



# Anaerobic Digestion of Hemp and Flax Straw and Shives and Rapeseed Straw by the Ruminal Microbiota

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## Abstract

To mitigate greenhouse gas emissions and improve energy supply security, there is an increasing effort toward the use of non-fossil energy sources. Crop residues have a great potential to be exploited as biomass for biogas production. However, due to their lignocellulosic structures they are difficult to degrade and do not reach competitive performance. A feasible option to mine these substrates is present in the forestomach of ruminants. Therefore, the aim of the present study was to use rumen microorganisms to improve anaerobic digestion (AD) of crop residues. For this purpose, hemp straw, mechanically pre-treated hemp fibers and shives, flax straw, flax shives, and aged and fresh rapeseed straw were evaluated using the rumen simulation technique. The AD of the substrates was divided into three batches. In two batches, hay was added as a control substrate. In summary, none of the analyzed substrates had an equivalent performance as the control hay, but pre-treated hemp fibers and shives had better AD parameters compared to all other alternative substrates, with the lowest pH (mean: 6.81), highest short chain fatty acid (20.0 mmol/day) and H<sub>2</sub> production (25.6 mM) and highest degradability (25.2%). Flax straw had the second-best performance (6.81, 17.4 mmol/day, 20.6 mM and 22.2%, respectively), followed by fresh rapeseed straw, hemp straw, aged rapeseed straw and flax shives. Therefore, hemp fibers and shives demonstrated to be the most suitable substrates for AD. However, since pre-treatment can represent significant additional costs for biogas production, flax straw also demonstrated to be a good alternative.

**Keywords** Biogas · Rumen Simulation Technique (RUSITEC) · Straw · Anaerobic digestion · Crop residues

## Introduction

Since the industrial revolution, a significant number of human activities has been driven by fossil fuels. These reserves are limited and have a harmful potential, since their combustion is associated with the emission of greenhouse gases to the atmosphere, which is directly associated with global warming [1, 2]. In order to improve energy supply security, there is an increasing effort toward the use of non-fossil energy sources [3, 4]. One alternative is biofuels (biodiesel, bioalcohol, biogas and biomethane) derived from various kinds of biomass. Biofuels are increasingly

used in transportation, heat and power development [4]. The biomass used for biofuel production can include the conventional agricultural food-based crops, exploiting sugars, starch and vegetable oils as direct feedstocks to the conversion processes (so-called ‘first generation’ biofuel technologies), or non-food based biomass, such as agricultural residues (leaves, stalks, rice husk, straw, etc.), perennial grasses and animal waste (described as the ‘second generation’ biofuel technologies) [5, 6].

The majority of biofuel currently produced is first generation. The proteins, fats and simple sugars present in this biomass are converted within an economically reasonable retention time [1]. However, the use of such biomass is responsible for the competition with food production [7]. Therefore, there is a growing interest in the use of non-food-based biomass for energy production. In particular, crop residues (wheat straw, rice straw, corn straw, etc.) are ideal candidates to be used as raw materials for biofuel production, since they are often treated as a waste or by-product with no

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or low agricultural use, resulting in a high availability at low costs [8]. The use of such biomass may reduce competition among different forms of land use and contribute to effective waste management and could play a very significant future role for renewable energy production if processed anaerobically or with more complex technologies [9, 10].

Crop residues consist mainly of substrates rich in lignin, hemicellulose and cellulose, so called cellulose-based substrates (cbS), and are difficult to degrade [9]. Within the different types of biofuels that can be produced from cbS is the biogas, which can be directly applied as fuel for on-site heat, steam and electricity generation in industries or used in natural gas grids and as fuel in vehicles when refined [11]. Anaerobic digestion (AD) represents the most widely applied process for biogas production due to its high efficiency, operational flexibility and overall environmental benefits offered as compared to other biorefinery processes [12, 13]. However, the recalcitrant nature of this biomass limits the AD performance, requiring additional strategies such as physical, chemical or biological pre-treatments, as well as co-digestion in order to increase both the rates and the yields of biomethane production [9, 12]. Some pre-treatment methods have been demonstrated to induce the production of other toxic byproducts (furan aldehyde, organic acid and phenolic compounds) that can negatively affect AD [14, 15]. Moreover, those additional processes may increase costs for biogas production, decreasing its economic competitiveness with first generation biomass and fossil fuel [14, 16]. Therefore, second generation biofuels are still not a viable economic solution. In order to take them to a true commercial level extensive research and investment are required to improve the efficiency of cbS use and plant technology for biogas production, to anchor the principles of sustainability.

