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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 5mg 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 $p < 0.05$, 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

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

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 CO_2 and CH_4. The protein contained in the microbial biomass is digested by the host once it flows out of the reticulorumen (RR) into the lower gastrointestinal 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 (Eun et al., 2004; Herbert et al., 1956; Isaacson et al., 1975; Meng et al., 1999; Pfau et al., 2021). In live animals, several studies investigated the effect of an increased dilution rate in the 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 (Bird et al., 1993; Froetschel et al., 1989; Harrison et al., 1975; Wiedmeier, Arambel, Lamb, et al., 1987; Wiedmeier, Arambel, & Walters, 1987). 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 et al., 1997). Theoretical considerations indicate that the increase in microbial biomass production could be linked to a decrease in CH_4 production (Czerkawski, 1986; Ramin & Huhtanen, 2013), which was also confirmed in in vitro studies (Isaacson et al., 1975; Pfau et al., 2021; Van Nevel & Demeyer, 1979). 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 CH_4 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 CH_4 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, Arambel, & Walters, 1987) and goats (Castellano et al., 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 CH_4 yield.

2 | MATERIALS AND METHODS

2.1 | Animals, feeding and treatment

The study was approved by the veterinary office of the Swiss Canton of Zurich (license no. ZH247/18) and carried out from August 2020 to January 2021 at the research station AgroVet-Strickhof (Eschikon). 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, $C_{11}H_{16}N_2O_2 \times HCl$; Fagron GmbH & Co. KG). 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 m^2, 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 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 m^2 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) and 50 g salt (Schweizer Salinen AG). In Weeks 3 and 4, the amount of hay fed per animal was assigned according to their metabolic body weight (body weight$^{0.75}$), 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 (±20kg). 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 Weeks 3 and 4. Water flow meters (GWF MessSysteme AG) 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) onto the skin. 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 Grovum and Williams (1973), Huhtanen and Kukkonen (1995), and Thielemans et al. (1978), described in detail in Zhang et al. (2022). Selectivity factors were calculated as ratio of $MRT_{particles}/MRT_{fluids}$ and ratio of $MRT_{large\ particles}/MRT_{small\ particles}$ 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) as described by Braun et al. (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) by counting contractions of reticulum within 3 min at 1 h and 4 h 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). The RC volume was about 40.9 m^3 in total, a cuboid room with an additional space of about 2.9 m^3 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 m^2), 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 4:00 AM to 90% of max light intensity at 12:00 AM and at last turned off at 12:00 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 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°C 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), coupled with a frequency controller (VLT 3.3 KW, HWAC Drive; Danfoss); this pulled fresh air into the RC. The system was maintained at a slight negative pressure. The concentrations of O_2, CO_2 and CH_4 in fresh and extracted air were determined with an MGA 3500 (ADC Gas Analysis) 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. 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) was injected at 0.36 NLPM (normal litre per minute) via a tube through the outside wall into the empty RC for 4h. The measured CH_4 concentration reached a plateau after 2–2.5 h. The flow rate was controlled by a mass flow controller (MC-5SLPM-RD, Alicat Scientific). 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 (Hellwing et al., 2014; Verstegen et al., 1987). Subsequently, the flow rate of incoming air was estimated based on the principle that N_2 is inert, that is, 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, and fixated using the self-locking feed barrier and a Vink cattle head support (Albert Kerbl GmbH). 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), which was via T-connection attached to both, a containing bottle and a milk-line vacuum in the barn. The first 200ml 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 concentration. About 15 ml of samples were centrifuged at 4000*g* for 5 min at 24°C (Centrifuge 5810 R, Eppendorf AG). 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 CO_2 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; AOAC index no. 968.06) (AOAC, 2016). The metabolizable energy (ME) of the hay was calculated according to equation of GfE Gesellschaft für Ernährungsphysiologie (1995):

$$\begin{aligned}\text{ME (MJ)} = {} & 0.0312 \times \text{digestible EE (g)} + 0.0136 \\ & \times \text{digestible CF (g)} + 0.0147 \\ & \times (\text{digestible OM} - \text{digestible EE} \\ & - \text{digestible CF})\,\text{(g)} + 0.00234 \times \text{CP (g)}.\end{aligned}$$

