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Title

Effect of tannic acid combined with fluoride and lignosulfonic acid on anaerobic digestion in the agricultural waste management chain.

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Abstract

Livestock waste is stored and used as soil fertilizer or directly as substrate for biogas production. Methane emissions from manure storages and ammonia inhibition of anaerobic digesters fed with manure, are well-known problems related to manure management. This study examines the effect of adding tannic acid with fluoride (TA-NaF) and lignosulfonic acid (LS) on methanogenic activity in batch reactors with ammonia inhibited maize silage digestate and in batch reactors with manure. Lignosulfonic acid counteracted urea induced ammonia inhibition of methanogenesis, whereas TA-NaF inhibited methanogenesis itself. Stable carbon isotope ratio analysis and methanogen community analysis suggested that TA-NaF affected acetoclastic methanogens the most. The combined findings suggest that TA-NaF could be used to reduce methane emissions from stored manure. Conversely, LS could be used as supplement in anaerobic digesters prone to urea induced ammonia inhibition.

Keywords

Anaerobic digestion, Ammonia inhibition, Stable isotope, Methanogens, Tannic acid
1. Introduction

Agricultural waste holds a vast potential as nutrient and energy source if managed properly (Westerman and Bicudo, 2005), whereas improper management may result in increased environmental pollution and greenhouse gas emissions (Holly et al., 2017). Anaerobic digestion (AD) may extract value from agricultural waste by producing biogas, which fuels heat or power generation at combined heat and power units (Wu et al., 2016). The digestate from AD maintains its value as a fertilizer, and it has been suggested to emit less odorants upon subsequent landspreading (Hansen et al., 2006). Under some circumstances, the waste is not used for AD owing to challenges with manure transport to an AD facility, national regulations or AD inhibiting constituents such as ammonia. Ammonia in AD arises mainly from dietary protein catabolism and enzymatic urea hydrolysis by urease (Elzing and Monteny, 1997), and it exhibits an inhibitory effect on methanogenesis (Lv et al., 2019). Urease is produced in fecal material by ureolytic bacteria, and urea is excreted in urine (Elzing and Monteny, 1997). Consequently, manure fed AD frequently suffers from ammonia inhibition (Sun et al., 2016). In this scenario, composting or manure storage followed by landspreading is the conventional management route (Hou et al., 2015). However, manure storage and subsequent soil application is associated with methane emissions and significant N-loss in the form of ammonia or nitrous oxide, which reduce the fertilizer value and contribute to global warming (Lee et al., 2017; Sørensen and Amato, 2002).

It is imperative that agricultural operations manage waste materials and reduce the environmental footprint in all parts of the waste management chain. In this regard, manure waste treatment with polyphenols may be a key strategy that can alter its applicability in AD or for storage and landspreading activities, thereby offering more
flexibility in the management chain. Polyphenols are a wide group of secondary plant metabolites containing large numbers of di and/or trihydroxyphenyl units (Bravo, 2009; Quideau et al., 2011), and have been identified as antimicrobial agents (Papuc et al., 2017). Tannic acid (TA) is a polyphenol consisting of gallic acid and polyol units (Bravo, 2009) that in combination with sodium fluoride (NaF) was found to mitigate ammonia, methane and volatile organic compounds emissions from manure (Dalby et al.) The mitigation of ammonia emissions was partially attributed to a synergism between TA and NaF that directly inhibited the urease enzyme, but it was also attributed to the inherent antimicrobial effects of TA, as claimed in other studies (Al-Jumaili et al., 2017; Whitehead et al., 2013). The urease inhibiting effect of TA could potentially be of value in ammonia inhibited AD. However, TA-NaF reduces methanogenic activity, obscuring the potential positive effect of urease inhibition. Therefore, TA-NaF may be more adequate as supplement to stored manure from which methane emissions should be abated. The pulp and paper industry byproduct, lignosulfonic acid (LS) (Calvo-Flores and Dobado, 2010), which contains similar functional hydroxyl groups to TA, may be an alternative supplement to urea rich AD substrates. Despite reported anti-methanogenic activity of some lignin derivatives, the inhibitory effect of these seems to be linked to low molecular weight lignins (Sierra-Alvarez, 2007).

The aim of this study was to elucidate the effect of TA-NaF and LS on methane production and methanogenic pathways in a standard biogas reactor (fed with inoculum from a maize silage fed reactor) and in stored swine manure, cattle manure, and poultry litter. Owing to the urease inhibiting activity of TA-NaF, it was hypothesized that LS would exhibit a similar urease inhibiting effect in urea loaded biogas reactor slurry and thereby increase the methane yield. Furthermore, it was hypothesized that TA-NaF may
inhibit acetoclastic methanogenesis, which would counteract the positive effect of
urease inhibition in biogas reactors. The interpretation of methane yield, compound-
specific stable isotope analysis of the produced biogas, and microbial community
structure analysis were used to test these hypotheses. The findings hint to possible
application areas of TA-NaF and LS in the manure management chain, which could
advance the sustainability of agricultural activities.

