Effect of carbohydrate composition of the diet and ruminal fluid passage on microbial transformations in the rumen

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List of abbreviations

[2H]	metabolic hydrogen
aD CP	apparent digestibility of crude protein
ADFom	acid detergent fiber (exclusive residual ash)
aNDFom	neutral detergent fiber (assayed with heat-stable amylase, exclusive residual ash)
ATP	adenosine triphosphate
BG	barley grain
BGhigh	barley grain with high dilution rate (treatment chapter 5)
BGlow	barley grain with low dilution rate (treatment chapter 5)
BM	body mass
BP	beet pulp
BPhigh	beet pulp with high dilution rate (treatment chapter 5)
BPlow	beet pulp with low dilution rate (treatment chapter 5)
C2	acetate
C3	propionate
C4	butyrate
CS	carbohydrate source
CP	crude protein
deOM	organic matter degradability (used in chapter 4 only)
DL	dilution rate
DM	dry matter
DMI	dry matter intake
dOM	degraded OM (used in chapter 4 for digestibility of organic matter)
DOM	digested organic matter
FOMr	fermentable organic matter in the rumen
GIT	gastrointestinal tract
GP	gas production
HFT	Hohenheimer Futterwerttest
HGT	Hohenheim gas test
hrsm	hay and rapeseed meal
LAM	liquid-associated microorganisms
MCP	microbial crude protein
MP	microbial protein
ME	metabolizable energy

MRT	mean retention time
MRT_{fluid}	mean retention time of the fluid phase
$MRT_{fluid}RR$	mean retention time of the fluid measured in the ruminoreticulum
$MRT_{particle}$	mean retention time of the particles
$MRT_{part}RR$	mean retention time of the particles measured in the ruminoreticulum
NDFD	NDFom degradability
NDS	neutral detergent soluble
NFC	non-fiber carbohydrates
NSC	nonstructural carbohydrates
OM	organic matter
OMD	organic matter degradability (except chapter 4)
OMD_{cs}	OMD of the carbohydrate source
OMD_{hrsm}	OMD of the hay and rapeseed meal together
rDMI	relative dry matter intake
RR	reticulorumen
RSM	rapeseed meal
SAM	solid-associated microorganisms
SBH	soybean hulls
SBHhigh	soybean hulls with high dilution rate (treatment chapter 5)
SBHlow	soybean hulls with low dilution rate (treatment chapter 5)
SCFA	short chain fatty acid
SEM	standard error of means
SD	standard deviation of means
SF	selectivity factor ($MRT_{particle}/MRT_{fluid}$)
$t_{1/2}$	estimate the time of half-maximal gas production
TMR	total mixed ration
tot	total
uCP	utilizable crude protein

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1 Summary

The aim of this study was to investigate certain effects on the microbial crude protein (**MCP**) production in the rumen and to investigate mechanisms which could promote the ruminal MCP yield to enhance the MCP supply to the ruminant. Due to the high protein requirements due to lactation, this is particularly important for high yielding dairy cows. First, the MCP yield of different pure carbohydrates and different feeds, which differ considerably in their carbohydrate composition, respectively, was investigated. Further, the impact of fluid passage rate (dilution rate [**DL]**) on MCP production was examined. The third question, which was explored, was if there is a different in mean retention time (**MRT**) of feed particles and fluid in the rumen of cattle and sheep and if relating thereto a difference in MCP production between the species could be obtained.

For the first part of this study, fermentation characteristics and MCP production of four different pure carbohydrates (sucrose, starch, cellulose and pectin) were investigated *in vitro* by the modified Hohenheim gas test (**HGT**). After 8 and 24 h of incubation in the modified HGT (3 runs × 2 syringes) measurements of gas production (**GP**), short chain fatty acid and ammonia concentration were conducted. Ammonia values were used for estimation of the MCP formation. Additionally, the substrates were incubated for 96 h in the HGT system (2 runs × 3 syringes) and GP was measured after 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 60, 72 and 96 h of incubation to obtain the fermentation kinetics and the time of half-maximal GP ($t_{1/2}$) of the substrates. The pure carbohydrates differed considerably in their fermentation kinetics. At $t_{1/2}$, MCP yield [g/kg dry matter] was higher for cellulose than for sucrose and pectin and higher for starch than for sucrose and MCP [g/L GP] was higher for starch and cellulose than for sucrose and pectin. These findings show, that different carbohydrates vary in their MCP yield, especially cellulose promotes higher MCP production. However, the slower fermentation rate of cellulose has to be kept in mind.

In the second part of this study, three commonly used feeds (barley grain [**BG**], beet pulp [**BP**], and soybean hulls [**SBH**]) were incubated together with a mixture of grass hay and rapeseed meal in two identical Rumen simulation technique (**Rusitec**) apparatuses (each 6 vessels). Additionally, differences in DL were simulated by infusing artificial saliva at two different rates (1.5% [**low**] and 3.0% [**high**] of fermenter volume per h). This resulted in six treatments tested in 3 runs. The system was adapted for 7 d, followed by 4 d of sampling. Production of MCP was estimated by ^{15}N analysis. Production of MCP (mg/g degraded organic matter [**dOM**]) was higher for SBH compared to both BG and BP and greater with high DL. High DL reduced organic matter degradability (**OMD**) and methane production (both /d and /g dOM) compared to low DL. Feeds with different carbohydrate composition varied in their MCP yield, especially SBH containing mostly cellulose promoted a higher MCP production. However, the

lower OMD of SBH have to be kept in mind. Additionally, increasing fluid passage rate showed the potential to increase MCP and decrease methane production at the same time.

In the third part of this study, the hypothesis of a systematical difference between cattle and sheep in MRT of particle and fluid and its ratio (**$MRT_{particle}/MTR_{fluid}$** = selectivity factor [**SF**]) as well as in the apparent digestibility of crude protein (**aD CP**) were investigated in a meta-analysis. Keeping the microorganism in a state of more intense growth due to a more pronounced digesta 'washing' by a higher SF should lead to an increase in MCP yield and therefore should increase the metabolic losses of fecal nitrogen of microbial origin and decrease the aD CP simultaneously (true digestibility of protein not being influenced). The datasets included 12 studies on MRT (of which 11 contained information on feed intake), yielding 102 (or 89) individual data; and 26 studies on protein digestibility (of which 18 contained information on intake), yielding 349 individual data. Only studies that investigated cattle and sheep simultaneously were used. Cattle had a higher SF than sheep, mainly due to longer $MRT_{particle}$. Only if body mass was included in the model, MRT_{fluid} was significantly shorter in cattle in the larger MRT dataset. Cattle had a significantly lower aD CP than sheep, while there was no such difference in overall (dry or organic matter) digestibility. These findings indicate that cattle are especially good in maximizing the ruminal MCP yield.

In conclusion, MCP production is affected by carbohydrate source or feedstuff composition, by DL and by SF. However, not all studies in the literature found the same effects and transferability from *in vitro* data into *in vivo* is challenging and not without limits. Also, optimizing diets for a maximal MCP yield is not easy as various other factors affecting MCP production in the rumen as well. Further research is needed for a more precise understanding of the complex relationships between diet, feedstuff and carbohydrate composition, MRT, animal individual factors and MCP production in the rumen.

2 Zusammenfassung

Ziel dieser Studie war es Faktoren zu untersuchen, die die Produktion des mikrobiellen Rohproteins (**MCP**) im Pansen beeinflussen können, und Mechanismen zu untersuchen, die den ruminalen MCP-Ertrag unterstützen können und dadurch die Versorgung des Wiederkäuers mit MCP erhöhen. Aufgrund des besonders hohen Proteinbedarfs für die Milchbildung ist dies besonders wichtig für hochleistende Milchkühe. Zuerst wurde die MCP-Produktion verschiedener Kohlenhydrate bzw. verschiedener Futtermittel, welche sich deutlich in ihrer Kohlenhydratzusammensetzung unterscheiden, untersucht. Des Weiteren wurde der Einfluss der Flüssigkeitspassagerate (Verdünnungsrate [**DL]**) auf die MCP-Produktion untersucht. Die dritte Fragestellung, die untersucht wurde, war, ob ein Unterschied zwischen Rindern und Schafen in der mittleren Verweildauer (mean retention time [**MRT]**) von Futterpartikeln und Flüssigkeit im Pansen besteht und sich dadurch ein Unterschied in der MCP-Produktion zwischen den Spezies ergibt.

Für den ersten Teil der Arbeit wurden vier verschiedene Kohlenhydrate (Saccharose, Stärke, Zellulose und Pektin; alle als Reinsubstanzen) mittels des erweiterten Hohenheimer Futterwerttestes (**HFT**) auf ihr Fermentationsverhalten und ihr Potential für die MCP-Bildung *in vitro* untersucht. Dafür wurden sie für 8 und 24 h für die Messung der Gasproduktion und des Ammoniumgehaltes im erweiterten HFT (3 Läufe × 2 Spritzen) inkubiert. Die Ammoniumgehalte wurden zur Schätzung der MCP-Bildung verwendet. Zusätzlich wurden die Substrate für 96 h im HFT mit detaillierter Gasproduktionsmessung nach 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 60, 72 und 96 h zur Bestimmung der Fermentationskinetik und des Zeitpunktes der halbmaximalen Gasbildung ($t_{1/2}$) inkubiert. Die Fermentationskinetik der Kohlenhydrate im HFT unterschieden sich deutlich voneinander. Bei $t_{1/2}$ war der MCP-Ertrag [g/kg Trockenmasse] der Zellulose größer als von Saccharose und Pektin und der MCP-Ertrag von Stärke größer als von Saccharose. Stärke und Zellulose hatten eine höhere MCP-Produktion [g/L Gasproduktion] als Saccharose und Pektin bei $t_{1/2}$. Die Ergebnisse zeigen, dass verschiedene Kohlenhydrate sich in ihrem MCP-Ertrag unterscheiden und besonders Zellulose eine höhere MCP-Produktion fördert. Allerdings muss hierbei die langsamere Fermentationsrate von Zellulose gegenüber den anderen Substraten berücksichtigt werden.

Im zweiten Teil der Arbeit wurden drei verschiedene Futtermittel (Gerste [**BG**], Zuckerrübenschnitzel [**BP**] und Sojaschalen [**SBH**]) zusammen mit einer Mischung aus Grasheu und Rapsextraktionsschrot in zwei identischen Pansensimulationsystemen (**Rusitec**) (jeweils mit 6 Fermentern ausgestattet) inkubiert. Zusätzlich wurden zwei verschiedene Flüssigkeitspassagen (Verdünnungsrate; DL) mittels niedriger (1.5% [**low DL**]) und hoher (3.0% [**high DL**] des Fermentervolumens pro h) Infusionsrate der Pufferlösung (künstlicher Speichel) in die Fermenter simuliert. Daraus ergaben sich sechs verschiedene

Behandlungen, die in jeweils 3 Läufen getestet wurden. Die Adaptationszeit betrug 7 d mit nachfolgenden 4 d Sammelperiode. Die MCP-Produktion wurde mittels ^{15}N-Analyse bestimmt. Die MCP-Bildung [mg/g abgebaute organische Masse (**dOM**)] der SBH war höher im Vergleich zu BG und BP und größer mit high DL. Die Abbaubarkeit der organischen Masse (**OMD**) und die Methanproduktion [sowohl pro Tag und als auch pro g dOM] war mit high DL verringert. Der MCP-Ertrag variierte mit den Futtermitteln, die sich deutlich in ihrer Kohlenhydratzusammensetzung unterschieden. Besonders die SBH, die größtenteils aus Zellulose bestehen, haben die MCP-Produktion gefördert. Allerdings muss die geringere OMD der SBH im Vergleich zu BG und BP berücksichtigt werden. Des Weiteren hat eine Erhöhung der Flüssigkeitspassagerate im Pansen das Potential gleichzeitig MCP-Bildung zu erhöhen und die Methanproduktion zu reduzieren.

Im dritten Teil der Arbeit wurde mittels Metaanalyse die Hypothese eines systematischen Unterschiedes zwischen Rindern und Schafen in der MRT von Partikeln und Flüssigkeit und deren Verhältnis (**$MRT_{particle}$/MRT_{fluid}** = selectivity factor [**SF**]) sowie der scheinbaren Verdaulichkeit des Rohproteins (**aD CP**) untersucht. Wenn die Mikroorgansimen durch ein vermehrtes „Waschen“ der Futterpartikel durch einen höheren SF mehr im Wachstumsstadium gehalten werden, sollte dies zu einem erhöhten MCP-Ertrag und gleichzeitig zu einer Erhöhung der metabolischen Verluste von mikrobiellem Stickstoff im Kot und damit zu einer geringeren aD CP führen (bei gleichzeitig nicht beeinflusster wahrer Verdaulichkeit des Rohproteins). Dazu wurde ein Datensatz mit 12 Studien zur MRT mit 102 Einzeldaten, von denen 11 Studien (89 Einzeldaten) Informationen zur Futteraufnahme enthielten, und ein weiterer Datensatz mit 26 Studien zur aD CP, von denen 18 Informationen zur Futteraufnahme enthielten, mit 349 Einzeldaten erhoben. Es wurden ausschließlich Studien verwendet, die gleichzeitig Daten zu Rindern und Schafen erhoben haben, die mit derselben Ration gefüttert wurden. Rinder hatten einen höheren SF als Schafe, hauptsächlich durch eine längere $MRT_{particle}$. Die aD CP der Rinder war geringer als die der Schafe, während sich die generelle Verdaulichkeit (der Trocken- oder organischen Substanz) nicht unterschieden. Die Ergebnisse weisen darauf hin, dass Rinder besonders gut den ruminalen MCP-Ertrag maximieren können.

Abschließend lässt sich festhalten, dass die MCP-Produktion von der Kohlenhydratquelle bzw. der Futtermittelzusammensetzung, der Flüssigkeitspassagerate und dem SF beeinflusst wird. Allerdings finden in der Literatur nicht alle Studien dieselben Effekte und der Übertragbarkeit der *in vitro* Ergebnisse auf das Tier sind naturgemäß gewisse Grenzen gesetzt. Zusätzlich ist es nicht einfach, Rationen für eine optimale MCP-Bildung zu optimieren, da auch viele andere Faktoren die MCP-Bildung beeinflussen. Für ein genaueres Verständnis der komplexen Zusammenhänge zwischen Ration, Futtermittel- und Kohlenhydratzusammensetzung, MRT, tierindividuellen Faktoren und der MCP-Bildung im Pansen besteht weiterer Forschungsbedarf.

3 General introduction

One great advantage of ruminants are the microorganisms in their rumen. These microbes are able to use non protein nitrogen to synthesize essential amino acids and protein. As a result, ruminants are largely independent of the protein quality of their feedstuff. For high yielding dairy cows this might not apply completely. Due to their high performance, it is challenging to meet their protein requirements because the protein synthesis of the microbes could be limited by various factors. For example, energy, nitrogen and mineral supply, ruminal pH and passage rate of fluid and feed particles affect the synthesis of microbial crude protein (**MCP**) in the rumen (Owens and Goetsch, 1986).

3.1 Factors affecting microbial growth in the rumen

3.1.1 Energy supply to the microbes

The feedstuffs which ruminants consume represent also the feed for the microorganisms in the rumen. These feedstuffs contain various nutrients of which carbohydrates are regarded as the main energy source for rumen microorganisms. The structure of carbohydrates can vary from monosaccharides like glucose to very complex polysaccharides like starch, pectin or cellulose. Fermentability and therefore the availability of energy for the rumen microbes differs between carbohydrates and depend on factors like solubility, complexity and embedding in other plant structures (Russell and Hespell, 1981). While cellulose is insoluble, pectin is soluble and starch could be both (Van Soest et al., 1991). The insolubility contributes partly to slower fermentation and degradation rates. Additionally, native starch granules may escape ruminal fermentation (Russell and Hespell, 1981). Structural factors like lignification affect degradation mostly of forage fiber due to a close linkage between lignin and cellulose (Russell and Hespell, 1981). Among complex carbohydrates, pectin is most rapidly and very completely degraded in the rumen (Van Soest et al., 1991; Hatfield and Weimer, 1995).

For microbial growth, is it important that particularly energy, nitrogen and amino N are available at the same time and speed (Russell and Strobel, 2005). If energy supply is limited, a large proportion of the energy is used for maintenance (Figure 3.1, Russell and Strobel, 2005). The expenses for maintenance are diluted if more energy is provided. The digestion rate of the feedstuff determines the amount of energy that is available for the rumen microorganisms. Higher digestion rates provide more energy to the microbes per time unit (Van Soest et al., 1991). Digestion rates differ between carbohydrates. Cellulose is fermented at lower rates than pectin or some starch, but can yield more bacteria per gram digested organic matter (**DOM**) due to the lower maintenance costs of cellulolytic bacteria (Van Soest et al., 1991).

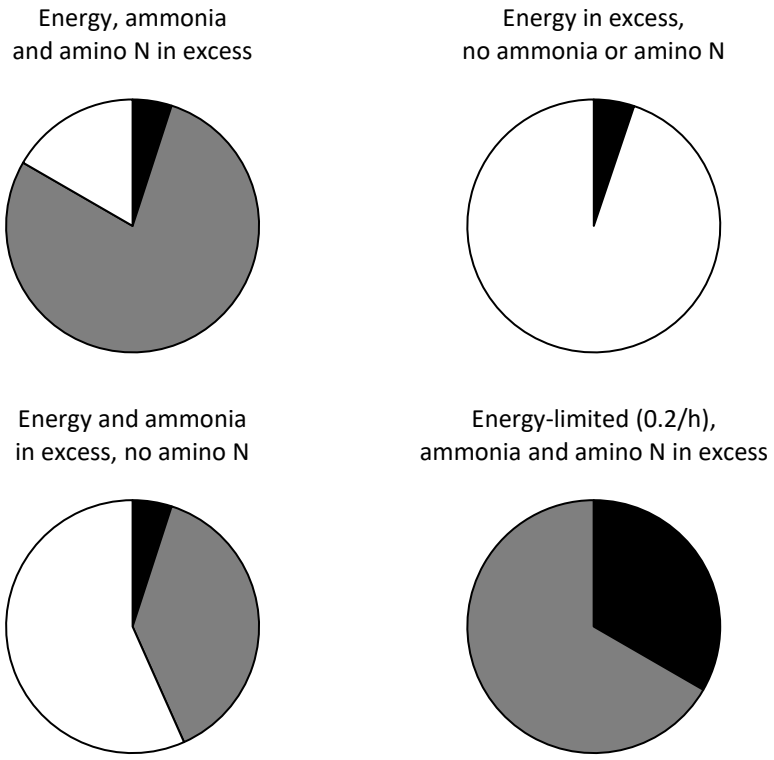

Figure 3.1: A schematic showing the effect of energy, ammonia and amino N on the relative distribution of energy utilization by Streptococcus bovis. Black: maintenance energy; grey: growth; white: energy spilling. (Russell and Strobel, 2005)

Cell wall components like cellulose and pectin are mostly fermented to acetate, whereas fermentation of starch and sucrose leads to higher proportions of propionate and butyrate, respectively. Since acetate provides more adenosine triphosphate (**ATP**) to the microbes than propionate or butyrate, fermentation of cellulose and pectin could provide more energy for the microbes resulting in more MCP (Bergner and Hoffmann, 1996).

3.1.2 Ruminal pH

In general, a lower ruminal pH decreases carbohydrate fermentation and bacterial cell yield even of species which are more tolerant towards a low ruminal pH (Russell and Dombrowski, 1980; Strobel and Russell, 1986). Also, efficiency of carbohydrate utilization decreases as some ATP is used for maintenance or energy spilling reactions (Strobel and Russell, 1986).

Because starch degrading bacteria can switch from acetate to lactate production, feeding large amounts of rapidly degradable starch and sucrose can lead to rumen acidosis (Van Soest et al., 1991) (Figure 3.2). Lactate production leads only to halve the amount of ATP for the microbes as acetate production, but the lower ruminal pH favors starch degrading microorganisms, as other microorganisms, especially cellulose and pectin degrading bacteria, are more sensitive to low ruminal

pH (Strobel and Russell, 1986; Van Soest et al., 1991; Russell and Strobel, 2005). While starch can be degraded to lactate, especially at lower pH, pectins are not fermented to lactate (Strobel and Russell, 1986; Van Soest et al., 1991; Hatfield and Weimer, 1995). Additionally, pectin consists of galacturonic acids which could have some buffering effect in the rumen itself (Van Soest et al., 1991).

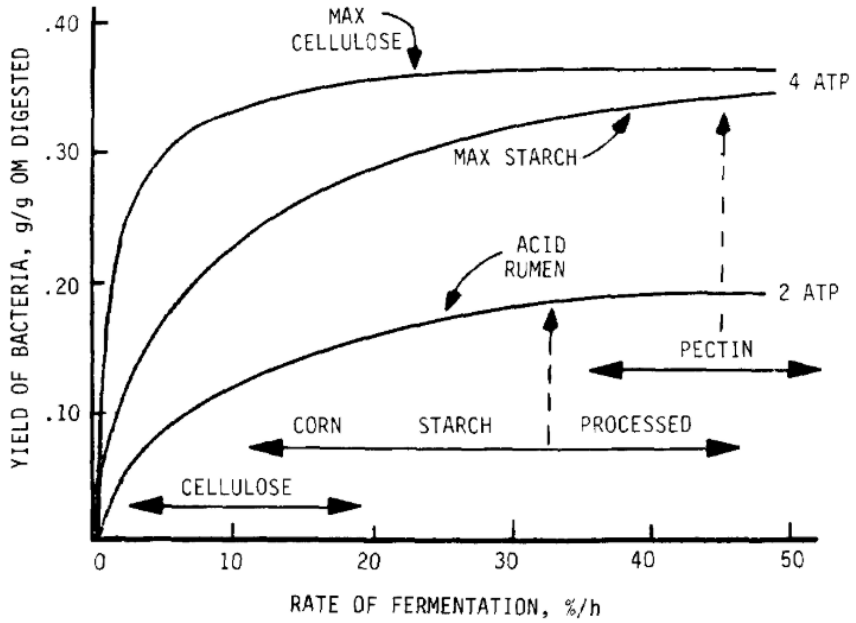

Figure 3.2: The relationship between the amount of microbial protein produced per unit of feed fermented in the rumen in relation to rate of fermentation. Bacteria in a normal rumen ferment carbohydrate to short chain fatty acids with a yield of 4 adenosine triphosphate (ATP) from 1 glucose. Lactic acid production (lower curve) is characteristic of acidic rumens and yields only 2 ATP/mol of glucose. (Van Soest et al., 1991)

3.1.3 Passage rate/ dilution rate/ mean retention time

With increased dilution rate (**DL**), the microbial yield increases due to faster growing microorganisms which use more of the available energy for growth than for maintenance (Figure 3.3) (Isaacson et al., 1975; Van Soest, 1994). Therefore, the efficiency of microbial cell and protein formation increases with higher DL (Van Soest, 1994). According to Isaacson et al. (1975), energy used for maintenance can vary considerably. At high DL only 15% of the energy was used for maintenance whereas at low DL 55% of the energy derived from glucose was used for maintenance (Isaacson et al., 1975). Higher washout rates of microbial cells from the rumen keep the microbial population in a growing state and result in less microbial cell lyses and N turnover within the rumen (Nolan and Leng, 1983; Van Soest, 1994).

A greater formation of microbial biomass and consequently microbial protein and a higher washout rate of microbial cells from the rumen lead to an increased MCP supply for the ruminant (Hungate, 1966; Isaacson et al., 1975; Van Soest, 1994). Additionally, Isaacson et al. (1975) observed a higher N content in microbial cells at higher DL.

According to Isaacson et al. (1975) short chain fatty acid (**SCFA**) pattern shifted with increased DL. While total SCFA and acetate production was not effected, propionate production increased and butyrate production decreased with increased DL (Table 3.1). Carro et al. (1995) observed similar changes for propionate and butyrate. Also, methane production was reduced with increased DL (Isaacson et al., 1975). In contrast, Martínez et al. (2009) observed an increase in methane and total SCFA production with higher DL and Carro et al. (1995) determined an decrease total SCFA production and no effect on methane production.

While DL is the term in *in vitro* systems, in live animals, the mean retention time (**MRT**) of the fluid is regarded. As *in vivo* the particle flow through the gastrointestinal tract as a whole cannot be hindered, MRT of the particle and the ratio $MRT_{particle}$ to MRT_{fluid}, the so-called selectivity factor (**SF**) (Lechner-Doll et al., 1990), should be regarded as well. An increased DL is comparable with an increased SF.