Measurement of urinary purine derivates (PD) were conducted by reverse-phase HPLC (Prominence LC-20A, Shimadzu Europe GmbH) 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). The microbial N was estimated according to Chen and Ørskov (2004), using the following equations:

$$\begin{aligned}\text{Microbial N(g/days)} &= \frac{\text{PD absorption(mmol/day)} \times 70}{0.116 \times 0.83 \times 1000} \\ &= 0.727 \times \text{PD absorption(mmol/day)},\end{aligned}$$

$$\begin{aligned}\text{PD absorption (mmol/day)} = {} & (\text{PD excretion in the urine(mmol/day)} \\ & - 0.385 \times \text{BW}^{0.75}) \div 0.85.\end{aligned}$$

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 concentration 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). SCFA were analysed with high-performance liquid chromatography (LaChrom L 7000, Hitachi) equipped with an

UV detector, using the column HPX-87H (7.8× 300mm; Bio-Rad Laboratories). Composition of gas samples collected from HGT syringes were analysed with a gas chromatograph (Agilent 6890N Network Gas Chromatograph; Agilent Technologies) equipped with a thermal conductivity detector and a flame ionisation detector. The column (Carboxen-1000, Fluka) 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 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 $p = 0.034$, respectively). Faecal and urine output were not affected by pilocarpine. When receiving the highest pilocarpine dose, the animals appeared, subjectively, mildly depressed, and were 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 $p = 0.042$, respectively) and linearly decreased DM contents of faecal samples ($p = 0.002$) when pilocarpine dosage was increased (Table 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 1).

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

Increasing pilocarpine dosages linearly and quadratically decreased the $MRT_{solutes}$ in the total tract ($p < 0.001$ and $p = 0.019$, respectively; Figure 1a); for the average of pilocarpine treatments, $MRT_{solutes}$ was shorter than for the control at a magnitude of 7.8% (Table 2). A linear relationship between pilocarpine dosages and $MRT_{2\,mm\ particles}$ was found ($p = 0.046$; Figure 1b), while between pilocarpine dosages and $MRT_{1\,cm\ particles}$, there was only a trend of a linear relationship ($p = 0.095$; Figure 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 $p = 0.044$, respectively). The selectivity factors were not affected by treatment (Table 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). 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).

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 4). No quadratic and cubic relationship were found between pilocarpine and these CH_4 variables. Methane emissions per digested DM were reduced at a magnitude of 6.5% for the average of pilocarpine treatments (Table 4). There was no significant correlation between CH_4 yield and microbial N yield per digested DM ($p = 0.348$), nor 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 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 1 Effect of graded levels of pilocarpine on body weight, intake and leftovers, excreta output, apparent digestibility and chewing behavior

