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PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including the Commission Services)	
CO	Confidential, only for members of the consortium (including the Commission Services)	

D13: Characterisation of optimised silage starter cultures

Introduction

The ensiling of forage is an alternative way to use the crops even in the seasons when the material is unavailable. The principle of silage preservation is based on anaerobic fermentation achieved by the compression of the material, followed by airtight sealing to ensure the absence of oxygen. The anaerobic conditions lead to the growth of lactic acid bacteria, which convert the available substrate (soluble carbohydrates) to lactic acid, which reduces the pH and hence inhibits undesirable pathogenic organisms (McDonalds *et al.*, 1991). In addition, acetic acid, that is sometimes also formed during the silage fermentation process can be useful to inhibit yeasts and moulds after the opening of the silos (Holzer *et al.*, 2003).

Due to the regulations on energy crops production in Austria (AMA, 2005), silages that are produced from areas that are subsidized for non-food or non-feed use have to be doped in order to prevent misuse. The most common way to denature these crops is the addition of pig or cattle manure during ensiling. Since animal manure consists of biomass rich in organic content its utilization in the production of bioenergy is economically and environmentally important (Saha, 1994, Augenstein *et al*., 1994).
The objective was to determine the potential for enhancing the methane yield of different energy crops observing their suitability for silage preparations and to find out optimal starter cultures to ensure good silage quality and methane production. In a further experiment also the effects of mixing pig or cattle manure with whole crop maize on the silage fermentation process and on methane yields after anaerobic digestion were determined.

For this purpose, silage using different substrates were treated with different inoculants: homofermentative lactic acid bacteria, a mixture of homo- and heterofermentative lactic acid bacteria and spoilage organisms (*Clostridium tyrobutyricum*). The effects on silage quality, storage losses and methane yields were studied.

Materials and Methods

Ensiling

Chopped whole crop maize (M), triticale (T), clover (Cl), oat (O), sunflower (SF) and manure were obtained from farms in Austria. The silage material was prepared according to Danner et al. (2003).
The three different treatments applied for maize, triticale and clover were a control silage without additives (C), silage inoculated with homofermentative lactic acid bacteria (# LAB) and silage inoculated with *Clostridium tyrobutyricum* (# C.tr.). Parallel of this a second experiment using manure was done inoculating it instead of an inoculant in which also the three different treatments were applied: a control maize silage (K), silage mixed with pig manure (#1) and silage mixed with cattle manure (#2). 33 g of manure were added per kg of chopped whole crop maize during ensiling.
The homofermentative lactic acid (LAB) were obtained as freeze dried powder and applied at a concentration of 1 mg/kg of fresh material and clostridia (*Clostridium tyrobutyricum* – DSMZ 2637), at concentration of $1.15 \bullet 10^5$spore per kg of fresh material. In the whole crop maize silage #C.tr., to ensure the growth of *Clostridium tyrobutyricum*, which is a pathogenic bacteria undesirable in the silage. These maize (M04) samples were buffered with $CaCO_3$ at a concentration of 15 g/kg of fresh material and the concentration of dry solids was lowered to 27.77 % by the addition of 267 g of water per kg fresh material.
The obtained amount of oat were already very dry which was the reason to shift the experiment just doing a control of silage to observe the effect caused in anaerobic digestion.
The amount of obtained sunflower were not enough to prepare the same three different treatments applied to M, T and Cl. In this case, 25 % of sunflower was mixed with 75 % of maize, called M05, and also a control maize (C) was done.
All treatments were performed in duplicate.

Analytical methods

The silos were opened after 60 days of ensiling for the substrates experiment and after 7, 14, 32 and 90 days for the second experiment. The silos were weighed before opening to estimate losses.
To observe the effect of the addition of manure on the fermentation, the dry matter (DM), volatile solids (VS), total Kjeldahl nitrogen (TKN) and ammonia were measured for all opening days.
The ammonia and TKN analysis were performed according to Horwitz (1980) utilizing a Gerhardt Vapotest device.
The content of volatile solids (VS) was calculated from the ash content, that was obtained using 5 g of ground dry samples after 5 h in a muffle furnace at 505°C.
The determination of pH, DM and chemical analysis was performed as described by Danner et al. (2003).