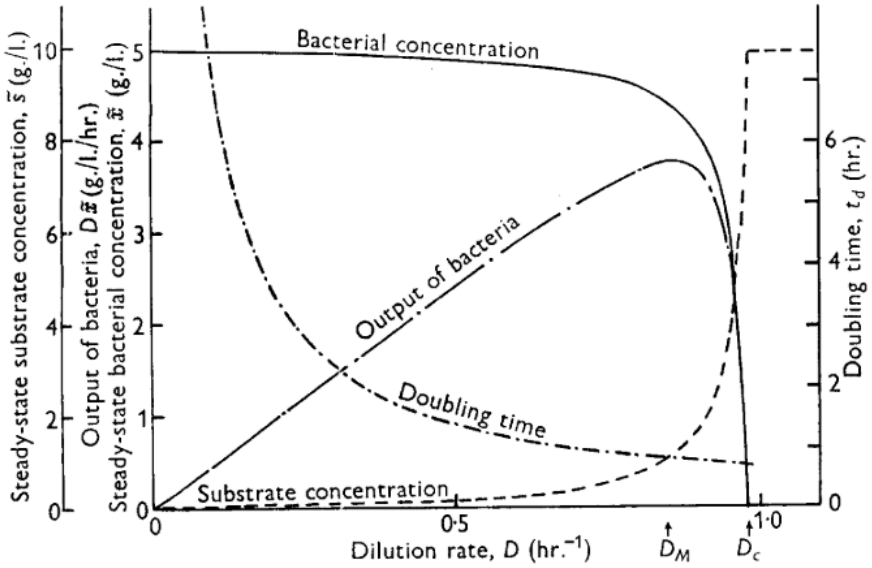

Figure 3.3: Steady-state relationship in a continuous culture (theoretical). (Herbert et al., 1956)

Table 3.1: Effect of dilution rate on fermentation parameters of glucose. (Means of four glucose concentrations at each dilution rate) (Isaacson et al., 1975)

		Dilution rate [h^{-1}]							
Parameter	**Unit**	**0.02**	**SD**	**0.06**	**SD**	**0.12**	**SD**	**Mean**	**SD**	**Significance**
SCFA	moles produced per mole glucose fermented	1.57	0.33	1.50	0.11	1.53	0.19	1.53	0.13	n. s.
Acetate	moles produced per mole glucose fermented	1.18	0.31	1.11	0.17	1.13	0.06	1.14	0.11	n. s.
Propionate	moles produced per mole glucose fermented	0.16	0.05	0.22	0.02	0.26	0.11	0.21	0.05	tend.
Butyrate	moles produced per mole glucose fermented	0.23	0.11	0.18	0.09	0.15	0.09	0.18	0.06	tend.
Methane	moles produced per mole glucose fermented	1.67	0.53	1.34	0.32	1.04	0.15	1.35	0.26	sign.
Dry cell concentration	mg/ml	0.61	0.40	0.86	0.59	1.07	0.60	0.84	0.51	sign.
Viable cells	x 10^{13} per mole glucose fermented	5.6	1.7	13.5	9.1	15.8	2.9	11.6	3.60	sign.
$Y_{glucose}$	g cells per mole glucose fermented	42.2	3.2	60.2	5.0	83.9	12.5	62.1	4.70	sign.
Y_{ATP}	g cells per mole ATP	7.5	1.6	11.6	1.5	16.7	1.4	11.7	1.60	sign.

SD – standard deviation of mean
Significances were taken from the text (n. s. – not significant; tend. – tended do differ; sign. – significant)
ATP – adenosine triphosphate

3.2 Microbial cell composition

The cell composition of ruminal microorganisms can vary between species and host animals (Van Soest, 1994). Carbohydrates and maybe also peptides stored in the cells alter the measured microbial cell composition; complete separation of microbial cells from external plant material can also represent a methodological challenge (Van Soest, 1994). Also, the growth stage of the cells at collection time can affect cell composition of the microbes (Hespell and Bryant, 1979). Additionally, the true microbial protein content is affected by applied conversion factor if only the N content is measured and the protein content is calculated (Van Soest, 1994). Considering a N content of 15% for true microbial protein (Van Soest, 1994), the calculated conversion factor would be 6.67. In Table 3.2, composition of microbial cells are presented with data summarized by Hespell and Bryant (1979) and Van Soest (1994).

Table 3.2: Composition of microbes (on dry matter basis unless otherwise indicated) (Van Soest, 1994)

	Bacteria		Protozoa
Constituent	**Probable most representative value[a]**	**Range**	**Range**
Total nitrogen	10[b]	5.0[c]-12.4[d]	3.8-7.9[d]
True protein	47.5[e]	38-55	-
RNA	24.2[e]	-	-
DNA	3.4[e]	-	-
Lipid	7.0[e]	4[f]-25[e]	-
Polysaccharide	11.5[e]	6-23[e]	-
Peptidoglycan	2	-	0
Nitrogen digestibility	71[g]	44-86[g]	76-85[h]

[a] Many discordant values have been recorded, possibly reflecting contamination or inclusion of plant material
[b] Isaacson et al. (1975)
[c] Smith and McAllan (1973)
[d] Weller (1957)
[e] Summarized by Hespell and Bryant (1979)
[f] Abdo et al. (1964); also reported 6% crude fiber.
[g] Bergen et al. (1968); values as percentage of total N.
[h] Bergen et al. (1967)

3.3 Species effect

Within ruminants, the MRT of fluid and particles in the gastrointestinal tract as well as the SF can differ between species. Such differences in SF have been used to divide ruminants into two types, the 'cattle-type' and the 'moose-type' (Clauss et al., 2010; Przybyło et al., 2019). Ruminates of the 'moose-type' are associated with a low SF (Clauss et al., 2010; Przybyło et al., 2019) and a smaller variation in SF

(1.14-1.80) (Clauss and Lechner-Doll, 2001). Ruminants of the 'cattle-type' are associated with a high SF (Clauss et al., 2010; Przybyło et al., 2019) and therefore with a higher washing of the particulate digesta in the rumen by the fluid phase (Müller et al., 2011). This is suggested to function in the same way as an increased DL in *in vitro* systems which leads to a higher microbial yield through an increased microbial outflow from the fermenter triggering an increased microbial growth (Herbert et al., 1956; Isaacson et al., 1975; Hummel et al., 2008, 2015; Müller et al., 2011) (see also chapter 3.1.3). While the 'moose-type' occurs only in exclusively browsing ruminants, grazing and intermediate ruminants both belong to the 'cattle-type' (Clauss et al., 2006, 2010). According to Clauss et al. (2006) domestic cattle seem to have an especially short MRT_{fluid} per unit $MRT_{particle}$ or – the other way around – an especially long $MRT_{particle}$ per unit MRT_{fluid} among grazing and intermediate ruminants yielding in a particularly high SF (Figure 3.4). Potential differences between the two domestic grazers cattle and sheep are investigated in chapter 6 in further detail.

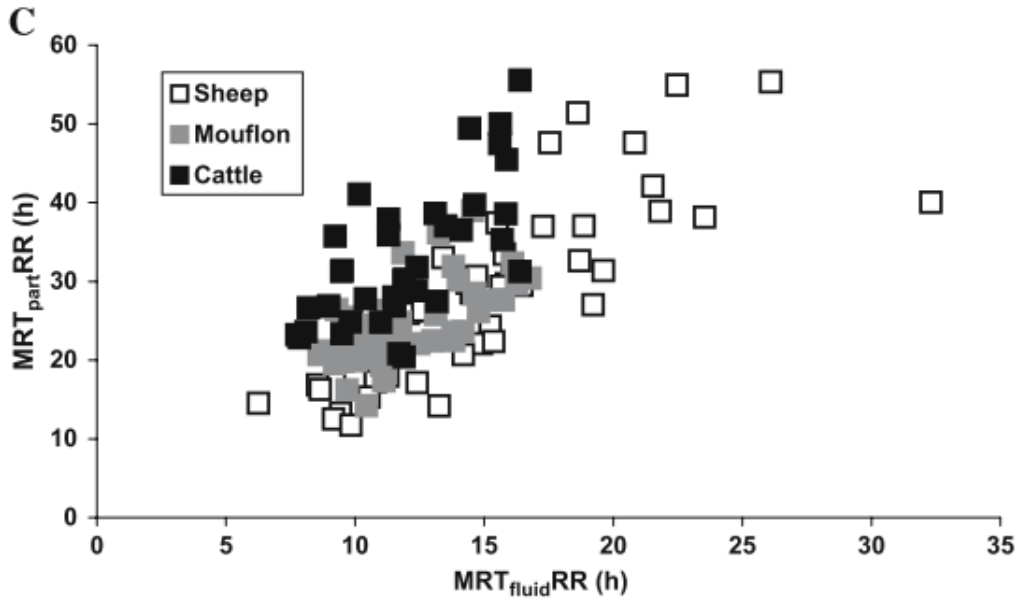

Figure 3.4: Correlation between mean retention time of the fluid measured in the ruminoreticulum ($MRT_{fluid}RR$) and mean retention time of the particles measured in the ruminoreticulum ($MRT_{part}RR$) in different ruminant species of grazers; only data were used where both values were determined simultaneously in individual animals (Clauss et al., 2006)

3.4 *In vitro* methods

In vitro cultures of rumen microbes are a way to investigate for example their growth and behavior without direct animal experiments. *In vitro* systems are designed to mimic the rumen as good as they can. Besides the general challenge to create a rumen-like environment in the lab, their biggest disadvantage is their lack of absorption of fermentation products like the rumen wall is doing it (Van Soest, 1994). However, compared to *in vivo* experiments, *in vitro* cultures have the advantage to allow

the investigation of otherwise hard to evaluate variables (like SCFA composition) and the analysis of a large number of treatments in a relatively short period of time (Deitmers et al., 2022).

3.4.1 Batch cultures

Batch cultures as described by Tilley and Terry (1963) and Menke and Steingass (1988) with mixed rumen microbes are used to measure the degradability of feedstuff as well as their fermentation kinetics. Small amounts (0.2-0.5 g) of feed sample are incubated in a mixture of strained rumen liquor and buffer solution for 24-48 h (Tilley and Terry, 1963; Menke and Steingass, 1988). At short incubation times (6-24 h) anaerobic conditions for the rumen inoculum are particularly crucial (Van Soest, 1994). There is no insertion of new feed or buffer or removal of fermentation products from the fermentation solution over the incubation time (Tilley and Terry, 1963; Menke and Steingass, 1988). Therefore, the provided buffer in the inoculum has to be sufficient to maintain normal ruminal pH over the whole incubation time. To measure complete degradation incubation times of 96 h are used. At such long incubation times, the fermentation comes to an end when the substrate is completely used up.

Additionally, the modified Hohenheim gas test (**HGT**) (Leberl et al., 2007; Edmunds et al., 2012) can be used to estimate utilizable crude protein (**uCP**) consisting of the undegraded feed protein and the microbial protein of feed stuff.

3.4.2 Continuous cultures

Continuous culture systems with rumen inoculum are a step closer to the live animal. Unlike batch cultures, they have the possibility to infuse buffer solution (artificial saliva) continuously, to simulate the feeding of the animal and to wash out fermentation products via overflow. Quick absorption of fermentation products through the rumen wall still remains reserved for the live ruminant as well as the very selective retention of feed particles (Van Soest, 1994). But continuous culture systems are able to maintain a stable fermentation over several weeks (Czerkawski and Breckenridge, 1977; Deitmers et al., 2022).

Currently, two continuous culture systems are primarily in use (Van Soest, 1994; Deitmers et al., 2022). One, a semi-continuous culture system, is the rumen simulation technique (Rusitec) developed by Czerkawski and Breckenridge (1977) where the fluid exits via overflow and the feed is contained in nylon bags; a second system is described by Hoover et al. (1976) where both fluid and feed particles exit via overflow. In both systems the fluid turnover or DL is controlled by the inflow of liquid. In the Rusitec system the microbes in the fermenters normally receive new feed substrate every 24 h while the buffer solution or artificial saliva usually according to McDougall (1948) enters the fermenters continuously (Czerkawski and Breckenridge, 1977).

4 Microbial protein formation of different carbohydrates *in vitro*

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Abstract

The aim of this study was to investigate the microbial protein yield of different pure carbohydrates to contribute to a more precise prediction of the microbial protein formed in the rumen. In a first experiment, sucrose, wheat starch, microcrystalline cellulose and citrus pectin were incubated for 8 and 24 hr in the modified Hohenheim gas test (**HGT**) system (3 runs × 2 syringes) including gas production, ammonia and short-chain fatty acid concentration measurements. Ammonia values were used for estimation of the microbial protein formation. In a second experiment, the same substrates were incubated for 96 hr in the HGT system (2 runs × 3 syringes) and gas production was measured after 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 60, 72 and 96 hr of incubation to obtain the fermentation kinetics and the time of half-maximal gas production ($\boldsymbol{t_{1/2}}$) of the substrates. The substrates differed considerably in their fermentation kinetics, and therefore, comparison on the basis of $t_{1/2}$ was chosen as the most meaningful. At $t_{1/2}$, microbial protein yield [g/kg dry matter] was higher for cellulose than for sucrose and pectin and higher for starch than for sucrose. The microbial protein expressed in g/L gas production was higher for starch and cellulose than for sucrose and pectin at $t_{1/2}$. Effects of carbohydrates related to ruminal pH may remain undetected in *in vitro* trials.

KEYWORDS

cellulose, fermentation, Hohenheim gas test, microbial growth, pectin, starch, sucrose

1 | INTRODUCTION

High yielding dairy cows have a particularly high protein demand. Since most of their protein supply comes from microbial protein (**MP**) built in the rumen, the process of microbial growth deserves particular attention. In addition, ruminal N metabolism has been identified as the major process influencing N efficiency of the ruminant (Calsamiglia, Ferret, Reynolds, Kristensen, & Van Vuuren, 2010). Besides nitrogen, micro-organisms need energy for their growth; the amount of MP formed depends on the energy available for the microbes. In general, carbohydrates can be regarded as the main energy source for rumen micro-organisms, but they obviously differ considerably, for example in their structure, solubility and fermentation characteristics. Among complex carbohydrates, pectin is most rapidly and very completely degraded in the rumen (Hatfield & Weimer, 1995; Van Soest, Robertson, & Lewis, 1991). Formation of MP is reported to be more efficient with a rapid rate of degradation (Van Soest et al., 1991). While starch-degrading bacteria can switch from acetate to lactate production resulting in less energy available for microbes and decreased ruminal pH value, pectin is not fermented to lactate (Van Soest et al., 1991; Strobel & Russell, 1986). In consequence, starch-rich diets have been said to lead to ruminal acidosis and inefficient MP production more often than pectin-rich. In contrast, Hall and Herejk (2001) found a greater maximal MP yield for starch than for pectin and isolated neutral detergent fibre in *in vitro* experiments. According to Van Soest et al. (1991), cellulose can yield more bacteria per g digested organic matter (**DOM**) at lower rates of fermentation than starch. Due to their lower maintenance costs, cellulolytic bacteria are more efficient (Van Soest et al., 1991). Since acetate provides more adenosine triphosphate (**ATP**) to the microbes than propionate or butyrate, cellulose and pectin as mostly fermented to acetate could provide more energy for the microbes resulting in more MP (Bergner, 1996; Van Soest et al., 1991).

Detailed knowledge on carbohydrates and their effects on microbial growth can be regarded as a very important factor when ruminal MP formation is to be predicted. The prediction of MP yield is crucial for an adequate protein supply for high yielding dairy cows. Until now predictions are general (e.g. GfE, 2001: prediction of 162 g MP/kg fat-free digestible organic matter (**dOM**), irrespective of the carbohydrate composition of the diet), but more accurate values would be desirable for a more specific diet composition. The aim of this study was to investigate MP yield of different pure carbohydrates (sucrose, starch, pectin and cellulose) with the modified Hohenheim gas test *in vitro*.

2 | MATERIAL AND METHODS

2.1 | In vitro gas production measurements

For the first experiment sucrose (CAS 57-50-1, VRW International GmbH, Darmstadt, Germany), wheat starch (unmodified, CAS 9005-25-8, Sigma-Aldrich, St. Louis, USA), microcrystalline cellulose (for thin-layer chromatography, Merck, Darmstadt, Germany) and citrus pectin (degree of esterification ≥ 69%; CAS 9000-69-5, Carl Roth GmbH & KG, Karlsruhe, Germany) were incubated at 39°C for 8 and 24 hr in the modified Hohenheim gas test **(HGT**) system (method 25.1; Menke & Steingass, 1988; VDLUFA, 2012; Leberl, Gruber, Steingaß, & Schenkel, 2007). Three runs were conducted on different days with two repetitions per run resulting in six replicates per substrate and time. Each syringe was filled with 200 mg dry matter (**DM**) and 30 ml inoculum. Three blank syringes per sampling time were incubated without substrate. The buffer contained sodium hydrogen carbonate at 33 g/L and ammonium hydrogen carbonate at 6 g/L. The whole buffered rumen inoculum had an ammonium-N concentration of 173.2 mg/L (±11.3 *SD*). The N content of the substrates was measured by Dumas method (method 4.1.2; VDLUFA, 2012; TruMac N, Leco Instrumente GmbH, Mönchengladbach, Germany). The substrates contained minor amounts of N (0.03, 0.06, 0.04 and 0.20% N for sucrose, starch, cellulose and pectin respectively), and which were not taken into account further. The source of the N is unknown. Rumen fluid for HGT was obtained from a rumen- cannulated jersey steer before morning feeding. The steer was fed with a constant ration consisting of grass hay and concentrate twice a day. *In vitro* gas production (**GP**) was measured after 8 and 24 hr of incubation.

In a second experiment, same substrates were incubated in HGT for 96 hr (3 syringes × 2 runs) to obtain the cumulative GP curves of the substrates and to estimate the time of half-maximal GP ($t_{1/2}$). Buffer, rumen fluid collection and further handling were done as in the first experiment. *In vitro* GP was measured after 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 60, 72 and 96 hr of incubation. Best curve fitting was obtained with the equation by France et al. (1993) $GP = A\left(1 - e^{-b(t-T)-c\left(\sqrt{t}-\sqrt{T}\right)}\right)$ where t is the incubation time [hr], T the lag time [hr], b [hr^{-1}] and c [$hr^{-1/2}$] are parameters of the fractional rate of degradation and A the maximal cumulative GP [ml]. Same equation was used to estimate $t_{1/2}$ for each substrate. $t_{1/2}$ represented the time (hr) when half of the asymptotic gas volume was produced (Grings, Blümmel, & Südekum, 2005).

Approximately 50% of the GP in the HGT system represents CO_2 resulting from the buffering reaction of bicarbonate with short-chain fatty acids (**SCFA**) but also with any other acid present in the feed (Blümmel, Aiple, Steingass, & Becker, 1999). Since pectins are known to have acidic components (galacturonic acid), the effect of acid on GP was simulated by incubating all substrates and a blank (three syringes each) with an inoculum where rumen fluid was replaced by water. Cumulative GP was

measured after 2, 4 and 6 hr of incubation. Incubation was terminated after 6 hr since there was no further GP after 4 hr of incubation. There was no gas produced with sucrose, starch and cellulose as substrate, while 10.0, 13.3 and 13.3 ml/g DM were produced from pectin after 2, 4, and 6 hr of incubation, respectively. Measured GP was subtracted from the GP of the first and second experiments.

2.2 | Microbial protein estimation

To estimate MP, an approach to estimate utilizable crude protein (**uCP**) based on changes in ammonium-N values between blank incubation and incubation with substrate was used (Edmunds, Südekum, Spiekers, Schuster, & Schwarz, 2012; Leberl, Gruber, Steingass, & Schenkel, 2007). Therefore, ammonium-N content of the whole syringes after 8 and 24 hr of incubation (experiment 1) was measured (3 × 2 replicates) by steam distillation (VAPODEST® 300, C. Gerhardt GmbH & KG, Königswinter, Germany) with 2 ml 1 molar NaOH solution and subsequent titration (TitroLine® 6000/7000 Titrator, SI Analytics GmbH, Mainz Germany) with 0.05 molar HCl solution. Utilizable crude protein [g/kg DM] for and 24 hr was calculated by subtracting the ammonium-N content of the substrate containing syringes from the ammonium-N content of corresponding blank syringes, multiplying the result by 25 (conversion from N to crude protein) and relating it to the substrate weight in the syringe. Utilizable CP for $t_{1/2}$ was estimated via linear regression of uCP values for 8 and 24 hr at logarithmized $t_{1/2}$. Since no feed sample was included, changes in ammonium-N content must be due to net MP production alone and, therefore, uCP equals MP.

To relate the MP yield to the digestibility of organic matter (**dOM**), the equation dOM [%] = 31.55 + 0.8343 GP (No. 40f, Menke & Steingass, 1988) with GP as corrected gas production [ml/200 mg DM] after 24 hr of incubation (experiment 1) was used for estimation of dOM. The dOM value was then used to estimate the metabolizable energy (**ME**) with equation ME [MJ/kg DM] = −1.15 + 0.1600 dOM (No. 36f, Menke & Steingass, 1988).

2.3 | Apparent organic matter degradability

After 8 and 24 hr of incubation, fermentation for assigned syringes (6 replicates per substrate and time, experiment 1) was stopped by immediately cooling them down with ice. For substrate degradability determination, the incubation residue from syringes was quantitatively transferred into centrifuge tubes and centrifuged by 20,000 × *g* at 4°C for 30 min. The supernatant was stored and used for ammonium and SCFA analysis. Syringes were washed three times with 10 ml sodium chloride solution (4 g NaCl/L), and washing solution was returned into the respective centrifugation tube. Tubes were centrifuged by 20,000 × *g* at 4°C for 30 min again. The second supernatant was discarded. Tubes including the pellet were frozen, freeze-dried and weighed back. Pellets were dried overnight at 103°C for analytical DM and afterwards ashed for organic matter (**OM**) quantification. Apparent organic

matter degradability (**deOM**) was calculated for each syringe by subtracting the sample pellet OM corrected for mean blank pellet OM from sample OM and divided by sample OM.

2.4 | Short-chain fatty acids

For SCFA analysis, 1 ml of the centrifugation supernatant was acidified with 150 µl meta-phosphoric acid (25%) and 50 µl formic acid which contained 2-methylpentanoic acid (4%) and centrifuged in 1.5-ml Eppendorf tubes with 16,600 × *g* at ambient temperature for 10 min. 2-methylpentanoic acid was used as the internal standard in the following gas chromatographic (GC) analysis. The SCFA analysis was carried out using GC-14B (Shimadzu Corporation, Kyoto, Japan) with flame ionization detection. The detector temperature was 220°C, the injector temperature was 170°C, and the oven temperature was 130°C. The carrier gas was hydrogen. An external standard containing a certain amount of SCFA and the internal standard were used to calibrate the GC. 1.4 µl sample was manually injected in GC. Peak recording and area calculation were conducted by an integrator (D-2000, Merck, Hitachi). All SCFA values were adjusted for SCFA production from blank incubations.

2.5 | Statistical analyses

A nonlinear regression after France et al. (1993) (see 2.1) was used for curve fitting using GraphPad Prism 6. Parameters of GP, MP related to dOM and ME were analysed using the mixed model procedure of SAS with substrate as fixed and run as random effect. A two-factorial analysis of variance (substrate, time and their interaction) with run as random effect was conducted for MP related to DM and GP, deOM and SCFA using the mixed model procedure of SAS 9.4. All data are presented as least square means. Separation of treatment means was accomplished using the Tukey-Kramer procedure ($p \leq .05$) for all analysis.

3 | RESULTS

3.1 | Gas production and fermentation kinetics

Cumulative GP curves of sucrose, starch, cellulose and pectin are shown in Figure 4.1 (experiment 2). Substrates differed in their fermentation kinetic. Sucrose and pectin were very rapidly fermented with a sharp increase in GP within the first hours of incubation, whereas GP of cellulose was extremely low in this period. This was reflected in a lag time of 7 hr for cellulose (Table 4.1). Fermentation of starch started not as rapid as sucrose or pectin (starch lag time 2.1 hr; Table 4.1) but significantly earlier than fermentation of cellulose. Estimated maximal GP after 96 hr of incubation was significantly different between substrates (pectin > sucrose > cellulose = starch; Table 4.1). However, maximal GP of starch was underestimated with the equation of France et al. (1993) as actually measured GP was on average 31 ml/g higher. Differences in GP curves between the carbohydrates were also shown in estimates of

$t_{1/2}$. With 5.7 hr, sucrose needed less time to reach this point than starch and pectin, which did not differ (8.2 and 8.1 hr), and cellulose, which needed significantly longer (17.7 hr; Table 4.1).

3.2 | Microbial protein

There was a significant effect of substrate, time and their interaction for the MP yield, irrelevant if expressed as g/kg DM or g/L GP (experiment 1). Microbial protein yield after 24 hr of incubation was higher for cellulose and starch than for sucrose and pectin. MP yield at $t_{1/2}$ was also higher for cellulose and starch (135 and 130 g/kg DM respectively) than for sucrose (98 g/kg DM). Pectin MP yield (110 g/kg DM) was only significantly different from cellulose (Table 4.2).

Microbial protein yield expressed as g/L GP was higher for starch and cellulose than for sucrose and pectin (0.71, 0.74, 0.40 and 0.42 g/L GP respectively) at the time of $t_{1/2}$. After 24 hr of incubation, cellulose had the highest value of MP per L GP (0.55 g/L GP) followed by starch (0.42 g/L GP) and sucrose (0.33 g/L GP) while pectin had the lowest (0.28 g/L GP) not different from sucrose (Table 4.2).

MP yield per kg DOM after 24 hr of incubation resulted in the same ranking between the carbohydrates as MP yield expressed as g/MJ ME (cellulose > starch > sucrose > pectin) with values between 111 and 198 g/kg DOM and 7.33 and 13.51 g/MJ ME (Table 4.2).

3.3 | Organic matter degradability

After 8 hr of incubation, apparent deOM was highest for sucrose (68.7%) and pectin (68.5%) and lowest for cellulose (8.3%) with starch (41.5%) in between (experiment 1). After 24 hr of incubation, there was no significant difference between sucrose, starch and pectin for apparent deOM, but deOM of cellulose was still significantly lower (Table 4.2).

