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Separating the effects of pre-ensiled chemical and microbial composition on silage fermentation and aerobic stability

K. Mogodiniyai Kasmaei, R. Spörndly & P. Udén
Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Box 7024, 750 07 Uppsala, Sweden Correspondence:
Kamyar.mogodiniyai.kasmaei@slu.se

Introduction

Ensiling results of different forage types usually differ. For instance, legume silages usually contain more acetic acid, butyric acid and ammonia-N and are aerobically more stable than maize silages (Muck & O'Kiely, 1992; O'Kiely & Muck, 1992; Wilkinson & Davies, 2013). However, as to what extent these differences are related to chemical or microbial composition is yet unknown. In recent work by Mogodiniyai Kasmaei *et al*. (2015), a new ensiling methodology was introduced that enables sterilization of forages and inoculation with original microfloras. In the present experiment, we have further utilized this methodology to separate the confounding effects of microbial and chemical composition on silage fermentation and aerobic stability.

Materials and Methods

Samples used were one second-cut of a mixed timothy (*Phleum pratense*)-meadow fescue (*Festuca pratensis*) sward harvested at early maturity (grass), one second-cut red clover (*Trifolium pratense*) (clover) at late flowering and one whole-crop maize (*Zea mays*) (maize) at early dent. Perennial forage samples were collected from fields around Uppsala, Sweden (59°51′N, 17°37′E) and maize sample was collected from southern Sweden (55°41′N, 14°10′E). Samples were collected in the autumn of 2013. The perennial forage samples were chopped to a length of 3-5 cm by a stationary chopper and the maize sample was chopped by a precision chopper before being frozen at -20°C.

Sampling procedure

An amount of 3 kg of frozen samples was divided into two replicates which were thawed at room temperature before sampling of 150 g for microbial isolation and 500 g for drying at 60°C in a forced draught oven for 18 h.

Sterilization

The dried samples were milled on a hammer mill to pass a 1-mm screen before weighing 130 g in glass beakers. Samples covered loosely with aluminium foil were sterilized by the heating procedure described by Mogodiniyai Kasmaei *et al*. (2015), i.e. heating at 60°C for 3 h followed by heating at 103°C for 15 h in a forced draught oven. Thereafter, samples were tightly sealed with aluminium foil and were transferred into a desiccator to reach the room temperature.

Microbial isolation procedure

A volume of 900 mL of 0.25-strength Ringer solution fortified with Tween®80 (Merck KGaA, Darmstadt, Germany) at 0.5 mL/L (O'Brien *et al*., 2007) was added to 150-g samples. The suspensions were kept on a bench for 30 min before being pummelled for 2 min in a laboratory stomacher (Seward 3500, Seward Ltd, Worthing, UK). Thereafter, an amount of 750 mL of the microbial solution, accounting for 75% of the total volume of Ringer solution and sample water, was centrifuged at 15,500 *g* for 90 min. The supernatant was discarded and

the pellet was re-suspended in 15 mL sterile 0.25-strength Ringer solution and stored at 4°C overnight.

Reconstitution and ensiling

To estimate the amount of forage fresh matter corresponding to the isolated microbes two assumptions were made: i) microbial population was completely removed from each 150-g forage sample and evenly distributed into the Ringer solution after stomaching, ii) based on our preliminary observations (data not shown), recovery proportion of microbial population after centrifugation was considered to be 90%. Further, half the hypothetical ratio of microbes:fresh matter in original forage samples was targeted at reconstitution and all samples were reconstituted to a DM content of 40%.

The microbial isolates (n=6) were each divided into 3 equal volumes. The 5-mL inocula were added to the calculated amounts of sterilized DM so that each forage type received each of the three kinds of inoculum. Final reconstitution was carried out with addition of sterile distilled water. An amount of ~65 g was then put in sterile glass tubes (20 and 3 cm in length and inner diameter, respectively) and sealed with water locks. Silos were kept at 20±2°C for 71 day.