Batch experiments

The methane production from anaerobic digestion was assessed in batch tests according to the modified norm DEV S6, DIN 38 414-S6 (Deutsche Einheitsverfahren guidelines).
The volume of produced methane was measured almost every day until no further production could be observed (4–5 weeks). The batch tests were performed in duplicate for each silage sample.

Results and Discussion

Effect of different treatments from substrates in the anaerobic fermentation

Figure 1 shows the DM analyzed for all different treatments using five different substrates (maize, triticale, clover, oat and sunflower). The values correspond to a measurement on the day of ensiling (day 0), indicated for each fresh material respectively.
Since the optimum DM content for a silage is about 30-40 %, the maize, triticale and unexpectedly sunflower had adequate DM content necessary for the silage. Clover, as already expected, had the lowest DM.

The harvest time seemed to have influence on the properties of the plant. It can been observed by the oat, which was later harvested showing higher DM content than expected. The same effect can be observed for the sunflower, which usually has 12% DM (McDonalds *et al.*, 1991) content which shifted the initial goals of the experiment.
Besides, it is worth mentioning that the weather conditions play an important role considering the DM content for the both maize used in the assay. In the first experiment, the maize (M04) was harvested in August 2004 and the second one (M05), which were mixed with sunflower, were harvested later in September 2005.

Figure 2 shows pH values for the different substrates measured at the day of ensiling (day 0, fresh material), after 119 days for maize (M04) and after 60 days of ensiling for the others.
It can be seen that in almost all control samples, silage without additives, the pH could decrease till pH 4.0 which is suitable for the silage since its decrease is an important factor to avoid the growth of pathogenic microorganisms.
Even for the clover, inoculation with LAB seemed to result in slightly lower decrease of the pH comparing to control silage.
Inoculation with *Clostridium tyrobutyricum* showed results in agree with the silage treatment. For M04, it could not decrease since it was previous buffered. In contrast to M04, silage from triticale