A feasible option to degrade these high-fiber and cellulosic substrates is present in the forestomach system of ruminants, which can be used as a model to improve methane yield in biogas plants [17, 18]. The digestive strategies of ruminants have been studied for years for implementation in industrial anaerobic reactors of biogas plants; however, no system has yet reached animal digestive performances [19, 20]. The rumen microbial consortium is a very effective, co-evolved ecosystem containing a wide range of enzymes and microorganisms involved in an efficient breakdown of cbS [21, 22]. Organic matter is fermented to short-chain fatty acids (SCFA), with carbon dioxide (CO<sub>2</sub>) and methane (CH<sub>4</sub>) produced as by-products [23]. In the rumen, methane is mainly formed via the hydrogenotrophic pathway from H<sub>2</sub> and CO<sub>2</sub> released during fermentation [24]. In the biogas plant, organic matter is also first hydrolysed (hydrolysis phase), then degraded into organic acids and alcohols (acidogenic phase), but then, these are mainly converted to acetate by acetogenic microorganisms (acetogenic phase) which serve as main substrate for methane production via the

acetogenic pathway (methanogenic phase) [1]. Acetogenesis and methanogenesis are often physically separated from the hydrolysis/acidogenesis step to optimize performance [25]. By replacing animal manure, sewage sludge or organic waste which are still commonly used as starter cultures (inoculum) in biogas plants [12] with rumen contents in the first reactor (so-called DAUMEN system [26, 27]), hydrolysis of cbS and production of organic acids for acetogenesis and methane production can be enhanced.

To provide a better understanding on the microbial fermentation of different crop residues and their potential for anaerobic digestion in a rumen hydrolysis reactor, fermentation parameters of different crop residues were evaluated by using the rumen simulation technique (RUSITEC) [28]. The RUSITEC is a well-established model for long-term studies on rumen fermentation patterns and complex microbial interactions, highly similar to *in vivo* conditions [29] that can be used to determine the extent to which each substrate can be degraded by rumen microorganisms. The substrate with the best performance in the RUSITEC will consequently be used in a larger-scale setup of a rumen hydrolysis reactor combined with a UASB reactor for quantification of methane production.

## Material and Methods

### Ethics Statement

The two donor cows were housed at the Department for Physiology and Cell Biology, University of Veterinary Medicine, Hanover. The animals were kept and treated according to the guidelines of the German Animal Welfare Act. The Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) approved the previous fistulation of the donor cows by the experiment number AZ 33.4-42505-04-13A373.

### Substrates

The substrates analyzed were hemp straw (HSt), flax straw (FSt), fresh and aged rapeseed straw (FRSt and ARSt, respectively), which were cut into pieces of about 2 cm, and flax shives (FSh). Additionally, hemp pre-treated once with the BMS-Flaksy® device (HessenLeinen GmbH, Zierenberg, Germany) was used. This resulted in an incomplete separation of fibers and shives, and the whole material was mixed for the trial (HF + Sh). This mechanical pre-treatment leads to the opening of the lignocellulosic structure of the substrate resulting in a larger specific surface area, thus making cellulose more accessible to hydrolysis [16].

Due to the great number of substrates to be analyzed, they were separated into three groups which were run separately.

Hemp straw and flax substrates were analyzed in the first trial, while Flasky-treated hemp was analyzed in a second trial and the fresh and aged rapeseed in a third trial. The same hay which was fed to the cattle was added as a control substrate (named H1 and H2) in two of the trials, since it is a high-quality forage widely used as cattle feed and the rumen microbiota was adapted to it.

### Rusitec Experiment

The present study is part of an *in vitro* experiment using a RUSITEC system [28] to observe effects of different substrates on fermentation parameters. Three fermenters were used for each substrate type ( $n=3$ ). At the beginning of the experiment, the fermenters were inoculated with mixed rumen contents collected in the morning (about 3 h after feeding) from two ruminal fistulated German Black-Pied cows, following the procedure described by Wetzels et al. [29]. Briefly, solid and liquid phases of the rumen were separated by gauze filtration. Fermenters were filled with the liquid phase, while 70 g of the solid phase was placed into a nylon mesh bag of 12 cm × 6.75 cm, pore size 50 μm (R712 Ankom Technologies, Gesellschaft für Analysentechnik, Salzwedel, Germany). Similar nylon bags were prepared and filled with 10 g dry matter of solid, coarsely chopped substrate. One bag with the solid rumen content and one with the substrate were then placed into the perforated inner vessel and inserted into the fermenter. The inner vessels were constantly moved up and down by an electric motor (6 times/min), to ensure the mix with the liquid phase. Fermentation vessels were kept in a water bath at 39 °C to imitate rumen conditions. After 24 h, the nylon bag containing the solid phase of the rumen was removed, rinsed with 40 ml of pre-warmed buffer solution for 1 min to dislodge the attached microorganisms and replaced by a substrate-filled one. The rinsing solution was returned to the fermenter. The nylon bags were replaced alternately with a new substrate bag every 24 h, such that the retention time for each nylon bag was 48 h. The fermentation vessels were continuously infused with a buffer solution, which mimics bovine saliva in pH and mineral composition [29]. The effluent was collected in conical glass flasks kept on ice. After the daily exchange of substrate bags, effluent flasks were flushed with nitrogen to maintain the anaerobic conditions.