Item	Pilocarpine (mg/kg body weight and day)				Contrast (*p* value)[a]		
	0	1	2.5	5	*L*	*Q*	*C*
Body weight (kg)	748 ± 79	745 ± 84	750 ± 77	758 ± 76	0.104	0.267	0.793
Intake (kg/day)							
DM	12.4 ± 1.0	12.3 ± 1.3	12.4 ± 0.7	12.5 ± 0.8	0.740	0.598	0.703
Water	58.8 ± 10.8	58.6 ± 10.2	58.8 ± 6.2	64.4 ± 2.9	0.011	0.034	0.357
ME (MJ)	117 ± 9	117 ± 15	119 ± 4	117 ± 11	0.802	0.757	0.696
Hay leftovers (kg DM/day)	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.4	0.6 ± 0.2	0.538	0.794	0.919
Faecal output (kg DM/day)	4.5 ± 0.5	4.3 ± 0.4	4.3 ± 0.4	4.4 ± 0.1	0.861	0.197	0.842
Urine output (kg/day)	15.7 ± 2.5	16.3 ± 1.3	15.5 ± 1.0	16.8 ± 1.1	0.323	0.506	0.177
DM content (%)							
Hay leftovers	67.7 ± 10.8	64.7 ± 10.1	62.5 ± 7.9	45.3 ± 10.3	0.001	0.042	0.250
Faeces	13.4 ± 0.7	12.8 ± 1.5	12.1 ± 0.5	11.7 ± 0.7	0.002	0.671	0.723
Apparent digestibility (%)							
DM	64.2 ± 2.7	65.3 ± 2.0	65.6 ± 1.8	64.5 ± 2.4	0.704	0.180	0.868
OM	67.4 ± 2.7	68.2 ± 2.3	68.4 ± 2.2	67.4 ± 2.7	0.931	0.192	0.795
NDF	71.4 ± 2.4	72.0 ± 1.5	72.1 ± 1.3	71.3 ± 1.7	0.950	0.315	0.935
ADF	64.8 ± 4.8	65.7 ± 3.5	66.8 ± 4.5	65.7 ± 3.9	0.310	0.211	0.507
CF	74.7 ± 3.1	75.1 ± 4.1	75.1 ± 3.5	73.9 ± 4.0	0.534	0.422	0.831
Chewing behavior[b]							
Ingestion							
Chews per kg DMI	1693 ± 142	1893 ± 481	1731 ± 203	1823 ± 336	0.483	0.455	0.090
Chews per min	67.9 ± 4.7	67.0 ± 4.0	67.1 ± 5.3	65.4 ± 6.3	0.061	0.620	0.412
Rumination							
Chews per kg DMI	2672 ± 957	2614 ± 588	2588 ± 402	2558 ± 699	0.521	0.912	0.946
Chews per min	66.5 ± 10.2	66.6 ± 9.3	65.1 ± 7.8	64.3 ± 9.1	0.034	0.504	0.500
Ratio of rumination: ingestion time	1.6 ± 0.4	1.4 ± 0.3	1.6 ± 0.3	1.5 ± 0.4	0.525	0.783	0.274

Note: Values are arithmetic means ± standard deviation; $n = 4$ for each treatment group.

Abbreviations: 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.

[a]Contrasts: L, linear, Q, quadratic, C, cubic.

[b]Data on chewing behaviour were based on dry matter intake during measurement 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.