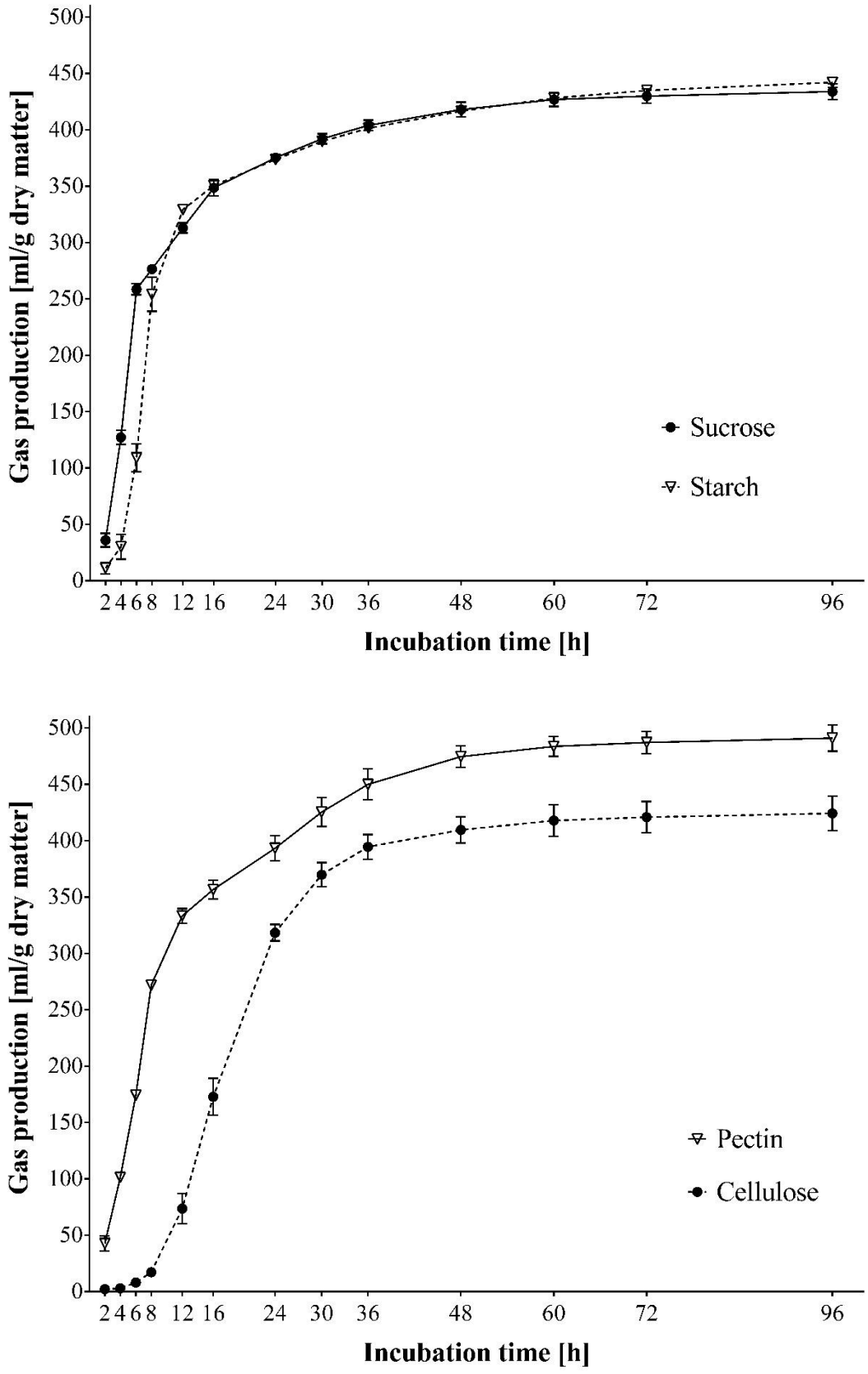

FIGURE 4.1 Cumulative gas production curve (mean ± *SD*) of sucrose, starch, cellulose and pectin over 96 hr of *in vitro* incubation

3.4 | Short-chain fatty acids

Total SCFA production was higher after 24 than 8 hr of incubation for all carbohydrates (experiment 1). After 8 hr of incubation, pectin had the highest total SCFA values (6.66 mmol/g) and cellulose the lowest (0.65 mmol/g) with sucrose and starch in between, which did not differ (Table 4.3). Percentage of acetate was significantly higher for pectin than for the other carbohydrates. Percentage of propionate after 24 hr of incubation was higher for cellulose than for the other carbohydrates and lowest for pectin. Percentage of butyrate was highest for starch after 24 hr of incubation. The acetate:propionate ratio (**C_2:C_3 ratio**) after 8 hr of incubation was significantly higher for starch and pectin than for sucrose and cellulose. After 24 hr of incubation the C_2:C_3 ratio for starch was the same as for sucrose and cellulose.

TABLE 4.1 Fermentation characteristics of sucrose, wheat starch, microcrystalline cellulose and citrus pectin (estimates after France et al., 1993) (Lsmeans and parameter for goodness of fit)

	Substrate					
	Sucrose	**Starch**	**Cellulose**	**Pectin**	**SEM**	***p*-value**
max. GP [ml g^{-1} DM]	447^{b}	411^{c}	425^{c}	493^{a}	5.231	<0.001
Lag time [h]	1.7^{ab}	2.1^{b}	6.8^{c}	1.6^{a}	0.12	<0.001
$t_{1/2}$ [h]	5.7^{a}	8.2^{b}	17.7^{c}	8.1^{b}	0.18	<0.001
b [h^{-1}]	0.16^{a}	0.13^{b}	0.02^{c}	0.03^{c}	0.004	<0.001
c [$h^{-1/2}$]	0.24^{b}	0.55^{a}	0.10^{d}	0.14^{c}	0.011	<0.001
R^2	0.986	0.975	0.992	0.986		
Sy.x	14.6	24.1	16.4	18.1		

Note: Means within a line with different superscripts ($^{a-d}$) differ ($p < .05$).
Abbreviations: b and c, parameters of the fractional rate of degradation; DM, dry matter; Max. GP, maximal gas production (A); SEM, standard error of mean; $t_{1/2}$, time of half-maximal gas production.

TABLE 4.2 Microbial protein (MP) yield and apparent organic matter degradability (deOM) of different carbohydrates after 8 and 24 hr of *in vitro* incubation and at the estimated substrate individual time of half-maximal gas production ($t_{1/2}$)

		Substrate						
	Time	**Sucrose**	**Starch**	**Cellulose**	**Pectin**	***SEM***		***p*-value**
MP [g/kg DM]	8 h	103^{a}	130^{aB}	37^{bC}	110^{a}	7.642	Substrate	<0.001
	24 h	125^{b}	161^{aA}	172^{aA}	111^{b}	7.642	Time	<0.001
	$t_{1/2}$	98^{c}	130^{abB}	135^{aB}	110^{bc}	7.642	Substrate × Time	<0.001
MP [g/l GP]	8 h	0.38^{c}	0.71^{bA}	1.22^{aA}	0.42^{cA}	0.029	Substrate	<0.001
	24 h	0.33^{bc}	0.42^{bB}	0.55^{aC}	0.28^{cB}	0.029	Time	<0.001
	$t_{1/2}$	0.40^{b}	0.71^{aA}	0.74^{aB}	0.42^{bA}	0.029	Substrate × Time	<0.001
MP [g/kg dOM]	24 h	127^{c}	161^{b}	198^{a}	111^{d}	4.496	Substrate	<0.001
MP [g/MJ ME]	24 h	8.60^{c}	10.86^{b}	13.51^{a}	7.33^{d}	0.310	Substrate	<0.001
deOM [%]	8	68.7^{a}	41.5^{bB}	8.3^{cB}	68.5^{a}	2.23	Substrate	<0.001
	24	74.0^{a}	71.8^{aA}	63.0^{bA}	75.5^{a}	2.23	Time	<0.001
							Substrate × Time	<0.001

Note: Means with different superscripts within a line ($^{a-d}$) or within a column ($^{A-C}$) differ ($p < .05$).
Abbreviations: DM, dry matter; DOM, digested organic matter; GP, gas production; ME, metabolizable energy; SEM, standard error of mean.

TABLE 4.3 *In vitro* short-chain fatty acid (SCFA) production (adjusted for SCFA production from blank incubations) of different pure carbohydrates after 8 and 24 hr of incubation

		Substrate						
	Time [h]	**Sucrose**	**Starch**	**Cellulose**	**Pectin**	**SEM**		***p*-value**
Total SCFA [mmol/g]	8	4.04^{bB}	3.30^{bB}	0.65^{cB}	6.66^{aB}	0.459	Substrate	<0.001
	24	8.70^{A}	8.00^{A}	7.66^{A}	8.39^{A}	0.459	Time	<0.001
							Substrate × Time	<0.001
Acetate (C_2)	8	53.1^{c}	67.7^{bA}	50.0^{c}	78.3^{a}	2.142	Substrate	<0.001
[% of total SCFA]	24	48.7^{b}	51.8^{bB}	45.3^{b}	75.6^{a}	2.142	Time	<0.001
							Substrate × Time	0.002
Propionate (C_3)	8	36.8^{a}	16.8^{bB}	42.6^{aB}	15.9^{b}	1.880	Substrate	<0.001
[% of total SCFA]	24	39.2^{b}	29.2^{cA}	51.4^{aA}	16.8^{d}	1.880	Time	<0.001
							Substrate × Time	0.013
Butyrate (C_4)	8	10.1^{b}	15.5^{a}	7.4^{bc}	5.9^{c}	1.914	Substrate	<0.001
[% of total SCFA]	24	12.1^{b}	19.1^{a}	3.3^{d}	7.6^{c}	1.914	Time	0.256
							Substrate × Time	0.001
C_2:C_3 ratio	8	1.45^{b}	4.35^{aA}	1.28^{b}	4.94^{a}	0.227	Substrate	<0.001
	24	1.26^{b}	1.79^{bB}	0.89^{b}	4.53^{a}	0.227	Time	<0.001
							Substrate × Time	<0.001

Note: Means with different superscripts within a line ($^{a-d}$) or within a column ($^{A-B}$) differ ($p < .05$).
Abbreviation: SEM, standard error of mean.

4 | DISCUSSION

4.1 | Gas production and fermentation kinetics

Fermentation of pectin started comparably fast as fermentation of sucrose with the same estimated lag time but higher GP after 2 hr of incubation. Fermentation of starch and cellulose started slower. This observation is consistent with the statement made by Van Soest et al. (1991) that pectins are the most rapidly fermentable complex carbohydrates. The used citrus pectin seems to contain two chemical compounds which ferment in a different way since the GP curve of pectin has a second increase after 24 hr of incubation which was present in all six individual incubations. Pectins are composed of different covalently linked galacturonic acid structures like homogalacturonan, rhamnogalacturonan I and II, xylogalacturonan and apiogalacturonan which are varying in their complexity (Mohnen, 2008). These differences could lead to variation in fermentation kinetics.

According to Menke and Steingass (1988), there is a difference in fermentation kinetics between amorphous and crystalline cellulose. Amorphous cellulose as found in plants had a greater *in vitro* GP and therefore was more readily fermented than crystalline cellulose (Menke & Steingass, 1988). The microcrystalline cellulose we used seems to ferment not like crystalline cellulose as GP after 24 hr was comparable to values of amorphous cellulose according to Menke and Steingass (1988).

4.2 | Microbial protein

Hall and Herejk (2001) stated that the MP yield of the non-neutral detergent fibre carbohydrates sucrose, starch and pectin depended considerably on the sampling time. Concerning the substrates of our study, at 8 hr cellulose still had a very low GP (4% of 96 hr GP) while sucrose reached already 64% of the 96 hr GP. To compare the MP formation capacity of substrates with very different fermentation kinetics, $t_{1/2}$ can be considered to be the best time. It measures MP at a time when only approximately 50% of the energy totally available has been used, which means that the microbial population has used a considerable proportion, but is far from being starved (Grings et al., 2005).

We measured the net protein formation of microbes since it is hardly possible to quantify the exact amount of formation and degradation of MP separately. In contrast to feeds where the amount of uCP declines from 8 to 24 hr of incubation due to degradation of the feed protein, the uCP amount of the pure substrates – equalling the MP yield in this case – is greater after 24 hr of incubation than after 8 hr, especially for cellulose but except of pectin. Since MP yields of pectin after 8 and 24 hr were nearly the same, MP formation and degradation of pectin seem to be equally expressed while the MP formation of sucrose seems to be greater than the degradation. This is a little surprising because both substrates are very rapidly fermented. Hall and Herejk (2001) found a much slower decrease in MP yield for sucrose in relation to starch and pectin, once the time point of maximal MP yield has been

surpassed. They explained this by the ability of rumen micro-organisms to store water-soluble carbohydrates and use them later when the substrate supply will be declining. This could also explain the differences we found between sucrose and pectin.

In our study, MP yield [g/kg DM] at $t_{1/2}$ was higher for starch than for sucrose which is consistent with maximal MP yield for those substrates according to Hall and Herejk (2001). Hall and Herejk (2001) also found a greater maximal MP yield for starch than for pectin. This was different from our results where pectin equalled starch and sucrose when MP related to kg DM. When MP yield was expressed in g per L GP, we found the same ranking among the substrates as Hall and Herejk (2001) did (starch > pectin = sucrose). A study by Strobel and Russell (1986) found no differences between starch, pectin and sucrose in microbial cell protein after 10 hr of *in vitro* incubation. Our MP values [g/kg DM] after 8 hr of incubation showed no difference between those three carbohydrates as well. Pure carbohydrates as sole substrate surely exclude rumen micro-organisms which need other substrates to survive and grow. In a study using diets including pure carbohydrates in proportions of app. 50% of the diets, greater MP amounts for a sucrose-rich than for a corn starch or cellulose-rich diet were found after 4 and 12 hr of *in vitro* incubation (Kand, Bagus Raharjo, Castro-Montoya, & Dickhoefer, 2018). After 24 hr of incubation, there was no difference between the diets anymore. This contrasts results of Hall and Herejk (2001), Strobel and Russell (1986) and of this study.

Also, some studies (Hall & Herejk, 2001; Strobel & Russell, 1986) supplemented the inoculum with amino acids (cysteine) and protein (casein acid hydrolysate) instead of using ammonia as only nitrogen source for the micro-organisms. This likely results in at least some differences in the absolute values of MP yield between our results and the literature. Experiments with mixed rumen bacteria and different peptide sources showed that bacterial growth can be enhanced by additional peptides and amino acids (Argyle & Baldwin, 1989). We did not supplement amino acids or protein because we wanted the pure carbohydrates to be the only energy source for the microbes.

The hypothesis that pectin as very rapidly fermentable complex carbohydrate, which at the same time does not negatively affect rumen pH could be particularly beneficial for microbial growth (Van Soest et al., 1991) is not supported by our results as well as those of Hall and Herejk (2001). However, we only used pure citrus pectin and no complete feed or formulated ration. A very well buffered *in vitro* system where negative or positive effects on pH value have no impact on the fermentation may not detect advantages of substrates like pectin in this respect.

The MP yield at $t_{1/2}$ of cellulose equalled the MP yield of starch if expressed per kg DM or L GP. Thus, at this point of fermentation cellulose provided the same amount of energy as starch for the microbes. However, it should be kept in mind that cellulose needed more than twice as much time than starch

to reach this point. In another study, the MP yield of isolated bermudagrass NDF was significantly lower per g OM than that of starch, sucrose and pectin (Hall & Herejk, 2001).

The energy supply to the microbes is considered to be the major driver of microbial growth. The degradation of feed to acetate is described to provide more energy as ATP for the micro-organisms than propionate (Bergner, 1996). According to Bergner (1996), 1 mol hexose provides 2 mol acetate with an overall energy supply of 4 mol ATP for the microbes, while the formation of 2 mol propionate provides only 2 mol ATP and the formation of 1 mol butyrate yields 3 mol ATP in total. We calculated the yields of ATP available to the microbes according to the measured values of SCFA. The calculated amounts of ATP for sucrose, starch, cellulose and pectin were 7.0, 6.5, 1.1 and 12.7 mol/g DM after 8 hr of incubation and 15.0, 15.2, 11.7 and 16.0 mol/g DM after 24 hr of incubation respectively. The differences in MP yield could not be explained by the calculated ATP yields since MP [g/mol ATP] after 8 hr of incubation was highest for cellulose (36.2) and lowest for pectin (8.8) with sucrose (15.1) and starch (21.0) in between and MP [g/mol ATP] after 24 hr of incubation was higher for cellulose (15.2) than for sucrose (8.2) and pectin (7.0) with starch (10.6) not differing from the other substrates.

Looking at the efficiency of the MP formation, it seems to be greater at the beginning of the fermentation. The effect was particularly high for MP values [g/L GP] of cellulose but also clearly visible for starch and pectin. For sucrose, the first MP measurement was probably not early enough to see such big differences.

If the MP is expressed per kg DOM or MJ ME after 24 hr of incubation, there is a clear ranking (cellulose > starch > sucrose > pectin). Because we calculated the dOM and MJ ME on the basis of the corrected 24 hr GP, we had only a value for 24 hr of incubation. Due to the very different fermentation progress of the substrates, this time point is not optimal for their comparison. According to Blümmel, Makkar, and Becker (1997), at a given degradability there is a negative relationship between the *in vitro* GP and the microbial biomass yield per gram of truly degraded substrate which could explain the lower MP yield of pectin because GP was highest for pectin. The German recommendation for the nutrient and energy supply for dairy cows (GfE, 2001) gives MP values of 10.8 ± 1.7 g/ MJ fat-free ME and 162 ± 26 g MP/kg fat-free dOM. Our MP values of starch per MJ ME or kg DOM are located nearly in the middle of the recommendation range while the MP values of cellulose are even greater than the recommendation value. MP values of sucrose and pectin are below this range.

Finally, multiplication of N content with 6.25 as done for feeds seems not the most accurate way to express MP but is still often used (e.g. GfE, 1995).

4.3 | Organic matter degradability and short-chain fatty acids

Measured deOM reflected the different fermentation kinetics and the amount of gas produced of the substrates. The deOM of cellulose was much slower than the deOM of the other substrates and not complete after 24 hr of incubation.

The total amount of produced SCFA after 8 and 24 hr of incubation reflected the fermentation progress of the different substrates. After 8 hr of incubation, pectin had the highest amount of all substrates while sucrose had an identical GP. Thus, the GP did not reflect completely the higher SCFA amount of pectin. According to Blümmel et al. (1999), 1 mmol SCFA releases approximately 1 mmol CO_2 in the HGT which is equal to 25.6 ml CO_2 gas and therefore higher SCFA values of pectin should provide higher GP. Also, there should be a lower GP if higher propionate values occur due to less CO_2 production (Menke & Steingass, 1988).

There was a great shift in the acetate:propionate ratio of starch from 8 to 24 hr of incubation. Maybe this could be partly explained by findings of Marounek, Bartos, and Brezina (1985). They found a higher C_2:C_3 ratio for starch after 8 hr of incubation with neutral pH values than in acidic medium (pH 6.79 to 5.25 at the beginning and end of the experiment respectively). Since the HGT is a strongly buffered system, such acidic pH values are highly unlikely and could only occur at advanced incubation time. As expected, the C_2:C_3 ratio was low for sucrose and high for pectin which is known to be mostly fermented to acetate (e.g. Marounek et al., 1985). We did not expect the very low C_2:C_3 ratio of cellulose after 8 hr as well as after 24 hr of incubation because forage with high cellulose proportion normally results in high acetate and low propionate contents leading to a high C_2:C_3 ratio. Pure extracted cellulose without its natural embedding in the plant material as we incubated it in the HGT seems to be fermented in a different way. In earlier *in vitro* experiments, Beuvink and Spoelstra (1992) found approximately the same C_2:C_3 ratio for crystalline cellulose as we did. Also, Senshu, Nakamura, Sawa, Miura, and Matsumoto (1980) found a C_2:C_3 ratio of 0.83 for cellulose after 16 hr of *in vitro* incubation, which is similar to 0.89 as we found after 24 hr, while Senshu et al. (1980) found a C_2:C_3 ratio of 2.95 after 8 hr of incubation for hay. Soya bean hulls which contain a large amount of cellulose had a C_2:C_3 ratio of 3.40 after 24 hr of *in vitro* incubation (Blümmel et al., 1999). For incubations with cellulose as main substrate, propionate could be produced through succinate which is the main pathway for propionate production in the rumen (Wolin, 1975). *Bacteroides succinogenes* ferments cellulose to succinate and *Selenomonas ruminantium* uses the succinate to produce propionate (Scheifinger & Wolin, 1973). Therefore, it is not surprising that in our experiment pure cellulose as the only substrate leads to higher values of propionate.

5 | CONCLUSION

Some variance was present regarding the MP yield of different pure carbohydrates when incubated in the HGT system. Considering a comparable stage of fermentation by using $t_{1/2}$, cellulose and starch yielded more MP than sucrose and pectin (only MP in g/L GP). Fermentable cellulose seems to have a particular potential for MP formation. Postulated positive effects of pectin on MP yield could not be confirmed *in vitro*; however, any differences related to pH are likely to be missed in *in vitro* experiments. Further investigations including different types/origins of the respective carbohydrates are necessary for a more precise prediction of MP formation in the rumen with different carbohydrates as energy source.

ANIMAL WELFARE STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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5 Effects of dilution rate on fermentation characteristics of feeds with different carbohydrate composition incubated in the rumen simulation technique (Rusitec)

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This study investigated the impact of carbohydrate source and fluid passage rate (dilution rate) on ruminal fermentation characteristics and microbial crude protein (**MCP**) formation. Three commonly used feeds (barley grain [**BG**], beet pulp [**BP**], and soybean hulls [**SBH**]), which differ considerably in their carbohydrate composition, were incubated together with a mixture of grass hay and rapeseed meal in two identical Rusitec apparatuses (each 6 vessels). Differences in fluid passage rate were simulated by infusing artificial saliva at two different rates (1.5% [**low**] and 3.0% [**high**] of fermenter volume per h). This resulted in six treatments (tested in 3 runs): **BGhigh**, **BGlow**, **BPhigh**, **BPlow**, **SBHhigh** and **SBHlow**. The system was adapted for 7 d, followed by 4 d of sampling. Production of MCP (mg/g degraded organic matter [**dOM**]; estimated by 15N analysis) was greater with high dilution rate (**DL**; $p < 0.001$) and was higher for SBH compared to both BG and BP ($p < 0.001$). High DL reduced OM degradability (**OMD**) compared to low DL ($p < 0.001$), whereas incubation of BG resulted in higher OMD compared to SBH ($p < 0.002$). Acetate:propionate ratio decreased in response to high DL ($p < 0.001$). Total gas and methane production (both /d and /g dOM) were lower with high DL ($p < 0.001$). In our study increasing liquid passage rate showed the potential to increase MCP and decrease methane production simultaneously. Results encourage further studies investigating these effects on the rumen microbial population.

Keywords: fermentation products, microbial protein synthesis, fluid passage rate, methane, carbohydrate source

INTRODUCTION

Sufficient post-ruminal supply of microbial crude protein (**MCP**) is crucial especially for high-yielding dairy cows, which have particularly high amino acid requirements. Numerous factors such as energy, nitrogen and mineral supply, pH, and passage rate affect the synthesis of MCP in the rumen (Owens and Goetsch, 1986).

An increase in fluid passage rate commonly leads to greater formation of microbial biomass through a stimulation of fast-growing bacteria and a higher washout rate from the rumen, which is expected to result in an increase in post-ruminal MCP supply (Hungate, 1966; Van Soest, 1994). The formation of microbial cells is more efficient in response to higher passage rates of the fluid phase, since a fast-growing microbial population utilizes more of the available energy for growth than a slower-growing, which will use more energy for maintenance of cell functions (Hespell and Bryant, 1979). Furthermore, the efficiency of microbial cell formation is decreased by higher cell lysis at longer retention time (Nolan and Leng, 1983). The N-efficiency may be further improved since faster growing microorganisms have been found to contain more nitrogen per unit of microbial biomass (Hespell and Bryant, 1979).

In addition to the factors outlined above, the carbohydrate composition of feeds has also been reported to impact rumen fermentation and MCP. *In vitro* experiments used different carbohydrates (sugar, starch, pectin and cellulose) as energy source for rumen microbes and showed variations in MCP formation, organic matter degradability (**OMD**) and short chain fatty acid (**SCFA**) production (Hall and Herejk, 2001; Pfau and Hummel, 2019). At the time point of half-maximal gas production, Pfau and Hummel (2019) detected higher MCP yield from cellulose compared to sucrose and pectin. In addition, a higher MCP was found for starch compared to sucrose. Similarly, Hall and Herejk (2001) reported higher MCP yield for starch compared to sucrose and pectin.

The effects of ruminal fluid passage rate and carbohydrate source and their interaction are not extensively understood to date; a more detailed understanding must be regarded a prerequisite to more accurate predictions of post-ruminal MCP supply. Therefore, the objective of this study was to investigate the impact of three commonly used feeds (barley grain, beet pulp, soybean hulls), which differ considerably in their carbohydrate composition, and differences in fluid passage rate on fermentation characteristics and MCP formation.

Inducing defined changes to ruminal fluid passage rates *in vivo* is challenging, since a number and complexity of factors such as level of feed intake, diet composition, saliva flow rate, rumination and mastication behavior affect ruminal passage rate. For that reason, we chose to employ the rumen simulation technique (Rusitec) in this study and induced two different fluid passage or dilution rates (**DL**) by infusing artificial saliva (i.e., buffer) at two different rates, while keeping the retention of feed particles and therefore the energy supply of microbial populations constant.

MATERIALS AND METHODS

Experimental Design and Treatments

The experiment was conducted as completely randomized block design with two blocks (Rusitec apparatuses). The treatment factors were carbohydrate source (barley grain [**BG**], beet pulp [**BP**], and soybean hulls [**SBH**]) and DL (high and low), resulting in six treatments: **BGhigh**, **BGlow**, **BPhigh**, **BPlow**, **SBHhigh**, and **SBHlow**. For each fermenter and day, the incubated basal substrates consisted of a mixture of 5 g grass hay and 2 g solvent-extracted rapeseed meal (**RSM**; dry matter [**DM**] basis). The basal substrate was incubated together with either 4 g of BG, BP, or SBH (DM basis).

The hay was ground through a 10-mm screen aperture (Retsch SM 200, Retsch GmbH, Haan, Germany). After grinding, fine particles were separated and discarded by sieving the hay manually through a 1.18-mm screen aperture. The RSM, BG, BP, and SBH were ground through a 3-mm screen aperture (Retsch SM 200, Retsch GmbH, Haan, Germany). The hay and RSM were weighed into one set of nylon bags and the respective carbohydrate sources (BG, BP, or SBH) in a second set of nylon bags (all bags: 6.5 ×

12 cm; 50 ± 10 µm mean pore size; R 712 Forage Bags, Ankom Technology Corp., Macedon, NY, USA). The targeted DL of the infused buffer were 1.50 and 3.00%/h (400 and 800 ml/d). Actual mean infusion rates were 1.56 and 2.90%/h (411 and 764 ml/d) for low and high DL, respectively. The experiment consisted of three separate Rusitec runs carried out under identical conditions. The system was adapted for 7 d, followed by 4 d of sampling.

Rumen Simulation Technique and Sampling

The rumen fluid and solid digesta used in the experiment were obtained from two rumen fistulated Holstein-Friesian heifers housed at the Institute for Physiology and Cell Biology, University of Veterinary Medicine Hannover (Germany). The heifers were housed and sampled in accordance with the German Animal Welfare Act. Housing and sampling protocols were reviewed and approved by the State Office for Consumer Protection and Food Safety of the state of Lower Saxony (reference number: AZ 33.4-42505-04-13A373).

