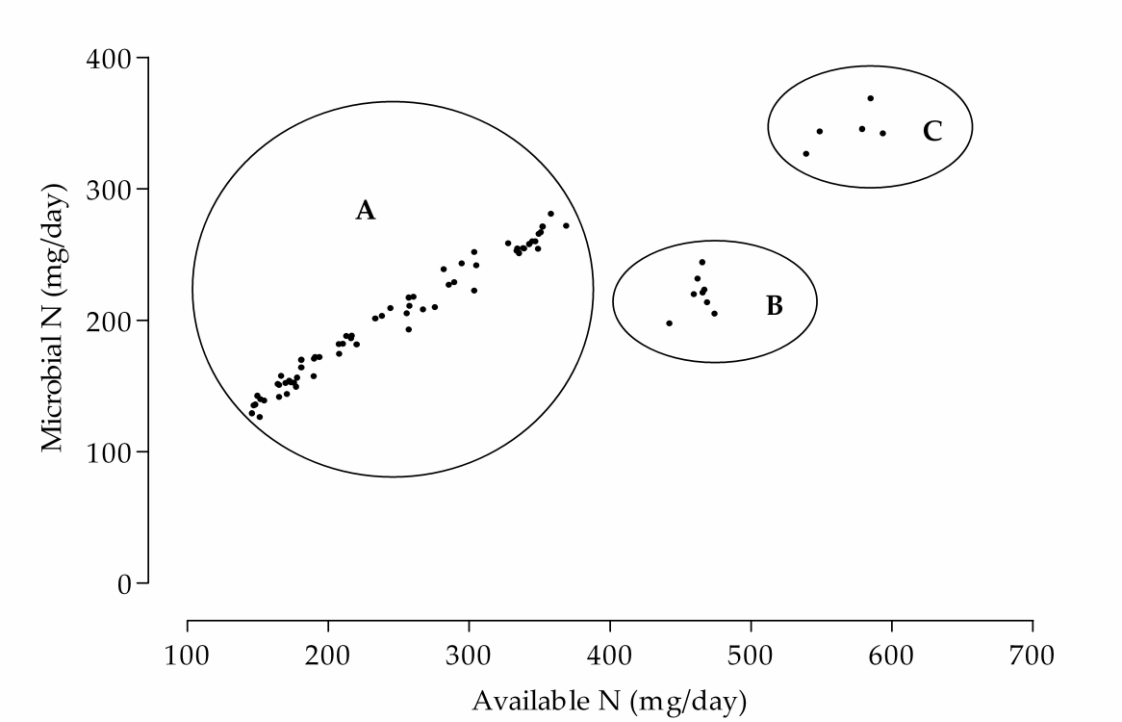

Figure D-3 Relationship between daily amounts of available N and microbial N flow for all data sets; (A) diets M100, M79, M52, M24, M0, pure MS, pure GS; (B) MS + urea; (C) pure soybean meal (Hildebrand, unpublished work)

As recently reported in the RUSITEC study of Gast (2010), GS with high CP content (>16%) led to an increase in bacterial mass in comparison to the control silage (16% CP). Moreover, the partial inclusion of clover in grass-based silages, subsequently leading to an increase in CP content, was reported to result in an increase in microbial CP yield in continuous culture (Merry *et al.* 2006) and *in vivo* (Dewhurst *et al.* 2003). In contrast no clear effect of CP content and botanical composition of grass-based silages on microbial CP yield was found by Seng *et al.* (2008), using a RUSITEC system. In contrast to the own studies, EMPS was reported to be higher with a MS-based diet than with a GS-based diet in a RUSITEC system (Jalč *et al.* 2009a, Jalč *et al.* 2009b). Figure D-3 also indicated that the source of dietary N had an effect on microbial N yield. As discussed in Paper II + III other N sources than NH_3-N are needed for maximal microbial growth. Several studies have demonstrated that the supply of non-ammonia-N sources promote microbial protein synthesis to a higher extent than supplying NH_3-N exclusively *in vitro* (Maeng *et al.* 1989, Carro and Miller 1999, Meng *et al.* 2000). It was

shown that a deficit in availability of several amino acids (AA) limits microbial growth (Kajikawa *et al.* 2002) and that individual AA are incorporated to different proportions into microbial protein (Atasoglu *et al.* 2004) The dietary concentration of all analysed AA was higher in GS than in MS (Paper III), and it is very likely that the total amount of available AA was higher in GS-based diets than in MS-based diets. As shown by Von Keyserlingk *et al.* (1998) the ruminal disappearance of AA in GS after 12 h of incubation *in situ* ranged from 35 to 61% for individual AA, indicating differences in availability or utilisation between AA.

Besides CP content and availability of AA, other nutritional factors can be assumed to have affected microbial protein yield. It was discussed in Paper II + III that the ratio of NSC content and degraded CP were closely related to microbial N flow and EMPS. However, this close relationship was only stated *in vitro* (Stokes *et al.* 1991) and the effect of ruminal pH was discussed to lead to differing results *in vivo* (Hoover and Stokes 1991). Moreover, the content of fibre fractions in MS and GS could have played a role. The amount of degraded NDF was nearly three times higher in GS than in MS (1.2 vs. 0.4 g after 48 h; Paper III). An increase in dietary fibre content was reported to promote microbial N yield and EMPS in some continuous culture studies (Rodríguez-Prado *et al.* 2004, Martínez *et al.* 2010a), however no clear effect of CF content in total mixed rations on microbial N yield was found by Boguhn *et al.* (2006b) in a RUSITEC system.

Proportions of minerals and trace elements were not analysed in the present feedstuffs, but wide ranges for MS and GS are reported (Leberl 2006). As pointed out by Durand and Komisarczuk (1988) a deficit in phosphorus, sulfur and magnesium can impair ruminal fermentation and microbial protein flow. Phosphorus and magnesium were additionally supplemented via buffer solution, but sulfur was supplied only with feedstuffs. It has been shown for the RUSITEC system that an increase of sulfur supply increased microbial N in the effluent and EMPS (Durand *et al.* 1986). As sulfur is linked to CP supply in form of the AA cystine and methionine it can be assumed that sulfur supply was higher in GS than in MS, and a deficit in availability of these AA may also have contributed to the lower microbial N flow in MS-based compared to GS-based diets.

For estimation of the microbial N flow originating from liquid-associated microbes in the effluent (LAM-E) losses of N were assumed to be negligible. Possible losses of NH_3-N might have occurred in cause of aerobe conditions in the effluent flasks (Kohn and Dunlap 1998) or during oven-drying of feed residues after incubation. Therefore an overestimation of microbial N flow with the LAM-E fraction cannot be excluded. As discussed in Paper II the

amount of microbial N originating from the SAM fraction may be underestimated if microbial samples isolated from the feed residues were not representative for the whole microbial mass in the feed residues. On the other hand an overestimation of SAM-N in the feed residues could have been induced due to adhering ammonium salt, containing ^{15}N.

Microbial amino acid flow

The microbial AA profiles determined in the present studies were close to the mean values reviewed by Clark *et al.* (1992). Nevertheless, for comparison of AA profiles from different studies the number of analysed AA has to be taken into account. In the present study the AA tryptophan, histidine and tyrosine were not analysed and therefore the percentage of individual AA (% of analysed AA) can be assumed to be slightly higher than in studies with a higher number of analysed AA.