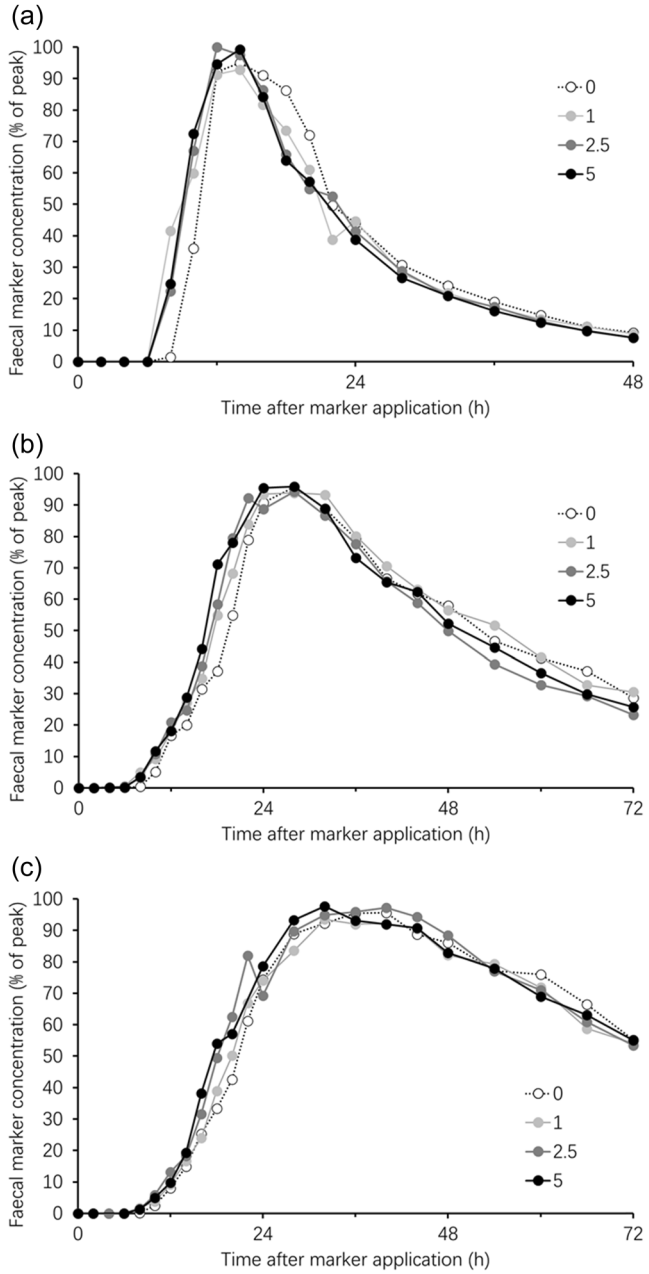

FIGURE 1 The effect of pilocarpine treatment (0, 1, 2.5 and 5 mg/kg BW) on faecal marker elimination pattern: (a) solute marker (Co); (b) 2 mm particle marker (Cr); (c) 1 cm particle marker (La). Faecal markers were collected for totally 7 days.

4.1 | Pilocarpine as a saliva stimulant

Pilocarpine is a parasympathomimetic agonist widely used in human medicine; it is most likely absorbed through the RR 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 (Bird et al., 1993; Castellano et al., 1986; Froetschel et al., 1987; Jacques et al., 1989). Individual differences in reaction to these drugs occur. Gurnsey et al. (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 license; however, the first application of this higher dose 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, than done in the present study. 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 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, Arambel, & Walters (1987) 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 a comparable effect was reported in no other study (Table 6). This is likely due to the relatively high amount of

TABLE 2 Effect of graded levels of pilocarpine on mean retention time in the gastrointestinal tract, selectivity factor, gut fill and reticular contractions

Item	Pilocarpine (mg/kg, body weight and day)				Contrast (*p* value)[a]		
	0	1	2.5	5	*L*	*Q*	*C*
Mean retention time (h)							
Solutes GIT	27.2 ± 3.1	25.7 ± 3.0	24.7 ± 4.1	24.8 ± 4.0	<0.001	0.019	0.574
2 mm GIT	48.2 ± 4.9	47.4 ± 5.4	45.0 ± 5.1	45.4 ± 8.3	0.046	0.576	0.367
1 cm GIT	57.0 ± 6.5	56.2 ± 5.7	54.6 ± 6.2	54.9 ± 8.9	0.095	0.527	0.520
Solutes RR	13.3 ± 1.4	13.7 ± 0.6	13.1 ± 1.2	13.0 ± 1.3	0.104	0.264	0.126
2 mm RR	34.4 ± 3.2	35.5 ± 3.2	33.4 ± 2.6	33.5 ± 5.4	0.287	0.596	0.247
1 cm RR	43.2 ± 4.9	44.3 ± 3.5	43.0 ± 3.2	43.1 ± 6.3	0.715	0.619	0.414
Distal GIT	13.8 ± 1.8	11.9 ± 2.6	11.6 ± 3.0	11.9 ± 2.9	0.018	0.044	0.627
Selectivity factor							
2 mm/solute GIT	1.78 ± 0.05	1.85 ± 0.07	1.84 ± 0.11	1.82 ± 0.08	0.399	0.231	0.593
1 cm/2 mm GIT	1.18 ± 0.02	1.19 ± 0.03	1.21 ± 0.03	1.22 ± 0.05	0.116	0.912	0.540
2 mm/solute RR	2.58 ± 0.08	2.58 ± 0.16	2.56 ± 0.12	2.58 ± 0.25	0.920	0.897	0.898
1 cm/2 mm RR	1.25 ± 0.03	1.25 ± 0.03	1.29 ± 0.05	1.29 ± 0.06	0.166	0.863	0.419
Total DM gut fill (kg DM)	20.1 ± 3.2	19.5 ± 3.4	19.0 ± 2.6	19.4 ± 3.4	0.086	0.138	0.646
Reticular contractions per 3 min							
1 h after dosage	4.3 ± 0.5	4.5 ± 0.6	4.5 ± 1.0	5.0 ± 0.8	0.205	0.745	0.663
4 h after dosage	4.0 ± 0.0	4.0 ± 0.0	4.3 ± 0.5	4.5 ± 0.6	0.049	0.485	0.752