2. Materials and methods

Figure 1 presents a schematic of the experimental work conducted in this study. The
study consists of two parallel experiments in which digestate obtained from a biogas
reactor fed with maize silage (hereinafter denoted as maize silage digestate) or different
manure types were used as inocula. The maize silage digestate batch reactors were
treated with TA-NaF, LS, urea, and/or cellulose as substrate, whereas manure reactors
were treated with TA-NaF or LS. Besides the methods displayed in Figure 1, the
inocula were also analyzed for relevant chemical characteristics.

2.1 Maize silage digestate batch reactor experiments

Two consecutive batch experiments were conducted using an automatic methane
potential test system (AMPTS, Bioprocess control, Lund, Sweden) under mesophilic
conditions (38 °C). Batch 1 and batch 2 was performed to test the effect of TA-NaF and
LS under high urea load and without urea load, respectively. Table 1. compiles the
reactor treatments of batch 1 and batch 2. As inoculum, degassed digestate from a large-
scale biogas plant, operating with maize silage as a main substrate, was used (referred to
as maize silage digestate). Each batch experiment lasted 30 days and included 15 x 500
mL reactors incubated with 350 g inoculum. The experiment was conducted under anaerobic conditions by initially flushing the reactors with nitrogen. Methane production was measured volumetrically, and carbon dioxide was removed by channeling the produced gas from the batch reactors through the headspace of a 100 mL 3M sodium hydroxide solution with a thymolphthalein pH indicator prior to the volumetric gas detection unit according to the AMPTS default recommendations. The measured gas volumes were corrected to standard temperature (273.15 K) and pressure (101.32 kPa), then reported as normalized milliliters. A detailed description of the AMPTS setup is provided in the Supplementary materials. The reactors were supplemented with 1% (w/w) cellulose (Sigma Aldrich CAS 9004-34-6) as substrate and 1% (w/w) urea-N (Sigma Aldrich, CAS 57-13-6) to induce ammonia inhibition. As a treatment, either 1% (w/w) lignosulfonic acid sodium salt (LS) (Sigma Aldrich, CAS 8061-51-6) or 2.5 to 10 mM tannic acid (TA) (Sigma Aldrich, CAS 1401-55-4) combined with 1 mM sodium fluoride (NaF) (Sigma Aldrich, CAS 7681-49-4) was added. Demineralized water was added to level out volume differences between reactors. Each experimental treatment was performed in triplicates.

**2.1.1 Sampling and analyzes of maize silage digestate batch reactors.** The pH, volatile solids (VS), and total solids (TS) were measured at the beginning and end of the experiments. Samples for microbial community structure analysis, total ammonia nitrogen (TAN), and volatile fatty acids (VFA) analysis were collected at experiment start and end and stored at -18 °C until analysis. Gas samples for biogas composition and compound-specific isotope analysis were collected every 2-4 day through a customized gas sampling port with rubber septa inserted in TYGON tubings between...
the reactors and the CO$_2$ trap of the AMPTS. The gas samples were stored in 20 mL argon flushed vials until analysis.

2.2 Manure batch reactor experiments

Swine manure (2% VS), cattle manure (7.5% VS), and poultry litter (50% VS) was initially diluted to the same VS content (2%) with demineralized water. Then 36 x 200 mL serum bottles were each inoculated with 120 g of the diluted manure without additional cellulose substrate. The reactor headspaces were flushed with nitrogen to obtain an anaerobic environment. Eighteen of the reactors were incubated at room temperature (~23 °C) and 18 reactors were incubated at 38 °C using a heating chamber. Incubation at room temperature simulated manure storage conditions, whereas incubation at 38 °C was prepared to enhance methanogenic activity and simulate a mesophilic biogas reactor fed with manure. Cattle manure was collected from an operating biogas reactor’s manure storage at the Deutsches Biomasseforschungszentrum, Leipzig, Germany. Swine manure and poultry litter were collected from local farmers and stored at 5 °C for one month before experiment start. The manure reactors were supplemented with either 5:1 mM TA-NaF or 1% (w/w) LS as treatments prior to incubation. All reactor setups were performed in duplicates.

2.2.2 Sampling and analyzes of manure batch reactors. The headspace pressure development was measured frequently with a LEO 5 digital manometer (Omni instruments, Dundee, UK), and samples for biogas composition and compound-specific isotope analysis were collected from the headspace and stored as described for the maize silage reactors. After gas sampling, the headspace was flushed with nitrogen for 1 minute at ~5 L min$^{-1}$ via syringe needles through rubber septa. The pH, TAN, VFA, VS,
TS, and samples for microbial community analysis were collected as described for the maize silage reactors.