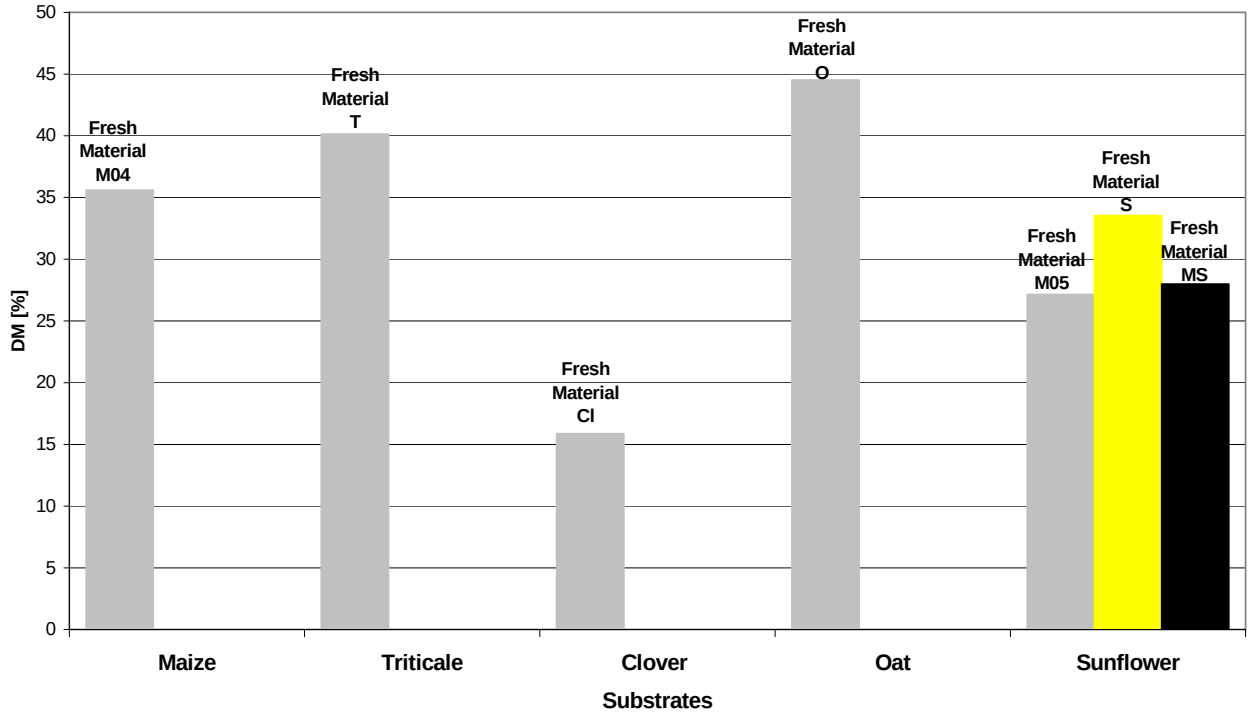

Figure 1: DM content of the different substrates on the day of ensiling.

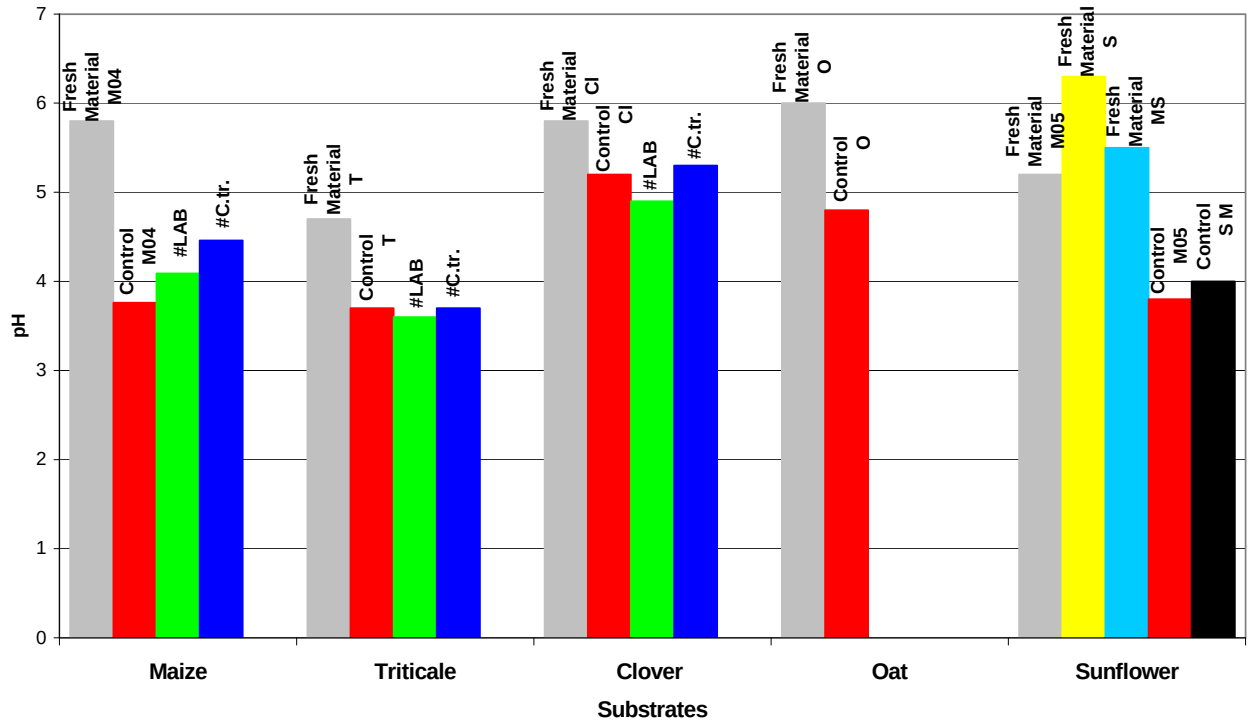

Figure 2: pH of the of the different substrates on the day of ensiling (day 0) and after 119 days for maize (M04), and after 60 days of ensiling for triticale (T), clover (Cl), oat (O), maize (M05), sunflower (S), mixture of sunflower (SM). Control: substrate silage without any further treatment, #LAB: silage with LAB, #C.tr. silage with *Clostridium tyrobutyricum*

could decrease pH to values that had minimized the growth of Clostridia (see table 6 in appendix). As already expected, clover could not decrease promoting the increase of clostridia noted by the production of butyric acid (see Table 6).