### Sampling and Sample Analysis

The pH values and redox potential of the liquid phase were measured daily using a pH and a redox electrode (Polyplast pH Sensors, Polyplast ORP Sensors, Hamilton Bonaduz AG, Bonaduz, Switzerland) during the whole experiment, to ensure adequate environmental conditions for microbial survival and anaerobic fermentation. Likewise,

effluent volumes were measured daily. After an equilibration phase of 7 days, during which the microbiome adapts to the substrate, effluent samples were collected daily for the next 7 days of experiment for the analysis of NH<sub>3</sub>-N and SCFA concentrations. The NH<sub>3</sub>-N concentrations were determined photometrically at 546 nm in a spectrometer (DU 640, Beckman Coulter GmbH, Krefeld, Germany) as described by Riede et al. [30]. Analysis of SCFA concentrations was performed by gas chromatography. Shortly, 1.5 ml of the effluent sample was centrifuged at 40,000 g for 20 min at 4 °C. From each supernatant fraction, 1 ml of fluid was acidified by adding 0.1 ml 98% formic acid, and the sample was incubated for 10 min for protein precipitation. After that, the sample was centrifuged again at 40,000 g for 10 min at 4 °C. The supernatant was then filled into glass vials for GC analysis. The GC analyses were conducted on a Shimadzu GC-2025 system (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a split injector with SH-Stabilwax-DA column (30 m × 0.32 mm × 0.50 μm). Nitrogen was used as carrier gas. The following temperature program was used: stepwise rise in temperature from 100 to 240 °C (15 °C/min) and hold for 2.66 min. Fatty acid was identified and quantified by comparison of their retention times and area under the curve with fatty acid standards, also prepared with formic acid (Sigma-Aldrich, Munich, Germany). Production rates of SCFA were calculated by multiplying SCFA concentration with daily effluent volume. Substrate residues from the feed bags were collected and dried at 65 °C for 48 h. To determine degradation, dried substrates were incinerated in a muffle furnace at 600 °C for 24 h.

Hydrogen production was calculated using the formula by Wang et al. [31] derived from the equations from Demeyer [32]:

$$\text{Net H}_2(\text{mM}) = 2(\text{acetate} + \text{n-butyrate} + \text{isobutyrate}) - (\text{propionate} + \text{isovalerate} + \text{valerate}) \quad (1)$$

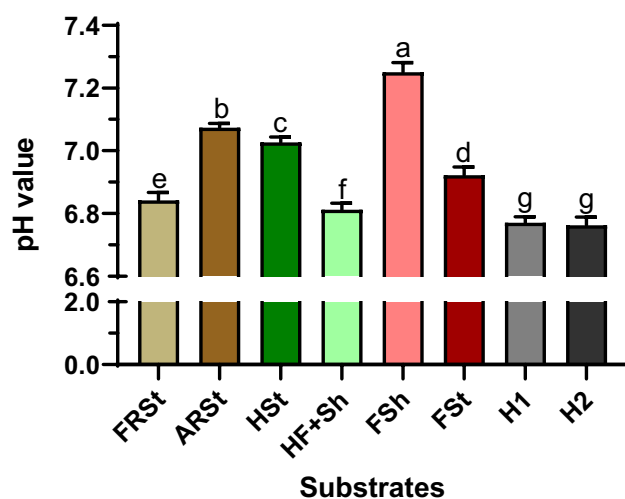
To determine the chemical composition of the substrates used, the crude fibers (Xfi), N-free extracts (Nfe), acid detergent fibers (ADF), neutral detergent fibers (NDF), crude ash (XA), crude protein (XP) and crude fat (XF) were analyzed by Weender analysis (Institute of Animal Nutrition, University of Veterinary Medicine Hannover, Germany). For this a representative sample was collected from the undigested substrates. For the digested substrates the dried residues from the feed bags were pooled per treatment to gain enough material for the analysis. The degradation of the chemical fractions was calculated based on the mass of the fraction analyzed in the digested samples compared to the undigested ones. The missing samples are due to the lack of enough material to perform all analyses.

## Statistical Evaluation of the Rusitec Results

The results of the Rusitec tests were statistically evaluated by a two-factorial analysis of variance. For this purpose, the software GraphPad Prism 9 was used. Data were assessed for normal distribution of residuals by applying the Kolmogorov–Smirnov test. Two-way analysis of variance (ANOVA) for repeated measurements was applied to detect effects of Time, Treatment or interactions of Time  $\times$  Treatment. In case of significant interaction, Tukey post-test was used to identify significant differences among treatments within time-points and between time-points within treatment. In case of significant effects of treatment without interaction, Tukey post-test was applied to detect differences among treatments. Significance levels were set at  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$ . The presented graphs were created using GraphPad Prism 9.

