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Effect of Iodoform in Maize and Clover Grass Silages: An In Vitro Study

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Simple Summary: The implementation of new strategies for the reduction of methane emissions by cattle is necessary. Iodoform is an additive that has the potential to inhibit methane production during digestion in ruminants. The present research investigated the effect of iodoform on maize silage and clover grass silage in four 22 h in vitro gas production fermentations with periodic gas chromatography sampling. Iodoform decreased organic matter degradation in both substrates but only significantly in grass silage. The total volatile fatty acid production increased, and the profile was altered, but only significantly in grass silage. There were consistently opposite effects of 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite on total gas production and methane production in the two substrates, although not all differences were significant. Iodoform decreased total gas production and methane yield when added to grass silage but consistently and significantly increased both total gas and methane yield when added to maize silage. The efficiency of microbial conversion decreased significantly when iodoform was added to both substrates. These results show differential effects of iodoform on maize silage and clover grass silage and suggest that more research is needed to study the mechanism of methane reduction by iodoform in enteric methane production.

Abstract: Iodoform has been shown to be an enteric methane-reducing agent. This study aimed to investigate if iodoform differentially affected maize (MS) and clover grass silage (GS). These substrates were used in four 22 h in vitro gas production fermentations with and without 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite (IZ). The results of organic matter degradation (dOM, g/g), volatile fatty acids (VFAs, mMol/L), partitioning factor (PF, mg/mL), total gas production (TGP, mL/g OM), and periodic hydrogen and methane yield (mL/g OM) were evaluated. The interaction between substrate and IZ for dOM was significant with significantly less in GS. The interaction between substrate and additive was continuously significant for TGP. The additive in GS numerically decreased the TGP, but significantly increased TGP in MS. The interaction between substrate and additive was sporadically significant with methane, increasing the yield when IZ was added to MS, but a consistent, non-significant, decrease in yield was found when IZ was added to GS. Total VFA production and propionate content increased in both substrates with the addition of IZ, but only significantly in GS. Iodoform significantly decreased the efficiency of microbial conversion (PF) in both substates. Differential effects of iodoform on GS and MS were evident. More research is needed to determine the modes of action of iodoform.

Keywords: degradation; fermentation; in vitro gas production; iodoform; methane

Citation: Massaro, S.; Jantzen, B.; Axel, A.M.D.; Tagliapietra, F.; Hansen, H.H. Effect of Iodoform in Maize and Clover Grass Silages: An In Vitro Study. *Ruminants* **2024**, *4*, 418–432. https://doi.org/10.3390/ruminants4030030

Received: 7 June 2024
Revised: 9 August 2024
Accepted: 29 August 2024
Published: 30 August 2024

1. Introduction

A major contributor to climate change is the greenhouse gas methane (CH_4). The global warming potential of non-fossil methane is about 80 ± 26, on a 20-year time horizon [1]. The

agricultural sector is responsible for 41% of the total anthropogenic methane emissions [2]. Ruminant enteric methane production from livestock is a major contributor within the agricultural sector [2]. This indicates the need to reduce methane from enteric emissions.

Cattle produce CH_4 during rumen enteric microbial fermentation of ingested feed during the process of methanogenesis. Carbon dioxide (CO_2) and hydrogen (H_2) are by-products of microbial fermentation that are used by archaea microbes during methanogenesis to produce CH_4. The continuous use of H_2 is necessary to delete the known inhibitory effects of an accumulation of H_2 on the rumen microorganisms [3–5].

In recent years, research has proposed different methane mitigation strategies based on manipulating animal diets with various additives (e.g., ionophores, essential oils, plant extracts, probiotics, and halomethane compounds) [2,6]. The efficacy of these strategies was reviewed recently [1]. Among others, halomethane compounds have been shown to reduce enteric CH_4 production when added to the diet of dairy cows [3,5]. Chemical inhibitors were found to result in the greatest absolute methane reduction of all mitigation strategies in a large-scale review of methane mitigation strategies including forms of rumen manipulation by ionophores, chemicals (including 3-NOP), immunization, bromoform-containing and other seaweeds, defaunation, alternative electron acceptors, essential oils, tannin extracts, saponins, biochar, direct-fed microbials, early-life interventions, and phage and lytic enzymes active against methanogens [1]. These compounds reduce CH_4 formation by (i) irreversibly binding vitamin B_{12}, by competitively inhibiting the action of the coenzyme M methyltransferase, and by blocking the activity of methyl coenzyme M reductase; (ii) inhibiting CH_4 formation by serving as a competing terminal acceptor of the electrons donated by H_2; or (iii) reducing the abundance of the methanogenic archaea [3,7,8]. Bromoform is the most-studied halomethane compound [2,7]. Even if iodoform has been less tested as a feed additive, it showed a potential in the reduction of enteric methane. It has been shown to inhibit enteric methane production in vitro, but only a few studies have investigated the effect in in vivo trials with production animals [3,9]. Thorsteinsson et al. [3] found that iodoform dramatically decreased methane emissions from dairy cows. A decrease in methane production causes an increase in hydrogen because for each molecule of methane produced by the archaea microbes, four hydrogen molecules are used. The simple stoichiometric equation followed during the methanogenesis process is $CO_2 + 4H_2 \rightleftarrows CH_4 + 2H_2O$ [10]. Decreased activity and an abundance of archaea have been proposed as the reaction when using iodoform in the feed. This leads to a significant increase in H_2 emission by eructation [3]. However, Thorsteinsson et al. [3] did not recover all the theoretical excess of H_2 in eructed gas, suggesting that other hydrogenotrophic pathways must be involved. The lack of recoverable H_2 may be accounted for by an increase in the production of valerate and propionate VFAs that are both hydrogen sinks.

While the effect of iodoform has been shown in vitro and in vivo, no studies were found that compared the magnitude of effect when using different substrates. Maize silage (MS) and grass silages (GS) are commonly used as cattle feed worldwide, yet they differ in their chemical compositions and have been shown to result in different methane levels [11–13]. Grass silage tends to have more lignin, ash, and protein, while maize silage has more starch. To the best of our knowledge, the reaction of methanogenesis to these differences in the presence of iodoform has not been documented.

