FINAL REPORT
Amaferm Effects on *in sacco* NDF degradation
Prepared for BioZyme, Inc.

by

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The information in this report replaces the prior report.

Executive Summary

An extensive data set from 300 Corn Silage and Haylage samples that included *in sacco* DM and NDF disappearance data from Dr. Jim Nocek at Spruce Haven and nutrient composition data from Mr. Ralph Ward at Cumberland Valley Analytical Services was provided. The data were analyzed using multiple, robust statistical procedures to determine whether Amaferm altered NDF degradation, which forage types it was most effective on, and whether nutrient composition would be a significant effector of Amaferm success.

Amaferm was found to be most effective on Haylages, and worked equally well on grass and legume Haylages to increase rates of NDF degradation. Across all Haylage samples, Amaferm increased degradation rates by 28%. Amaferm was also effective in increasing NDF degradation rates of Corn Silages by 11% as compared to the control treatment.

While chemical composition does affect NDF degradation in Haylages and Corn Silages, the individual nutrients are not robust predictors of NDF degradation rates.

The effect of Amaferm on NDF degradation can be modeled in ration formulation software based on the CPM Dairy/CNCPS and NRC 2001 systems. In CPM Dairy/CNCPS, when feeding Amaferm at the recommended feeding levels, the effects can be accounted for by increasing the CHO-B3 k_d by 10% for corn silages and 20% for haylages. In NRC 2001, when feeding Amaferm at the recommended feeding levels, the effects can be accounted for by increasing NDF digestility by 7% for corn silages and 14% for haylages.

According to Oba & Allen, 1999, as forage NDF digestibility increases in response to Amaferm feeding, lactating dairy cows are expected to increase dry matter intake when rumen fill is limiting, increase milk yield, and increase feed efficiency.

Sales Summary

- 75 corn silage and 75 haylage samples were incubated in Dacron bags in rumen-fistulated cows fed a Control diet (without Amaferm) or a diet containing Amaferm, and NDF disappearance between the Control and Amaferm samples was compared.

- Amaferm increased NDF degradation. The absolute amount of the increase is a function of time and dependent on the NDF concentration.

- Amaferm increased <u>rates</u> of degradation by 11% in corn silages and 28% in haylages.

- Amaferm increases rates of degradation the same across all levels of NDF and lignin in both corn silage and haylage. Thus, Amaferm is expected to be as effective in poorer quality forages as higher quality forages. Also, Amaferm worked equally well on legume vs. grass haylages.

- The effect of Amaferm can be modeled in CPM Dairy 3.0 and CNCPS 6.1 by increasing the CHO-B3 k_d by 10% for corn silage and 20% for haylage. Examples:

  Corn Silage CHO-B3 k_d = 3.6%/hr without Amaferm → k_d = 4.0% with Amaferm

  Mixed haylage CHO-B3 k_d = 5.0%/hr without Amaferm → k_d = 6.0%/hr with Amaferm

- The effect of Amaferm can be modeled in NRC 2001 by increasing NDF digestibility by 7% for corn silage and 14% for haylage. Examples:

  Corn Silage NDF digestibility = 55% without Amaferm → 58.8% with Amaferm

  Mixed haylage NDF digestibility = 45% without Amaferm → 51.3% with Amaferm

- In early lactation cows whose dry matter intake is limited by rumen fill, the increased NDF digestibility seen with feeding Amaferm would be expected to increase dry matter intake, increase energy availability to the animal, decrease loss of body condition, and allow the cows to reach greater peak milk yields.

- In mid- to late-lactation cows who are not limited by rumen fill, but who eat to meet energy requirements, the increased NDF digestibility seen with feeding Amaferm would be expected to decrease dry matter intake, increase energy availability, maintain milk yields, and increase feed efficiency.

- In mid- to late-lactation cows who are limited by rumen fill, the increased NDF digestibility seen with feeding Amaferm would be expected to increase dry matter intake, energy availability, milk yield, and feed efficiency.

Analytical Approach, Statistical Methods, & Detailed Results

1. Data quality evaluation

Data were checked for outliers and appropriate forage type identification. Outliers were defined as those samples where the NDF remaining at a given time point was greater that the NDF in the initial, intact sample. 70 samples (30 Corn Silages and 40 Haylages) had more NDF remaining at time 0 than in the intact sample. 154 samples had NDF remaining that was less than 95% of the intact sample. This appears to be a result of the *in sacco* and/or NDF analysis procedures. Samples at 12h with NDF remaining greater than the intact sample were removed from the data set and are shown in Table 1. No such samples were identified at 24 or 36 hours of incubation.

Table 1. Outliers with NDF remaining at 12 hours of incubation that were greater than the initial, intact sample and removed from further data analysis.

ID1	ID2	Forage type	Trt	Intact sample NDF (g)	Residue NDF (g)
7639	78	Corn Silage	Amaferm	1.828	2.337
7763	17	Hay	Control	1.704	1.766
7784	5	Corn Silage	Control	1.655	1.669
7784	40	Corn Silage	Amaferm	2.623	2.665
7784	75	Corn Silage	Control	1.835	1.975
7784	75	Corn Silage	Amaferm	1.841	2.074
7824	141	Corn Silage	Amaferm	1.882	1.882

Mistyped samples were identified by analysis of their chemical composition and by comparison to the Cumberland Valley Analytical Services (CVAS) feed type designation. Forage type identification was corrected before subsequent data analysis. Mistyped feed samples are given in Table 2.

Table 2. Misidentified forage types of feed samples in data set.

ID1	ID2	Original forage type	Corrected forage type
7803	67	Corn Silage	Haylage
7824	148	Haylage	Corn Silage

Lastly, many samples had chemical composition data where the analytical value was 0. It was clarified that these values actually were not determined or were below the detectable range, and so were recoded as missing, eliminating the bias that would occur if the values were 0.

2. Analyis of Variance on Observed Data

Two-way analysis of variance was conducted on the proportion of NDF remaining at each time point as function of treatment (Control vs. Amaferm), time, and the interaction of time x treatment for corn silages and haylages. The initial amount of NDF placed in the nylon bags was set equal to 1, and the NDF remaining at the subsequent time points expressed as a proportion of the initial NDF. Results are given in Table 3.

Table 3. Observed differences in proportion of NDF remaining at set time points during *in sacco* incubation. NDF remaining is a proportion of the initial NDF amount. *p*=probability that the NDF remaining in Control vs. Amaferm fermentations are equal at a given time point.

Corn Silage

time (hr)	Control	SE	Amaferm	SE	Difference	*p*
12	0.8135	0.0104	0.8304	0.0106	-0.0169	0.2548
24	0.6751	0.0103	0.6580	0.0104	0.0171	0.2431
36	0.5790	0.0103	0.5392	0.0110	0.0398	0.0084

Haylage

time (hr)	Control	SE	Amaferm	SE		*p*
12	0.7344	0.0097	0.6836	0.0105	0.0508	0.0008
24	0.6047	0.0097	0.5279	0.0097	0.0768	0.0001
36	0.5005	0.0099	0.4455	0.0099	0.0550	0.0001

Over all three time points, Amaferm numerically increased the disappearance of NDF in corn silages (p=0.12) and significantly increased it in haylages (p=0.0001) compared to the Control treatment. As expected, time had a significant effect on NDF disappearance in both corn silages and haylages (p=0.0001). The interaction of Amaferm treatment x time is significant for corn silages (p=0.0266), but not for haylages (p=0.3184).