## Results

In general, all pH values were within the range of 6.8 to 7.2. No significant changes in pH were observed for any of the substrates along the course of the experiment. Therefore, pH is presented as the mean value along the 7 days of sampling, for each substrate (Fig. 1). Significant differences were observed among the different substrates ( $p < 0.0001$ ) in the following order: flax shives (FSh) > aged rapeseed straw (ARSt) > hemp straw (HSt) > flax straw (FSt) > fresh rapeseed straw (FRSt) > hemp fibers and shives (HF + Sh) > hay (H1 and H2). The pH value of both hay groups did not differ.

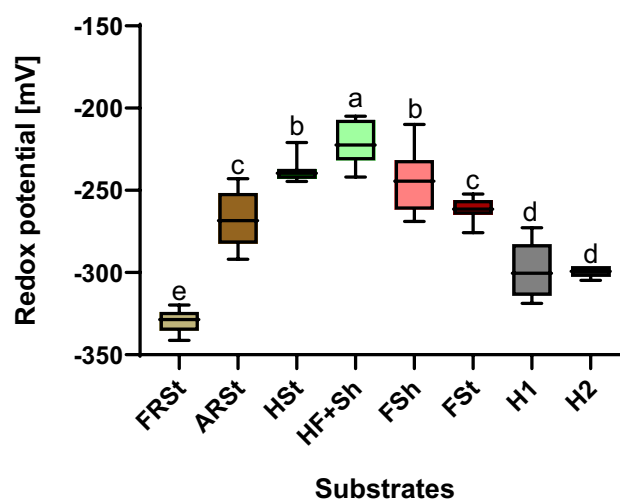


**Fig. 1** Mean values of 7 days of consecutive pH measurements for fresh rapeseed straw (FRSt), aged rapeseed straw (ARSt), hemp straw (HSt), hemp fibers and shives (HF + Sh), flax shives (FSh), flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among the substrates. Data are presented as mean + SD

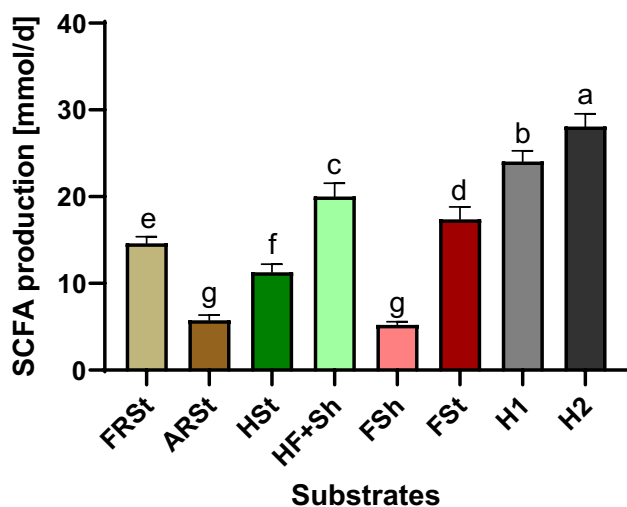
The redox potential varied significantly along the course of the experiment for some substrates (effect of time  $p = 0.0076$ ), such as ARSt and FSh. For ARSt, the redox potential decreased from  $-243$  mV on day 7 to  $-292$  mV on day 14, while the opposite occurred for FSh with an increase from  $-269$  mV on day 7 to  $-210$  mV on day 13. For all other substrates, no significant differences occurred along the time. However, the observed variations on ARSt and FSh are likely to have a negligible biological relevance.

The redox potential differed significantly among substrates ( $p < 0.0001$ , Fig. 2). In general, values ranged from  $-221$  to  $-329$  mV and with the exception of the FRSt, all other substrates exhibited a more positive redox potential than the control hay. Hemp had the most positive redox potential ( $-221$  mV and  $-238$  mV for HF + Sh and HSt, respectively), followed by FSt, ARSt and FRSt with the lowest value ( $-329$  mV). Treating hemp with the retting device significantly increased its redox potential, compared to the non-treated straw.

Again, no significant changes on the total daily production of SCFA occurred along the timeline of the experiment, but the SCFA production rates of the different substrates were significantly different from each other ( $p < 0.0001$ , Fig. 3), except for ARSt and FSh that had similar values. The H2 had a significantly greater SCFA production rate compared to H1, and both had higher production rates compared to all other analyzed substrates. Treating hemp with the retting device resulted in a higher SCFA production compared to the untreated samples and resulted in treated hemp samples to have the highest SCFA of all test substrates. The amount of SCFA produced by HF + Sh was followed



**Fig. 2** Mean redox potential [mV] of fresh rapeseed straw (FRSt), aged rapeseed straw (ARSt), hemp straw (HSt), hemp fibers and shives (HF + Sh), flax shives (FSh), flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among substrates. Data are presented as box and whiskers



**Fig. 3** Mean SCFA production (mmol d<sup>-1</sup>) of fresh rapeseed straw (FRSt), aged rapeseed straw (ARSt), hemp straw (HSt), hemp fibers and shives (HF+Sh), flax shives (FSh), flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among substrates. Data are presented as mean + SD

by FSt, FRSt, HSt and ARSt. FSh produced significantly less SCFA compared to the FSt sample, and together with ARSt, they produced significantly less SCFA compared to all other samples.