Therefore, the aim of this study was to investigate the effect of iodoform when fermenting the two chemically different substrates, MS and GS, with the same rumen fluid in vitro. We hypothesized that supplemental iodoform would affect fermentation parameters differentially in MS and GS due to fiber content differences.

2. Materials and Methods

2.1. Substrate Information

Maize silage and grass silage were used as substrates. Both substrates were collected in Denmark in 2019 and freeze-dried immediately after the collection (Hetosicc CD 8, Heto Lab Equipment A/S, Allerød, Denmark) at −20 °C for 24 h at 0.1000 mbar with a final

drying pressure at 0.0010 mbar. The samples were thereafter ground through a 2 mm screen using a cyclone mill (CT 193 Cyclotex™, FOSS, Hillerød, Denmark) and stored for later use.

Before fermentation or chemical analyses, the final dry matter (DM) content was determined, in triplicate, by drying the ground plant material in a forced-air chamber (Binder GmbH, Bohemia, NY, USA) at 100 °C for 12 h, cooled in a desiccator until room temperature, and weighed. Ash content was determined by placing the samples in a muffle oven (Carbolite RWF 1100, Carbolite Gero Ltd., Hope Valley, UK) at 525 °C for 16 h and weighed after cooling to environment temperature in a desiccator. The crude protein content (CP) was measured in triplicate freeze-dried samples using the Kjeldahl protocol, in the VELP Kjeldahl system (VELP Scientifica, New York City, NY, USA). Fiber content was sequentially determined using the cell wall methodology [14] in an Ankom Fiber Analyzer 200 (Ankom, Macedon, NY, USA). Neutral detergent fiber was determined with sulfite and α-amylase ($aNDF_{om}$) and acid detergent fiber (ADF_{om}) thereafter [15,16]. Acid detergent lignin ($ADL_{(SA)}$) was determined using the Ankom procedures [17] in a Daisy Incubator (Ankom, Macedon, NY, USA). The chemical compositions of the substrates are shown in Table 1.

Table 1. Chemical compositions of the substrates. The percentages are expressed on organic matter (OM) basis unless otherwise stated.

Item [1]	Clover Grass Silage (GS)	Maize Silage (MS)
Organic matter, % (DM)	92.9	96.8
CP, %	18.2	8.8
$aNDF_{om}$, %	47.7	42.0
ADF_{om}, %	26.8	22.8
$ADL_{(SA)}$, %	2.4	1.7
Ash, % (DM)	7.1	3.2
NSCs, %	27.0	46.0
Cellulose, %	24.4	21.1
Hemicellulose, %	20.9	19.2

[1] CP: crude protein; $aNDF_{om}$: neutral detergent fiber with α-amylase; ADF_{om}: acid detergent fiber; $ADL_{(SA)}$: acid detergent lignin, including ash; NSCs: non-structural carbohydrates, OM—(CP + $aNDF_{om}$); Cellulose: ADF_{om}—$ADL_{(SA)}$; Hemicellulose: $aNDF_{om}$—ADF_{om}.

2.2. Experimental Design and In Vitro Procedure

The effects of adding iodoform in two different substrates (MS and GS) were determined during four independent fermentations (runs) of 22 h. Iodoform (Sigma-Aldrich, 99%) was diluted in zeolite (aluminosilicate). Zeolite was chosen as an inert, non-nutritional carrier for the iodoform. It consisted of 96% ash. The dose chosen to test was based on the results from the in vivo research by Thorsteinsson et al. [3] in which iodoform was given intraruminally twice daily in 4 doses per day. The dose of 640 mg/day reduced yield and intensity significantly compared to the control. This dose, with a 20 kg DM intake, is the same as the 0.032 mg/g DM as used in the present research. A dilution was made in a stepwise series with the final concentration of 0.064% iodoform in zeolite (IZ). Four analytical replicates were included in each of the fermentations. In addition to the four treatments (MS and GS with or without IZ), bottles without substrate (blanks) were incubated. The blanks were used to correct for the baseline minimum microbial activity present in the rumen fluid.

Before a run, rumen fluid was collected from two cannulated late third- and fourth-parity Danish Red cows from Assendrup farm (Haslev, Denmark). The use of the cannulated animals was authorized by Danish law (license no. 2012-15-2934-00648). The lactating cows were fed with total mixed ratio of NaOH-treated wheat, rapeseed, maize silage, grass silage, wheat straw, and rapeseed cake (6.15 MJ/kg DM), for 6 weeks before the fermentation trials. For the first two fermentations, four bottles containing IZ with GS or with MS, four samples of each of the substrates without IZ, and six blanks were used

for a total of 22 bottles. During the last two fermentations, two extra blank samples were added for a total of 24 bottles.

2.2.1. Sample Preparation

Before each run, 500 mg (±10 mg) of substrate was added to each bottle. Assignment of samples to sequential bottle numbers, within a fermentation, was randomized so that placement in the fermentation chamber was not sequential. The bottles were completely randomized in the first and second fermentations, and a stratified randomization ensured that the replicates were equidistance in the third and fourth runs. Twenty-five mg of IZ was added to all bottles assigned to receive the additive. All bottles were placed in a thermoshaker (Gerhardt Analytical Systems, Königswinter, Germany) at 39.5 °C on the morning of the fermentation to reduce cold shock to the microbes when the rumen fluid was added.

2.2.2. Buffer Preparation

The buffer media were prepared as described by Menke and Steingass [18] before fermentation. The final media solution consisted of a mixture of buffer, macrominerals, and microminerals that was mixed with reazurin. This solution was kept on a magnetic stirring hotplate (IKA RCT Standard Pt1000, Lab Logistic Group, Meckenheim, Germany) to maintain the solution at 39.5 °C. The media were flushed with CO_2 for two hours before the arrival of the rumen fluid in the laboratory. Approximately 15 min before the arrival of the rumen fluid, a reduction agent consisting of sodium sulfide and sodium hydroxide (0.1 M) was added, to ensure that anaerobic conditions were achieved.