2.3 Chemical analysis

Biogas composition was analyzed on a Clarius 580 GC system (PerkinElmer, Washington, USA) using a 7' HayeSep N 60/80, 1/8" Sf column followed by a 9' Molecular Sieve 13x mesh 45/60, OD 1/8" using a thermal conductivity detector (Agilent Technologies, Germany). Samples for VFA analysis were esterified as described in Mulat, D. et al., (Mulat et al., 2016) and analyzed on a GC 7890A GC System (Agilent Technologies, Germany) equipped with a DB-FFAP column (Agilent technologies, Germany) (length 60 m, ID 0.25 mm and film thickness of 0.5 µm) and a flame ionization detector (Agilent Technologies, Germany). Total VFA was calculated as the sum of C1-C10 linear carboxylic acids, lactic acid, benzoic acid, phenylacetic acid, and phenylpropanoic acid. For TAN analysis, samples were diluted in demineralized water (1000 – 4000 times) and determined by the standard Nessler method using a DR 3900 benchtop spectrometer (Hach-Lange, Loveland, CO, USA) (Koch and McMeekin, 1924). The VS and TS were determined gravimetrically by heating at 105 °C (Binder oven, Germany) for 24 h followed by burning at 550 °C (P300 Nabertherm furnace, Germany) for 6 h. The sugar content in lignosulfonic acid was determined according to (Sluiter et al., 2012).

2.4 Compound-specific isotope analysis

The δ^{13}C isotope signature was analyzed by gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). A GC 7890A (Agilent Technologies, Germany)
equipped with a GC IsoLink interface coupled via a ConFlo IV open split system to a MAT 253 IRMS (Thermo Scientific, Waltham, Massachusetts, USA) was used. Samples of 0.2 to 1.0 mL headspace were injected with a split ratio of 1:5.

Chromatographic separation was done on a PoraBOND Q column (50 m length, 0.32 mm inner diameter, 5 μm film thickness; Agilent technologies, Germany) at a constant helium carrier gas flow of 2.0 mL/min with the following temperature program: 40 °C (hold 120 min isotherm); increasing at a 20 °C min\(^{-1}\) rate to 250 °C (hold 10 min isotherm). The injector temperature was set to 250 °C. The bulk δ\(_{13}\)C signatures of cellulose, TA, LS, and urea were measured with an elemental analyzer - isotope ratio mass spectrometry system (EA-IRMS) as described in Supplementary materials.

Stable carbon isotope ratios were reported as delta notations relative to the international standard Vienna Pee Dee Belemnite (VPDB) (Coplen, 2011; Werner and Brand, 2001).

\[
\delta^{13}C = \left( \frac{^{13}C/^{12}C \text{ (sample)}}{^{13}C/^{12}C \text{ (VPDB)}} - 1 \right) 
\]  

(1)

The isotope fractionation factor, \(\alpha_{AB}\), for the reaction, \(A \rightarrow B\), is defined according to (Conrad, 2005).

\[
\alpha_{AB} = \frac{\delta A + 1000}{\delta B + 1000} \quad (2)
\]

Alternatively, isotope fractionation can be expressed as an enrichment factor (Conrad, 2005), \(\varepsilon_{AB}\), as:

\[
\varepsilon_{AB} = (1 - \alpha_{AB}) \cdot 10^3 \quad (3)
\]

However, the apparent fractionation factor, \(\alpha_c\), between CO\(_2\) and CH\(_4\) (Conrad, 2005; Whiticar, 1999) is more convenient to use when dealing with mixed cultures:
\[ \alpha_c = \frac{\delta^{13}\text{C}_{\text{CO}2} + 1000}{\delta^{13}\text{C}_{\text{CH}4} + 1000} \]  

(4)

If \( \delta^{13}\text{C}_{\text{CO}2} - \delta^{13}\text{C}_{\text{CH}4} < 100\% \), the equivalent apparent enrichment factor, \( \epsilon_c \), can be approximated (Conrad, 2005; Fry, 2003) as:

\[ \epsilon_c \approx \delta^{13}\text{C}_{\text{CO}2} - \delta^{13}\text{C}_{\text{CH}4} \]  

(5)

Hydrogenotrophic methanogenesis displays larger isotope fractionation than acetoclastic methanogenesis, yielding more depleted (negative) \( \delta^{13}\text{C}_{\text{CH}4} \) values (Conrad, 2005). Thus, characteristic \( \epsilon_c \) values for hydrogenotrophic methanogenesis and acetoclastic methanogenesis dominated environments are \( \epsilon_c > 65\% \) and \( \epsilon_c < 55\% \), respectively (Conrad, 2005; Whiticar, 1999).

2.5 Microbial community analysis

2.5.1 DNA extraction. Samples were defrosted and 400-500 mg were used to extract DNA using a NucleoSpin soil kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The DNA quality was checked with a 0.8% agarose gel electrophoresis, and the DNA concentration was determined using a NanoDrop ND-1000 UV/visible spectral photometer (PeqLab, Germany) and a Qubit dsDNA BR Assay kit (Invitrogen, Waltham Massachusetts, USA).

