Influence of formic acid and dry matter on protein degradation in the tanniniferous legume sainfoin

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Abstract

Improved protein utilization due to protein tannin binding has been observed in sainfoin and other tannin containing legumes. Since it is well known that binding of protein to tannins and dissociation of the resulting complexes are pH dependant, effects of low pH after treatment with unusually high amounts of acidic silage additives and different dry down stages on fresh herbage prior to ensiling was investigated. Criteria for tannin binding effects were based on N fractionation in an *in vitro* ruminal digestion system. A mixture of sainfoin varieties were wilted to different dry matter (DM) levels and treated with 0, 4 and 8 ml formic acid per kg fresh matter prior to ensiling for 60 days. Nitrogen fractionation included measurement of total N, buffer soluble N, non-protein N, α-amino acid N and ammonia N. Protein degradation was measured by an *in vitro* ruminal protein digestion system. Results show beneficial effects of moderate and high acidification particularly for buffer soluble N at 180 and 400 g/kg DM ($p<0.0001$) while these effects level off at 500 and 600 g/kg DM ($P<0.852$). However, no negative effect related to impaired tannin protein binding in the silages or the *in vitro* protein degradation occurred.

Keywords: formic, pH, silage, inhibited, *in vitro*, additives

Abbreviations: AA-N, amino acid N; BSA, buffer soluble N; DM, dry matter; FA, formic acid; FD, fraction degraded protein N; FM, fresh matter; FUD, fraction undegraded protein NNPN, non protein N; RF, rumen fluid; TCA, trichloroacetic acid

1. Introduction

Forage conservation as hay or silage is accompanied by decreased nutritive value and loss of dry matter (DM) due to oxidation and fermentation of the forage. These losses occur during harvesting, ensiling and feed-out due to respiration, rain damage, mechanical losses and silage effluents. (McDonald et al., 1991). This is an economical loss to farmers and an environmental problem due to increased carbon emissions and pollution of ground water with nitrates (Woolford et al., 1983; Jones and Jones, 1995). To reduce these losses, a variety of ensiling techniques with various types of silage containments and silage

48 additives, have been implemented (Buxton et al., 2003). Wilting and application of acids are two
49 conservation methods widely used in ensiling. Wilting decreases plant protease activity and clostridial
50 growth, lowers the amount of silage effluent and increases the concentration of sugars in the crops thereby
51 improving silage nutritional quality (Gross and Grub, 1968; McDonald et al., 1991; Henderson, 1993).
52 This is particularly important when ensiling legumes, which generally have low sugar and high protein
53 concentrations. Acidifiers, such as formic acid (FA), quickly lower pH at the beginning of ensiling.
54 Depending on the crop ensiled and on the amount of FA used, conditions for bacterial activity and growth
55 are altered (McDonald and Henderson, 1974; Randby, 2000). Trends to lower fermentation acid
56 concentrations, decreased lipolysis and higher water soluble carbohydrate concentrations have been
57 reported from FA treatment of legumes (Henderson et al., 1972; Adesogan and Salawu, 2004). Studies by
58 Pursiainen and Tuori (2008) and Lorenz (2010) showed decreased protein degradation in acidified legume
59 silages.
60 Drawbacks of using FA have been observed in studies where FA was applied to low DM grass
61 silage, and include increased amounts of effluent (Henderson and McDonald, 1971) and higher DM losses
62 due to yeast growth (Henderson et al., 1972). Very low concentrations of FA may also promote growth of
63 clostridia (Beck, 1968). Forages which are difficult to ensile, such as cocksfoot or legumes, benefit from
64 the addition of up to 6 ml FA per kg fresh matter (FM), as shown by Lancaster and Brunswick (1977).
65 This study also showed that higher levels of FA addition, up to 8 ml FA/kg FM increased DM intake, daily
66 weight gain and organic matter digestibility in lambs, although this was not considered to be economically
67 feasible.
68 Effects of tannins in legumes on protein degradation, resulting from tannin-protein binding and
69 precipitation, has been described in many publications (Karnezos et al., 1994; Koivisto and Lane, 2001)
70 and were also reviewed by Mueller-Harvey (2006) and Rochfort et al. (2008). Tannin-protein binding may
71 increase the nutritive value of the forage for ruminants by an increase in rumen escape proteins which can

72 subsequently be released at the low abomasal pH (Oh and Hoff, 1987) for absorption in the small intestine
73 (Thomson et al., 1970; Jones and Mangan, 1977).