2.2.3. Rumen Fluid Preparation

The rumen fluid with feed particulates was collected from several locations in the rumen and directly transported to the laboratory in pre-heated thermoses. Upon arrival in the laboratory, the rumen fluid was filtered through two layers of commercial cheesecloth to eliminate large feed particles and gently squeezed to include microbes attached to the particles. An equal amount of fluid from each cow was used. The rumen fluid was mixed in a ratio of 1:2 (rumen fluid/media solution), and this inoculum (rumen fluid mixed to the media solution) was kept on a magnetic stirrer hotplate with a continuous flow of CO_2 above the liquid to remove oxygen. Dosing of the inoculum into the warm bottles commenced within 5 min of the final rumen fluid addition.

2.2.4. In Vitro Preparation

Precisely eighty-five mL of the inoculum was added to each bottle, a similar procedure used to that described in Jantzen and Hansen [19]. In short, the headspace of the bottle was immediately flushed with N_2 to remove other gasses and then closed with the Ankom^{RF} module (Ankom Technology®, Macedon, NY, USA). The modules are comprised of a circuit board, microchip, pressure sensor (pressure range: –10 to +4996 psi; resolution: 3.34 psi; accuracy ± 0.1% of measured values), vent valve that allows pressure release, and a radio frequency antenna that sends signals to the modules and receives a measure of absolute and ambient pressure from the modules via a base station. The base station is connected to a dedicated computer in which Ankom software is installed (RF GPM software version 11.4, Ankom Technology®, Macedon, NY, USA). The software was set to connect to the bottles every 60 s and release gas for 500 ms when the pressure within the headspace was more than 0.75 PSI over ambient pressure. Absolute and cumulative pressures were recorded every two minutes.

All the bottles were incubated in a rotating water bath (Thermo Scientific, Thermo Fisher Scientific, Waltham, MA, USA) for 22 h. The water heater was calibrated to maintain the fluid inside the bottle at 39 °C and set to 40 movements per minute. The vent valve of each module was attached to a gas-tight bag (50 mL; HedeTech Co., Ltd., Dalian, China) with silicon tubing. The gas bag was connected itself with another silicon tubing to

the Micro Gas Chromatograph (GC) system (990 MicroGC, Agilent Technologies, SRA Instruments Sas, Marcy l'Etoile, France), for periodic measurement of N_2, CO_2, H_2, and CH_4 concentrations in the bottle's headspace gas. A gas-tight valve between the gas bag and the GC is closed during gas collection from the bottle. Another valve located between the bag and module is closed during the injection of gas from the bag to the GC. After sampling, the bag is evacuated by suction and flushed with N_2 and the GC system is flushed with ambient air to ensure that no gas remained from the previous period. The bottles were sampled sequentially with 2.4 min between each GC measurement. In the first two fermentations, a GC injection was taken between 2 and 22 min from each replicate of a sample, while in the second two fermentations, the GC results for replicates were 14 min apart.

2.3. Post-Fermentation Sample Collection and Analyses

The bottles were placed in ice at the end of fermentation immediately after gas bag detachment. The undegraded feed material in each bottle was filtered into a F57 filter bag with pore size of 25 µm (Ankom Technology®, Macedon, NY, USA) by vacuum suction (max −0.32 bar pressure). These bags were used to determine the degradation of organic matter (dOM). Eighteen 5 mL samples of the filtrate were collected and immediately frozen for volatile fatty acid (VFA) analysis. The remaining filtrate was used to determine sample pH (sensION + PH31, Hach, Düsseldorf, Germany).

The samples for VFA analyses were immediately frozen to −20 °C. At the time of the analyses, the samples were defrosted and mixed by inversion. A total of 1 mL of aliquot was placed in Eppendorf tubes; 40 µL of crotonic acid was added as an internal standard, and 200 µL of metaphosporic acid was added [20]. The mixture was, again, mixed by inverting 3 times and allowed to incubate for 30 min at room temperature. After incubation, the samples were centrifuged for 10 min at 12,000 rpm with a centrifuge (Eppendorf AG 5417R, Hamburg, Germany) and the supernatant filtered through a 0.2 µm filter (CHROMAFIL® RC-20/25, Macherey-Nagel GmbH & Co. KG, Düren, Germany) into a GC vial for analysis. Determination of VFAs was undertaken on a GC (Nexis GC-2030, Shimadzu Scientific Instruments Inc., Kyoto, Japan) with a 30 m long wall-coated open-tubular fused-silica capillary column (Stabilwax-DA; 30 m × 0.32 mm i.d., 0.25 µm film thickness; Shimadzu, Riverwood Drive, Columbia, USA). The internal temperature of the machine was set to 145 °C for three minutes, then increased by 16.6 °C/min from 145 to 245 °C. The injector and the flame ionizer detector were kept at 250 °C. The gas flows were 24, 32, and 200 mL/minute for N_2, H_2, and synthetic air, respectively. The standard curve for the VFA detection was made using the standard mix from Sigma-Aldrich (St. Louis, MO, USA), and the run time for each sample was 12 min.

2.4. Calculations

The biomass filtered from blank samples was considered as minimum microbial biomass, and the average weight of the residue in the blanks was subtracted from the residue of all other samples. Organic matter degradation was calculated as

$$dOM\ (g/g) = 1 - \frac{dry\ weight\ of\ the\ bag\ after\ fermentation - empty\ bag\ weight}{sample\ organic\ matter\ (OM)}$$

(corrected for the minimum microbial biomass growth)

Microbial efficiency was estimated by calculating a partitioning factor (PF, mg dOM/mL TGP), using the ratio between the dOM and the final TGP at 22 h of fermentation [21].

The average gas pressure produced by the bottles containing only rumen fluid was considered as baseline gas production. This pressure was subtracted from the gas pressure produced by all the bottles until the cumulative gas in the blanks began decreasing, indicating absorption of gas into the inoculum. At this point, the maximum gas produced by the blanks was subtracted from all sample bottles [19]. Total gas production (TGP) was calculated for each sample from the recorded cumulative gas production. Blank-corrected

cumulative gas production was converted to mL of gas at STP (standard temperature and pressure)/g incubated OM by using the ideal gas law.

$$V = \frac{nRT}{P}$$

Thereafter, the yield of gas for each bottle was calculated from the gas volume (V).

$$TGP\ (mL\ gas/g\ OM) = \frac{V}{g\ OM\ in\ the\ sample} (\text{corrected for the blank})$$

The concentrations of the methane and hydrogen measured (%, v/v) at a given sampling point by the GC was multiplied by the TGP that had accumulated in the bag since the last measurement and summed across all measurements (mL/g OM).