The results of lactic acid produced in the silage can be observed in Figure 3. Except for clover, silage seemed to be stable indicated by the formation of lactic acid. Comparing the amount of soluble carbohydrates showed in the table 6 and 7 (appendix), almost all the sugars were metabolized to products of fermentation (see table 6 and 7).
Treatment with homofermentative LAB did not show significant difference in comparison with their respective control of each substrate.

Even though in treatments inoculated with *Clostridium tyrobutyricum*, sufficient acid formation was observed, which is probably due to the availability of additional carbon sources by hydrolysis process (Neureiter, 2004). Further studies are being made on it.

As already expected, clover showed the lowest lactic acid production due to the fact that it is a sort of legume which is usually difficult to ensilage because of their often low DM and low water soluble carbohydrate content and highly buffered capacity contributing for a fermentation dominated by a clostridia (McDonalds *et al.*, 1991) which can be observed by the results in the table 6 in appendix.

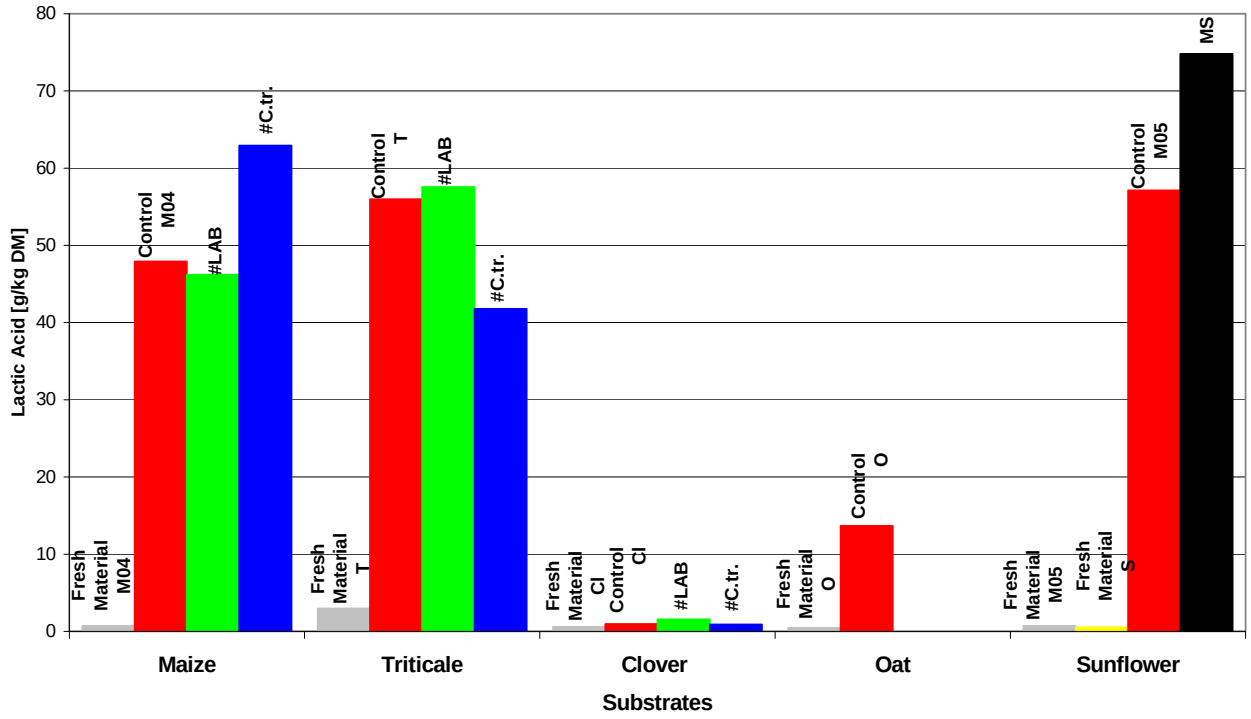

Figure 3: Lactic acid of the of the different substrates on the day of ensiling (day 0) and after 119 days for maize (M04), and after 60 days of ensiling for triticale (T), clover (Cl), oat (O), maize (M05), sunflower (S), mixture of sunflower (SM). Control: substrate silage without any further treatment, #LAB: silage with LAB, #C.tr. silage with *Clostridium tyrobutyricum*