Within the individual types of fatty acids produced, acetate was the one with the highest molar proportion for all substrates, representing around 60–70% of the total SCFA (Supplementary Fig. 1). Propionate was the fatty acid produced in second highest proportion in all substrates, representing 21–27% of the total SCFA. Butyrate varied between 4 and 11%. Valerate and isovalerate represented up to 2% of the total SCFA, while isobutyrate had the smallest proportion with up to 1% of the total SCFA.

**Table 1** Daily production rates [mmol d<sup>-1</sup>] of the individual short chain fatty acids produced during the AD of the different substrates (fresh rapeseed straw (FRSt); aged rapeseed straw (ARSt); hemp straw (HSt); hemp fibers and shives (HF+Sh); flax shives (FSh); flax

Substrate	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate
	[mmol d <sup>-1</sup> ]					
H1	17.03 ± 1.16 <sup>a</sup>	6.64 ± 0.43 <sup>a</sup>	3.14 ± 0.23 <sup>a</sup>	0.26 ± 0.03 <sup>a</sup>	0.71 ± 0.04 <sup>a</sup>	0.67 ± 0.09 <sup>a</sup>
H2	14.63 ± 2.45 <sup>b</sup>	5.17 ± 0.87 <sup>b</sup>	2.79 ± 0.55 <sup>b</sup>	0.16 ± 0.02 <sup>b</sup>	0.54 ± 0.05 <sup>b</sup>	0.40 ± 0.09 <sup>b</sup>
ARSt	4.01 ± 0.85 <sup>g</sup>	1.37 ± 0.29 <sup>f</sup>	0.30 ± 0.07 <sup>e</sup>	n.d.*	0.02 ± 0.02 <sup>e</sup>	0.04 ± 0.04 <sup>e</sup>
FRSt	10.28 ± 1.09 <sup>e</sup>	3.17 ± 0.36 <sup>d</sup>	0.75 ± 0.10 <sup>d</sup>	n.d.	0.18 ± 0.03 <sup>d</sup>	0.24 ± 0.05 <sup>c</sup>
FSt	11.71 ± 1.90 <sup>d</sup>	4.48 ± 0.74 <sup>c</sup>	0.87 ± 0.17 <sup>d</sup>	0.06 ± 0.04 <sup>c</sup>	0.16 ± 0.04 <sup>d</sup>	0.08 ± 0.02 <sup>d</sup>
FSh	3.52 ± 0.47 <sup>g</sup>	1.41 ± 0.21 <sup>f</sup>	0.23 ± 0.05 <sup>d</sup>	n.d.	0.04 ± 0.02 <sup>e</sup>	n.d.
HSt	7.67 ± 1.29 <sup>f</sup>	2.60 ± 0.39 <sup>e</sup>	0.80 ± 0.09 <sup>d</sup>	n.d.	0.17 ± 0.04 <sup>d</sup>	0.02 ± 0.03 <sup>ef</sup>
HF+Sh	13.66 ± 1.73 <sup>c</sup>	4.47 ± 0.51 <sup>c</sup>	1.49 ± 0.18 <sup>c</sup>	0.06 ± 0.03 <sup>c</sup>	0.25 ± 0.03 <sup>c</sup>	0.08 ± 0.02 <sup>d</sup>

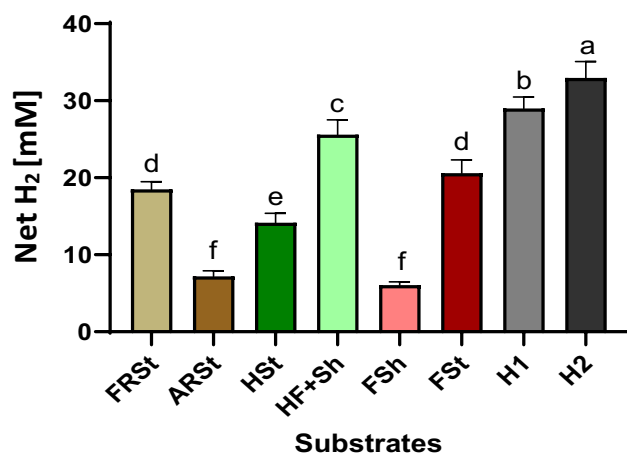
\* n.d. not detectable

Acetate production followed the very same pattern as observed for the total SCFA, with the highest production rate for hay and the lowest for ARSt and FSh (Table 1). Propionate followed a similar pattern compared to acetate and total SCFA, with the exception that propionate production of FSt and HF+Sh did not differ. Butyrate and valerate had similar pattern and were produced in high rates in H1 and H2, followed by HF+Sh. The FSt, HSt and FRSt had similar values and did not differ among each other but had significantly lower amounts of butyrate and valerate than the previously mentioned substrates and significantly higher amounts compared to ARSt and FSh. Isovalerate production was highest in H1 and H2, followed by FRSt. The FSt and HF+Sh had similar values, but significantly higher concentration compared to ARSt and HSt. The isovalerate concentration from FSh was below the detection limit. Similarly, isobutyrate could only be detected for hay, FSt and HF+Sh. The FSt and HF+Sh had similar values and both significantly lower than H1 and H2.