2.5. Statistical Analyses

Statistical analyses were carried out using the software R v.4.1.1 [22]. The different traits were tested with two different models, first with a linear mixed model and then with a normal linear model. After verifying all assumptions of homoscedasticity, linearity of the data, and normality of the residuals, the best-fitting model was chosen. This was performed after testing the significance of the random effect with "exactLRT" function of the "RLRsim" package [23]. If the random effect was not found to be significant, the model was reduced by eliminating the random effect.

The first model selected was a linear mixed model implemented with function "lmer" of the "lme4" package [24]:

$$y_{ijkl} = \mu + Substrate_i + Additive_j + (Substrate \times Additive)_{ij} + Run_k + e_{ijkl}$$

where y_{ijkl} is the investigated trait (blank-corrected dOM, VFA concentrations, (acetate + n-butyric acid)/proprionate ratio, PF, and blank-corrected TGP); μ is the overall mean; $Substrate_i$ is the fixed effect of the ith type of substrate incubated (i = GS and MS); $Additive_j$ is the fixed effect of the jth presence or absence of the IZ additive in the treatment (j = 2; presence or absence of IZ); $(Substrate \times Additive)_{ij}$ is the 2-way interaction between $Substrate_i$ and $Additive_j$; Run_k is the random effect of the kth fermentation (k = 4); and e_{ijkl} is the residual random error term. The random effect and residuals were tested to be normally distributed with a mean equal to zero and variance σ_h^2 and σ_e^2, respectively. Separation of the least square means (LSM) were undertaken using "emmeans" package in R [25].

When the random effect was not significant for the model, it was deleted. The linear model was used to analyze the methane and hydrogen yield (mL/g OM) response variables.

3. Results

3.1. Substrate Differences

There was no significant difference between dOM of GS and MS after 22 h of fermentation (Table 2). Total VFA production in the filtrate after 22 h did not differ between substrates with 58.7 and 59.7 mMol/L produced during GS and MS fermentation, respectively. Only n-butyric, iso-valeric, and caproic acid contents in the filtrate differed significantly between substrates with a 14, 22, and 32% (6.57 vs. 7.50, 0.93 vs. 1.14, and 0.40 vs. 0.53, respectively) increase in the MS filtrate compared to the GS on the base of the contrasts. On the other hand, the (A + B)/P ratio ((acetate + n-butyric acid)/propionate ratio) and the PF did not differ at the end of the run. Maize silage tended (p = 0.086) to produce more gas than grass silage at the 6 h timepoint, but the difference was not significant (Table 2 and Figure 1).

Table 2. In vitro fermentation responses (degradability, volatile fatty acids, total gas) of fermenting maize silage and grass silage with and without the addition of iodoform.

	Treatments [2]				Substrate × Additive	Contrasts [3]		
Traits [1]	**GS**	**MS**	**GS + IZ**	**MS + IZ**	***p*-Value**	**GS vs. MS**	**GS vs. GS + IZ**	**MS vs. MS + IZ**
dOM, g/g	0.58	0.55	0.45	0.53	**0.014** [4]	0.273	**<0.001**	0.487
Volatile fatty acids (VFAs), mMol/L								
Acetate	37.8	37.1	38.8	37.6	0.734	0.538	0.339	0.629
Propionate	11.5	11.8	12.0	12.0	0.398	0.195	**0.018**	0.202
iso-butyric acid	0.54	0.63	0.66	0.55	**0.034**	0.138	0.070	0.209
n-butyric acid	6.57	7.50	7.20	7.45	**0.049**	**<0.001**	**0.012**	0.821
iso-valeric acid	0.93	1.14	1.09	1.06	**0.012**	**0.003**	**0.016**	0.229
n-valeric acid	0.98	1.04	1.10	1.01	**0.014**	0.142	**0.007**	0.435
Caproic acid	0.40	0.53	0.47	0.46	**0.026**	**0.004**	0.097	0.115
Total VFA	58.7	59.7	61.3	60.2	0.157	0.340	**0.019**	0.660
(A + B)/P	3.88	3.81	3.84	3.77	0.986	0.554	0.730	0.749
PF, mg/mL	3.93	3.70	3.20	3.31	0.223	0.227	**<0.001**	**0.044**
Cumulative total gas production (TGP), mL/g OM								
TGP, 6 h	59.6	65.2	58.0	72.8	**0.047**	0.086	0.611	**0.023**
TGP, 12 h	100	104	98	112	**0.034**	0.292	0.495	**0.021**
TGP, 18 h	129	130	126	139	**0.036**	0.696	0.544	**0.019**
TGP, 22 h	144	144	142	155	**0.029**	0.935	0.568	**0.013**

[1] dOM: degraded organic matter; (A + B)/P: (acetate + n-butyric acid)/propionate ratio; PF: partition factor; [2] GS: grass silage; MS: maize silage; GS + IZ: grass silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; MS + IZ: maize silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite. [3] Differences analyzed by linear mixed model and separation of LSM using Kenward–Roger's F-test. [4] Bold text highlights significant values.