2.5.2 Amplicon sequencing of 16S rRNA and mcrA. The bacterial community structure was assessed by PCR amplifying the V3-V4 variable regions of the archaeal and bacterial 16S rRNA gene using the 341f (5'-CCTACGGGNGGCWGCAG-3') and 785r (5'-GACTACHVGGGTATCTAATCC-3') primer set (Klindworth et al., 2013). The methanogenic community structure was assessed by PCR amplifying the methyl coenzyme reductase A gene (mcrA) by using the mlas (GGTGGTGTGMDGD TTCACMCARTA) and mcrA-rev (CGTTCATBGCGTAGTTVGGRTAGT) primer set.
The PCR products were purified with Agencourt AMPure XP magnetic beads and a magnetic stand (Beckman Coulter, Brea, California, USA). An index PCR on the purified PCR products was carried out using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, California, USA). The cleaned index PCR products were diluted and sequenced with the Illumina MiSeq amplicon sequencer (Illumina V3, 2X300bp).

2.6 Data analysis and statistics

2.6.1 Microbial community data analysis. For 16S rRNA and mcrA gene sequencing, the raw sequencing data was processed in QIIME2 bioinformatics platform 2018.11 (Bolyen et al., 2018). Denoising of paired-end reads, dereplication, chimera filtering, and generation of Amplicon Sequence Variants (ASVs) were made with the DADA2 plugin according to instructions in (Callahan et al., 2016). For 16S rRNA the taxonomy was assigned to the ASVs using the MiDAS 2.1.3 reference database built for the V3 – V4 hypervariable regions, respectively (McIlroy et al., 2015). The methanogen taxonomy was assigned using a custom database of mcrA genes (Popp et al., 2017). For the 16S rRNA and mcrA amplicons, the amplicon sequence variants frequency table, taxonomy, and DNA sequences were exported from QIIME2 objects to text and FASTA files for data analysis. Non-metric multidimensional scaling plots were generated in R (R Core Team, Vienna, Austria) using the vegan package and the “envfit” function.

2.6.2 Statistics. Errors reported in tables and figures are presented as sample standard deviations, and statistical significances were based one-way ANOVA with a Tukeys HSD post hoc test to test pairwise differences between group means. For one-way
ANOVA and Tukeys HSD the level of significance (α) was 0.05. The statistical tests were done in Microsoft Excel 2016.

3. Results and Discussion

3.1 Chemical characterization

3.1.1 Maize silage digestate batch reactors. The characterization of the maize silage digestate is shown in Table 1 at experiment initialization (day 0) and end (day 30). The TAN increased considerably for LS, 10:1 mM TA-NaF, and urea treated reactors from after 30 days compared to the control reactors (Inoc+sub) (batch 1). This TAN increment was clearly a consequence of urea hydrolysis. The acetic acid concentration in 10:1 mM TA-NaF treated samples increased to 4250 mg/L by day 30. This indicated sustained acetate production and concurrent inhibition of syntrophic acetate oxidation and acetoclastic methanogenesis. The acetic acid content of urea amended control reactors increased by day 30 and was significantly higher than the acetic acid content in reactors with urea+LS (batch 1). This suggests that LS counteracted the urea induced ammonia inhibition of methanogenesis.

3.1.2 Manure batch reactors. The characterization of the swine manure, cattle manure and poultry litter, which were prediluted to the same initial volatile solids content, is shown in Table 2 at experiment initialization (day 0) and end (day 30). In Table 2, the acetic acid concentrations were generally higher for reactors incubated at room temperature. Treatment with TA-NaF decreased the acetic acid content in the poultry litter batch reactors, contrasting the increase of acetic acid in the swine and cattle manure batch reactors. Nevertheless, high acetic acid content in the poultry litter control and poultry litter+LS reactors were observed. Hence, it was speculated that acetic acid...
accumulation resulted from a lack of methanogenic communities in the poultry litter. Methanogens have only been reported in poultry excreta in a few studies (Miller et al., 1986; Saengkerdsub et al., 2007), and the high TAN content in the undiluted poultry litter of 6.8 g/L combined with a relatively aerobic environment in poultry litter could limit the initial abundance of methanogens in the poultry litter reactors. Low acetic acid concentration in poultry litter+TA-NaF reactors, suggested that microbial inhibition possibly affected acetogenic and acidogenic bacteria, which normally produce acetate and other VFAs. However, the slight TAN increment in the TA-NaF inhibited poultry litter by day 30 was indicative of a sustained uric acid and urea hydrolysis activity. The environmental parameters in general portrayed similar tendencies for swine and cattle manure digestion. The environmental parameters for poultry litter diverged from those of swine and cattle manure, which was attributed to the lack of methanogenic activity.