3. Equation fitting

NDF degradation *in sacco* was modeled as the classical three-pool model, where A represents the rapidly solubilized and highly degraded material, B represents the more slowly and potential degradable material, and C represents the undegradable material. With respect to NDF, the A pool is assumed to be zero; that is none of the NDF is rapidly solubilized and degraded, leaving only the B and C pools. In the first pass, the degradation of NDF over time was fit to the NDF degradation of forage type x treatment groups using the exponential decay or first-order degradation curve $NDF_{remaining} = \beta * e^{(-kdeg*t)} + C$ where β represents the fraction of NDF in the B pool at the beginning of the incubation (time 0), t is time (in hours), kdeg is the degradation rate, and C is the undegradable NDF fraction. C was fit according to the Cornell equation (Van Amburgh et al. CNC 2008) as 2.4 x lignin/NDF, where lignin and NDF are given on a %DM.

The equation was fit both with and without the observed time 0 data. For 70 samples, the proportion of NDF remaining in the time 0 sample was significantly different than the initial amount placed in the nylon bags. These observed differences lack a rational biological explanation. It was found that the equation fit without the time 0 data gave the best fit to the NDF remaining at the other time points. Secondly, C was found to be greater than the NDF remaining at 36 hours of incubation for many samples. In the second pass, C was estimated jointly for both the control and Amaferm treated samples by the equation C = P*lignin/NDF with P allowed to vary from 1.0 to 4.0. This approach makes the assumption that P is a function of the forage chemistry and is not altered by Amaferm treatment.

Figure 1. Amaferm increases NDF degradation *in sacco*. First-order equations for Corn Silage and Haylage samples as a function of Amaferm (gold line) vs. Control (black line) treatment. Note that y axes are not equal. Full page graphs can be seen in Appendix A.

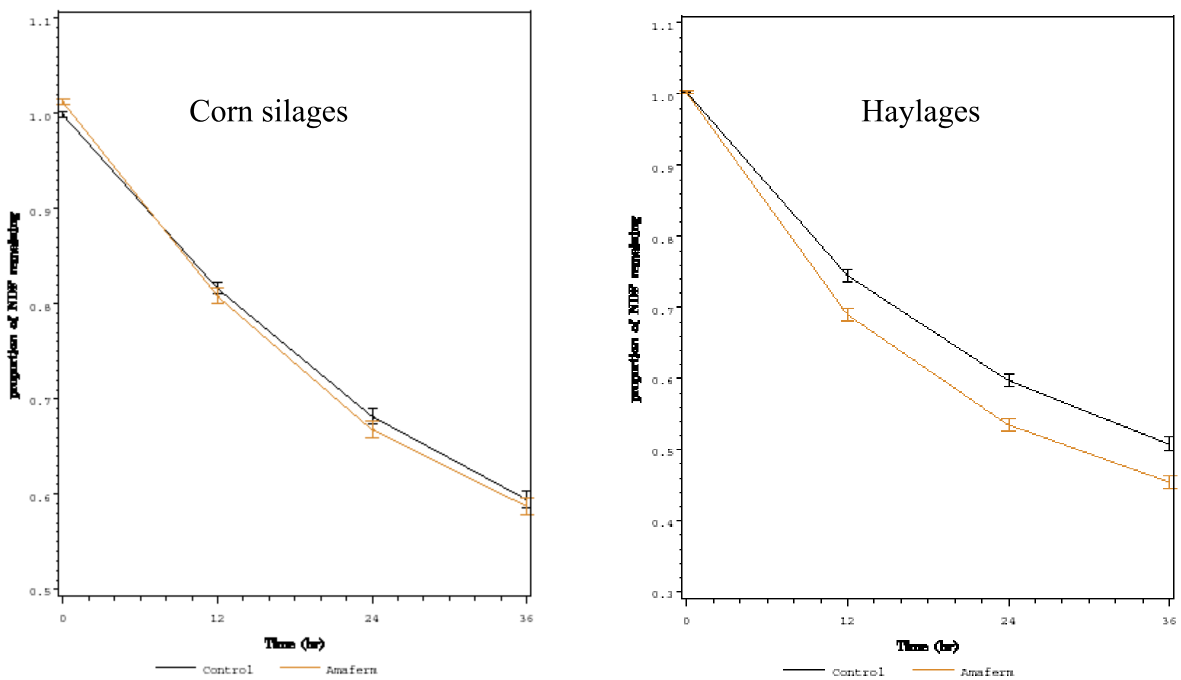


Table 4. Least square means of parameter estimates for *in sacco* NDF disappearance fit with the equation NDF remaining = $\beta_0 * e^{(-kdeg*t)} + C$ as a function of forage type and treatment, where β_0 estimates the potentially degradable NDF pool, kdeg is the NDF degradation rate, t is time (hours) and C = P*lignin/NDF estimates the undegradable NDF pool.

Corn Silage

	Control	SE	Amaferm	SE	Difference	*p**
B0	0.8091	0.0150	0.8217	0.0154	0.0126	0.5573
kdeg	0.0226	0.0012	0.0258	0.0012	0.0032	0.0647
P	2.41	0.17	2.41	0.17	--	--

Haylage

	Control	SE	Amaferm	SE		*p**
B0	0.6886	0.0174	0.6877	0.0174	0.0009	0.9707
kdeg	0.0450	0.0030	0.0578	0.0030	0.0128	0.0027
P	2.16	0.10	2.16	0.10	--	--

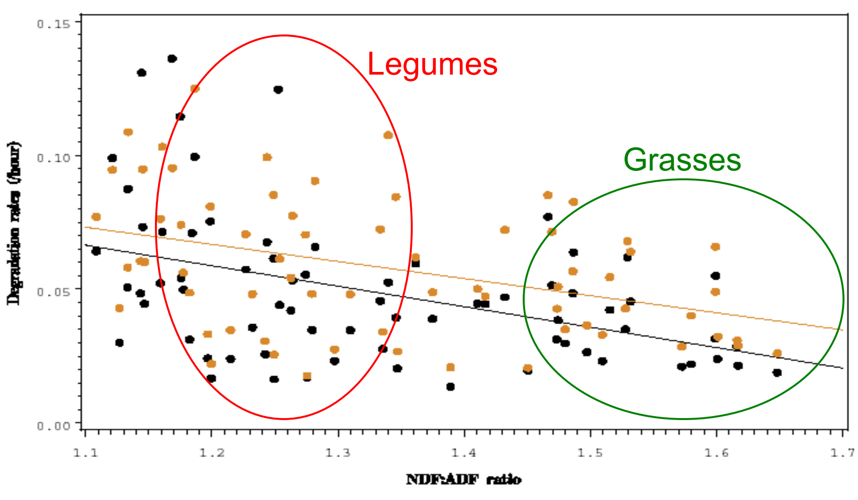
These equations fit the data well (Figure 1); $R^2 > 0.98$ for the four equations (forage type x treatment) and the residuals were found to be unbiased. Parameter estimates for each forage type x treatment equation are given in Table 4. There are high correlations between β_0 and kdeg, ranging from 0.5-0.7. This is expected and not unusual for the first-order degradation equation. The β_0 estimates of the potentially degradable NDF fraction were not significantly different between Control and Amaferm treatments (details in Section 4). Significant differences in estimates of the degradation rate kdeg between Control and Amaferm treatment are detailed in Section 4. Because the C pool was estimated as P*lignin/NDF, it is the same for both the Control and Amaferm treatments on a given sample, and there were no treatment differences for this parameter. However, there are differences by forage type in the estimated size of the C pool. Note that for haylages, P=2.16 and is significantly lower than the 2.40 factor in the Cornell equation.