Note: Values are arithmetic means ± standard deviation; $n = 4$ for each treatment group.

Abbreviations: DM, dry matter; GIT, gastrointestinal tract; RR, reticulorumen.

[a]Contrasts: L, linear; Q, quadratic; C, cubic.

TABLE 3 Effect of graded levels of pilocarpine on microbial nitrogen (N) yield and N balance

Item	Pilocarpine (mg/kg, body weight and day)				Contrast (*p* value)[a]		
	0	1	2.5	5	*L*	*Q*	*C*
Microbial N yield							
g/day	116 ± 11	117 ± 3	105 ± 11	131 ± 30	0.364	0.154	0.186
g/kg digested DM	14.5 ± 1.6	14.7 ± 1.6	12.9 ± 1.6	16.2 ± 2.7	0.445	0.108	0.108
MFN (g/kg DM)	17.8 ± 0.9	18.0 ± 0.8	18.1 ± 0.5	18.6 ± 0.8	0.020	0.462	0.463
N balance (g/d)							
N intake	299 ± 56	296 ± 66	299 ± 59	299 ± 41	0.969	0.739	0.647
Total faecal N output	112 ± 12	108 ± 11	109 ± 12	115 ± 7	0.343	0.058	0.887
MFN output	79 ± 8	77 ± 8	78 ± 10	82 ± 6	0.269	0.090	0.957
Urinary N	119 ± 39	118 ± 30	109 ± 28	116 ± 35	0.382	0.411	0.264

Note: Values are as arithmetic means ± standard deviation; $n = 4$ for each treatment group.

Abbreviation: DM, dry matter; MFN, metabolic faecal N.

[a]Contrasts: L, linear; Q, quadratic; C, cubic.

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

TABLE 4 Effect of graded levels of pilocarpine on methane (CH_4) emissions

Item	Pilocarpine (mg/kg body weight and day)				Contrast (*p* value)[a]		
	0	1	2.5	5	*L*	*Q*	*C*
CH_4 per day							
g	332 ± 33	321 ± 52	311 ± 42	314 ± 45	0.108	0.396	0.729
g/$BW^{0.75}$	2.33 ± 0.25	2.25 ± 0.24	2.18 ± 0.29	2.17 ± 0.15	0.023	0.391	0.812
CH_4 per nutrient intake (g/kg)							
DM	26.5 ± 2.8	25.7 ± 2.4	24.9 ± 2.7	24.7 ± 2.5	0.018	0.514	0.732
OM	28.7 ± 3.0	27.9 ± 2.5	27.0 ± 2.8	26.8 ± 2.5	0.022	0.528	0.698
NDF	45.0 ± 5.5	43.7 ± 4.6	42.2 ± 5.0	42.0 ± 4.3	0.021	0.490	0.685
ADF	84.0 ± 9.4	81.5 ± 8.1	78.9 ± 8.5	78.5 ± 7.3	0.024	0.507	0.740
CH_4 per digested nutrient (g/kg)							
DM	41.0 ± 3.2	39.2 ± 4.0	37.8 ± 4.2	38.0 ± 3.9	0.006	0.163	0.780
OM	42.5 ± 3.7	40.8 ± 4.2	39.4 ± 4.3	39.5 ± 3.8	0.011	0.229	0.713
NDF	62.7 ± 5.3	60.3 ± 6.1	58.5 ± 7.3	58.5 ± 5.2	0.009	0.259	0.778
ADF	130 ± 19	124 ± 16	119 ± 18	119 ± 12	0.010	0.275	0.728

Note: Values are arithmetic means ± standard deviation; $n = 4$ for each treatment group.