Both heifers were fed 9 kg mixed grass hay, 600 g compound feed and 75 g mineral supplement per heifer and day (as fed basis). Forestomach contents were collected manually 2 h after the morning feeding from three sites (reticulum, dorsal, and ventral sac) within the reticulo-rumen of each heifer. The contents were strained through two layers of gauze and the resulting fluid was transferred into two pre-warmed plastic canisters. The canisters containing rumen fluid and rumen digesta were placed in a pre-warmed (39◦C) polystyrene box for transportation to the Department of Animal Science at the University of Goettingen (ca. 1 h of drive). Two different buffer solutions were used for the high and low DL; both supplied 3.27 g/d of $NaHCO_3$, 3.10 g/d of $Na_2HPO_4 \times 12\ H_2O$, 0.155 g/d of NaCl, 0.188 g/d of KCl, 0.017 g/d of CaCl × 2 H2O, 0.042 g/d of MgCl × 6 H_2O (McDougall, 1948). Buffer solutions had a pH of 8.25–8.27. They were modified to supply 0.210 g NH_4Cl/d to increase the minimum ruminal nitrogen balance (which was present in BG) to 20 g N/kg DM. In addition, 0.002 g $^{15}NH_4Cl$/d were infused as microbial marker to estimate MCP. The amount of infused salts per day and fermenter was identical for both buffer infusion rates (high and low) to ensure equal mineral supply and buffering capacity among treatments.

Two Rusitec apparatuses (Czerkawski and Breckenridge, 1977), each equipped with six identical fermenters (1.1 l volume), were used in this study. Before the onset of the experiment, each fermenter was filled with 690 ml strained rumen fluid and 410 ml pre-warmed buffer solution. To promote the inoculation with particle associated microorganisms one reusable nylon bag containing 80 g of solid rumen digesta (wet weight) was placed in each fermenter. In addition, each fermenter was equipped with one nylon bag containing hay+RSM and a second nylon bag containing one of the three carbohydrate sources. On the following morning (900 h), the nylon bag containing the solid digesta was replaced by two fresh nylon bags containing hay + RSM and the carbohydrate source, respectively.

Every following day at 900 h, the pair of substrate bags which was incubated for 48 h was exchanged with a new pair of substrate bags. The bags withdrawn from each fermenter were placed in a polypropylene bag and washed manually with 60 ml of pre-warmed buffer for 1 min, to remove some of the microbial attachment. The buffer solution was added back into the fermenter. The effluent from each individual fermenter was collected in graduated flasks, which were cooled permanently/24 h using ice packs to stop further microbial activity. The quantity of effluent from each fermenter was measured once per day during the exchange of substrate bags. On sampling days (d 8 to 11), the effluent was subsampled for analysis of SCFA, ammonium-N and MCP (15N). During the daily exchange of substrate bags, the pH of the fermenter fluid was measured (pH-Meter CG 825, SCHOTT Instruments, Mainz, Germany) to ensure that pH conditions were stable and near neutral. The pH data were not recorded.

The fermentation gas was collected in gas-tight bags (5L TECOBAG, Tesseraux Spezialverpackungen GmbH, Bürstadt, Germany) attached to the flasks. The volume of total gas was measured once per day using a water displacement apparatus. The total gas volume was corrected for differences in air pressure and temperature among sampling days. Before the gas volume was measured, 15 ml gas samples (3 repetitions) were collected directly from each gas bag using a 25 ml syringe and injected into evacuated exetainer vials (Labco Ltd., Lampeter, United Kingdom) for methane analysis.

Chemical Analysis of the Substrates and Residues

All feed samples were analyzed prior to incubation according to VDLUFA (2012) for DM (method 3.1), ash (method 8.1), ether extract (method 5.1.1), CP (method 4.1.1, Kjeldahl, N × 6.25), neutral detergent fiber (**aNDFom**, method 6.5.1, assayed with heat-stable amylase, exclusive residual ash) and acid detergent fiber (**ADFom**, method 6.5.2, exclusive residual ash). Both, aNDFom and ADFom were analyzed using an Ankom 200 Fiber Analyzer (ANKOM Technology, Macedon, NY, United States). The starch content of the barley grain was analyzed polarimetrically (VDLUFA, 2012; method 7.2.1). The sugar content of the dried beet pulp was analyzed according to method 7.1.3 (VDLUFA, 2012). After removing the substrate bags from the fermenter after 48 h of incubation, the bags were freeze-dried. For each fermenter, residues were pooled separately for hay+RSM and carbohydrate source for 4 d to ensure sufficient sample for chemical analysis. Unfortunately, we lost two sampling days in the first run. Therefore, residues from that run were only pooled over two sampling days. The residues of the hay+RSM bags were ground through a 2-mm screen aperture using a laboratory mill (Polymix PX-MFC 90 D, Kinematica AG, Luzern, Switzerland) designed to handle small volumes, which matched the Retch mill grinding through a 1-mm screen aperture closely. The residues of the carbohydrate sources were manually crumbled and not ground because of the small quantities and the fact that they were already ground through a 3-mm screen prior incubation. All feed residues were analyzed for DM (VDLUFA,

2012; method 3.1), ash (VDLUFA, 2012; method 8.1) and NDFom (VDLUFA, 2012; method 6.5.3; Ankom 2000 Fiber Analyzer, ANKOM Technology Corp., Macedon, NY, USA). Analysis of NDFom was conducted to remove the feed associated microorganisms and obtain the truly degraded substrate.

The OMD and the degradability of NDFom (**NDFD**) were calculated as the proportion of OM or NDFom that disappeared during incubation from the amount of these components in the substrate before incubation. Results were expressed for the degradability of the hay and RSM mixture (**OMD_{hrsm}** and **$NDFD_{hrsm}$**), of the carbohydrate source (**OMD_{cs}** and **$NDFD_{cs}$**), and of all feeds combined (**OMD_{tot}** and **$NDFD_{tot}$**).

Microbial Crude Protein Synthesis

Microbial crude protein synthesis was estimated using three different methods: (1) ^{15}N analysis (**MCP1**), (2) an N-balance approach comparable to that used in the modified Hohenheim gas test (**MCP2**; Leberl et al., 2007; Edmunds et al., 2012), and (3) solely based on the N content of the effluent after centrifugation (**MCP3**). The effluent samples (50 ml) for NH_4^+ analysis were acidified with 1% (wt/vol) H2SO4 (10 mL).

For method (1), a steam distillation of 10 ml effluent was conducted (VAPODEST® 300, C. Gerhardt GmbH & Co.KG, Königswinter, Germany) with 2.5 ml 1 M NaOH solution to obtain samples for ^{15}N analyses. The distillates were vaporized at 40°C. Aliquots of the remainder and sub-samples of the microbial pellets (see below), substrates and substrate residues after incubation were weighed into tin cups for analyzes of ^{15}N. These were carried out at the Center for Stable Isotope Research and Analysis of the University Goettingen (KOSI). Isotope ratios of solid samples were determined by elemental analysis isotope ratio mass spectrometry.

For method (2), ammonium-N content of the effluent was analyzed; an aliquot (10 ml) of the daily effluent was subjected to steam distillation (VAPODEST® 300, C. Gerhardt GmbH & Co.KG, Königswinter, Germany) with 2.5 ml 1 molar NaOH solution and subsequent titration (TitroLine® 6000/7000 Titrator, SI Analytics GmbH, Mainz, Germany) with 0.05 molar HCl solution. The N contents of feed residues were available from 15N analyses.

For method (3), aiming to quantify MCP synthesis in the fluid phase/effluent, an aliquot of 40 ml of the daily effluent was centrifuged at 500 × *g* and 4°C for 10 min to separate the feed particles. The supernatant was then centrifuged again at 20,000 × *g* and 4°C for 30 min. The resulting supernatant was discarded and the remaining microbial pellet was washed three times with NaCl solution (9 g/l) followed by centrifugation (20,000 × *g*, 4°C for 30 min; Romero-Pérez et al., 2015). The resulting microbial pellet was freeze-dried and weighed.

For the three methods, MCP was calculated according to the following equations:

(1) MCP1 = [(^{15}N intake – $^{15}N_{feed\ residues}$ – $^{15}N_{effluent}$)/enrichment of microbial N] × 6.25 (modified after Nolan and Leng, 1983)
with ^{15}N intake being the input from the buffer solution and the naturally occurring traces of 15N in the substrates, $^{15}N_{feed\ residues}$ being the amount of ^{15}N in the incubation residuals (after NDF boiling), $^{15}N_{effluent}$ being the amount of ^{15}N in the effluent and enrichment of microbial N being the ^{15}N enrichment in the microbial pellet from the effluent

(2) MCP2 = (N intake – $N_{feed\ residues}$ – NH_4^+-$N_{effluent}$) × 6.25 with N intake being the N input from buffer solution and substrates, $N_{feed\ residues}$ being the N amount in incubation residues (after NDF boiling) and NH_4^+-$N_{effluent}$ being the N covered in NH_4^+ in the effluent (and therefore the N not used by microbes).

(3) MCP3 = Microbial mass × effluent volume × N $content_{microbial\ pellet}$ × 6.25
with microbial mass being the weight of the centrifugation pellet in the centrifuged effluent volume, effluent volume being the 24 h amount of effluent and N $content_{microbial\ pellet}$ being the N concentration in the centrifugation pellet.

Short Chain Fatty Acids

For SCFA analysis, 1.5 ml of the effluent was centrifuged at 16.600 × *g* and 4°C for 10 min. The resulting supernatant (1 ml) was acidified with 150 µl meta-phosphoric acid (25%) and 50 µl formic acid which contained 2-methylpentanoic acid (4%) and centrifuged again (16.600 × *g*, ambient temperature, 10 min). The 2-methylpentanoic acid was used as the internal standard. Short chain fatty acids were analyzed using a gas chromatograph (model GC-14B, Shimadzu Corporation, Kyoto, Japan) equipped with flame ionization detector. The injector temperature was 170°C, the detector temperature was 220°C. The oven temperature was 130°C. The carrier gas was hydrogen. Peak recording and area calculation were conducted by an integrator (D-2000, Merck Hitachi, Tokyo, Japan).

Methane

The methane content of the gas samples was measured using an infrared analyzer (Advanced Gasmitter®, PRONOVA Analysentechnik GmbH & Co. KG, Berlin, Germany). Since the infrared analyzer required a minimum of 10 ml of total gas for accurate measurements, 8 ml of gas was withdrawn from each of the three exetainers taken per fermenter and sampling day. The gas was pooled in one syringe and injected twice (12 ml) into the analyzer (Wild et al., 2019).

Statistical Analyses

Yield of MCP, OMD and NDFD, SCFA, total gas production and methane were analyzed using the mixed model procedure of SAS (version 9.4) with substrate, DL and substrates × DL as fixed and Rusitec run as random effect. Sampling day was considered as repeated measurement. All data are presented as least squares means. Separation of treatment means was accomplished using the Tukey-Kramer procedure. For the repeated measurements, several covariance structures were tested (unstructured, variance components banded, autoregressive, heterogeneous autoregressive, compound symmetry, and heterogeneous compound symmetry). The best-fitting covariance structure (variance components banded) was chosen based on the lowest Akaike's information and Bayesian information criteria. Statistical significance was declared at $p < 0.05$ and trends are discussed at $0.05 \geq p \leq 0.10$.

RESULTS

Substrate Composition

The treatments contained between 151 g (BPlow and BPhigh) and 177 g (SBHlow and SBHhigh) crude protein/kg DM (Table 5.1). The aNDFom and ADFom content of the incubated substrate mixtures were between 380 and 528 g aNDFom/kg and 200 and 331 g ADFom/kg (DM basis).

Organic Matter and Neutral Detergent Fiber Degradability

Overall, OMD_{tot} was high and on a range of 68–78% (Table 5.2). Among the three carbohydrate sources, BG had a higher OMD_{tot} compared to SBH, no matter which DL was applied ($p = 0.002$). Considering OMD_{cs}, SBHlow (74.9%) was degraded less extensively compared to BGlow (90.0%) and BPlow (90.0%; $p < 0.001$). Within the high DL treatments, OMD_{cs} from all three carbohydrate sources differed from each other (SBHhigh [68.1%] < BPhigh [81.6%] < BGhigh [88.7%]; $p < 0.01$). A significant interaction was present between the factors CS and DL; in fact, dilution rate had a decreasing effect on OMD_{cs} for BP and SBH (by about 10%), but not for BG.

The differences in $NDFD_{hrsm}$ among treatments were the same as for OMD_{hrsm} (Table 5.2). Considering only the carbohydrate source, incubation of BGlow resulted in lower $NDFD_{cs}$ compared to BPlow ($p < 0.001$) and SBHlow ($p = 0.026$). There was no difference in $NDFD_{cs}$ among BGhigh, BPhigh and SBHhigh.

Microbial Crude Protein

Due to the differences in DOM, values are given in mg/d and mg/g dOM. Total MCP1 formation per day was only affected by carbohydrate source (Table 5.3). Incubation of BPlow resulted in a reduction of MCP1 (mg/d) compared to SBHlow; while BPhigh reduced MCP1 (mg/d) compared to SBHhigh and BGhigh ($p < 0.001$). Yield of MCP1 per unit degraded organic matter (**dOM**) was affected by carbohydrate source ($p < 0.001$) and DL ($p < 0.001$), with a trend for an interaction ($p = 0.092$).

Incubation of SBH resulted in higher MCP1 (mg/g dOM) compared to BG and BP no matter which DL was applied ($p < 0.001$). High DL resulted in higher MCP per gram dOM compared to low DL for BGhigh ($p = 0.006$) and SBHhigh ($p = 0.020$) but not for BPhigh.

Estimation of MCP2 resulted in ~15% higher values than for MCP1, but the pattern of the effects of carbohydrate source and dilution rate was basically comparable (Table 5.3). The amount of MCP3 (mg/d and mg/g dOM) in the effluent was foremost affected by DL (Table 5.3). The MCP3 yield (mg/g dOM) from the different carbohydrate sources differed only within low DL treatments, with higher values for SBHlow (17.6 mg/g dOM) compared to BGlow (13.6 mg/g dOM; $p = 0.040$).

Table 5.1 Chemical composition of the incubated substrates containing a mixture of 5 g grass hay, 2 g rapeseed meal (RSM) and 4 g barley grain (BG), beet pulp (BP) or soybean hulls (SBH) as carbohydrate source (all basis DM).

Item, g/kg DM	Treatment			Carbohydrate source				
	BGlow, BGhigh	BPlow, BPhigh	SBHlow, SBHhigh	BG	BP	SBH	Hay	RSM
Organic matter	942	927	931	979	936	949	920	924
Crude protein	154	151	177	105	96	168	105	375
Ether extract	28	18	36	33	7	57	21	35
aNDFom	380	434	528	175	324	585	571	310
ADFom	200	239	331	57	165	418	306	221
Starch	227	---	---	625	---	---	---	---
Sugar	---	83	---	---	227	---	---	---

aNDFom – neutral detergent fiber assayed with heat stable amylase and expressed exclusive residual ash; ADFom – acid detergent fiber expressed exclusive residual ash

Short Chain Fatty Acids

For total SCFA, DL had a decreasing effect on mmol/d values ($p < 0.001$; Table 5.4) while CS had an effect on mmol/g dOM values ($p = 0.009$). There was a decreasing effect of DL on acetate production (mmol/d) ($p < 0.001$), which was less prominent for mmol/g dOM values. Acetate production was higher for SBH and BP compared to BG ($p = 0.001$) (and for high DL also for SBH compared to BP). For propionate (in mmol/d and mmol/g dOM), there was an interaction of CS and DL; in fact, the increasing effect of DL was only present in BG. Changes were also reflected in the acetate to propionate ratio, which decreased in response to high DL for all carbohydrate sources ($p < 0.001$), with a particularly prominent effect in BG. For butyrate (both, mmol/d and mmol/g dOM) we observed an interaction between carbohydrate source and DL ($p < 0.001$). Among the CS, dilution rate had a significantly decreasing effect in BG; concerning an effect of CS, butyrate was lowest in SBH.

Table 5.2 *In vitro* organic matter degradability (OMD) and neutral detergent degradability (NDFD) of different carbohydrate sources incubated at high and low dilution rate for 48 h.

Item	Treatment						SEM	*P*-value		
	BGlow	BGhigh	BPlow	BPhigh	SBHlow	SBHhigh		CS	DL	CS × DL
OMD_{hrsm} [%]	70.4^{aA}	65.5^{xyB}	66.2^{b}	63.4^{y}	71.5^{a}	68.0^{x}	1.00	<0.001	<0.001	0.451
OMD_{cs} [%]	90.0^{a}	88.7^{x}	90.0^{aA}	81.6^{yB}	74.9^{bA}	68.1^{zB}	1.40	<0.001	<0.001	0.030
OMD_{tot} [%]	77.9^{aA}	74.4^{xB}	75.0^{abA}	70.3^{yB}	72.8^{bA}	68.2^{yB}	0.91	<0.001	<0.001	0.785
$NDFD_{hrsm}$ [%]	45.1^{aA}	36.0^{xyB}	37.1^{b}	32.1^{y}	47.1^{a}	40.7^{x}	1.85	<0.001	<0.001	0.451
$NDFD_{cs}$ [%]	45.6^{b}	38.1	70.9^{aA}	47.7^{B}	59.4^{a}	48.3	3.36	<0.001	<0.001	0.039
$NDFD_{tot}$ [%]	45.2^{A}	36.6^{B}	46.6^{A}	36.6^{B}	52.1^{A}	44.0^{B}	1.85	0.001	<0.001	0.863

hrsm – hay and rapeseed meal; cs – carbohydrate source (barley grain, beet pulp or soy bean hulls only); tot – total; SEM – standard error of means; DL – dilution rate; means with different superscripts within low dilution rate ($^{a-b}$), high dilution rate ($^{x-z}$) or within a carbohydrate source ($^{A-B}$) differ ($p < 0.05$)

Table 5.3 *In vitro* microbial crude protein (MCP) formation of different carbohydrate sources incubated at high and low dilution rate estimated using three different methods.

Item	Treatment						SEM	*P*-value		
	BGlow	BGhigh	BPlow	BPhigh	SBHlow	SBHhigh		CS	DL	CS × DL
MCP (1)										
mg/d	890^{ab}	935^{x}	854^{b}	818^{y}	948^{a}	971^{x}	24.6	<0.001	0.539	0.140
mg/g dOM	110.2^{bB}	121.4^{yA}	111.6^{b}	114.1^{y}	127.8^{aB}	138.4^{xA}	2.61	<0.001	<0.001	0.092
MCP (2)										
mg/d	1056^{abB}	1150^{xA}	1009^{b}	1031^{y}	1131^{a}	1191^{x}	23.6	<0.001	0.001	0.223
mg/g dOM	131.1^{bB}	149.2^{yA}	132.2^{bB}	144.3^{yA}	153.1^{aB}	170.4^{xA}	2.28	<0.001	<0.001	0.278
MCP (3)										
mg/d	111^{B}	200^{A}	134^{B}	200^{A}	138^{B}	187^{A}	14.6	0.195	<0.001	0.015
mg/g dOM	13.8^{bB}	26.9^{A}	16.3^{abB}	28.3^{A}	17.6^{aB}	27.1^{A}	1.75	0.036	<0.001	0.115

SEM – standard error of means; CS – carbohydrate source; DL – dilution rate; MCP (1) – ^{15}N analysis; MCP (2) – N-balance approach used in the modified Hohenheim gas test; MCP (3) – solely based on the N content of the effluent after centrifugation; dOM – degraded organic matter; means with different superscripts within superscripts within low dilution rate ($^{a-b}$), high dilution rate ($^{x-y}$) or within a carbohydrate source ($^{A-B}$) differ ($p < 0.05$)

Table 5.4 *In vitro* short chain fatty acid (SCFA), methane and total gas production of different carbohydrate sources incubated at high and low dilution rate.

Item	Treatment						SEM	*P*-value		
	BGlow	BGhigh	BPlow	BPhigh	SBHlow	SBHhigh		CS	DL	CS × DL
Total SCFA										
mmol/d	54.7	50.2	53.8	49.5	52.2	48.8	1.17	0.222	<0.001	0.856
mmol/g dOM	6.79	6.60	7.10	6.98	7.05	7.14	0.191	0.009	0.538	0.567
Acetate (C_2)										
mmol/d	27.2^{bA}	23.9^{yB}	30.6^{aA}	26.5^{xyB}	31.5^{aA}	28.0^{xB}	0.72	<0.001	<0.001	0.817
mmol/g dOM	3.40^{b}	3.18^{z}	4.06^{a}	3.74^{y}	4.22^{a}	4.11^{x}	0.105	<0.001	0.002	0.455
Propionate (C_3)										
mmol/d	11.9^{B}	13.6^{xA}	12.9	12.6^{xy}	11.8	12.0^{y}	0.32	0.007	0.039	0.003
mmol/g dOM	1.46^{bB}	1.78^{A}	1.69^{a}	1.79	1.61^{ab}	1.75	0.046	0.019	<0.001	0.024
Butyrate										
mmol/d	12.1^{aA}	7.3^{xB}	7.4^{b}	7.2^{x}	6.0^{c}	5.7^{y}	0.32	<0.001	<0.001	<0.001
mmol/g dOM	1.50^{aA}	0.97^{xyB}	0.99^{b}	1.00^{x}	0.82^{c}	0.84^{y}	0.051	<0.001	<0.001	<0.001
C_2:C_3 ratio	2.30^{bA}	1.76^{zB}	2.39^{bA}	2.12^{yB}	2.71^{aA}	2.33^{xB}	0.051	<0.001	<0.001	0.018
Total gas										
ml/d	1798^{A}	1375^{B}	1728^{A}	1352^{B}	1657^{A}	1242^{B}	53.8	0.035	<0.001	0.892
ml/g dOM	225^{A}	179^{B}	223^{A}	187^{B}	207^{A}	179^{B}	5.9	0.116	<0.001	0.342
Methane										
mmol/d	8.08^{A}	4.24^{B}	7.71^{A}	4.75^{B}	7.78^{A}	4.93^{B}	0.362	0.833	<0.001	0.243
mmol/g dOM	1.02^{A}	0.56^{yB}	0.98^{A}	0.64^{xyB}	0.95^{A}	0.71^{xB}	0.041	0.542	<0.001	0.011

dOM – degraded organic matter; SEM – standard error of means; CS – carbohydrate source; DL – dilution rate; means with different superscripts within low dilution rate ($^{a-b}$), high dilution rate ($^{x-z}$) or within a carbohydrate source ($^{A-B}$) differ ($p < 0.05$)

Total Gas and Methane

Total gas production (ml/d and ml/g dOM) was affected by DL ($p < 0.001$; Table 5.4).

Methane production (ml/d and ml/g dOM) was influenced by DL with considerably lower values at the higher dilution rate. No effect of CS was present in the ANOVA but an interaction between CS and DL for the ml/g dOM values. In fact, the influence of DL appeared more prominent in BG compared to BP and SBH.

DISCUSSION

Influence of Carbohydrate

For rumen microbial growth, fermentable energy is generally considered a very relevant factor. In a further step, the origin of this energy may also become relevant. Since carbohydrates are the main energy source for rumen microbes, even minor differences in their efficiency for microbial growth will result in relevant changes in MCP yield. A link between type of carbohydrate and microbial growth can be explained in different ways, which are not exclusive. For example, differences in microbial yield could be primarily due to the microbial group supported by the particular carbohydrate, e.g., amylolytes vs. cellulolytes (Van Soest, 1994). Different carbohydrate sources can also provide varying amounts of energy (ATP) to microbes; acetate formation has been described to result in higher microbial ATP gain than propionate (Bergner and Hoffmann, 1996). In higher fermenting carbohydrates such as sucrose, some ATP will need to be spent on glycogen synthesis for temporary storage of oversupply, leading to lower overall efficiency (Hall and Weimer, 2016). Microbial yield can also be affected by differences in ruminal pH, which may explain beneficial effects of pectin compared to starch (Van Soest et al., 1991).

Hall and Herejk (2001) reported that the *in vitro* MCP yield from starch was the highest, followed by pectin, sucrose, and NDF. All values in that study are based on MCP/g OM and therefore include differences due to fermentation rate, which explains the low MCP from NDF. To some degree in contrast to this, a ranking of cellulose^{a}, starch^{a,b}, pectins^{b}, and sucrose^{c} (different superscripts = statistical differences $p < 0.05$) in terms of MCP/g DM was found when MCP was measured at half-maximal gas production (time points of 17.7, 8.2, 8.1, and 5.7 h for the respective carbohydrate) (Pfau and Hummel, 2019). In the present study, using feeds rich in particular carbohydrates, MCP yields of cellulose (SBH) and starch (BG) were superior to pectin + NDF (BP) when related to DM, while cellulose (SBH) was superior to both others when related to dOM. In summary, under the given conditions (e.g., particle incubation for 48 h), this study supports the finding of a particularly efficient MCP production from cellulose, producing 10–15% more MCP from SBH compared to BP (but not BG) when expressed per unit of incubated DM. When expressed per unit of dOM, SBH produced app. 10–15% more MCP

than both BG and BP. It should added that the ranking of pectins may be influenced by the high buffering capacity in *in vitro* fermentations, not reacting to differences of carbohydrates in this trait, which was considered a major reason for a beneficial effect of pectins on MCP (Van Soest et al., 1991).

The C2:C3 ratio was the highest for SBH, followed by BP, and BG, respectively. This supports the general view that fermentation of cell wall components favors the production of acetate, while the fermentation of grains favors propionate as fermentation product. The fact that methane yield was higher in SBH (rich in cellulose) compared to BG (rich in starch) also fits in with expectations.

Of the components used, SBH was characterized by a lower OMD_{cs} compared to BP or BG after 48 h of incubation, which was expected due to the considerably lower fermentation rate of its major carbohydrate cellulose of ~4%/h (Hall et al., 1998). Surprisingly, some interaction between degradability of forage and protein supplements and the type of component used was present. In treatments containing BP, a lower OMD_{hrsm} was measured.