3.2 Methane production

3.2.1 Maize silage digestate batch reactors. The methane production was measured from biogas slurry with cellulose as standard substrate under the influence of urea, TA-NaF, and LS addition. The methane recovery in all positive control reactors (Inoc+sub) were 83.1 ± 6.5% of the theoretical methane yields according to Boyles extended formula, refined from (Buswell and Mueller, 1952). In Figure 2a, the methane yield of the positive urea control (inoc+cellulose+urea) was significantly lower than the positive control (1488 ± 26 mL vs 1863 ± 19 mL), indicating urea induced inhibition of the methanogenic activity (Lv et al., 2018). Interestingly, in the presence of LS, the urea inhibiting effect on methanogenesis was counteracted yielding 1867 ± 44 mL methane. This could be explained by either inhibition of urea hydrolysis to ammonia or chelation
of ammonium ions by LS. The latter theory was rejected from rough estimates of a LS chelating capacity of 3.5 mmole cations/g LS, which is insignificant in comparison to the total abundance of cations in the batch reactors. The methane spike, observed within the first 24 hours from LS supplemented reactors (Fig. 2b), was attributed to degradation of simple sugars (Glucose + galactose + xylose) contained in the LS powder (16% of the LS). The 10:1 mM TA-NaF treatment completely inhibited methanogenic activity (Fig. 2a). However, 5:1 mM TA-NaF amended reactors without cellulose substrate (Fig. 2b) produced 602 ± 23 mL extra methane compared to the inoculum control from day 7 to day 30, suggesting microbial degradation of TA. Based on Figure 2, it was concluded that TA-NaF supplementation was unsuited as treatment for ammonia inhibited AD given the general negative or delaying effect on methane production. Moreover, the biogas quality of TA-NaF amended reactors (Fig. 2a) were poor (35:65 methane:carbon dioxide ratio) compared to other treatments (Fig. 2a), with final methane:carbon dioxide ratios around 65:35 (Supplementary materials). Nevertheless, TA-NaF utilization for reducing methane production would be beneficial in manure storage tanks, storage of AD digestate used as fertilizer, and in livestock buildings.

### 3.2.2 Manure batch reactors.

To investigate the potential of TA-NaF as methane mitigation agent in a manure storage scenario, TA-NaF was added to reactors with swine manure, cattle manure, or poultry litter. Although LS was not expected to mitigate methanogenic activity, it was included as a treatment for comparative purposes. The methane production from the manure batch reactors are shown in Figure 3. Methane production was greater at 38 °C for all manure types, as expected from methanogen growth rate studies (Lin et al., 2016). The methane production was delayed
only briefly in swine manure with 5:1 mM TA-NaF at 38 °C and resulted in final yields equal to or exceeding the untreated controls. This is consistent with the observations of TA degradation in 5:1 mM TA-NaF treated maize silage reactors (Fig. 2b). Treatment with TA-NaF, inhibited methane production more efficiently in cattle manure than in swine manure at both temperatures, suggesting a less resilient microbial community in the cattle manure. This finding highlights the potential relevance of using TA-NaF as a methane-mitigating agent in cattle manure. Reactors with LS exceeded the methane yields of the untreated swine manure control at both incubation temperatures. This trend occurred concordantly with the high TAN concentration in the swine manure (Table 2), which supports the observed counteractive effect of LS on urea hydrolysis to ammonia. A similar effect of LS was not observed in cattle manure, which did not contain nearly as high TAN concentrations either. One of the reactors with poultry litter and LS at 38 °C produced significant amounts of methane (354 mL) from day 17 to 28, whereas the remaining parallel reactors inoculated with poultry litter produced less than 1 mL methane during the entire experiment. This suggested a very limited methanogen population in the poultry litter inoculum at experiment start or an extremely long lag phase of the methanogens to accommodate to the conditions in poultry litter batch reactors.

3.3 Carbon isotope signatures

Carbon isotope signatures were measured as a proxy for the relative contribution of acetoclastic and hydrogenotrophic methanogenesis (Conrad, 2005), as described in section 2.4. By conducting compound specific isotope analysis, further insight into the effect of TA-NaF and LS was acquired.
3.3.1 Maize silage digestate batch reactors. In Figure 4, the δ\textsuperscript{13}C signatures are presented for batch 1 (Fig. 4a) and batch 2 (Fig. 4b) reactors. The relatively negative δ\textsuperscript{13}C\textsubscript{CO2} signatures of urea amended reactors (Fig. 4a) were likely related to ureolytic activity characterized by an isotope enrichment factor of around 12.5‰ (Millo et al., 2012), and the fact that the urea was already 13\textsuperscript{C} depleted (δ\textsuperscript{13}C = -41‰) compared to the cellulose (δ\textsuperscript{13}C = -24.9‰). For δ\textsuperscript{13}C\textsubscript{CH4}, the urea amended reactors (Fig. 4a) were also relatively depleted, indicating dominance of hydrogenotrophic methanogenesis (Conrad, 2005; Nikolausz et al., 2013). However, with urea + 10:1 mM TA:NaF treatment (Fig. 4a), the δ\textsuperscript{13}C\textsubscript{CH4} values reached as low as -82‰ after 27 days, indicating that TA-NaF inhibited acetoclastic methanogenesis in addition to the urea induced ammonia inhibition of acetoclastic methanogenesis. In comparison, Figure 4b shows δ\textsuperscript{13}C\textsubscript{CH4} values around -30‰ to -35‰ for the 5:1 mM TA:NaF reactors, coinciding with the period where methane was produced (see Fig. 2b). Hence, bacterial degradation of TA that fueled acetoclastic methanogenesis must have occurred. However, at high TA concentrations, complete inhibition of acetoclastic methanogenesis probably results in acetate accumulation, as reported in Table 1. Lignosulfonic acid seemed to affect δ\textsuperscript{13}C\textsubscript{CH4} values in the positive direction compared to the urea controls (inoc+sub+urea in Fig. 4a). Hydrogenotrophic methanogen predominance has previously been observed under inhibitory or otherwise environmental stressing conditions (Buhlmann et al., 2019; Webster et al., 2016), supporting the deductions made here. The δ\textsuperscript{13}C signatures of TA and LS are -27.5‰ and -28.5‰, respectively, closely resembling the δ\textsuperscript{13}C signature of cellulose, and thereby ruling out their potential contribution to the depleted δ\textsuperscript{13}C values.
3.3.2 **Manure batch reactors.** Carbon signatures in the manure batch reactors were measured to evaluate whether the observations from the maize silage digestate batch reactors were associated with the inoculum. As presented in Figure 4c, clear differences in carbon isotope signatures were measured for the different manure types. Carbon dioxide from swine manure reactors was significantly enriched in $^{13}$C compared to the carbon dioxide from cattle manure reactors and poultry litter reactors ($\delta^{13}C_{CO_2}$ of 19.81 ± 0.87‰ for swine manure vs -9.64 ± 1.02‰ for cattle manure and poultry litter, errors as 95% confidence intervals). Only a few samples with TA-NaF treatment were measurable due to extremely limited quantities of biogas produced in these reactors.