4. <u>Treatment and Forage Type Effects on NDF Degradation: Analysis of Variance</u>

The parameter estimates β_0 and kdeg from the degradation equations fit to individual samples were subjected to analysis of variance to test for difference between Amaferm treatment, forage type, and any interactions. With regards to β_0, the potentially degradable NDF fraction, there was no difference between Control and Amaferm Treatments within a forage type. This fits our expectation, as Amaferm treatment would not be expected to decrease the undegradable NDF (Pool C), as the size of this pool is a function of the amount of lignin in the plant and the degree of crosslinking between lignin and hemicellulose. Forage type was a significant effector of the potentially degradable NDF fraction β_0, with estimates of 0.82 for Corn Silage 0.65 for Haylage.

Amaferm treatment increased NDF degradation by 11% in Corn Silage and 28% in Haylage samples, respectively (Table 4; Figure 1). In Haylages, this degradation rate is similar across legumes and grasses (Figure 2). Amaferm works as well on grasses as it does on legumes in increasing NDF degradation.

Figure 2. Amaferm works equally well on legume and grass haylages to increase NDF degradation.



5. Correlations between NDF degradation rates (kdeg) and Chemical Composition

Correlations between kdeg, dry matter, corrected NDF, lignin, crude protein (CP), ash, and non-structural carbohydrate (NSC) contents reported by CVAS for each forage type are given in Tables 5 & 6. Corrected NDF was calculated as NDF – ADFICP – lignin and would represent the true carbohydrate portion of NDF. There are significant correlations between several of the chemical components, as would be expected. However, correlations between the chemical components and NDF degradation rates are not very high (-0.30 to 0.30).

Table 5. Correlations between chemical composition for Corn Silage samples (n=144 observations). All nutrients given on a %DM basis.

	kdeg	DM	cNDF	lignin	CP	Ash	NSC
kdeg	1.00	-0.061	0.179	0.217	0.153	0.284	-0.241
DM		1.00	-0.689	-0.363	-0.341	-0.454	0.699
cNDF			1.00	0.725	0.143	0.619	-0.967
lignin				1.00	0.123	0.687	-0.772
CP					1.00	0.205	-0.315
Ash						1.00	-0.754
NSC							1.00

Table 6. Correlations between chemical composition for Haylage samples (n=144 observations). All nutrients given on a %DM basis.

	kdeg	DM	cNDF	lignin	CP	Ash	NSC
kdeg	1.00	-0.0001	-0.320	0.323	0.284	0.293	0.200
DM		1.00	-.0643	0.135	0.551	0.235	0.674
cNDF			1.00	-0.222	-0.861	-0.584	-0.892
lignin				1.00	0.089	0.113	0.108
CP					1.00	0.601	0.611
Ash						1.00	0.243
NSC							1.00

6. Treatment and Chemical Composition Effects on NDF Degradation: Analysis of Covariance

Following the determination that forage types had a significant effect on NDF degradation rates, analysis of covariance was used to determine if there were any relationships between the NDF degradation rate, Amaferm treatment, and the chemical composition determined at CVAS within a given forage type. Dry matter (DM), ash, corrected NDF (cNDF), lignin, NSC (non-structural carbohydrate) and crude protein (CP) were investigated as covariates. Corrected NDF was calculated as NDF – ADFICP – lignin and would represent the true carbohydrate portion of NDF. If the linear effects of the covariates were significant, quadratic and cubic effects were also tested.

For Corn Silage samples, Amaferm treatment significantly increased NDF degradation rates and there were also linear effects of DM, cNDF, and NSC content, but there was no interaction between these nutrient measurements and Amaferm treatment (data graphs in Appendix B). Given the high correlations between DM, cNDF and NSC in corn silage samples (Table 5), it is not surprising that all are significant. No quadratic or cubic effects of these nutrient measurements were apparent. Also, there were no apparent effects of ash, CP, or lignin content (data not shown).

For Haylage samples, Amaferm treatment significantly increased NDF degradation rates and there were also linear effects of DM, ash, lignin, and CP content, but there was no interaction between these nutrient measurements and Amaferm treatment (data graphs in Appendix B). No quadratic or cubic effects of these nutrient measurements were apparent. There were no apparent effects of cNDF or NSC content (data not shown).

While these linear effects of chemical composition are statistically significant, their contribution to the overall variation is quite small. In other words, they do not explain much of the variation seen in degradation rates between samples.

7. Recommendations for adjusting NDF degradation constants in CPM Dairy 3.0 and CNCPS 6.1, and NDF digestibility in NRC 2001.

In CPM Dairy 3.0 and CNCPS 6.1 the potentially degradable pool defined as B_0 in this report is defined as the CHO-B3 pool in the ration formulation software. These models assume first-order kinetics, the same as the approach in this analysis. Thus, the results from the equation fitting and statistical analysis are directly applicable to these nutrition models. When cows are fed Amaferm at the recommended levels, the recommendation is to increase the k_d for the CHO-B3 fraction by 10% for corn silages and 20% for haylages in CPM Dairy 3.0 and CNCPS 6.1. For example:

Corn Silage
CHO-B3 k_d = 3.6%/hr without Amaferm → k_d = 4.0% with Amaferm

Mixed grass/legume haylage
CHO-B3 k_d = 5.0%/hr without Amaferm → k_d = 6.0%/hr with Amaferm

In NRC 2001, NDF digestion is modified by changing NDF digestibility. This NDF digestibility represents both reticulo-rumen and hindgut fermentations. When the equations developed in the current analysis are used to predict NDF digestibility at 24 hours, Amaferm is found to increase NDF digestibility in corn silages by 7% and in haylages by 14% as compared to the Control treatment. It is assumed that Amaferm acts only in the reticulo-rumen, and is not active in the hind-gut. When cows are fed Amaferm, the recommendation is to increase NDF digestibility for forages by 7% for corn silages and 14% for haylages in ration formulation software based on NRC 2001. For example,

Corn Silage
NDF digestibility = 55% without Amaferm → NDF digestibility = 58.8% with Amaferm