Dilution Rate and Microbial Crude Protein

The major question of this study was the effect of DL (fluid passage) on fermentation variables, in particular MCP, when feed retention is kept constant at the same time. This approach helps to learn about variables relevant in steering fermentation, which can become important to understand the phenotype of individual animals with a particularly high microbial production. It is also relevant to understand differences between ruminant species in this regard. The mean retention time (**MRT**) of fluid and particles for example differs between sheep and cattle, the latter being characterized by a significantly higher ratio of $MRT_{particle}/MRT_{fluid}$ (Udén et al., 1982; Colucci et al., 1990).

Increasing the DL of the buffer resulted in higher MCP formation (MCP/g dOM) with MCP1+2, both covering total MCP. These findings are consistent with earlier studies conducted in continuous culture systems with DL between 2 and 20%/h (Isaacson et al., 1975; Meng et al., 1999; Eun et al., 2004). Efficiency of MCP production (MCP/g dOM) was greater with higher DL in these studies as well as in our experiment. A likely explanation is the shift in microbial metabolism from maintenance to expedited growth as stated by Isaacson et al. (1975). While this explanation is in line with fundamentals in microbiology (Herbert et al., 1956), other studies reported conflicting results. Using DL of 3.8 and 5.4 %/h in a Rusitec, Martínez et al. (2009) found no effect of higher DL on the total microbial N flow (MCP/d) and the daily N flow of the liquid-associated microorganisms (**LAM**), but the N content of LAM increased with increasing DL. Regarding the N flow of the solid-associated microorganisms (**SAM**) and the efficiency of microbial growth, the opposite effect to our study was observed. The SAM and the efficiency of microbial growth decreased with increasing DL. Also, the proportion of SAM of the total microbial N flow decreased with increased DL (Martínez et al., 2009).

The cellular mechanisms through which an increase in DL would enhance MCP yield are likely of some complexity. One potential factor might be the increased circulation around microbes creating a microclimate of increased nutrient supply and an efficient removal of fermentation end products and potential deterrents. Additionally, the efficiency of MCP formation is increased with increasing DL due to less microbial cell lysis and intraruminal N-recycling (Nolan and Leng, 1983).

While in general, OMD was high in this experiment (68-78%), an unexpected effect of higher DL was the lowered OMD, in particular for BP and SBH (please note that retention of solids was not different between DL and can be excluded as an explanation). It appears that the substrates rich in cell wall components were more affected. A potential explanation could be that attachment of microbes to the fibrous substrate was hindered by increased washout due to the higher DL. The fact that total SCFA production per day was lower with high DL is a logic consequence of these results. In fact, DL had no impact on total SCFA when SCFA were adjusted for differences in dOM. In contrast to our study, Martínez et al. (2009) reported higher total SCFA production and higher apparently fermented OM (estimated from SCFA production) in response to an increase in DL.

Impact of Dilution Rate on Methane Production

Besides the 10-15% increase in MCP yield, high DL also was linked to a reduction in methane production by 35% (CH_4/g dOM). This was one of the most striking results of this study. While Isaacson et al. (1975) also found a decrease in methane formation (CH_4/unit fermented glucose) by 20% when DL increased, others report no influence (Martínez et al., 2009) or even an increase in methane production when DL was augmented (Eun et al., 2004).

When trying to explain a link between DL and methane in this study, the first and most obvious argument must be the reduction in dOM observed with high DL. However, while this explains a part of the difference in methane formation, the effect of DL is still detectable after expressing methane per g dOM. A further explanation could be a shift in SCFA toward propionate (as observed in this study) with its known decreasing effect on methane synthesis due to less development of reduction equivalents in its metabolic pathway. An increasing effect of DL on propionate proportion was also found by Isaacson et al. (1975), Martínez et al. (2009) and Hoover et al. (1984), while Eun et al. (2004) report the opposite. Increased propionate amounts have been associated with shifts in the microbial population, like e.g., for protozoa depleted populations (Mobashar et al., 2019). As a third potential mechanism linked to methane reduction, fatty acid synthesis of growing microbes can be considered (Hackmann and Firkins, 2015; Cabezas-Garcia et al., 2017). Cell membranes contain considerable amounts of fatty acids [total fat content of bacterial cells being 8-14% (Czerkawski, 1986)], which need to be synthesized for bacterial cell proliferation and which can be considered a relevant sink for carbon and reduction equivalents. Estimations by Mills et al. (2001) assume 0.41 moles [2H] (metabolic

hydrogen) being used per gram microbial biomass produced. This level already explains the differences in methane production seen between dilution rates in the present study. This is true even if a potentially larger effect by a change in composition of fatty acids in microbial membranes from unsaturated to saturated at higher [2H] pressures (Ungerfeld, 2015; Guyader et al., 2017) is not considered yet.

A strategy of high fluid turnover and constant particle retention may have the potential to maximize MCP production, while decreasing ruminal methane production to some extent. While some support for this concept has been found in this study, further research is necessary to confirm its relevance *in vivo*, e.g., as a potential physiological explanation for individuals emitting less methane. If this concept proves to be valid, a likely limit for a strategy of increasing fluid turnover to increase ruminal efficiency will be a concomitant reduction of the ruminal barrier function against anti-nutritive substances ranging from mycotoxins to plant toxins (Müller et al., 2011).

Considerations on Methods

While the Rusitec is obviously a long- and well-established method, methodological refinements to further evolve the fermentation system and involved measurements still appear justified. The following points may deserve to be mentioned here. The first is the approach to estimate microbial production in the system, a measure that is of high importance, but notoriously challenging, since quantifiable only indirectly via a marker. While the ^{15}N method (MCP1) is well established, few studies appear to have used NH_4^+ content (MCP2) as a measure of N fixation by microbes. Its principle is straightforward. The difference between daily N input [N from substrates + N from buffer] and microbially unused N [NH_4^+ in effluent + undegradable N in substrate] was used to estimate microbial production. This approach was adapted from a method to estimate utilizable CP (CP available at the small intestine) from N input and changes of NH_4^+ concentration in the modified Hohenheim Gas Test (Leberl et al., 2007; Edmunds et al., 2012). Both MCP estimation methods led to concordant results, the ^{15}N method arriving at approx. 15% lower numbers (e.g., average of 121 vs. 147 mg MCP/g dOM). In our study, growth efficiency (MCP/dOM) was close to a value of 156 mg/g dOM suggested in official German recommendations (GfE 2001), albeit the latter being based on *in vivo* apparent digestibility experiments (and not ruminal degradability). But our values also fit well into the range of 142 to 173 mg MCP/g fermented OM based on theoretical considerations (Czerkawski, 1986; p. 142) and a value of 148 mg/g fermented OM (SD ± 33) as estimated in a more recent meta-analysis (Cabezas-Garcia et al., 2017). Due to the lack of a direct quantitative measure of microbial growth in the Rusitec, a final evaluation of methods appears challenging; based on the largely similar reaction of MCP1 and MCP2, the closeness of MCP2 with published values and the straightforwardness of its principle, we consider this approach to be a valid alternative for MCP measurements in Rusitec.

It is well established that the major part of microbial activity and growth is directly linked to the biofilm of substrate surfaces; however, the fluid phase also contributes substantially to microbial growth. In this study, the value of MCP in the effluent represented 16% of total microbial production. This is considerably lower than values of 29-41% estimated by Guyader et al. (2017) and than ∼50% of total MCP in the effluent as measured by Ribeiro et al. (2015), who used a similar method to estimate MCP in the soluble phase. While MCP3 is an interesting measure, it surely represents an additional measure to MCP1 or MCP2 only.

In contrast to the standard Rusitec procedure (Czerkawski and Breckenridge, 1977), a N source (NH_4Cl) was added to the artificial saliva/buffer solution in the present study, like e.g., also done by Romero-Pérez et al. (2015). In our view, this modification represents an important modification of the Rusitec, since a continuous supply of rapidly available N resembles an important aspect of ruminant physiology, where some supply of urea N via saliva and the rumen mucosa is constantly present and contributes to level out diurnal fluctuations in non-protein N supply in response to feeding. This is of particular importance in a study investigating effects of carbohydrates on microbial growth, which, according to the principle of synchrony (Sinclair et al., 1993), will benefit from simultaneous availability of N and energy.

CONCLUSIONS

Both dilution rate and carbohydrate source were shown to be relevant factors for microbial production in the Rusitec. Among the carbohydrate sources, SBH (rich in fermentable cellulose) lead to a higher microbial production per unit of dOM than BG and BP. Higher fluid dilution rate (1.5 vs. 3.0 %/h) increased production of MCP per unit dOM by about 10%, the shift in microbial metabolism from maintenance to growth probably playing some role. Among the concomitant changes in fermentation was a substantial reduction in methane [ml/g dOM] at high DL. This can be explained by changes in SCFA production partly, but increased microbial production may also be involved. The significance of microbes as hydrogen sink deserves further attention.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the State Office for Consumer Protection and Food Safety of the state of Lower Saxony (reference number: AZ 33.4-42505-04-13A373).

AUTHOR CONTRIBUTIONS

FP, JH, and MH designed the experiment. FP and MH conducted the experiment and analyzed the data. FP prepared the tables and drafted the manuscript together with JH and MH. K-HS facilitated the CH_4 analysis and contributed in data interpretation and manuscript. GB gave access to the donor cows needed for the inoculum and contributed in data interpretation and manuscript. MC contributed in project concept, data interpretation, and manuscript.

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6 Is there a difference in ruminal fermentation control between cattle and sheep? A meta-analytical test of a hypothesis on differential particle and fluid retention

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ABSTRACT

Ruminant species differ in digestive physiology. The species-specific ratio of mean retention time of particles and fluid (**$MRT_{particle}/MRT_{fluid}$**) in the reticulorumen has been interpreted as controlling ruminal fermentation: a higher ratio indicates of a more distinct 'washing' of particulate digesta by liquid. This should increase the harvest of microbes from the reticulorumen, and keep the microbiome in a state of more intense growth; at the same time, this should increase the metabolic losses of faecal nitrogen of microbial origin, leading to lower values for the apparent digestibility of crude protein (**aD CP**). A systematic difference has been hypothesized between cattle (higher ratio) and sheep (lower ratio), with a lower MRT_{fluid} in cattle due to a higher saliva production. Here, we test these hypotheses in a meta-analysis, using only studies that investigated cattle and sheep simultaneously. The datasets included 12 studies on MRT (of which 11 contained information on feed intake), yielding 102 (or 89) individual data; and 26 studies on protein digestibility (of which 18 contained information on intake), yielding 349 individual data. Cattle had a higher $MRT_{particle}/MRT_{fluid}$ (2.1) than sheep (1.7), mainly due to longer $MRT_{particle}$; only if body mass was included in the model, MRT_{fluid} was significantly shorter in cattle in the larger MRT dataset (and tended to be shorter in the slightly smaller dataset). Cattle had a significantly lower aD CP than sheep, while there was no such difference in overall (dry or organic matter) digestibility. The dataset confirms a shift in fermentation strategy towards microbial production in cattle. While this has been suggested for ruminants in general, cattle appear particularly far on an evolutionary trajectory of maximizing microbial yield from the forestomach. The application

of more specific digestive physiology data (like endogenous losses) gained from sheep to cattle should be done bearing these differences in mind.

1. Introduction

As foregut fermenters, ruminants digest plant material, including plant cell walls, in the forestomach with the help of a microbiome that releases extensive amounts of short chain fatty acids that are absorbed by the host (Stevens and Hume, 1995). The microbiome sustained by the supply of fermentable substrate serves, at the same time, as an important source of protein for foregut fermenters (Van Soest, 1994). Ruminants have a sorting mechanism in their forestomach that selectively retains large particles (Dittmann et al., 2015), to submit them to repeated chewing via rumination (Kovacs et al., 1997). Thus, ruminants achieve an unprecedented chewing efficiency that sets them apart from other herbivores, including nonruminant foregut fermenters (Fritz et al., 2009; Clauss et al., 2015).

By contrast, ruminants share another feature of forestomach digesta kinetics with most nonruminant foregut fermenters: a shorter mean retention time of fluid (**MRT_{fluid}**) as compared to that of particles (**$MRT_{particle}$**), or, in other words, a washing of the particulate digesta by the liquid digesta phase (Müller et al., 2011). The main function suggested for this 'digesta washing' is the mechanical removal of microbes from the fermentation chamber and their transport to the lower digestive tract, or, in other words, a more intensified harvest of the forestomach microbiome (Hummel et al., 2008; Müller et al., 2011; Hummel et al., 2015). In *in vitro* systems, an increased dilution typically leads to a higher outflow of microbes, and a concomitant increased microbial growth in the fermenter (Herbert et al., 1956; Pfau et al., 2021). However, actual tests of this function in live animals are largely missing. Very few studies have increased the fluid throughput through the gastrointestinal tract and thus achieved an increased microbial harvest from the reticulorumen (reviewed in Zhang et al., 2022).

Ruminants represent an interesting test case for this mechanism, because ruminants vary distinctively in the degree of digesta washing. The ratio of the $MRT_{particle}$ to the MRT_{fluid}, the so-called selectivity factor (**SF**) (Lechner-Doll et al., 1990), has been used to categorize ruminants as either 'moose-type' with a low ratio or 'cattle-type' with a higher ratio (Clauss et al., 2010; Przybyło et al., 2019). Among the domestic ruminants, cattle have been suggested to have a particularly high SF, due to a combination of particularly long $MRT_{particle}$ and particularly short MRT_{fluid} (Clauss et al., 2006). In contrast to the large number of nondomestic species for which MRT data (Przybyło et al., 2019) but no large datasets on protein digestibility are available, this limitation does not apply to cattle and sheep. Therefore, cattle and sheep represent ideal cases to test the hypothesis of a concomitant difference in digesta washing and microbial harvest and growth.

Following the logic of the faecal nitrogen method for an estimation of organic matter (**OM**) digestibility in ruminants (Lancaster, 1949; Lukas et al., 2005; Gálvez-Cerón et al., 2015), more microbial harvest from, and more microbial growth in, the reticulorumen (**RR**) will lead to an increase in metabolic faecal nitrogen losses. While the true digestibility of crude protein should not be influenced, a decreased apparent digestibility (**aD**) of crude protein (**CP**) can be expected in ruminants with an increased microbial production in the RR. Note that it is exactly for these very difficult-to-measure metabolic losses that the conventional measure of digestibility based on quantifying feed intake and faecal output is called 'apparent' – because it cannot differentiate between faecal components of dietary, metabolic (microbial) or endogenous origin. Therefore, aD CP is a value of questionable relevance when trying to gauge the true CP digestibility in ruminants (GfE, 2001). However, while caution arguing against the naive interpretation of the aD CP value as an indicator of true digestibility of CP is warranted, the dependence of the aD CP value on the contribution of metabolic (microbial) protein gives it considerable potential as a proxy for the quantity of microbial production in ruminants.

In this study, we aimed to investigate whether a significant difference in terms of the SF (ratio of $MRT_{particle}/MRT_{fluid}$) and in consequence ruminal microbial harvest exists between two domestic ruminant species (cattle and sheep), for which a difference in SF has long been postulated. The following hypotheses were tested:

1) Cattle have a significantly higher ratio of $MRT_{particle}/MRT_{fluid}$

2) To an important part, this is due to a shorter MRT_{fluid} in cattle

3) The assumed shift in ruminal fermentation products towards more microbial growth will result in higher metabolic faecal N losses and in consequence a reduced aD CP in cattle.

2. Materials and methods

Only data from studies were included that assessed both cattle and sheep in parallel, in order to maintain comparability of the experimental diets and husbandry conditions. In order to achieve sufficiently large datasets, two separate ones had to be compiled – one for MRT, and one for aD CP. Whenever available, data on body mass (**BM**) and dry matter intake (**DMI**) were also extracted.

For the dataset on $MRT_{particle}$, MRT_{fluid} and their ratio ($MRT_{particle}/MRT_{fluid}$), 12 studies (Table 6.1) representing 49 values for cattle and 53 values for sheep were available. The sheep were all males or animals without information on sex, and no animal was lactating. The cattle comprised 14 females, of which 8 were in lactation. The descriptive statistics of the dataset are given in Table 6.2. Only studies that had data on both, $MRT_{particle}$ and MRT_{fluid}, were used. Ten studies gave data for the MRT in the RR and two only for the whole gastrointestinal tract (**GIT**). As particle markers, six studies used Cr-fiber, three Yb-fiber, one Ce-fiber, and two used no marker but a rumen evacuation method. As fluid

marker, Co-EDTA was used in eight studies, Cr-EDTA in three and both together in one study. One study did not measure feed intake or diet composition. Diets varied, but were mostly forage based.

For the comparison of aD CP and overall diet digestibility in cattle and sheep, 26 studies representing values for 173 cattle and 164 sheep were used (Alexander et al., 1962; Buchman and Hemken, 1964; Vander Noot et al., 1965; Buchanan-Smith et al., 1968; Jentsch et al., 1969; Colovos et al., 1970; Schiemann et al., 1972; Wilkins et al., 1972; Greenhalgh and Reid, 1973; Arman and Hopcraft, 1975; Christopherson, 1976; Bergner and Weissbach, 1983b; Bergner and Weissbach, 1983a; Prigge et al., 1984; Vona et al., 1984; Amaning-Kwarteng et al., 1986; Bowman and Paterson, 1988; Colucci et al., 1989; Jentsch et al., 1992; Rooke et al., 1992; Jentsch et al., 1993; Jentsch and Wittenburg, 1993; Steingass et al., 1994; Südekum et al., 1995; O'Mara et al., 1999; Woods et al., 1999; Burns et al., 2005; Pearson et al., 2006). In cattle, the ratio of males:females was 1.6, and only 7 individuals were noted as lactating. In sheep, only males or animals without information on sex had been used, and no individual was lactating. Studies with pasture as feeding basis were excluded, because of the different grazing behavior (selectivity) of cattle and sheep. For overall diet digestibility, either DM or OM digestibility was used because neither of the two values was reported in all studies; the type of digestibility was therefore included in the statistical model. If both values were stated, OM digestibility was chosen for analysis. Eleven studies representing 67 values for each species reported only DM digestibility. For eight studies, no information on DMI was available. Absolute DMI information was available for 16 studies (173 measurements); the relative DMI (**rDMI**) was available for 18 (per $BM^{0.75}$, 253 measurements), 16 (per $BM^{0.85}$, 173 measurements) and 16 studies (per BM, 177 measurements), respectively. Note that different studies use different exponents for the expression of rDMI; a higher exponent than the traditional one of 0.75 has repeatedly been suggested for ruminants or large herbivores (Graham, 1972; Hackmann and Spain, 2010; Müller et al., 2013). The descriptive statistics of the dataset are given in Table 6.3.

The data collection on MRT was analyzed in two datasets (one including all twelve studies; the other including only the eleven studies that also quantified feed intake). $MRT_{particle}$, MRT_{fluid} and SF were the independent variables. Data were analyzed using mixed effects models. As our main target of investigation, species was included in these models as a fixed factor. To ensure species were compared within studies, and also to account for inter-study effects, study was included as a random factor. Similarly, to ensure species were compared on the same diets per study and to control for intra-study effects, diet was also included as a random factor (because in some studies, several diets were used). The site of MRT measurement (RR or GIT) was also added as a random factor. To test for species effects dissociated from BM or intake differences, additional models were performed that added BM as a random factor, or - for the datasets with feed intake – models that used either BM and absolute feed intake together, or only the relative feed intake (per $BM^{0.75}$, $BM^{0.85}$ or BM), as random factors.

Table 6.1 Characteristics of the studies included in the meta-analysis of mean retention times

Study	animals/ species	measure-ments/ species	Lactat-ion	$Marker_{particle}$	Particle size $marker_{particle}$	$Marker_{fluid}$	MRT data site	Feed intake measured	Roughage [% of total feed]	Feed
1	4 (cattle), 6 (sheep)	2	no	(rumen evacuation)	no information	Cr-EDTA	RR	yes	100	Lablab purpureus leafs or stems
2	4 (cattle), 8 (sheep)	8	no	(rumen evacuation)	no information	Cr-EDTA	RR	yes	100	grass leaves or stems
3	5 (cattle), 4 (sheep)	5/4	no	Cr-fiber	rumen content from dorsal sac (cattle), <6 mm (sheep)	Co-EDTA	RR	yes	100	grass hay
4	4	4	no	Yb-fiber	< 4 cm	Co-EDTA	RR	yes	100	grass hay
5	6	1	no	Yb-fiber	< 4 cm	Cr-EDTA	RR	yes	?-100	NaOH-straw, cottonseed meal, barley
6	2	2	yes/no	Yb-fiber	not chopped	Co-EDTA/PEG	RR	yes	40	alfalfa hay, wheat bran/beet pulp
7	6	6	yes	Cr-fiber	not chopped	Co-EDTA	RR	yes	30 - 80	alfalfa hay, corn and soy bean meal
8	4 (cattle) 9 (sheep)	4/9	no	Ce-fiber	< 2mm	Cr- / Co-EDTA	RR	no	100	pasture
9	4	4	no	Cr-fiber	not chopped	Co-EDTA	RR	yes	50 - 88	alfalfa hay, corn silage and concentrate
10	4 (cattle), 2-4 (sheep)	2	no	Cr_2O_3 / Cr-mordanted soy hulls	no information	Co-EDTA	RR	yes	14 - 18	soy bean hulls, hay, soy bean meal
11	4	8	no	Cr-fiber	pelleted	Co-EDTA	GIT	yes	100	grass hay, alfalfa hay or barley straw
12	3	3*	no	Cr-fiber	1-2 mm	Co-EDTA	GIT	yes	100	grass hay

1 Hendricksen et al. (1981), 2 Poppi et al. (1980; 1981), 3 Udén et al. (1982) , 4 Prigge et al. (1984), 5 Amaning-Kwarteng et al. (1986), 6 Doreau et al. (1988), 7 Colucci et al. (1989); Colucci et al. (1990), 8 Lechner-Doll et al. (1990)*, 9 Bartocci et al. (1997); Terramoccia et al. (2000), 10 Mulligan et al. (2001), 11 Pearson et al. (2006), 12 Steuer et al. (2011)*; *original data provided by the authors

Table 6.2 Descriptive statistics of the complete dataset on mean retention time (MRT) (n = 102)

Measure mean ±SD (min-max)	**Cattle** (n = 49)	**Sheep** (n = 53)
Body mass (kg)	515 ±224 (220-1310)	48 ±16 (18-99)
Dry matter intake (kg/d)*	8.3 ±4.6 (3.1-22.5)	1.0 ±0.3 (0.4-2.0)
MRT particles (h)	39.9 ±16.1 (17.7-78.0)	33.2 ±14.1 (11.7-72.6)
MRT fluid (h)	19.4 ±11.7 (6.8-46.2)	20.0 ±12.7 (6.8-60.4)
Selectivity factor	2.3 ±0.7 (1.1-3.8)	1.8 ±0.4 (1.1-2.7)

*n = 89

The second data collection on apparent protein and organic/dry matter digestibility was also assessed using mixed effects models, with species as fixed factor as our main target of investigation. As in the evaluation of the MRT datasets, study (inter-study) and diet (intra-study) were included as random factors. Additionally, the type of overall digestibility (OM or DM digestibility) was added as a random factor for the models assessing overall digestibility. Again, to test for species differences beyond the effect of feed intake, the relative dry matter intake (rDMI; per $BM^{0.75}$) was additionally assessed as random factor in the corresponding dataset; similarly, either BM and DMI together, or rDMI (per $BM^{0.75}$, $BM^{0.85}$ or BM) were added as random factors in the corresponding datasets. To check for a potential difference in intake level (which has potential to influence results), a further model with intake as dependent variable (DMI or rDMI per BM, $BM^{0.85}$ and $BM^{0.75}$), species as fixed factor and study and diet as random factors was performed (for DMI, an additional model was run with BM as a further random factor).

Table 6.3 Descriptive statistics of the complete dataset on protein digestibility (n = 349)

Measure mean ±SD (min-max)	**Cattle** (n = 179)	**Sheep** (n = 170)
Body mass (kg)*	473 ±169 (116-766)	50 ±13 (28-78)
Dry matter intake (kg/d)°	7.2 ±3.4 (2.9-22.5)	1.0 ±0.3 (0.5-2.1)
Relative dry matter intake (kg/($BM^{0.75}$ d))#	82 ±28 (42-180)	58 ±16 (33-94)
Apparent digestibility of organic/dry matter (%)	68.7 ±8.3 (39.6-86.1)	67.9 ±10.2 (40.6-89.1)
Apparent digestibility of crude protein (%)	61.2 ±12.2 (19.3-84.4)	64.5 ±12.5 (30.3-87.9)

*n = 214, °n = 175, #n = 255

Normal distribution of model residuals was assessed by Shapiro-Wilks-test; if residuals using the original data were not normally distributed, we used first a log-transformation of all quantitative data in MRT models and an arcsine-squareroot-transformation for digestibility data, and if that did not lead to normally distributed residuals, we repeated the model with ranked data. For models with original

or log- transformed data, least square means for the species are presented; for models using arcsine-squareroot-transformation or ranked data, only the direction of a significant difference was indicated. If percentages are reported to quantify the size of differences, the approach has been to set the higher value as 100% and to use the difference to the percentage of the lower value as a quantification of the difference. Statistical analyses were performed in R (R Core Team, 2020) using the packages lmerTest (Kuznetsova et al., 2017) and emmeans (Lenth et al., 2018), with a significance level set at $p \leq 0.05$.

3. Results

Generally, the random effects of study and diet were significant in the majority of analyses (Tables 6.4-6.6).

The effect of species was clearly significant for $MRT_{particle,}$ with longer $MRT_{particle}$ for cattle ($p < 0.001$). This was true for all eight models applied (Table 6.4 and 6.5). Some reduction of the level of significance was observed at the inclusion of BM ($p \sim 0.04$). The range of values was 43-47 h for cattle and 36-40 h for sheep, which corresponds to a 15-17% larger $MRT_{particle}$ in cattle. Note that all MRT values represent a mixture of data for the RR and the GIT and should not be directly compared to data in other studies.