Effect of different treatments from substrates on methane production

Figure 4 shows the methane yields based on volatile solids performed as batch tests in laboratory scale. Comparing the methane production for the different treatments and substrates, they seemed to have the similar tendency where the treatment with *Clostridium tyrobutyricum* showed higher specific methane than fresh material samples probably due to the released compounds that were not available in the normal silages (Neureiter *et al*., 2004). Besides, slightly low methane yields can be observed for the treatment inoculated with LAB. Moreover, clover appears as an exception here, where it could be due to the highest amount of clostridia predominated in the silage.

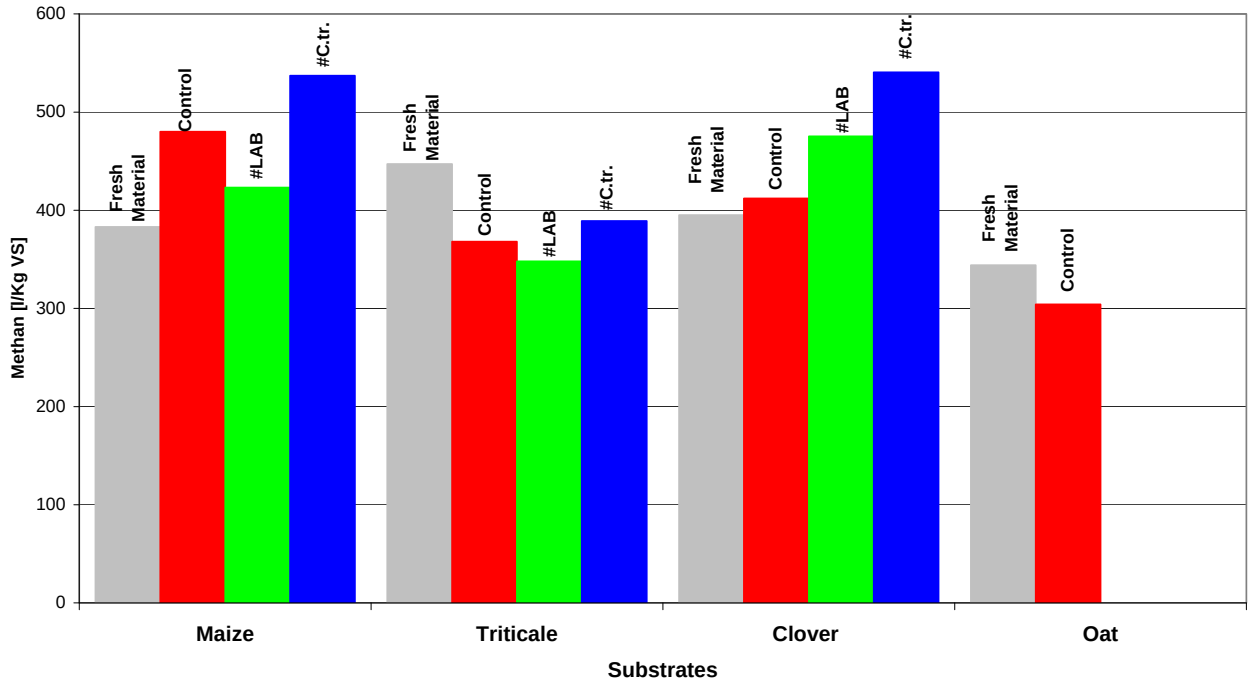

Figure 4: Methane yields of fresh material and silage for the different substrates after 119 days (M04) and 60 days (T, Cl, O, M05 and S). Control: substrate silage without any further treatment, #LAB: silage with LAB, #C.tr.: silage with *Clostridium tyrobutyricum*

Effects of the addition of manure before ensiling

Table 1 shows TKN, ammonia, weight losses, DM, and VS analyzed for whole crop maize, the mixture of it with pig and cattle manure, and samples that contained only pig and cattle manure. The values correspond to a measurement on the day of ensiling (day 0) and after 90 days (figures in parentheses).