Abbreviations: 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.

[a]Contrasts: L, linear; Q, quadratic; C, cubic.

TABLE 5 Effect of graded levels of pilocarpine on rumen fluid characteristics and on gas production in the Hohenheim Gas Test

Item	Pilocarpine (mg/kg, body weight and day)				Contrast (*p* value)[a]		
	0	1	2.5	5	*L*	*Q*	*C*
Rumen fluid properties (mmol/L)							
pH	7.15 ± 0.07	7.23 ± 0.24	7.32 ± 0.10	7.23 ± 0.26	0.334	0.271	0.565
NH_3	9.6 ± 3.3	9.2 ± 4.7	7.8 ± 2.8	8.1 ± 2.6	0.085	0.638	0.342
Total short-chain fatty acids (SCFA)	98.4 ± 29.41	92.4 ± 28.22	91.8 ± 24.36	95.5 ± 18.69	0.776	0.590	0.953
Individual SCFA (% of total SCFA)							
Acetate	72.5 ± 3.0	72.0 ± 4.5	72.0 ± 3.2	73.0 ± 3.1	0.847	0.179	0.906
Propionate	17.1 ± 3.7	17.1 ± 5.0	17.8 ± 4.1	16.9 ± 3.2	0.993	0.542	0.417
Iso-butyrate	0.8 ± 0.3	1.2 ± 0.5	1.0 ± 0.3	0.9 ± 0.2	0.759	0.106	0.235
Butyrate	7.6 ± 1.7	7.8 ± 1.9	6.8 ± 0.8	7.2 ± 1.7	0.441	0.941	0.219
Iso-valerate	1.1 ± 0.4	1.3 ± 0.3	1.3 ± 0.3	1.1 ± 0.4	0.759	0.106	0.235
Valerate	0.9 ± 0.4	1.1 ± 0.4	1.1 ± 0.5	0.9 ± 0.4	0.716	0.087	0.608
Acetate to propionate ratio	4.39 ± 1.10	4.48 ± 1.24	4.22 ± 1.00	4.45 ± 0.96	0.895	0.716	0.391
Gas produced (ml/200 mg DM)							
Total in 8 h	16.2 ± 1.9	14.7 ± 4.3	13.7 ± 1.5	15.5 ± 4.1	0.448	0.095	0.533
Total in 24 h	39.9 ± 1.5	38.5 ± 2.1	38.6 ± 1.7	38.5 ± 1.8	0.033	0.103	0.271
CH_4 in 24 h	6.0 ± 0.5	6.4 ± 1.4	5.9 ± 1.0	6.4 ± 1.9	0.692	0.943	0.447
CO_2 in 24 h	31.9 ± 0.7	31.3 ± 2.9	31.5 ± 2.3	31.1 ± 2.3	0.526	0.876	0.660

Note: Values are arithmetic means ± standard deviation; $n = 4$ for each treatment group.

Abbreviation: DM, dry matter.

[a]Contrasts: L, linear; Q, quadratic; C, cubic.

TABLE 6 Collection of studies that manipulated saliva inflow into the reticulorumen