74 Tannin-protein complexes in silage have been exposed to a low pH before entering the rumen.
75 Hence, if pH is too low, the positive protein sparing effect could be impaired if the complexes have
76 dissociated. However, numerous reports have shown reduced ruminal protein degradation in tannin-rich
77 legume silages such as sainfoin (*Onobrychis viciifolia*; Wang et al. (2007) and Lorenz et al. (2010)),
78 birdsfoot trefoil (*Lotus corniculatus;* Albrecht and Muck (1991)) and tannin supplemented perennial
79 ryegrass (*Lolium perenne*) silage (Salawu et al., 1999).

80 In light of these known dissociative effects on the protein-tannin complex associated with low pH,
81 coupled with current recommendations for increased FA application to improve silage storage quality,
82 investigations into FA application limits are appropriate. Therefore, our objectives were to assess effects
83 of acidification levels, in combination with different DM contents, on the protein protecting effect of
84 tannins in sainfoin during silage fermentation and digestion on the basis of N-fractionation and *in vitro*
85 ruminal degradation of sainfoin silages.

86

87 **2. Material and methods**

88 *2.1. Plant material*

89 Thirteen sainfoin varieties, grown at the National Institute of Agricultural Botany, Cambridge,
90 Great Britain (NIAB) were harvested in August 2008, frozen and transported to the Kungsängen Research
91 Center (Uppsala, Sweden). The varieties were: *Onobrychis viciifolia SCOP* var. CPI63761, var. CPI63815,
92 var. CPI63826, var. Sparceto, var. CPI63825, var. CPI63752, var. CPI63808, var. Dnepropetrovsk, var.
93 Fizes, var. Palio, var. AR-111 and *Onobrychis antasiatica* var. Sisiani Local and var. Akhurian-107.

94 *2.2. Ensiling*

95 All samples were chopped (~3 cm), pooled and mixed together thoroughly to make one large
96 forage mass. The mixture was ground in a meat grinder (Bankeryd A90B, Bankeryd, Sweden) and divided

97 into four batches which were either not dried (180 g/kg DM) or dried in a force draft oven at 35°C to a
98 DM content of 400 g/kg, 500 g/kg or 600 g/kg. Each batch was further divided into three portions and put
99 into separate plastic bags. Formic acid was added at levels of either 0, 4 (i. e. moderate acidification) or 8
100 ml (i. e. high acidification) FA/kg FM by spraying acid on the chopped plant material followed by
101 thoroughly shaking the bags. The plant material was packed in glass mini-silos (20 * 3 (inner diameter)
102 cm) in duplicate. After filling the silos with 80 to 90 g FM, leaving a 1 cm free headspace, the tubes were
103 closed with a rubber stopper and a water lock. Silos were incubated in a dark 20°C room for 60 days, after
104 which the content of each silo was divided into two batches, one of which was freeze dried and ground on
105 a cutter mill (Brabender, Type 880803, Brabender OHG, Duisburg, Germany) to pass a 1 mm screen
106 awaiting further analysis and the other batch was frozen for analysis of α-amino acid N (AA-N) and
107 ammonia NH_3) N.
108 *2.3. Analytical methods*
109 Representative 5 g subsamples of each silo were collected immediately after application of FA and
110 after opening the silos after 60 d and mixed with 10 ml of de-ionized water for pH measurement.
111 Dry matter of fresh samples was determined by drying at 105°C to constant weight in a forced
112 draught oven and for ensiled material by freeze drying. Dry matter contents were corrected for remaining
113 water and volatile losses by multiplying by a correction factor of 0.94. Nitrogen analyses were in duplicate
114 on the freeze dried material by a Kjeldahl procedure using a Kjeltec Analyzer 2400 and a 2020 Digestor
115 (Foss, Hillrød, Denmark) with Cu as a catalyst. Buffer soluble N (BSN) was determined by extracting
116 freeze dried samples with a borate phosphate buffer, pH 6.75, at 39ºC for 1 h according to a modified
117 method by Licitra et al. (1996): 1.5 g of the freeze dried plant material was weighed into a 50 ml tube
118 (Sarstedt, Nümbrecht, Germany) and mixed with 50 ml of borate phosphate buffer. Tubes were sealed,
119 shaken and incubated for 1 h in a 39ºC water bath with an additional thorough shaking every 15 min.
120 Thereafter, tubes were centrifuged at 3000 x *g* for 10 min at 20°C on a swing-out rotor centrifuge (G4.11,
121 Jouan, Saint Herbain, France). Twenty ml of the supernatant was transferred to a Kjeldahl tube and

