Assessing the relevance of fluid dilution rate for ruminal metabolism in cattle and sheep

Dissertation

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Alle aber, auch das banalste, trafen mit leisem stetigem Hammerschlag auf denselben Punkt in mir, alle halfen an mir bilden, alle halfen Häute von mir abstreifen, Eierschalen zerbrechen, und aus jedem hob ich den Kopf etwas höher, etwas freier, bis mein gelber Vogel seinen schönen Raubvogelkopf aus der zerstrümmerten Weltschale stieß.

— Hermann Hesse, Demian
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List of papers and conference contributions

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ADF</td>
<td>Acid detergent fibre</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BW</td>
<td>Body weight</td>
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<td>CF</td>
<td>Crude fibre</td>
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<td>CL</td>
<td>Crude lipid</td>
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<td>CP</td>
<td>Crude protein</td>
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<td>CoA</td>
<td>Coenzyme A</td>
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<td>DM</td>
<td>Dry matter</td>
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<td>DMI</td>
<td>Dry matter intake</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
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<td>EE</td>
<td>Ether extract</td>
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<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>FADH$^2$</td>
<td>Flavin adenine dinucleotide</td>
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<tr>
<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GHG</td>
<td>Greenhouse gas</td>
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<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
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<td>GWP</td>
<td>Global warming potential</td>
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<td>HGT</td>
<td>Hohenheim Gas Test</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IPCC</td>
<td>Intergovernmental Panel on Climate Change</td>
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<tr>
<td>LCFA</td>
<td>Long chain fatty acid</td>
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<tr>
<td>LMM</td>
<td>Linear mixed model</td>
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<tr>
<td>MBW</td>
<td>Metabolic body weight</td>
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<td>ME</td>
<td>Metabolizable energy</td>
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<td>MFN</td>
<td>Metabolic faecal nitrogen</td>
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<td>MRT</td>
<td>Mean retention time</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (NADP) + hydrogen (H)</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fibre</td>
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<tr>
<td>OM</td>
<td>Organic matter</td>
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<tr>
<td>PD</td>
<td>Purine derivate</td>
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<tr>
<td>PF</td>
<td>Partitioning factor</td>
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<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoate</td>
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<tr>
<td>RC</td>
<td>Respiration chamber</td>
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<tr>
<td>RR</td>
<td>Reticulorumen</td>
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<tr>
<td>Rusitec/RUSITEC</td>
<td>Rumen Simulation Technique</td>
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<tr>
<td>SARA</td>
<td>Sub-acute Ruminal Acidosis</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SCFA</td>
<td>Short chain fatty acid</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VDLUFA</td>
<td>Association of German Agricultural Analysis and Research Centers</td>
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<tr>
<td>2H</td>
<td>Metabolic hydrogen</td>
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<td>3-NOP</td>
<td>3-Nitrooxypropanol</td>
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Abstract

Ruminants evolved a forestomach in which plant materials are fermented with the help of ruminal symbiotic microbes. Ruminal microbes use the ingested materials to proliferate, and produce volatile fatty acids which are absorbed by host animals as energy and nutrient substrates, and carbon dioxide and methane as fermentation end products. The ingested solid materials are mixed with rumen fluid, and generally, the solid phase retain longer than the fluid phase. Both in vitro and animal studies indicated that a higher fluid dilution rate is related to a more efficient microbial growth and a lower methane emission. Additionally, in vitro studies as well as theoretical considerations suggested a reverse relationship between microbial yield and methane production, since both microbial synthesis and methanogenesis consume metabolic hydrogen.

To test whether an increased fluid dilution rate is associated with increased microbial yield and decreased methane production in live animals, this study applied a saliva stimulant, pilocarpine, to modify liquid flow rate in cattle and sheep. Four non-lactating cattle and three non-pregnant sheep were fed forage only (restricted to constant intake) in Latin square designs with oral pilocarpine doses. Measurements included feed and water intake, mean retention time (MRT) of fluid and particles in the reticulorumen and the total tract, ruminal microbial yield (via urinary purine bases or metabolic faecal nitrogen), methane emission, digestibility, rumen fluid parameters for both cattle and sheep, and chewing behaviour as well as reticular motility for cattle. Data were investigated using orthogonal polynomial contrasts.

The fluid MRT was decreased via treatments for both cattle and sheep. The methane production via treatments for cattle was decreased, but not for sheep. The microbial yield based on urinary purine bases was not affected, but increased based on metabolic faecal nitrogen only for cattle. The ratio between particulate and fluid MRT was smaller for sheep than that for cattle, and was not affected by treatment. The apparent digestibility was not affected by treatment. In addition, this study suggested a correlation between chewing measures and MRT measures as indicated by the cattle experiment.

The relationship between chewing behaviour and fluid MRT could be linked through saliva production via the masticatory-salivary reflex, which indicates that
chewing measures could emerge as easy-to-measure proxies for MRT characteristics. Our results showed methane production decreased, and microbial N yield increased indicated by metabolic faecal nitrogen by applying pilocarpine but only for cattle. The discrepancy between cattle and sheep might be related to the difference in ratios of the MRT of particles to fluid, which has been shown to be largely resistant to changes in diet type or feed intake. Whether setting the breeding target of shorter fluid MRT could contribute to less methane production and more microbial yield remains to be further investigated.
Chapter 1

General introduction
1. General introduction

1.1. Necessity of reducing methane emissions from domestic ruminants

Methane has gained attention of animal nutritionists since long because of the energy loss (2-18% of the total ingested energy, (von Engelhardt et al., 2015); 6% of the ingested energy, Johnson et al., 1995). Nowadays, more attention is paid to its potential contributing to the greenhouse effect. As one of the most important greenhouse gases (GHG) listed by the Intergovernmental Panel on Climate Change (IPCC), methane has a high radiative forcing while relative short lifetime (8.6-12.5 years; reviewed by Moss et al., 2000; Broucek, 2014; Beauchemin et al., 2020; Arndt et al., 2022). The 2021 IPCC report lists the global warming potential (GWP) of methane as 83 over a time scale of 20 years, 30 over 100 years and 10 over 500 years. Thus, mitigating methane emission might help to achieve significant progress in stopping or slowing down global warming, especially in a relative short period.

Recently, the importance of the GHG emissions from the food system has been highlighted; to achieve the Paris Agreement’s goal of limiting the increase in global temperature, adopting mitigation strategies of GHG-emission in food system is essential (Clark et al., 2020). Crippa et al. (2021) estimated that between 1990 and 2015, a third of the total anthropogenic GHG emissions originated from food system globally, of which the enteric fermented methane (mostly from the domestic ruminants) accounted for 17%; thus, the domestic ruminants contributed 5-6% of the total anthropogenic GHG emissions. In addition, these emissions are expected to continually increase in the coming years, especially in developing countries (IPCC, 2022).

Methane production from domestic ruminant individuals could vary dramatically, primarily determined by feed intake and feed characteristics. Eggleston et al. (2006) estimated the methane production factor, which for dairy cattle ranged from 46 to 128 kg CH$_4$ per head and year depending on the region of the world, while for sheep it was 5-8 kg and for goats 5 kg CH$_4$ per head and year. The methane production per unit of feed was also variable among many factors, which might imply the potential of mitigating methane emissions from domestic ruminants.

In summary, methane emissions from domestic ruminants constitute considerable GHG emissions and are suggested to grow in the future. To achieve the Paris Agreement’s goal, mitigation strategies of methane emission from domestic...
1. General introduction

Ruminants is essential.

1.2. Methanogenesis in the rumen

Methane is produced by ruminants mostly in the forestomach (rumen) as fermentation end product, and thus emitted through eructation. The rumen is basically a large anaerobic fermenter, inhabited by microbes including bacteria, archaea, protozoa, and fungi. With help of these ruminal microbes, the plant cell wall components, primarily structural polysaccharides, are degraded to monosaccharides, and then primarily further to short chain fatty acids (acetate, propionate, and butyrate) and gases (CH₄ and CO₂).

The breakdown of macromolecules, namely catabolism, releases energy for the ruminal microbes; additionally, the reducing power is produced, in the form of reduced co-factors, such as NADH and FADH₂. Hence, to maintain the redox balance – since the reduced co-factors cannot diffuse across the cell membrane, the reduced co-factors must be re-oxidized within the microbial organisms. In the rumen, different organisms build the electron flow chain and coordinate to degrade substrates and conserve energy while neither can degrade alone; this syntrophy plays a major role in maintaining normal rumen function. The typical example is the interspecies hydrogen transfer during methanogenesis. Figure 1.1 shows the interspecies hydrogen transfer between Ruminococcus albus and methanogens (Wolin et al., 1997). When in monoculture, R. albus degrades glucose, produces ethanol, acetate, H₂, CO₂, and sometimes formate. In the presence of methanogens, the H₂ can be consumed through interspecies hydrogen transfer – therefore, the NADH made during glycolysis will be consumed forming H₂; as a result, acetyl-CoA is no longer reduced to ethanol. The molar ATP yields for the monoculture and with methanogens are 3 and 4, respectively; additionally, methanogens will gain extra energy using H₂ to produce CH₄. Therefore, the energetics is favourable with interspecies hydrogen transfer.
1. General introduction

Ultimately, the rumen is a place to ferment feedstuff, where catabolism exceeds anabolism; therefore, to maintain the normal rumen function, the consumption of reducing power during catabolism is an overriding requirement, while methanogenesis is a major metabolic pathway in consuming reducing power. Some methane mitigation strategies have tried to directly prohibit the methanogens, including vaccination against methanogens (Wright et al., 2004; Cook et al., 2008), halogenated methane analogues (which were indicated to be the effective methane-reducing compounds in red seaweeds, reviewed by Min et al., 2021); however, rare studies could successfully modify the ruminal fermentation in a production way, while realizing permanently reduced methane emissions to a considerable extent. The 3-NOP is the inhibitor for methyl coenzyme-M reductase (the enzyme that catalyses the last step of methanogenesis), and recently was approved by EFSA panel on FEEDAP (European Food Safety Authority on Additives and Products or Substances used in Animal Feed) as feed additive that potentially reduces enteric methane production under the proposed conditions of use (EFSA FEEDAP, 2021). However, Jayanegara et al. (2018) reported by applying 3-NOP, higher dihydrogen emissions were detected, which should also be borne in mind as energy loss.

The ruminal microbial community demonstrates both redundancy (overlap of function among various microbial species) and resilience (ability to recover from perturbation), which provide remarkable stability, but also bring challenge to modify the rumen function, particularly to mitigate methane emissions (Weimer, 2015). Methanogenesis is a fundamental process situated at the end of rumen electron flow; when modifying rumen fermentation in the direction of less methane, either we

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**Figure 1.1.** Interspecies hydrogen transfer between *Ruminococcus albus* and methanogens (Wolin et al., 1997).
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successfully redirect the electron flow (or in a practical way concerning methanogenesis, hydrogen flow) while maintaining the normal rumen function, or we to some extent deviate from the normal rumen function such as lower fibre digestibility or accumulation of dihydrogen. Therefore, the key to mitigate methane is to redirect the metabolic hydrogen (Czerkawski, 1986; McAllister et al., 2008; Janssen, 2010; Martinez-Fernandez et al., 2016; Greening et al., 2019; Ungerfeld, 2020). In the following, the potential to mitigate methane by increasing microbial yield will be discussed in detail.

1.3. Microbial synthesis as 2H consumer

Czerkawski (1986) suggested that 48% of metabolic hydrogen is used for methanogenesis, 33% is used for production of short chain fatty acids (SCFA), and 12% is used for microbial synthesis. Several processes of biosynthesis consume reducing power, like the biosynthesis of fatty acid consumes NADPH, and some bacteria consume NADH to assimilate ammonia into amino acids (both NADPH and NADH serve as electron carriers) (Czerkawski, 1986; Madigan, 2012).

Fatty acids are major structural components of membranes. Thus, the biosynthesis of fatty acids is a major series of reactions in cells and accomplished by incorporation every two-carbon unit in the chain and consumption of two NADPH. The fatty acid composition of cells varies from species to species; it was reviewed by Hegarty (1999) that the microbial long chain fatty acid (LCFA) production could be regulated through changing the proportion of gram positive to negative bacteria, the solid-related bacteria to fluid-related bacteria, defaunation treatment, and methionine supplementation.

Protozoa synthesizes glycogen in response to excess carbohydrates (Williams et al., 1992). For bacteria, polyhydroxyalkanoates (PHAs) serve as energy source and carbon store; the content of PHA can account for up to 90% of the cell dry matter (Hai et al., 2004). Macrae et al. (1958) and Senior et al. (1972) suggested that PHA synthesis could be regulated by hydrogen and oxygen potentials and the synthesis of PHA is an alternative electron sink.

Demeyer et al. (1972) tried to quantify the amount of consumed 2H for microbial synthesis; based on the measured microbial element composition and rumen
fermentation balance, they have calculated that synthesis of 1 g microbial cellular contents required 0.0061 mol 2H. Though it is rather a rough estimation based on assumption and fermentation balance, it helps to better understand the capacity to mitigate methane through increasing microbial synthesis. Assuming 200 mg substrate was fermented with true substrate degradability of 80%, it contributed to net microbial yield as 40 mg and methane production as 6 mL. When we manage to increase the microbial yield to the degree of 20%, according to the estimation of Demeyer et al. (1972), the increased 8 mg microbial mass will consume additional 0.048 mmol 2H, which would be used to produce 0.012 mmol CH₄ (in standard temperature and pressure equaling to 0.2688 mL). Thus, by increasing 20% of microbial yield, the methane production would decrease about 4.5%.

The magnitude of decreasing methane production by increasing microbial yield was reported larger by Ramin et al. (2013), who suggested the improvement in microbial cell yields triggered by increased feed intake would account for about 20% of the decline in methane-energy/gross energy. Nevertheless, optimizing ruminal microbial yield is suggested to be helpful to redirect the hydrogen flow to anabolism; additionally, increasing the microbial protein is also desirable.

Studies concerning the methane production and microbial yield have been based on different systems; Leng (1993) investigated the fermentation in different situations and imposed an inverse relationship between microbial mass and total produced SCFA as well as CH₄ production. In the following section, the relationship between microbial yield and methane production will be investigated from the closed batch culture system to real rumen, and when inducing a change of fluid dilution rate, how microbial yield and methane production will be affected.

1.4. Dilution rate, microbial yield and methane production— from batch culture to real rumen

Since the fermentation end products are diluted in continuous culture system or absorbed by animals in the rumen, it would be more meaningful to study the relationship between fermentation and microbial yield in the closed system, i.e. in batch culture system. Blümmel et al. (1997), based on roughage samples using a modified
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Hohenheim Gas Test (HGT), suggested a negative correlation between produced gas and microbial yield. They also defined the partitioning factor (PF) as an index of the variation of fermented end products per unit true degraded substrate, whereas a higher PF indicates that more degraded substrate is directed to microbial yield. We conducted a HGT and incubated twenty-three silage samples, measured produced microbial mass and methane, and found that the methane production and net microbial yield were negatively correlated (P<0.001) (Figure 1.2, not published).

![Figure 1.2](image.png)

Figure 1.2. Relationship between net microbial yield and methane production per unit of incubated substrate. Twenty-three silage samples were incubated based on Hohenheim Gas Test.

The batch culture system is a closed system, in which the metabolic activities of the growing organisms continuously alter the microbial growth; especially when cell numbers become quite large, the chemical and physical composition of the culture medium changes dramatically (Madigan, 2012). Therefore, the microbiologists induced the dilution rate by using a chemostat (a continuous culture system, with adjustable inflow of medium and outflow of effluent). When the system comes to a steady state, the microbial growth rate increases as the dilution rate increases (Figure 1.3; Herbert et al., 1956, Madigan, 2012). Early work from Hobson (1965), who used a chemostat and cultured the rumen bacteria *Streptococcus bovis*, suggested that with the help of continuous culture, we can better understand the behaviour of rumen bacteria.
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Figure 1.3. Theoretical relationship between dilution rate and bacterial harvest from continuous culture system (Herbert et al., 1956). The theoretical relationship was confirmed experimentally in the same publication.

However, different from monoculture, the natural milieu normally includes more than one microbial species, usually a microbial ecosystem, among which exist competition, predation and mutualism. Isaacson et al. (1975) incubated rumen fluid using a chemostat with continuously supplied glucose, and reported that with increased dilution rate, the efficiency of microbial synthesis increased, while the amount of methane yield per unit of substrate decreased. Afterwards, different continuous culture systems were designed to simulate the real rumen, including both influent fluid as well as solid matrix in the fermenter (Hoover et al., 1976; Teather et al., 1988; Meng et al., 1999 a). Meng et al. (1999) and Eun et al. (2004) have applied different continuous culture designs; both found that increased fluid dilution rate was related to increased microbial yield. Wenner et al. (2017) has kept fluid dilution rate constant and found that increased solid passage rate tended to increase microbial yield. Czerkawski et al. (1977) developed the rumen simulation technique (RUSITEC), which enables to maintain a stable fermentation for a relative long period. In RUSITEC, the solid phase is contained in the nylon bags and incubated in the fermenter for 48 hours. The fluid flow rate is adjustable via inlet of buffer fluid. Based on RUSITEC, Pfau et al. (2021) found that the increased fluid dilution rate was associated with increased microbial yield and decreased methane production.

Compared to the in vitro ruminal simulation systems, the real rumen has several characteristics of complexity summarized as followed: (a) stratification of the rumen contents; (b) rumen papillae; (c) no accumulation of end products; (d) selection mechanisms for particles; (e) feeding behaviour influence, leading to inconsistent saliva inflow and solid particle reduction; (f) variations between species and even individuals;
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(g) larger pH fluctuation; (h) measurement difficulties. Nevertheless, it was indicated that factors which increase the digesta passage rate or liquid dilution rate are associated with decreased methane production and increased microbial yield (Janssen, 2010). The consistent negative relationship between fluid dilution rate and the methane yield was also observed in live sheep (Pinares-Patiño et al., 2011; Goopy et al., 2014; Hammond et al., 2014).

Manual modification of fluid dilution or solid passage including cold explosion and putting weights in the rumen, resulted in a reduction of methane production from an increased digesta passage rate (Kennedy et al., 1978; Okine et al., 1989). Harrison et al. (1975) directly induced artificial saliva through fistulas, and found microbial yield increased. Several studies that used saliva stimulants like slaframine and pilocarpine, increased the fluid dilution rate and microbial yield (Froetschel et al., 1987; Wiedmeier et al., 1987 a; Jacques et al., 1989; Bird et al., 1993). However, no studies have simultaneously demonstrated that microbial yield and methane production are triggered by fluid dilution rate in live animals.

1.5. Research project and objectives of this dissertation

As described above, both in vitro and in vivo work have provided evidence that increased fluid dilution rate is related to increased microbial yield and decreased methane production. We have used a pharmacological saliva stimulant, pilocarpine, to induce saliva production and subsequently to increase the fluid dilution rate. Pilocarpine was used in cattle and goats to increase the saliva flow (Castellano et al., 1986; Wiedmeier et al., 1987 a), and was thought to be absorbed rapidly from the reticulum and not to affect ruminal fermentation (Ruckebusch et al., 1980). Wiedmeier et al. (1987) as well as several other studies applying slaframine (Froetschel et al., 1987; Jacques et al., 1989; Bird et al., 1993), successfully increased ruminal fluid dilution rate and mostly resulted in increased microbial yield; yet, no studies have measured the methane production. We applied pilocarpine in both cattle and sheep, measured chewing activity, ruminal motility, mean retention time (MRT) of fluid and particles (2-mm and 1-cm) in the reticulorumen (RR) and total gastrointestinal tract (GIT), ruminal microbial yield, total tract methane emission, apparent nutrient digestibility, and rumen fluid parameters. The results will provide an important link between current
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*in vitro* understanding of the effect of increased microbial biomass production and reduced methane emissions. A positive outcome would indicate one of many possible ways in which domestic ruminant physiology might be targeted by selective breeding to mitigate methane emissions.

The resulting papers can be found in the next three chapters as follows:

Chapter 2: the measurements of MRT are labour-intensive; this chapter focuses on the relationship between digesta retention and chewing in cattle based on individual differences, indicating the potential for chewing measures as the easy-to-measure proxy for MRT characteristics.

Chapter 3: this chapter describes the saliva-inducing experiment in cattle.

Chapter 4: this chapter describes the saliva-inducing experiment in sheep, and compares the results with those in cattle.

In the last chapter (Chapter 5), the achieved results are discussed from various aspects, outlook on future research and application in breeding are given.

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1. General introduction


1. General introduction


1. General introduction

Chapter 2

Individual differences in digesta retention and their relation to chewing in cattle – a pilot investigation

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2. Chewing and digesta retention

Abstract

While information on individual differences in digesta mean retention time (MRT) might be interesting when selecting phenotypes for digestive efficiency, MRT measurements are prohibitively labour-intensive for large-scale application. Therefore, more easily measured proxies of MRT might be helpful. We used the opportunity of an experiment applying saliva stimulant on cattle to investigate the effect of different individual chewing behaviour on fluid and particle MRT with a consistent diet. Four non-lactating cattle (670 - 850 kg body mass) were used in a 4×4 Latin square design, treated with the saliva stimulant pilocarpine in dosages of 0, 1, 2.5 and 5 mg/kg body mass per day. The cattle were fed hay with dry matter intake assigned according to their metabolic body weight. MRT in the whole gastrointestinal tract (GIT), the reticulorumen (RR) and the distal tract were measured using Co-EDTA, Cr-mordanted fibre and La-mordanted fibre as markers representing fluid, small particles (2 mm) and large particles (1 cm), respectively. The chewing behaviour was measured via noseband pressure sensor and expressed as chewing frequency (chews per time) and chewing intensity (chews per dry matter intake), both for total chewing (ingestion plus rumination) and rumination chewing alone. The animals differed considerably in chewing behaviour and MRT measures. Body mass did not show a significant effect on chewing behaviour and MRT measures, though it tended to negatively correlate to total chewing intensity. Chewing intensity exerted a significant negative influence on MRT of fluid and particles in the RR, which was not the case for chewing frequency. Chewing frequency showed a significant relationship with MRT of large particle in the GIT. We suggest that chewing behaviour could influence MRT in two ways: (i) by affecting saliva production via the masticatory-salivary reflex and subsequently, the fluid inflow to the RR; (ii) by contributing to particle size reduction. Should the link between chewing behaviour and MRT be corroborated in larger studies, chewing measures, with their large interindividual variation, could emerge as an easy-to-measure proxy for MRT characteristics.
2. Chewing and digesta retention

KEYWORDS

rumination, ingestion, passage, chewing halter, digesta kinetics
2. **Chewing and digesta retention**

## 1 | INTRODUCTION

The mean retention time (MRT), i.e. the time digesta require to pass through the digestive tract, is an important characteristic of the ruminant’s digestion efficiency. It is mainly affected by the food intake level and characteristics of the diet of an experiment, but also by the rumen capacity of the individual (Lechner-Doll, Kaske & Engelhardt, 1991). Large differences in MRT between ruminant species have been demonstrated in many studies (Lechner-Doll et al., 1990; Bartocci et al., 1997; Przybylo et al., 2019). While most studies in domestic animals were designed to assess effects of diet and intake (e.g. Colucci, Chase & Van Soest, 1982), ontogenetic and reproductive life stages (e.g. Linden, Titgemeyer, Olson & Anderson, 2014; Grandl et al., 2018), or climate (e.g. Kennedy & Milligan, 1978) on MRT, less attention has been paid to differences among individual animals. Nevertheless, such differences have been demonstrated, and it has been suggested that information on the phenotype-specific MRT could be useful for genetic selection: Digesta retention is generally positively related to digestibility and methane yield, and negatively to ruminal microbial yield (Janssen, 2010; Pinares-Patiño et al., 2011; Goopy et al., 2014), and could therefore theoretically be an important breeding target (Thompson, Dellow & Barry, 1989; Smuts, Meissner & Cronjé, 1995; Hegarty, 2004). However, measuring MRT is difficult and time-consuming; it normally takes 5-7 days of faeces collection after an adaptation period to the respective diet, and food intake must be measured in parallel to account for its distinct effect. Should differences in MRT between phenotypes be considered as an important factor for selective breeding, easier proxies to characterize the MRT phenotype on a large scale would be required.