Acetate represented the major SCFA produced in all substrates. Since H<sub>2</sub> is a by-product of the production of acetate and butyrate, the H<sub>2</sub> production followed the same patterns as observed for the SCFA production. Therefore, within the substrates analyzed, HF+Sh exhibited the highest H<sub>2</sub> production after the controls H1 and H2, followed by FSt and FRSt (Fig. 4). The FSh and ARSt had the lowest H<sub>2</sub> production among substrates.

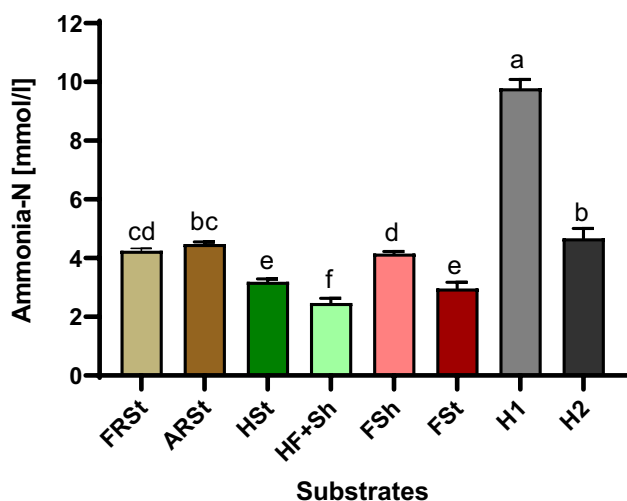
The concentration of NH<sub>3</sub>-N remained relatively stable (Time *p* > 0.05) for each substrate but significantly differed among the substrates (*p* < 0.0001, Fig. 5). H1 had the highest NH<sub>3</sub>-N production with 9.78 mmol/l, nearly two times higher compared to 4.67 mmol/l from H2. ARSt and FRSt had comparable NH<sub>3</sub>-N concentrations to H2, followed by FSh, HSt and FSt. HF+Sh had the lowest NH<sub>3</sub>-N concentration of all substrates.

straw (FSt) and hay (H1 and H2)) in the RUSITEC system. Different letters indicate significant differences among the substrates. Data are presented as mean ± SD

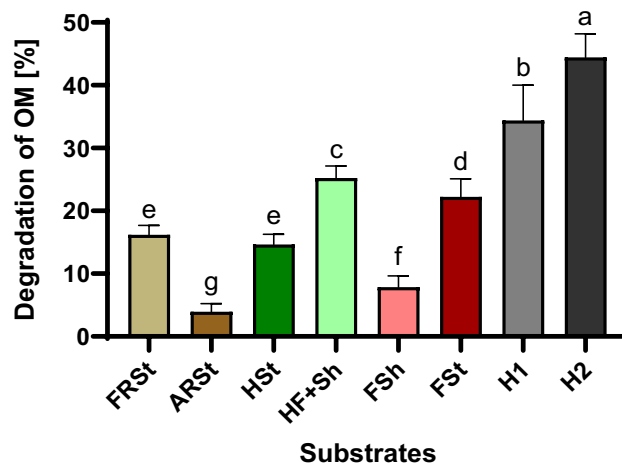


**Fig. 4** Calculated net H<sub>2</sub> [mM] produced during AD of fresh rapeseed straw (FRSt); aged rapeseed straw (ARSt); hemp straw (HSt); hemp fibers and shives (HF+Sh); flax shives (FSh); flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among substrates. Data are presented as mean + SD

The degradation of organic matter of the substrates after 48 h of incubation did not change along the time of the experiment, but it differed among the different types of substrates ( $p < 0.0001$ , Fig. 6), except for FRSt and HSt which had a similar degradation. All substrates were less degradable compared to both controls hay, but FSt exhibited the highest degradation of all analyzed substrates, followed by FRSt, HSt and FSh. The ARSt had the lowest degradation rate of all substrates. Treating HSt with the retting device significantly affected degradability. While the degradation of



**Fig. 5** Ammonia-N [mmol l<sup>-1</sup>] concentration during AD of fresh rapeseed straw (FRSt); aged rapeseed straw (ARSt); hemp straw (HSt); hemp fibers and shives (HF+Sh); flax shives (FSh); flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among samples. Data are presented as mean + SD



**Fig. 6** Degradation of organic matter (%) after 48 h of incubation of fresh rapeseed straw (FRSt); aged rapeseed straw (ARSt); hemp straw (HSt); hemp fibers and shives (HF+Sh); flax shives (FSh); flax straw (FSt) and hay (H1 and H2). Different letters indicate significant differences among samples. Data are presented as mean + SD

HSt after 48 h in the Rusitec was 14.63%, HF+Sh of treated hemp had a better performance reaching 25.22%.