122 analyzed for BSN. To avoid floating particles, a polyester cloth with 20-µm openings was wrapped around
123 the tip of the pipette and the liquid was pipetted slowly. A stepwise increase of the temperature of the
124 Kjeltec digestion block was needed when analyzing the aqueous samples to prevent extensive foaming
125 during digestion. Initially, temperature was slowly increased to evaporate excess water. In the last step, Cu
126 containing K_2SO_4 tablets were added and the standard procedure described above for solid samples was
127 completed. For analysis of non-protein N (NPN), another 15 ml of the BSN extract was transferred to a 30
128 ml polypropylene centrifuge tube and 1.5 ml of trichloroacetic acid (TCA; 200 g/L) was added and
129 incubated for 1 h in ice water to precipitate polypeptides and proteins. After incubation, the sample was
130 centrifuged at 27000 x *g* for 15 min at 20°C in a Suprafuge 22 centrifuge with fixed-angle rotor (Heraeus
131 Sepatech GmbH, Osterode, Germany) and the aqueous supernatant was analyzed for NPN according to the
132 procedure for aqueous samples described above.

133 Aqueous extracts from fresh-frozen samples were analyzed for NH_3-N and AA-N using phenol-
134 hypochlorite and ninhydrin (Broderick and Kang, 1980), respectively, on a Technicon Auto Analyser
135 (Dublin, Ireland). Leucine was used as a standard for amino acids and ammonium sulphate (Merck,
136 Darmstadt, Germany) for NH_3-N. Both were expressed as a proportion of BSN.

137 Tannins were measured by a HCl/butanol method: Triplicates of 50 mg freeze dried sample were
138 weighed into 30 ml polypropylene test tube. Five ml of HCl/butanol reagent (HCl:butanol=1:20) were
139 added to the samples and they were incubated in a water bath set at 100°C for 60 min. Tubes were cooled
140 to 20°C and absorbance read on a spectrophotometer (Pharmacia LKB, Uppsala, Sweden) at 550 nm.
141 Tannin values were reported on the basis of absorbance units at 550 nm per 50 mg DM ($AU_{(550)}$).

142 *2.4. Inhibited in vitro (IIV) protein degradation*

143 Protein degradation was measured by a modified IIV method from Broderick (1987). An
144 equivalent of 4 mg N of freeze dried, not ensiled material or freeze dried silage was weighed in a 30 ml
145 polypropylene centrifuge tube in duplicate. The material was wetted for 60 min at 20°C in 10 ml
146 McDougal´s buffer. Rumen fluid was collected from two fistulated cows in the morning after feeding and