For MRT_{fluid}, the model on the complete dataset including BM as random factor resulted in significant effect of species ($p = 0.019$), with shorter MRT_{fluid} for cattle (Table 6.4). In the reduced dataset (excluding the study that did not give intake data), a similar effect when including BM was evident as a statistical trend ($p = 0.054$). The five other models did not result in a significant effect (Table 6.4 and 6.5).

There was a significant effect of species ($p < 0.001$) for SF (the ratio of $MRT_{particle}/MRT_{fluid}$) for all eight models investigated, irrespective of the inclusion of factors like BM or intake (Table 6.4 and 6.5; Fig. 6.1). The SF was 20-26% larger in cattle than in sheep. Values were within the range of 2.1-2.2 for cattle and 1.6-1.7 for sheep.

In the MRT dataset, rDMI was higher in sheep if related to $BM^{1.0}$, but lower if related to $BM^{0.75}$; no significant species effect was present if intake was related to $BM^{0.85}$ ($p = 0.207$). Absolute DMI least square means were 7.7 kg DM for cattle and 1.0 kg DM for sheep (Table 6.5).

In the aD CP dataset (on CP and overall digestibility; Table 6.6), aD CP was lower in cattle than sheep in all models (at least on the level of $p = 0.002$). As expected, aD CP increased with dietary CP content (Fig. 6.2). For overall digestibility, no species effect was present in the full dataset and the most reduced dataset that included DMI or rDMI ($p = 0.377$-0.782). Just for the reduced dataset that included the rDMI (per $BM^{0.75}$) only, a higher overall digestibility for cattle was observed ($p = 0.047$).

In the aD CP dataset, intake was higher in sheep if related to $BM^{1.0}$, and lower if related to $BM^{0.75}$ and – in contrast to the MRT dataset – also if related to $BM^{0.85}$ (Table 6.6).

Table 6.4 Results of mixed effects linear models for the complete dataset on MRT (n = 102)

Dependent variable	Model	Species		Least square means*	
		F	P	Cattle	Sheep
MRT particle	Species[1sdl]	23.628	<0.001	42.7	35.5
	Species and BM[2sdl#]	4.717	0.037	Cattle >	Sheep
MRT fluid	Species[2sl]	0.895	0.348	-	-
	Species and BM[2sdl#]	6.088	0.019	Cattle <	Sheep
SF	Species[s]	59.949	<0.001	2.21	1.70
	Species and BM[1sd#]	50.645	<0.001	2.17	1.61

[1]Log-transformed data
[2]Ranked data
[sdl]Random factors significant (s = study, d = diet, l = location (RR or GIT), [#] = additionally mentioned factors in model)
*Note that LSmeans are a mixture of MRT data referring to the RR and the GIT, and are de-logged if necessary

Table 6.5 Results of mixed effects linear models for the MRT dataset with feed intake (n = 89)

Dependent variable	Model	Species		Least square means*	
		F	P	Cattle	Sheep
MRT particle	Species[sdl]	23.884	<0.001	46.6	40.3
	Species and BM[2sdl#]	4.694	0.042	Cattle >	Sheep
	Species, BM and DMI[2sdl#]	4.694	0.042	Cattle >	Sheep
	Species and rDMI (BM)[sdl]	23.883	<0.001	46.6	40.3
	Species and rDMI ($BM^{0.85}$)[1sdl]	24.694	<0.001	42.7	35.5
	Species and rDMI ($BM^{0.75}$)[sdl]	23.884	<0.001	46.6	40.3
MRT fluid	Species[2sl]	0.077	0.782	-	-
	Species and BM[2sdl#]	4.171	0.054	Cattle <	Sheep
	Species, BM and DMI[2sdl#]	4.171	0.054	Cattle <	Sheep
	Species and rDMI (BM)[2sl#]	0.197	0.659	-	-
	Species and rDMI ($BM^{0.85}$)[2sl]	0.139	0.711	-	-
	Species and rDMI ($BM^{0.75}$)[2sl]	0.027	0.870	-	-
SF	Species[s]	39.245	<0.001	2.18	1.73
	Species and BM[1sd#]	24.544	<0.001	2.14	1.64
	Species, BM and DMI[1s#]	25.297	<0.001	2.14	1.64
	Species and rDMI (BM)[1s#]	45.511	<0.001	2.09	1.68
	Species and rDMI ($BM^{0.85}$)[2s#]	45.327	<0.001	Cattle >	Sheep
	Species and rDMI ($BM^{0.75}$)[s]	35.523	<0.001	2.17	1.74
DMI	Species[1d]	1150.9	<0.001	7.69	1.00
	Species and BM[1d#]	461.1	<0.001	7.93	0.96
rDMI (BM)	Species[1d]	61.514	<0.001	15.1	20.4
rDMI ($BM^{0.85}$)	Species[1d]	1.638	0.207	38.9	37.2
rDMI ($BM^{0.75}$)	Species[1d]	48.371	<0.001	72.4	55.0

[1]Log-transformed data
[2]Ranked data

[sdl]Random factors significant (s = study, d = diet, l = location (RR or GIT), [#] = additionally mentioned factors in model)
*Note that LSmeans are a mixture of MRT data referring to the RR and the GIT, and are de-logged if necessary

Table 6.6 Comparison of digestibility data for cattle and sheep

Dependent variable	Model	Species		Least square means*	
		F	P	Cattle	Sheep
full dataset (n = 349)					
aD CP	Species[2sd]	32.237	<0.001	Cattle < Sheep	
aD OM/DM	Species[2sdm]	0.077	0.782	-	-
reduced dataset (n = 255)					
aD CP	Species[2sd]	11.749	<0.001	Cattle < Sheep	
	Species and rDMI ($BM^{0.75}$)[2sd]	10.301	0.002	Cattle < Sheep	
aD OM/DM	Species[2sd]	4.035	0.047	Cattle > Sheep	
	Species and rDMI ($BM^{0.75}$)[2sd]	4.035	0.047	Cattle > Sheep	
more reduced dataset (n = 175)					
aD CP	Species[2sd]	31.007	<0.001	Cattle < Sheep	
	Species and BM[2sd#]	25.901	<0.001	Cattle < Sheep	
	Species, BM and DMI[2sd#]	25.901	<0.001	Cattle < Sheep	
	Species and rDMI (BM)[2sd]	30.223	<0.001	Cattle < Sheep	
	Species and rDMI ($BM^{0.85}$)[2sd]	29.969	<0.001	Cattle < Sheep	
	Species and rDMI ($BM^{0.75}$)[2sd]	30.182	<0.001	Cattle < Sheep	
aD OM/DM	Species[2sd]	0.647	0.423	-	-
	Species and BM[2sd]	0.813	0.377	-	-
	Species, BM and DMI[2sd]	0.813	0.377	-	-
	Species and rDMI (BM)[2sd]	0.647	0.423	-	-
	Species and rDMI ($BM^{0.85}$)[2sd]	0.647	0.423	-	-
	Species and rDMI ($BM^{0.75}$)[2sd]	0.647	0.423	-	-
DMI	Species[2s]	1035.2	<0.001	Cattle > Sheep	
	Species and BM[2d#]	402.85	<0.001	Cattle > Sheep	
rDMI (BM)	Species[1sd]	71.637	<0.001	17.4	20.9
rDMI ($BM^{0.85}$)	Species[2sd]	17.905	<0.001	Cattle > Sheep	
rDMI ($BM^{0.75}$)	Species[2sd]	257.37	<0.001	Cattle > Sheep	

[1]Log-transformed data
[2]Ranked data
[sdl]Random factors significant (s = study, d = diet, m = measurement (OM or DM), [#] = additionally mentioned factors in model)
*Note that LSmeans are de-logged if necessary

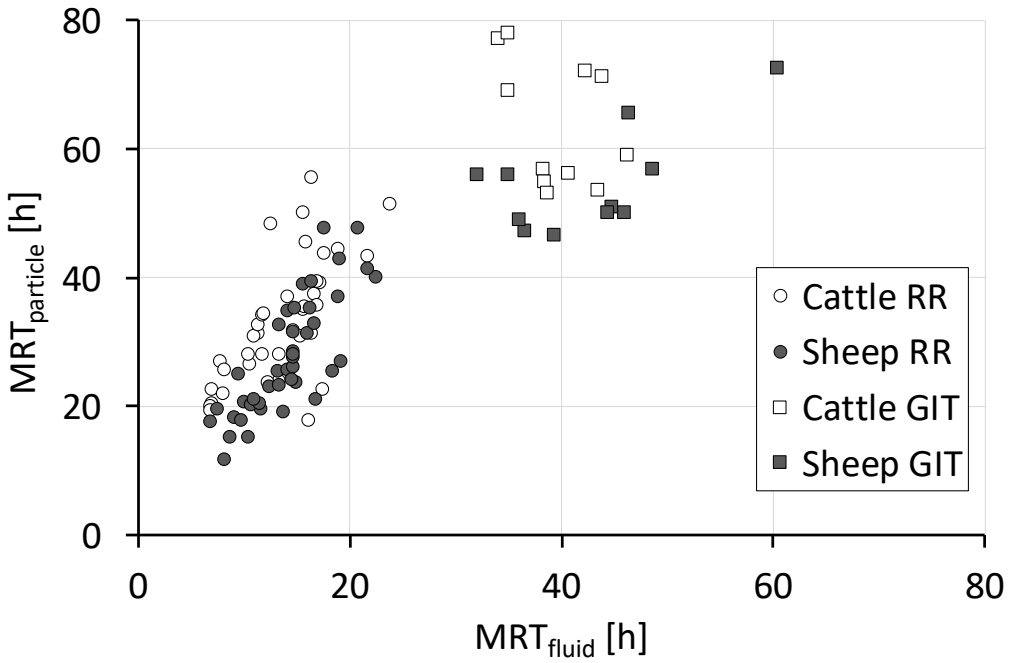

Figure 6.1 The mean retention time (MRT) of fluid and particles in the reticulorumen (RR) or whole gastrointestinal tract (GIT) in cattle and sheep compiled in this study. Note the similar range of MRT_{fluid} between the species, and the general offset of a higher $MRT_{particle}$ in cattle.

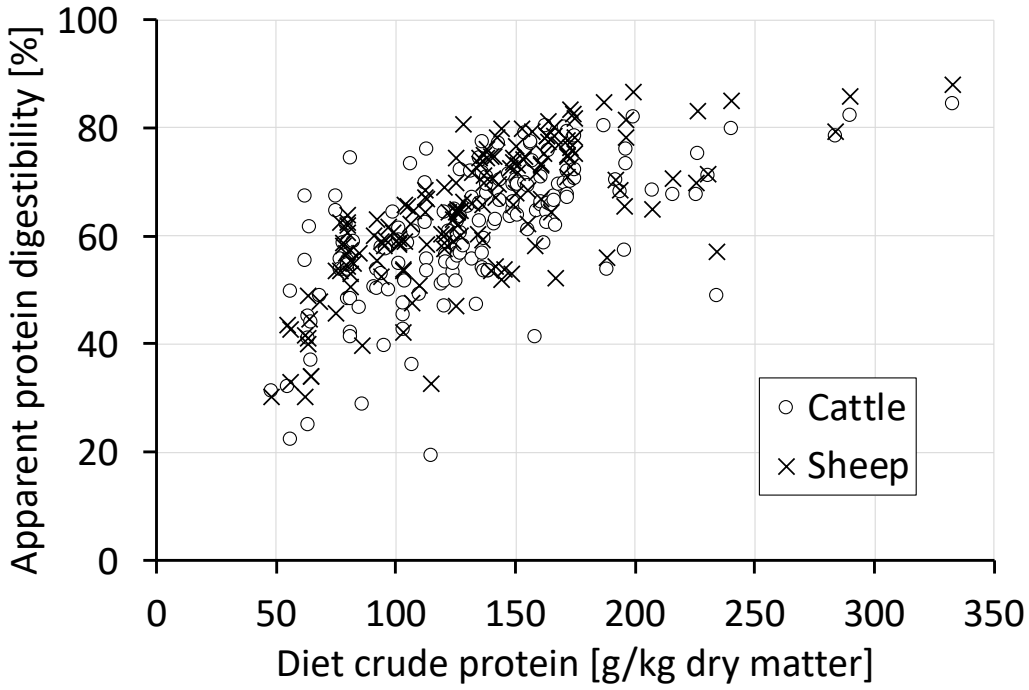

Figure 6.2 Apparent crude protein digestibility in relation to the crude protein content of the feed for cattle and sheep. As expected, the apparent protein digestibility increases with dietary crude protein content.

4. Discussion

Ruminants have been described to vary in their strategy to influence ruminal fermentation via the ratio of ruminal particle and fluid passage rate ('digesta washing') (Clauss et al., 2006; Clauss et al., 2010). High fluid outflow in relation to particle retention has been interpreted as a strategy to maximize microbial growth, while slow ruminal fluid throughput is assumed be a consequence of using a protein-rich, viscous saliva to neutralize tannins (Hofmann et al., 2008) and to have the benefit to minimize effects of plant toxins due to increased toxin degradation by ruminal fermentation. Cattle (Bovini) appear to be the ruminant most distinctively following the former strategy (microbial output), even if compared to other dominantly grazing species like sheep (Przybyło et al., 2019). If rumen microbial output is maximized, a lower apparent crude protein digestibility is an expected consequence, resulting from increased faecal losses of undigested microbial protein. For example, a digestibility of 85% is assumed for microbial protein at the small intestine of ruminants (GfE, 2001).

4.1. Retention time patterns

The data collection for retention time patterns included a variety of passage markers. The overall sample size did not allow a reasonable evaluation of differences between marker systems; however, because we only selected studies that assessed both cattle and sheep at the same time (i.e., with the same respective marker system), and accounted for study in the statistical model, the species comparison is nevertheless valid.

In line with one of our major hypothesis, a very clear difference in SF of cattle and sheep was evident, with the SF in sheep on average being lower on the size of 22%. In conclusion, a difference in retention time pattern could be clearly demonstrated in our study and was present in almost all comparisons in the individual studies. This higher SF can be interpreted as a higher degree of washing of particles, particularly in the forestomach.

In general, MRT depends on intake, with shorter MRT at higher levels of intake; this has been demonstrated both within and across species (Przybyło et al., 2019), and intake was therefore part of our statistical models. However, it has been shown that the species-specific SF is basically independent of the intake level (Grandl et al., 2018; Przybyło et al., 2019). Therefore, even though the range of intake for cattle in the MRT studies was comparatively low, the SF derived from these studies can be considered representative for the species. Although the level of water intake might be suspected to have an effect on MRT, especially on MRT_{fluid}, the – admittedly limited number of – existing studies do not indicate an effect but suggest that in ruminants, MRT_{fluid} is independent of water intake (Bernabucci et al., 2009; Hebel et al., 2011).

We had expected that the difference in SF between cattle and sheep was partly due to a difference in $MRT_{particle}$, which was longer in cattle compared to sheep at a magnitude of approximately 16%. This effect indicates a superior potential of cattle for fiber digestion, which, due its fermentation rate being on the same order of magnitude than rate of passage from the rumen, is reacting most sensitive to changes in MRT (Mertens, 1993). This effect will at least partially be offset by a slightly higher particle size reduction in sheep: For example, on the same diet, a faecal particle size of 640-830 µm has been described in cattle heifers while it was 460 µm in sheep (Udén and Van Soest, 1982).

While this species difference in $MRT_{particle}$ was unequivocal in our sample, our second hypothesis had been a shorter MRT_{fluid} in cattle, which, if it was present, would be best explained by a higher saliva output. Given the high saliva production of ruminants in general (estimations: cattle 180-220 L/day, sheep 6-16L/day (Piatkowski et al., 1990)) and its promoting effects on ruminal fermentation processes (e.g. supply of buffer, nitrogen and phosphorus into the fermentation chamber), some control function of saliva on ruminal fermentation appears a reasonable scenario. Saliva input into the RR can be considered as subject to a tradeoff. On the one hand, the inclusion of a high level of defensive proteins will constrain the salivary production rate (Hofmann et al., 2008), and a short MRT_{fluid} may flush soluble toxins into the lower digestive tract before they are deactivated by the RR microbiome. On the other hand, a high production rate of a saliva with little defensive proteins and the resulting short MRT_{fluid}, will intensify the harvest of microbes from the RR and hence microbial production due to an intensified regrowth. Therefore, the species-specific natural diet could be expected to relate to the species' salivary strategy, with an in increasing use of toxin-poor grasses being associated with a 'cattle-type' high-fluid-throughput RR physiology (Codron and Clauss, 2010). However, recent observations that 'cattle-type' ruminants can also ingest major proportions of browse (Przybyło et al., 2019) suggest that alternative ways to compensate for the potentially toxic nature of non-grass material must exist. Additionally, the possibility should not be neglected that in evolutionary terms, optimizing RR microbe production is still ongoing, with different ruminant genera being on different levels of a trajectory towards high RR fluid throughput. Compared to other large ruminants such as moose (*Alces alces*) or giraffe (*Giraffa camelopardalis*), cattle have distinctively shorter MRT_{fluid} (Clauss et al., 2006).

The results for our hypothesis that cattle have shorter MRT_{fluid} than sheep were equivocal. For the total data set, a shorter MRT_{fluid} was found for cattle than for sheep if BM was included in the model; inclusion of BM also led to near-significant *p*-values in the MRT_{fluid} models on the smaller data set, at a level of $p = 0.054$. All other models (not including BM as a factor) did not indicate an inter-specific difference in MRT_{fluid}. An equivocal outcome of a cattle-sheep comparison in this respect had already been mentioned by Dulphy et al. (1995), and a similar data compilation in (Clauss et al., 2006) had not yielded a significant difference between cattle and sheep, even though the cattle average had been numerically shorter than the sheep average MRT_{fluid}. Across ruminant species, no effect of BM on the

MRT_{fluid} in the RR has been reported (Clauss et al., 2006; Dittmann et al., 2015). This is in contrast to $MRT_{particle}$ in the RR, for which an increase with BM was found in the same evaluations (Clauss et al., 2006; Dittmann et al., 2015). The constancy of MRT_{fluid} across species of different BM is remarkable, because due to the more than linear scaling of RR volume with BM (Codron et al., 2019), constancy of MRT_{fluid} can only be attained by disproportionately increasing saliva flow at higher BM.

When taking a careful look at the results of the MRT_{fluid} as reported in the individual studies, it can be stated that for five of twelve studies, a relatively clear difference in the order MRT_{fluid} sheep > MRT_{fluid} cattle was present; this includes some classic cattle-sheep comparisons (Udén et al., 1982; Amaning-Kwarteng et al., 1986; Colucci et al., 1990; Lechner-Doll et al., 1990; Pearson et al., 2006), while in five further studies there were no or only marginal (< ±5%) differences between sheep and cattle (Prigge et al., 1984; Doreau et al., 1988; Bartocci et al.,1997; Mulligan et al., 2001; Steuer et al., 2011). In an earlier comparison of MRT_{fluid} between cattle and sheep, Dulphy et al. (1995) did not only compile some of the publications also used by us, but mentioned also two separate unpublished observations by C. Poncet and R. Baumont that claim shorter MRT_{fluid} in cattle. The two studies clearly contradicting our hypothesis are Poppi et al. (1980) and Hendricksen et al. (1981). While these studies did not use the usual method of sampling faeces after a single marker dose to estimate $MRT_{particle}$, but a method using the evacuation of total rumen contents, MRT_{fluid} was measured in the conventional way and hence, these results cannot be discarded. In previous data comparing MRT_{fluid} in cattle and sheep, it seemed that whereas both species can have similarly short MRT_{fluid}, long values were only reported in sheep (Fig. 4c in Clauss et al., 2006). This impression is clearly not corroborated by the comparative data of the present study (Fig. 6.1). To date, we either have to conclude that the number of experiments is still too low to come to a decisive conclusion, or we need to accept that the difference in SF between cattle and sheep is mainly due to longer $MRT_{particle}$ in cattle.

4.2. Apparent digestibility of crude protein

Our third hypothesis was that of a lower aD CP digestibility in cattle, which can be interpreted as a result of higher microbial production in the forestomach. From our data, it appears that this difference in aD CP in fact represents a very constant and reliable result in comparative cattle-sheep studies. This difference in aD CP between cattle and sheep has already been commented on by other authors (Südekum et al., 1995). The interpretation as a result of higher microbial production appears a parsimonious explanation based on general concepts of ruminant digestive physiology, requiring relatively little assumptions. While a lower aD CP in cattle cannot be regarded a final proof for a higher microbial production, it would surely be perfectly in line with such a concept. Further experimental studies looking at the reason for the aD CP differences in more detail would be helpful to elucidate underlying mechanisms. In fact, it has been reported that more ruminal NH_3 is incorporated in

microbes in cattle compared to sheep (Prigge et al., 1984). Whereas Amaning-Kwarteng et al. (1986) measured a higher bacterial N flow at the small intestine of sheep than in cattle, Rooke et al. (1992) reported numerically higher microbe flows (both per OM intake and per apparently digested OM in the rumen) from the abomasum in cattle than in sheep. More comprehensive evaluations of ruminal microbe production across the species would be welcome.

One important methodological issue related to endogenous and metabolic faecal nitrogen losses is feed intake. The GfE (2001) recommends to account for 5 g metabolic faecal N loss per kg intake in ruminants, consisting largely of microbial debris. This mechanism could partially explain the observed differences between cattle and sheep, if one would assume a generally higher feed intake in cattle. However, when the effect of feed intake was taken into account in the models, none of the results changed, irrespective of whether absolute feed intake and BM, or various expressions of relative feed intake were used. Therefore, the generally lower aD CP in cattle as compared to sheep emerges as a robust finding from the present analysis.

4.3. General differences between cattle and sheep

Besides insights in potential digestive strategies of ruminants in general, differences in digestive capacity between cattle and sheep are of interest also from a very practical point of view, since sheep are often used as model animals for the economically more important cattle. Therefore, differences in digestive physiology have repeatedly been discussed (Steingass et al., 1994; Südekum, 2002). The identical overall (organic or dry matter) digestibility shown in our dataset confirms the practice of using sheep data to draw conclusions on the feed value for cattle. Nevertheless, systematic differences on a more detailed level than feed digestibility are still of relevance and interest to explain every phenomenon seen in cattle-sheep comparisons.

Sometimes, different scaling exponents for the expression of relative feed intake are suggested for cattle than for sheep; Riaz et al. (2014), for example, suggested that *within* species, intake scaled at $BM^{0.88}$ in cattle and at $BM^{0.64}$ in sheep. This discrepancy raises the evident question which scaling exponent to use when intending to compare intake *across* species. Rather than using the conventional metabolic exponent of $BM^{0.75}$ or the traditional linear scaling when expressing intake per BM or in %BM (which both assumes a scaling of $BM^{1.0}$), scaling exponents of $BM^{0.9}$ (Graham, 1972; Hackmann and Spain, 2010) or $BM^{0.84–0.89}$ (Müller et al., 2013) have been suggested for ruminants and large herbivores, respectively. As previously described (Südekum, 2002), relative intake was higher in cattle than sheep when expressed per $BM^{0.75}$ and lower in cattle than sheep when expressed per $BM^{1.0}$ (Tables 6.5 and 6.6). By contrast, in the MRT dataset, there was no difference between the species when intake was expressed per $BM^{0.85}$. This could be taken as an indication for an 'ideal' allometric exponent. However, the argument that average performance potential and breeding level is higher in cattle than in sheep

also applies here and potentially interacts with BM as a factor for intake. In fact, while any breeding for performance will indirectly also lead to higher intake potential, this will be more significant in dairy breeds, which were more prominent in cattle than in sheep in our dataset.

Besides intake, MRT and aD CP, several other variables of digestive physiology differ between cattle and sheep. Based on faecal particle size, the food comminution process appears slightly more comprehensive in sheep than in cattle (Udén and Van Soest, 1982); sheep also comminute whole grains such as corn more comprehensively than cattle do (Kirchgessner et al., 1989). These observations are in line with a general scaling of chewing efficiency with BM (Fritz et al., 2009).

Based on the difference in SF and potential consequences for the partitioning of degraded feed into SCFA and methane on the one hand and microbial protein on the other (Isaacson et al., 1975; Pfau et al., 2021), an influence of this characteristic on methane production (as related to digested OM or digested fiber) could be postulated. Based on the higher SF and the lower aD CP shown in this study for cattle, it can be hypothesized that their microbiome should be more tuned to growth, and hence produce more microbial matter and less methane per kg digested OM/fiber. Though individual studies arrived at no difference (Blaxter and Wainman, 1961) or a higher methane production in cattle (20.6 g/kg DMI) compared to sheep (18.6 g/kg DMI) (Swainson et al., 2008), a meta-analysis arrived at 18% higher values for sheep (23.4 ± 5.73 g/kg DMI for SF6 data and 23.1 ± 2.89 g/kg DMI for calorimetry) than for cattle (19.1 ± 3.70 g/kg DMI) (Hammond et al., 2009). In a study comparing the effect of the inoculum on in vitro fermentation, Boguhn et al. (2013) found that cattle inoculum produced less gas and methane on the same feed compared to sheep inoculum, which is in line with our general interpretation. Further comparative studies on differences in the microbiome's metabolic state between cattle and sheep are encouraged. So while basic variables values like OM digestibility appear very comparable between sheep and cattle, more specific physiological variables like endogenous N losses or microbial production should be transferred from sheep to cattle with more caution and having the outlined differences in mind.

4.4. Beyond cattle and sheep: Bovini and Caprinae

While focusing on the major domestic species of cattle and sheep, the question arises whether general differences in digestive physiology are also present in other members of the respective groups, like water buffalo (*Bubalus bubalis*), African buffalo (*Syncerus caffer*), banteng (*Bos javanicus*), bison (*Bison bonasus*, *Bison bison*) or yak (*Bos grunniens*) from the bovini and mouflon (*Ovis ammon musimon*), ibex (*Capra ibex*) or the domestic goat from the Caprinae. The SF data available for Bovini and Caprinae is still somewhat restricted, but available information indicates that particulary large SF are ubiquitously present in cattle genera like water buffalo, anoa (*Bubalis depressicornis*), banteng, African forest buffalo or European bison (Bartocci et al., 1997; Flores-Miyamoto et al., 2005; Schwarm et al., 2008;

Steuer et al., 2011; Przybyło et al., 2019), while SF appear uniformly lower for members of Caprinae like ibex, mouflon or domestic goats (Udén et al., 1982; Gross et al., 1996; Behrend et al., 2004).