The AA profile differed between the microbial fractions isolated from the feed residues, the liquid inside the fermenter and the effluent (Paper II). This observation was in accordance with other RUSITEC studies (Boguhn *et al.* 2006a, Molina-Alcaide *et al.* 2009). In order to decrease analytical costs it would be worthwhile to use only one representative microbial fraction for investigations on the chemical composition of microbial mass. The concentrations of N and ^{15}N were lowest in the SAM fraction, highest in the LAM-E fraction and intermediate in the fraction of liquid-associated microbes inside the fermenter (LAM-F) across all treatments (Paper II). In contrast, no consistent trend was found for the proportion of AA-N and the individual AA (Figure D-4). The variations in AA profile between the three microbial fractions indicated differences in the microbial flora. But, differences in chemical composition do not prove diversity in bacterial community structure. In the companion study of Witzig (2009) the LAM-F and LAM-E fraction were shown to differ in their proportions of the bacterial groups *Bacteroides-Prevotella*, *Firmicutes* and *Proteobacteria*. Moreover, differences in the bacterial community structure between the LAM-F and the SAM fraction (Martínez *et al.* 2010b) and between the LAM-E and the SAM fraction (Boguhn *et al.* 2008) were reported for the RUSITEC system. In accordance with others (Martín-Orúe *et al.* 1998, Ranilla and Carro 2003, Trabalza-Marinucci *et al.* 2006), these authors pointed out that representativeness of the microbial fractions is limited and primarily is determined by the technique of isolation, particularly in regard to the SAM fraction.

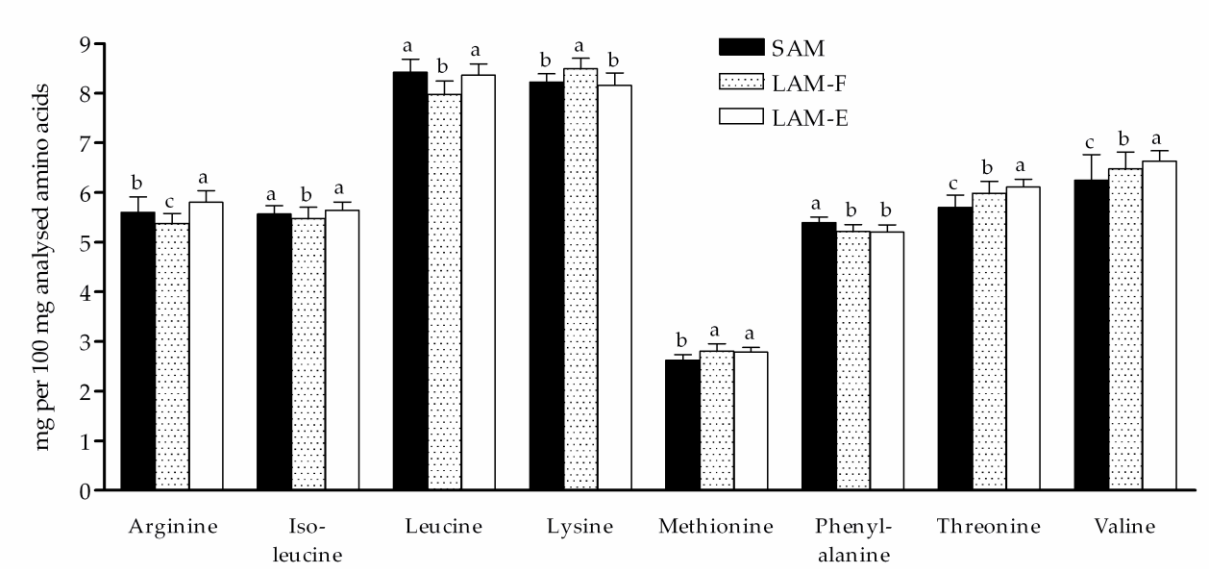

Figure D-4: Contents of essential amino acids (AA) in the fractions of solid associated microbes from feed residues (SAM) and liquid associated microbes from fermenter (LAM-F) and effluent (LAM-E) pooled across all treatments presented in Paper II (mg per 100 mg analysed AA; means and SD, $n = 35$). Different letters (abc) indicate significant differences within each AA (t-test, $P \leq 0.05$)

It was shown that the AA profile of the microbial fractions was affected by forage source and MSS (Paper II). However, in accordance with Rodríguez-Prado *et al.* (2004) only small effects of diet and feed particle size, and marginal interactions of both factors on the AA profile were detected. Measurements from continuous culture studies are used as indication for the situation *in vivo*. As shown in Table D-3 the daily outflow of all AA, as a sum of the SAM and the LAM-E fraction, was significantly affected by diet and MSS and no significant interactions ($P \leq 0.05$) occurred (Table D-3). Because AA flow is highly determined by the total microbial N flow, the daily amount of all AA increased with ascending proportion of GS in the diet. Nevertheless, it is questionable to what extent quantitative values measured *in vitro* are transferable to the conditions *in vivo* (Blanchart *et al.* 1989, Carro *et al.* 2009). Moreover, it has to be reminded that the AA profiles in the present study only referred to the bacterial fraction. Although bacterial AA contribute the majority of microbial AA flow *in vivo* the contribution of protozoa has to be considered (Shabi *et al.* 2000), because the AA profile of protozoa leaving the rumen was shown to be different from that of bacteria (Martin *et al.* 1996, Volden *et al.* 1999, Jensen *et al.* 2006).

Table D-3: Daily outflow of amino acids (AA) with the solid associated microbes from feed residues and the liquid associated microbes from effluents (mg/day; means, pooled SE, $n \geq 3$)

Diet	M100		M79		M52		M24		M0			*P* values		
MSS [2]	1	4	1	4	1	4	1	4	1	4	SE	Diet	MSS	Diet×MSS
Alanine	63.3	69.0	75.9	80.9	78.3	84.7	92.2	92.4	102.3	102.0	3.05.	***	***	0.08
Arginine	41.1	43.1	47.5	50.0	53.1	55.8	59.5	58.9	68.8	69.0	1.74	***	***	0.35
Aspartic acid	89.2	94.4	111.0	118.2	111.8	119.6	135.2	134.4	145.3	143.6	4.83	***	***	0.09
Cystine	7.5	8.2	8.7	9.6	9.0	10.4	10.8	10.8	11.7	12.6	0.37	***	***	0.45
Glutamic acid	101.8	111.2	120.1	126.9	128.4	140.2	147.7	146.0	167.4	169.8	4.89	***	***	0.07
Glycine	42.8	45.3	51.9	55.1	53.3	57.4	63.5	63.7	69.2	69.4	2.11	***	***	0.19
Isoleucine	40.4	43.5	47.0	50.7	49.9	55.4	57.4	58.7	65.1	67.5	2.18	***	***	0.41
Leucine	60.5	64.7	69.9	73.3	76.4	81.9	85.6	85.3	99.1	99.2	2.62	***	***	0.19
Lysine	59.6	63.6	71.2	75.0	73.4	79.6	84.7	84.0	91.8	93.8	2.91	***	***	0.16
Methionine	19.3	20.8	23.1	25.3	24.0	26.7	28.7	29.1	32.0	32.7	0.96	***	***	0.38
Phenylalanine	37.5	39.6	45.3	47.0	47.2	50.2	55.8	54.7	61.4	60.5	2.13	***	***	0.13
Proline	31.2	34.2	32.0	33.7	38.8	43.1	38.2	40.2	49.4	52.1	1.34	***	***	0.89
Serine	32.0	33.3	41.8	43.6	40.4	41.0	51.0	49.8	52.8	50.2	2.10	***	***	0.10
Threonine	43.1	45.8	52.8	55.8	53.4	56.5	63.0	62.7	68.3	67.6	2.21	***	***	0.09
Valine	47.7	51.5	54.0	57.2	59.0	65.2	64.9	65.8	76.7	79.3	2.14	***	***	0.26
Total AA	717	768	852	902	897	968	1038	1037	1161	1169		***	***	0.13

[1] Maize silage to grass silage ratio of 100:0 (M100), 79:21 (M79), 52:48 (M52), 24:76 (M24) and 0:100 (M0)

[2] Milling screen size (mm)

*** $P < 0.001$

Standardisation of grinding in the RUSITEC system?

As shown in the present studies (Paper I + III) and as illustrated before (Figure D-2), several fermentation characteristics of silage-based diets were improved by the increase in MSS in the RUSITEC system. Moreover, the present results indicated that interactions of forage source and MSS on fermentation in a semi-continuous culture system are rare, and therefore supported the findings of Rodríguez-Prado *et al.* (2004), who found no interactions between fibre content and feed particle size (1 vs. 3 mm) in continuous culture. However, the fermentation characteristics of GS tended to respond more sensitively to an increase in MSS than the fermentation characteristics of MS and an effect of lower differences in bulk density between MSS in MS compared to GS was assumed (Paper III). The measurements in bulk density confirmed that interactions between forage source and MSS on particle size distribution and mean particle size have to be expected (Emanuele and Staples 1988, Michalet-Doreau and Cerneau 1991).