The substrate composition within the analyzed substrates differed (Table 2). ARSt had the highest XA and lowest Xfi of all test substrates, FRSt had the second highest XA and second lowest Xfi. Therefore, XA and Xfi were the main components affected by the storage/harvesting time. The other four test substrates (HSt, HF+Sh, FSh, FSt) were quite similar in chemical composition. All of the test substrates analyzed had higher amounts of Xfi, ADF and NDF, compared to the control hays (H1, H2), demonstrating the recalcitrant properties of those substrates.

Despite similar ADF and NDF amounts, the degradation of those components varied among the substrates. FSh had a low Xfi, ADF and NDF degradation, while

**Table 3** Degradation of the chemical components in 48 h for each of the different substrates: hemp straw (HSt); hemp fibers and shives (HF+Sh); flax shives (FSh); flax straw (FSt) and hay (H1). The chemical analysis was performed on pool samples for each treatment

Substrate	XP	Xfi	Nfe	ADF	NDF
Degradation (%)					
H1	35.28	34.81	48.75	34.47	32.63
FSt	25.07	11.49	32.32	13.90	14.67
FSh	36.32	3.92	8.48	4.08	3.91
HSt	n.d.	5.90	24.00	9.24	4.06
HF+Sh	7.32	16.65	n.d.	17.44	13.43

crude fibers (Xfi), N-free extracts (Nfe), acid detergent fibers (ADF); neutral detergent fibers (NDF), crude ash (XA); Crude protein (XP); crude fat (XF); dry matter (DM); n.d. not determined

**Table 2** Chemical composition of the different substrates fresh rapeseed straw (FRSt), aged rapeseed straw (ARSt), hemp straw (HSt), hemp fibers and shives (HF + Sh), flax shives (FSh), flax straw (FSt) and hay (H1 and H2) before AD

Substrate	Xfi % (DM basis)	Nfe	ADF	NDF	XA	XP	XF
H1	30.77	55.25	35.54	64.90	5.63	7.22	1.12
H2	30.22	48.13	36.04	63.74	4.92	15.27	1.41
ARSt	46.18	31.44	66.38	82.97	17.90	3.96	0.53
FRSt	50.06	36.78	60.73	77.87	7.43	5.23	0.53
FSt	58.63	32.79	66.99	83.39	4.92	2.63	0.99
FSh	61.70	31.77	69.75	88.47	2.36	2.94	1.23
HSt	61.32	33.48	71.94	86.57	2.90	1.82	0.52
HF + Sh	59.38	n.d	69.61	81.68	4.00	2.56	n.d

crude fibers (Xfi), N-free extracts (Nfe), acid detergent fibers (ADF); neutral detergent fibers (NDF), crude ash (XA); crude protein (XP); crude fat (XF); dry matter (DM); n.d. not determined

HF + Sh and FSt had a much higher degradation of those components (Table 3). None of the substrates reached a comparable degradation to the control hays.

## Discussion

The RUSITEC system is an *in vitro* technology that has been considered as a valuable tool to investigate the potential of different substrates to be used in anaerobic fermentation for biogas production. The aim of the present study was to investigate the decomposition parameters of different crop residues, with and without mechanical pre-treatment, that may represent a potential second generation biomass for large-scale biogas production when combined with a new forestomach reactor. For this, AD parameters were evaluated for six different substrates: aged rapeseed straw, fresh rapeseed straw, hemp straw, hemp fibers + shives, flax straw and flax shives. Hay was used as control.

During the experiment, pH fluctuated between 6.8 and 7.2 for all samples. Ruminal pH is particularly driven by the amount of fermentable carbohydrates, which are converted to SCFA and decrease pH. The level of SCFAs indicates the efficiency of hydrolytic and acidogenic processes; therefore, higher SCFA concentrations reflect a greater amount of fermented substrate by rumen microorganisms [33, 34]. In the present study, HF + Sh had the lowest pH, highest SCFA production and OM degradation, demonstrating to be the most suitable substrate for AD by rumen microbiota. Similarly, FSt had the second best AD performance, being also a good alternative to be used for AD, followed by FRSt. On contrary, FSh had the highest pH of all samples, which was above the normal biological range for the rumen (5.6–7.0), the lowest SCFA production and the second lowest degradation of OM, therefore, demonstrating to be a poor substrate for AD. Likewise, ARSt and HSt also had pH values above the normal biological

range, and low SCFA production and OM degradation. Within the different SCFA produced during fermentation, acetate proportions are a good indicator for ruminal methane production, since acetate production is associated with the release of H<sub>2</sub>, which can be used by hydrogenotrophic methanogens to form methane [31, 35]. Moreover, the produced acetate can further be used for methanogenesis by acetoclastic methanogens in a second-step reactor. In this context, the retted HF + Sh had the highest acetate and H<sub>2</sub> production, significantly higher than the untreated HSt, therefore demonstrating a great potential for both first-step (rumen reactor) and second-step (e.g. UASB reactor) methane production.