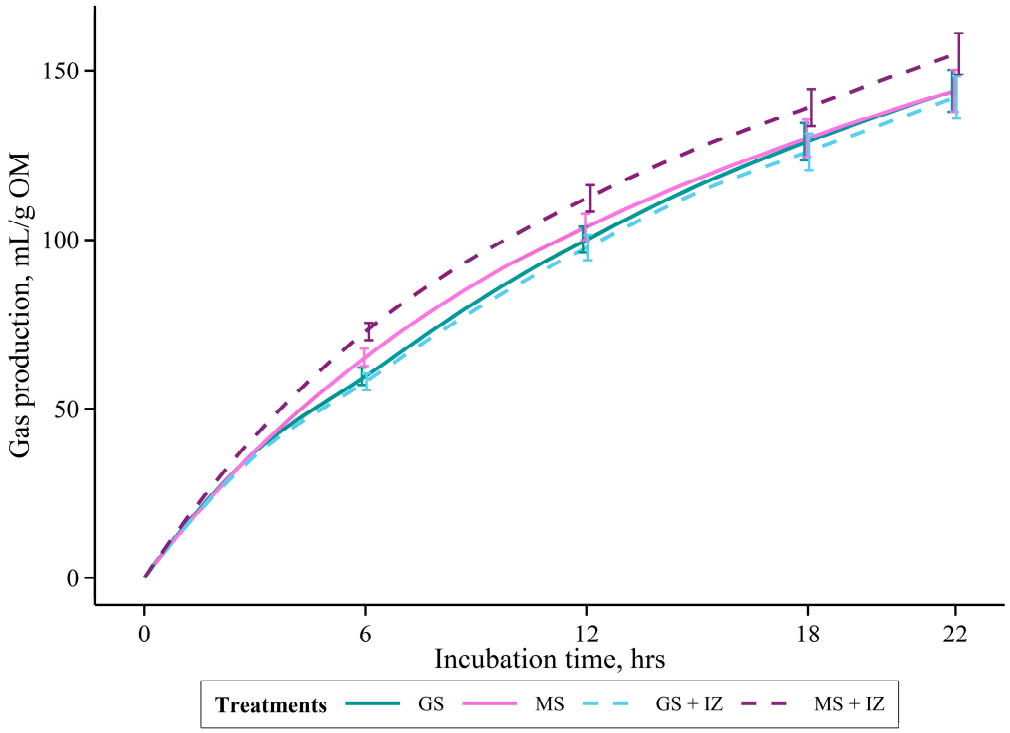

Figure 1. Cumulative total gas production (TGP, mL/g OM) of in vitro fermentation of maize and grass silage with and without iodoform. GS: grass silage; MS: maize silage; GS + IZ: grass silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; MS + IZ: maize silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite. The perpendicular bars report the standard error.

Methane yield (mL/g OM) was significantly higher for MS than GS, from the third hour until the seventh hour but not before or after (Table 3 and Figure 2A,B). Hydrogen yield (mL/g OM) differed significantly by substrate with a significantly higher production for MS from the third hour to the end of the sixteenth hour (Table 3 and Figure 3A,B). The total cumulative production of methane and hydrogen was significantly higher in MS than in GS with no significant additive effects or interaction (Table 3).

Table 3. In vitro fermentation responses (hydrogen and methane yield) of fermenting maize silage and grass silage with and without the addition of iodoform.

	Treatments [1]				**Substrate × Additive**	**Contrasts** [2]		
Traits	**GS**	**MS**	**GS + IZ**	**MS + IZ**	***p*-Value**	**GS vs. MS**	**GS vs. GS + IZ**	**MS vs. MS + IZ**
Methane yield, mL/g OM [3]								
1 h	0.04	0.03	0.02	0.03	0.313	0.287	0.068	0.686
2 h	0.16	0.19	0.11	0.21	0.461	0.609	0.449	0.771
3 h	0.37	0.70	0.31	0.75	0.508	**0.011** [4]	0.622	0.656
4 h	0.58	0.94	0.51	1.07	0.180	**0.002**	0.490	0.228
5 h	0.66	1.00	0.57	1.21	**0.027**	**0.001**	0.338	**0.030**
6 h	0.75	0.99	0.68	1.17	**0.044**	**0.008**	0.404	**0.043**
7 h	0.76	1.00	0.70	1.03	0.557	**0.010**	0.562	0.799
8 h	0.80	0.88	0.75	1.02	0.098	0.359	0.552	0.082
9 h	0.79	0.88	0.72	0.85	0.744	0.229	0.394	0.703
Hydrogen yield, mL/g OM [5]								
1 h	0.015	0.006	0.014	0.005	0.918	**0.002**	0.726	0.625
2 h	0.018	0.014	0.026	0.017	0.420	0.193	**0.044**	0.377
3 h	0.012	0.022	0.020	0.024	0.275	**0.007**	**0.037**	0.576
4 h	0.011	0.024	0.015	0.028	0.960	**<0.001**	0.243	0.280
5 h	0.010	0.023	0.014	0.027	0.888	**<0.001**	0.165	0.241
6 h	0.011	0.021	0.013	0.025	0.826	**<0.001**	0.331	0.208
7 h	0.009	0.019	0.014	0.020	0.348	**0.002**	0.133	0.864
8 h	0.012	0.016	0.013	0.018	0.898	0.051	0.507	0.406
9 h	0.010	0.016	0.013	0.015	0.300	**0.042**	0.282	0.691
10 h	0.009	0.014	0.011	0.014	0.676	0.127	0.516	0.957
11 h	0.009	0.015	0.012	0.010	0.081	**0.048**	0.362	0.118
12 h	0.008	0.025	0.019	0.008	0.075	0.128	0.299	0.136
13 h	0.007	0.012	0.019	0.017	0.588	0.600	0.225	0.660
14 h	0.021	0.012	0.008	0.035	0.256	0.694	0.572	0.297
15 h	0.028	0.018	0.004	0.030	0.180	0.612	0.211	0.513
16 h	0.007	0.087	0.003	0.018	0.076	**0.003**	0.874	**0.009**
Cumulative total yield, mL/g OM								
Methane	9.79	11.4	9.23	11.8	0.375	0.070	0.496	0.564
Hydrogen	0.356	0.594	0.258	0.508	0.950	0.085	0.464	0.527

[1] GS: grass silage; MS: maize silage; GS + IZ: grass silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; MS + IZ: maize silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite. [2] Differences analyzed by linear mixed model and separation of LSM using Kenward–Roger's F-test. [3] All values for methane yield after ninth measurement not significantly different and not shown. [4] Bold text highlights significant values. [5] All values for hydrogen yield after sixteenth measurement not significantly different and not shown.