Cattle and swine manure reactors incubated at room temperature yielded more negative $\delta^{13}C_{CH_4}$ values, and hence yielded larger $\varepsilon_c$ than cattle and swine manure reactors incubated at 38 °C. Based upon $\varepsilon_c$, swine manure reactors were dominated by hydrogenotrophic methanogenesis regardless of incubation temperature. Mostly, hydrogenotrophic methanogenesis was dominant in cattle manure reactors incubated at room temperature, while in poultry litter reactors the dominant pathway was acetoclastic methanogenesis. The latter finding was unexpected, considering the fact that biogas from poultry manure fed AD is normally characterized by more depleted $\delta^{13}C_{CH_4}$ values, which indicate the predominance of hydrogenotrophic methanogens (Nikolausz et al., 2013). In general, LS and TA-NaF supplemented manure reactors were characterized by slightly more negative $\delta^{13}$C signatures compared to the untreated manure reactors. The seemingly small effect of TA-NaF and LS on $\delta^{13}$C signatures suggested that the methanogenic community was already dominated by hydrogenotrophic methanogens, possibly as a consequence of adaptation to the naturally high TAN concentrations in manure. Another explanation is that the gut conditions of
these animals do not support acetoclastic methanogenesis, therefore such methanogens are negligible in the manure (Ozbayram et al., 2020).

3.4 Microbial community analysis

A dual approach was used for the microbial community structure analysis by targeting both the mcrA and 16S rRNA genes. The mcrA gene approach strictly targets methanogens, as this gene is unique to this group of microorganism (Friedrich, 2005). On the other hand, the domain-specific 16S rRNA gene is ubiquitous in all bacteria and archaeal cells, and hence is not restricted to only methanogens (Janda and Abbott, 2007).

3.4.1 Methanogens in maize silage digestate batch reactors. Figure 5 shows the non-metric multidimensional scaling (NMDS) plot (Fig. 5a) and the relative abundance of methanogens (Fig. 5b). *Methanoculleus* and *Methanothrix* (formerly *Methanosaeta*) were highly represented in all samples (Fig 5b). The acetoclastic genus *Methanothrix* (Holmes and Smith, 2016) was negatively correlated with TA-NaF, whereas *Methanogenium* and methanogens belonging to the class of Thermoplasmata was positively correlated with TA-NaF. The latter methanogenic lineages exhibit both hydrogenotrophic and methylotrophic methanogenesis and is consistent with the depleted $\delta^{13}$C$_{CH_4}$ values observed with TA-NaF treatment (Fig 4a). The genus *Methanoculleus* was more tolerant to high urea and TAN concentrations. Both *Methanoculleus* and *Methanogenium* genera belong to the Methanomicrobiaceae family, which has shown to be resilient to environmental stress and ammonia inhibition (Bonk et al., 2018; Esquivel-Elizondo et al., 2016).
3.4.2 Methanogens in manure batch reactors. Figure 6 shows the non-metric multidimensional scaling plot (Fig. 6a) and the relative abundance (Fig. 6b) of methanogens in manure batch reactors. The methanogen community structure was correlated with manure type rather than manure treatment. Swine manure was dominated by *Methanoculleus* and Thermoplasmata, whereas cattle manure was dominated by *Methanocorpusculum* and various genera belonging to the Methanomassiliicoccus order. The Methanomassiliicoccus order is taxonomically classified under Thermoplasmata and rely on an external H₂ source to reduce methyl-compounds to methane (Borrel et al., 2014). Methanomassiliicoccales was previous found to be abundant in the rumen fluid (Ozbayram et al., 2020) and the results presented here support and highlight the significance of hydrogen dependent methylotrophic methanogenesis in manure as well. Methylotrophic methanogenesis carried out by *Methanosarcina* species are characterized by large fractionation factors (Penger et al., 2012), and hence it is likely that the large apparent fractionation factors of swine and cattle manure (Fig. 4c) were to a significant degree a consequence of hydrogen dependent methylotrophic methanogenesis. Methanogens in poultry litter were assigned to Thermoplasmata (Fig. 6b) but this correlation was not significant (Fig. 6a) and methanogens were only detected in some of the poultry litter reactors. The low abundance or absence of methanogens in poultry litter reactors is consistent with the minimal or absent methane yields (Fig. 3).