One factor that theoretically should influence MRT but has, to our knowledge, not been investigated in detail so far in this respect (but see Gindri, Moraes & Teixeira, 2021), is chewing characteristics: chewing intensity, i.e., chews per dry matter intake; chewing frequency, i.e., chews per time; and chewing time per dry matter intake; chewing refers to both ingestive and rumination mastication. It is known that individual cattle differ in chewing characteristics (Dado & Allen, 1994). Assuming similarity in
dental anatomy, a higher chewing intensity in phenotypes of a species should affect MRT in at least two ways (Watt et al., 2015; López-Paredes et al., 2020): (i) Increased chewing activity both during eating and ruminating should lead to more saliva production and inflow into the reticulorumen (RR), which should decrease the MRT of fluid. (ii) Increased chewing activity should achieve a faster particle size reduction, which should decrease particle MRT, as large particles are reduced faster to below the critical size threshold for leaving the RR.

We used the opportunity of an experiment performed to investigate the effect of a pharmacological saliva stimulant, pilocarpine, on methane emissions; in this experiment, food intake was kept constant per metabolic body weight (MBW, kg\(^{0.75}\)), and the MRT of a solute and two different-sized particle markers as well as chewing characteristics were measured. Although the study was explorative, we expected that MRT should differ between individuals, and that this should be related to chewing intensity and chewing frequency, with shorter MRT in individuals with higher chewing intensity.

2 | METHODS

2.1 | Animals, treatment and management

The experiment lasted from August 2020 to January 2021 at the research station AgroVet-Strickhof (Eschikon, Lindau, Switzerland). Four multiparous cattle (two black Holstein, one red Holstein and one Brown Swiss, non-pregnant and non-lactating, body weight from 670 to 850 kg) were used in 4×4 Latin square design with four treatments, consisting of one placebo and three oral dosages of pilocarpine (Fagron GmbH&Co. KG, Glinde, Germany) of 0, 1, 2.5 and 5 mg/kg body weight per day at 0600, 1400 and 2200 h. All animals were clinically healthy with no evident chewing problems; however, the state of their dentitions was not documented. With respect to the results reported here, each treatment round consisted of four weeks: during the first week, animals were kept as a group without treatment (10×5 m\(^2\), half the area with straw bedding), fed with hay for ad libitum consumption. In the second week, animals
were kept in the same place, fed 60 kg (as fed) of hay daily in total spread across the feed bins, and individually dosed for the respective pilocarpine treatment. In the third (used for determining chewing data) and fourth week (used to determine MRT), animals were kept individually (tie-stall barn, 2×1.33 m² are of rubber mat with chopped straw bedding). The amount of hay fed per animal was restricted according to their MBW in these two weeks, aimed to maintain the body weight, and reduce the effect of intake on the measurements. The total amount of hay allotted to each animal was distributed into three portions, offered after the three daily dosages of pilocarpine or placebo. The pilocarpine was mixed with a small amount of corn silage (Table 1) for better acceptance. The daily silage dry matter intake accounted for 4.8±1.7% of daily total dry matter intake. The placebo treatment consisted of the application of a similar amount of silage without pilocarpine. The hay used in the experiment originated from a single batch (Table 2.1) during the whole experiment. The animals were given 100 g multivitamin and mineral supplement (Künzle Farma AG, Oberaach, Switzerland) and 50 g salt (Schweizer Salinen AG, Pratteln, Switzerland) per day. Water was provided at *ad libitum* access during the whole experiment.

**Table 2.1** Nutrient composition (g/kg dry matter) of provided hay and whole-plant corn silage during the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Grass hay</th>
<th>Silage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic matter</td>
<td>917</td>
<td>910</td>
</tr>
<tr>
<td>Crude protein</td>
<td>150</td>
<td>129</td>
</tr>
<tr>
<td>Ether extracts</td>
<td>23</td>
<td>32</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>595</td>
<td>411</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>316</td>
<td>273</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>279</td>
<td>232</td>
</tr>
</tbody>
</table>

**2.2 | Preparation and application of digesta passage marker**

Markers for ingesta retention, Co-ethylenediaminetetraacetic acid (Co-EDTA; solute marker), Cr-mordanted fibre (2 mm particle marker) and La-mordanted fibre (1 cm particle marker) were prepared according to Udén, Colucci & Van Soest (1980). The grass hay was dried and cut in a cutting mill to pass a 2-mm and 4-mm screen separately.
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The milled hay particles were sequentially screened by shaking on a particle size separator to obtain two size fractions (2 mm and 1 cm; note that milling through a 4-mm screen results in a certain proportion of 1-cm particles). The particles were washed in a washing machine with washing powder for two hours at room temperature and thoroughly rinsed with water and then incubated with the respective mordant for 24 hours (76 g LaCl$_3$·7H$_2$O per 100 g 1-cm particles based on dry matter at 37°C; 33 g Na$_2$Cr$_2$O$_7$·2H$_2$O per 100 g 2-mm particles based on dry matter at 100°C). The particles were washed after mordanting, and the Cr-mordanted particles were treated with ascorbic acid and washed again. All particles were then dried at 65°C to constant weight.

Co-EDTA was prepared as described by Udén et al. (1980). The dosage provided to the animals was approximately 0.1 g of particle marker and 0.01 g of Co-EDTA per kg of body weight. The passage marker was given in the morning of the first day of week four. For that, Co-EDTA was dissolved with hot water and mixed with the particle markers and the morning pilocarpine dose in the silage.

2.3 Data recording, sampling and analysis

The chewing activity was monitored using a noseband pressure sensor (MSR Electronics GmbH, Seuzach, Switzerland) as described by Braun, Trösch, Nydegger & Hässig (2013); the sensor was mounted on the noseband of a halter for two separate days during the third week. A data logger recorded pressure signals (10 Hz) from an oil-filled tube that was integrated in the noseband. The recorded signals were firstly cut to 24 h using the software MSR Cutter V6.05.00 (MSR Electronics GmbH), then evaluated by the software Viewer2 V2.02.00 and differentiated into ingestion, rumination and other activities (such as drinking, scratching, etc.). Then the results were visually evaluated and corrected if needed using the software Editor V2.02.00. The chewing behaviour was expressed as total and rumination chewing frequency (number of chews per time) and total and rumination chewing intensity (number of chews per dry matter intake [DMI]), where total chewing included ingestion and rumination. Body mass (BM) was measured before and at the end of each 4-week-run using a vehicle.
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scale (± 20 kg). The amount of water consumed was recorded during weeks three and four by water flow meters (GWF MessSysteme AG, Luzern, Switzerland), which were installed on each individual water pipe to the water trough. Representative samples of the hay, the silage used for treatment dosing, and the individual leftovers of each animal were taken daily in weeks three and four. After each run, these samples were pooled per cow, dried at 60°C overnight and milled through a 0.75 mm sieve for later analysis. They were analysed for the contents of dry matter, organic matter, crude protein, ether extract, crude fibre, neutral detergent fibre and acid detergent fibre according to the standard methods of the Association of German Agricultural Analysis and Research Centers (VDLUFA, 2006). Neutral detergent fibre was analysed after adding amylase, and all detergent fibre values are corrected for residual ash. Faecal samples for MRT determination were collected before marker application (three samples per animal for baseline marker values) and 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 54, 60, 66, 72, 80, 88, 96, 104, 112, 120, 128, 136, 144, 152, 160 and 168 hours after marker application. These faecal samples were dried at 105°C to constant weight and ground through a 1-mm screen with a cutting mill. The passage marker concentrations in the faeces were analysed as described by Frei et al. (2015) using inductively coupled plasma optical emission spectrometry (Optima 8000, PerkinElmer Inc., Rodgau, Germany) after wet ashing. The baseline concentrations in faecal samples measured before the marker application were used to correct for background levels.

2.4 | Calculations and statistics

Dry matter intake was calculated as the difference in food offered and leftover, both expressed as dry matter. The MRT in the whole digestive tract (GIT) was calculated according to Thielemans et al. (1978) as:

\[
MRT = \frac{\sum t_i C_i dt_i}{\sum C_i dt_i}
\]

with \(C_i\) = marker concentration in the faecal samples from the interval represented by time \(t_i\) (hours after marker administration, using the midpoint of the sampling interval), and \(dt_i\) = the interval (h) of the respective sample:
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\[ dt_i = \frac{(t_{i+1} - t_i) + (t_i - t_{i-1})}{2} \]

The MRT of fluid in the RR was calculated following Grovum & Williams (1973), and the MRT of the distal GIT as the difference of MRT GIT – MRT RR of the fluid marker. The MRT RR of the particles was calculated as MRT RR particles = MRT GIT particles – MRT distal GIT. This approach is based on the empirically confirmed (Huhtanen & Kukkonen, 1995; Mambrini & Peyraud, 1997; Wylie et al., 2000) assumption that there is no differential passage of fluid and particles in the distal GIT.

To investigate differences between individuals (as opposed to a traditional analysis for a difference between treatments), analysis of variance was conducted with a linear mixed model (LMM) using R version 3.5.2 with experiment rounds and treatments as random factor and animals as fixed factor. The Shapiro-Wilk test was used to assess residual normality; if needed, the data were ln-transformed (indicated in the results tables). Tukey’s procedure was used for multiple comparisons when the LMM was significant (P<0.05). Results are presented as numeric means and standard deviations.

The relationships between chewing intensity and chewing time per DMI were investigated through LMM as:

*Chewing intensity*

\[ = \mu + \text{Chewing time per DMI} + \text{Treatment}_i + \text{Animal}_j + \text{Round}_k + e; \]

Chewing measures as well as MRT measures as dependent variables were fitted into LMM to investigate the effect of BM as:

*Chewing or MRT measures* = \[ \mu + \text{BM} + \text{Treatment}_i + \text{Animal}_j + \text{Round}_k + e; \]

MRT measures as dependent variables into LMM to investigate the effect of water intake as:

*MRT measures* = \[ \mu + \text{Water Intake} + \text{Treatment}_i + \text{Animal}_j + \text{Round}_k + e; \]

the relationship between MRT measures and chewing measures were
investigated through LMM as:

\[ MRT \text{ measures} = \mu + \text{Chewing measures} + \text{Treatment}_i + \text{Animal}_j + \text{Round}_k + e; \]

where \( \mu \) is the overall mean, treatment is the fixed factor (\( i = 1, 2, 3, 4 \)), round is a random factor, as is animal to account for repeated measures (\( j, k = 1, 2, 3, 4 \)), \( e \) is the random residual error. In order to compare the inter-individual variability of our study with that of Dado & Allen (1994), we calculated the coefficient of variation between our four cows.

3 | Results

The animals maintained a relatively constant BW during the experiment irrespective of pilocarpine dosage. As planned, no significant differences between treatments were observed for feed intake (data not shown). However, water intake (kg/d) was higher at the highest pilocarpine dose (data not shown).

Total ingestion time, rumination times and the ratio of rumination to ingestion were not affected by treatment; the same was true for total and rumination chewing frequency as well as chewing intensity (data not shown). However, the individual animals differed distinctively in these characteristics (Table 2.2). The chewing intensity (chews per DMI) was significantly, positively related to time spent chewing per DMI, for ingestion, rumination, and total chewing behaviour (\( P<0.001 \)). However, there were clear differences between animals, which are explained by the differences in chewing frequency (Fig. 2.1). The coefficient of variation between the four animals was low to moderate, between 7.4 and 13.7%, for most MRT and chewing frequency measures. By contrast, it was higher, between 12.8 and 25.1%, for measures of chewing time and chewing intensity. The only MRT measure with a similarly high coefficient of variation was that for the distal GIT at 20.0%. For each chewing measure, the coefficient of variation was higher for rumination than for ingestive chewing, indicating that the four cows were particularly variable in their rumination behaviour (Table 2.2).

Marker elimination showed the typical cattle pattern, with a clear distinction of
2. Chewing and digesta retention

solute, small and large particle excretion (Fig. 2.2). MRT measures were different among the individuals (Table 2.2). Body mass did not have a significant effect in the models on chewing or MRT measures (Table 2.3), although total chewing intensity tended to decrease when BM increased (P=0.084). Drinking water intake had no effect on MRT measures (Table 2.4).

Significant negative relationships were evident between some chewing and MRT measures, for example between rumination chewing intensity and MRT RR of fluid (Fig. 2.3A) and between total chewing intensity and MRT RR of large particles (Fig. 2.3B). Rumination chewing frequency was negatively related to the MRT GIT of large particles, but there was only a trend for a negative relation with the MRT GIT of fluid and small particles (Table 2.5). The same negative relation was also seen for total chewing frequency with large particle MRT GIT. For the RR, total as well as rumination chewing intensity had a negative relation with the fluid, small and large particle MRT RR, while chewing frequency showed no relation on MRT RR of fluid, small and large particles (Table 2.5). The MRT in the distal GIT was not affected by the chewing measures (Table 2.5).

When using the time spent chewing rather than the number of chews, MRT RR was negatively related with rumination time but not with total chewing time per DMI; no relationships were detected between the rest of MRT measures and measures of chewing time per DMI (Table 2.5). Chewing intensity, as based on the number of chews, had a distinctively higher correlation to MRT measures than chewing time per DMI (Fig. 2.3BC).
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Table 2.2 Individual means (±SD) and the inter-individual coefficient of variation for body weight, intake, chewing and mean retention time (MRT) measures; different superscripts indicate significant differences (P<0.05) between individuals.

<table>
<thead>
<tr>
<th>Animal</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>P#</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>683±5</td>
<td>688±15</td>
<td>843±10</td>
<td>788±13</td>
<td>&lt;0.001</td>
<td>10.4</td>
</tr>
<tr>
<td>Hay intake (kg/d)</td>
<td>11.1±0.5</td>
<td>11.1±0.2</td>
<td>13.1±0.4</td>
<td>12.0±0.2</td>
<td>&lt;0.001</td>
<td>8.0</td>
</tr>
<tr>
<td>Dry matter intake (DMI) (kg/d)</td>
<td>11.8±0.3</td>
<td>11.7±0.5</td>
<td>13.7±0.2</td>
<td>12.6±0.3</td>
<td>&lt;0.001</td>
<td>7.4</td>
</tr>
<tr>
<td>Water intake (kg/d)</td>
<td>56.5±8.7</td>
<td>60.0±5.2</td>
<td>69.2±2.7</td>
<td>54.9±5.0</td>
<td>&lt;0.001</td>
<td>10.6</td>
</tr>
<tr>
<td>Ingestion time (min/kg DMI)</td>
<td>32.3±3.8</td>
<td>26.3±4.5</td>
<td>22.9±1.6</td>
<td>25.7±2.3</td>
<td>0.009</td>
<td>14.8</td>
</tr>
<tr>
<td>Rumination time (min/kg DMI)</td>
<td>37.3±1.8</td>
<td>48.8±3.8</td>
<td>32.3±1.1</td>
<td>40.2±2.1</td>
<td>&lt;0.001</td>
<td>17.4</td>
</tr>
<tr>
<td>Total chewing time (min/kg DMI)</td>
<td>69.5±4.3</td>
<td>75.2±5.5</td>
<td>55.1±1.2</td>
<td>65.8±3.3</td>
<td>&lt;0.001</td>
<td>12.8</td>
</tr>
<tr>
<td>Ratio rumination:ingestion time</td>
<td>1.17±0.2</td>
<td>1.89±0.3</td>
<td>1.42±0.1</td>
<td>1.57±0.2</td>
<td>0.007</td>
<td>19.9</td>
</tr>
<tr>
<td>Ingestion chewing intensity (/kg DMI)</td>
<td>2131±228</td>
<td>1839±294</td>
<td>1627±84</td>
<td>1543±130</td>
<td>&lt;0.001</td>
<td>17.6</td>
</tr>
<tr>
<td>Rumination chewing intensity (/kg DMI)</td>
<td>2426±155</td>
<td>3574±395</td>
<td>2299±106</td>
<td>2133±113</td>
<td>&lt;0.001</td>
<td>25.1</td>
</tr>
<tr>
<td>Total chewing intensity (/kg DMI)</td>
<td>4557±289</td>
<td>5412±502</td>
<td>3926±92</td>
<td>3676±184</td>
<td>&lt;0.001</td>
<td>17.6</td>
</tr>
<tr>
<td>Ingestion chewing frequency (/min)</td>
<td>66.1±1.4</td>
<td>69.9±0.9</td>
<td>71.2±1.7</td>
<td>60.1±2.5</td>
<td>&lt;0.001</td>
<td>7.4</td>
</tr>
<tr>
<td>Rumination chewing frequency (/min)</td>
<td>65.1±1.3</td>
<td>73.1±2.9</td>
<td>71.2±1.5</td>
<td>53.1±1.2</td>
<td>&lt;0.001</td>
<td>7.4</td>
</tr>
<tr>
<td>Total chewing frequency (/min)</td>
<td>65.5±0.4</td>
<td>71.9±1.8</td>
<td>71.2±1.5</td>
<td>55.8±1.6</td>
<td>&lt;0.001</td>
<td>13.7</td>
</tr>
<tr>
<td>MRT GIT Co (h)</td>
<td>26.3±1.4</td>
<td>21.1±1.5</td>
<td>25.3±2.1</td>
<td>29.5±0.5</td>
<td>&lt;0.001</td>
<td>13.6</td>
</tr>
<tr>
<td>MRT GIT Cr (h)</td>
<td>46.8±1.9</td>
<td>39.2±2.8</td>
<td>46.7±2.5</td>
<td>53.3±2.2</td>
<td>&lt;0.001</td>
<td>12.4</td>
</tr>
<tr>
<td>MRT GIT La (h)</td>
<td>55.1±2.1</td>
<td>47.7±2.8</td>
<td>55.8±1.0</td>
<td>64.2±1.5</td>
<td>&lt;0.001</td>
<td>12.1</td>
</tr>
<tr>
<td>MRT RR Co (h)</td>
<td>13.8±0.1</td>
<td>11.8±0.8</td>
<td>13.4±0.6</td>
<td>14.1±0.4</td>
<td>&lt;0.001</td>
<td>7.7</td>
</tr>
<tr>
<td>MRT RR Cr (h)</td>
<td>34.4±0.9</td>
<td>29.8±2.1</td>
<td>34.7±2.1</td>
<td>37.9±2.4</td>
<td>0.005</td>
<td>9.7</td>
</tr>
<tr>
<td>MRT RR La (h)</td>
<td>42.7±1.3</td>
<td>38.3±2.5</td>
<td>43.9±1.4</td>
<td>48.8±1.7</td>
<td>0.001</td>
<td>9.9</td>
</tr>
<tr>
<td>MRT distal GIT (h)</td>
<td>12.4±1.5</td>
<td>9.4±1.3</td>
<td>12.0±1.8</td>
<td>15.4±0.2</td>
<td>&lt;0.001</td>
<td>20.0</td>
</tr>
</tbody>
</table>

*P-value for the fixed factor ‘animal’ in linear mixed models; *ln-transformed data; GIT=gastrointestinal tract; RR=reticulorumen
2. Chewing and digesta retention

Table 2.3 Effect of body weight on chewing and mean retention time (MRT) measures using linear mixed models, with treatment as fixed factor, animal and round as random factor.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Body weight (kg)</th>
<th>Treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate (Standard error)</td>
<td>p#</td>
</tr>
<tr>
<td>Chewing frequency</td>
<td>Total chewing</td>
<td>-0.025 (0.029)</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.056 (0.040)</td>
</tr>
<tr>
<td>Chewing intensity</td>
<td>Total chewing</td>
<td>-10.07 (3.884)</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-6.18 (3.803)</td>
</tr>
<tr>
<td></td>
<td>GIT Co</td>
<td>-0.001 (0.019)</td>
</tr>
<tr>
<td></td>
<td>GIT Cr</td>
<td>0.024 (0.036)</td>
</tr>
<tr>
<td></td>
<td>GIT La</td>
<td>0.008 (0.039)</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>RR Co</td>
<td>0.004 (0.007)</td>
</tr>
<tr>
<td></td>
<td>RR Cr</td>
<td>0.025 (0.022)</td>
</tr>
<tr>
<td></td>
<td>RR La</td>
<td>0.027 (0.026)</td>
</tr>
<tr>
<td></td>
<td>distal GIT</td>
<td>-0.016 (0.019)</td>
</tr>
</tbody>
</table>

*P-value for the independent variable ‘body weight’ in linear mixed models; †P-value for the effect of pilocarpine treatment; *ln-transformed data

DMI=dry matter intake; GIT=gastrointestinal tract; RR=reticulorumen

Table 2.4 Effect of water intake on mean retention time (MRT) measures using linear mixed models, with treatment as fixed factor, animal and round as random factor.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Water intake (kg)</th>
<th>Treatment effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate (Standard error)</td>
<td>p#</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>GIT Co</td>
<td>-0.064 (0.074)</td>
</tr>
<tr>
<td></td>
<td>GIT Cr</td>
<td>0.063 (0.129)</td>
</tr>
<tr>
<td></td>
<td>GIT La</td>
<td>-0.036 (0.128)</td>
</tr>
<tr>
<td>RR Co</td>
<td>0.037 (0.027)</td>
<td>0.249</td>
</tr>
<tr>
<td>RR Cr</td>
<td>0.032 (0.124)</td>
<td>0.808</td>
</tr>
<tr>
<td>RR La</td>
<td>-0.040 (0.111)</td>
<td>0.727</td>
</tr>
<tr>
<td>distal GIT</td>
<td>-0.013 (0.070)</td>
<td>0.862</td>
</tr>
</tbody>
</table>