147 strained through four layers of cheese cloth. Cows were fed daily 3.4 kg DM grass hay with a crude
148 protein content (CP) of 155 g/kg DM and 1.6 kg concentrates (CP: 200 g/kg DM). Strained rumen fluid
149 was pre-incubated with 8 g/L maltose, 4 g/L soluble starch, 4 g/L sucrose, 4 g/L xylose, 4 g/L pectin and 5
150 g/L $NaHCO_3$ in a 39°C water bath for 3 h during constant stirring. After pre-incubation, pH was adjusted
151 to 6.7 with 3 N NaOH. Bacterial growth was inhibited by adding 41.67 ml hydrazine sulphate solution (72
152 mM/L), 41.67 ml chloramphenicol solution (10 mM/L) and 0.234 g mercaptoethanol per liter pre-
153 incubated rumen fluid. After additional 30 min, the sample fermentation was initiated by adding 20 ml of
154 rumen fluid to the sample tubes. Final concentrations of hydrazine sulphate and chloramphenicol were 30
155 and 1.3 µM/L in each incubation vessel. Shaking and a constant temperature of 39°C were maintained
156 under anaerobic conditions. Samples of 1 ml for NH_3-N and AA-N were collected every hour for 4 h and
157 mixed with 0.1 ml TCA solution (550 g/L) to stop fermentation.

158 The fraction of undegraded protein N at the respective time point *i* ($FUD_{(i)}$) was calculated as:

159 $FUD_{(i)} = 1 - FD_{(i)}$

160 $FD_{(i)} = (NH_3\text{-}N_{(i)} + (AA\text{-}N_{(i)}/0.7003))/N_{(sample)}$

161 0.7003 is the average proportion of α-amino N in total N determined for a number of protein
162 sources, which derives from an average of 50 µmol total amino acids in 1 mg total N for most feed
163 proteins after acid hydrolysis, and

164 $N_{(sample)}$ is the amount of N in mg weighed into the incubation flask, and

165 $NH_3\text{-}N_{(i)}$ is in mg present in the sample, and

166 $AA\text{-}N_{(i)}$ in mg present in the sample corrected for the blank at time=0.

167 *2. 5. Biometric analysis*

168 Silage data was analyzed using Proc Mixed of SAS (SAS, 2003). The experiment had a factorial
169 design with four DM levels (i.e. 180, 400, 500, 600 g/kg) and three FA levels (i. e. 0, 4, 8 ml FA/kg FM).
170 Dependent variables were N, BSN, NPN, AA-N, NH_3-N and pH. Analysis of variance with means
171 separations by pairwise comparisons using Tukey´s test (not shown in table form) was completed.

Proc Mixed in SAS was used for repeated measurements with unstructured covariance matrix used to test for effect of DM and FA in the IIV digestion. Class variables were time, DM and FA. Response variable was FUD at respective time points. According to the technique of Dhanoa (1988), the MLP program (Ross, 1987) was used to fit all curves of FUD *versus* time to the equation:

FUD=a+b*Exp(c*t)

where: ‘a’ = undegradable protein fraction; ‘b’ = potentially degradable protein fraction, ‘c’ = rate (/h) and t = time (h).

One curve out of 12 was excluded due to poor fit. To test for differences in parameter values, the approximate analysis of variance provided by the MLP program (i. e. parallel curve analysis option; CPCA = 2) was applied. Four residual sums of squares were estimated being i) within individual curves, ii) within curves fitted using a common non-linear parameters (‘c’), iii) within curves fitted using a common ‘c’ and ‘b’-value and iv) for the combined data set. Tests for curve displacement (‘a’), common linear (‘b’) and common non-linear (‘c’) parameters among the curves used an F-test and the mean residual sums of squares were calculated according to Ross (1987).

3. Results

Good nutritional silage quality was anticipated since there were no visible moulds or yeast growth and NH_3-N ranged from 4.67 to 12.14 g/kg N. A photometric value of 1.244 $AU_{(550)}$ confirmed the presence of tannins in sainfoin.

3.1. pH

Formic acid addition resulted in pH values from 4.05 to 5.48 prior to ensiling and values from 3.67 to 4.76 for the 60-d silage. Silage pH was increased by higher DM ($P<0.001$) and increased FA level lowered pH ($P<0.001$; Table 1). Lowest silage pH was at the highest acidification level and lowest DM content, whereas the highest pH was measured in the non-acidified, high DM silages.