3.4.3 Bacteria in maize silage digestate and manure batch reactors. In maize silage digestate, *Fastidiosipila*, Hydrogenisporalis, Ruminococcaceae, and VadimBC27 wastewater sludge group was dominant (Supplementary materials). *Fastidiosipila* and Hydrogenisporalis decreased with TA-NaF treatment, whereas VadimBC27 wastewater
sludge group and Ruminococcaceae were more resilient to TA-NaF. Ruminococcaceae is found in animal gut systems and is suited to degrade recalcitrant plant materials (Biddle et al., 2013) possibly explaining its adaption to TA-NaF. For swine manure VadimBC27 wastewater sludge group and Clostridium senso stricto 1 were dominant taxa (Supplementary materials). In cattle manure Sphaerochaeta, Proteiniphilum, and Acholeplasma were abundant genera (Supplementary materials). Acholeplasma abundance in cattle manure was substantially reduced with TA-NaF treatment and replaced by Pseudobutyrivibrio at 38 °C (Supplementary materials). Pseudobutyrivibrio ferments carbohydrates to lactic and butyric acid, which was also reflected in the high VFA content seen in Table 2. Poultry litter was dominated by Ruminoclostridium 5 at 38 °C and by Bacteroides at 23 °C (Supplementary materials). There was no significant correlation with LS treatment for any of the manure types. In general, Clostridium senso stricto 1 and VadimBC27 wastewater sludge group were the taxa mostly correlated with TA-NaF treatment (Supplementary materials), which suggest better adaptation capabilities of these microbial groups. Most of the microbial groups were affiliated to taxa without cultured members. This highlights the importance of further exploration of the microbial diversity in anaerobic digestion systems.

4. Conclusion

Lignosulfonic acid (LS) could be a promising supplement to anaerobic digesters suffering from urea induced methanogenesis inhibition. This statement is linked to a lesser inhibition of acetoclastic methanogens upon high urea loads in anaerobic digestion. Tannic acid with fluoride impairs methane production at high concentrations and is suitable for mitigating methane emissions from manure storages. Methanothrix
was very susceptible to TA-NaF inhibition, whereas hydrogenotrophic methanogens
were more resilient to TA-NaF treatment. These observations strongly suggest that the
methanogen community influences the efficacy of both LS and TA-NaF treatment on
the anaerobic digestion process.

Acknowledgements

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Appendix A. Supplementary materials

E-supplementary materials for this work can be found in e-version of this paper online

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Figure Captions
Figure 1. Schematic presentation of experimental work. TA-NaF = tannic acid with sodium fluoride, LS = lignosulfonic acid, Sub = Substrate (cellulose), GC/IRMS = gas chromatography combustion isotope ratio mass spectrometry, AMPTS = automatic methane potential test system.

Figure 2. Methane potential tests of maize silage batch reactors. The reactors were amended with tannic acid with sodium fluoride (TA:NaF) or lignosulfonic acid (LS) (10 g/L) in batch experiment 1 (a) and batch experiment 2 (b). Curves labeled with the same letter were not significantly different by experiment end (p-value is for ANOVA).

Figure 3. Methane production from swine manure, cattle manure, and poultry litter. Manures were incubated at 38 °C and at room temperature (~23 °C).

Figure 4. Isotope signatures of CH$_4$ and CO$_2$ from maize silage digestate. Maize silage digestate from batch experiment 1 (a) and batch experiment 2 (b). Inoc= inoculum (maize silage digestate), Sub= substrate (cellulose), TA:NaF= tannic acid: sodium fluoride, LS= lignosulfonic acid (10 g/L). Curves labeled with the same letter were not significantly different by experiment end (p-value is for ANOVA). (c) Carbon isotope signatures of CH$_4$ and CO$_2$ from manure batch reactors. Dashed lines indicate the apparent enrichment factor, $\varepsilon_c$. Time resolution was omitted as there was no significant $\delta^{13}$C development over time. TA-NaF=5:1 mM tannic acid: sodium fluoride, LS= lignosulfonic acid (10 g/L). The numbers 23 and 38 indicate incubation temperatures in °C.