*P-value for the independent variable ‘water intake’ in linear mixed models; †P-value for the effect of pilocarpine treatment; *ln-transformed data

GIT=gastrointestinal tract; RR=reticulorumen
## 2. Chewing and digesta retention

### Table 2.5 Effect of chewing measures on mean retention time (MRT) measures using linear mixed models, with treatment as fixed factor, animal and round as random factor.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Chewing measure</th>
<th>Treatment effect</th>
<th>Estimate (Standard error)</th>
<th>P#</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MRT GIT Co</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.209 (0.144)</td>
<td>0.283</td>
<td><strong>0.006</strong>*</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.230 (0.102)</td>
<td>0.079</td>
<td><strong>0.002</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chewing intensity (/kg DMI)</td>
<td>Total chewing</td>
<td>0.000 (0.001)</td>
<td>0.958</td>
<td><strong>0.004</strong>*</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>0.000 (0.001)</td>
<td>0.998</td>
<td><strong>0.006</strong>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chewing time (min/kg DMI)</td>
<td>Total chewing</td>
<td>-0.008 (0.060)</td>
<td>0.741</td>
<td><strong>0.004</strong>*</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>0.016 (0.078)</td>
<td>0.702</td>
<td><strong>0.004</strong>*</td>
<td></td>
</tr>
<tr>
<td><strong>MRT GIT Cr</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.660 (0.241)</td>
<td>0.066</td>
<td><strong>0.041</strong></td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.544 (0.182)</td>
<td>0.052</td>
<td><strong>0.035</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MRT GIT La</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.874 (0.240)</td>
<td><strong>0.017</strong></td>
<td><strong>0.022</strong></td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.636 (0.187)</td>
<td><strong>0.026</strong></td>
<td><strong>0.044</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MRT RR Co</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.004 (0.002)</td>
<td>0.056</td>
<td>0.108</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.004 (0.002)</td>
<td>0.121</td>
<td>0.129</td>
<td></td>
</tr>
<tr>
<td><strong>MRT RR Cr</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.004 (0.001)</td>
<td>0.055</td>
<td><strong>0.055</strong>*</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.001 (0.000)</td>
<td><strong>0.005</strong></td>
<td><strong>0.026</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MRT RR La</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.039 (0.034)</td>
<td>0.282</td>
<td>0.099</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.113 (0.033)</td>
<td><strong>0.008</strong></td>
<td><strong>0.036</strong></td>
<td></td>
</tr>
<tr>
<td><strong>MRT distal GIT</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.004 (0.001)</td>
<td><strong>0.019</strong></td>
<td>0.177</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.004 (0.001)</td>
<td><strong>0.025</strong></td>
<td>0.250</td>
<td></td>
</tr>
<tr>
<td><strong>MRT distal GIT</strong> (h)</td>
<td>Chewing frequency (/min)</td>
<td>Total chewing</td>
<td>-0.005 (0.002)</td>
<td><strong>0.021</strong></td>
<td>0.513</td>
</tr>
<tr>
<td></td>
<td>Ruminating</td>
<td>-0.005 (0.002)</td>
<td><strong>0.021</strong></td>
<td>0.606</td>
<td></td>
</tr>
</tbody>
</table>

*P-value for the independent variable ‘chewing measure’ in linear mixed models; †P-value for the effect of pilocarpine treatment; ln-transformed data

DMI=dry matter intake; GIT=gastrointestinal tract; RR=reticulorumen
2. Chewing and digesta retention

Figure 2.1 Relationship between (A) the time spent chewing during ingestion (per kg dry matter intake DMI) and the ingestive chewing intensity (number of ingestive chews per kg DMI) and (B) the time spent chewing during rumination (per kg dry matter intake DMI) and the rumination chewing intensity (number of rumination chews per kg DMI). Note the difference between animals 3 and 4, which is due to the distinct difference in chewing frequency between these animals (Table 2.2).

Figure 2.2 Faecal marker elimination pattern based on average of all cattle during the trial (Co for solute marker, Cr for 2 mm particle marker, La for 1 cm particle marker)
2. Chewing and digesta retention

Figure 2.3 Relationship between (A) MRT fluid in RR (MRT: mean retention time; RR: reticulorumen) and rumination chews per DMI (dry matter intake), (B) MRT of 1-cm-particle in RR and total chews per DMI, and (C) MRT of 1-cm-particle in RR and total chewing time per DMI; simple linear regression coefficient of determination ($R^2$) as well as $P$ value are indicated; for a full statistical evaluation, see Table 2.5.
2. Chewing and digesta retention

4 | Discussion

The results of our pilot study suggest that individual differences in chewing intensity as well as chewing frequency exist when fed the same hay-only diet, and that chewing activity may affect fluid and particle MRT in the RR. In contrast to MRT measurements, chewing measurements take much less time and effort. Future studies need to corroborate their suitability as a proxy for MRT measurements, at least in the sense of a ranking of phenotypes. In doing so, it should be further assessed whether it is necessary to count the number of chews, or whether a measure of ‘time spent chewing’ is already sufficient. Our preliminary data suggests that, due to individual differences in chewing frequency, ‘time spent chewing’ is a less informative measure than the actual number of chews made in that time. This is relevant because many recent studies that include a measure of chewing behaviour usually only report (and possibly, also only record) chewing times (Watt et al., 2015; Byskov, Fogh & Løwendahl, 2017; Zetouni et al., 2018).

4.1 | Factors influencing MRT and chewing activity

Intake level is considered the primary factor that affects MRT; a higher relative intake level is generally accompanied by a decrease of fluid and particle MRT (Thornton & Minson, 1972; Mudgal, Dixon, Kennedy & Milligan, 1982; Shaver, Nytes, Satter & Jorgensen, 1986; Lechner-Doll et al., 1991). A higher intake level is also related to an increased gut fill, which partly, but not completely, mitigates the MRT-decreasing effect of intake (Findeisen, Südekum, Hummel & Clauss, 2021). The diet type and physical form also exert an influence on MRT (Shaver, Nytes, Satter & Jorgensen, 1988; Udén, 1988; Zebeli et al., 2007); however, in order to ensure a diet characteristic is really a factor of influence, an effect of diet on food intake level must be excluded (Levey & Martínez del Rio, 1999). In the present study, diet composition as well as the relative food intake level was kept constant throughout, excluding these potential factors. Although BM as such, which varied among the experimental animals of the present study, was repeatedly discussed to be positively related to MRT (Illius & Gordon, 1992; Gordon & Illius, 1994), the effect might have been overestimated (Clauss et al., 2007; Steuer et al., 2011; Müller et al., 2013; Abraham et al., 2021). Our pilot results showed that even for two individuals with similar BM and food intake, the
2. Chewing and digesta retention

MRT can still differ considerably.

Intake level was also suggested to be related to chewing behaviour; a higher relative intake level often leads to a decreased chewing intensity (Welch & Smith, 1969; Bae, Welch & Smith, 1981; Dias et al., 2011). Physical diet characteristics also affect chewing activity. A reduced diet particle size was related to either a decreased rumination time (Krause, Combs & Beauchemin, 2002; Beauchemin, Yang & Rode, 2003) or a decreased total chewing intensity (Kononoff, Heinrichs & Lehman, 2003). Body weight also might affect chewing measures, as studies showed that larger individuals or species spent less time chewing per ingested cell wall constituents (Welch, 1982; Bae, Welch & Gilman, 1983; Druzinsky, 1993; Shipley et al., 1994). Our preliminary results were not consistent with these findings, even though total chewing intensity tended to negatively relate to BM. As indicated by Janis et al. (2010) when using a small sample of horses and cattle, chewing frequency may much more likely lead to misrepresentation in low sample size datasets than chewing intensity.

Apart from body mass, the dental status (e.g., whether all teeth have already erupted or not) could also be relevant to chewing behaviour (Grandl et al., 2018), although this has not been studied systematically in domestic ruminants. This is a general phenomenon in the cattle literature. Although dental abnormalities or pathologies had a high occurrence in the few studies that addressed this issue (Scheler, 1953; Fiedler, 1967; Simmerstetter, 1994; Ingham, 2001; Borsanelli et al., 2016; Fadden et al., 2016; Probst, Kauf, Schellenberger & Spengler Neff, 2016; Borsanelli et al., 2021), and although it is known from humans (e.g. Helkimo, Carlsson & Helkimo, 1978; Van der Bilt, Olthoff, Bosman & Oosterhaven, 1993; Ikebe et al., 2011) as well as from ruminants (Pérez-Barbería & Gordon, 1998; Grandl et al., 2018) that a lack of chewing surface is compensated by increased chewing intensity, dental status is typically not assessed in studies that measure chewing behaviour. In theory, this makes chewing behaviour an ambiguous signal: a high chewing intensity can indicate both – a specimen with healthy teeth and an above-average propensity for chewing, or a specimen with a compromised dental status and a compensatory chewing investment. The present pilot study is no exception: While no chewing problems were observed in the animals, their dental status was not recorded, and an influence on the chewing behaviour cannot be excluded.

Therefore, when investigating the effect of chewing on MRT, it is necessary to
2. Chewing and digesta retention

control both intake level and diet composition and account for body mass effects (and ensure dental integrity). To our knowledge, the effect of differences in chewing behaviour between individuals on a consistent diet on MRT measures have not been reported so far. Our results show that for two animals with similar body weight, the chewing behaviour can vary considerably, and the chewing measures can significantly relate to the MRT measures. The low number of animals makes this a pilot finding. Dado & Allen (1994) reported inter-individual variability in measures of chewing activity in six primiparous and six multiparous cows. The coefficients of variation of the multiparous cows for ingestion, rumination and total chewing time per DMI are, at 14.1, 18.9 and 13.6%, very similar to those of our four multiparous cows (14.8, 17.4 and 12.8%, respectively; Table 2.2). These findings support the concept that individual cattle may vary distinctively in chewing characteristics.

The chewing behaviour of ruminants differs considerably between ingestive and rumination mastication; the latter is more uniform and consistent (Deswysen & Ehrlein, 1981; Dittmann, Kreuzer, Runge & Clauss, 2017), usually represents the larger proportion of chewing activity (Beauchemin, 2018), and is responsible for the majority of particle size reduction (McLeod & Minson, 1988). Therefore, assessing not only total chewing activity, but also separately rumination activity only, is justified – especially so as individuals apparently differ most in this aspect of chewing behaviour (Table 2.2).

4.2 | Relationship between chewing activity and MRT

The relationship between chewing activity and MRT found in the present study may have several reasons (Watt et al., 2015; López-Paredes et al., 2020), and these are different for MRT of particles and MRT of fluid. Evidently, in mammalian herbivores, chewing plays the predominant role in the reduction of particle size. Kennedy (1985) collected the regurgitated material during rumination and found that about 70% of the large particles in the mouth were comminuted to small particles during one cycle of rumination, and rumination contributed about 85% of the comminution of the large particles which disappear from the RR. A higher chewing intensity should simply facilitate a faster passage of particulate matter from the RR.

Compared to the escape of particles, the passage of fluid is based on different mechanisms. Liquid is dependent to a large extent to absorption and to secretion at various sites of the GIT. Absorption is mainly influenced by osmolality; water
absorption occurs secondarily to osmotic gradients generated by the active transport of ions and solutes, and passively by hydrostatic or oncotic forces (Masyuk, Marinelli & LaRusso, 2002). In studies that investigated both drinking water intake and the MRT of fluid (in the RR or the GIT) in ruminants, no correlation was found (Bernabucci et al., 2009; Hebel et al., 2011), indicating that drinking water intake is no relevant factor influencing RR fluid passage. Adding mineral salts or hypertonic solutions to the RR, but not (hypotonic) water, typically decreases RR fluid retention (Harrison, Beever, Thomson & Osbourn, 1975; Rogers, Marks, Davis & Clark, 1979; Rogers & Davis, 1982), suggesting that the absence of an effect of drinking water intake, as observed in the present study, is probably due to its rapid absorption. By contrast, saliva production is linked to fluid MRT. This was demonstrated repeatedly by the pharmacological stimulation of salivation (Froetschel et al., 1987; Wiedmeier, Arambel, Lamb & Marcinkowski, 1987; Wiedmeier, Arambel & Walters, 1987; Bird et al., 1993), including the present study. A physiological factor that regulates saliva inflow into the RR is chewing behaviour. Chewing activity, whether for ingestion or rumination, is linked to a high salivary flow (Méot, Cirio & Biovin, 1997), and therefore diets that require more chewing trigger more salivary flow (Kaufmann & Orth, 1966; Đuric, Zhao, Ørskov & Chen, 1994). Individual differences in saliva production in cattle have been reported and linked to the occurrence of frothy bloat (Gurnsey, Jones & Reid, 1980). Chewing behaviour stimulates salivation through the masticatory-salivary reflex, which is based on intra-oral mechanoreceptors (Hector & Linden, 1999). Compared to the ‘resting flow’, salivation rate increases by 2-4 times in cattle during ingestion and rumination (Bailey, 1961). Cattle can produce more than 180 L saliva per day (Van Soest, 1994), which is around three-fold of the drinking amount (by our study 50-70 L). Individual differences in chewing behaviour could contribute greatly to saliva inflow and, subsequently, the fluid MRT.

5 | CONCLUSION

Our results underline the relevance of using the number of chews rather than chewing time as the quantity when relating chewing behaviour to other data. In future studies, the dental status of animals should ideally be accounted for to exclude it as a main causative factor for differences in chewing intensity. Chewing behaviour is a potential trait that is definitively easier to acquire than measurements of mean retention time, and
may only require some standardized test meal offered for a limited period of time. While it would be premature to suggest predictive equations for MRT from chewing measures based on the present study, our pilot results may justify more detailed studies into the link between chewing behaviour and digestive physiology to use the former as a predictor for the latter.

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2. Chewing and digesta retention


2. Chewing and digesta retention


2. Chewing and digesta retention


2. Chewing and digesta retention


2. Chewing and digesta retention

Chapter 3

Effect of induced saliva flow on fluid retention time, ruminal microbial yield and methane emission in cattle

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Abstract

Both in vitro and animal studies indicated that a higher dilution rate is related to a more efficient microbial synthesis and a lower methane (CH$_4$) yield. The latter could be a consequence of the former, as an increase in microbial cell synthesis offers an alternative hydrogen sink competing with methanogenesis. To test this assumption in live animals, we applied a saliva stimulant, pilocarpine, to modify liquid flow rate in cattle. Four non-lactating cows (750 ± 71 kg) were fed forage only (restricted to constant intake) in a 4 × 4 Latin square design with oral doses of 0, 1, 2.5 and 5 mg pilocarpine/kg body weight and day. We quantified feed and water intake, ruminal and total tract mean retention time (MRT) of solute and particle markers, ruminal microbial yield (via urinary purine bases or metabolic faecal nitrogen), CH$_4$ emission, digestibility, chewing behaviour, reticular motility and rumen fluid parameters. The effect of induced saliva flow was evident by visibly increased salivation and water intake. Increasing the pilocarpine dosages resulted in a linearly decreased MRT of fluid and small particles ($p < 0.001$ and $< 0.05$, respectively) and methane yield as related to digested DM ($p < 0.05$), the latter at a magnitude of 5%. No effect of treatment was found on ruminal microbial yield estimated via purine derivates. Metabolic faecal N as an indicator of microbial growth linearly correlated with pilocarpine dosages ($p < 0.05$). No significant relationship was found between pilocarpine dosages and large particle MRT, nutrient digestibility, ruminal pH and short-chain fatty acids. In conclusion, different from some in vitro studies, there was little indication of a reciprocal effect of CH$_4$ and microbial biomass production in cows fed a forage-only diet.

KEYWORDS

salivation, pilocarpine, passage rate, digestion, methanogenesis, microbial synthesis, ruminant
1 INTRODUCTION

Ruminants evolved a digestive system with a voluminous forestomach, which selectively retains large particles for rumination and where plant matter is fermented by symbiotic microbes. Microbes produce short-chain fatty acids (SCFA) that the host uses as energy source, and collateral metabolites such as CO₂ and CH₄. The protein contained in the microbial biomass is digested by the host once it flows out of the rumen into the lower gastro-intestinal tract (GIT) (Van Soest, 1994).

In vitro studies provided evidence that an increased dilution of fermenter contents (the equivalent of a higher throughput of fluid through the rumen, relative to particulate matter) leads to an increased yield ('harvest') of microbial biomass from the fermenter, putting the microbial population into a metabolic state of increased regrowth (Herbert, Elsworth, & Telling, 1956; Isaacson, Hinds, Bryant, & Owens, 1975; Meng, Kerley, Ludden, & Belyea, 1999; Eun, Fellner, & Gumpertz, 2004; Pfau et al., 2021).

In live animals, several studies investigated the effect of an increased dilution rate in the reticulorumen (RR) via saliva stimulants or artificial saliva infusion on microbial yield. They found that an increased dilution rate was (or tended to be) associated with an increased microbial yield and enhanced efficiency of microbial protein synthesis (Harrison, Beever, Thomson, & Osbourn, 1975; Wiedmeier, Arambel, Lamb, & Marcinkowski, 1987a; Wiedmeier, Arambel, & Walters, 1987b; Froetschel, Amos, Evans, Croom, & Hagler, 1989; Bird et al., 1993). This led Croom and Hagler (1987, 1989) to register patents for one of the pharmacological substances used in these experiments, slaframine, for the use in intensively-fed cattle. However, it seems that this approach has not been widely pursued since.

Increasing microbial biomass yield by dilution had an additional effect in in vitro assays. It was inversely related to the amount of gas produced per unit of digested substrate (Blümmel, Steingaß, & Becker, 1997). Theoretical considerations indicate that the increase in microbial biomass production could be linked to a decrease in CH₄ production (Czerkawski, 1986; Ramin & Huhtanen, 2013), which was also confirmed in in vitro studies (Isaacson et al., 1975; Van Nevel & Demeyer, 1979; Pfau et al., 2021). This phenomenon could be explained by the synthesis of fatty acids for microbial cell membranes, which act as hydrogen sinks. Such effects have been estimated to have the capacity to diminish CH₄ outputs at a magnitude of 20% (Ramin & Huhtanen, 2013). However, to which extent these mechanisms are also operative in live animals has, to
our knowledge, not yet been tested empirically.

We aimed to test the effect of a pilocarpine-induced increased fluid dilution on ruminal microbial yield and in consequence CH₄ emission as well as apparent digestibility, chewing behaviour and rumen fluid parameters, in a Latin Square design where each animal served as its own control. We chose pilocarpine as it is a well-known saliva stimulant that does not affect microbial fermentation (Ruckebusch, 1980), and has been used in cattle (Wiedmeier et al., 1987b) and goats (Castellano, Moreno, Raggi, Victoria, & Mataix, 1986) to increase the flow of saliva into, and hence also the throughput of fluid through, the RR. To ensure that observed effects were due to the presumed dilution and not to differences in feed intake, we kept the feed intake constant for each animal across all treatments. We predicted that increasing pilocarpine dosages should lead, due to more salivation, (1) to shorter solute marker MRT at little effect on small and large particle MRT, and (2) to a higher microbial N and a lower CH₄ yield.

2 | MATERIALS AND METHODS
2.1 | Animals, feeding and treatment

The study was carried out from August 2020 to January 2021 at the research station AgroVet-Strickhof (Eschikon, Lindau, Switzerland). Four cattle (two black Holstein, one red Holstein and one Brown Swiss, all non-pregnant and non-lactating, body weight from 670 to 850 kg) were subjected to a randomized 4×4 Latin square design with four treatments. The treatments consisted of oral supplementation with pilocarpine (pilocarpine hydrochloride, C₁₁H₁₆N₂O₂ × HCl, Fagron GmbH&Co. KG, Glinde, Germany). Dosages of 0, 1, 2.5 and 5 mg/kg body weight per day were used. They were divided into three portions and given every 8 h with a small amount of silage mixture (around 0.6 kg DM per day and animal). To allow for a gradual adjustment, the cows receiving pilocarpine always received the lowest dose on day 1 at the beginning of each treatment period. The two higher-dosed animals were then given the intermediate dose on day 2, and the highest-dosed animal received its full dose from day 3 onwards. The control treatment consisted of the application of a similar amount of silage mixture without pilocarpine.

Each treatment run consisted of 4 weeks: during week 1, the animals were kept as a group without treatment (10 × 5 m², half the area with straw bedding), fed with hay for ad libitum consumption. In week 2, they were provided with 60 kg of hay per day
3. Saliva and methane in cattle

for the whole group and individually fed the respective pilocarpine dose. In week 3, the animals were transferred to individual places in tie-stall barn (2 × 1.33 m² area of rubber mat with chopped straw bedding), receiving a fixed daily amount of hay and their pilocarpine treatment. In week 4, the procedures applied in week 3 were continued, but the straw bedding was removed. Exact food and water intake was recorded in weeks 3 and 4, and total faeces and urine were collected during week 4. In the last 2 days of week 4, the cows were placed into respiration chambers (RC).

The animals were fed the same hay during the whole experiment and were given daily 100 g mineral-vitamin supplement (Künzle Farma AG, Oberaach, Switzerland) and 50 g salt (Schweizer Salinen AG, Pratteln, Switzerland). In weeks 3 and 4, the amount of hay fed per animal was assigned according to their metabolic body weight (body weight0.75), aimed to maintain the body weight and reduce the effect of intake on the measurements. The total amount of hay allotted to each animal per day was distributed into three portions, which were offered to the animals after the three daily dosages of pilocarpine or placebo. Body weight was measured before and at the end of each 4-week run using a vehicle scale (± 20 kg). Water was provided for ad libitum access during the whole experiment from automated self-drinkers.

2.2 | Sampling

The amounts of feed offered and of leftovers were recorded daily during week 3 and 4. Water flow meters (GWF MessSysteme AG, Luzern, Switzerland) installed on each individual water pipe to the water trough allowed recording the daily individual water consumption of the animals during week 4. Representative samples of hay, silage mixture and individual leftovers were taken daily. After each run, these samples were pooled per cow, dried at 60°C for approximately 18 h and milled through a 0.75 mm sieve for later analysis.