The amount of previous data on fermentability of these substrates by ruminal microorganisms is limited and mostly restricted to digestibility data derived from feeding experiments or *in situ* degradation. Inclusion of hemp stems in an oaten chuff diet in sheep increased apparent digestibility of OM, CP, NDF and ADF and acetate and butyrate concentrations in the rumen [36], underlining the ability of the rumen microorganisms to degrade hemp straw effectively despite its high fiber content. For flax straw an organic matter digestibility of 33.9% was reported in an *in vitro* assay [37], while lambs were able to digest 49% of flax straw organic matter [38]. In a comparison of barley straw, canola straw, timothy and alfalfa hay, *in situ* NDF and ADF digestibility of canola straw reached about 27% after 48 h which was considerably lower than for the other substrates [39]. Although a quantitative comparison is not possible due to different experimental conditions, these reports indicate a better rumen fermentability of hemp straw and flax straw compared to rapeseed straw.

Anaerobic digestion of these substrates with manure or sludge microbiota has mainly been studied in batch cultures with methane production as main parameter (biomethane potential test, BMP). In these experiments hemp fibers performed better than shives, with untreated stem being intermediate [40]. Therefore, mixing the high hemicellulosic

fibers with the lignin rich shives can significantly increase anaerobic digestion performance of shives residues, while it may still provide a better performance than the untreated straw once the hemicellulosic structures of fibers are much more exposed to microbial degradation after retting [41]. Here, the whole material was used (fiber content: 30.4%); an increase of the fiber proportion should further increase AD performance but would require further retting and again result in the accumulation of shives as a waste material. Flax and rapeseed straw did not reach the same methane production in BMP experiments compared to hemp [42–45]. However, in contrast to our study, where fresh rapeseed straw performed slightly worse than flax and aged rapeseed straw performed poor, rapeseed seemed to perform a bit better in the mentioned reports; however, as the absolute values varied from study to study and the inoculums for the batch cultures differed, this difference could also be linked to the inoculum.

The differences in AD performance of the substrates investigated in the present study can be correlated with their chemical properties. Despite similar proportions of fiber fractions between all test substrates, the degradation of those fractions varied. Again, HF + Sh had the highest Xfi and ADF degradation of all analyzed substrates, quite superior compared to the values obtained for the untreated straw, while FSh had the lowest degradation. The ADF is the least digestible plant components, including cellulose and lignin. Therefore, higher degradation of such fraction in HF + Sh might have been decisive for its better AD performance. In addition, the higher XP and ADF degradation of HF + Sh compared to the untreated straw corroborates the expectation that the physical treatment would result in a larger surface area exposed to microbial attachment, thus enhancing the AD process.

Structural components, such as lignin content, can also affect other important parameter for AD of the substrates, the  $\text{NH}_3\text{-N}$  concentration. The  $\text{NH}_3\text{-N}$  concentration is dependent on the degradation of crude protein and the synthesis of microbial biomass [46]. Therefore, the content of crude protein together with the arrangement of such structural components in the substrate might limit cell-wall protein degradation decreasing  $\text{NH}_3\text{-N}$  production. In the present experiment, the control hay had the highest  $\text{NH}_3\text{-N}$  concentration of all substrates, which can also be explained by its highest XP concentration and its high degradability. Within the tested substrates, rapeseed straw, both fresh and aged, had higher  $\text{NH}_3\text{-N}$  concentration during AD, which is also in accordance with its high XP concentration, although information regarding the degradability of the protein could not be obtained. Nevertheless, except for H1, values varied between 2.47 and 4.67  $\text{mmol l}^{-1}$ , which is within the range reported to favor bacterial growth [33, 47]; therefore, none of the substrates tested in the present study had inhibition patterns that could negatively affect AD.

## Conclusion

The main objective of the present work was to evaluate the potential of different crop residues for anaerobic digestion by rumen microorganisms to select suitable substrates to be further used as energy source for biogas production and repowering of existing agricultural biogas plants. A hemp fiber and shives mix obtained by retting exhibited the best anaerobic digestion performance, followed by flax straw. These substrates are candidates for further testing of methane production in an up-scaled two-stage biogas system.

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**Author Contribution** MB, DW and LI developed the concept of the project and designed the experiments. JH and LR performed the experiments and acquired and analyzed the data, AF evaluated the data and wrote the manuscript. MB revised the manuscript. All authors read and approved the final version of the manuscript.

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**Data Availability** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Competing Interests** The authors declare no competing interests.

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