196 The interaction between DM stage and FA addition (P<0.001) indicates that increasing FA addition
197 decreased the effect of DM.

198 *3.2. N and BSN*

199 Data on the distribution of N in different fractions are in Table 1. Total N content was lower in
200 acidified compared to non-acidified silages (P<0.008). The increase from moderate to high FA addition
201 did not further decrease total N content (P=0.916). The only reduction in total N content as an effect of
202 DM content was seen when low DM (180 g/kg) silage was compared to high DM (600 g/kg) silage
203 (P<0.045).

204 The proportion of BSN to total N was influenced by both FA (P<0.01) and DM level (P<0001).
205 The response of FA addition was dependant on DM, as effects of FA tended to be lower at high DM stages
206 (Interaction FA*DM P=0.001). Wilting from 180 g/kg DM to 400 g/kg DM in non-acidified silage had the
207 highest impact on BSN (-33%) followed by FA addition to 180 g/kg DM silages (-20%). Overall,
208 moderate and high FA addition decreased BSN (P<0.01) in silages whereas there was no difference
209 between moderate and high FA addition (P<0.224). Lowest levels of BSN were in samples dried to 500
210 and 600 g/kg DM (P<0.002; Figure 1)

211 *3.3. NPN, AA-N and NH_3-N*

212 The NPN proportion, expressed relative to total N was altered by DM (P<0.001) and FA addition
213 (P<0.015; Table 1). Increasing FA from medium to high had no effect on NPN, AA-N and NH_3-N.Wilting
214 was effective for AA-N and NH_3-N up to 500 g/kg and 400 g/kg DM, respectively.

215 *3.4. In vitro protein degradation*

216 Dry matter content (P<0.001), FA addition (P<0.001) and their interaction (FD*DM, P<0.001)
217 affected protein degradation expressed as FUD (Table 2).

218 The highest protein degradation was for silages with low DM and no FA addition. Overall, FUD
219 was high for all treatments and ranged from 0.56 to 0.63 (Figure 2). A pairwise comparison of treatments
220 show differences in FUD between silages at all DM (P<0.001) except for silages of 400 and 500 g/kg DM.

221 Parameter estimates from fitting the degradation data are also in Table 2. Rates ('c') did not differ among
222 treatments in spite of a wide range in rate from 0.12 to 1.2 /h and neither did the potentially degradable
223 fractions ('b') but there was curve displacement ($P<0.001$), signifying differences in amounts of
224 undegradable protein ('a'). The curve fitted to the combined data sets gave estimates for 'a', 'b' and 'c' of
225 0.59, 0.21 and 0.40 /h, respectively (not shown in table).
226
227 **4. Discussion**
228 *4.1. Effect of treatments on pH*
229 Wilting and FA addition both altered pH in the silage as expected (Table 1).
230 Addition of 4 or 8 ml FA/kg FM represents a moderate and a high level of acid application compared to
231 other studies on forages using 2.5 to 4.5 ml FA/kg FM (Adesogan and Salawu, (2004); Lorenzo and
232 O'Kiely, (2008); Mann and McDonald, (1976). Surprisingly, silage pH was not decreased in our study by
233 addition of 4 ml FA/kg FM on the low-DM forage, probably as a result of compensatory inhibition of
234 lactic acid fermentation as previously suggested (Henderson and McDonald, 1971; McDonald and
235 Henderson, 1974). This explains the strong interaction between DM and FA level, and the influence of
236 DM on the efficacy of FA to decrease pH level.
237 *4.2. Effect of treatments on N fractions*
238 A treatment effect of DM and FA addition on all N-fractions occurred, in accordance with other
239 studies (Valentine and Brown, 1973; Charmley, 2001). Higher DM resulted in higher total N probably due
240 to loss of carbohydrates from plant respiration. The change in total N by FA addition cannot be explained,
241 but changing total N values have been observed in studies by Salawu (2001), Adesogan and Salawu (2004)
242 or Lorenzo and O'Kiely (2008).
243 The most pronounced effect of FA addition and DM was on the soluble N fraction, which
244 decreased with increasing FA addition and DM up to 500 g/kg. No effects of FA addition on BSN in silage
245 with 500 or 600 g/kg DM occurred. The reducing effect of FA on NH_3-N has been previously described,

246 reviewed by McDonald et al. (1991), and was also observed in our study with a 50% NH_3-N decrease in
247 comparison with non-acidified silages.