Mixed grass/legume haylage
NDF digestibility = 45% without Amaferm → NDF digestibility = 51.3% with Amaferm

8. Predicting responses in dry matter intake (DMI) and milk yield in rations when feeding Amaferm

Increased NDF digestibility is expected to increase DMI and milk yields in lactating dairy cattle. From a published literature review, Oba and Allen (1999; JDS 82:589) predicted that for every 1% increase in NDF digestibility *in vitro* or *in situ*, cows increased DMI by 0.37 lb/day and 3.5% fat-corrected milk yield by 0.55 lb/day. Based on these predictions and the estimated increase in NDF digestibility at 24 hours from the current analysis, Amaferm feeding would be predicted to increase DMI by 1.0 – 2.3 lb/day and milk yield by 1.5 – 3.4 lb/day in corn silage/haylage based diets, depending on the proportion of corn silage to haylage, total NDF content, and the proportion of dietary NDF from forages. The consequences of improved NDF digestibility on milk yield in corn silage/haylage based rations can be modeled in the ration formulation software by adjusting the degradation rates (k_d) or NDF digestibility as described above in section 7 and the impact on ME/NE_l and MP allowable milk predicted (Table 7):

Table 7. Energy and protein allowable milk predicted for a 1500 lb. Holstein cow producing 75 lb/day milk with 3.8% milkfat and 3.0% true protein at 48.3 lb/day dry matter intake. Diets were based on 54% forage mixture of 50:50 corn silage:mixed haylage, and were not optimized to be least-cost rations.

	CNCPS 6.1		NRC 2001	
	- Amaferm	+ Amaferm	- Amaferm	+ Amaferm
ME/NE_l allowable milk (lb/d)	77.9	79.0	76.5	77.4
MP allowable milk (lb/d)	74.6	76.0	77.5	78.0

Note that neither CNCPS 6.1 or NRC 2001 are able to predict changes in DMI in response to improved NDF digestibility. In both systems, DMI is predicted as a function of nutrient requirements with stage of lactation adjustments.

The predicted responses in DMI and milk yield from Oba & Allen, 1999 may be conservative. More recent research where the proportion of alfalfa haylage and corn silage of differing NDF digestibilities was varied while total forage content and forage NDF were held constant showed larger responses in DMI and milk yield (Weiss et al., 2009 JDS 92:5595). For each 1% increase in observed total tract NDF digestibility, DMI increased 0.7 lb/day and milk yield increased 1.7 lb/day.

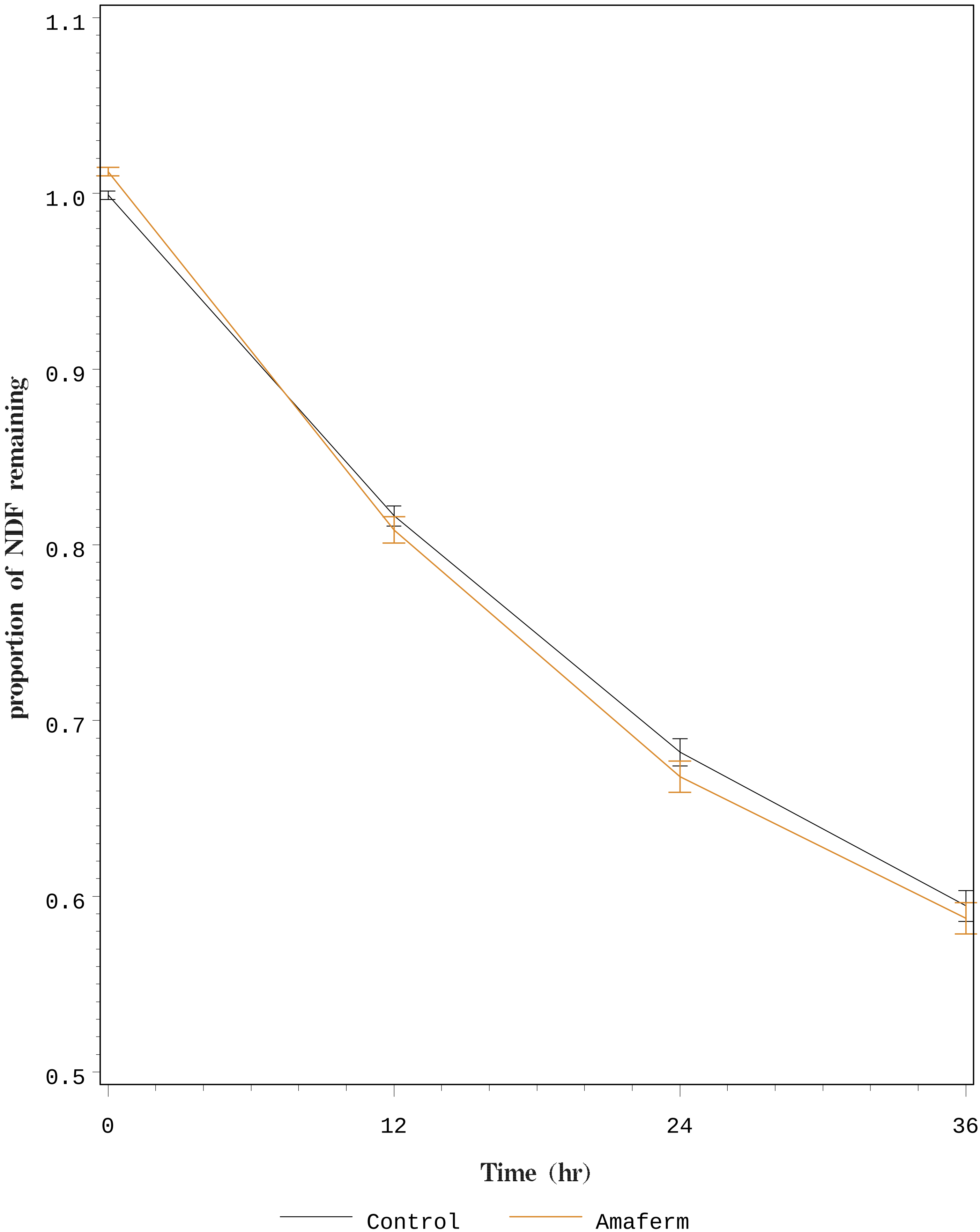
The expected increase in NDF digestibility with Amaferm feeding should increase DMI in early lactation cows (before peak milk yield and maximal DMI are attained) as the impact of rumen fill on DMI is lessened. As these cows are able to consume more DMI, they should respond with better peak yields. They may also lose less body condition as more of their energy requirements are met from dietary NDF digestion. In mid- to late-lactation cows that eat to meet their energy requirements, Amaferm feeding should allow the cows to meet their energy requirements by consuming less DM. This would be reflected in higher feed efficiencies (lb. milk/lb. feed).