During week 4, the entire faeces were collected in trays under the grid at the rear end of the tie stalls. Faeces were either pushed through the grid or collected into an additional container regularly, often in synch with the sampling for the passage marker. The total amount of faeces was recorded and representative samples (10%) were taken and frozen immediately. Urine was separated from faeces with urinals custom-made from diving suits attached around the vulva of the cows and fixed by hook-and-loop fastener straps glued (Ergo 5011; Kisling AG, Wetzikon, Switzerland) onto the skin.
3. Saliva and methane in cattle

The urinals were connected through a pipe to a canister on the ground for total urine collection, with an additional outlet to a 1-L bottle for collecting acidified urine samples. The bottles were first filled with 30 mg of 50% sulfuric acid for later nitrogen determination. In the course of each sampling day, the bottles were changed and filled with 30 mg of 10% sulfuric acid for later determination of purine derivates. Determination of total faecal and urinal amounts and sampling was also accomplished in the final 2 days of week 4 in the respiration chambers. Faecal and urine samples were pooled later to one sample per cow and treatment. The faecal samples were dried at 60°C overnight and milled through a 0.75 mm sieve for later analysis. Feed intake and leftover amount data were based on weeks 3 and 4, while faecal amount was based on week 4; dry matter (DM) intake (DMI) and amounts digested to be related to the respiratory data were based on 5 days (2 days in the RC and 3 days before entering).

To measure MRT, Co-EDTA, Cr-mordanted fibre and La-mordanted fibre were applied as markers for fluid, 2-mm particle and 1-cm particle, respectively. The markers were given together in the morning of the day 1 of week 4, and faeces were collected regularly during the whole week. Marker preparation and the sampling regime were described in detail by Zhang et al. (2022). Because we expected differences especially soon after marker feeding, the collection interval was set to 2 h for day 1. The MRT through the entire GIT and the RR was calculated according to Thielemans et al. (1978), Grovum and Williams (1973) and Huhtanen and Kukkonen (1995), described in detail in Zhang et al. (2022). Selectivity factors were calculated as ratio of MRTparticles/MRTfluids and ratio of MRTlarge particles/MRTsmall particles in GIT or RR. Dry matter gut fill was calculated following Holleman and White (1989) considering DMI, apparent DM digestibility, and the MRT GIT of the 1-cm particle marker (La).

2.3 | Chewing activity and reticular motility

The chewing activity was monitored using a noseband pressure sensor (MSR Electronics GmbH, Seuzach, Switzerland) as described by Braun, Trösch, Nydegger, and Hässig (2013). Data collection and calculation of the chewing data are described in detail in Zhang et al. (2022). The reticular motility of cattle was monitored using a mobile ultrasound system (DP-50Vet; Mindray Bio-Medical Electronics Co. Ltd., Shenzhen, China) by counting contractions of reticulum within 3 min at 1 h and 4 h
3. Saliva and methane in cattle

after receiving a pilocarpine or a placebo dose.

2.4 | Respiration chamber measurements

The individual animals’ gas exchange data were measured in four RC (No Pollution Industrial Systems Ltd., Edinburgh, United Kingdom). The RC volume was about 40.9 m³ in total, a cuboid room with an additional space of about 2.9 m³ in the back of the RC beneath the floor for collection of faeces and urine. The size of the tie stalls, also equipped with rubber mats, was similar to those used in the days before. The RC were equipped with a tie stand with rubber mat (1.9×1.3 m²), tubular steel sides to the standing, a feed bin and water through. The chamber walls consisted of glass panels on both sides so the animals could see each other. The light program was set automatically changing the light intensity every 4 h, 50% of max light intensity at 4 am to 90% of max light intensity at 12 am and at last turned off at 12 pm.

The animals were familiarized with the chambers before the first experimental run. For the measurements, the cows were moved to the chambers for 48 h directly after the morning collection of faeces and urine, and the urinals were attached again in the RC. The RC were opened three times a day for feeding, pilocarpine dosing and faeces collection for passage marker analysis, which took less than 10 min in total. In the mornings, after 1 day and 2 days in RC had passed (the latter after removal of the animals), all faeces and urine were removed completely, and the total amounts were recorded. The chamber doors were kept closed during these activities and only opened for personnel to enter and exit or pass out the faecal trays and urine canisters.

The temperature was maintained between 10.9 and 18.1°C and the relative humidity was 60±15%. Spent air was extracted at rates of 39.6 to 40.7 L/s (equivalent to an air exchange of about 3.5 times the chamber volume per h) with an extraction fan (K06-MAS Blower, FPZ Blower Technology, Concorezzo, Italy), coupled with a frequency controller (VLT 3.3 Kw, HWAC Drive, Danfoss, Offenbach, Germany); this pulled fresh air into the RC. The system was maintained at a slight negative pressure. The concentrations of O₂, CO₂ and CH₄ in fresh and extracted air were determined with an MGA 3500 (ADC Gas Analysis Ltd., Hertfordshire, UK) using nondispersive infrared absorption and an electrochemical oxygen sensor, respectively, in a 10-min sample cycle for each RC. Calibrations were performed directly before onset of measurement and in the morning of the second day of each experimental run.
3. Saliva and methane in cattle

Subsamples of ingoing and outgoing air were pumped to the analyzer and the gas composition was measured. A recovery test (total calibration) was performed three times during the experiment for each RC. While data collection was performed as described, CH$_4$ (99.9%; PanGas AG, Dagmersellen, Switzerland) was injected at 0.36 NLPM (normal litre per minute) via a tube through the outside wall into the empty RC for 4 h. The measured CH$_4$ concentration reached a plateau after 2 to 2.5 h. The flow rate was controlled by a mass flow controller (MC-5SLPM-RD, Alicat Scientific, Tucson, AZ, USA). The recovery test provided a recovery factor for each RC and experiment round, which was used to adjust the data. The recovery rates (average values plus standard deviation) for CH$_4$, CO$_2$ and O$_2$ were 96.5±1.1%, 83.2±0.9% and 100.0±0.5%, respectively.

Respiration chamber data were first standardized for temperature, barometric pressure and moisture (Verstegen, Van der Hel, Brandsma, Henken, & Bransen, 1987; Hellwing, Lund, & Weisberg, 2014). Subsequently, the flow rate of incoming air was estimated based on the principle that N$_2$ is inert, i.e., entering and leaving the RC in the same amount, where the fractional concentration of N$_2$ was calculated as 1-O$_2$-CO$_2$-CH$_4$. Using incoming and outcoming air flow rates and the respective concentrations, gas production or consumption was calculated by subtraction. This approach yields identical results to that proposed by Lighton (2018) for pull-systems, even though the two sets of equations cannot be transformed into one another.

2.5 | Assessment of rumen fluid composition and in vitro gas production

The last dose of pilocarpine of each run was administered at 0600 in the RC, but the animals were not fed with hay at this time to facilitate rumen fluid sampling by oesophageal tubing. The animals were taken out of the chambers at 0800, led to the barn, fixed using the self-locking feed barrier and cattle head support. Rumen fluid was collected using a rumen fluid extractor (a metal spiral tube connected with a suction head, H. Hauptner und Richard Herberholz GmbH&Co. KG, Solingen, Germany), which was via T-connection attached to both, a containing bottle and a milk-line vacuum in the barn. The first 200 mL of rumen fluid was discarded to minimize contamination with saliva. A total of 500 mL rumen fluid was collected from each cow and stored in a prewarmed thermos bottle. The rumen fluid was then strained through four layers of cheesecloth. Samples were taken for measurement of pH and ammonia
3. Saliva and methane in cattle

concentration. About 15 mL of samples were centrifuged at 4000 g for 5 min at 24°C (Centrifuge 5810R, Eppendorf AG, Hamburg, Germany). The supernatant was taken for later SCFA analysis.

The remaining strained rumen fluid was used for incubation in a Hohenheim Gas Test (HGT) apparatus. The hay used for HGT was the same as that fed to the animals and milled through a 1 mm sieve. Four 1-L glass bottles were prepared, each containing a buffer solution and rumen fluid from the assigned cow. The mixture was prepared according to Menke et al. (1979), with the ratio of the volume of rumen fluid to medium 1:2. Each bottle was equipped with a centrifuge stirrer and a rubber tube connected with an extended individual CO₂ cylinder. All four bottles were set in the same water bath, with a thermostat to maintain the water temperature as 39°C, and clamp holders and stand clamps to fix the bottles. For each bottle, four replicates of HGT syringes with about 200 mg hay as well as two blank syringes without substrates were used. The syringes were prepared, filled and set into the incubator as described by Soliva and Hess (2007). The incubation lasted for 24 h at 39°C. The volume of fermentation gas produced was read after 8 and 24 h. After 24 h, the incubation was stopped and fermentation gas sampled through the outlet covered with polyfluoroethylene-layer.

2.6 | Laboratory analyses

Hay, silage mixture, leftovers and faeces were analysed according to the standard methods of the Association of German Agricultural Analysis and Research Centers (VDLUFA, 2006) for DM, organic matter (OM), crude protein (CP; N × 6.25), ether extract (EE), crude fibre (CF) as well as neutral detergent fibre (NDF) and acid detergent fibre (ADF). The NDF was analysed after adding amylase, and NDF and ADF were corrected for residual ash. The N in acidified urine was analyzed with a C/N analyser (TruMac CN, Leco Corporation, St. Joseph, Michigan, USA; AOAC index no. 968.06) (AOAC, 2016). The metabolizable energy (ME) of the hay was calculated according to equation of GfE (1995):

\[
ME (MJ) = 0.0312 \times digestible \ EE \ (g) + 0.0136 \times digestible \ CF \ (g) + 0.0147 \times (digestible \ OM - digestible \ EE - digestible \ CF) \ (g) + 0.00234 \times CP \ (g).
\]

Measurement of urinary purine derivates (PD) were conducted by reverse-phase
3. Saliva and methane in cattle

HPLC (Prominence LC-20A, Shimadzu Europe GmbH, Duisburg, Germany) coupled to the SPD-M10Avp (DAD) detector according to the method of Shingfield and Offer (1999). The column used was Spherisorb ODS 2 C18-RP (5 µm, 4.6 × 250 mm; Waters GmbH, Eschborn, Germany). The microbial N was estimated according to Chen and Ørskov (2004), using the following equations:

\[
\text{Microbial N (g)} = \frac{PD \text{ absorption} (\text{mmol/d}) \times 70}{0.116 \times 0.83 \times 1000} = 0.727 \times PD \text{ absorption (mmol/d)}.
\]

\[
PD \text{ absorption (mmol/d)} = (PD \text{ excretion in the urine (mmol/d)} - 0.385 \times BW^{0.75}) + 0.85.
\]

As an additional, less sophisticated proxy for microbial growth, metabolic faecal N (MFN) was analyzed as described by Steuer et al. (2014). For this, the NDF-N was quantified in faeces. Then MFN was calculated as total faecal N minus NDF-N.

Ammonia concentration and pH value of the rumen fluid were determined with a potentiometer (pH: model 913; ammonia: model 713; Metrohm AG, Herisau, Switzerland). Short-chain fatty acids (SCFA) were analysed with high-performance liquid chromatography (LaChrom L 7000, Hitachi, Tokyo, Japan) equipped with an UV detector, using the column HPX-87H (7.8 × 300 mm; Bio-Rad Laboratories, Hercules, California, USA). Composition of gas samples collected from HGT syringes were analysed with a gas chromatograph (Agilent 6890N Network Gas Chromatograph, Agilent Technologies, Wilmington, DE, USA) equipped with a thermal conductivity detector and a flame ionisation detector. The column (Carboxen-1000, Fluka, Buchs, Switzerland) was 4.5 m × 2.1 mm in size, and argon was used as a carrier gas.

2.7 | Statistical analysis

Data were analyzed using a linear mixed model using R version 3.5.2 with treatments as fixed factor and animal and experimental run as random factors. Orthogonal polynomial contrasts (linear, quadratic, and cubic) were used to test the effect of pilocarpine dosages on concerning variables. An effect is declared significant for \( p \leq 0.05 \) and declared a trend if \( 0.05 < p < 0.10 \). Results are presented as arithmetic means and standard deviations.
3 | RESULTS

3.1 | Intake, digestibility and chewing behavior

The nutrient composition (g/kg dry matter) of the grass hay and of the silage mixture fed during the experiment was OM, 917 and 910; CP, 150 and 129; EE, 23 and 32; NDF, 595 and 411; ADF, 316 and 273; CF, 279 and 232, respectively. The animals maintained a relatively constant body weight during the experiment irrespective of pilocarpine dosage (Table 3.1). No significant effects of treatments were observed for DM and ME intake. However, water intake linearly and quadratically increased when pilocarpine dosage was increased ($p = 0.011$ and $0.034$, respectively). Faecal and urine output were not affected by pilocarpine. When receiving the highest pilocarpine dose, the animals appeared, subjectively, mildly depressed, which was visually characterized by continuous drooling of salivation and apparently more liquid faeces. This was indicated by linearly and quadratically decreased DM contents of the hay residues ($p = 0.001$ and 0.042, respectively) and linearly decreased DM contents of faecal samples ($p = 0.002$) when pilocarpine dosage was increased (Table 3.1). No effects were detected for the apparent digestibility of DM, OM, NDF, ADF and CF. The ingestion chews and rumination chews per DMI were not affected by treatment. However, increasing the pilocarpine dosages linearly decreased the rumination chews per minutes ($p = 0.034$) and tended to linearly decrease the ingestion chews per minutes ($p = 0.061$). The ratio of rumination to ingestive mastication was not affected by treatment (Table 3.1).

3.2 | Digesta retention, selectivity factor, total gut fill and reticular contractions

Increasing pilocarpine dosages linearly and quadratically decreased the MRT$_{\text{solute}}$ in the total tract ($p < 0.001$ and $= 0.019$, respectively; Figure 3.1a); for the average of pilocarpine treatments, MRT$_{\text{solute}}$ was shorter than for the control at a magnitude of 7.8% (Table 3.2). A linear relationship between pilocarpine dosages and MRT$_{2\text{mm particles}}$ was found ($p = 0.046$; Figure 3.1b), while between pilocarpine dosages and MRT$_{1\text{ cm particles}}$, there was only a trend of a linear relationship ($p = 0.095$; Figure 3.1c). In terms of MRT in the RR, no significant relationship was detected for solutes, 2 mm and 1 cm particles. The MRT in the distal GIT linearly and quadratically decreased when pilocarpine dosages were increased ($p = 0.018$ and 0.044, respectively). The selectivity factors were
3. Saliva and methane in cattle

not affected by treatment (Table 3.2). Total dry matter gut fill tended to linearly decrease with pilocarpine dosages ($p = 0.086$). The contractions of the reticulum per 3 min measured at 1 h after pilocarpine application showed no treatment effect, while at 4 h after treatment, there was a linear correlation with pilocarpine dosages, with more contractions at higher dosages ($p = 0.049$).

3.3 Microbial N yield, N balance and CH$_4$ emissions

The microbial N yield in the rumen, estimated by the concentration of urinary purine derivates, was not affected by pilocarpine. However, the MFN concentration linearly increased with pilocarpine dosages ($p = 0.020$) (Table 3.3). The daily N excretion via faeces tended to quadratically correlate with pilocarpine dosages ($p = 0.058$). The daily N intake and excretion via urine was not affected by treatment (Table 3.3).

The mean absolute daily CH$_4$ was not significantly affected by treatment, while CH$_4$ decreased linearly when expressed per intake or digested amounts of DM, OM, NDF and ADF when pilocarpine dosages were increased ($p < 0.05$; Table 3.4). No quadratic and cubic relationship were found between pilocarpine and these CH$_4$ variables. Methane emission per digested DM was reduced on a magnitude of 6.5\% for the average of pilocarpine treatments (Table 3.4). There was no significant correlation between CH$_4$ yield and microbial N yield per digested DM ($p = 0.348$), or between CH$_4$ yield and MFN output per digested DM ($p = 0.142$).

3.4 Rumen fluid characteristics and fermentation gas production

The main variables of the rumen fluid collected, including pH, SCFA concentration and composition showed no significant relationship with pilocarpine dosages, while the ammonia concentration tended to linearly decrease when pilocarpine dosages increased ($p = 0.085$; Table 3.5). The total fermentation gas produced within 24 h showed a linear negative relationship with pilocarpine treatments ($p = 0.033$), while gas production after 8 h of incubation and production of CH$_4$ and CO$_2$ within 24 h were not correlated with pilocarpine.
### Table 3.1

Effect of graded levels of pilocarpine on body weight, intake and leftovers, excreta output, apparent digestibility and chewing behavior. Values are arithmetic means ± standard deviation; n = 4 for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>Contrast (p-value)$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>748±79</td>
<td>745±84</td>
</tr>
<tr>
<td>Intake (kg/day)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>12.4±1.0</td>
<td>12.3±1.3</td>
</tr>
<tr>
<td>Water</td>
<td>58.8±10.8</td>
<td>58.6±10.2</td>
</tr>
<tr>
<td>ME (MJ)</td>
<td>117±9</td>
<td>117±15</td>
</tr>
<tr>
<td>Hay leftovers (kg DM/day)</td>
<td>0.5±0.3</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td>Faecal output (kg DM/day)</td>
<td>4.5±0.5</td>
<td>4.3±0.4</td>
</tr>
<tr>
<td>Urine output (kg/day)</td>
<td>15.7±2.5</td>
<td>16.3±1.3</td>
</tr>
<tr>
<td>DM content (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay leftovers</td>
<td>67.7±10.8</td>
<td>64.7±10.1</td>
</tr>
<tr>
<td>Faeces</td>
<td>13.4±0.7</td>
<td>12.8±1.5</td>
</tr>
<tr>
<td>Apparent digestibility (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>64.2±2.7</td>
<td>65.3±2.0</td>
</tr>
<tr>
<td>OM</td>
<td>67.4±2.7</td>
<td>68.2±2.3</td>
</tr>
<tr>
<td>NDF</td>
<td>71.4±2.4</td>
<td>72.0±1.5</td>
</tr>
<tr>
<td>ADF</td>
<td>64.8±4.8</td>
<td>65.7±3.5</td>
</tr>
<tr>
<td>CF</td>
<td>74.7±3.1</td>
<td>75.1±4.1</td>
</tr>
<tr>
<td>Chewing behavior$^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ingestion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chews per kg DMI</td>
<td>1693±142</td>
<td>1893±481</td>
</tr>
<tr>
<td>Chews per min</td>
<td>67.9±4.7</td>
<td>67.0±4.0</td>
</tr>
<tr>
<td>Rumination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chews per kg DMI</td>
<td>2672±957</td>
<td>2614±588</td>
</tr>
<tr>
<td>Chews per min</td>
<td>66.5±10.2</td>
<td>66.6±9.3</td>
</tr>
<tr>
<td>Ratio of rumination:ingestion time</td>
<td>1.6±0.4</td>
<td>1.4±0.3</td>
</tr>
</tbody>
</table>

ADF, acid detergent fibre corrected for residual ash; CF, crude fibre; DM, dry matter; DMI, dry matter intake; ME, metabolizable energy; NDF, neutral detergent fibre corrected for residual ash, with heat stable amylase; OM, organic matter.

$^1$ Contrasts: L = linear, Q = quadratic, C = cubic.

$^2$ Data on chewing behaviour were based on dry matter intake during measurement days.
## Table 3.2 Effect of graded levels of pilocarpine on mean retention time in the gastrointestinal tract, selectivity factor, gut fill and reticular contractions. Values are arithmetic means ± standard deviation; n = 4 for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>Contrast (p-value)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Mean retention time (h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solutes GIT</td>
<td>27.2±3.1</td>
<td>25.7±3.0</td>
</tr>
<tr>
<td>2 mm GIT</td>
<td>48.2±4.9</td>
<td>47.4±5.4</td>
</tr>
<tr>
<td>1 cm GIT</td>
<td>57.0±6.5</td>
<td>56.2±5.7</td>
</tr>
<tr>
<td>Solutes RR</td>
<td>13.3±1.4</td>
<td>13.7±0.6</td>
</tr>
<tr>
<td>2 mm RR</td>
<td>34.4±3.2</td>
<td>35.5±3.2</td>
</tr>
<tr>
<td>1 cm RR</td>
<td>43.2±4.9</td>
<td>44.3±3.5</td>
</tr>
<tr>
<td>Distal GIT</td>
<td>13.8±1.8</td>
<td>11.9±2.6</td>
</tr>
<tr>
<td>Selectivity factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mm/solute GIT</td>
<td>1.78±0.05</td>
<td>1.85±0.07</td>
</tr>
<tr>
<td>1 cm/2 mm GIT</td>
<td>1.18±0.02</td>
<td>1.19±0.03</td>
</tr>
<tr>
<td>2 mm/solute RR</td>
<td>2.58±0.08</td>
<td>2.58±0.16</td>
</tr>
<tr>
<td>1 cm/2 mm RR</td>
<td>1.25±0.03</td>
<td>1.25±0.03</td>
</tr>
<tr>
<td>Total DM gut fill (kg DM)</td>
<td>20.1±3.2</td>
<td>19.5±3.4</td>
</tr>
<tr>
<td>Reticular contractions per 3 min</td>
<td>4.3±0.5</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>1 h after dosage</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
</tr>
</tbody>
</table>

GIT, gastrointestinal tract; RR, reticulum rumen.

¹Contrasts: L = linear, Q = quadratic, C = cubic.
3. Saliva and methane in cattle

Table 3.3 Effect of graded levels of pilocarpine on microbial nitrogen (N) yield and N balance. Values are as arithmetic means ± standard deviation; n = 4 for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Microbial N yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/day</td>
<td>116±11</td>
<td>117±3</td>
</tr>
<tr>
<td>g/kg digested DM</td>
<td>14.5±1.6</td>
<td>14.7±1.6</td>
</tr>
<tr>
<td>MFN (g/kg DM)</td>
<td>17.8±0.9</td>
<td>18.0±0.8</td>
</tr>
<tr>
<td>N balance (g/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N intake</td>
<td>299±56</td>
<td>296±66</td>
</tr>
<tr>
<td>Total faecal N output</td>
<td>112±12</td>
<td>108±11</td>
</tr>
<tr>
<td>MFN output</td>
<td>79±8</td>
<td>77±8</td>
</tr>
<tr>
<td>Urinary N</td>
<td>119±39</td>
<td>118±30</td>
</tr>
</tbody>
</table>

MFN, metabolic faecal N.

\(^1\)Contrasts: L = linear, Q = quadratic, C = cubic.
### Table 3.4 Effect of graded levels of pilocarpine on methane (CH\textsubscript{4}) emissions. Values are arithmetic means ± standard deviation; n = 4 for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>Contrast (p-value)\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>CH\textsubscript{4} per day</td>
<td></td>
<td>L</td>
</tr>
<tr>
<td>g</td>
<td>332±33</td>
<td>321±52</td>
</tr>
<tr>
<td>g/BW\textsuperscript{0.75}</td>
<td>2.33±0.25</td>
<td>2.25±0.24</td>
</tr>
<tr>
<td>CH\textsubscript{4} per nutrient intake (g/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>26.5±2.8</td>
<td>25.7±2.4</td>
</tr>
<tr>
<td>OM</td>
<td>28.7±3.0</td>
<td>27.9±2.5</td>
</tr>
<tr>
<td>NDF</td>
<td>45.0±5.5</td>
<td>43.7±4.6</td>
</tr>
<tr>
<td>ADF</td>
<td>84.0±9.4</td>
<td>81.5±8.1</td>
</tr>
<tr>
<td>CH\textsubscript{4} per digested nutrient (g/kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>41.0±3.2</td>
<td>39.2±4.0</td>
</tr>
<tr>
<td>OM</td>
<td>42.5±3.7</td>
<td>40.8±4.2</td>
</tr>
<tr>
<td>NDF</td>
<td>62.7±5.3</td>
<td>60.3±6.1</td>
</tr>
<tr>
<td>ADF</td>
<td>130±19</td>
<td>124±16</td>
</tr>
</tbody>
</table>

ADF, acid detergent fibre corrected for residual ash; BW, body weight; DM, dry matter; NDF, neutral detergent fibre corrected for residual ash, with heat stable amylase; OM, organic matter.