5. Conclusion

Ruminal mean retention time can be regarded as a major controlling variable for fermentation processes in the forestomach. A higher ratio $MRT_{particle}/MRT_{fluid}$ was found for cattle as compared to sheep, which was dominantly due to a longer $MRT_{particle}$ in cattle. In line with this, indications of a slightly higher microbial production and higher metabolic faecal nitrogen losses as indicated by a lower apparent protein digestibility were found. Any increased washing of forestomach digesta by fluid can be interpreted as an adaptation to harvest microbes and keep the microbiome in an escalated state of growth, and selective pressures for a high SF can therefore be postulated to act on any ruminant species during evolution. Cattle appear to be particularly far on this trajectory, following a further shift in partitioning of fermentation products towards microbial production.

CRediT authorship contribution statement

Friederike Pfau: Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Marcus Clauss:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Jürgen Hummel:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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7 General discussion

7.1 Range of carbohydrates in forages and diets for ruminants

Variation of carbohydrate fractions in silages and other roughages can be high. Starch content of all analyzed corn silages by LUFA Nord-West in 2022 ranged between 16.2 and 40.2% DM and sugar content of grass silages varied between 5.0 and 19.1% DM (LUFA, 2022) (Table 7.1 and Table 7.2). Evaluating 78 round-bale grass silages from farms in Norway, Weiby et al. (2022) found 40.8-66.5% NDF (mean 53.7% ± 5.79 SD) and 0.03-13.7% water soluble carbohydrates (mean 4.26% ± 3.68 SD) on DM basis. In a study investigating 10 different grass silages and 4 different clover silages, NDF, ADF, ADL and non-fiber carbohydrate (**NFC**) content ranged between 40.1-51.0%, 23.8-29.9%, 1.1-2.1% and 22.1-37.7% of DM for grass silages and 29.5-32.2%, 23.2-29.1%, 1.9-3.6% and 31.3-38.1% of DM for clover silages, respectively (Parnian-Khajehdizaj et al., 2023).

Also, nutrient composition of by-product feedstuffs can vary considerably between sources and between mean values of official feedstuff tables and analytical value of the respective feedstuff (Belyea et al., 1989; Arosemena et al., 1995). Analysis on DM basis of nine different BP sources resulted in a range of 32.9-41.1% NDF (mean 35.8% ± 2.82 SD), 17.0-21.2% ADF (mean 18.8% ± 1.54 SD) and 15.3-19.1% cellulose (mean 16.5% ± 1.31 SD) (Arosemena et al., 1995). Citrus pulp (n = 4) analyzed in the same study varied in NDF (14.4-22.4% DM, mean 17.7% ± 3.76 SD), ADF (13.8-21.6% DM, mean 16.8% ± 3.77 SD) and cellulose content (12.8-20.7% DM, mean 15.9 ± 3.76 SD) (Arosemena et al., 1995). Content of NDF, ADF and cellulose of three different SBH resulted in average 57.5% (± 1.17 SD), 45.4% (± 3.19 SD) and 42.5% (± 3.32 SD) on DM basis, respectively. Cellulose was calculated by subtraction of acid detergent lignin (**ADL**) from ADF content (Arosemena et al., 1995), whereas Miron et al. (2001) determined cellulose by analysis of NDF-glucans because determination of cellulose via neutral and acid detergent fractions may not separate cellulose or hemicellulose completely. In investigations of Miron et al. (2001), BP and citrus pulp contained high amounts of neutral detergent soluble (**NDS**) carbohydrates (54 and 75% of total carbohydrates on DM basis, respectively), while SBH contained 75% of the carbohydrates as NDF (DM basis). They determined pectin as NDS uronic acid and found 20.7, 12.7 and 11.0% NDS uronic acid (DM basis) for citrus pulp, BP and SBH, respectively. Analysis of NDF-glucans (= cellulose) resulted in 36.6, 21.9 and 11.8% of DM and analysis of NDF-non-glucose polysaccharides (= hemicellulose) resulted in 24.0, 13.1 and 6.98% of DM for SBH, BP and citrus pulp, respectively, while calculation of cellulose (ADF-ADL) resulted in 51.2, 29.7 and 20.9% of DM and calculation of hemicellulose in 15.9, 12.9 and 0.4% of DM for SBH, BP and citrus pulp, respectively (Miron et al., 2001). A more recent study found on average 64.4% nonstructural carbohydrates (**NSC**), 20.4% NDF, 17.5% ADF and 22.1% sugar on DM basis for citrus pulp (n = 6) and 11.7% NSC, 69.6% NDF,

53.8% ADF and 2.2% sugar on DM basis for SBH (Ünlü et al., 2022). Examining six lots of BG, Engstrom et al. (1992) detected differences in content of β-glucans, starch, NDF and ADF (3.5-4.8, 56.5-65.6, 14.4-22.1 and 5.7-9.7% of DM, respectively).

Table 7.1: Chemical fractions [% dry matter] of silages analyzed by LUFA Nord-West (Germany) in 2022

	Corn silage (n = 2534)		**Grass silage** (n = 5827)		**Cereal whole-plant silage** (n = 138)	
	Mean	**Variation**	**Mean**	**Variation**	**Mean**	**Variation**
Starch	28.6	16.2-40.2				
Sugar	1.6	<1.5-3.5	6.9	5.0-19.1		
NFC					28.6	5.9-48.0
Crude fiber	21.3	16.6-26.2	25.9	20.0-33.0		
aNDFom	41.1	32.5-50.8	47.4	37.6-61.7	54.5	38.7-70.1
ADFom	23.7	18.7-29.2	29.4	23.4-37.0	32.2	21.8-42.3
ADL			2.5	1.4-4.0		

Variation: ± 2 standard deviations
NFC: non-fiber carbohydrates
aNDFom: neutral detergent fiber (assayed with heat-stable amylase, exclusive residual ash)
ADFom: acid detergent fiber (exclusive residual ash)
ADL: acid detergent lignin

Table 7.2: Chemical fractions [% dry matter] of fresh grass and grass hay analyzed by LUFA Nord-West (Germany) in 2022

	Fresh grass (n = 501)		**Grass hay** (n = 1372)	
	Mean	**Variation**	**Mean**	**Variation**
Sugar	19.5	4.5-36.8	10.3	3.4-17.5
NFC	28.6	14.6-48.7	20.1	8.0-30.2
Crude fiber	20.9	13.6-29.9	33.0	25.7-39.1
aNDFom	45.9	35.1-59.3		
ADFom	24.1	17.6-32.0		

Variation: ± 2 standard deviations
NFC: non-fiber carbohydrates
aNDFom: neutral detergent fiber (assayed with heat-stable amylase, exclusive residual ash)
ADFom: acid detergent fiber (exclusive residual ash)

Total mixed rations (**TMR**; n = 16) differing in ingredient composition, varied from 29.0-55.2% NDF of DM (mean = 38.7), 16.3-31.4% ADF of DM (mean = 22.9) and 15.6-28.2% crude fiber of DM (mean = 21.1) (Boguhn et al., 2006). Starch, sugar or pectin content were not analyzed, although most TMR contained corn silage, many grains and some beet pulp silage. Considering the large variations in carbohydrate composition of single feeds, carbohydrate composition of whole diet for ruminants can also vary considerably. Nutrient analysis of the individual feedstuffs used in the diet is recommended to balance the diet according to nutritional requirements the ruminant. Practical recommendations for a diet for high producing dairy cows in Germany are a maximum of 7.5% sugar on DM basis, at least

15%, but maximal 25% on DM basis rumen degradable starch and sugar, 3-5% on DM basis rumen undegradable starch and a minimum of 28% on DM basis aNDFom from forage (Bayerische Landesanstalt für Landwirtschaft, 2019). Calculated under the assumption of 600 kg body weight and 20 kg DM intake, Varga et al. (1998) classified 22.5, 27.0, 28.5, 30.0 and 36.0 % forage NDF as minimum, moderately low, average, moderately high and maximum of total NDF, respectively. For TMR consisting mainly of corn or alfalfa silage as forage and ground corn grain as starch source, the National Research Council (2001) recommended at least 19% NDF from forage, at least 25% total dietary NDF and a maximum of 44% NFC on DM basis for high producing dairy cows. If the diet contains a lesser amount of forage NDF or more rumen degradable starch or forage with small particle size or if the diet is no TMR or is not calculated from analyzed nutrient values of the used feedstuff, dietary NDF content should be higher (National Research Council, 2001). This shows that several factors influence diet composition and must be considered during diet formulation. Also, diet formulation and feedstuff availability influence the carbohydrate composition of the diet.

7.2 Influence of carbohydrate source or feedstuff on fermentation kinetics

For the range of different carbohydrates, considerable differences in fermentation rate are obvious. Overall fermentation kinetics have an influence on effective ruminal degradation and therefore potentially also on microbial growth in the fermentation chamber, particularly if energy release kinetics are largely different from nitrogen availability.

In the preparatory work for the HGT results presented in chapter 4, fermentation kinetics of pure carbohydrates were investigated in some detail for the carbohydrates and feeds used in the study, and a closer look at these results appears rewarding at this point. Comparing the fermentation kinetics of the pure substrates with that of the feedstuffs that contain certain amounts of the specific carbohydrates, total gas production after 96 h of incubation is constantly higher for pure substrates (Figure 7.1 and Figure 7.2). This is not surprising because the degradable carbohydrates extracted from the feedstuffs represent pure energy for microbes and do not contain any amounts of ash or lipids, both known to be of no energetic value for ruminal microbes. In addition, plant components that hinder fermentation or make the plant material unavailable for microbial degradation are absent. Comparing gas production of soybean hulls (known for their high contents of digestible cellulose) and pure cellulose, soybean hulls produced considerably more gas in the first eight hours of incubation. However, gas production of cellulose was equal or higher after 16 h of incubation. Nevertheless, pure cellulose needed more time to reach $t_{1/2}$ than SBH. After 6 h of incubation, cumulative gas production of pure citrus pectin was notably higher than gas production of the pectin rich feeds citrus pulp or BP (despite the latter containing 23.8% sugar, which is usually very fast fermented). This underlines once again how fast pectin can be fermented. Until 12 h of incubation, gas production of citrus pulp is lower

than BP, which changes to the opposite after 24 h of incubation (with some degree of variability in our sample of citrus pulp). In general, sucrose is more rapidly fermented than BP. However, after 2 h of incubation gas production of BP is at least the same as gas production of sucrose. Maybe this is due to the amount of pectin in the BP, which is very rapidly fermented, or because the potentially extremely fast ("explosive") fermentation of pure sucrose is not realized in the incubation due to some lag phase in the fermentation of the freshly started *in vitro* fermentation; this effect at the beginning of fermentation will influence the fermentation of potentially very fast fermenting substrates like sucrose in particular. Until 8 h of incubation, wheat grain and BG is potentially faster fermented than pure wheat starch. However, after 8 h of incubation gas production of starch is higher than gas production of the grains, perhaps due to similar reasons as described above. Considering $t_{1/2}$, the pure substrates sucrose and pectin needed less time to reach this point than BP or citrus pulp, respectively; wheat grain needed almost as much time as pure starch.

While the ruminal microbiome generally thrives on simple nitrogen sources, among other factors, the presence or absence of more complex molecules like amino acids or peptides may have an influence on fermentation. In the preparatory analyses described here also a modified HGT buffer solution with varying N sources was used. Tryptone (pancreatic digest of casein; 17.32 g/L) and cysteine-hydrochloride (1.53 g/L) (Hall and Herejk, 2001; Hall and Weimer, 2016) were added to the buffer solution as well as the threefold amount of ammonium hydrogen carbonate (= 18 g/L). The usual HGT buffer solution contains ammonia as the only N source. Since the investigated pure carbohydrates contain no protein, the total lack of amino acids and peptide could affect microbial growth and therefore fermentation kinetics (Argyle and Baldwin, 1989). While it should be considered that the added peptides and amino acids could also be used as energy source for the rumen microbes and therefore alter fermentation, according to Argyle and Baldwin (1989), this is rather unlikely, because in their studies additional amino acids in contrast to an additional energy source improved microbial growth efficiency without altering intercept values. On the other hand, Maeng et al. (1976) found that 14% of the amino acids were actually fermented when rumen microbes were provided with the optimum ratio of nonprotein (75%) to amino acid (25%) nitrogen for microbial growth.

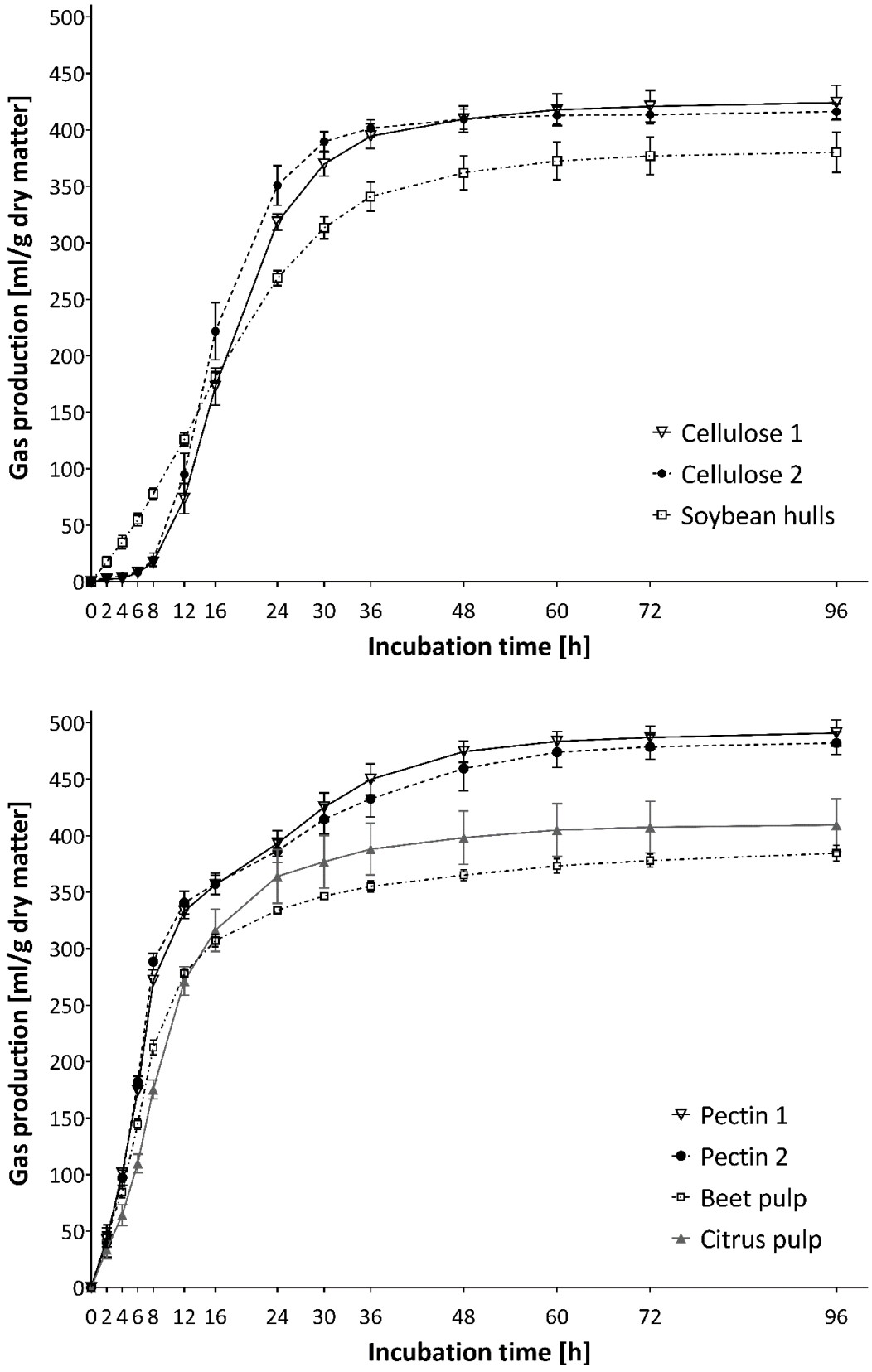

Figure 7.1: Cumulative gas production curve (mean ± SD) of cellulose and pectin (1 = normal buffer; 2 = buffer with additional tryptone and cysteine) and soybean hulls, molassed beet pulp and citrus pulp (normal buffer) over 96 h of in vitro incubation.

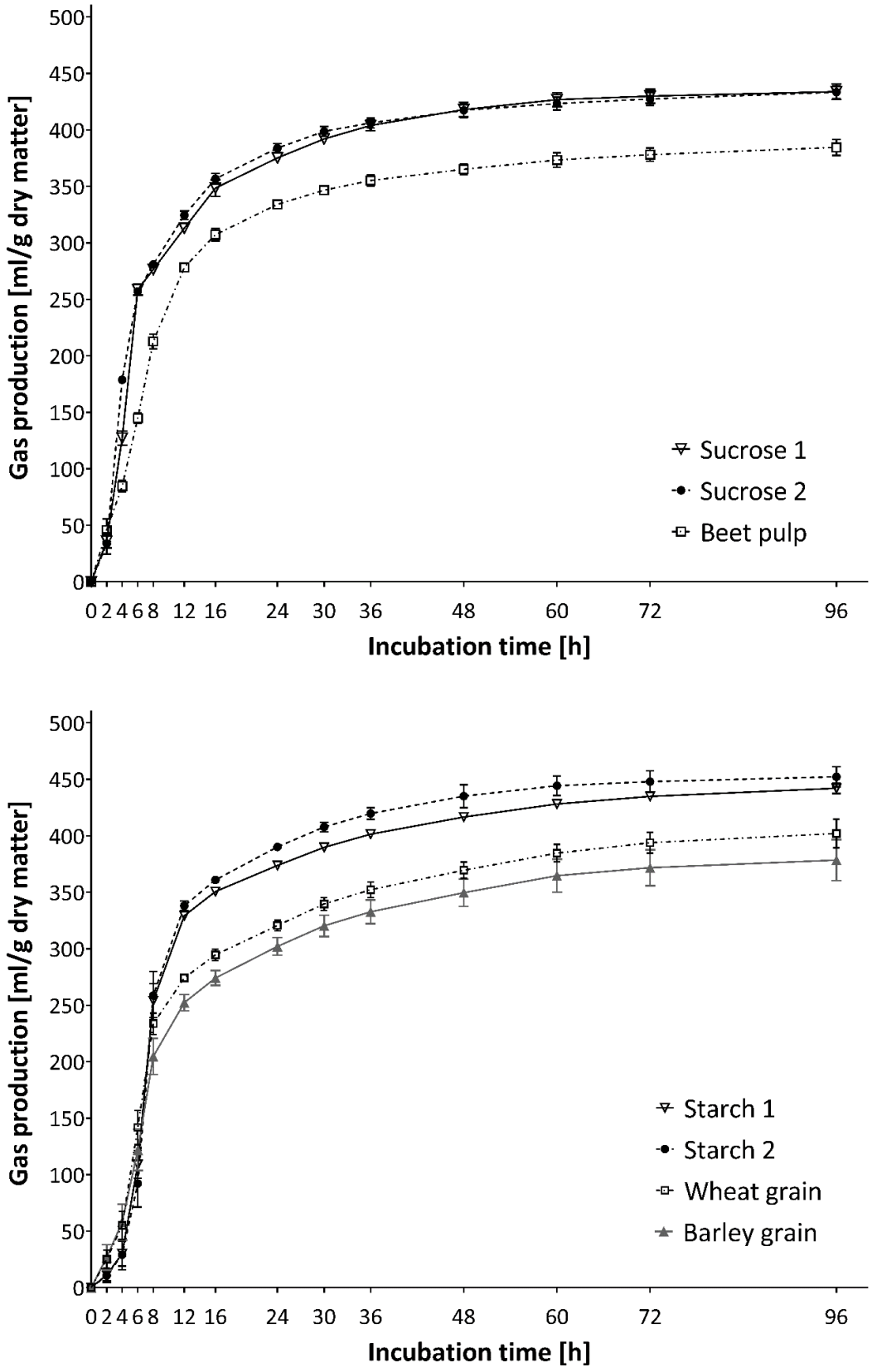

Figure 7.2: Cumulative gas production curve (mean ± SD) of sucrose and starch (1 = normal buffer; 2 = buffer with additional tryptone and cysteine) and molassed beet pulp, wheat grain and barley grain (normal buffer) over 96 h of in vitro incubation.

Table 7.3: Estimated time of half maximal gas production (mean) of different substrates incubated with additional tryptone and cysteine in the Hohenheim gas test (HGT) buffer (modified buffer) and HGT buffer without addition (normal buffer).

	Time of half-maximal gas production [hr]	
Substrate	**Normal buffer**	**Modified buffer**
Sucrose	5.8	5.2
Starch	8.2	8.3
Cellulose	17.8	16.0
Pectin	7.7	7.1
Beet pulp	7.6	-
Citrus pulp	9.7	-
Soybean hulls	16.5	-
Wheat grain	8.0	-
Barley grain	8.5	-

By adding tryptone and cysteine to the HGT buffer, fermentation of cellulose and sucrose were enhanced as shown in Figure 7.1 and Figure 7.2; gas production of sucrose and cellulose were considerably higher at 4 h and 16 h, respectively. Furthermore, times needed to reach $t_{1/2}$ were numerically shorter for sucrose, cellulose and pectin incubated with the modified buffer (Table 7.3). However, in contrast to gas production rate, no effect on total gas production was obvious for incubations with and without additional peptides/amino acids (Figure 7.1, Figure 7.2). Apparently especially fermentation of sucrose and cellulose benefits from added tryptone and cysteine. Please note that all differences reported were only visual or numerical as no statistic for the additional data were conducted.

7.3 Range of microbial crude protein production and effects on it

As various factors affect MCP production in the rumen, the range of MCP production is quite high. Reviewing 64 observations of numerous studies, Stern and Hoover (1979) found a range of 63 to 307 g MCP/kg OM digested with a mean of 169 g MCP/kg dOM in the rumen. In another review with 61 different rations, efficiency of MCP production was 156 g/kg digestible OM and 162 g/kg fat-free digestible OM (GfE, 2001). In a more recent meta-analysis of Rusitec studies, estimated *in vitro* MCP yield was 154 g/kg dOM (calculated from 24.7 g N content × 6.25) (Hristov et al., 2012). Also, Boguhn et al. (2006) found 141 to 286 g MCP/kg fermented OM of 16 different TMRs *in vitro* in Rusitec fermenters. Investigating the MCP yield of different forage types, Verbic (2002) found 115-158 for grass silages, 126 in hay, 145-199 for fresh forage and 165-217 g MCP/kg fermentable OM for corn silages. Fresh forages seem to provide more fermentable OM for the microbes than grass silages; fermentable OM represents the energy available to rumen microbes (Verbic, 2002).

In diets differing in content of NSC and degradable protein, MCP production efficiency was not affected (averaged 24 g of microbial N/kg of OM digested [150 g MCP/kg of OM digested] for all treatments) (Stokes et al., 1991). However, OM digestion was lower for the diet lowest in NSC (24% of DM) and degradable protein (9% of DM), resulting in a lower microbial N flow per day for this diet (Stokes et al., 1991). Also, very high dietary NSC content (39%) paired with a lack of conventional forage in the diet results in a decrease of MCP production efficiency (21.8 and 33.5 g microbial N/kg OM digested [136 and 209 g MCP/kg OM digested] for 39% and 29% NSC diet, respectively) (Feng et al., 1993). Investigating combinations of high and low rumen-available NSC and protein *in situ*, Aldrich et al. (1993) found higher bacterial N efficiency [g/kg OM truly digested in the rumen] for diets, where both NSC and protein were highly rumen-available (17.9 g N = 112 g MCP/kg OM truly digested) or both were low rumen-available (17.8 g N = 111 g MCP g/kg OM truly digested), while diets with either high NSC and low protein or low NSC and high protein rumen-availability had lower bacterial N efficiency (14.5 g N = 91 g MCP/kg OM truly digested and 15.2 g N = 95 g MCP/kg OM truly digested, respectively). This demonstrates the demand for synchronous availability of energy and protein for the rumen microbes.

According to McCarthy et al. (1989), there was no difference in microbial N efficiency in early lactating dairy cows for diets containing either ground shelled corn or steam rolled barley (35.2 g N/kg dOM [220 g MCP/kg dOM] and 31.2 g N/kg dOM [195 g MCP/kg dOM], respectively). Efficiency of bacterial synthesis in continuous culture fermenters was higher for BP than for corn (34.9 and 32.0 g N/kg OM truly digested [218 and 200 g MCP/kg OM truly digested], respectively) (Stern et al., 1994). In studies stating bacterial or microbial N efficiency only, MCP values were calculated by multiplying with 6.25.

The MCP values in this study are within the range of the literature. In the HGT study presented in chapter 4, MCP production efficiency after 24 h of incubation was higher for cellulose than starch, sucrose and pectin (198, 161, 127 and 111 g/kg dOM, respectively) resulting in the following ranking cellulose > starch > sucrose > pectin. However, it should be noted that OMD after 24 h of incubation was considerably lower for cellulose than OMD of sucrose, pectin and starch. Also, OMD of starch was lower than OMD of sucrose and pectin. In the Rusitec study (chapter 5), MCP production efficiency of the cellulose rich SBH was higher than MCP of BG and BP associated with also lower OMD_{cs} for SBH than BG and BP. Zhao et al. (2013) found an increase in daily N flow of solid-associated microorganisms in Rusitec fermenters with increasing amounts of BP replacing corn starch and wheat bran (resulting in an increasing level of neutral detergent-soluble fiber) and a trend of increasing efficiency of microbial synthesis. In contrast, partially replacing corn grain with soybean hulls (normal starch diet, 27% starch/kg DM and reduced starch diet, 18% starch/kg DM) did not affect bacterial protein flow in lactating dairy cows (Fredin et al., 2015).