Table 1 Composition of the samples and weight losses from the different treatments and manure

Samples	TKN	NH_4-N	Weight losses	DM	VS
	(g/kg DM)	(g/kg DM)	(%)	(%)	(%)
K	74.50 (65.05)	0.14 (0.64)	1.24	30.62 (28.56)	29.50 (27.47)
1	84.40 (67.35)	0.42 (0.96)	1.37	29.77 (27.11)	28.61 (26.05)
2	77.16 (66.95)	0.28 (0.86)	1.35	29.76 (26.92)	28.61 (25.82)
Pig Manure	26.31	2.28	-	1.37	1.36
Cattle manure	25.81	1.44	-	5.75	4.60

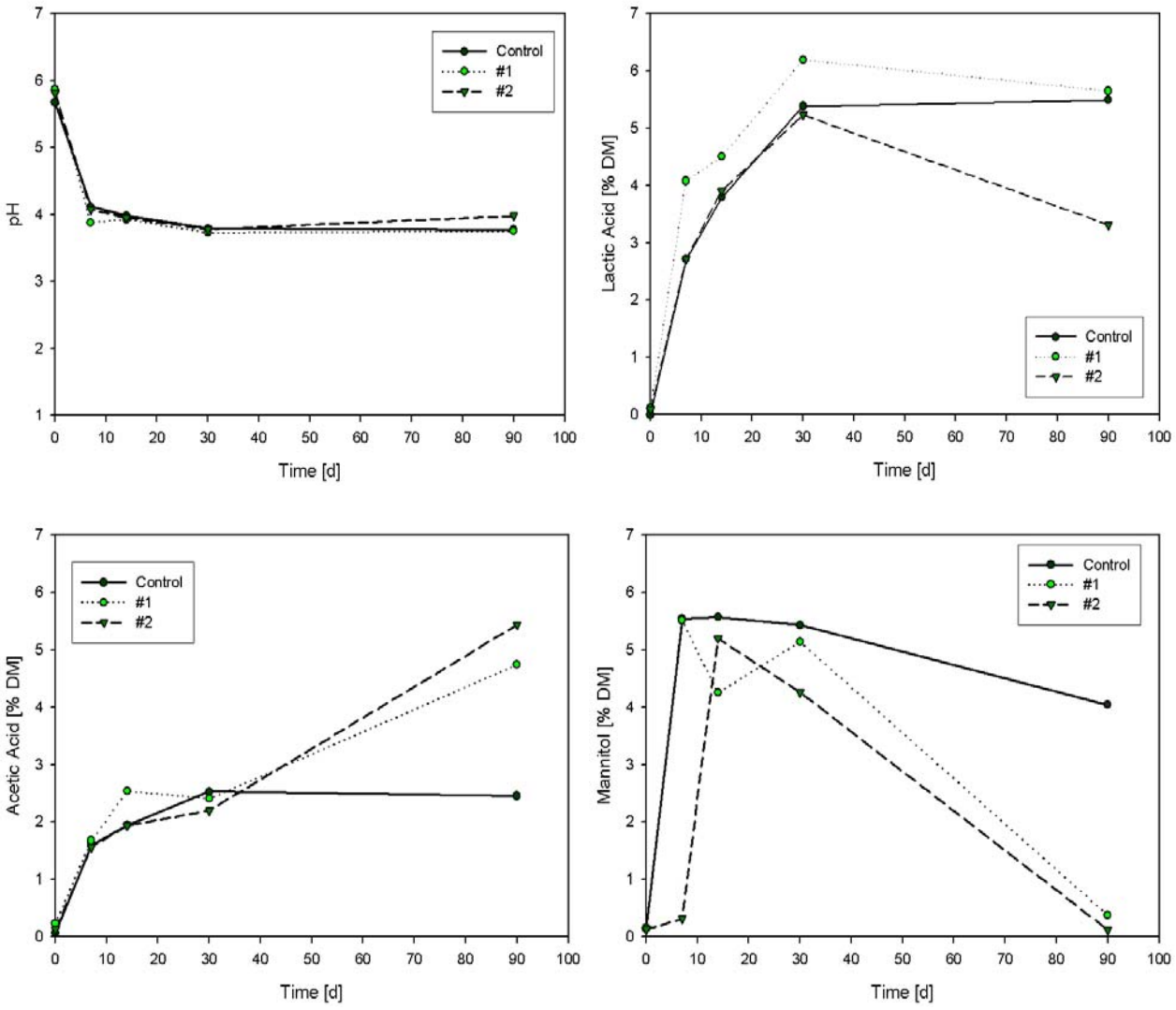

Figure 5: Time course of pH and fermentation products (lactic acid, acetic acid and mannitol) of the different silage treatments. Control: whole crop maize silage without any further treatment, #1: silage with pig manure, #2 silage with cattle manure

Figure 5 shows important parameters for describing the fermentation process during the ensiling process. Observing the effect of addition of manure on the fermentation process, it seems to have no effect on the ensiling process since the pH decreased to values of approximately 4.0 in all experiments during the first 7 days after ensiling due to the production of lactic acid.

The production of large amounts of acetic acid can be explained by the presence of heterofermentative lactic acid bacteria, which are usually acting in the last steps of ensiling. Some authors (Beck, 1972; Beck *et. al.*, 1986) have reported that in well-preserved silages the fermentation process is initiated by homofermentative lactic acid bacteria and is dominated by heterofermentative species at the end of the process.

It is worth mentioning that the detected amount of butyric acid found at the end of the experiment was the same as in the applied manure (e.g. 0.15 g/kg DM pig manure and 0.16 g/kg DM cattle manure). Besides, mannitol could only be degraded in the doped silages. This could be explained by the high diversity of microorganisms that are present in the added manure. Before ensiling the amount of free sugars in DM was 9.52 %, 9.80 %, and 8.98 % (samples K, 1 and 2, respectively). After 90 days of ensiling, those amounts were almost completely converted into fermentation products (0.062 % DM; 0.54% DM and 0.58 % DM, respectively).