\textsuperscript{1}Contrasts: L = linear, Q = quadratic, C = cubic.
3. Saliva and methane in cattle

Table 3.5 Effect of graded levels of pilocarpine on rumen fluid characteristics and on gas production in the Hohenheim Gas Test. Values are arithmetic means ± standard deviation; n = 4 for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>Contrast (p-value)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Rumen fluid properties (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.15±0.07</td>
<td>7.23±0.24</td>
</tr>
<tr>
<td>NH₃</td>
<td>9.6±3.3</td>
<td>9.2±4.7</td>
</tr>
<tr>
<td>Total short-chain fatty acids (SCFA)</td>
<td>98.4±29.41</td>
<td>92.4±28.22</td>
</tr>
<tr>
<td><strong>Individual SCFA (% of total SCFA)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>72.5±3.0</td>
<td>72.0±4.5</td>
</tr>
<tr>
<td>Propionate</td>
<td>17.1±3.7</td>
<td>17.1±5.0</td>
</tr>
<tr>
<td>Iso-butyrate</td>
<td>0.8±0.3</td>
<td>1.2±0.5</td>
</tr>
<tr>
<td>Butyrate</td>
<td>7.6±1.7</td>
<td>7.8±1.9</td>
</tr>
<tr>
<td>Iso-valerate</td>
<td>1.1±0.4</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.9±0.4</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>Acetate to propionate ratio</td>
<td>4.39±1.10</td>
<td>4.48±1.24</td>
</tr>
<tr>
<td><strong>Gas produced (ml/200 mg DM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total in 8 h</td>
<td>16.2±1.9</td>
<td>14.7±4.3</td>
</tr>
<tr>
<td>Total in 24 h</td>
<td>39.9±1.5</td>
<td>38.5±2.1</td>
</tr>
<tr>
<td>CH₄ in 24 h</td>
<td>6.0±0.5</td>
<td>6.4±1.4</td>
</tr>
<tr>
<td>CO₂ in 24 h</td>
<td>31.9±0.7</td>
<td>31.3±2.9</td>
</tr>
</tbody>
</table>

DM, dry matter.

¹Contrasts: L = linear, Q = quadratic, C = cubic.
Figure 3.1 The effect of pilocarpine treatment (0, 1, 2.5 and 5 mg/kg BW) on faecal marker elimination pattern: (a) solute marker (Co); (b) 2 mm particle marker (Cr); (c) 1 cm particle marker (La). Faecal markers were collected for totally 7 days.
4 | DISCUSSION

The results of the present study agree with previous *in vitro* findings that increased dilution rates, equivalent to shorter solute marker retention in live animals, are linked with a reduction of CH$_4$ yield. While the magnitude found in this study was smaller than expected from the *in vitro* assays, it should be kept in mind that the change in dilution rate in the *in vitro* studies was also considerably larger than the increase induced by the saliva stimulant *in vivo* in the present study. In contrast to our expectations based on the *in vitro* and previous *in vivo* studies, only little indication for an increase in microbial yield as a potential link between passage rate and methane production was detected. The major limitations of this study are: (i) a replicated Latin square design would have provided more statistical power (but was beyond the logistically realizable); (ii) the pharmacologically induced saliva might not be able to increase the fluid dilution rate to the degree of that *in vitro*. Thus, in the following, we discuss these limitations, and how our results relate to the current knowledge of cattle digestive physiology.

4.1 | Pilocarpine as a saliva stimulant

Pilocarpine is a parasympathomimetic agonist widely used in human medicine; it is most likely absorbed through the rumen wall after ingestion. Pilocarpine acts on muscarinic receptors and it stimulates secretion by exocrine glands such as the salivary, sweat, lacrimal and respiratory mucous glands (Braga et al., 2009).

Pilocarpine and slaframine, another parasympathomimetic agonist, have been shown to increase saliva secretion (of the parotid and mandibular gland) and saliva flow through the oesophagus in goats, cattle and sheep (Castellano et al., 1986; Froetschel et al., 1987; Jacques, Harmon, Croom, & Hagler, 1989; Bird et al., 1993). Individual differences in reaction to these drugs occur. Gurnsey, Jones, and Reid (1980) concluded that pilocarpine does not always stimulate saliva flow and they found that the salivation of one individual cattle was actually inhibited by pilocarpine. Estimation of saliva flow obviously represents a challenge and no objective direct measure of saliva production or flow was available in the present study. Thus, we could only gauge the effect by (i) subjective visual observation of saliva drooling; (ii) DM content of feed leftovers, which supported the visual impression of saliva drooling; (iii) more watery faeces; (iv) higher water expenditure. An increased water intake may have represented a reaction
to increased saliva and faecal water losses, or attempts to clear the oral cavity of the excessive saliva.

The visual impression of saliva drooling took only place at the highest dosage, where also water expenditure was significantly increased, but not at the lower dosages. We initially planned to apply a higher dose, which had been approved by the experimental licence; however, the first application led to severe behavioral depression, so that – subjectively – we think that pilocarpine cannot be dosed higher in cattle, at least not when divided into only three individual doses per day. The behavioral depression was, to our subjective observation, still visible at the highest dose applied in the present study, which was more objectively also supported by a lower chewing frequency at this dosage. Thus, investigating the effects of an increased liquid flow rate experimentally in vivo using pharmacological stimulation might be limited, and continuous infusion of artificial saliva in fistulated animals (Harrison et al., 1975) might be required for further proof of concept studies.

4.2 | Importance of feed intake and diet for pilocarpine effects

Pilocarpine or slaframine have induced salivary excretion in several animal experiments, which subsequently increased fluid dilution rate (Table 3.6). Inconsistent results across studies on digesta retention, digestibility, microbial yield and rumen fluid might be due to different dosages, feeding regimes, or individual animal predisposition. Feed intake and diet type are the primary factors that influence the respective variables. Therefore, to prevent ambiguity of interpretation with respect to an effect of saliva stimulant vs. increased intake, we applied a restricted feeding level in the present study. The absence of body weight changes indicates that the intake level was sufficient for covering maintenance requirements. Other studies showed either that the application of a saliva stimulant had no effect on intake, or the intake was also controlled in the experiment.

With respect to the influence of diet type, the study of Wiedmeier et al. (1987b) is indicative. This study stands out due to the clear effects of the saliva stimulant, with a 44% reduction in MRT fluid, and a concomitantly increased digestibility of DM and cellulose, while in no other study a comparable effect was reported (Table 3.6). This is likely due to the relatively high amount of concentrate used in that study — when the saliva stimulant was applied, the ruminal pH increased significantly because of the
buffering effect of saliva, which supports fibre fermentation. In those studies where the pH of the control group (without saliva stimulant) was below 6.4, the application of the saliva stimulant increased ruminal pH, while when the control group pH was higher than 6.5, the values remained constant. In studies which used larger amounts of forages in the diet, where animals intensively ruminate and hence already salivate more on the control treatment, the buffering effect of saliva may be far less relevant. Therefore, it was expected that in our own experiment with a forage-only diet digestibility and rumen fluid pH were not affected by applying pilocarpine.
### Table 3.6 Collection of studies that manipulated saliva inflow into the reticulorumen

<table>
<thead>
<tr>
<th>Source</th>
<th>Treatment</th>
<th>Highest dose (\mu g/kg) (portions/day)</th>
<th>Species</th>
<th>F prop.</th>
<th>Ruminal retention time Fluid</th>
<th>Part.</th>
<th>Digestibility DM</th>
<th>Cell.</th>
<th>Microbial yield Total</th>
<th>Rumen fluid</th>
<th>pH(^1)</th>
<th>NH(_4^+)</th>
<th>SCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>Saliva infusion</td>
<td>-</td>
<td>S</td>
<td>90</td>
<td>⬇️ (45.0%)</td>
<td>-</td>
<td>-</td>
<td>(15.9%)</td>
<td>(6.01)</td>
<td>-</td>
<td>Propionate ⬇️ (16.9%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b)</td>
<td>Slaf</td>
<td>48</td>
<td>S</td>
<td>n.a.</td>
<td>NS</td>
<td>-</td>
<td>(♀️) (27%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>b)</td>
<td>Slaf</td>
<td>24 (3)</td>
<td>C</td>
<td>91</td>
<td>⬇️ (20.6%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>⬆️ (6.26)</td>
<td>-</td>
<td>(♀️) (15.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c)</td>
<td>Slaf</td>
<td>20 (2)</td>
<td>C</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>(♀️) (16.5%)</td>
<td>NS (6.55)</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>d)</td>
<td>Slaf</td>
<td>24 (3)</td>
<td>C</td>
<td>50</td>
<td>⬇️ (4.2%)</td>
<td>-</td>
<td>⬆️ (47.2%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NS (6.57)</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>e)</td>
<td>Slaf</td>
<td>8 (2)</td>
<td>S</td>
<td>100</td>
<td>(♀️) (11%)</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>NS</td>
<td>⊖ (6.4)</td>
<td>(♀️) (44.7%)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>f)</td>
<td>Carb</td>
<td>10 (1)</td>
<td>C</td>
<td>50</td>
<td>⬇️ (15.4%)</td>
<td>⬇️ (22.0%)</td>
<td>⬇️ (8.9%)</td>
<td>(♀️) (4.9%)</td>
<td>(♀️) (12.0%)</td>
<td>(♀️) (3.7%)</td>
<td>NS (6.53)</td>
<td>(♀️) (18.9%)</td>
<td>(♀️) (7.1%)</td>
</tr>
<tr>
<td>f)</td>
<td>Pilo</td>
<td>100 (1)</td>
<td>C</td>
<td>50</td>
<td>⬇️ (15.4%)</td>
<td>⬇️ (24.3%)</td>
<td>⬇️ (8.5%)</td>
<td>(♀️) (4.0%)</td>
<td>(♀️) (7.5%)</td>
<td>(♀️) (4.2%)</td>
<td>NS (6.53)</td>
<td>(♀️) (12.8%)</td>
<td>(♀️) (9.1%)</td>
</tr>
<tr>
<td>g)</td>
<td>Pilo</td>
<td>4000 (2)</td>
<td>C</td>
<td>53</td>
<td>⬇️ (44.1%)</td>
<td>⬇️ (46.0%)</td>
<td>NS</td>
<td>⬆️ (9.3%)</td>
<td>⬆️ (9.5%)</td>
<td>NS</td>
<td>⊖ (6.34)</td>
<td>⬇️ (37.7%)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^1\)pH on the control diet (without saliva stimulant) given in brackets; ⬇️/⬆️ means significant decrease/increase (p<0.05); (♀️)/(♂️) means a trend of a decrease/increase. C, cattle; Cell., cellulose or fibre; F prop., forage proportion; Carb., carbachol; n.a., not available; NS, not significant; Pilo., pilocarpine; RR liquid vol., reticular rumen liquid volume; S, sheep; SCFA, short-chain fatty acids; Slaf., slaframine.

3. Saliva and methane in cattle

4.3 | Effects of the saliva stimulant

In our study, we had intended to test the effect of an increase in ruminal fluid throughput on a natural diet, based on our previous speculations that the expected effects might represent an advantage that could have led to the widespread evolution of high fluid throughput in ruminants (Clauss & Hummel, 2017; Przybyło et al., 2019). While its effect on MRT fluid (decrease of 8%) was below our expectations concerning the magnitude, it can still be stated that a relevant decrease was induced. Most previous studies that employed a saliva stimulant successfully increased fluid dilution rate (Table 3.6), except for the sheep experiment of Froetschel et al. (1987) using slaframine. We found that the saliva stimulant also correlated – albeit at a lower significance level – with MRT of small particles, while for MRT of large particles, only a trend was detected. Therefore, we speculate that the decreased MRT of particles was primarily induced by the increased fluid dilution. For studies that measured both fluid and particle retention of cattle, the selectivity factor of particles vs. fluid remained numerically constant, as in our experiment. The selectivity factor is a comparatively fixed characteristic of ruminant physiology; it is species-specific and largely independent of the experimental diet, intake and – as was the case here – also of saliva flow, suggesting that RR morphophysiology and interaction with contents ensures a constant relationship between \( \text{MRT}_{\text{solutes}} \) and \( \text{MRT}_{\text{particles}} \) (Przybyło et al., 2019).

This could be the major cause that contributed to the difference between results obtained \textit{in vitro} and \textit{in vivo}: In animal studies, it seems that a higher fluid throughput is partly linked to a higher particle outflow. By contrast, it is much easier to increase the dilution rate in \textit{in vitro} fermenters while keeping the particle incubation time constant. For example, Pfau et al. (2021) nearly halved the liquid retention in an \textit{in vitro} system (from 64.1 h to 34.5 h of liquid MRT). Theoretically, a change of MRT at such a magnitude in live animals is not unrealistic: For dairy cows with (energy corrected) milk yields < 15 L/d, the MRT RR for concentrates is estimated at 50 h, where it is only 12.5 h for milk yields >30 L/d (Spiekers, Nußbaum, & Potthast, 2009); however, this is evidently linked to a corresponding difference in feed intake.

Besides the direct effect of saliva volume on MRT fluid, changes in muscular activity of the lower gut cannot be excluded as influencing factors. As a parasympathomimetic applied in human medicine, pilocarpine has side effects on smooth muscles and may cause bowel spasm (Brunton, Chabner, & Knollmann, 2018).
3. Saliva and methane in cattle

This seems to be also the case in ruminants. Gurnsey et al. (1980) observed an increased tonus of the RR; by contrast, Froetschel et al. (1986) found that slaframine decreased ruminal contraction frequency in both cattle and sheep. Kelly, McBride, Froetschel, Croom, and Hagler (1991) found that slaframine prolonged the duration of the opening of the reticulo-omasal orifice, which might also contribute to a higher liquid/digesta outflow from the RR. By quantifying reticular contractions in a 3-min period, we detected that pilocarpine increased the reticular contractions 4 hours after application. And in particular, we found that digesta retention time in the GIT beyond the RR, i.e. in the omasum, abomasum, small and large intestine, was decreased by pilocarpine, suggesting an increased peristalsis of one or several of these sections of the digestive tract. Additionally, the higher faecal water content at higher doses suggests less water absorption in the spiral colon, which could be due to a higher motility in the lower GIT. Unfortunately, we could not assess the effect on the motility of the lower GIT directly. The fact that in spite of the detected higher peristalsis of the reticulum, no effect on the MRT of the RR was detected, suggests that a major effect of the pilocarpine was on the lower digestive tract.

No other studies have tested the effect of saliva stimulant on chewing activity. We found that even though there was an effect on chewing frequency (chews per min), chewing intensity (chews per DMI) was not affected. As discussed in Zhang et al. (2022), our results showed that chewing behavior varies much more between individuals than between treatments.

Most of the studies that applied a saliva stimulant reported no effect on SCFA concentration and profile in rumen fluid. Harrison et al. (1975) increased dilution rate by intraruminal infusion of artificial saliva and found that the proportion of propionate concomitantly decreased; this was also reported by Wiedmeier et al. (1987a). However, considering the effect of induced liquid dilution, the yield (rather than the concentration) of SCFA might increase, but this would have to be measured by more invasive methods.

Wiedmeier et al. (1987b) and Bird et al. (1993) reported a decreased ammonia concentration in rumen fluid (which can be interpreted as an indication of a reduced ruminal protein degradation or more ammonia use by more microbial growth), while Jacques et al. (1989) and Froetschel et al. (1989) detected no such effect. Our results similarly indicated a trend of linearly decreased ammonia concentrations in rumen fluid when pilocarpine dosages were increased, while microbial yield indicated by purine
3. Saliva and methane in cattle
derivatives were not affected. As another microbial N indicator, MFN concentration increased with increasing pilocarpine dosages. This should not be interpreted as an effect in the hindgut, but likely derives from a higher outflow of microbial N from the RR (Lukas, Südekum, Rave, Friedel, & Susenbeth, 2005). While MFN has long been used as a proxy of OM digestibility, i.e. more energy leading to more microbial production (Steuer et al., 2014), this value should react due to any change in microbial output, irrespective of the reason for more intensive microbial growth (increase in digestibility or, in our case, increase of dilution rate). In several other studies it was reported that microbial mass or microbial synthesis efficiency seemed to increase due to a saliva stimulant, but for most of these the effects were not statistically significant (Table 3.6). The reported magnitudes of increased microbial yield measures were comparable with our MFN finding, except for Wiedmeier et al. (1987b) where it was higher. Additionally, Bird et al. (1993) found that for sheep on a forage-only-diet, the ruminal cellulolytic bacteria numbers per unit of rumen fluid increased on slaframine, while total bacteria and protozoa counts were not affected.

Isaacson et al. (1975) and Blümmel et al. (1997) indicated a reverse relationship of in vitro gas production and microbial yield per unit of truly degraded substrate. Theoretically, microbial cell synthesis consumes metabolic hydrogen, which is a precursor for methane production by methanogens – therefore, increasing microbial yield may potentially help to decrease methane emission (Ramin & Huhtanen, 2013). The key factor triggered by increasing feed intake might be the increased digesta flow rate. In the present study, in which the feed intake level was kept constant, no reverse relationship between microbial yield and methane yield was evident.

Nevertheless, irrespective of the limited evidence for a change in microbial protein production, methane yield was reduced by 5%. Our in vitro gas production study showed that this was not resulting from a direct action of the molecule pilocarpine. Since fibre digestibility was not influenced in the cattle by the pilocarpine treatment, variation in digestibility can be safely ruled out as an explanation for the CH$_4$ mitigation in the present study. Sheep characterized by a shorter retention time in the RR have a lower CH$_4$ yield (Pinares-Patiño et al., 2011; Goopy et al., 2014). However, studies where liquid or digesta retention were experimentally modified to investigate the effect on CH$_4$ in live animals are rare. We found that by application of a saliva stimulant in cattle, the CH$_4$ emission significantly decreased concomitantly to a decreased liquid
3. Saliva and methane in cattle

Retention time, though at a much smaller magnitude compared to our expectation based on the *in vitro* study of (Pfau et al., 2021). It must be kept in mind that the change in dilution rate in that *in vitro* study was much higher than what could be realized in the present *in vivo* study. Pfau et al. (2021) found that the decreased liquid MRT in fermenters was linked to a 35% reduction of methane per unit of digested OM. Based on the estimation of Ramin and Huhtanen (2013), the maximal effect to be expected is of a magnitude of a 20% decline in CH₄-energy per gross energy intake, which means that at least 1/3 of this range in mitigation had been achieved in the present study. One possibility that cannot be ruled out in the present study is that the reduction in methane yield mainly occurred in the lower GIT. While to our knowledge, detailed data for cattle are missing, in sheep CH₄ produced in the hindgut (which is mainly excreted by exhalation) accounts for 10 to 16% of all enteric CH₄ (Murray, Bryant, & Leng, 1976). When digesta MRT is reduced due to increasing feed intake, this proportion may increase (Murray, Bryant, & Leng, 1978), possibly due to a lower digestibility in the rumen and a higher inflow of fermentable material into the hindgut. In the present study, where pilocarpine specifically reduced the MRT in the lower GIT, the opposite might have occurred, and the shorter MRT in the hindgut might have reduced CH₄ production at this site to an extent that the overall CH₄ yield (which is, in respiration chambers, a composite of CH₄ produced in the foregut and the hindgut) and MFN yield (which is an effect at the level of the foregut) were decoupled.

Besides the ‘stimulation of microbes as hydrogen sink’ hypothesis, there is another theoretical argument for more saliva leading to less methane: oxygen is a very strong hydrogen consumer (Czerkawski, 1969) and saliva is one of the ways by which oxygen is introduced into the rumen (together with feed and diffusion from blood and rumen tissues). Assuming that cattle produce 180 L saliva per day, and that the solubility of oxygen in saliva is 5 mL/L (Czerkawski, 1969), the amount of oxygen delivered via saliva per day is 0.9 L. However, assuming all delivered oxygen would consume hydrogen, which would otherwise have been used to produce methane, then 0.9 L oxygen would contribute to mitigate 0.45 L methane – compared to daily methane production indicated by our dataset (more than 400 L per day), this is negligible.

5 | CONCLUSION

We applied pilocarpine on cattle and thus stimulated salivation and increased fluid flow
rate, although to a lesser degree than expected from literature. Still, methane yield decreased, though to an extent much smaller than found in in vitro studies where rumen fluid dilution was accomplished. Microbial N yield as estimated by urinary metabolites was not affected but metabolic faecal N as a further indicator increased, yet again at a smaller extent than expected from in vitro studies. This shows that effects observed in in vitro assays are more difficult to demonstrate in live animals, maybe particularly so on forage-dominated diets that already trigger substantial chewing and hence saliva flow. The CH₄-sparing effect of an increased ruminal microbial yield observed in vitro remains to be demonstrated in vivo. Whether selectively breeding cattle for an increased saliva production could contribute to overall production efficiency remains to be further investigated.

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Chapter 4

A pilot investigation on the effect of induced saliva flow on digestive parameters in sheep, and a comparison with cattle

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4. Increasing saliva flow in sheep and its effects

ABSTRACT
Sheep with a relatively low methane yield were observed to have shorter fluid and particle mean retention times (MRT). Because the application of pilocarpine, a saliva stimulant, was successful in reducing retention times in ruminants in previous studies, we applied this substance to sheep, expecting a reduction in MRT and methane yield. Three non-pregnant sheep (74±10 kg) were fed a hay-only diet in a 3×3 Latin square design with oral doses of 0, 2.5 and 5 mg pilocarpine/kg body weight and day. Measurements included feed and water intake, MRT of liquid and particulate phases in the reticulorumen (RR) and total gastrointestinal tract (GIT), ruminal microbial yield (via urinary purine bases and metabolic faecal nitrogen), total tract methane emission, apparent nutrient digestibility and rumen fluid parameters. Data were investigated for linear and quadratic effects using orthogonal polynomial contrasts. The MRT of liquid and small particles in the RR and total GIT, and the short chain fatty acid concentration in rumen fluid, linearly declined with increasing pilocarpine dosage, while no quadratic relationship was detected. Intake of feed DM and water, apparent nutrient digestibility, methane yield and microbial yield were not affected by pilocarpine. When combining the sheep data with that of a similar experiment in cattle, we found that the MRT of the liquid phase was positively associated with estimated NDF digestibility and with methane production per digested NDF, but was not associated with microbial yield or the ratio of acetate to propionate. The ratio between MRT of the particulate and the liquid phase was smaller for sheep than that for cattle, and was not affected by treatment. Differences in this ratio might explain why species reacted differently to the saliva-inducing agent, which might help to explain the discrepancy between species in the effect of induced saliva flow on digestive parameters.