Amaferm Effects on *in sacco* NDF degradation

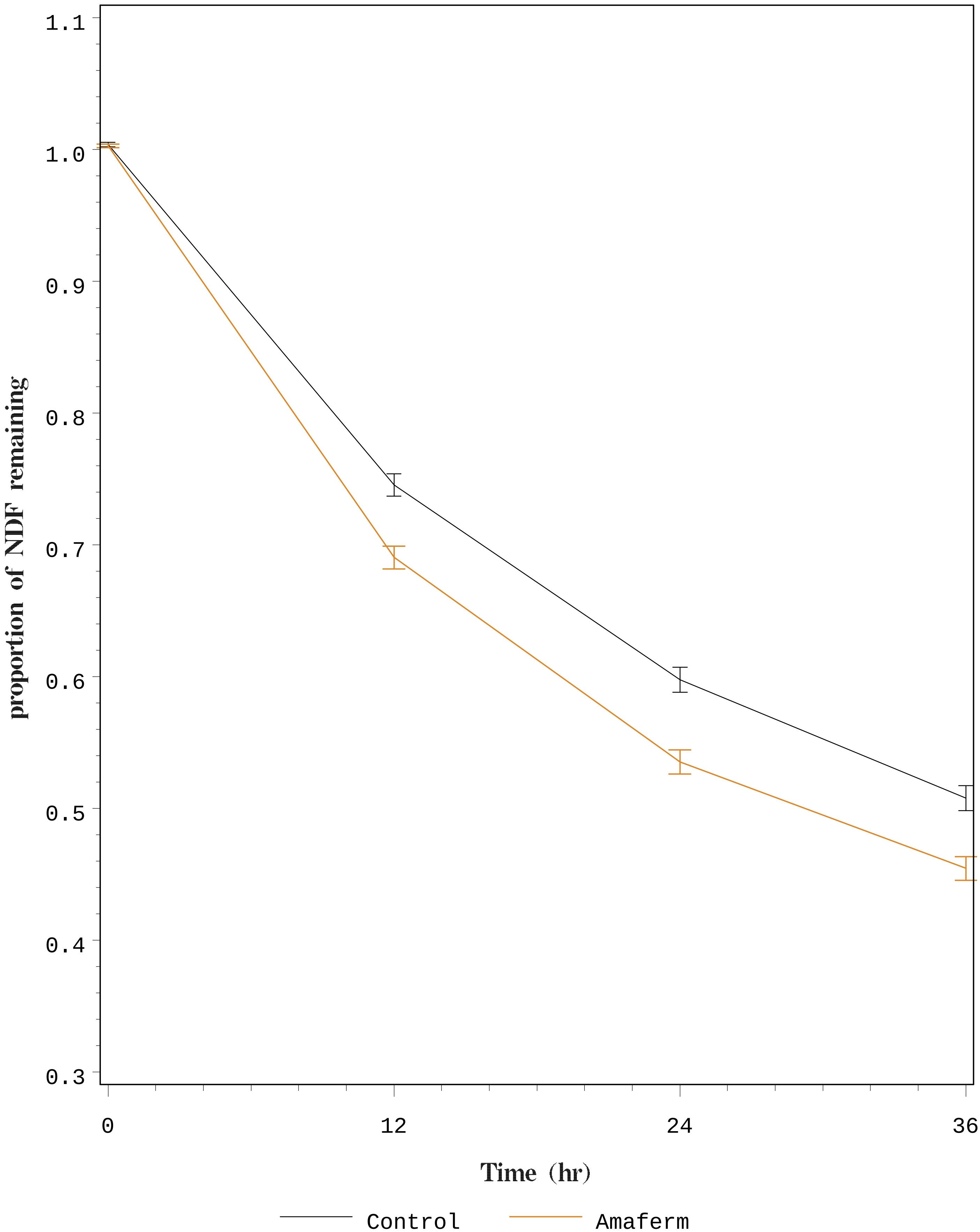
Appendix A

Graphs of NDF remaining
Comparison of first-order equations
By forage type and treatment

Amaferm treatment effects on NDF degradation



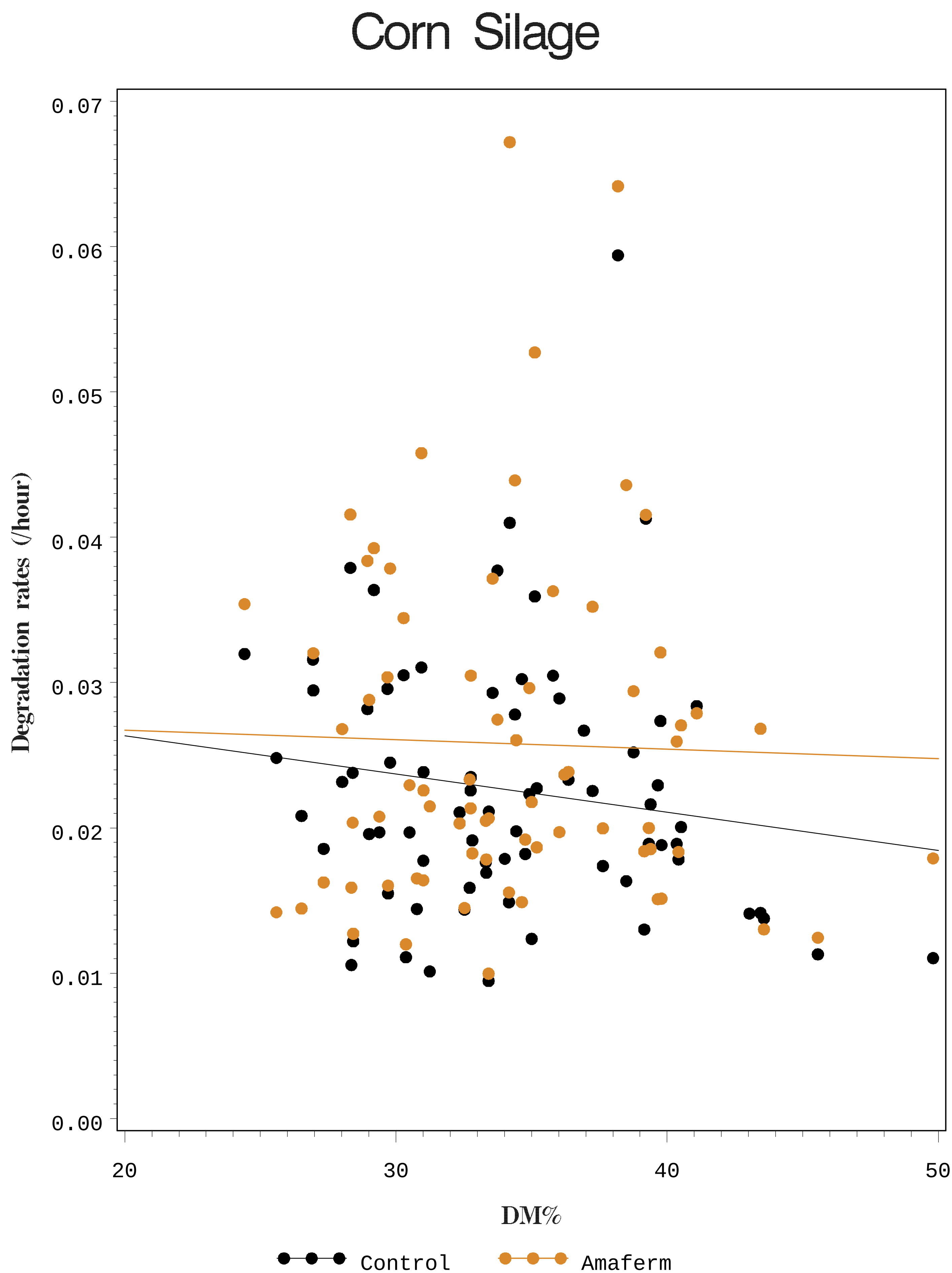
Amaferm treatment effects on NDF degradation