Table 2: Methane yield from different treatments

	Fresh Material	Control Silage	Pig Manure	Cattle Manure	Silage + Pig Manure	Silage + Cattle Manure
				(l/kg VS)		
Measured	321.28	415.09	531.26	558.12	404.91	424.31
Estimated					432.23	433.09

To compare the methane yields, batch tests using only manure were performed as well. The ratio of 1:30 (600 g of manure in 18 kg silage) was taken into account to estimate the expected methane production. Table 2 shows the amount of methane that was actually produced and estimated (theoretical) in terms of organic dry matter.

A comparison of the control silages (containing just whole crop maize) with the silage that were treated with manure using ANOVA did not show any significant differences with respect to the methane yield. Therefore the results show that the samples ensiled with manure tend to yield the same amount of methane as the control. Besides, the expected methane yield does not differ between the samples, that were ensiled with pig or cattle manure, although they have different properties (Table 1).

Conclusions

The use of conventional starter cultures as the are employed for forage conservation in animal feed had no significant effect on methane production. However, it is known that such products can help to minimise storage losses, especially with respect to aerobic deterioration, which occurs during the removal of the material from the silo.

Spoilage organisms like *Clostridium tyrobutyricum* showed significant improval of methane production, however, such organisms may contribute to enhanced losses during storage (anaerobic spoilage). Under current circumstances Clostrida cannot be recommended as starter cultures for several reasons. In conventional silos odour emissions due to the formation of butyric acid will occur. Furthermore it has been shown that these microorganisms will only have an effect when they find proper conditions, which is low TS (below 30%) and/or pH above 5 (by buffering). Still, for improvement of the overall process it is worth considering, whether similar microorganisms can be used in a pre-treatment or fermentation step.