One main objective of grinding feedstuffs prior to *in vitro* incubations is to generate homogenous samples. Increasing the MSS leads to a shift in particle size distribution, and large particles rather originate from the stem fraction than from the leaf fraction of forages (Emanuele and Staples 1988, Kennedy and Doyle 1993). It can be assumed that the risk of inhomogeneity in chemical composition of small feed samples increases with increasing MSS. Consequently, repeatability of the measurements might be impaired. This in turn would increase the number of required replicates per treatment. Coefficients of variation (CV) of nutrient degradation were high within the individual treatments, particularly for the fibre fractions (Table D-4). However, no clear effect of MSS on the relative SD of fermentation characteristics seems to exist. The low level of degradation in fibre fractions (sometimes negative values were calculated) and the high standard deviation induced very high values of CV, particularly at ADF degradation in MS. In some characteristics CV tended to be even higher at fine milling (1 mm-MSS) than at coarse milling, e.g. degradation of OM or total SCFA. Consequently, other factors than MSS can be expected to have contributed to high variation between replicates of the same treatment. A high effect of experimental run was observed and therefore taken into account in statistical analysis in Paper I – III. A better comparability of RUSITEC studies, when performed in different laboratories, was taken for one reason to investigate the effect of grinding. In Paper III results from two labs were shown (Experiment 1 and 2). MS and GS, milled at 4 mm screen size, were used in both

experiments. However, most of the measured fermentation characteristics of these treatments differed between laboratories (Figure D-2). Especially the fermentation of the carbohydrate fractions was different between laboratories, whereas degradation of CP was similar. However, reasons for the effect of laboratory could not be identified. Several factors might have been involved, e.g. diet of donor animal, environmental conditions in the laboratory, differences in the individual steps of sample preparation and analysis.

Table D-4 Coefficients of variation (%) of fermentation characteristics in maize silage and grass silage at varying milling screen sizes (MSS) measured by the RUSITEC system in two laboratories

Forage source	Maize silage				Grass silage			
Laboratory	Halle/S.		Hohenheim		Halle/S.		Hohenheim	
MSS (mm)	1	4	4	9	1	4	4	9
Nutrient degradation								
OM	7.6	3.3	5.5	4.9	6.6	3.8	2.0	4.0
CP	0.6	2.0	3.2	4.9	5.8	3.9	0.7	3.0
CF	39.1	38.2	25.7	85.7	19.6	37.1	12.5	8.7
NDF	30.8	4.7	12.8	7.3	18.6	11.2	4.7	5.8
ADF	248.7	121.7	28.8	119.3	40.2	6.3	10.4	9.4
NSC	13.4	3.8	6.8	5.6	6.6	5.1	2.5	4.6
Starch	-	-	5.1	2.8	-	-	-	-
Fermentation products								
NH_3-N	18.5	5.4	7.3	7.0	7.5	3.4	1.5	2.1
Total SCFA	15.9	3.4	7.1	2.1	10.2	4.2	3.9	0.5
Microbial N								
mg/d	5.2	0.5	3.9	7.3	7.3	2.7	0.7	4.4
mg/g degraded OM	8.5	3.0	1.6	5.3	1.5	1.1	1.7	1.7

In terms of improving the simulation of ruminal fermentation the present results suggest that using a MSS of 4 or 9 mm is more favorable than using a 1 mm-MSS. In the present study the capacity limit of the fermentation vessel was reached when GS, milled at 9 mm screen size, was incubated. Using feed samples with an even higher bulk density would require a reduction in amount of incubated feed, when using the present RUSITEC system. On the one hand this would additionally contribute to a reduction in homogeneity of feed samples. On the other hand the proportion of solid phase to liquid phase inside the fermenter would be another. As shown by Czerkawski and Breckenridge (1977) a reduction in the amount of incubated feed leads to an increase in pH value inside the vessel and a lower degradation of DM might occur.

References

Akin DE 1993. Perspectives of cell wall biodegradation. In Forage cell wall structure and digestibility (eds. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 73-82, American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin, USA.

Akin DE and Borneman WS 1990. Role of rumen fungi in fiber degradation. Journal of Dairy Science 73, 3023-3032.

Arhab R, Laadjimi K, Driss D, Djabri B and Bousseboua H 2010. Evaluation of feed mixture interactions by using in vitro gas production method. Livestock Research for Rural Development 22.

Asao N, Ushida K and Kojima Y 1993. Proteolytic activity of rumen fungi belonging to the genera Neocallimastix and Piromyces. Letters in Applied Microbiology 16, 247-250.

Atasoglu C, Newbold CJ and Wallace RJ 2001. Incorporation of [15N]Ammonia by the Cellulolytic Ruminal Bacteria Fibrobacter succinogenes BL2, Ruminococcus albus SY3, and Ruminococcus flavefaciens 17. Applied and Environmental Microbiology 67, 2819-2822.

Atasoglu C, Guliye AY and Wallace RJ 2004. Use of stable isotopes to measure de novo synthesis and turnover of amino acid-C and -N in mixed micro-organisms from the sheep rumen in vitro. British Journal of Nutrition 91, 253-261.

Bauchop T 1989. Biology of gut anaerobic fungi. Biosystems 23, 53-64.

Blanchart G, Durand M, Barry JL, Bouiller-Oudot M and Jouany JP 1989. Advantages and limits of the semi-continuous artificial rumen (Rusitec). Annales de Zootechnie 38, 285-314.

Boguhn J, Kluth H and Rodehutscord M 2006a. Effect of total mixed ration composition on amino acid profiles of different fractions of ruminal microbes in vitro. Journal of Dairy Science 89, 1592-1603.

Boguhn J, Kluth H and Rodehutscord M 2006b. Effect of total mixed ration composition on fermentation and efficiency of ruminal microbial crude protein synthesis in vitro. Journal of Dairy Science 89, 1580-1591.

Boguhn J, Strobel E, Witzig M, Tebbe CC and Rodehutscord M 2008. Description of the structural diversity of rumen microbial communities in vitro using single-strand conformation polymorphism profiles. Archives of Animal Nutrition 62, 454-467.

Bossen D, Mertens DR and Weisbjerg MR 2008. Influence of fermentation methods on neutral detergent fiber degradation parameters. Journal of Dairy Science 91, 1464-1476.

Carro MD and Miller EL 1999. Effect of supplementing a fibre basal diet with different nitrogen forms on ruminal fermentation and microbial growth in an *in vitro* semi-continuous culture system (RUSITEC). British Journal of Nutrition 82, 149-157.

Carro MD, Lebzien P and Rohr K 1995. Effects of pore size of nylon bags and dilution rate on fermentation parameters in a semi-continuous artificial rumen. Small Ruminant Research 15, 113-119.

Carro MD, Ranilla MJ, Martín-García AI and Molina-Alcaide E 2009. Comparison of microbial fermentation of high- and low-forage diets in Rusitec, single-flow continuous-culture fermenters and sheep rumen. Animal 3, 527-534.

Chai WZ, van Gelder AH and Cone JW 2004. Relationship between gas production and starch degradation in feed samples. Animal Feed Science and Technology 114, 195-204.

Clark JH, Klusmeyer TH and Cameron MR 1992. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. Journal of Dairy Science 75, 2304-2323.

Cone JW, Van Gelder AH and Valk H 1998. Prediction of nylon bag degradation characteristics of grass samples with the gas production technique. Journal of the Science of Food and Agriculture 77, 421-426.

Czerkawski JW and Breckenridge G 1977. Design and development of a long-term rumen simulation technique (Rusitec). British Journal of Nutrition 38, 371-384.

De Boever JL, Vanacker JM and De Brabander DL 2002. Rumen degradation characteristics of nutrients in maize silages and evaluation of laboratory measurements and NIRS as predictors. Animal Feed Science and Technology 101, 73-86.