Aerobic stability test

An amount of 30 g of silage was placed in glass filter crucibles. The crucibles were insulated in foam polyethylene insulation pipe (15 mm wall thickness). Pipes were covered with aluminium foil and the samples were kept at 20°C with sample temperature being recorded at 2 h intervals for 8 days.

Chemical analyses

The sterilized forage samples were subjected to N determination by the Kjeldahl method, with Cu as a catalyst. Extracted juice was analyzed for short chain organic acids and alcohols by HPLC and ammonia-N by flow injection analysis. The pH of silage and aerated silage samples was measured also on the extracted juice by a laboratory pH-meter (Metrohm 654, Metrohm AG, Herisau, Switzerland). The DM content of the silage samples were estimated after drying at 103°C and corrected for volatiles as described by Mogodiniyai Kasmaei *et al*. (2015).

Statistical analyses

The effect of inoculum type (n=3), forage type (n=3) and their interactions on fermentation quality and aerobic stability variables was tested by the General Linear Model procedure. Total number of observations was 18. The significant levels for the main and interaction effects were declared at $P < 0.05$ and $P < 0.10$, respectively. If significant, pairwise comparisons were made by the Tukey method.

Results and Discussion

The effects of forage type, inoculum type and their interaction on silage variables are depicted in Table 1. Butyric acid concentrations were below 1 g/kg DM and are therefore not shown. Formation of all the end-products was affected by the forage type. Clover silages had the highest contents of lactic and acetic acid. Silages made from maize had higher amounts of propionic, 2,3-butanediol and ammonia-N than grass silages. They also formed more ethanol than clover silages.

Inoculum type only affected the formation of ethanol where, the grass inoculum gave the highest ethanol concentration. This is surprising considering that counts and species of epiphytic lactic acid bacteria (LAB) differ among forage types (Andrieu & Gouet, 1991; Li *et al*., 1992). It may suggest that differences in fermentation quality of silage crops are mostly attributed to their chemical composition. It should be interesting to find out whether this also can be seen within the same forage type. An elaborate chemical analyses (e.g. water activity, amino acid and sugar composition) is then needed as chemical variables commonly measured (e.g. dry matter, crude protein, water soluble carbohydrates) poorly explained variations in fermentation results (Mogodiniyai Kasmaei *et al.,* 2013). Clover samples inoculated with grass and maize inocula had the highest concentration of acetic acid, with inoculation having no negative effects on lactic acid formation.

Table 1 The effect of forage, inoculum and their interaction on silage variables

Treatment	pH	Lactic acid	Acetic acid	Propionic acid	Ethanol	2,3-butanediol	Ammonia-N
		g/kg DM					g/kg N
Forage (F)							
Grass	4.12^{b}	40.3^{b}	12.4^{b}	2.6^{b}	4.3^{ab}	0.9^{b}	13.6^{b}
Clover	4.55^{a}	57.0^{a}	26.7^{a}	3.0^{a}	3.4^{b}	2.0^{ab}	15.5^{ab}
Maize	3.94^{c}	27.5^{c}	10.7^{b}	3.0^{a}	5.0^{a}	3.0^{a}	20.1^{a}
Inoculum (I)							
Grass	4.28^{a}	40.8	17.1	2.9	6.6^{a}	1.5	15.4
Clover	4.25^{a}	39.5	15.9	2.9	3.3^{b}	2.2	17.7
Maize	4.08^{b}	44.6	16.8	2.8	2.8^{b}	2.2	16.1
SEM[1]	0.02	1.52	0.51	0.06	0.33	0.35	1.57
Interaction							
Grass×Grass	4.20	36.1^{def}	12.1^{c}	2.6	7.0^{ab}	0.2	12.7
Grass×Clover	4.18	39.5^{cde}	12.4^{c}	2.7	3.0^{c}	1.0	16.3
Grass×Maize	3.99	45.4^{bcd}	12.8^{c}	2.6	2.8^{c}	1.4	11.7
Clover×Grass	4.59	62.5^{a}	28.5^{a}	3.1	3.9^{bc}	1.2	14.6
Clover×Clover	4.59	51.0^{abc}	23.2^{b}	2.9	3.8^{bc}	2.5	13.7
Clover×Maize	4.47	57.6^{ab}	28.4^{a}	3.0	2.6^{c}	2.4	18.2
Maize×Grass	4.05	23.8^{f}	10.7^{c}	2.8	8.9^{a}	3.0	18.9
Maize×Clover	3.99	28.1^{ef}	12.1^{c}	3.2	3.0^{c}	3.0	23.0
Maize×Maize	3.78	30.7^{def}	9.2^{c}	2.8	3.0^{c}	3.8	18.3
SEM[1]	0.03	2.64	0.88	0.11	0.57	0.61	2.71
P value							
F	<0.01	<0.01	<0.01	0.01	0.03	0.01	0.04
I	<0.01	0.10	0.25	0.48	<0.01	0.31	0.59
F×I	0.22	0.06	0.01	0.16	0.01	0.75	0.47