For the employment of starter cultures in silage preparation for anaerobic digestion, two strategies are possible, which are to some extent contradictory: Minimisation of storage losses and improvement of methane production.
For improving of the storage conventional starter cultures can be used. As shown in the experiment, the need to use such additives will very much depend on the plants and the harvest conditions. E.g. with maize generally good silage quality could be achieved in most experiments without the addition of starter cultures.
In order to improve hydrolysis processes during ensiling with respect to enhance methane production, spoilage organisms were found to be more effective. In order to use such organisms on a technical scale the currently used techniques for storage will have to be adapted so that losses can be minimised and the growth of such cultures can be ensured.

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Appendix

Table 1: Composition of the samples and weight losses from different treatments

	DM				***VS***				***Weight losses***			
					(%)							
	M	**T**	**Cl**	**O**	**M**	**T**	**Cl**	**O**	**M**	**T**	**Cl**	**O**
FM	35.59	40.13	15.85	44.51	34.29	36.42	13.54	41.14	-	-	-	-
C	34.92	37.16	17.08	42.82	33.56	31.88	14.15	39.56	0.95	1.06	1.56	1.28
1	32.70	40.19	16.57	-	31.39	33.83	13.74	-	1.28	1.04	1.00	-
2	27.40	38.80	14.63	-	25.31	33.01	11.68	-	2.37	0.71	1.54	-

Table 2: Composition of the samples from different treatments

	TKN				***NH_4-N***			
				(g/kg DM)				
	M	**T**	**Cl**	**O**	**M**	**T**	**Cl**	**O**
FM	8.6	5.54	30.56	15.89	1.0	0.19	0.11	0.24
C	8.9	11.99	26.40	16.64	0.6	1.57	5.01	1.03
1	8.6	11.38	25.59		0.6	1.22	4.52	
2	9.2	12.16	26.13		0.9	1.39	8.28	

Table 3: Composition for sunflower samples from different treatments

	DM	***VS***	***Weight losses***	***TKN***	***NH_4-N***
	(%)	***(%)***	***(%)***	***(g/kg DM)***	***(g/kg DM)***
FM M	27.14	25.97	-	11.65	0.13
FM SF	33.53	29.64	-	14.75	0.28
C M	26.60	25.46	1.02	11.81	0.83
M SF	29.37	27.47	0.71	12.40	0.90

Table 4: Fermentation products (acetic acid, sugars, mannitol and butyric acid) of the different silage treatments.

	Acetic Acid				***Sugars***			
	(g/kg DM)							
	M	**T**	**Cl**	**O**	**M**	**T**	**Cl**	**O**
FM	0.84	1.22	0.85	1.00	52.42	24.04	3.89	9.11
C	16.5	28.05	32.45	10.16	3.14	1.43	2.28	3.41
1	39.2	19.07	58.58	-	2.02	0.32	7.07	-
2	20.7	24.29	35.70	-	2.00	0.62	3.93	-

	Mannitol				***Butyric Acid***			
	(g/kg DM)							
	M	**T**	**Cl**	**O**	**M**	**T**	**Cl**	**O**
FM	0.86	0.75	0.11	1.49	n.d.	n.d.	n.d.	n.d.
C	19.90	2.44	1.28	0.63	0.30	0.24	42.82	0.92
1	4.80	0.70	4.01	-	n.d.	0.75	13.59	-
2	n.d.	0.40	2.16	-	45.00	8.63	61.39	-

Table 5 Fermentation products for sunflower samples (acetic acid, sugars, mannitol and butyric acid) of the different silage treatments.

	Acetic acid	***Sugars***	***Mannitol***	***Butyric acid***
	(g/kg DM)	***(g/kg DM)***	***(g/kg DM)***	***(g/kg DM)***
FM M	1.67	73.17	n.d.	n.d.
FM SF	0.83	59.24	n.d.	n.d.
C M	20.93	2.70	1.10	n.d.
M SF	19.02	8.50	5.31	n.d.