De Boever JL, Aerts JM, Vanacker JM and De Brabander DL 2005. Evaluation of the nutritive value of maize silages using a gas production technique. Animal Feed Science and Technology 123, 255-265.

De Boever JL, Cottyn BG, De Brabander DL, Vanacker JM and Boucqué CV 1996. Prediction of the feeding value of grass silages by chemical parameters, in vitro digestibility and near-infrared reflectance spectroscopy. Animal Feed Science and Technology 60, 103-115.

De Boever JL, Cottyn BG, De Brabander DL, Vanacker JM and Boucque CV 1997. Prediction of the feeding value of maize silages by chemical parameters, in vitro digestibility and NIRS. Animal Feed Science and Technology 66, 211-222.

Dewhurst RJ, Evans RT, Scollan ND, Moorby JM, Merry RJ and Wilkins RJ 2003. Comparison of grass and legume silages for milk production. 2. In vivo and in sacco evaluations of rumen function. Journal of Dairy Science 86, 2612-2621.

DLG, Deutsche Landwirtschafts-Gesellschaft 1997. DLG-Futterwerttabellen Wiederkäuer. DLG-Verlag, Frankfurt am Main.

Dunker M 2006. Gras- und Maissilagequalitäten des Jahres 2005 in Mecklenburg-Vorpommern. In Jahresbericht zur Futterproduktion, Landesforschungsanstalt für Landwirtschaft und Fischerei Mecklenburg-Vorpommern, Gülzow.

Durand M and Komisarczuk S 1988. Influence of major minerals on rumen microbiota. Journal of Nutrition 118, 249-260.

Durand M, Hannequart G, Beaumatin P, Dumay C and Tassery F 1986. Influence du niveua d'apport de soufre sur la synthèse d'acide ribonuclèique et de protéines par les

microorganismes du rumen en fermenteur semi-continu. Reproduction Nutrition Development 26, 299-300.

Emanuele SM and Staples CR 1988. Effect of Forage Particle Size on In Situ Digestion Kinetics. Journal of Dairy Science 71, 1947-1954.

Fernandez I, Nozière P and Michalet-Doreau B 2004. Site and extent of starch digestion of whole-plant maize silages differing in maturity stage and chop length, in dairy cows. Livestock Production Science 89, 147-157.

Ferreira G and Mertens DR 2005. Chemical and physical characteristics of corn silages and their effects on in vitro disappearance. Journal of Dairy Science 88, 4414-4425.

Ferret A, Gasa J, Plaixats J, Casanas F, Bosch L and Nuez F 1997. Prediction of voluntary intake and digestibility of maize silages given to sheep from morphological and chemical composition, in vitro digestibility or rumen degradation characteristics. Animal Science 64, 493-501.

García-Rodriguez A, Mandaluniz N, Flores G and Oregui LM 2005. A gas production technique as a tool to predict organic matter digestibility of grass and maize silage. Animal Feed Science and Technology 123-124, 267-276.

Gast A 2010. Untersuchungen zum Einfluss von Grassilagen mit auffälligen Reineiweißgehalten auf die Zahl der Bakterien und der Protozoen im Pansensaft (in vitro). Dissertation, Tierärztliche Hochschule Hannover.

Getachew G, Robinson PH, DePeters EJ and Taylor SJ 2004. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. Animal Feed Science and Technology 111, 57-71.

GfE, Gesellschaft für Ernährungsphysiologie 1991. Leitlinien für die Bestimmung der Verdaulichkeit von Rohnährstoffen an Wiederkäuern. Journal of Animal Physiology and Animal Nutrition 65, 229-234.

GfE, Gesellschaft für Ernährungsphysiologie 1998. Mitteilungen des Ausschusses für Bedarfsnormen: Formeln zur Schätzung des Gehaltes an Umsetzbarer Energie in Futtermitteln aus Aufwüchsen des Dauergrünlandes und Mais- Ganzpflanzen. Proceedings of the Society of Nutrition Physiology 7, 141-150.

GfE, Gesellschaft für Ernährungsphysiologie 2008. Mitteilungen des Ausschusses für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie: Neue Gleichungen zur Schätzung der Umsetzbaren Energie für Wiederkäuer von Gras- und Maisprodukten. Proceedings of the Society of Nutrition Physiology 17, 191-197.

Givens DI, Everington JM and Adamson AH 1989. The digestibility and metabolisable energy content of grass silage and their prediction from laboratory measurements. Animal Feed Science and Technology 24, 27-43.

Givens DI, Cottyn BG, Dewey PJS and Steg A 1995. A comparison of the neutral detergent-cellulase method with other laboratory methods for predicting the digestibility in vivo of maize silages from three European countries. Animal Feed Science and Technology 54, 55-64.

Gizzi G, Zanchi R and Sciaraffia F 1998. Comparison of microbiological and fermentation parameters obtained with an improved rumen in vitro technique with those obtained in vivo. Animal Feed Science and Technology 73, 291-305.

Griswold KE, Hoover WH, Miller TK and Thayne WV 1996. Effect of form of nitrogen on growth of ruminal microbes in continuous culture. Journal of Animal Science 74, 483-491.

Griswold KE, Apgar GA, Bouton J and Firkins JL 2003. Effects of urea infusion and ruminal degradable protein concentration on microbial growth, digestibility, and fermentation in continuous culture. Journal of Animal Science 81, 329-336.

Hoffman PC, Brehm NM, Bauman LM, Peters JB and Undersander DJ 1999. Prediction of laboratory and in situ protein fractions in legume and grass silages using near-infrared reflectance spectroscopy. Journal of Dairy Science 82, 764-770.

Hoover WH and Stokes SR 1991. Balancing carbohydrates and proteins for optimum rumen microbial yield. Journal of Dairy Science 74, 3630-3644.

Hoover WH, Kincaid CR, Varga GA, Thayne WV and Junkins LL, Jr. 1984. Effects of solids and liquid flows on fermentation in continuous cultures. IV. pH and dilution rate Journal of Animal Science 58, 692-699.

Jalč D, Lauková A, Varadyova Z, Homolká P and Koukolová V 2009a. Effect of inoculated grass silages on rumen fermentation and lipid metabolism in an artificial rumen (RUSITEC). Animal Feed Science and Technology 151, 55-64.

Jalč D, Váradyová Z, Lauková A, Homolka P and Jančík F 2009b. Effect of inoculated corn silage on rumen fermentation and lipid metabolism in an artificial rumen (RUSITEC). Animal Feed Science and Technology 152, 256-266.

Jančík F, Koukolová V, Kubelková P and Čermák B 2009. Effects of grass species on ruminal degradability of silages and prediction of dry matter effective degradability. Czech Journal of Animal Science 54, 315-323.

Jensen C, Weisbjerg MR and Hvelplund T 2006. Evaluation of methods for estimating the amino acid supply to the duodenum of microbial, endogenous and undegraded feed protein on maize silage diets fed to dairy cows. Animal Feed Science and Technology 131, 1-24.

Jensen C, Weisbjerg MR, NÃ¸rgaard P and Hvelplund T 2005. Effect of maize silage maturity on site of starch and NDF digestion in lactating dairy cows. Animal Feed Science and Technology 118, 279-294.

Joblin KN, Campbell GP, Richardson AJ and Stewart CS 1989. Fermentation of barley straw by anaerobic rumen bacteria and fungi in axenic culture and in co-culture with methanogens. Letters in Applied Microbiology 9, 195-197.

Juniper DT, Browne EM, Bryant MJ and Beever DE 2008. Digestion, rumen fermentation and circulating concentrations of insulin, growth hormone and IGF-1 in steers fed diets based on different proportions of maize silage and grass silage. Animal 2, 849-858.

Kajikawa H, Mitsumori M and Ohmomo S 2002. Stimulatory and inhibitory effects of protein amino acids on growth rate and efficiency of mixed ruminal bacteria. Journal of Dairy Science 85, 2015-2022.

Kennedy PM and Doyle PT 1993. Particle-size reduction by ruminants - Effects of cell wall composition and structure. In Forage cell wall structure and digestibility (eds. HG Jung, DR Buxton, RD Hatfield and J Ralph), pp. 499-534, American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin, USA.