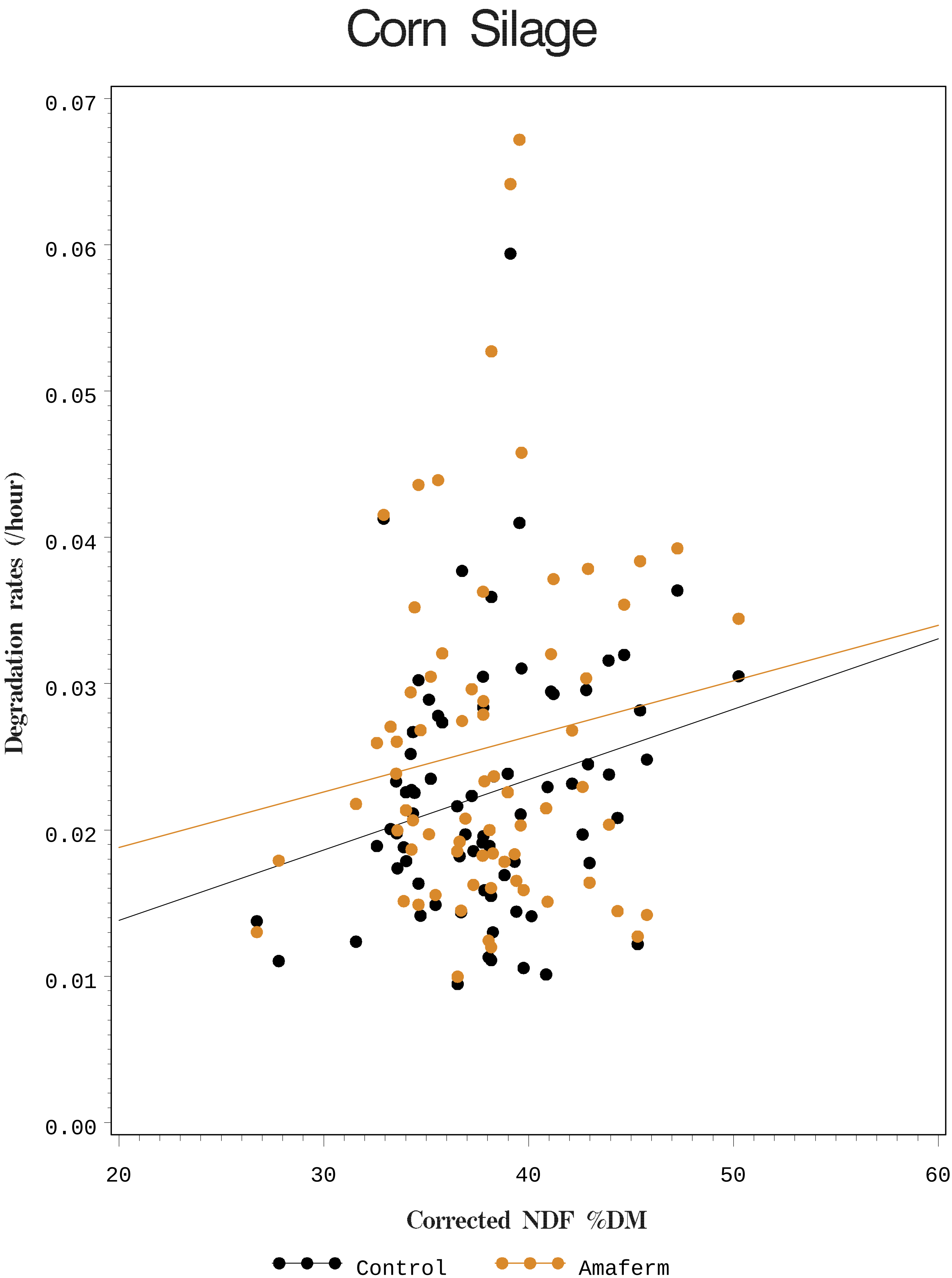
Amaferm Effects on *in sacco* NDF degradation

Appendix B

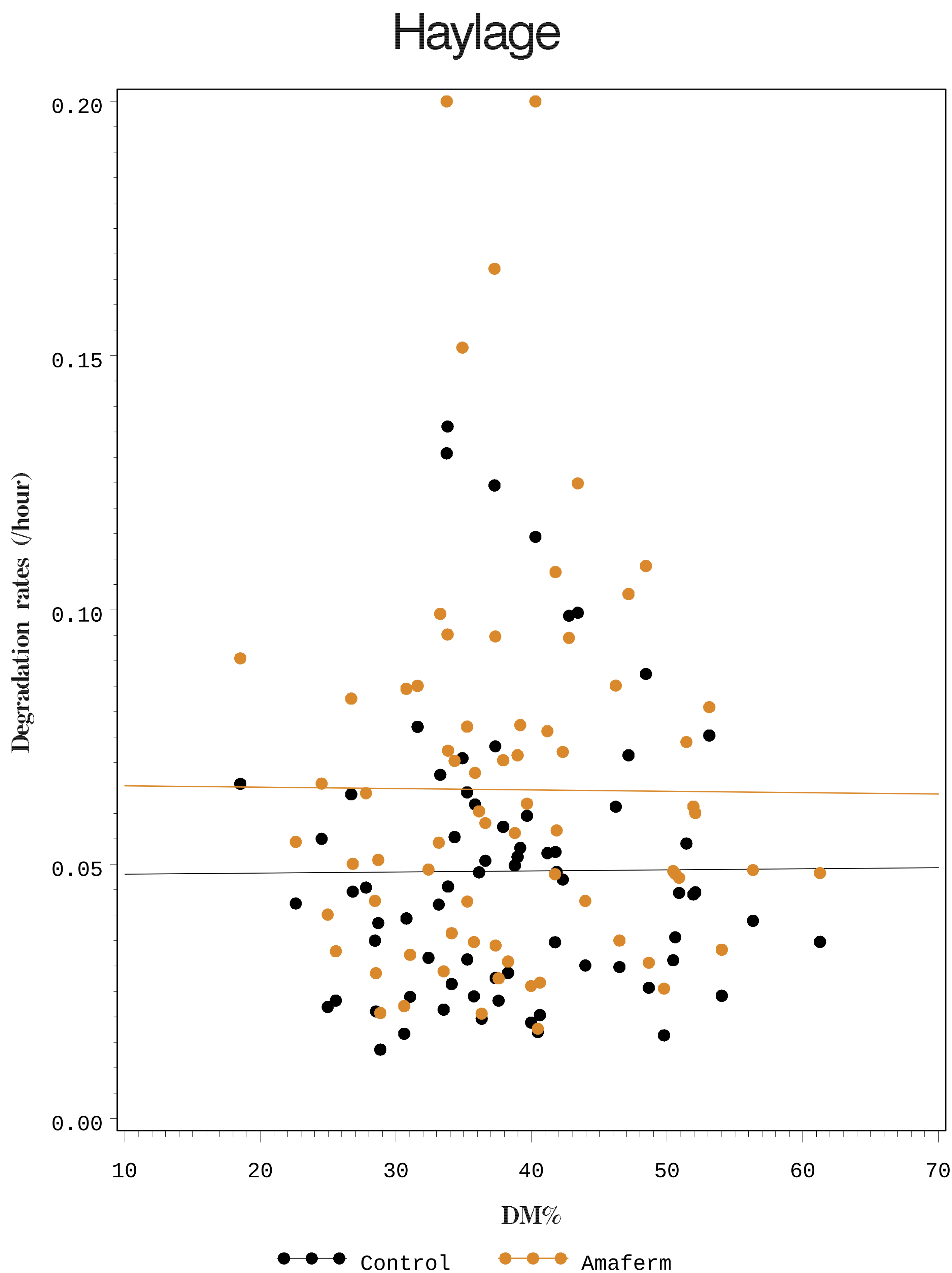
Graphs of Amaferm Effects on NDF Degradation Rates
as Affected by Chemical Composition