KEYWORDS
salivation, digestion, methane, microbial synthesis, passage rate, ruminal fluid balance, ruminant
1 INTRODUCTION

According to the recent estimation by Crippa et al. (2021), a third of the total anthropogenic GHG emissions originated from global food production between 1990 and 2015, of which the enteric methane (mostly from the domestic ruminants) accounted for 17%; thus, domestic ruminants contributed 5-6% of the total anthropogenic GHG emissions, and these emissions are projected to continually increase. For decades, scientists have been trying to mitigate the methane emissions from ruminant livestock, and developed a set of effective feeding strategies and feed additives (Arndt et al., 2022). Recently, based on the observations of individual between-animal differences in methane production, it was suggested that the trait of methane yield, i.e., the amount of methane formed per unit of feed or digested matter, might be heritable and repeatable, and that genetic selection for genotypes with low methane yield might be another option to mitigate methane emissions (Pinares-Patiño et al., 2011; Huhtanen et al., 2016 a). However, it is not fully understood how methane production and animal physiological traits are associated, irrespective of the feeding conditions.

Goopy et al. (2014) compared high- and low-methane yield sheep, and found that those with low methane yield had lower rumen capacities and shorter mean retention times (MRT) of particulate and liquid digesta. Using a modelling approach, Huhtanen et al. (2016) concluded that among-animal variation in MRT can markedly contribute to among-animal variation in methane emissions from ruminants. These authors attributed the cause of the variation to the increased efficiency of microbial synthesis trigged by an increased dilution rate, since the microbial cell synthesis acts as a H₂ sink which competes with methanogenesis (Demeyer et al., 1972; Czerkawski, 1986). For example, the biosynthesis of fatty acids consumes NADPH, which acts as electron carrier. Fatty acids are a fundamental part of cell membranes, which need to be formed in the process of cell division. Clauss et al. (2010) suggested that a high fluid turnover in the rumen helps to optimise the harvest of microbial yield from the forestomach. Additionally, many in vitro experiments with continuous culture systems showed that an increased liquid dilution was associated with increased microbial yield and/or decreased methane emission (Isaacson et al., 1975; Meng et al., 1999 b; Pfau et al., 2021 b). However, studies that directly manipulated MRT in live animals to observe changes in methane and microbe yields are rare.
Pilocarpine is a parasympathomimetic agonist, and was reported to increase saliva flow in ruminants, either given intravenously (Castellano et al., 1986) or orally (Wiedmeier et al., 1987). Treating cattle orally with pilocarpine resulted in a reduction of MRT of the liquid phase in the GIT by 7.8% and the methane yield per digested dry matter (DM) by 6.5%, while the microbial yield, other rumen fluid parameters and the apparent nutrient digestibility were not affected (Zhang et al., 2022). Sheep differ from cattle in terms of the relationship between MRT of the particulate and the liquid phase in the reticulorumen; this ratio is smaller in sheep compared to cattle, suggesting comparatively longer MRT of the liquid phase or comparatively shorter MRT of the particulate phase or both in sheep (reviewed by Pfau et al., 2023). This has been interpreted as an indication of a particularly high basic salivation rate in cattle (Clauss et al., 2010). Therefore, in theory, cattle, operating already at very high salivary output, might be less reactive to a pilocarpine-induced salivation increase. In order to test whether a salivary stimulant could have a more pronounced effect in a ruminant with a putatively lower basic saliva production, we performed a similar experiment in sheep. Considering the limited animal number, this was a pilot investigation.

While applying pilocarpine as a salivary stimulant to sheep, we measured the MRT of the liquid and the particulate phase, methane and microbial yield, apparent digestibility and rumen fluid parameters. We predicted again that the stimulant would lead to a shorter MRT of the liquid phase, a higher microbial and a lower methane yield. By analysing the data in conjunction with those in cattle, we additionally aimed to illustrate some more general principles of digestive physiology across ruminant species.

2 | MATERIALS AND METHODS

2.1 | Animals, feeding and treatment

The experiment lasted from October 2020 to January 2021 and was carried out at the research station AgroVet-Strickhof (Eschikon, Lindau, Switzerland). Three adult non-pregnant and non-lactating Swiss Black-Brown Mountain sheep (one, three and four years old) were used in a Latin square design with three treatments in three runs. Each run lasted for four weeks. During the first two weeks, the sheep were kept together in an 11 m² indoor area with straw bedding, and additionally with 52 m² concrete outdoor area. In the first week, the animals were fed with hay for ad libitum consumption and not treated with pilocarpine. In the second week, animals were fed a fixed amount of
hay, aimed to best maintain a constant feed intake and animal body weight during the whole experiment. The restricted amount of hay was allocated according to the individual metabolic body weight (around 85 g/kg$^{0.75}$ body weight). From the second week on, the animals were subjected to the respective treatment. During the following third week, the animals were kept individually in 2.8×1.4 m$^2$ boxes, around 3 m$^2$ of which were covered with straw, aimed to measure individual feed and water intake. With beginning of the fourth week, the sheep were fed the retention markers and transferred to metabolic crates on wheels, suited for the total collection of faeces and urine. Before the fourth week, the sheep were already adapted to the crates, and normal feeding, drinking and ruminating behaviour was observed. These crates had transparent acrylic glass side panels on three sides and an open grid on the fourth side to which feed and water troughs were attached. Therefore, the animals had visual, acoustic and olfactory contact amongst each other. The floor area was 2 m$^2$ covered with a rubber mat with slits, allowing faeces and urine to fall into the funnel beneath. A screen in the funnel retained faeces, but not urine. During the last 2 days, these crates were moved to respiration chambers. The animals were familiarized with the chambers before the first measurement week by putting the crates in the chambers for hours.

The daily dosages of pilocarpine (Fagron GmbH&Co. KG, Glinde, Germany) were 0, 2.5 and 5 mg/kg body weight and given in three portions at 0600, 1400 and 2200; the same dosages had also been applied in Zhang et al. (2022). The pilocarpine was dissolved in approximately 15 ml of orange juice and then given orally by a syringe. For the zero treatment a similar amount of orange juice was given without pilocarpine. After this treatment, the sheep received the allotted amount of hay.

Body mass was measured before the experiment and at the end of each run using a mechanical animal scale (± 0.5 kg). A mineral lick stone (Schweizer Salinen AG, Pratteln, Switzerland) and water were provided for ad libitum access during the whole experiment. The persons performing the animal experiment could not be blinded to the treatment because of the visually distinct higher salivation with the highest pilocarpine dosages. However, the laboratory analyses of samples were done by blinded personnel.

2.2 | Sampling

The amounts of feeds and water ingested were measured by manually weighing feed and water offered and leftovers. Representative samples of the hay and the individual
leftovers of individuals were collected daily. After each run, daily samples were pooled (for hay and individual leftovers of each animal), dried at 60°C overnight and milled through a 0.75 mm sieve for later analysis.

The total daily faeces and urine production was recorded, and representative faecal samples were taken and frozen immediately. At the end of the experiment, the individual faecal samples were thawed, pooled to one sample per animal and treatment, dried at 60°C overnight and milled through a 0.75 mm sieve for later analysis. For urine sampling, one bottle was set in the basin and directly below the outlet of the screen. The bottle contained either 30 mg 50% sulfuric acid for nitrogen determination or 30 mg 10% sulfuric acid for later purine derivative determination.

The markers for determining digesta retention, Co-ethylenediaminetetraacetic acid (Co-EDTA; solute marker), Cr-mordanted fibre (2-mm particle marker) and La-mordanted fibre (1-cm particle marker) were prepared according to Udén et al. (1980). A detailed description about preparation and application was made in Zhang et al. (2022). Faecal samples for MRT determination were collected before marker application as correction for background levels (three samples per individual). The MRT markers were applied in the morning of first day during the fourth week, and collected 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 36, 40, 44, 48, 54, 60, 66, 72, 80, 88, 96, 104, 112, 120, 128, 136, 144, 152, 160 and 168 hours after marker application. At each of these time points, all faeces were removed from the metabolism crates, mixed, and a representative sample (~10%) was taken. The calculation of MRT data is described in Zhang et al. (2022). The selectivity factors (SF) was calculated as the ratios between the MRT data obtained with different markers. Dry matter gut fill was calculated using DM intake, DM digestibility and the MRT of large particles following Holleman et al. (1989).

Rumen fluid was collected via oesophageal tube at the end of each run prior to the subsequent regular morning feeding. The tube was composed of a 10-cm-long metal probing head with several holes and an around-150-cm-long plastic tube, which ended in a manual pull-push suction apparatus and an outlet for rumen fluid collection. The collected rumen fluid was stored in a prewarmed thermos bottle.

2.3 | Respiratory chamber measurement

The methane emissions of the individual sheep were measured in three open circuit
Increasing saliva flow in sheep and its effects

respiration chambers with a volume of about 21 m$^3$ (RC, No Pollution Industrial Systems Ltd., Edinburgh, United Kingdom). The entire metabolic crates were moved into the RC. The RC walls consisted of glass panels on both sides so that the sheep could see each other. The temperature was maintained between 13.3 and 16.6°C and the relative humidity at 55±10%. The light program was set to automatically change the light intensity every four hours, 50% of max light intensity at 4 a.m. to 90% of max light intensity at 12 a.m. and at last turned off at 12 p.m. For detailed parameters of the RC, the calibration procedure, and the calculation of the respiratory data see Zhang et al. (2022). The recovery was determined to adjust the data; the recovery rates (average values ± standard deviation) for CH$_4$, CO$_2$ and O$_2$ were 90.7±2.5%, 91.2±2.9% and 99.5±0.2%, respectively.

During measurements, the animals were kept in RCs for 48 hours. Researchers entered the RC three times a day at 6 am, 2 pm and 10 pm for feeding, pilocarpine dosing and sampling, opening the doors only for quick entry and exit; no effect of these procedures on the gas measurements could be detected. The daily total faeces and urine amounts were recorded also during the time the sheep stayed in the RC.

2.4 | Laboratory analysis

Experimental hays, feed residuals and faeces were analysed for DM, organic matter (OM), crude protein (CP), crude lipid (CL), crude fibre (CF), neutral detergent fibre (NDF) and acid detergent fibre (ADF) according to the standard methods of the Association of German Agricultural Analysis and Research Centres (VDLUFA, 2006). The concentration of NDF was analysed after adding amylase; NDF and ADF concentrations were corrected for residual ash. Feed intake and leftover measurements were based on a measurement period of 14 days, while faecal amount was based on the collection period of 7 days; DMI and digested amount regarding to respiratory data was based on 5 days (2 days in the RC and the preceding 3 days).

The metabolizable energy (ME) content of the hay was calculated according to equation of GfE (1995):

$$ME (MJ) = 0.0312 \times digestible \text{ CL (g)} + 0.0136 \times digestible \text{ CF (g)}$$
$$+ 0.0147 \times (digestible \text{ OM} – digestible \text{ CL} – digestible \text{ CF}) (g)$$
$$+ 0.00234 \times CP \text{ (g)},$$
4. Increasing saliva flow in sheep and its effects

where the digestibilities had been determined from intakes and excretion of the respective fractions.

Measurements of urinary purine derivates (PD) were conducted according to the method of Shingfield et al. (1999) by reverse-phase HPLC (Prominence LC-20A, Shimadzu Europe, Duisburg, Germany), coupled to the SPD-M10Avp (DAD) detector. The column used was Spherisorb ODS 2 C18-RP (5 µm, 4.6 × 250 mm; Waters, Eschborn, Germany). The microbial N synthesis by sheep was estimated from PD absorption according to Chen et al. (2004):

\[
\text{Microbial N (g/d)} = \frac{PD\text{ absorption (mmol/d)} \times 70}{0.116 \times 0.83 \times 1000} = 0.727 \times PD\text{ absorption (mmol/d)};
\]

where the PD absorption was estimated through the formula:

\[
PD\text{ excretion in the urine (mmol/d)} = 0.84 \times PD\text{ absorption (mmol/d)} + (0.15 \times BW^{0.75} \times e^{-0.25 \times PD\text{ absorption (mmol/d)}});
\]

To solve the equation, the Newton-Raphson iteration process was performed:

\[
X_{n+1} = X_n - \frac{f(X_n) - Y}{f'(X_n)} = X_n - \frac{0.84 \times X_n + 0.15 \times BW^{0.75} \times e^{-0.25 \times PD\text{ absorption (mmol/d)}} - Y}{0.84 - 0.0375 \times BW^{0.75} \times e^{-0.25 \times PD\text{ absorption (mmol/d)}}}.
\]

where \( Y \) refers to PD excretion in the urine (mmol/d), \( X_0 = Y / 0.84 \).

As another proxy for microbial yield, metabolic faecal N (MFN) was calculated as total faecal N minus faecal NDF-N as described by Steuer et al. (2014).

The pH of the rumen fluid was determined with a potentiometer (pH: model 913; Metrohm AG, Herisau, Switzerland). Short-chain fatty acids (SCFA) in rumen fluid were analysed with high-performance liquid chromatography (LaChrom L 7000, Hitachi, Tokyo, Japan), equipped with an UV detector, using the column HPX-87H (7.8 × 300 mm; Bio-Rad Laboratories, Hercules, California, USA).

2.5 | Statistical analysis

Data were analysed by a linear mixed model using R version 3.5.2 with pilocarpine treatments as fixed factor and animal and experimental run as random factors. Based on the results of the analysis of variance, orthogonal polynomial contrasts were established to recognize linear and quadratic effects of pilocarpine dosages. The data of ME intake were exponentially transformed to assess residual normality (indicated in
4. Increasing saliva flow in sheep and its effects

Table 1). Combined with data of cattle from Zhang et al. (2022), a linear mixed model was conducted with methane or microbial yield as the dependent variable, and MRT of the liquid phase in the total GIT as the independent variable, species as fixed factor, animals and experiment runs as random factor. The interaction between MRT of the liquid phase in the total GIT and species was also considered, but since the effect of this interaction was not significant, it was removed from the final model. Data was presented as arithmetic mean ± standard deviation. The significance level was set to < 0.05, p values of 0.05 to 0.1 were considered as a trend.

3 | RESULTS

3.1 | Intake and digestibility

The nutrient composition (g/kg dry matter) of the experimental grass hay was OM: 920, CP: 142, CL: 24, NDF: 513, ADF: 275, CF: 247, respectively. The average ME intake was 12.2 MJ/day per animal. The body weight of animals remained constant during the experiment irrespective of pilocarpine dosage (Table 1). In each run, the individual receiving the highest pilocarpine was often observed with foamy saliva at the corners of the mouth, which is similar to observations in cattle (Zhang et al., 2022 b); however, the DM contents of the hay leftovers and faeces did not change with the use of pilocarpine. Intakes of feed DM, ME and water were not affected by treatments. Similarly, the apparent digestibility in the total GIT of DM, OM, NDF and ADF was not influenced by the treatments.

3.2 | Digesta retention and selectivity factor

The marker elimination patterns indicate an increasing passage rate of liquid and particles with increasing doses of pilocarpine (Fig. 1). Accordingly, the MRT of the liquid phase in the total GIT was linearly (and tended to be quadratically) decreased with increasing pilocarpine dosage ($p = 0.015$ and 0.088, respectively). The MRT of the 2-mm-particles in the total GIT decreased linearly with increasing pilocarpine dosage ($p = 0.032$). The MRT of the liquid phase and 2-mm particles in the reticulorumen (RR) linearly decreased with increasing pilocarpine dosage ($p = 0.027$ and 0.049, respectively). The MRT in the distal GIT was not affected by the treatments. The SF between 2-mm particles and liquid, and between 1-cm and 2-mm particles were not affected by the treatments; however, quadratic relationships were detected for the
4. Increasing saliva flow in sheep and its effects

SF between 1-cm particles and liquid both in the GIT and RR. The total DM gut fill (kg) tended to linearly decrease with increasing pilocarpine dosage ($p = 0.054$, Table 2).

3.3 | Microbial N and methane emission

The microbial N yield, indicated by both, urinary purine derivates and microbial faecal N, was not affected by the treatments (Table 3). The daily methane production tended to linearly decrease with increasing pilocarpine dosage ($p = 0.077$). The methane yield per DM or NDF intake, as well as per digested DM or NDF, was not affected by the treatments (Table 3).

3.4 | Rumen fluid parameters

The pH of rumen fluid increased ($p = 0.035$), whereas the concentration of total SCFA linearly decreased with increasing pilocarpine dosage ($p = 0.013$) (Table 4). The proportions of the individual SCFA of the total SCFA were not affected by the treatments, except that the butyrate was quadratically related to the pilocarpine dosage. The acetate-to-propionate ratio was not affected by the treatments.

3.5 | Effect of fluid dilution in the total GIT and species differences

By grouping the data from sheep and cattle together, the MRT of the liquid phase showed no effect on the apparent OM digestibility (Table 5). In line with the different hay qualities used for sheep and cattle, sheep tended to have a higher digestibility of OM than the cattle ($p = 0.077$). A longer MRT of the liquid phase was related to a higher apparent digestibility of NDF ($p = 0.016$), while species showed no effect. The methane yield per digested OM and NDF was positively related to MRT of the liquid phase in the total GIT. Species did not differ in methane yield. The microbial N yield per digested OM, indicated by urinary purine derivates and MFN, was not affected by MRT of the liquid phase or species.
4. Increasing saliva flow in sheep and its effects

Table 4.1 Effect of pilocarpine dosages on body weight, daily intake, dry matter content of feeds and faeces, urine output and apparent digestibility. Values are presented as arithmetic mean ± standard deviation; n = 3 sheep for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>p-value</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>74.6±10.8</td>
<td>73.4±11.8</td>
<td>73.6±12.9</td>
</tr>
<tr>
<td><em>Intake (kg/day)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay (DM)</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Water</td>
<td>2.9±1.0</td>
<td>3.3±0.8</td>
<td>3.0±0.6</td>
</tr>
<tr>
<td>ME (MJ)</td>
<td>12±1</td>
<td>13±2</td>
<td>12±2</td>
</tr>
<tr>
<td><em>DM content (%)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hay leftovers</td>
<td>76.5±1.3</td>
<td>76.0±3.2</td>
<td>77.0±1.2</td>
</tr>
<tr>
<td>Faeces</td>
<td>30.2±1.6</td>
<td>29.4±3.1</td>
<td>29.3±2.9</td>
</tr>
<tr>
<td>Urine output (kg/day)</td>
<td>1.0±0.3</td>
<td>1.1±0.7</td>
<td>1.0±0.6</td>
</tr>
<tr>
<td>Apparent digestibility (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>71.0±3.0</td>
<td>69.8±3.6</td>
<td>67.9±3.8</td>
</tr>
<tr>
<td>OM</td>
<td>73.0±2.7</td>
<td>71.8±3.2</td>
<td>69.9±3.4</td>
</tr>
<tr>
<td>NDF</td>
<td>73.4±3.8</td>
<td>71.6±5.2</td>
<td>69.5±4.9</td>
</tr>
<tr>
<td>ADF</td>
<td>70.9±3.6</td>
<td>68.0±6.0</td>
<td>65.1±4.9</td>
</tr>
</tbody>
</table>

*Determined with exponentially transformed (power of -4) data.
DM, dry matter; ME, metabolizable energy; OM, organic matter; NDF, neutral detergent fibre corrected for residual ash; ADF, acid detergent fibre corrected for residual ash.
4. Increasing saliva flow in sheep and its effects

Table 4.2 Effect of pilocarpine dosages on mean retention time in digestive tract, selectivity factor and gut fill. Values are presented as arithmetic mean ± standard deviation; n = 3 sheep for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>p-value</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Mean retention time (h)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid phase in GIT</td>
<td>29.6±1.1</td>
<td>25.8±5.3</td>
<td>25.1±5.0</td>
</tr>
<tr>
<td>2 mm particles in GIT</td>
<td>42.1±4.7</td>
<td>33.8±1.6</td>
<td>33.0±4.7</td>
</tr>
<tr>
<td>1 cm particles in GIT</td>
<td>43.3±2.7</td>
<td>42.8±8.6</td>
<td>37.5±9.5</td>
</tr>
<tr>
<td>Liquid phase in RR</td>
<td>15.9±1.3</td>
<td>11.6±0.8</td>
<td>11.4±2.9</td>
</tr>
<tr>
<td>2 mm particles in RR</td>
<td>28.4±3.7</td>
<td>19.6±6.4</td>
<td>19.3±2.6</td>
</tr>
<tr>
<td>1 cm particles in RR</td>
<td>29.6±3.1</td>
<td>28.6±4.0</td>
<td>23.9±7.7</td>
</tr>
<tr>
<td>Distal GIT</td>
<td>13.7±2.2</td>
<td>14.2±4.9</td>
<td>13.6±2.1</td>
</tr>
</tbody>
</table>

Selectivity factor

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>p-value</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>2 mm particles/liquid phase in GIT</td>
<td>1.42±0.11</td>
<td>1.35±0.32</td>
<td>1.32±0.07</td>
</tr>
<tr>
<td>1 cm particles/liquid phase in GIT</td>
<td>1.46±0.07</td>
<td>1.66±0.04</td>
<td>1.49±0.14</td>
</tr>
<tr>
<td>1 cm/2 mm particles in GIT</td>
<td>1.03±0.06</td>
<td>1.28±0.31</td>
<td>1.13±0.15</td>
</tr>
<tr>
<td>2 mm particles/liquid phase in RR</td>
<td>1.79±0.27</td>
<td>1.70±0.58</td>
<td>1.72±0.19</td>
</tr>
<tr>
<td>1 cm particles/liquid phase in RR</td>
<td>1.87±0.13</td>
<td>2.46±0.22</td>
<td>2.08±0.30</td>
</tr>
<tr>
<td>1 cm/2 mm particles in RR</td>
<td>1.05±0.09</td>
<td>1.62±0.80</td>
<td>1.22±0.26</td>
</tr>
<tr>
<td>Total gut fill (kg dry matter)</td>
<td>1.41±0.13</td>
<td>1.44±0.22</td>
<td>1.21±0.16</td>
</tr>
</tbody>
</table>

GIT, gastrointestinal tract; RR, reticulorumen.