Kohn RA and Dunlap TF 1998. Calculation of the buffering capacity of bicarbonate in the rumen and in vitro. Journal of Animal Science 76, 1702-1709.

Kostyukovsky V, Inamoto T, Ando T, Nakai Y and Ogimoto K 1995. Degradation of hay by rumen fungi in artificial rumen (RUSITEC). Journal of General and Applied Microbiology 41, 83-86.

Landwirtschaftskammer N-W 2005. Tabellen: Maissilage 2005, published by: Landwirtschaftskammer Nordrhein-Westfalen, online document: http://www.landwirtschaftskammer.de/landwirtschaft/tierproduktion/rinderhaltung/pdf/tabellen-maissilage-2005.pdf, date of access: 08.05.2011

Leberl P 2006. Ergebnisse der Grundfutteruntersuchung des Jahres 2005: hohe Grundfutterleistung ist Meßlatte für die Wirtschaftlichkeit. Landinfo 04/2006, 42-45.

Legay-Carmier F and Bauchart D 1989. Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. British Journal of Nutrition 61, 725-740.

Legleiter LR, Mueller AM and Kerley MS 2005. Level of supplemental protein does not influence the ruminally undegradable protein value. Journal of Animal Science 83, 863-870.

Licitra G, Hernandez TM and VanSoest PJ 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Animal Feed Science and Technology 57, 347-358.

Maeng WJ, Chang MB, Yun HS and Choi I 1989. Dilution rates on the efficiency of rumen microbial growth in continuous culture. Asian Australasian Journal of Animal Science 2, 477-480.

Mansfield HR, Endres MI and Stern MD 1995. Comparison of microbial fermentation in the rumen of dairy cows and dual flow continuous culture. Animal Feed Science and Technology 55, 47-66.

Martín-Orúe SM, Balcells J, Zakraoui F and Castrillo C 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure. Animal Feed Science and Technology 71, 269-282.

Martin C, Bernard L and Michalet-Doreau B 1996. Influence of sampling time and diet on amino acid composition of protozoal and bacterial fractions from bovine ruminal contents. Journal of Animal Science 74, 1157-1163.

Martínez ME, Ranilla MJ, Ramos S, Tejido ML and Carro MD 2009. Effects of dilution rate and retention time of concentrate on efficiency of microbial growth, methane production, and ruminal fermentation in Rusitec fermenters. Journal of Dairy Science 92, 3930-3938.

Martínez ME, Ranilla MJ, Tejido ML, Ramos S and Carro MD 2010a. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of

sheep and Rusitec fermenters. I. Digestibility, fermentation parameters, and microbial growth. Journal of Dairy Science 93, 3684-3698.

Martínez ME, Ranilla MJ, Tejido ML, Saro C and Carro MD 2010b. Comparison of fermentation of diets of variable composition and microbial populations in the rumen of sheep and Rusitec fermenters. II. Protozoa population and diversity of bacterial communities. Journal of Dairy Science 93, 3699-3712.

Meng QX, Xia ZG and Kerley MS 2000. The requirement of ruminal degradable protein for non-structural carbohydrate-fermenting microbes and its reaction with dilution rate in continuous culture. Asian-Australasian Journal of Animal Sciences 13, 1399-1406.

Menke KH and Steingass H 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Animal Research and Development 28, 7-55.

Merry RJ and McAllan AB 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. British Journal of Nutrition 50, 701-709.

Merry RJ, Lee MRF, Davies DR, Dewhurst RJ, Moorby JM, Scollan ND and Theodorou MK 2006. Effects of high-sugar ryegrass silage and mixtures with red clover silage on ruminant digestion. 1. In vitro and in vivo studies of nitrogen utilization. Journal of Animal Science 84, 3049-3060.

Michalet-Doreau B and Cerneau P 1991. Influence of foodstuff particle size on in situ degradation of nitrogen in the rumen. Animal Feed Science and Technology 35, 69-81.

Molina-Alcaide E, Moumen A, Martín-García I and Carro MD 2009. Comparison of bacterial pellets and microbial markers for the estimation of the microbial nitrogen and amino acids flows from single flow continuous culture fermenters fed diets containing two-stage olive cake. Journal of Animal Physiology and Animal Nutrition 93, 527-537.

Nousiainen J, Rinne M, Hellämäki M and Huhtanen P 2003. Prediction of the digestibility of primary growth and regrowth grass silages from chemical composition, pepsin-cellulase solubility and indigestible cell wall content. Animal Feed Science and Technology 110, 61-74.

Orpin CG 1974. The rumen flagellate Callimastix frontalis: does sequestration occur? Journal of General Microbiology 84, 395-398.

Orpin CG and Letcher AJ 1979. Utilization of cellulose, starch, xylan, and other hemicelluloses for growth by the rumen phycomycete Neocallimastix frontalis. Current Microbiology 3, 121-124.

Owens D, McGee M, Boland T and O'Kiely P 2009. Rumen fermentation, microbial protein synthesis, and nutrient flow to the omasum in cattle offered corn silage, grass silage, or whole-crop wheat. Journal of Animal Science 87, 658-668.

Pries M 2004. Tabellen: Nur beste Grassilagen bereiten, published by: Landwitschaftskammer Nordrhein-Westfalen, online document: http://www.landwirtschaftskammer.de/landwirtschaft/tierproduktion/rinderhaltung/pdf/tabellen-grassilagen-bereiten.pdf, date of access: 08.05.2011

Ranilla MJ and Carro MD 2003. Diet and procedures used to detach particle-associated microbes from ruminal digesta influence chemical composition of microbes and estimation of microbial growth in Rusitec fermenters. Journal of Animal Science 81, 537-544.

Robinson PH, Getachew G and Cone JW 2009. Evaluation of the extent of associative effects of two groups of four feeds using an *in vitro* gas production procedure. Animal Feed Science and Technology 150, 9-17.

Rodríguez-Prado M, Calsamiglia S and Ferret A 2004. Effects of fiber content and particle size of forage on the flow of microbial amino acids from continuous culture fermenters. Journal of Dairy Science 87, 1413-1424.

Rodríguez CA, González J, Alvir MR, Repetto JL, Centeno C and Lamrani F 2000. Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. British Journal of Nutrition 84, 369-376.

Seng M, Bonorden S, Nissen J, Isselstein J and Abel H 2008. Fermentation patterns and nutrient contents of forb-containing silages and their effects on microbial fermentation in the artificial rumen system RUSITEC. Journal of Agricultural Science 146, 333-341.

Shabi Z, Tagari H, Murphy MR, Bruckental I, Mabjeesh SJ, Zamwel S, Celik K and Arieli A 2000. Partitioning of amino acids flowing to the abomasum into feed, bacterial, protozoal, and endogenous fractions. Journal of Dairy Science 83, 2326-2334.

Spiekers H, Nußbaum H and Potthast V 2009. Erfolgreiche Milchviehfütterung. DLG-Verlag, Frankfurt am Main.

Spiekers H, Lindermayer H, Moosmeyer M, Obermaier A, Preißinger W, Rauch P, Richter W, Rutzmoser K, Spann B and Steinberger S 2006. Bayrische Landesanstalt für Landwirtschaft (LfL), Institut für Tierernährung und Futterwirtschaft - Jahresbericht 2005.

Steinhöfel O and Krieg D 2006. Futterwert der sächsischen Grobfuttermittel aus der Ernte 2005. INFODIENST, Sächsische Landesanstalt für Landwirtschaft 5/2006, 92-95.

Stern MD, Bach A and Calsamiglia S 1997. Alternative techniques for measuring nutrient digestion in ruminants. Journal of Animal Science 75, 2256-2276.

Stokes SR, Hoover WH, Miller TK and Manski RP 1991. Impact of carbohydrate and protein levels on bacterial metabolism in continuous culture. Journal of Dairy Science 74, 860-870.

Trabalza-Marinucci M, Poncet C, Delval E and Fonty G 2006. Evaluation of techniques to detach particle-associated microorganisms from rumen contents. Animal Feed Science and Technology 125, 1-16.