Source	Treatment	Highest dose, (μg/kg, portions/day)	Species	*F* prop.	Ruminal retention time		RR liquid vol.	Digestibility		Microbial yield	Rumen fluid		
					Fluid	Part.		DM	Cell.	Total	pH[a]	NH_4^+	SCFA
(a)	Saliva infusion	-	S	90	⇩ (45.0%)	-	-	-	-	⇧ (15.9%)	⇧ (6.01)	-	Propionate ⇩ (16.9%)
(b)	Slaf	48	S	n.a.	NS	-	(⇧) (27%)	-	-	-	-	-	-
(b)	Slaf	24 (3)	C	91	⇩ (20.6%)	-	-	-	-	-	⇧ (6.26)	-	(⇩) (15.5%)
(c)	Slaf	20 (2)	C	40	-	-	-	NS	NS	(⇧) (16.5%)	NS (6.55)	NS	NS
(d)	Slaf	24 (3)	C	50	⇩ (4.2%)	-	⇧ (47.2%)	-	-	-	NS (6.57)	NS	-
(e)	Slaf	8 (2)	S	100	(⇩) (11%)	NS	-	NS	NS	NS	⇧ (6.4)	(⇩) (44.7%)	NS
(f)	Carb	10 (1)	C	50	⇩ (15.4%)	⇩ (22.0%)	⇩ (8.9%)	(⇧) (4.9%)	(⇧) (12.0%)	(⇧) (3.7%)	NS (6.53)	(⇩) (18.9%)	(⇩) (7.1%)
(f)	Pilo	100 (1)	C	50	⇩ A(15.4%)	⇩ (24.3%)	⇩ (8.5%)	(⇧) (4.0%)	(⇧) (7.5%)	(⇧) (4.2%)	NS (6.53)	(⇩) (12.8%)	(⇩) (9.1%)
(g)	Pilo	4000 (2)	C	53	⇩ (44.1%)	⇩ (46.0%)	NS	⇧ (9.3%)	⇧ (9.5%)	NS	⇧ (6.34)	⇩ (37.7%)	-

Note: (a) Harrison et al. (1975), (b) Froetschel et al. (1987), (c) Froetschel et al. (1989), (d) Jacques et al. (1989), (e) Bird et al. (1993), (f) Wiedmeier, Arambel, Lamb, et al. (1987), (g) Wiedmeier, Arambel, and Walters (1987).

Abbreviations: C, cattle; Carb., carbachol; Cell., cellulose or fibre; F prop., forage proportion; 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.

[a]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.

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.

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 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 $MRT_{solutes}$ and $MRT_{particles}$ (Przybyło et al., 2019).

This could be the major cause that contributed to the difference between results obtained in vitro and 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 in vitro fermenters while keeping the particle incubation time constant. For example, Pfau et al. (2021) nearly halved the liquid retention in an in vitro system (from 64.1 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/day, the MRT RR for concentrates is estimated at 50 h, where it is only 12.5 h for milk yields >30 L/day (Spiekers et al., 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 et al., 2018). 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 et al. (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 h after application. And in particular, we found that digesta retention time in the GIT beyond the RR, that is, 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, Arambel, Lamb, et al. (1987). 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, Arambel, and Walters (1987) 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 derivates 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 et al., 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 6). The reported magnitudes of increased microbial yield measures were comparable with our MFN finding, except for Wiedmeier, Arambel,

and Walters (1987) 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 fiber 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 (Goopy et al., 2014; Pinares-Patiño et al., 2011). 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 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 CH_4-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 CH_4 produced in the hindgut (which is mainly excreted by exhalation) accounts for 10% to 16% of all enteric CH_4 (Murray et al., 1976). When digesta MRT is reduced due to increasing feed intake, this proportion may increase (Murray et al., 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 CH_4 production at this site to an extent that the overall CH_4 yield (which is, in respiration chambers, a composite of CH_4 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 CH_4-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.

AUTHOR CONTRIBUTIONS

Melissa Terranova, Michael Kreuzer, Jürgen Hummel and Marcus Clauss designed the study; Xiaoyu Zhang, Yang Li and Marcus Clauss performed the study with support from Christian Gerspach; Sylvia Ortmann, Saskia Kehraus, Michael Kreuzer and Jürgen Hummel supervised the laboratory analyses; Xiaoyu Zhang and Marcus Clauss analysed the data; Xiaoyu Zhang and Marcus Clauss wrote the manuscript with input from all coauthors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The original data measured in this study are available as an electronic supplement linked to this article.

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