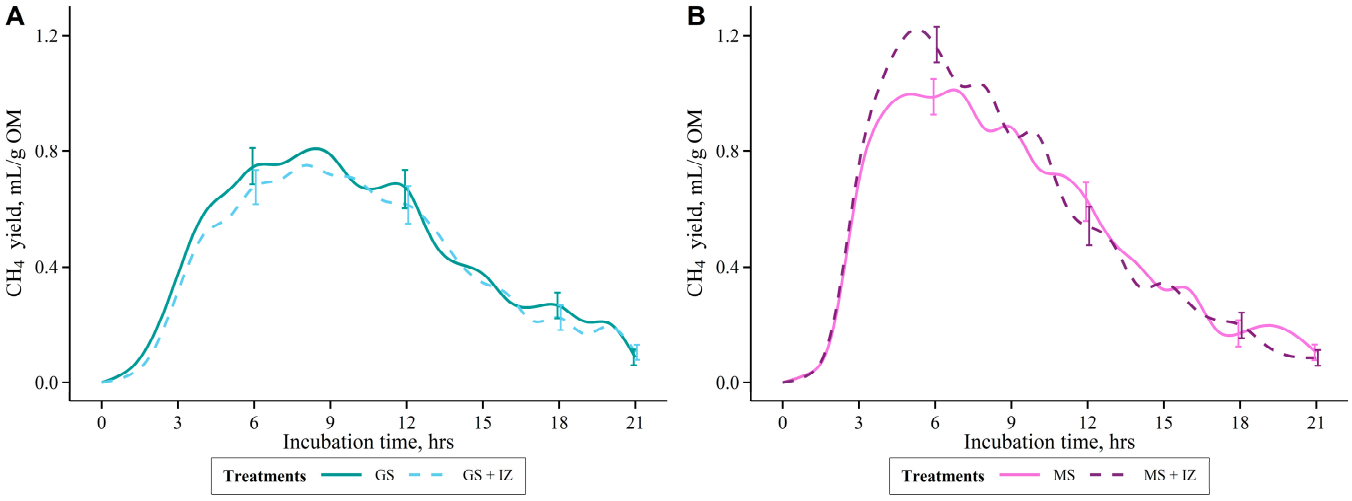

Figure 2. Methane yield (mL/g OM) from in vitro fermentations with and without iodoform (IZ): (**A**) grass silage; (**B**) maize silage. GS: grass silage; MS: maize silage; GS + IZ: grass silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; MS + IZ: maize silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; hrs: hours.

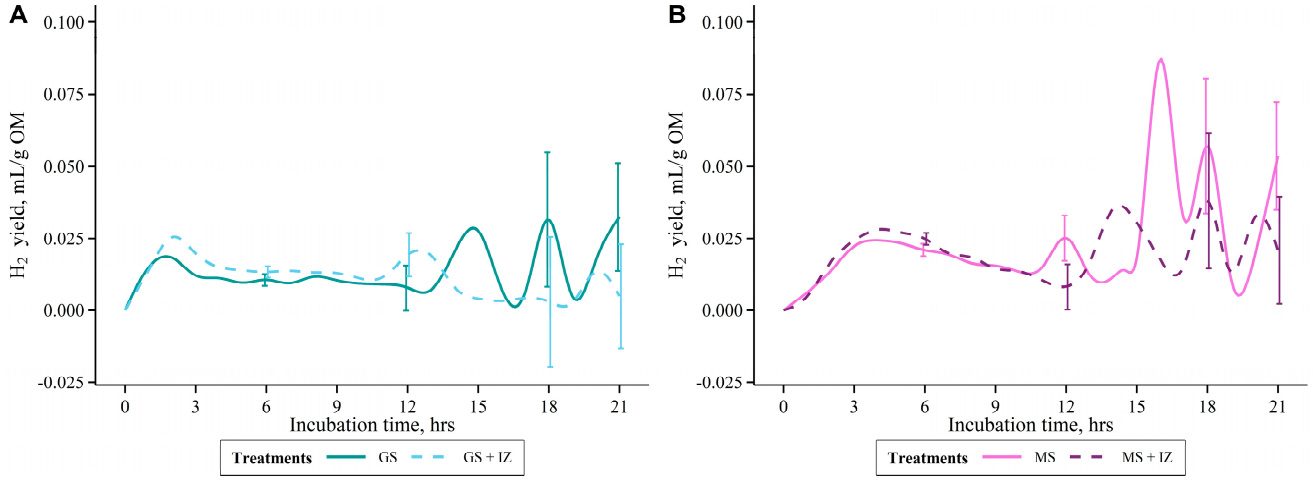

Figure 3. Hydrogen yield (mL/g OM) from in vitro fermentations with and without iodoform (IZ): (**A**) grass silage; (**B**) maize silage. GS: grass silage; MS: maize silage; GS + IZ: grass silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; MS + IZ: maize silage with 0.032 mg/g of dry matter substrate of 0.064% iodoform in zeolite; hrs: hours.

3.2. Differences between Substrate with and without Iodoform IZ

The addition of IZ decreased dOM, but with a much larger reduction in GS (22% and $p < 0.001$; 0.58 vs. 0.45) than in MS (4% and $p > 0.05$; 0.55 vs. 0.53). The addition of IZ to GS resulted in a consistent numerical increase in the production of all VFAs. When IZ was added to GS, this increased both propionate and total VFAs significantly by about 4.3% (12.0 vs. 11.5) and 4.4% (61.3 vs. 58.7), respectively, when compared to pure GS. Also, n-butyric and iso-and n-valeric acids increased significantly in GS with IZ. The PF significantly decreased for GS when IZ was included.

No significant effect of IZ in GS was seen on methane yield, despite a consistent, numerical decrease in production. The addition of IZ increased the hydrogen yield from GS from the second hour to the thirteenth hour, but the increase was only significant at the second and third hours.

The differential effect of IZ on GS and MS resulted in a significant interaction of substrate and additive (Table 2) for dOM, iso-butyric, n-butyric, iso-valeric, n-valeric,

caproic acids, and TGP. On the individual substrate level, the addition of IZ did not significantly affect VFA traits in MS. The addition of IZ to MS consistently and significantly increased TGP. The PF significantly decreased for MS when IZ was included.

The addition of IZ to MS increased the methane yield significantly at the fifth and sixth hours (Table 3, Figure 2A,B). However, a non-significant increase in hydrogen was only seen from the end of the second hour to the eighth hour.