Van Vuuren AM, Klop A, Van Der Koelen CJ and De Visser H 1999. Starch and stage of maturity of grass silage: Site of digestion and intestinal nutrient supply in dairy cows. Journal of Dairy Science 82, 143-152.

Volden H, Harstad OM and Mydland LT 1999. Amino acid content and profile of protozoal and bacterial fractions isolated from ruminal contents of lactating dairy cows fed diets differing in nitrogen supplementation. Acta Agriculturae Scandinavica, Section A, Animal Science 49, 245-250.

Von Keyserlingk MAG, Swift ML, Puchala R and Shelford JA 1996. Degradability characteristics of dry matter and crude protein of forages in ruminants. Animal Feed Science and Technology 57, 291-311.

Von Keyserlingk MAG, Shelford JA, Puchala R, Swift ML and Fisher LJ 1998. In situ disappearance of amino acids from grass silages in the rumen and intestine of cattle. Journal of Dairy Science 81, 140-149.

Wallace RJ and Joblin KN 1985. Proteolytic activity of a rumen anaerobic fungus. FEMS Microbiology Letters 29, 19-25.

Windschitl PM and Stern MD 1988. Effects of urea supplementation of diets containing lignosulfonate-treated soybean meal on bacterial fermentation in continuous culture of ruminal contents. Journal of Animal Science 66, 2948-2958.

Witzig M 2009. Molekularbiologische Untersuchungen zum Einfluss der Grobfutterquelle und Futterpartikelgröße auf die ruminale Mikroorganismengemeinschaft in vitro. Dissertation, Martin-Luther-Universität Halle-Wittenberg.

Wubah DA, Akin DE and Borneman WS 1993. Biology, fiber-degradation, and enzymology of anaerobic zoosporic fungi. Critical Reviews in Microbiology 19, 99-115.

Yan T and Agnew RE 2004a. Prediction of nutritive values in grass silages: I. Nutrient digestibility and energy concentrations using nutrient compositions and fermentation characteristics. Journal of Animal Science 82, 1367-1379.

Yan T and Agnew RE 2004b. Prediction of nutritive values in grass silages: II. Degradability of nitrogen and dry matter using digestibility, chemical composition, and fermentation data. Journal of Animal Science 82, 1380-1391.

Zhang Y, Gao W and Meng Q 2007. Fermentation of plant cell walls by ruminal bacteria, protozoa and fungi and their interaction with fibre particle size. Archives of Animal Nutrition 61, 114-125.

General conclusions and outlook

A stepwise replacement of maize silage (MS) by grass silage (GS) in a mixed diet with a constant forage proportion of about 0.88 induced a linear response in total tract digestibility coefficients and most *in vitro* fermentation characteristics. Therefore it can be assumed that nutritional values of individual silages are additive in mixed diets and associative effects on criteria of fermentation are unlikely to occur in a semi-continuous rumen simulation technique (RUSITEC).

The present study suggests that available nutrients from GS promote rumen microbial growth more than those from MS when both forages are incubated under the same conditions in a RUSITEC system. No positive associative effects of combinations of MS and GS on microbial protein synthesis can be expected.

The amount of available N significantly increased microbial N flow in the RUSITEC system. Urea-N supply to MS improved microbial N flow and efficiency of microbial protein synthesis. However, further research in identifying the accountability of dietary factors, e.g. the type of dietary crude protein (CP) and carbohydrate sources, on microbial protein synthesis in continuous culture systems is needed.

The supply of urea-N to MS indicated that fermentation of starch, but not that of fibre fractions, was limited by N availability in the system. But further work concerning the optimal level of N supply for maximising fermentation of maize forages in the RUSITEC system is required. The form of N supplementation, singly dosed or continuously infused, has only marginal effects on microbial fermentation processes in the RUSITEC system.

The degradation of carbohydrate fractions in MS- and GS-based diets in general was lower than values reported in literature, based on *in situ* and *in vivo* investigations, and degradation of the acid detergent fibre fraction in MS was close to zero. Therefore the present results were unsatisfying in regard to simulation of ruminal fermentation of fibre fractions and further investigations to identify limiting factors are needed.

Although it was not the objective of the present study to investigate the effect of soybean meal in MS- and GS-based diets, a comparison of results from pure silages and diets with soybean meal inclusion indicated that interactions between forage source and soybean meal on degradation of CP and microbial N flow can occur *in vitro*. Moreover, data from the Hohenheim gas production test implicated positive associative effects of silages and soybean meal, particularly in the first hours after the start of incubation.

An increase in mean feed particle size of oven-dried silage-based diets improves the simulation of several fermentation characteristics in the RUSITEC system. Therefore, variations in milling screen size (MSS) and feed particle size distribution have to be taken into account when evaluating feeds by rumen simulation systems. However, possible mechanisms that exist behind these results remained unclear and have to be clarified.

The largest MSS (9 mm) benefited fermentation in the RUSITEC system to the highest extent and investigations on a further increase in MSS would be very interesting. However, the volume of fermentation vessels is limited and investigations with MSS above 9 mm would require a reduction in amount of incubated feed. It has to be considered that both, the reduction in sample size *per se* and the increase in MSS, induce a reduction in homogeneity of chemical composition of feed samples.

Forage samples were ground by a cutting mill in the present study. The increase in mean particle size due to increasing MSS primarily can be attributed to an increase in size of particles originating from the stem fraction of plants. This aspect has to be considered when comparing the own results with those from continuous culture studies using another procedure to reduce particle size of samples, e.g. manual cutting.

A negative effect of coarse milling on fermentation in the Hohenheim gas production test was confirmed. This observation underlines the importance of using a standardised MSS in feed evaluation methods.

The amino acid profile of microbial fractions as well as the ratio of solid-associated and liquid-associated bacteria was affected by forage source and degree of milling. These observations indicate that a shift in bacterial community structure due to variation of both

dietary factors occurs in the RUSITEC system. This hypothesis was confirmed by a companion study using different molecular techniques. Identifying bacterial species in continuous-culture systems seems to be a worthwhile objective for further investigations in order to improve understanding of the observations made in this study.

The composition of microbial protein was shown to differ between the microbial fractions. Therefore the present results stated the concept that the origin of the bacterial samples is very important for measurements on microbial protein synthesis, and that both solid-associated as well as liquid-associated bacteria have to be considered.

The present results gave reason to speculate that other microbial groups than bacteria, e.g. anaerobe fungi, were involved in the fermentation process, particularly when mean particle size increased. Investigations on the presence of fungi in the RUSITEC system could help to explain the findings of this study.

Only one batch of MS and GS, respectively, was used in the present study. As chemical composition can highly vary within both silage types, the transferability of the own results to other silage batches is limited. The effect of variation in silage quality on fermentation characteristics in the RUSITEC system remains to be investigated.

Summary

The synthesis of microbial protein in the rumen has a major impact on protein- and amino acid supply in ruminants. The amount and amino acid composition of the protein that enters the small intestine primarily depends on diet formulation. In the present studies the effects of maize silage (MS) and grass silage (GS) on ruminal fermentation and microbial protein synthesis were investigated, considering methodical aspects of *in vitro* studies, particularly grinding of feed samples.

In the first experimental series five mixed diets with different proportions of MS and GS (100:0, 79:21, 52:48, 24:76 and 0:100) and a constant proportion of soybean meal (11%) were used. The content of crude protein (CP) and fibre fractions increased, whereas the content of organic matter (OM) and starch decreased with increasing proportion of GS in the diet. It was hypothesised that a combination of MS and GS can benefit microbial growth and thus fermentation of nutrient fractions to a higher extent than using only one forage source separately. It was also to be investigated how changes in diet composition affect the amino acid profile of microbial protein. A well standardised semi-continuous rumen simulation technique (RUSITEC) was used, which is a commonly accepted experimental model in investigations on ruminal fermentation. Changes in fermentation characteristics, as a result of changing the MS-to-GS ratio, were tested for linear and quadratic effects in order to identify possible associative effects. Prior to the *in vitro* incubation, feedstuffs were dried and ground. It was aimed to investigate in which way fermentation in the RUSITEC system is influenced by mean feed particle size. Therefore two milling screen sizes (MSS; 1 vs. 4 mm) were used in all diets and results on fermentation characteristic were tested for possible interactions of forage source and MSS.