Figure 5. (a) Non-metric multidimensional scaling plots (NMDS) of the methanogenic community structures in maize silage digestate batch reactors based on mcrA amplicon sequencing. (b) Relative abundances of methanogens from batch 1 and batch 2.
Sub=substrate (cellulose), TA:NaF=tannic acid:sodium fluoride, LS=lignosulfonic acid (10 g/L). The taxonomic level in (a) and (b) is denoted with (s) for species level, (g) for genus level, (f) for family level, (o) for order level, and (c) for class level.

Figure 6. (a) Non-metric multidimensional scaling plot (NMDS) of the methanogenic community structures in manure batch reactors based on mcrA sequencing. (b) Relative abundances of methanogens from manure batch reactors incubated at 23 °C and 38 °C. TA:NaF= tannic acid:sodium fluoride, LS = lignosulfonic acid (10 g/L). The taxonomic level in (a) and (b) is denoted with (s) for species level, (g) for genus level, (f) for family level, (o) for order level, and (c) for class level.
Table 1. Chemical analysis of maize silage digestate batch reactors. VS=volatile solids, TS=total solids, TAN=total ammonia nitrogen, Ac=acetic acid, VFA=volatile fatty acids.

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<td>VS (%)</td>
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<tr>
<td>Inoc</td>
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<tr>
<td>Inoc+TA:NaF (2.5:1 mM)</td>
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<td>Inoc+TA:NaF (5.1 mM)</td>
<td>7.19±0.06</td>
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Table 2. Chemical analysis of manure batch reactors. VS=volatile solids, TS=total solids, TAN=total ammonia nitrogen, Ac=acetic acid, VFA=volatile fatty acids. Values at day 0 are given for the diluted manure.

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<th>TAN (g/L)</th>
<th>pH</th>
<th>Ac (mg/L)</th>
<th>VFA (mg/L)</th>
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<th>TS  (%)</th>
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<th>pH</th>
<th>Ac (mg/L)</th>
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</table>
Figure 1.

Maize silage digestate batch reactors

Urea
TA-NaF
LS
Sub

Batch 1
15 x
38 °C

Batch 2
15 x

Sampling

Methane production by AMPTS

Microbial analysis
mcrA and 16s rRNA
Illumina sequencing

13C Isotope ratio
m/z 46
m/z 45
m/z 44

Biogas composition
GC/IRMS

Gas production by pressure readings

Manure batch reactors

TA-NaF
LS

Swine
6 x

Cattle
6 x

Poultry
6 x

23 °C

Swine
6 x

Cattle
6 x

Poultry
6 x

38 °C

Sampling
Figure 2.
Figure 3.
Figure 5.

Thermo (c) = Thermoplasmata (c)
M.gen (g) = Methanogenium (g)
M.nic (c) = Methanomicrobia (c)
M.vorans (g) = Methanomethylovorans (g)
M.c.bour (s) = Methanoculleus bourgensis (s)
M.culleus (g) = Methanoculleus (g)
M.t.soehngenii (s) = Methanotherrix soehngenii (s)
Figure 6.

- **M. planus (g)** = *Methanoplanus (g)*
- **M. celius (g)** = *Methanocellatus (g)*
- **M. t. soehngenii (g)** = *Methanothrix soehngenii (s)*
- **M. bact (g)** = *Methanobacterium (g)*
- **M. s. therm (s)** = *Methanosarcina thermophila (s)*
- **M. c. bour (s)** = *Methanoculleus bourgensis (s)*
- **M. massi (s)** = *Methanomasillikoccaceae (s)*
- **M. c. tab (s)** = *Methanococcum tabaceum (s)*
- **M. corpus (g)** = *Methanococcus (g)*
- **C. M. phill (g)** = *Candidatus Methanomethylophillus (g)*
- **C. M. alvis (s)** = *Candidatus Methanomethylophilus alvus (s)*
- **M. gen (g)** = *Methanogenium (g)*
- **M. sac (g)** = *Methanocarcinaceae (g)*
- **M. s. (f)** = *Methanococcaceae (f)*
- **M. s. smithi (s)** = *Methanobrevibacter smithii (s)*
- **M. s. stadt (s)** = *Methanospirillum stadtmanae (s)*
- **Thermococcus (c)** = *Thermoplasma (c)*
- **C. M. plas (g)** = *Candidatus Methanoplanus (g)*
- **C. M. gran (g)** = *Candidatus Methanoplanus (g)*

Legend:
- Thermoplasma (c)
- Candidatus Methanoplanus (g)
- Methanococcus (g)
- Methanococcus fur (g)
- Methanococcus mineralis (g)
- Methanococcales (c)
- Methanospirillum hungatei (s)
- Methanomassiliicoccales (c)
- Methanogenium (g)
- Methanococcus bourgensis (s)
- Methanococcus (g)
- Methanococcus thermophili (g)
- Methanobacterium (g)
- Methanobrevibacter smithii (s)
- Methanobrevibacter ruminantium (s)
- Methanobrevibacter (g)
- Methanosarcina thermophila (s)
- Methanosarcina mazei (s)
- Methanosarcina (g)
- Methanospirillum (g)