4. Discussion

4.1. Differences between Substrates

Plant variety, plant maturation, soil conditions, prevailing weather, compression during ensiling, and duration of ensiling are factors that affect the nutrient contents of maize silage and clover grass silage. Typically, the contents of cellulose, hemicellulose, and lignin increase with plant maturity in GS, while the relative ratio of starch to fiber increases until maturation in MS [12,26]. The organic matter in Danish MS typically contains 8–9% crude protein (CP), 89% carbohydrates, 36–42% neutral detergent fiber (NDF), 28% starch, and 2% sugar. Clover grass silage organic matter, on the other hand, is reported to contain between 17 and 21% CP, 74–77% carbohydrates, 37–42% NDF, 4.4% starch, and 4–5% sugar [27]. The major nutrients of the substrates used in the present study are in agreement with the composition found in the literature, as shown in Table 1. However, the NDF content of GS was a bit higher than expected.

Given the differences in NDF, lignin, and ash measured in the substrates and expected differences in cellulose and sugar, we might expect to observe a higher dOM for MS with respect to GS. However, in our study, no significant difference was observed, despite 70% more non-structural carbohydrates (NSCs) in MS. This is most likely due to the 41% more lignin in GS which, while part of the OM content, is not degraded. The NSCs are the easiest and most rapidly degraded carbohydrates and are almost completely fermented in the rumen [14]. In contrast, cellulose and hemicellulose are slowly digested because they are linked to the lignified matrix of the sample. The slow degradation of this fraction is fundamental to maintain the ruminal environment [14]. However, the focus of this research was to evaluate the effect of iodoform in the early stage of fermentation. The Ankom in vitro system does not allow the passage of feed, rumen fluid, and a buffer exchange. Therefore, longer incubation times could misrepresent the whole process of fermentation in live animals [28].

The total quantity of VFAs did not differ between GS and MS, but differences in specific VFAs were seen (i.e., n-butyric, iso-valeric, and caproic acids). A high NDF content in the substrate has been shown to decrease total VFA concentration and change the pattern of VFAs produced to more acetic acid and less propionic acid production [29]. In our research, we did not find these differences, despite 12% more cellulose and hemicellulose and 41% more lignin in GS. As reported by Pecka-Kiełb et al. [29], Liu et al. [30], and Dijkstra [31], the branched chain fatty acids (iso-butyric, iso-valeric, and 2-methylbutyrate) derived from the degradation of feed protein, reflect proteolytic activity of ruminal microorganisms and the oxidative deamination process. In the present research, the iso-valeric content was significantly higher in MS than in GS. The higher content of iso-valeric in MS does not reflect a higher protein content in MS compared to GS, but could, however, reflect microbial lysis after 22 h in MS as proposed by Cattani et al. [28]. Microbial lysis occurs during what is considered prolonged incubation. Substrate-specific fermentation duration has been suggested as microbial growth differs between substrates [28]. Although there were the basic differences in cell wall and NSC contents between GS and MS, the acetic acid and butyric acid ratio to propionic [(A + B)/P] ratio and the efficiency of microbial biomass production (PF) were not significantly different. The (A + B)/P ratio falls within the suggested range of 3:1–4:1 for a healthy rumen environment [32]. However, no comparable PF values were found in the literature.

The TGP depends primarily on the NSCs (i.e., starch and sugars), which are easily digested, and thereafter on the amount of cellulose and hemicellulose of the substrate. Lignin

is not degraded, while the protein content has been shown to retard gas production [33]. Usually, increasing the content of fiber reduces digestibility and therefore total gas. This was seen in the recent results by Pecka-Kiełb et al. [29] who found that sorghum silage produced less TGP and reduced digestibility compared to GS and MS. The sorghum silage contained more fiber, measured as crude fiber, NDF, and ADF. Total gas production was, however, not significantly different between pure GS and MS in the present study, and there was only a tendency ($p = 0.086$) for MS to produce more TGP than GS. This difference was expected to be higher due to the larger quantity of NSCs in MS that is quickly fermented as suggested by Cone and Van Gelder [33]. A 10% difference in TGP from GS and MS was seen, but only in the first 6 h, and the difference was not significant.

Methane and hydrogen yields (mL/g OM) were numerically higher in MS than for GS. Methane yield is affected by the TGP and methane concentration, which is in turn, affected by cellulose, hemicellulose, and protein contents during early fermentation of the sample [34]. The decreased TGP of GS is related to the delay in fiber breakdown compared to the immediate NSC breakdown in MS, and therefore gas production. This means that the methane yield during the early fermentation will also be delayed. In the same interval (3–7 h), the hydrogen production was significantly less in GS compared to MS. This was not expected as higher contents of cellulose and hemicellulose in GS would be expected to produce proportionally more methane and therefore use the hydrogen during methanogenesis [35].

4.2. The Effect of the Additive in the Substrates on All the Evaluated Traits

4.2.1. Addition of IZ in GS

Despite the non-significant differences in the substrates for dOM and VFA production, the addition of IZ did not elicit the same responses in GS and MS. The inclusion of IZ to the GS reduced dOM and resulted in a significantly higher total VFA production and a shift in the VFA profile at the end of the fermentation. This is in contrast to Thorsteinsson et al. [3] who found that the total amount of VFAs decreased in the rumen with an increasing dose from 320 mg to 800 mg/day of iodoform in an in vivo feeding trial with cows fed a partial mixed ration with maize (25% DM) and clover grass (28% DM) silages where the iodoform was solubilized in ethanol, mixed into concentrate, and dosed directly into the rumen. The total VFA content in the control and the two lowest doses tested (320 and 640 mg/day) were not significantly different. This is not aligned with the results from the present study; however, no PFs were calculated in the in vivo study [3]. The significant increase in total VFAs seen in the present study when IZ was added to GS may be due to the decreased efficiency of microbial conversion, as seen by the decreased PF. The addition of IZ to GS decreased the PF significantly by about 19% (3.20 vs. 3.93). A decrease in PF is associated with a decreased microbial efficiency during OM conversion to microbial biomass [36]. The lower PF aligns with the decrease in dOM of GS with IZ. This decrease in conversion efficiency with the addition of IZ is reflected in the different proportions and final amount of VFAs. This decrease was significant even though the (A + B)/P ratio was not affected by this shift. This decrease occurred despite the fact that the (A + B)/P ratio has been shown to be a determining factor in gas production [37]. While the TGP was not significantly affected by IZ in GS, the addition of IZ significantly increased n-butyric and iso-valeric acids. This is the same result found by Thorsteinsson et al. [3] between the control and the medium dose (640 mg/day). Valeric acid and propionate are a sink for the hydrogen produced in the rumen [38]; therefore, an increase in these should result in a methane reduction by decreasing the availability of the hydrogen to the methane-producing archaea microbes. The addition of IZ decreased the methane yield in GS, consistently but not significantly, as expected from the results of Thorsteinsson et al. [3]. This decrease in methane resulted in a significantly increased hydrogen yield.