One incubation period lasted for 13 days (6 days adaption period, 7 days sampling period), and each treatment was tested in at least three replicates. Ruminal digesta, obtained from rumen-fistulated wether sheep, was used as the inoculum for starting the incubation. Diets were fed once daily to the RUSITEC system, and nylon feed bags remained for 48 h inside the fermentation vessel. A buffer solution, containing $^{15}NH_4Cl$, was infused continuously into the vessel and the respective effluent was analysed for short chain fatty acids (SCFA) and NH_3-N. Solid- and liquid- associated microbial fractions were isolated from the feed residues, the liquid inside the vessel and the effluent by differential centrifugation. The flow

of microbial CP was quantified on the basis of N and ^{15}N balances. The feed residues were analysed for crude nutrients and detergent fibre fractions and the respective degradation rates were calculated. OS and CP in the feed residues were corrected for the contribution of solid-associated microbes.

The degradation of OM and fibre fractions, as well as amounts of NH_3-N increased linearly with stepwise replacement of MS by GS. Degradation of CP was unaffected by diet composition, as well as total SCFA production. The degradation of OM and CP was higher in coarse milled (4 mm-MSS) than in fine milled (1 mm-MSS) treatments, accompanied by higher amounts of NH_3-N and total SCFA. An improvement of growth conditions for some microbial groups, e.g. anaerobe fungi, was discussed. The amount of microbial CP increased linearly by the stepwise replacement of MS by GS, and was higher at 4 mm-MSS than at 1 mm-MSS. The amount of available N was assumed to advance microbial growth in the RUSITEC system. Efficiency of microbial CP synthesis was improved from 29 to 43 mg microbial N per g degraded OM by increasing the proportion of GS in the diet, but was unaffected by MSS. The N content and the profiles of amino acids of the three microbial fractions, as well as the ratio of solid- to liquid-associated microbes were affected by diet composition and MSS. Interactions of forage source and MSS were rare. However, the results indicated interactions between dietary factors and origin of microbial isolate on characteristics of microbial protein synthesis.

In order to provide additional information on the nutritional value, the mixed diets were evaluated by two further methods. The total tract digestibility of crude nutrients was determined in wether sheep. The content of metabolisable energy was similar between diets and averaged 11.5 MJ per kg dry matter. The *in vitro* gas production was measured within 93 h by using a modified Hohenheim gas production test, providing information on kinetics and extent of ruminal fermentation. Cumulative gas production decreased with increasing proportion of GS in the diet. A negative effect of coarse milling on fermentation in the Hohenheim gas production test was confirmed. Across all diets gas production was delayed at 4 mm-MSS compared to 1 mm-MSS. The results from both approaches supported the findings of the RUSITEC study that a stepwise replacement of MS by GS led to a linear response in degradation of nutrients. As indicated by the gas production data, positive associative effects might only occur in the first hours after starting an incubation.

When mixed diets are used effects cannot be clearly related to individual diet ingredients. Moreover, in the mixed diets interactions between soybean meal inclusion and forage source

or feed particle size cannot be excluded. Therefore pure silages were incubated separately in the RUSITEC system in the second experimental series and three milling screens of different size were used (1, 4 and 9 mm). In accordance with the first experimental series, degradation of OM, fibre fractions and non-structural carbohydrates, production of NH_3-N, as well as microbial CP flow and efficiency of microbial CP synthesis were higher in GS than in MS. A higher degradation of CP was found for MS than for GS, indicating interactions between forage source and soybean meal inclusion. An increase in MSS from 1 mm to 9 mm led to an improvement in the degradation of OM, CP and non-structural carbohydrates, particularly of starch in MS, as well as in the microbial CP flow for both silages. But the efficiency of microbial CP synthesis and microbial amino acid profile were less affected by MSS.

In the second experimental series additionally the effect of available N on fermentation of MS was investigated. The supplementation of urea-N improved the degradation of non-structural carbohydrates, especially starch, but not that of fibre fractions in MS. The efficiency of microbial CP synthesis was increased from 26 to 35 mg microbial N per g degraded OM by urea-N supplementation to MS. The way of urea administration, either supplied together with the feed once daily or infused continuously by buffer solution, had only marginal effects on fermentation characteristics.

It was concluded that microbial growth is improved by degradation of OM from GS compared to MS and by an increasing availability of N in the RUSITEC system. Meaningful associative effects of mixtures of MS and GS on ruminal fermentation characteristics are not likely to occur. However, transferability of results to other batches of MS and GS is limited, as high variations in chemical composition are known for both types of silage. Fermentation of MS- and GS-based diets in the RUSITEC system benefits more by coarse milling at MSS up to 9 mm than by fine milling at 1 mm-MSS. Consequently, variations in MSS and feed particle size distribution have to be taken into account when evaluating feeds by rumen simulation systems. The changes in composition and contribution of microbial fractions give indications to a shift in the microbial community as a result of variation of silage type and feed particle size, but further research on this aspect is needed. Moreover, the present results stated that the origin of the microbial samples is very important for measurements on microbial protein synthesis.

Zusammenfassung

Die Synthese mikrobiellen Proteins im Pansen hat einen entscheidenden Einfluss auf die Protein- und Aminosäurenversorgung der Wiederkäuer. Die Menge und die Aminosäurenzusammensetzung des am Dünndarm anflutenden Proteins sind vor allem von der Rationsgestaltung abhängig. In der vorliegenden Arbeit wurde der Einfluss von Maissilage (MS) und Grassilage (GS) auf die ruminale Fermentation und die mikrobielle Proteinsynthese untersucht. Die Betrachtung methodischer Aspekte, insbesondere der Vermahlungsgrad des Futters, wurde in den vorliegenden *in vitro* Untersuchungen einbezogen.

In der ersten Versuchsreihe (Paper I + II) wurden fünf Mischrationen mit unterschiedlichen Anteilen an MS und GS (100:0, 79:21, 52:48, 24:76 und 0:100) und einem konstanten Anteil an Sojaextraktionsschrot (11%) verwendet. Mit steigendem Anteil an GS in der Ration stieg der Gehalt an Rohprotein (XP) und Faserfraktionen an, wohingegen der Gehalt an organischer Substanz (OS) und Stärke abnahm. Es wurde die Hypothese aufgestellt, dass eine Kombination von MS und GS das mikrobielle Wachstum und somit den Nährstoffabbau in einem höheren Maße begünstigt als die Verwendung einer einzigen Grundfutterquelle. Zudem sollte geprüft werden, wie sich die Rationsänderung auf das Aminosäurenmuster des mikrobiellen Proteins auswirkt. Es wurde eine gut standardisierte semi-kontinuierliche Pansensimulationstechnik (RUSITEC) genutzt, welche als Modell für Untersuchungen zur Pansenfermentation weitestgehend akzeptiert ist. Die Veränderung der erfassten Fermentationsparameter, als Folge der Änderung des MS:GS-Verhältnisses in der Ration, wurde auf lineare und quadratische Effekte untersucht, um mögliche assoziative Effekte zu identifizieren. Vor der *in vitro* Inkubation der Futtermittel wurden diese getrocknet und vermahlen. Inwieweit die mittlere Futterpartikelgröße die Ergebnisse im RUSITEC-System beeinflusst, sollte durch eine Variation der Siebweite (SW; 1 vs. 4 mm) bei der Vermahlung geklärt werden. Mögliche Wechselwirkungen von Grundfutterquelle und SW auf die Kenngrößen der ruminalen Fermentation wurden geprüft.