[1]Standard error of mean; ^{abcdef}values with different superscribed letters within 'F', 'I' or 'F×I' and analyte differ (P<0.05 for 'F', 'I' and P<0.1 for 'F×I').

Data from the aerobic stability test is shown in Table 2. One of the replicates of grass with maize inoculum was discarded as the thermometer had been misplaced. The highest temperature and the greatest pH increase were observed in maize silages. At the same time, the maize inoculum had the best scores for the aerobic stability variables (Table 2).

Table 2 The effect of forage, inoculum and their interaction on temperature and pH rise (pH after – pH before) of silages aerated for 8 d (mean ambient temperature=20.2°C)

Treatment	Maximum temperature (°C)	pH increase
Forage (F)		
Grass	20.9^{b}	0.73^{b}
Clover	20.6^{b}	0.03^{c}
Maize	21.7^{a}	1.78^{a}
Inoculum (I)		
Grass	21.4^{a}	1.58^{a}
Clover	21.3^{a}	0.93^{b}
Maize	20.7^{b}	0.03^{c}
SEM[1]	0.1	0.07
Interaction		
Grass×Grass	21.5^{abc}	2.00^{b}
Grass×Clover	20.9^{bc}	0.20^{c}
Grass×Maize	20.4^{c,A}	0.00^{c}
Clover×Grass	20.5^{c}	0.03^{c}
Clover×Clover	20.6^{c}	0.01^{c}
Clover×Maize	20.7^{c}	0.04^{c}
Maize×Grass	22.0^{ab}	2.72^{a}
Maize×Clover	22.3^{a}	2.58^{ab}
Maize×Maize	20.8^{bc}	0.05^{c}
SEM[1]	0.2	0.12
P value		
F	<0.01	<0.01
I	0.01	<0.01
F×I	0.02	<0.01

[1]Standard error of mean; ^{abc}values with different superscribed letters within 'F', 'I' or 'F×I' and analyte differ (P<0.05 for 'F', 'I' and P<0.1 for 'F×I'); ^{A}SEM=0.3.

The results found here could be of a great potential in inoculant research. For instance, inoculating the difficult-to-ensile legume crops with strains of LAB obtained from grass or maize forages could be tested. Considering the effects of maize inoculum on silage pH (Table 1) and aerobic stability variables (Table 2), further characterization of maize LAB is warranted. It should be kept in mind the results presented were obtained from one forage sample of the forage types investigated and hence, caution needs to be undertaken when extrapolating. More studies of this kind are therefore needed before drawing firm conclusions.

Conclusions

Fermentation quality was affected by forage type but only to a very limited degree by inoculum type. Inoculation of clover sample with grass or maize inocula increased formation of acetic acid, but had no negative effect on lactic acid production. The maize inoculum improved aerobic stability. The study showed that the possibility to separate confounding effects of chemical and microbial composition can result in new insights as well as new research questions.

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Margareta.Norinder@slu.se