4.2.2. Addition of IZ in MS

When IZ was added to MS, no significant differences were found for dOM, and the VFA profile was not significantly different to MS without IZ. This is in contrast to Thorsteinsson et al. [3]. However, this study tested the effect of iodoform in grass and maize silages. No studies were found in the literature that tested iodoform in pure GS or MS. As in the addition of IZ to GS, the PF decreased significantly but only by about 12%, (3.31 vs. 3.70), albeit the dOM was not significantly affected. Total gas production increased consistently and significantly compared to MS without IZ. Therefore, the decrease in PF when adding IZ to MS is related to the increase in TGP.

The addition of IZ to MS consistently and significantly increased the methane yield, and this effect was not expected, given the results found by Thorsteinsson et al. [3]. With the addition of IZ to MS, the hydrogen yield numerically increased compared to MS, but it did not differ significantly, with the exception of the 16th hour. This means that the three mechanisms of methane reduction by halomethanes proposed by Thorsteinsson et al. [3], Glasson et al. [7], Patra et al. [8], and McAllister and Newbold [5] were not followed in our research in MS. After 22 h of fermentation, no differences were found between the normal substrate and those with IZ for the methane and hydrogen yields.

4.3. Differential Effect of IZ on Substrates

The differential effect of the addition of IZ to the two substrate is visible by the methane yield. When IZ was combined with GS consistently, even if not significantly, a reduction in CH_4 that is accompanied by the increase in H_2 yield was found as expected. On the other hand, when IZ was added to MS, the effect was not the same, but opposite. The addition of the additive to MS caused an inconsistent change in the hydrogen yield but a consistent increase in the methane yield, which was occasionally significant. The differential effect is also visible on other traits. When IZ was added to GS, a reduction in dOM, a shift in VFA profile production, a reduction in PF, and a numerical reduction of TGP were observed. On the contrary, when IZ was added to MS, a reduction in PF, and an increase in TGP were observed. The clearly differential and sometimes opposing effects of iodoform on GS and MS was unexpected when comparing the results with Thorsteinsson et al. [3] who used a mixture of GS and MS. When testing iodoform in the mixture of GS and MS, at a similar dose, a marked reduction of dOM and methane (g/hour) was seen. However, the response parameter methane yield was chosen in the current research to represent what is potentially delivered to the environment per gram OM of the substrate by enteric fermentation.

In summary, the findings from this study underscore the complex interplay between the substrate composition and the effect of iodoform (IZ) on ruminal fermentation traits. The responses to iodoform varied significantly between GS and MS. This variability highlights the need for further investigation into the specific mechanisms through which iodoform influences microbial fermentation and nutrient utilization.

5. Conclusions

This study assessed the effects of iodoform on the ruminal fermentative traits of degraded organic matter, volatile fatty acid profile and composition, and methane and hydrogen yields. The results show that iodoform (IZ) had different impacts when tested in vitro with GS and MS, with these effects varying in magnitude and sometimes showing opposite trends. When IZ was added to GS, there was a reduction in dOM, a shift in VFA profile, and a consistent numerical reduction in TGP and methane yield, with a slight increase in hydrogen production. Conversely, when IZ was added to MS, TGP increased significantly, while dOM and VFA production and profiles were not significantly affected. The methane yield increased significantly, whereas hydrogen production remained unchanged. Furthermore, when iodoform was added to both MS and GS, the partitioning factor (PF) decreased significantly, indicating a reduction in the efficiency of microbial biomass conversion. For GS, the lower efficiency was linked to the decreased degradation of organic matter, while in MS, the reduction in PF was attributed to the increased total gas

production. The significant interactions confirm that iodoform has different effects on MS and GS. Given the limited scientific research on the use of iodoform in animal nutrition, this study provides a foundation for the development of future projects that could study and explore, in more detail, the action of this halomethane compound in different types of substrates.

Author Contributions: Conceptualization, S.M. and H.H.H.; methodology, S.M. and H.H.H.; validation, S.M. and H.H.H.; formal analysis, S.M. and H.H.H.; investigation, S.M., B.J., A.M.D.A. and H.H.H.; resources, H.H.H.; data curation, S.M., B.J., A.M.D.A. and H.H.H.; writing—original draft preparation, S.M., B.J., A.M.D.A. and H.H.H.; writing—review and editing, S.M., B.J., A.M.D.A., F.T. and H.H.H.; visualization, S.M. and H.H.H.; supervision, F.T. and H.H.H.; project administration, H.H.H.; funding acquisition, H.H.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Innovations Fund Denmark grant. Project name: "Novel triple action feed additive to reduce enteric methane emission from cattle by blocking the enzymatic process, suppressing methanogens and draining the hydrogen substrate". Acronym "NoMethane". File number: 0224-00011B.

Institutional Review Board Statement: The use of cannulated animals was authorized according to the Danish law (license nr. 2012-15-2934-00648).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding authors upon reasonable request.

Acknowledgments: The authors want to thank the University of Padova, DAFNAE Department, for the opportunity given to the first author to spend her period abroad at the University of Copenhagen in Denmark. The authors also thank the Innovations Fund Denmark grant project "NoMethane" for the support of the laboratory trials. Many thanks also to the assistance given by the laboratory technicians Anni Christiansen and Lotte Ørbæk.

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

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