Eine Inkubationsperiode dauerte 13 Tage (6 Tage Adaptationsphase, 7 Tage Sammelphase) und jede Behandlung wurde mit mindestens 3 Wiederholungen getestet. Als Startmedium für die Inkubation diente feste und flüssige Phase der ruminalen Digesta von pansenfistulierten Hammeln. Das Futter wurde dem RUSITEC-System einmal täglich zugeführt und die Nylonfutterbeutel verblieben 48 h im Fermentationsgefäß. Eine mit $^{15}NH_4Cl$

versetzte Pufferlösung wurde kontinuierlich in das System infundiert. In der jeweiligen Überlaufflüssigkeit wurde der Gehalt an kurzkettigen Fettsäuren (SCFA) und NH_3-N bestimmt. Drei Mikrobenfraktionen wurden aus der festen und flüssigen Phase des Systems mittels Differentialzentrifugation isoliert; und zwar aus den Futterresten, aus der Flüssigkeit im Inneren des Fermentationsgefäßes und aus der Überlaufflüssigkeit. Die Menge an mikrobiellem XP, welches das System täglich verließ wurde mittels N- und ^{15}N-Bilanzen bestimmt. Die Futterreste wurden auf ihren Gehalt an Rohnährstoffen und Detergenzien-Faser-Fraktionen analysiert. Der Gehalt an OS und XP in den Futterresten wurde um den jeweiligen Anteil der Partikel-assoziierten Mikroben korrigiert.

Der stufenweise Austausch von MS durch GS bewirkte einen linearen Anstieg im Abbau der OS und der Faserfraktionen, sowie in der Menge an NH_3-N. Der XP-Abbau und die Gesamtmenge an gebildeten SCFA waren durch die Ration nicht beeinflusst. Eine grobe Vermahlung (4 mm SW) führte im Vergleich zu einer feinen Vermahlung (1 mm SW) zu einer Erhöhung im OS- und XP-Abbau, sowie zu einer erhöhten SCFA-Produktion. Eine Verbesserung der Wachstumsbedingungen für bestimmte Mikrobengruppen, z.B. anaerobe Pilze, wurde diskutiert. Die Menge an mikrobiellem XP stieg linear mit dem Austausch von MS durch GS an und war bei 4 mm-SW höher als bei 1 mm-SW. Es wurde vermutet, dass die Menge an verfügbarem N im RUSITEC-System das mikrobielle Wachstum fördert. Die Effizienz der mikrobiellen XP-Synthese (mg mikrobieller N/g abgebaute OS) stieg mit zunehmendem Anteil an GS in der Ration von 29 auf 43 an, war aber nicht durch die SW beeinflusst. Der N-Gehalt und das Aminosäurenprofil der drei Mikrobenfraktionen, ebenso wie das quantitative Verhältnis von Mikroben der festen und flüssigen Phase zueinander, waren durch die Rationsgestaltung und die SW beeinflusst. Wechselwirkungen von Grundfutterquelle und SW auf die untersuchten Merkmale waren kaum zu beobachten. Allerdings deuteten sich Wechselwirkungen zwischen den beiden Faktoren und der Herkunft der Mikrobenfraktion auf die Parameter der mikrobiellen Proteinsynthese an.

Um zusätzliche Informationen zum Futterwert zu erhalten, wurden zwei weitere Methoden zur Evaluierung der Mischrationen herangezogen. Eine Bestimmung der Rohnährstoffverdaulichkeit bei Hammeln ergab, dass der Gehalt an umsetzbarer Energie zwischen den Rationen ähnlich war und im Mittel 11.5 MJ je kg Trockensubstanz betrug. Zudem wurde *in vitro* die Gasbildung über 93 h mittels eines modifizierten Hohenheimer Futterwert-Testes gemessen, um Aussagen zur Kinetik und zum Umfang der ruminalen Fermentation zu erhalten. Die kumulative Gasbildung nahm mit steigendem Anteil an GS in der Ration ab.

Der negative Einfluss einer gröberen Vermahlung im Hohenheimer Futterwert-Test konnte bestätigt werden. In allen Rationen war die Gasbildung durch eine Erhöhung der SW von 1 mm auf 4 mm vermindert. Die Ergebnisse aus diesen beiden Untersuchungsansätzen untermauerten die Aussage der RUSITEC-Studie, dass ein stufenweiser Austausch von MS durch GS in der Ration zu einer linearen Änderung im Nährstoffabbau führt. Die Ergebnisse zur Gasbildung deuteten darauf hin, dass positive assoziative Effekte nur in den ersten Stunden nach Beginn der Inkubation auftreten.

Bei der Verwendung von Mischrationen können Effekte nicht eindeutig einzelnen Futterkomponenten zugeordnet werden. Zudem kann bei den Mischrationen nicht ausgeschlossen werden, dass Wechselwirkungen zwischen dem zugesetzten Sojaextraktionsschrot und der Grundfutterquelle oder der Futterpartikelgröße auftreten. Deshalb wurden in der zweiten Versuchsreihe ausschließlich die beiden Silagen getrennt inkubiert und drei verschiedene SW während der Vermahlung genutzt (1, 4, 9 mm). In Übereinstimmung mit den Ergebnissen der ersten Versuchsreihe war der Abbau der OS, der Faserfraktionen und der Nichtstruktur-Kohlenhydrate, die Menge an NH_3-N sowie der mikrobielle XP-Fluss und die Effizienz der mikrobiellen XP-Synthese bei GS höher als bei MS. Ein höherer XP-Abbau konnte bei MS im Vergleich zu GS festgestellt werden, was Hinweis auf eine Wechselwirkung zwischen Grundfutterquelle und der Zulage von Sojaextraktionsschrot gibt. Ein Anstieg in der Siebweite von 1 auf 9 mm führte in beiden Silagen zu einer Verbesserung im Abbau der OS, des XP und der Nichtstruktur-Kohlenhydrate, inbesondere von Stärke in MS, sowie zu einem erhöhten mikrobiellen XP-Fluss. Die Effizienz der mikrobiellen XP-Synthese, sowie das Aminosäurenmuster waren kaum von der SW beeinflusst.

In der zweiten Versuchsreihe wurde zusätzlich der Effekt der N-Verfügbarkeit auf die Fermentation von MS untersucht. Die Zugabe von Harnstoff-N führte zu einer Verbesserung im Abbau der Nichtstruktur-Kohlenhydrate, insbesondere der Stärke. Der Abbau der Faserfraktionen blieb hingegen unbeeinflusst. Die Effizienz der mikrobiellen XP-Synthese erhöhte sich von 26 auf 35 mg mikrobieller N/g abgebaute OS infolge der Harnstoffzugabe. Die Art und Weise der Harnstoffsupplementierung, entweder einmal täglich zusammen mit dem Futter oder als kontinuierliche Infusion über die Pufferlösung, hatte nur marginale Auswirkungen auf die untersuchten Fermentationsparameter.

Es wurde geschlussfolgert, dass das mikrobielle Wachstum im RUSITEC-System durch den Abbau der OS aus GS im Vergleich zu MS, sowie durch eine Erhöhung der verfügbaren N-Menge begünstigt wird. Nennenswerte assoziative Effekte auf das Fermentationsgeschehen

durch die Kombination von MS und GS in Mischrationen sind nicht zu erwarten. Allerdings ist die Übertragbarkeit dieser Ergebnisse auf andere Silage-Chargen begrenzt, da große Unterschiede in der chemischen Zusammensetzung bestehen können, sowohl bei MS als auch bei GS. Die Fermentation von MS und GS wird durch eine gröbere Vermahlung mit Erhöhung der SW von 1 mm bis auf 9 mm positiv beeinflusst. Infolgedessen sollten Unterschiede in der SW und der Futterpartikelgrößenverteilung bei der Bewertung von Silagen im RUSITEC-System berücksichtigt werden. Die Veränderungen in der Zusammensetzung und der Mengenanteile der einzelnen Mikrobenfraktionen, als Folge der Variation der Grundfutterquelle und der Futterpartikelgröße, weisen auf eine Verschiebung in der Zusammensetzung der mikrobiellen Gemeinschaft hin. Zudem bestätigen die vorliegenden Ergebnisse, dass die Herkunft der Mikrobenmasse einen großen Einfluss auf die Bewertung der mikrobiellen Proteinsynthese hat.

Lebenslauf

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt, keine anderen als die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche kenntlich gemacht habe.

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