Table 4.3 Effect of pilocarpine dosages on methane (CH\(_4\)) emission and microbial N yield. Values are presented as arithmetic mean ± standard deviation; n = 3 sheep for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>p-value</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td><strong>Microbial N yield</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/day</td>
<td>12.3±2.2</td>
<td>12.1±4.0</td>
<td>12.8±5.8</td>
</tr>
<tr>
<td>g/kg digested DM</td>
<td>14.3±1.5</td>
<td>13.6±2.9</td>
<td>15.3±4.3</td>
</tr>
<tr>
<td>MFN (g/kg DM)</td>
<td>21.3±2.1</td>
<td>21.2±2.8</td>
<td>21.1±0.3</td>
</tr>
</tbody>
</table>

| **Methane (g/day)**           |            |            |         |        |           |
| DM                            | 32.1±5.3   | 32.1±3.7  | 27.8±5.8 | 0.116  | 0.077     | 0.199   |
| NDF                           | 25.4±2.6   | 25.1±4.1  | 23.2±5.1 | 0.322  | 0.133     | 0.458   |

| **Methane per nutrient intake (g/kg)** |            |            |         |        |           |
| DM                            | 49.8±5.2   | 49.4±8.3  | 46.2±11.1 | 0.460  | 0.233     | 0.572   |
| NDF                           | 35.1±4.1   | 35.4±5.4  | 33.8±5.4 | 0.524  | 0.367     | 0.426   |

| **Methane per nutrient digested (g/kg)** |            |            |         |        |           |
| DM                            | 66.8±8.5   | 67.5±8.8  | 65.8±10.0 | 0.655  | 0.579     | 0.452   |

DM, dry matter; NDF, neutral detergent fibre corrected for residual ash; MFN, metabolic faecal N.
4. Increasing saliva flow in sheep and its effects

**Table 4.4** Effect of pilocarpine dosages on rumen fluid parameters. Values are presented as arithmetic mean±standard deviation; n=3 sheep for each treatment group.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pilocarpine (mg/kg body weight and day)</th>
<th>p-value</th>
<th>Contrast (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>pH</td>
<td>7.35±0.55</td>
<td>7.62±0.32</td>
<td>7.85±0.21</td>
</tr>
<tr>
<td>Total SCFA (mmol/L)</td>
<td>99.5±25.1</td>
<td>79.0±19.5</td>
<td>79.6±17.9</td>
</tr>
<tr>
<td><strong>Individual SCFA (% of total SCFA)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>68.0±12.3</td>
<td>69.2±3.2</td>
<td>68.6±1.7</td>
</tr>
<tr>
<td>Propionate</td>
<td>16.7±1.9</td>
<td>18.4±3.6</td>
<td>14.5±2.7</td>
</tr>
<tr>
<td>Butyrate</td>
<td>12.3±3.2</td>
<td>9.8±0.7</td>
<td>13.9±1.5</td>
</tr>
<tr>
<td>Iso-butyrate</td>
<td>0.9±0.6</td>
<td>0.8±0.5</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Valerate</td>
<td>0.8±0.4</td>
<td>0.6±0.2</td>
<td>1.0±0.8</td>
</tr>
<tr>
<td>Iso-valerate</td>
<td>1.5±0.4</td>
<td>1.3±0.3</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td><strong>Acetate-to-propionate ratio</strong></td>
<td>4.12±0.52</td>
<td>3.88±0.85</td>
<td>4.84±0.92</td>
</tr>
</tbody>
</table>

SCFA, short chain fatty acid.

**Table 4.5** Effect of species and mean retention time of fluid in the total GIT (MRT<sub>fluidGIT</sub>) on digestibility, methane (CH<sub>4</sub>) emission, ruminal microbial yield and acetate-to-propionate ratio. Values are presented as estimated slope or least square means.

<table>
<thead>
<tr>
<th>Item</th>
<th>MRT&lt;sub&gt;fluidGIT&lt;/sub&gt;</th>
<th>Species&lt;sup&gt;1&lt;/sup&gt;</th>
<th>p-value</th>
<th>MRT&lt;sub&gt;fluidGIT&lt;/sub&gt;</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cattle</td>
<td>Sheep</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Apparent digestibility (%)</strong></td>
<td></td>
<td>0.166</td>
<td>67.9</td>
<td>71.3</td>
<td>0.154</td>
</tr>
<tr>
<td>OM</td>
<td>0.354</td>
<td>71.8</td>
<td>71.0</td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>NDF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; emission (g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; per digested OM</td>
<td>0.555</td>
<td>40.5</td>
<td>36.9</td>
<td></td>
<td>0.007</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; per digested NDF</td>
<td>0.667</td>
<td>60.5</td>
<td>66.4</td>
<td></td>
<td>0.030</td>
</tr>
<tr>
<td><strong>Microbial N yield</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/kg digested OM</td>
<td>-0.183</td>
<td>15.1</td>
<td>15.2</td>
<td>0.265</td>
<td>0.810</td>
</tr>
<tr>
<td>MFN output (g/kg digested OM)</td>
<td>-0.030</td>
<td>10.2</td>
<td>9.8</td>
<td>0.588</td>
<td>0.581</td>
</tr>
<tr>
<td><strong>Acetate-to-propionate ratio</strong></td>
<td>0.045</td>
<td>4.39</td>
<td>4.28</td>
<td>0.329</td>
<td>0.795</td>
</tr>
</tbody>
</table>

<sup>1</sup>Cattle and sheep were fed different hays; the estimated organic matter digestibility of the hay was 72.0% and 80.3% for cattle and sheep, respectively.

OM, organic matter; NDF, neutral detergent fibre; MFN, metabolic faecal N.
4. Increasing saliva flow in sheep and its effects

**Figure 4.1** The effect of pilocarpine treatment (0, 2.5 and 5 mg/kg BW) on faecal marker elimination pattern: (a) solute marker (Co); (b) 2 mm particle marker (Cr); (c) 1 cm particle marker (La). Values represent the means of three sheep; note that the 100% peak is often not reached because the peak did not occur at the same time point in all animals.
4. Increasing saliva flow in sheep and its effects

4 | DISCUSSION

Stimulation of salivation in sheep reduced the MRT of the liquid phase as expected, but in contrast to our expectations, no effects on methane yield or on microbial yield were observed. Compared to our previous experiment in cattle, the sheep differed in several parameters in varying degrees in responding to the pilocarpine treatments, particularly the rumen fluid dilution, the particulate retention and the methane yield. However, the comparison of the sheep and cattle data is limited to some extent by the use of the different hays. The batch of type fed to the sheep tended to have a higher digestibility of OM than that fed to the cattle ($p = 0.077$), which can be attributed largely to the different hay qualities. This finding was supported by the estimated OM digestibility as, following the in vitro approach by Menke et al. (1988), OM digestibility was 72.0 % and 80.3 % for the hays fed to cattle and sheep, respectively. Thus, the effect of hay quality and animal species could not be statistically separated. Concerning the relationships of MRT of the liquid phase with the different target variables analysed, the influence of different hays would have been accounted for by including species into the model. In addition, the restricted sample size provides only limited statistical power and future investigations are indicated.

4.1 | Effects of the saliva stimulant in sheep and comparison with cattle

Generally, the rumen is filled with a large fibrous raft formed by undigested solid materials and rumen fluid; the rumen fluid occupies more than 60% of the total rumen volume (Kay, 1960; Colucci et al., 1984; Silanikove et al., 1989 a). Rumen fluid originates from two sources of inflow: water consumption and salivation; the major way of outflow is through reticulo-omasal orifice, and additionally liquid is exchanged with the blood through the rumen wall. Distinct differences in the amount of saliva produced are reported for individuals within a ruminant species (McDougall, 1948; Kay, 1960; Gurnsey et al., 1980), which indicates that there might be a potential of salivary modification by selective breeding. The stimulation of salivation via pilocarpine in our experiment was reflected by the foamy saliva observed at the corners of the mouth and the measured decrease in MRT of the liquid phase. Unlike in cattle, the increased salivation caused by pilocarpine did not fill the mouth of the sheep at the same pilocarpine level and saliva did not drop into the feed, which is confirmed indicated by the unaffected DM content of the hay leftovers.
4. Increasing saliva flow in sheep and its effects

The application of pilocarpine decreased the MRT of the liquid phase in the GIT as well as in the RR; the same decreasing effect was also true for the MRT of 2-mm particles in the GIT and in the RR, which could be attributed to an increased washout via the increased liquid outflow or the rumen motility. Measurement of rumen motility in sheep represents a challenge at the operational level; however, existing studies indicated that pilocarpine increases the tonus of the RR in cattle and sheep (Gurnsey et al., 1980; Zhang et al., 2022 b). Although the negative relationships between pilocarpine dosages and the MRT of the liquid phase in the GIT were both significant for cattle and sheep, the effect of pilocarpine in sheep was more evident in the RR, while for cattle it was more evident in the distal GIT (Zhang et al., 2022 b). This is also indicated by the unaffected DM contents of the sheep faeces, which indicates that colonic water absorption was not challenged by pilocarpine, while for cattle the faecal DM contents decreased when applying pilocarpine. The reasons for this difference remain unclear. Additionally, our results indicated, for the animals without treatments, that sheep possess a longer MRT of the liquid phase (15.9 h for sheep and 13.3 h for cattle) and shorter MRT of the particulate phase (2 mm particles: 28.4 h for sheep and 34.4 h for cattle; 1 cm particles: 29.6 h for sheep and 43.2 h for cattle) in the RR, a pattern previously described elsewhere (reviewed by Pfau et al., 2023).

In both our sheep and cattle experiments, the microbial yield indicated by urinary purines was not affected by pilocarpine or associated with MRT of the liquid phase. This is not consistent with our hypothesis and several in vitro observations. Czerkawski (1986) defined the ruminal contents as a free liquid phase, a liquid associated with solid phase and a solid phase. Similar to the compartmentation of the rumen contents, the ruminal microbial populations could also be divided into groups according to which they are associated with. Because of the compartmentation of the microbial population, the most likely affected microbial groups by increased liquid dilution are those which are liquid associated and probably also the loosely solid associated microbes. In in vitro continuous culture systems like Rusitec (Czerkawski et al., 1977), the proportion of the liquid part is much larger than in the real rumen, which might reinforce any effect of an increased fluid dilution rate on microbial yield.

Studies also indicate that the microbes from different compartments might considerably differ in metabolic processes and cellular contents (Czerkawski et al., 1982; Merry et al., 1983; Olubobokun et al., 1990; Rodríguez et al., 2000); thus,
exerting a disturbance on ruminal fluid balance might affect the composition of microbial compartmentation and subsequently the rumen fermentation. The treatment with pilocarpine showed no effect on methane yield in sheep, which is inconsistent with the decline in cattle reported by Zhang et al. (2022). The methane yield per unit of digested NDF was not significantly reduced in sheep. Even numerically the decrease is small with 1.5% at the highest pilocarpine dosage compared to the significant decline of 6.7% found in cattle. Therefore, the different response of methane production by cattle and sheep due to pilocarpine treatment may be attributed to distinct physiological characteristics of the two animal species and, possibly, to systematic differences between cattle and sheep in the rumen microbiome (Glendinning et al., 2021).

Meanwhile, the trend towards a decreased ruminal SCFA concentration and an increased pH with pilocarpine was another notable result found in the current sheep experiment. These results could be the result of both, a contamination of the rumen fluid with saliva during sampling, or a higher dilution of the rumen contents with buffering saliva.

4.2 | Relationship between selectivity factor or fluid dilution and digestive parameters

Compared to cattle, sheep are characterized by a significantly lower ratio between MRT of the particulate and the liquid phase, i.e. a lower SF of particles and liquid (Udé n et al., 1982; Colucci et al., 1990; Clauss et al., 2006). Our results indeed showed that the sheep had a smaller SF between particles and liquid in RR (1-cm particles and liquid: 2.13 for sheep and 3.27 for cattle; 2-mm particles and liquid: 1.74 for sheep and 2.58 for cattle) and a comparable SF between 1-cm particles and 2-mm particles in RR (1.30 for sheep and 1.27 for cattle). The SF between 2-mm particles and liquid is a species-specific ruminant physiology characteristic and is largely independent of the diet and intake (Przybyło et al., 2019). By contrast, the SF between large and small particles appears to be constant across various ruminant species (Dittmann et al., 2015 a), as shown in the present study between sheep and cattle. It can be interpreted as indication for a rather uniform critical particle size for ruminal outflow for different medium- and large-sized ruminant species. Based on our hypothesis, a higher SF of particles and liquid could optimize the microbial protein production and subsequently reduce methane emission. Hence this effect should be more prominent in cattle than in sheep.
4. Increasing saliva flow in sheep and its effects

Eventually, the microbial yield indicated by MFN was increased by pilocarpine dosages for cattle but not for sheep; yet ruminal microbial yield indicated by urinary purine derivates was not affected by pilocarpine treatments in either species (this study; Zhang et al., 2022). In a continuous culture system, where the solid phase is fixed in nylon bags and particle disappearance hence is less directly affected by liquid outflow, it is probably much easier to cause an increased microbial yield and a decreased methane yield by increasing the liquid dilution.

When combining sheep and cattle data, both microbial yield and the acetate-to-propionate ratio were not affected by MRT of the liquid phase. Consistent with this, Goopy et al. (2014) found that the urinary allantoin excretion was not associated with methane production. However, a reduction of methane yield with shorter MRT of the liquid phase was observed in our experiments. Other studies also observed a consistent positive relationship between MRT of the liquid phase and the methane yield in sheep (Pinares-Patiño et al., 2011; Goopy et al., 2014; Hammond et al., 2014). In our study, a reduction of 0.49 g (or 1.9%) in methane yield (per kg DM intake) was observed for a one-hour reduction of ruminal MRT of the liquid phase (at a range of 11.4-15.9 h). This is a similar magnitude as the difference found between sheep with high and low methane yield studied by Goopy et al. (2014). At a difference in ruminal MRT of the liquid phase of 18.5-22.8 h, these authors reported a difference of 0.54 g (or 2.3%) in methane yield (per kg DM intake) per one-hour difference in ruminal MRT of the liquid phase. By contrast, Pinares-Patiño et al. (2011) reported a twice-as-large difference of 1 g (or 4%) methane yield (per kg DM intake) per one-hour difference in ruminal MRT of the liquid phase in another group of sheep with high and low methane yield (at a ruminal MRT of the liquid phase range of 13.1-16.0 h). These findings suggest that additional factors than just the difference in ruminal MRT of the liquid phase are responsible for the difference in methane yield between high and low emitting sheep.

Goopy et al. (2014) also found that sheep with low methane yield have more clearly demarcated rumen gas and liquid phases, which points towards an influence of the interaction of solid and fluid compartments on methanogenesis. James et al. (1995) and Leng (2014) pointed out that biofilms provide a micro-environment for important interactions between microbes which play major role during methanogenesis. In a liquid medium, biofilms are easily formed (Guélon et al., 2011). Dilution rate was suggested to be an influencing factor on biofilm formation, although results are
4. Increasing saliva flow in sheep and its effects

inconsistent with respect to whether biofilm formation is favoured at higher or lower fluid dilution rate (Tijhuis et al., 1992; Moreira et al., 2013; Legner et al., 2019). Future work could focus more on the role of biofilms and the interaction among different compartments of microbes when methane is produced.

5 | CONCLUSION

In the present study pilocarpine stimulated the salivation and increased the fluid flow rate in sheep, effects similar to those found in a previous experiment in cattle. Different from cattle, methane yield and microbial yield did not respond to pilocarpine treatment. This different response might be related to the difference in ratios of the MRT of the particulate to the liquid phase between sheep and cattle. This ratio has been shown to be largely resistant to changes in diet type or feed intake. Nevertheless, due to the small sample size and the quantitatively small treatment effects in this pilot study, further research in the future is needed. Generally, in live animals, ruminal microbial yield might be less affected by changing liquid dilution rates than in in vitro systems, because in live animals, a separation of the particulate and liquid phase beyond that which is typical for the species in question may be difficult to achieve.

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4. Increasing saliva flow in sheep and its effects


Chapter 5

General discussion
5. General discussion

5.1. Significance and limitations of this study

Based on the hypothesis of this study that increased fluid dilution rate is associated with increased microbial yield and decreased methane production, pilocarpine was applied as saliva stimulant to modify ruminal fluid inflow in cattle and sheep. The induced salivation could be gauged through measurement of fluid dilution rate, and visual observation of saliva drooling for cattle and foamy saliva at the corners of the mouth for sheep.

The major achievement of the study was the decreased methane production via treatments for cattle; though to an extent relatively small, this provides evidence that increasing fluid dilution rate is in favour of less methane production for dairy cattle. The major limitation of this study was the relatively small sample size which provides limited statistical power; however, full measurements with large animal numbers were logistically not feasible. Additionally, the applied pilocarpine dosages reduced the fluid MRT only to a limited level, which might be attributed to the forage-based diet. Yet, the microbial yield as indicated by purine derivates was not affected by treatments, and methane production by sheep showed no significant change.

In the following, based on our results, we will firstly rethink the hypothesis of this study, then discuss the aspects that might lead to bias from the hypothesis, and at last give an outlook for future research and application.

5.2. Rethinking the relationship between dilution rate, microbial yield and methane production

5.2.1. Relationship between dilution rate and microbial yield

Microbial growth is the increase in the number of microbial cells. Basically, the ruminal microbes reproduce through binary fission. Different ruminal microbial populations have quite different generation times. For example, *Streptococcus bovis* has a doubling time of less than 30 minutes (Russell et al., 1984), while the generation time of rumen protozoa is much longer, generally more than 10 hours, and for some species over 24 hours (Dehority, 2004, 2010).

In batch culture systems, the growth rate increases along with increased nutrient
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centration till the limitation; while in a chemostat, the growth rate increases with
the increased dilution rate, probably because the influent liquid provides nutrients
(Madigan, 2012). The growth rate of the microbes is primarily determined by the
concentration of available energy sources (Janssen, 2010). An empirical model, the so-
called ‘Monod equation’, \( \mu = \frac{\mu_{\text{max}} \times S}{K+S} \) (\( \mu \), growth rate; \( \mu_{\text{max}} \), maximal growth rate;
S, energy concentration; K, constant), has described microbial growth as related to the
concentration of energy (Monod, 1949). However, both ruminal simulation systems as
well as the live rumen (during rumination) only introduce buffer to the chamber
containing the microbes, which principally provides no energy (but might, in the case
of saliva, provide nitrogen). On the contrary, the increased amount of buffer inflow
might lead to a dilution of soluble nutrients. Nevertheless, several \textit{in vitro} studies still
reported an increased efficiency of microbial synthesis along with an increased dilution
rate (Isaacson et al., 1975; Van Nevel et al., 1979; Pfau et al., 2021 a).

Principally, all the ATP produced during fermentation in the rumen is used by
ruminal microbes either for growth or maintenance. Maintenance energy is expended
on functions that are not directly related to growth, and refer to at least three functions:
(1) retention of ion gradients, (2) molecular turnover, and (3) motility (Russell et al.,
1995, 2009). Based on \textit{in vitro} continuous studies, the microbial yield efficiency
fluctuates with different dilution rates, and the fluctuation of microbial yield efficiency
was suggested to associate with maintenance energy demand among various microbial
species (Baldwin et al., 1983; Van Soest, 1994; Owens et al., 2016). Herbert et al. (1956)
mentioned that the higher dilution rate in continuous culture systems might help to
select fast-growing mutants of bacteria. Thus, it was also indicated by Meng et al.
(1999), based on rumen fluid incubation, that selecting fast-growing microbial species
might be the reason of higher microbial yield efficiency triggered by a higher fluid
dilution rate.

The ruminal microbial ecosystem constitutes of different microbial species,
among which competition, predation (food chains), and mutualism can exist. The
maybe best-known predation relationship is between protozoa and bacteria. Since the
protozoa number was reported to be easily reduced by a higher fluid dilution rate, this
predation pressure would be reduced (Meng et al., 1999 a). Thus, by shortening the
food chain in the rumen, the intermediate energy loss is expected to be lower, and the
overall efficiency of microbial synthesis would be higher. For this reason, defaunation
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was suggested to be beneficial for higher ruminal microbial yield and net protein output (Veira, 1986).

Another possible reason that might connect the fluid dilution rate and the microbial growth is the formation of biofilm. Biofilm is the matrix assembled by bacteria, typically when bacterial cells grow on dynamic surfaces (such as in flowing systems) (Madigan, 2012). Through formation of biofilms, the bacteria can better attach to the solid substrate and remain in their favourite niche (Madigan, 2012). According to the current studies about the formation of biofilm, dilution rate was indicated to be an influencing factor, although inconsistent results exist with respect to the question whether biofilm formation is favoured at higher or lower fluid dilution rate (Tijhuis et al., 1992; Moreira et al., 2013; Legner et al., 2019). Additionally, since biofilm is basically a mixture of polysaccharides, the formation might consume energy and metabolic hydrogen, while the magnitude compared to the total microbial cell synthesis is still unknown. The potential mass of biofilm could not be estimated by several measuring techniques including this study based on urinary purine derivates. In summary, knowledge on biofilm in the rumen is still rare, and how it will connect the ruminal fluid dilution rate and other concerning fermentation characteristics remains to be clarified in future research.

Overall, it seems that a higher dilution rate is in favour of higher microbial yield. Of course, the microbial growth must firstly overcome the turnover of ruminal contents, which means the microbial growth rate must override the passage rate; otherwise, the microbes would be washed out. This study did not indicate more ruminal microbe production as indicated by purine derivates, and some more reasons will be discussed in the next section. Nevertheless, optimizing microbial yield would be a consistent purpose for ruminant nutritionists, not just because the microbial synthesis is potentially a channel to consume metabolic hydrogen, but maybe more importantly, to increase protein output for the animals (Hoover et al., 1991; Hackmann et al., 2015).

5.2.2. Relationship between microbial yield and methane production

Microbial synthesis consumes metabolic hydrogen and thus competes with methanogenesis; some studies tried to quantify the magnitude of the potential to mitigate methane production by increasing microbial yield. Synthesis of one gram microbial mass consumes 0.0061 mol 2H according to Demeyer et al. (1972) or 0.0081
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mol 2H according to Czerkawski (1986). However, similar to the efficiency of microbial synthesis (expressed as $Y_{\text{glucose}}$ or $Y_{\text{ATP}}$), the amount of 2H consumption by microbial synthesis is strongly believed by authors to be not consistent – the most relevant influencing factor is the composition of the microbiome, and probably also the growth stage of the cells. As suggested before, in terms of the relationship between microbial yield and methane production, the batch culture systems would be preferred, since fermentation end products are accumulated, and measuring the composition of microbes combining stoichiometry can contribute to further understanding. In addition, as described before, besides new cells through binary fission, the microbial biosynthesis also includes other processes, such as biofilm matrix and storage polymers. Studies focusing on these are rare and the subject needs further investigation.