In the Rusitec study (chapter 5), an increased DL resulted in higher MCP production efficiency. These findings are consistent with earlier *in vitro* studies conducted in continuous culture systems (Isaacson et al., 1975; Meng et al., 1999; Eun et al., 2004), while in contrast to these results, Martínez et al. (2009) found a decrease in MCP production efficiency with increasing DL in Rusitec. Earlier *in vivo* studies which increased the saliva flow into the reticulorumen found a higher MCP yield (significant: Harrison et al., 1975; by trend: Wiedmeier et al., 1987 and Froetschel et al., 1989). In a more recent *in vivo* study with cattle, Zhang et al. (2023b) found an increasing effect of fluid passage rate on MCP production as indicated via metabolic faecal N, but not as estimated via purine derivates.

7.4 Effects on methane production

In the Rusitec study presented in chapter 5, increased DL reduced methane production [mmol/g dOM], while CS had no effect on methane production per se. However, an interaction between CS and DL was detected resulting in a lesser methane production [mmol/g dOM] for BG than SBH with high DL. Thus, in this study the starch-rich substrate had no direct advantage regarding methane production compared with fiber-rich substrates. In an *in vivo* study with dairy cows, starch-rich diets (231 g starch and 297 g NDF per kg DM) reduced methane production compared to fiber-rich diets (58.5 g starch and 419 g NDF per kg DM, respectively) on average by 15% measured in g/kg DMI, likely due to the reduction of protozoa number and a shift in SCFA composition from butyrate towards propionate (Bougouin et al., 2018), whereas MCP production was not measured. By contrast, methane production [g/kg digested OM] of dairy cows did not differ between diets containing molasses, wheat, apple pulp, SBH or oat hulls (Hindrichsen et al., 2005). However, if methane production was related to digested NDF, methane production of SBH diet was less than of wheat, apple pulp or molasses diet and methane production of molasses diet was higher than of wheat diet (Hindrichsen et al., 2005). Evaluating the same diets in the Rusitec, methane production [mmol/g dOM] increased from diets containing oat hulls (0.92), wheat (1.04), SBH (1.13), apple pulp (1.15), sugar BP (1.24) and molasses (1.37) with significantly higher values for molasses diet than wheat or oat hulls diets (Hindrichsen et al., 2004). Thus, under the condition of a high ruminal pH, diets high in sugar seem to have a greater methane producing potential than diets rich in lignified fiber (Hindrichsen et al., 2004). However, molassed BP in the Rusitec study (chapter 5), which resulted in the same dietary sugar content as the molasses diet according to Hindrichsen et al. (2004), did not have a higher methane production than BG. Feeding on different grass and clover silages, Holstein heifers fed clover silages had lower methane yields as related to DMI and digestible DMI and a higher DMI than heifers fed grass silages, maybe due to higher NFC content of clover silages (Parnian-Khajehdizaj et al., 2023).

Decreased methane production with higher DL in the Rusitec study (chapter 5) was associated with an increase in MCP production, potentially due to a higher washing of the feed particles by the fluid phase

and therefore keeping the rumen microorganisms more in the state of growth. This higher MCP yield was assumed to contribute to the reduction of methane as 0.41 moles [2H] (metabolic hydrogen) is being used per gram microbial biomass produced (Mills et al., 2001; chapter 5). In the meta-analysis study (chapter 6) investigating the fermentation control of cattle and sheep, cattle had a higher SF ($MRT_{particle}/MRT_{fluid}$) and simultaneously a lower aD CP than sheep (indicative of more MCP produced gastrointestinally), while overall digestibility was not affected by species. Thus, fermentation of cattle seems to be shifted slightly towards MCP production and therefore could be producing lower amounts of methane during fermentation than sheep. In *in vivo* studies with cattle and sheep on forage only diets, an induced increase in MRT_{fluid} led to an much smaller increase in MCP yield and reduction in methane production in cattle than *in vitro* and had no effect in sheep on MCP or methane production (Zhang et al., 2023b, 2023a). In a meta-analysis investigating methane production of sheep and cattle from fresh pasture via sulphur hexafluoride, methane yield [g/kg DMI] was in fact lower for cattle than for sheep and only 13% and <2% of the variation could be explained by chemical composition of the ryegrass, respectively (Hammond et al., 2009). Investigating individual differences in methane emission of sheep, Pinares-Patiño et al. (2011) found a shorter MRT_{fluid} in the rumen of low compared to high methane emitting sheep. According to Goopy et al. (2014), sheep with lower methane yield had both a shorter MRT_{fluid} and $MRT_{particle}$. Differences in MRT_{fluid} and $MRT_{particle}$ accounted for 59 and 70% of the variation in methane yield, respectively, while MCP production (estimated via urinary allantoin excretion) did not differ between low and high methane yielding sheep (Goopy et al., 2014) and therefore cannot be an explanation for the differences in methane yield. Additionally, low methane yielding sheep had smaller rumens and tended to have a more segregated rumen fluid and gas phase than high methane yielding sheep (Goopy et al., 2014). Also, DM digestibility tended be to be lower for low methane emitting sheep ($p = 0.05$) (Pinares-Patiño et al., 2011), while Goopy et al. (2014) found no difference. Further, the difference between sheep in methane yield was higher on a diet including wheat grain than a pasture only diet, whereas methane yield [g/kg DMI] was higher for pasture than wheat grain containing diet (Pinares-Patiño et al., 2011).

Differences in methane yield of dairy cows, which were selected according to low, medium or high methane yield (15.3, 19.2 , 24.8 g/kg DMI, respectively) during lactation disappeared when cows were non-lactating during the trial 11 month later (Pinares-Patiño et al., 2007). Methane yield [g/kg DMI] in the trial for all cows was as high as high methane yielding cows during lactation, while as expected, DMI was lower for dry cows than during lactation (Pinares-Patiño et al., 2007). Also, Münger and Kreuzer (2006) detected a higher methane yield [g/kg DMI] in dry period of cows compared to lactation, while there was no difference between breeds of Holstein Frisian, Jersey and Simmental. In contrast, Dutch and US Friesian dairy cows had lower methane yields [g/kg DMI] in early and mid-lactation than dairy cows from New Zealand (Friesian) but there was no difference in late-lactation

(Robertson and Waghorn, 2002). Also, methane production [g/kg DMI] increased from early to late-lactation for both dairy breeds and no matter if fed on pasture or TMR containing concentrates. Methane production was only higher for pasture than TMR in late-lactation (Robertson and Waghorn, 2002). Thus, it seems that the production state of dairy cows' influences methane production [g/kg DMI], maybe due to a difference in DMI and water intake to meet required amounts for respective production state and therefore changings in MRTs of particle and fluid. Methane production of heifers per day was strongly related to DMI (R^2 = 0.59) and digested OM (R^2 = 0.78) (Parnian-Khajehdizaj et al., 2023). Low methane-emitting dairy cows (247 days in milk; 15.5 g CH_4/kg DMI) had a lower C_2:C_3 ratio due to higher propionate and lower acetate proportion and larger quantity of bacteria which are positively correlated with propionate concentration in the rumen than high methane-emitting dairy cows (250 days in milk; 20.4 g CH_4/kg DMI) (Stepanchenko et al., 2023). While milk production, DMI and apparent total-tract digestibility of starch were not affected by methane yield, apparent total-tract digestibility of DM, OM, NDF and ADF were lower for low methane-emitting cows (Stepanchenko et al., 2023). So, it seems that reduced digestibility of the fiber fractions contributed to lower methane yield of low methane-emitting cows.

7.5 Species effect

Within ruminants, there are species which strictly browse or graze and intermediate feeders. The feeding types are found to differ in their SF and therefore could be categorized into the 'moose-type' (browsers with low SF and low SF range) or 'cattle-type' (grazers and intermediate feeders with higher SF and higher SF range) (Clauss and Lechner-Doll, 2001; Clauss et al., 2010). While the 'moose-type' seems strictly limited to browsing ruminants, the 'cattle-type' is not restricted to grazers and intermediate feeders (Przybyło et al., 2019). A higher SF is associated with greater digesta washing as MRT_{fluid} is lower per unit $MRT_{particle}$ or $MRT_{particle}$ is higher per unit MRT_{fluid}, yielding in a greater MCP production and therefore resulting in a greater supply of MCP to the ruminant (Clauss et al., 2006; Müller et al., 2011). Comparing two Asian antelopes, Hummel et al. (2015) found higher SF due to shorter MRT_{fluid} for the strictly grazing blackbuck compared to intermediate feeder nilgai. Both antelopes belong to the 'cattle-type' and the blackbuck showed a similarly high SF value as found for cattle before (Hummel et al., 2015). While the blackbuck data support the theory that 'cattle-type' ruminants with particularly high SF achieve this due to a lower MRT_{fluid} per unit $MRT_{particle}$ and as a result benefit from higher MCP yields, in the meta-analysis of this study (chapter 6), the dominating effect resulting in the higher SF of cattle compared to sheep was the greater $MRT_{particle}$ in cattle. Thus, in the next paragraph the individual studies are considered in greater detail.

As mentioned before in chapter 6, five of twelve studies of the meta-analysis lead to a higher MRT_{fluid} for cattle than for sheep, five further studies found no or only small differences in MRT_{fluid} between

species, while one study conducted in Australia found no difference in MRT_{fluid} between cattle and sheep (Amaning-Kwarteng et al., 1986) and two other studies conducted in Australia reported even higher MRT_{fluid} for cattle than for sheep (Hendricksen et al., 1981; Poppi et al., 1981). Animals used in these two studies were steers (no breed mentioned) and Merino weathers, while Amaning-Kwarteng et al. (1986) used Herford heifers and Merino and Border Leicester crossbred weathers. A possible influence of the breed is difficult to assess as not all studies provide detailed data on breeds and various combinations of breeds of cattle and sheep were used between the investigated studies. Examining differences of Churra and Merino breed sheep, Ranilla et al. (1998) found no differences in MRT of fluid or particle between breeds fed good-quality forage at a low intake level. Comparing Jersey and Holstein dairy cows with equal milk energy yield per kg $BM^{0.75}$, Jersey cows had a shorter $MRT_{particle}$ and higher NDFD than Holstein cows (Aikman et al., 2008). The authors suggested that Jersey comminuted their feed better as they spend more time ruminating per kg DMI and therefore enhance saliva production and fermentation of the feed particles by the rumen microbes and in conclusion enhance $MRT_{particle}$. However, MRT_{fluid} was not measured. Besides possible differences between breeds, Pinares-Patiño et al. (2011) and Goopy et al. (2014) found differences in MRT_{fluid} within a breeds of sheep and animals with shorter MRT_{fluid} were found to have smaller rumens. Among all investigated values of the meta-analysis (chapter 6), MRT_{fluid} values of sheep conducted by Hendricksen et al. (1981) were located at the lower edge of the dataset, while MRT_{fluid} values of cattle ranged from the center to the upper end of the dataset, whereas MRT_{fluid} values of sheep and cattle from Poppi et al. (1981) do not stand out. Low MRT_{fluid} values probably are linked to high DMI, but do not explain shorter MRT_{fluid} in sheep than in cattle, as DMI [g/kg $BW^{0.75}$] of cattle was equally high as for sheep (Hendricksen et al., 1981) and in other studies with high DMI (Prigge et al., 1984; Colucci et al., 1990) MRT_{fluid} values for both species were low and MRT_{fluid} were affected by intake level (Prigge et al., 1984).

While a higher ruminal fluid passage rate due to increased saliva production could enhance MCP production in the rumen, it may also increase the risk of soluble toxins escaping ruminal detoxification as the production of tannin-binding proteins is restricted and therefore their concentration in the saliva would be diluted (Hofmann et al., 2008; Codron and Clauss, 2010; Hummel et al., 2015). Some toxins may also be degraded in the rumen (Freeland and Janzen, 1974); if MRT_{fluid} is shorter, there is less time to degrade and more unchanged toxin is leaving the rumen immediately. In the grazing species sheep, the amount of saliva required to inhibit 50% of the tannin-protein binding was more than twice as high than for browsing mule deer (Robbins et al., 1991). Also, the domestic Suffolk sheep showed to be more vulnerable to the inclusion of quebracho tannin in the diet than mule deer. Despite the higher DM and CP digestibility of sheep compared to mule deer on the basal diet of alfalfa-grain pellets, the reduction in both digestibilities with inclusion of quebracho tannin in the diet was greater for the grazer sheep than for the browser mule deer (Robbins et al., 1991). However, the natural diet

of browsing ruminants contains more secondary plant compounds as tannins than the diet of grazing ruminants (Fickel et al., 1998). Also, Hummel et al. (2006) found a reduced relative gas production rate and reduced maximal gas production in browse leaves *in vitro* using the HGT with standardized sheep inoculum due to tannins. Besides tannins, other substances can be toxic like ochratoxin which is a mycotoxin produced by storage fungi and can be found in various grains and other plant products (Mobashar et al., 2010). An increased fluid passage rate could enhance the amount of bypass ochratoxin which is not degraded in the rumen and therefore lead to a higher risk for toxic effects on the ruminant. If breeding for dairy cows with increased fluid passage rate to enhance MCP supply, maybe diets and diet recommendations have to be adjusted and closer monitored for toxins, as the protective effect of the rumen may be reduced as a result of reduced MRT_{fluid}.

7.6 Comparison of methods and transferability to the *in vivo* situation

7.6.1 Hohenheim gas test vs. Rumen simulation technique

Both systems, Rusitec and HGT have their advantages and disadvantages for investigation of feedstuff and MCP. The HGT is designed for standardized evaluation of large numbers of feed samples in a relatively short period of time and with relatively little inoculum and sample quantities needed. Its main purpose is to estimate the energy value of feedstuff for the ruminant. Additionally, the modified version of the HGT allows determination of crude protein content available for the ruminant at the duodenum. Measurement of the fermentation kinetics of single feedstuffs via gas production is much easier than in the Rusitec or even *in vivo*. The Rusitec as semi-continuous culture system is closer to the animal but is also much more time-consuming and more resources e.g., feedstuff, buffer solution and inoculum, are needed. One Rusitec fermenter can be sampled over a longer period of time and various sample collection at the same time for different analyses is no problem, while analyzing e.g., the MCP, at a certain time point in a HGT syringe means the 'destruction' of the fermentation in this syringe.

During the experiments, two things attracted attention. First, during one of the modified HGT runs the pure cellulose, which was used as one of the substrates, used up nearly all ammonium provided with the inoculum (buffer + rumen fluid). This was unexpected since the buffer solution of the modified HGT is especially rich in ammonium hydrogen carbonate (contains 50% more than the original HGT). The HGT is designed to supply sufficient nitrogen even if energy concentrates like grains or BP are incubated. While such feeds still contain considerable amounts of nitrogen, pure carbohydrates obviously do not (measured N content was 0.03, 0.06, 0.04 and 0.20% N for sucrose, starch, cellulose and pectin respectively). For further experiments with pure carbohydrates in the HGT, additional ammonium hydrogen carbonate or other additional N sources in the buffer are recommended to

assure N supply not to become limiting for fermentation. It is interesting to note that some cellulose degrading bacteria are known to rely on simple N sources only (and cannot use amino acids), which may explain partly why ammonia was especially low in cellulose incubations (Van Soest, 1994).

Second, despite the larger amount of substrate (4 g DM for the CS; 11 g DM total) used in the Rusitec fermenters compared to the HGT syringe (200 mg DM substrate), the residual material of the CS after 48 h of incubation was limited. Therefore, residues of each fermenter were pooled for the four sampling days to ensure sufficient sample for chemical analysis and could not be analyzed separately. One reason is the high OMD_{cs} especially for BG and BP and the deliberated separation of CS and hay RSM mixture. In further investigations larger amounts of CS should be considered. Deitmers et al. (2022) recommend 13-16 g DM of diet per fermenter and day and they detected a range of 3 to 21 g DM (mean 13.1; median 14.4) per incubation bag in their meta-analysis. We used 11 g DM per day and fermenter in total. This amount would have produced sufficient residual material if we had made no separation for CS, but it was the aim to investigate CS separately. Substrate quantities were inspired by Romero-Pérez et al. (2015).

7.6.2 *In vitro* vs. *in vivo*

In vitro investigations of single substances and feeds are an important basis for the understanding of their fermentation characteristics and their MCP production potential. However, some adaptations and considerations are necessary when extrapolating *in vitro* results to live animals (*in vivo*); obviously, animals and their metabolic processes are much more complex than *in vitro* apparatuses. For example, Zhang et al. (2023b) found no effect on ruminal microbial yield estimated via purine derivates in cows when increasing MRT_{fluid} through increased saliva flow (stimulated with pilocarpine). The metabolic fecal N as a second indicator of MCP production in the rumen increased to a smaller magnitude than *in vitro* MCP production of our Rusitec trial (chapter 4; Zhang et al., 2023b).

Normally, pure carbohydrates as we investigated in the HGT are not fed to live animals. Diets for live animals contain complete feeds and are mostly combinations of different feedstuff. The possibility to investigate pure substances in the HGT is an advantage of this *in vitro* system but complete feeds containing certain amounts of the investigated carbohydrates may react in a different way (at least to some degree) as pure carbohydrates are not embedded in the cell matrix of the feeds. In the Rusitec experiments a simple substrate composition was chosen deliberately. The important criteria of diets for ruminants were met like a sufficient amount of coarse forage, a minimum crude protein content of 150 g/kg DM and an additional energy source which was the experimental feed. The benefit of reducing the used substrates (only three different substrates per Rusitec fermenter) to these criteria is to see possible effects and relationships more clearly. However, some discrepancy between *in vitro* and *in vivo* is always present and represents a dilemma between being close to the complex real

situation and simplifying conditions for investigation purposes. It can be regarded an advantage and disadvantage at the same time, therefore.

In the Rusitec trials the outcome of grinding of the grass hay through a 10 mm screen resulted in a particle spectrum apparently comparable to rumen contents. The grinding resulted in a large range of small and large particles with no straight cutting edge which is different when cutting the hay manually. After grinding, fine particles were separated and discarded by sieving the hay manually through a 1.18-mm screen aperture to avoid losses of undegraded substrate through the nylon bags. Visually there was no difference between the grinded hay we used as feed for the Rusitec fermenters and the sampled solid rumen content of the donor heifers mainly fed hay. Maybe this animal-like comminution of the hay was one factor affecting the OMD in our Rusitec fermenters positively. In general, OMD of the hay RSM mixture (63.4-71.5%; 496 g NDF/kg DM) and the CS (68.1-90.0%) were high in our Rusitec trials (see chapter 5). Measured OMD in Rusitec fermenters can be considerable lower as reported for example by Romero-Pérez et al. (2015) (56.6-57.7% for a mixture of barley silage, barley grain and supplements) and Zhao et al. (2013) (53,8% [333 g NDF/kg DM] for a mixture of alfalfa hay, ground corn, wheat bran, pelleted BP, soybean meal and mineral and vitamin supplements). Durand et al. (1988) found 74.3% OMD of BP in the Rusitec, while we found 81.6 and 90.0% OMD of BP for high and low dilution rate, respectively.

In order to compare the OMD of the hay RSM mixture with table data of the Dutch fermentable organic matter in the rumen (**FOMr**; based on *in situ* experiments) (CVB, 2018), a simple linear regression between NDF content and FOMr of the different grass hay quantities given in the CVB feedstuff table were conducted. To obtain a FOMr value of the hay used in the Rusitec its NDF content was insert in the regression equation. The related FOMr value for the hay RSM mixture was calculated afterwards. This calculation resulted in 47,1% FOMr, which is considerably lower than OMD measured in the Rusitec trials of this study.

In a meta-analysis conducted by Hristov et al. (2012), average OMD were 52.2 and 69.6% with an average NDF content of 44 and 32% for Rusitec and *in vivo* trials, respectively. However, the large difference in NDF content makes a comparison between Rusitec and *in vivo* trials very complicated. Additionally, comparability of *in vitro* OMD of Rusitec fermenters with *in vivo* OMD is difficult because of the different measurement site (rumen vs. total gastrointestinal tract). Disregarding these concerns, O'Mara et al. (1999) determined 69.8% OMD for a hay soybean meal mixture (622 g NDF/kg DM), 84.7% OMD for BG and 86.1% OMD for BP in cattle, which are in the same range as our Rusitec results.

7.7 Diet formulation for maximal microbial yield

For a maximal MCP production in the rumen, it is necessary to meet all nutritional and environmental requirements of the rumen microbes. For example an insufficient supply of energy or nitrogen leads to an increase in maintenance costs or an reduction in growth rate and therefore in an inefficient use of nutrients and energy by the microbes (Stern and Hoover, 1979; Russell and Strobel, 2005). Since the rumen microbes can only utilize the part of the feedstuff which is fermentable, it is not surprising that Boguhn et al. (2006) found a strong relationship betweeen the amount of fermentable OM in the diet and the yield of MCP produced (r = 0.75). Also, the fermentability of the OM was increased with an increased CP content (r = 0.79) and decreased with an increased NDF content (r = -0.76) of the diet in the same study. However, digestibility of NDF has to be considered, as forage high in digestible NDF could meet the fiber requirements of the ruminant and provide energy for the rumen microbes at the same time. In early-lactating cows, milk yield was higher when NDFD of the diet was enhanced (Dado and Allen, 1996; Kendall et al., 2009). The production of MCP per kg fermentable OM was higher for fresh forage than for grass silage or hay and comparable to MCP production of corn silage (Verbic, 2002), while in situ NDFD of corn silage decreased with in ensiling from 0 to 150 d due to a reduction of hemicelluose and NDF-bound protein (Hristov et al., 2020). However, if cellulose of feeds is well fermentable, cellulose has shown the potential to produce more MCP per kg dOM than starch, pectin and sucrose (chapter 4 and 5).

Additionally to providing the required amount of energy and nutrients to the microbes, energy and protein have to be available at the same time (Russell and Strobel, 2005) as an asynchronous rumen-availability of protein and energy reduce MCP production efficiency (Aldrich et al., 1993). Hence, diet formulation have to take the fermantation rates and the rumen-availability of the feedstuff into account and to optimize the diet for the rumen microbes and the ruminant itsself. Factors to achieve this goal are formulating diets with feeds differing in fermentation kinetics and source of energy and protein, using a TMR feeding regime as possible (Boguhn et al., 2006) and giving the ruminant the opportunity to consume the diet in frequent meals (Stern and Hoover, 1979).

Also, diet formulation affects the rumen enviroment for the microbes as for example starch rich diets can reduce ruminal pH due to fast fermentation and lactate production (Van Soest et al., 1991) and reduce diversity of bacteria and fungi in the rumen of lactating dairy cows compared to NDF rich diets (Belanche et al., 2012). A lower ruminal pH decreases MCP production and efficiency (Strobel and Russell, 1986). Thus, for optimizing diets for maximal MCP yield, to maintain an optimal ruminal pH for the rumen microbes is necessary. High degradability and high fermentation rates of the feedstuffs for great amounts of rumen-available energy and protein for the microbes on the one hand and sufficient supply of physically effective NDF of the forage for lasting maintenance of the rumen functions on the

other hand are needed to keep in balance. Firstly, well preserved and highly fermentable forages are desirable to meet the structural requirements of the ruminant (as diets low in actual forage among other things reduce MCP production (Feng et al., 1993)), to provide greater amounts of energy from the forage and to allow higher DMI which additionally benefits the nutrient supply in the rumen. Secondly, feeds that are fast fermented but not to lactate and therefore do lower the rumen pH less severe could be beneficial to maintain higher ruminal pH with high fermentation rates (Van Soest et al., 1991). As pectin meets these criteria (Van Soest et al., 1991; Hatfield and Weimer, 1995), feeds rich in pectin like BP could be used to replace starch rich feeds partly in the diet and could be beneficial for MCP production. Stern et al. (1994) found a higher bacterial yield for BP than for corn in continuous culture fermenters. However, in the Rusitec study (chapter 5), no difference was detected in MCP production for BP and BG, while the cellulose rich SBH had a higher MCP production. Also, by partly replacing corn silage, corn cob silage and BG in the diet with BP silage, MCP production efficiency was reduced in Rusitec fermenters, but did not affect milk production in dairy cows (Boguhn et al., 2010). The advantage of BP not affecting the ruminal pH negatively is not shown in very well buffered *in vitro* systems. In early-lactating dairy cows, ruminal pH and DMI were higher when 25% of the BG in the diet was replaced by BP, resulting in a starch content of 24.6% DM instead of 31.1% DM, respectively (Shahmoradi et al., 2016). Replacing high-moisture corn with pelleted BP up to 24% of diet DM (resulting in an increase of NDF from 24.3 to 31.6% diet DM and a decrease of starch from 34.6 to 18.4% diet DM), enhanced NDF fermentation rate, total tract NDF and OM digestibility without reducing total tract starch digestibility of lactating dairy cows (Voelker and Allen, 2003a), while the replacement did not affect daily mean or minimum ruminal pH and MCP production [per % OM truly digested] but tended to reduce pH range (Voelker and Allen, 2003b). Also, MCP production efficiency was positively correlated to passage rate of starch ($p < 0.05$; r = 0.63) and potential digestible NDF ($p < 0.10$; r = 0.36), but not with fluid passage rate or DMI (Voelker and Allen, 2003b). The authors suggested that the enhanced MCP production efficiency was caused by a reduced MCP turnover in the rumen due to a greater washing out of the rumen of solid-associated microorganisms with the feed particles, while fluid passage rate had no effect because individual values were very high (10.6 to 22.9%/h) and could not enhance MCP production efficiency further (Voelker and Allen, 2003b).

In conclusion, actual MCP production of diets remains difficult to be predicted as several factors of the feedstuff (e. g. digestibility), the diet composition (e. g. availability of feedstuffs, combination of nutrients and digestibility rates), the individual animal (e. g. DMI, rumen microbiome) and the farm environment (e. g. feeding regime) can influence fermentation kinetics and passage rate in the rumen and therefore affect MCP production. Diet formulation can only try to optimize MCP production in the rumen. To reduce uncertainties, detailed knowledge of nutrients and digestibility of the used feedstuff especially the forage is necessary. Also, further research is needed for a more precise understanding

of the complex relationships between diet, feedstuff and carbohydrate composition, MRTs, animal individual factors and MCP production in the rumen.

8 References (of general introduction and general discussion)

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