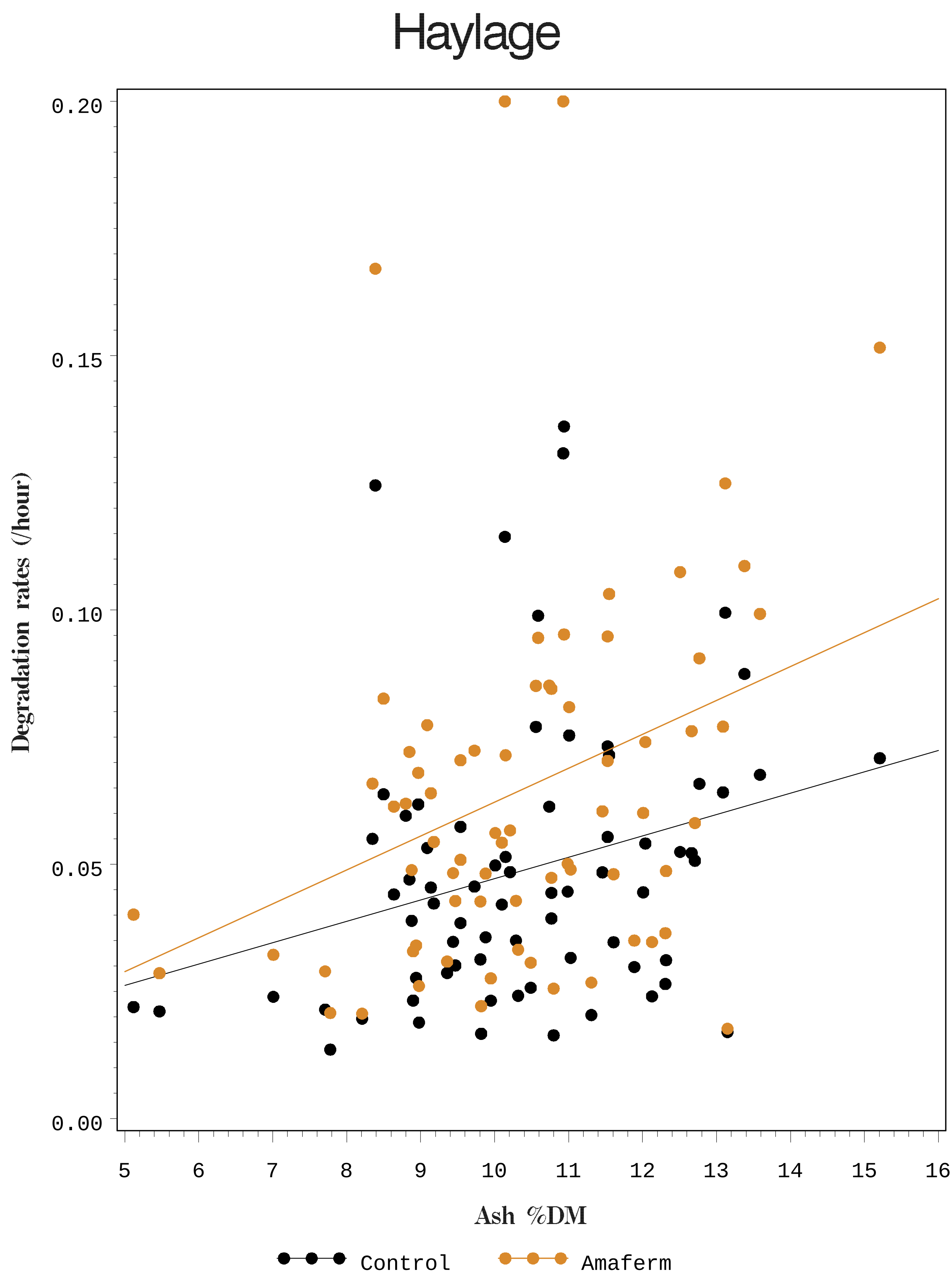


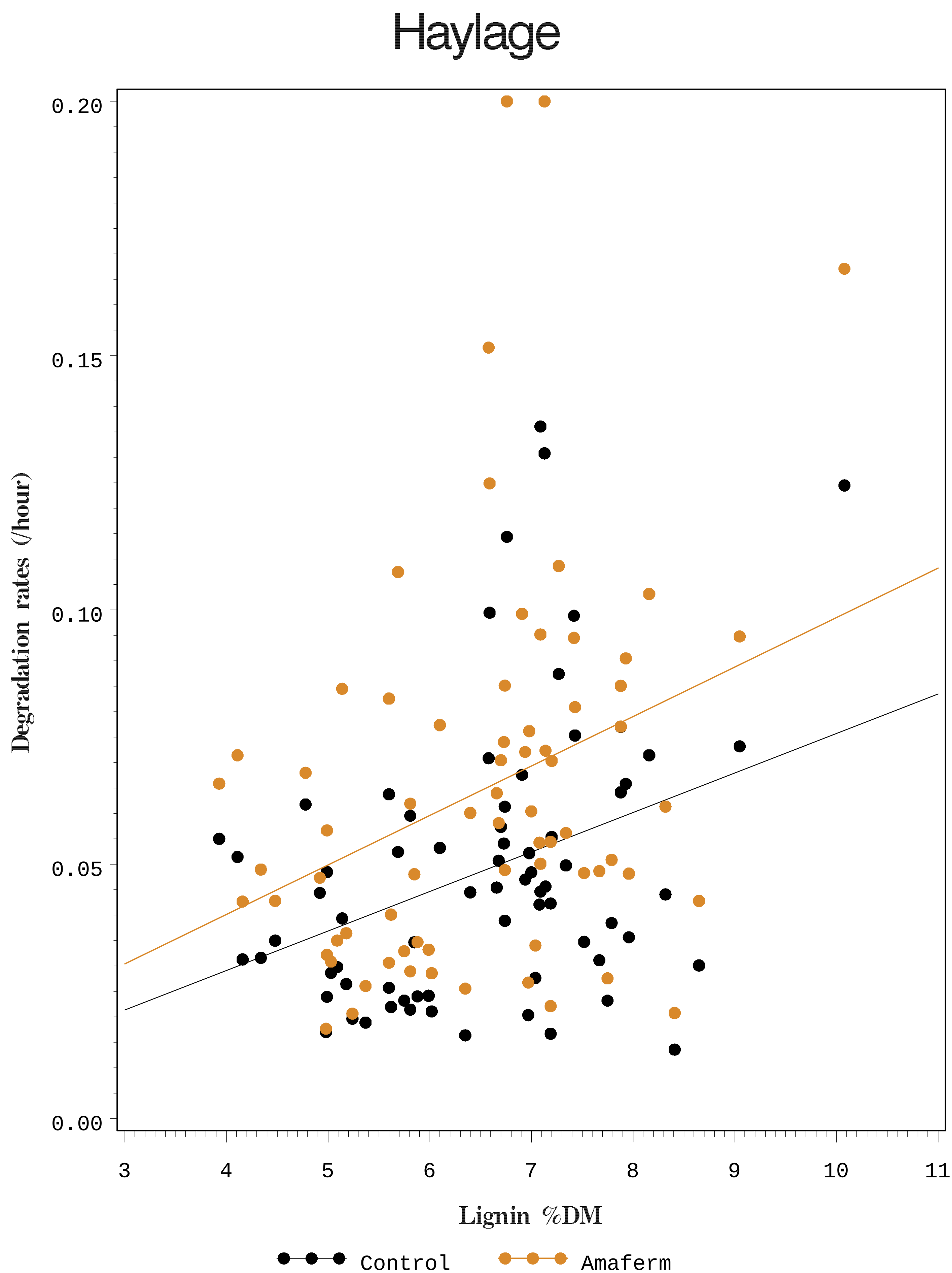
Corn Silage
Degradation rates (/hour)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
20
30
40
50
DM%
Control
Amaferm



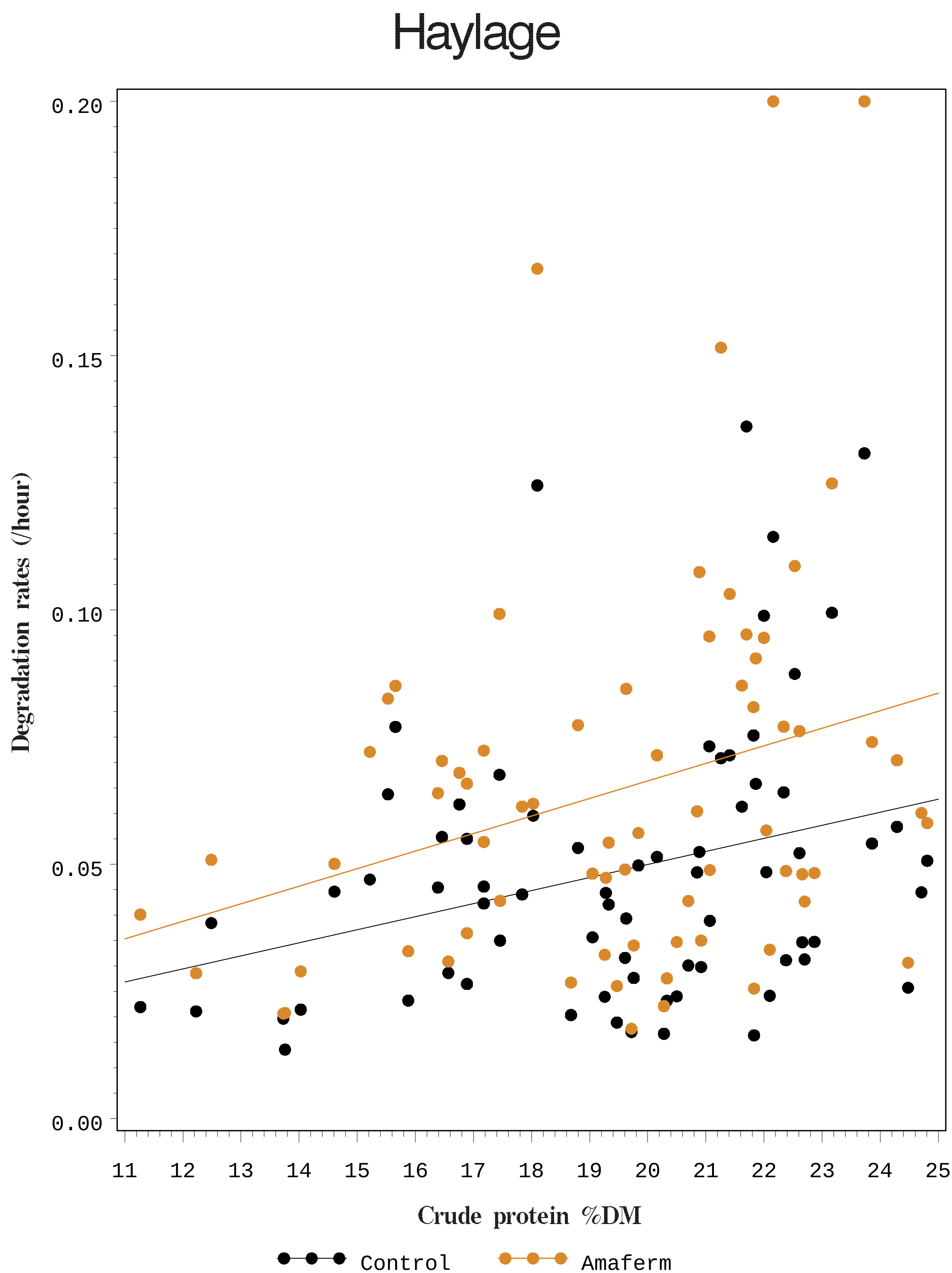
Corn Silage
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Degradation rates (/hour)
20
30
40
50
60
Corrected NDF %DM
Control
Amaferm


Haylage
Degradation rates (/hour)
0.00
0.05
0.10
0.15
0.20
10
20
30
40
50
60
70
DM%
Control
Amaferm


Haylage
Degradation rates (/hour)
0.20
0.15
0.10
0.05
0.00
5
6
7
8
9
10
11
12
13
14
15
16
Ash %DM
Control
Amaferm



Haylage
0.20
0.15
0.10
0.05
0.00
Degradation rates (/hour)
3
4
5
6
7
8
9
10
11
Lignin %DM
Control
Amaferm



Haylage
Degradation rates (/hour)
0.20
0.15
0.10
0.05
0.00
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
Crude protein %DM
Control
Amaferm