Ungerfeld (2015) reviewed studies that inhibited the methanogenesis and found microbial yield was increased (Van Nevel et al., 1969; Guo et al., 2007), or not affected (Lee et al., 2009). Ungerfeld (2015) suggested that when methanogenesis is inhibited, on the one hand, more metabolic hydrogen can be incorporated into microbial biomass, but on the other hand, the total energy supplementation for microbial synthesis is reduced, because the presence of methanogens is in favour of higher ATP generation. In conclusion, reduction of methane emissions by increasing microbial production is a robust hypothesis with a strong biochemical basis because of competition for metabolic hydrogen, but when considering the energetic advantage in the presence of methanogens, the reduction potential may be limited.

5.2.3. Relationship between dilution rate and methane production

The passage of liquid and solid digesta is one of the determining physical influencing factors for ruminal fermentation (Czerkawski, 1986; Van Soest, 1994). The mean retention time of digesta for dairy cattle in the rumen is less than 60 hours, and the fermentation end products are SCFA, $\text{CH}_4$ and $\text{CO}_2$; if the retention time would be prolonged until a week or longer, the SCFA would be further fermented to $\text{CH}_4$ and $\text{CO}_2$, just like sewers and bogs (Van Soest, 1994) – unless they were absorbed rapidly after their production. Even within the extent of reported ruminal retention time for domestic animals, fermentation patterns vary; Janssen (2010) suggested that higher passage rates are associated with fermentation pathways that lead to more propionate and less $\text{H}_2$ formed per unit of fermented substrate, and so less methane.
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The biochemical reactions are catalysed by enzymes, which are secreted by microorganisms. Microbial genomes encode many more enzymes than are actually present in the cell under any particular condition. Regulation is a major process for all microorganisms and helps to conserve energy and resources (Madigan, 2012). A well-known example for this is the function of lac operon – when glucose is not available, gene regulation of the lac operon helps the bacteria to effectively digest lactose. Thus, the physical and chemical factors derived from feed or animal might largely affect the expression of microbial genes, and subsequently, the ruminal fermentation pathways.

Shi et al. (2014) analysed metagenomic and metatranscriptomic data from low-methane-yield and high-methane-yield sheep, and found that the abundance of methanogens and methanogenesis pathway genes were similar, while transcription of these genes was substantially higher in high-methane-yield sheep than in low-methane-yield sheep. It was suggested by Huhtanen et al. (2016) that differences of methane production between animals were attributed to the ruminal digesta MRT. Thus, factors of animal origin could affect the ruminal microbial gene expression, namely the microbial metabolic pathways, via digesta retention.

The reticulorumen contains heterogeneous contents of free liquid, solid mass and gas phase, and the inhabited or suspended microorganisms. A compartmental model was used to describe the reticulorumen as follows: compartment 1, liquid associated; compartment 2, loosely solid associated; compartment 3, firm solid associated; compartment 4, rumen wall associated (Czerkawski, 1986; Cheng et al., 1997). Studies indicated that the different compartments of microbes possess considerable differences in metabolites and cellular contents, which might indicate the compartments of microorganisms or at the transcription level, which needs further research (Czerkawski et al., 1982; Merry et al., 1983; Craig et al., 1984; Olubobokun et al., 1990; Rodríguez et al., 2000). We have modified fluid dilution rate, which might primarily affect the microorganism in compartment 1, probably compartment 2, and indirectly also compartment 3, through the formation of biofilm. Goopy et al. (2014) also found that sheep with low methane yield have more clearly demarcated rumen gas and liquid phases, which points towards an influence of the interaction of solid and fluid compartments on methanogenesis. The interaction between compartments, as well as the attachment and biofilm formation play major roles in syntrophy, as well as the digestion of substrates; the gel-like biofilm matrix helps to exchange materials within
the microbial consortia (McAllister et al., 1994; James et al., 1995; Leng, 2014). As
described above, the formation of biofilm was suggested to be affected by dilution rate
(Moreira et al., 2013; Legner et al., 2019), and more detailed investigations are needed
in the future research.

5.3. The complexity in real animals, and comparison across
different systems

As discussed above, this study was based on a robust hypothesis with strong
biochemical basis, though more details concerning the mentioned aspects are needed;
and yet, several in vitro or in vivo studies have reported that a higher fluid dilution rate
was associated with higher microbial yield and less methane production. Nevertheless,
the ruminal microbial yield and methane emission are affected by many other factors,
primarily by feeds. When the effects of these factors dominate, bias in the results is
expected, and this is probably more the case in in vivo studies. After all, animals possess
more complex systems and could to some extent antagonize artificial modification.
Additionally, individual animals might react differently to the control, and small animal
numbers might also result in insignificant results. In the following, some factors that
might lead to bias against the hypothesis of this study are discussed, and a comparison
between systems is made to gain further understanding.

5.3.1. Shift the fermentation from foregut to hindgut

We used pilocarpine to increase saliva flow and subsequently fluid dilution rate;
however, the application of this saliva stimulant might also influence the ruminal
motility (Gurnsey et al., 1980; Froetschel et al., 1986; Brunton et al., 2018). Though
only a numerical trend was detected for reticular contractions by cattle, the possibility
has to be considered that by the application of pilocarpine, either via affected ruminal
motility, or flushed by increased fluid dilution, the fermentation was partly shifted from
rumen to the large intestine.

Generally, compared to ruminants, hindgut fermenters produce less methane
and harbour more reductive acetogens (Immig, 1996; von Engelhardt et al., 2015). As
another hydrogen consuming process, these acetogens reduce H₂ and CO₂ to acetate,
instead of methane by methanogens, which is more desirable, not just as a reduction of methane emissions, but also as an increase of SCFA yield. However, in the rumen, acetogens are out-competed by methanogens, and the reasons remain to be investigated (McAllister et al., 2008; Ungerfeld, 2020).

In ruminants, the large intestine as a secondary fermentation chamber produces much less methane (Murray et al., 1976); however, in some situations, the proportion of fermentation in the hindgut could increase (Hogan et al., 1971; Murray et al., 1978; Galyean et al., 1991). Though we detected that the distal MRT was also decreased by pilocarpine treatments in cattle, flushing the undigested particles partly to the lower tract might contribute to the reduction of methane emissions.

5.3.2. Effect of liquid dilution rate on methane emissions across systems

5.3.2.1. Comparison between cattle and sheep

A large amount of rumen volume is occupied by fluid, which provides buoyancy and is prerequisite of the selectivity mechanisms in the reticulum; it also helps to differentiate the ruminal microbial groups and build compartmentation (Czerkawski, 1986; Lechner-Doll et al., 1991). The ruminal liquid volume varies drastically as reported in different studies, primarily because of influence from feeds and individual differences (Kay, 1960; Colucci et al., 1984; Silanikove et al., 1989 b). As shown in Figure 5.1, it seems that the most variable factor which affects rumen liquid balance for cattle and sheep is salivation. Thus, individual differences might derive from chewing activities, as indicated in chapter two based on the four investigated cattle.
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**Figure 5.1** Ruminal fluid balance, comparing the daily inflow volume of saliva and water intake, and outflow volume through ROO, with the total rumen volume of cattle and sheep (reference volume for cattle, 100 L and for sheep, 10 L) (Kay, 1960; Colucci et al., 1984; Silanikove et al., 1989 b)

The results indicated that sheep possess a longer ruminal fluid MRT (15.9 h for sheep and 13.3 h for cattle, for the animals without pilocarpine treatments) in the RR. However, the individual animal might be able to adjust the saliva and subsequently the ruminal fluid dilution to some degree; Hammond et al. (2014) reduced the ruminal fluid MRT by sheep from 18.4 h to 7.5 h by increasing feed intake. The decreased fluid MRT was probably to a great degree attributed to salivary flow because of chewing behaviour, which is largely influenced by feed intake. Therefore, we kept the feed intake to a restricted amount. And yet, the extent of variation for individuals might be considerable and requires attention.

We applied pilocarpine and thus decreased methane production for cattle but not for sheep, although a numerical decrease could be observed. Other studies which have modified the fluid dilution rate or grouped the animals according to fluid dilution rate also suggested this reverse relationship between ruminal fluid dilution rate and methane production (or at least the numerical trend), as shown in Figure 5.2 (Pinares-Patiño et al., 2011; Sun et al., 2012; Goopy et al., 2014; Barnett et al., 2015; Grandl et al., 2016 a; b, 2018; Archimède et al., 2018). The ruminal fluid dilution rate, as an influence factor that partly originates from animal physiology and therefore is heritable (Hegarty, 2004; Pinares-Patiño et al., 2013; Donoghue et al., 2016), is indicated to exert a reducing effect on methane emissions for domestic ruminants.
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Figure 5.2 Relationship between fluid dilution rate in reticulorumen and $\text{CH}_4$ yield in cattle and sheep (Pinares-Patiño et al., 2011; Sun et al., 2012; Goopy et al., 2014; Barnett et al., 2015; Grandl et al., 2016; Archimède et al., 2018).

5.3.2.2. Comparison across other foregut fermenters

Methane production is ubiquitous among herbivore mammals, and fluid as well as digesta retention time should contribute significantly to the differences between species. For example, the fluid MRT in GIT for two-toed sloths (*Choloepus didactylus*) can be over 100 hours long and these animals produce a relatively large amount of methane (33 g/kg DMI) (Vendl et al., 2016a), while for red kangaroo (*Macropus rufus*) the fluid MRT in GIT can be 15 hours and methane production is less than 10 g/kg DMI (Schwarm et al., 2009; Vendl et al., 2015). Based on the dataset of Clauss et al. (2020), the relationship between fluid dilution rate and methane production from fourteen foregut fermenter species is shown in Figure 5.3. Thus, the inverse relationship between fluid dilution rate and methane production seems not universal among these investigated species, which indicates at least that it is not the only factor that influences methane production.
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Figure 5.3 Relationship between fluid dilution rate in reticulorumen and CH$_4$ yield across fourteen foregut fermenters (Clauss et al., 2020).

Among the investigated foregut fermenters, macropods produce much less methane. Godwin et al. (2014) investigated the microbiome in the kangaroo foregut, compared the fermentation pattern of the microbiome from kangaroo and cattle in in vitro cultures, and demonstrated that the activity of acetogens is the key of the lower methane output of kangaroos. Thus, the fermentation pattern of macropods is more similar to that in the hindgut like in equids, and different from that in many other foregut fermenters. Additionally, from a morphological view, the foregut of macropods is also more of a tubular shape like the colon; whether this is related to the specific fermentation pattern remains to be investigated.

Macropods also have a relatively high liquid dilution rate compared to other foregut fermenters. Some species of gazelle have a high liquid dilution rate, but this is primarily attributed to their small body weight (Dittmann et al., 2015 b). Vendl et al. (2015) suggested for macropods, in which a large difference exists between fluid MRT and particulate MRT, that forestomach microbes are more in the growth state and therefore produce less methane. Yet, the concept that a higher ratio of MRT$_{\text{particles}}$:MRT$_{\text{solute}}$ could optimize the microbial yield and reduce methane production was not corroborated in the pygmy hippo (Hexaprotodon liberiensis), which has a high ratio of MRT$_{\text{particles}}$:MRT$_{\text{solute}}$, but emits a higher amount of methane than
kangaroos (Clauss et al., 2004; Vendl et al., 2016 b). Thus, the respective species might possess different digestive physiological characteristics, for which more detailed investigations would be required. As for our dataset, with relatively strict control of variables apart from dilution rate, measurements for cattle resulted in smaller fluid MRT and larger particulate MRT compared to sheep; therefore, even though ruminal microbial yield as indicated by urinary purine derivates was not affected by pilocarpine treatments in either species, the microbial yield indicated by MFN was increased and methane production was decreased by applying pilocarpine in cattle but not in sheep.

5.3.2.3. **Comparison between in vitro and in vivo approaches**

The *in vitro* systems are much easier to measure and control. In continuous culture systems like Rusitec, a larger proportion of the fermentation chamber is occupied by fluid, which might reinforce any effect of an increased fluid dilution rate on microbial yield and methane production. Studies that have modified fluid dilution rate and measured methane production in continuous culture systems are shown in Figure 5.4. Isaacson et al. (1975), Van Nevel et al. (1979) and Pfau et al. (2021) reported an inverse relationship between fluid dilution rate and methane production, while Eun et al. (2004) reported a positive relationship, and Martínez et al. (2009) reported no significant relationship. The reason for the discrepancy among studies is not clear. It has to be pointed out that major differences exist between conditions of laboratory *in vitro* system and the rumen (Russell et al., 1981; Hristov et al., 2012).
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![Graph showing relationship between fluid dilution rate and CH₄ yield](image)

**Figure 5.4** Relationship between fluid dilution rate in reticulorumen and CH₄ yield via *in vitro* approaches (Isaacson et al., 1975; Van Nevel et al., 1979; Eun et al., 2004; Martínez et al., 2009; Pfau et al., 2021 a).

5.4. **Application, and outlook on future research**

5.4.1. **Application on breeding**

Breeding low-methane producing domestic ruminants has been stated as a permanent and cumulative way to reduce methane production over generations (Lassen et al., 2020; De Haas et al., 2021; Manzanilla-Pech et al., 2021). Methane production is a trait that might primarily be affected by feeds and microbiome, and secondly affected by animal physiological factors. Individual animals display considerable differences in respect to the ruminal microbiome (Jami et al., 2012; Shabat et al., 2016). The factors from feeds, microbiome, and animals themselves interact with each other, which may lead to the relatively low estimated heritability of methane emissions. The heritability estimation of methane production, as an indicator of the fraction determined by genetic effects, was from 0.05 to 0.27 for cattle and from 0.10 to 0.29 for sheep (Pinares-Patiño et al., 2013; Goopy et al., 2015; Brito et al., 2018; Lassen et al., 2020). Additionally, difficulties also exist for the methane measurement. Therefore, developing accurate, inexpensive and easy to use proxies for methane production might be helpful in
implementation on farms (Beauchemin et al., 2020).

To set methane production as a breeding target, a comprehensive understanding about the relationship between methane emission and other traits is needed. The greatest challenges might be the possible existence of undesirable associations with animal productivity (Beauchemin et al., 2022). Meaningful mitigation strategies should focus on redirecting metabolic hydrogen consumption, while not reducing digestibility. When digestibility is decreased, the energy conversion becomes less efficient, and the undigested feedstuff will be excreted and continues to produce methane.

This experiment has modified ruminal liquid dilution rate, reduced methane production in cattle, and found no effect on apparent digestibility. Although this was not confirmed in sheep, and the ruminal microbial yield as indicated by purine derivates was not increased, we might expect the effect to be more significant in a larger-scale animal experiment. Rowe et al. (2019) stated that low-methane sheep were also economically favourable, growing more wool and having smaller rumens. (Goopy et al., 2014; Bond et al., 2017) also reported that a smaller rumen is associated with higher fluid dilution rate and less methane production. (Elmes et al., 2014) reported that low-methane-yield sheep tended to have larger omasum; since the main function of omasum is fluid-absorption, this might also demonstrate the relation between fluid dilution rate and methane production. Therefore, the ruminal fluid dilution rate might be a proxy of methane production, and would also be potentially in favour of more microbial yield, both of which are desirable. Physiologically, this might also be associated with chewing activity as indicated in chapter two. Thus, whether chewing activity is potentially also heritable remains to be investigated. Yet, chewing intensity might be modified in practice.

In summary, selecting low-fluid-MRT breeds (or if possible, reducing fluid MRT in practice), might help to reduce methane and increase ruminal microbial yield; whether the fibre digestibility is affected should be born in mind. Additionally, it should be also kept in mind that for animals one important advantage of digesta retention in the reticulorumen is inactivation of plant toxins; thus, there should be trade-offs when considering manipulating ruminal fluid and particle MRT.

5.4.2. Outlook on future research

This study has modified the saliva flow for a forage-only diet. Compared to other
studies which have also applied pilocarpine, the relatively small decrease of ruminal fluid MRT might be attributed to the forage-only diet (Wiedmeier et al., 1987 a; b). Therefore, further research about the relationship between fluid dilution rate, microbial yield, and methane production by applying saliva stimulants might be based on concentrate diets, or through direct continuous infusion of artificial saliva in fistulated animals (Harrison et al., 1975).

A systematic study should be undertaken on the influences on methane production from the factors of feed, microbiome, and animal, among which interactions exist and remain to be clarified. Ruminal fluid dilution rate is largely affected by the factors of animal, and how this influences the expression of microbial genomes as well as metabolic activities, and if biofilm formation also plays a role, needs further research.

The potential to reduce methane production by redirecting metabolic hydrogen to microbial synthesis was based on a robust theory. However, this potential seems to be limited based on this animal study (methane decrease of 6-7% by cattle). With respect to this, experiments based on batch culture systems might be more helpful. Quantitative analysis combined with stoichiometry should reveal more unknown details about ruminal fermentation, and these should be firstly investigated based on easy-controlled batch culture systems.

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Main conclusions

1. Chewing behaviour could be linked to mean retention time through masticatory-salivary reflex and particle size reduction. Chewing measures are potentially an easy-to-acquire predictor for mean retention time.

2. By applying pilocarpine in cattle, the fluid dilution rate was increased, and methane production was decreased, though to an extent smaller than found in in vitro studies. Microbial N yield indicated by metabolic faecal N was increased, but not affected as indicated by urinary purine bases. By applying pilocarpine in sheep, the fluid dilution rate was increased, while the methane production and microbial N yield were not affected. This different response might be attributed to the difference in ratios of the MRT of particles to fluid between sheep and cattle, which is largely resistant to changes in diet type or feed intake. Yet, the decreased methane and increased microbial N yield triggered by increased fluid dilution rate might be more prominent in cattle than in sheep.

3. Generally, indicated by this study and through literature reviews, higher fluid dilution rate is related to increased microbial N yield and decreased methane production per unit of feed, although this is more difficult to demonstrate in live animals, probably because of the forage-only diets we have applied, and several complexities possessed by the real rumen.

4. The CH₄-sparing effect of an increased ruminal microbial yield observed in vitro remains to be investigated. The interaction between fluid phase and solid phase, and whether the formation of biofilm plays a role in ruminal metabolism and especially in methanogenesis, need further research.
Epilogue and acknowledgments

I still remembered my first semester in 2015, I sat in the first row of the lecture room and tried my best to follow the docent, but I failed. As a non-native speaker, it was difficult for me to understand the content of lectures. That feeling of depression and frustration has tortured me a lot. It was a tough time. Luckily, things were getting better.

In my fourth semester, I contacted Professor Jürgen Hummel and began my thesis for the master degree. At the same time, I have considered thoroughly whether I would be suitable for the academy. My parents wished me to continue the Ph.D., but I was not sure. Hence, I regarded this thesis as a trial. If I could do it well and enjoy the achievement, I would continue my research career. Therefore, I immersed myself fully in this project, in which I tried to figure out why the protozoa number decreased in Rusitec. Occasionally, I even figured out a mathematical model to simulate the fluctuation of protozoa number in Rusitec. Although the model was extremely primitive, it was the first time that I tried to figure out something with my own mind. I still feel the joy of doing something that has not been done before. It has motivated me to make up my mind.

The beginning of my doctorate has not gone well; I spent months to find a reasonable research topic. Then Prof. Jürgen Hummel and Prof. Marcus Clauss offered me a project based on animal experiments in Zurich. This project was about the influence of increased fluid dilution rate on microbial yield and methane production. Marcus and Jürgen have been working on such aspects for a long time. As I read their papers, I really felt that their work has expanded the boundaries of human knowledge. The project they offered me was a continuation of their work. I have never done such experiments before and I knew it would be a tough challenge, but I was extremely glad to take it. I felt honored that I could participate and make contributions, even tiny ones.

Well, to be honest, I think it is really hard to discover something in science. The researchers have to be extremely careful when making progress. Sometimes even if we were working really hard, we could probably only provide a perspective, which could be far from the truth. But if it is all we can do, I believe it is meaningful. And of course, some breakthroughs do require generations of effort.

Marcus told me once, that I can’t put everyone as co-author, but I could rather have a long acknowledgment list. Clearly, I cannot accomplish the experiments, the
published papers, and this dissertation only by myself. So, I would like to take up a few lines to write my acknowledgment list.

First of all, I would like to express my deepest appreciation to my Doktorvater, Prof. Dr. Jürgen Hummel. Leaving aside how much time and effort he has invested during my Ph.D., he himself is a role model for me in several aspects. Jürgen is very rigorous in research. Normally his feedback could point out the deficiencies very precisely and his creative suggestions usually solve my bottleneck problems. He is quite strict with himself but very kind and respectful to others. Then I would also like to extend my deepest gratitude to Prof. Dr. Marcus Clauss. During the animal experiments in Zurich, we spent so much time together. So I got a chance to know an experienced researcher, and I have learned a lot from him. Marcus is always energetic, and very frank and sincere. He always gives me feedback timely. And his encouragement has always motivated me to go through the tough times of my Ph.D.

I would also like to extend my gratitude to Prof. Dr. Eva Schlecht and Dr. Martin Hünerberg, who are also members of my thesis committee. They gave me helpful suggestions and feedback. I’m extremely grateful to Prof. Dr. Michael Kreuzer, for kindly supporting the animal experiments in Zurich. Dr. Melissa Terranova and Dr. Sergej Amelchanka have offered me a great amount of instrumental assistance in animal experiments. I would also like to thank Dr. Inga Mölder from GFA for her help and guidance in my doctoral degree.

I would like to acknowledge the assistance from all my colleagues in Göttingen and in Zurich; without your help I could not accomplish this work.

At last, I would like to give my most sincere thanks to my father, Yigong Zhang, and my mother, Huahong Tian -- thank you for always supporting me, and letting me choose my own way. And special thanks to my girlfriend, Siwen Yuan. Life is a great relief when I'm with you.
Declaration

Declaration by the doctoral candidate
at the Georg-August-Universität Göttingen
Surname: **Zhang Xiaoyu**
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(Country / Postcode / Place)
I intend to produce a dissertation on the topic of
**Assessing the relevance of fluid dilution rate for ruminal metabolism in cattle and sheep**
at Georg-August-Universität Göttingen.
In this, I shall be supervised by **Prof. Dr. Jürgen Hummel**
I submit the following declaration:

1. The opportunity for the existing doctoral project was not made commercially available to me. Especially, I have not engaged any organisation that seeks thesis advisers against a fee for the preparation of dissertations or performs my obligations with respect to examination components entirely or partly.

2. I have until now and shall in future accept the assistance of third parties only in a scope that is scientifically justifiable and compliant with the legal statutes of the examinations. I shall specifically complete all parts of the dissertation myself; I have neither, nor will I, accept unauthorised outside assistance either free of charge or subject to a fee.

Furthermore, I am aware of the fact that untruthfulness with respect to the above declaration repeals the admission to complete the doctoral studies and/or subsequently entitle termination of the doctoral process or withdrawal of the title attained.

......................................................, the
(Place)
...............................................................
(Signature)