248 That AA-N continued to decrease with higher DM content from 400 to 500 g/kg, while NH_3-N did
249 not decrease further, indicates that NH_3-N was not a good indicator of inhibition of protein degradation, as
250 suggested by Davis (1998). Ammonia N would overestimate protein degradation whereas AA-N seemed
251 to be more indicative of protein degradation. A previous study (Hristov and Sandev, 1998)) showed that
252 AA-N seems to be a good indicator of protein degradation.

253 *4.3. In vitro silage protein degradation*

254 Results from the IIV incubations resulted in an estimated degradation rate of 0.4/h for the
255 combined data sets and an undegradable fraction of 0.6. This may have been the result of the method with
256 a slowly dying microflora and a short incubation time. However, it seems clear that the ensiling treatments
257 had little effect on protein degradation rate. Protein degradation rates in tanniniferous plants such as
258 lespedeza or sainfoin, calculated by linear regression after ^{e}log transformation, have been found to be very
259 low compared to non-tannin containing plants, ranging from less than 0.01 to 0.06/h in un-ensiled material
260 (Broderick and Albrecht, 1997). As rate estimates may differ, depending on calculation method, we also
261 calculated them according to Broderick and Albrecht (1997). This resulted in degradation rates from 0.017
262 to 0.056/h (not shown in tables). These similarly low rates might be attributed to tannin binding and to
263 inhibitory tannin effects on the proteolytic bacteria and their enzymes (Jones et al., 1994). However, this
264 method estimates rate, assuming complete degradation of the protein by fermentation, something which
265 was not evident from curve analysis.

266 Acidification and DM had an impact on FUD (i. e. DM * FA effect) as shown earlier in
267 degradation studies by McDonald et al. (1991). This caused curves displacement as curves shifted upwards
268 in the low DM acidified silages relative to the non-acidified silages, which means that the protein fraction
269 'a' (i. e. undegradable protein fraction) increased (Fig. 2 a and b). However, no change in rate of
270 degradation or the potentially degradable fraction occurred (Table 2).

Addition of FA resulted in lower FUD values compared to non-acidified silages, with no signs of possible dissociation of tannin bound proteins, either during ensiling or *in vitro* degradation**.**

5. Conclusions

Effects of formic acid on N fractions were more pronounced at low than at higher dry down stages. The hypothesis that a dissociative effect of tannin protein binding and subsequent loss of dietary protein due to increased proteolysis occurs, could not be validated. A pH value as low as 3.67, which could result from use of high application levels of formic acid, is therefore unlikely to jeopardize the beneficial effects of sainfoin tannins either by increasing protein degradation in the silage or in the rumen.

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References

Adesogan, A.T., Salawu, M.B., 2004. Effect of applying formic acid, heterolactic bacteria or homolactic and heterolactic bacteria on the fermentation of bi-crops of peas and wheat. Journal of the Science of Food and Agriculture 84, 983-992.

Albrecht, K.A., Muck, R.E., 1991. Proteolysis in Ensiled Forage Legumes That Vary in Tannin Concentration. Crop Sci. 31, 464-469.

Beck, T., 1968. Die mikrobiologische Prüfung von Silierhilfsmitteln im Wachstumversuch - The microbiological examination of ensiling agents. Das betriebseigene Futter 14, 177-193.

Broderick, G.A., 1987. Determination of protein degradation rates using a rumen in vitro system containing inhibitors of microbial nitrogen metabolism. Brit. J. Nutr. 58, 463-475.

Broderick, G.A., 2009. Personal communication.

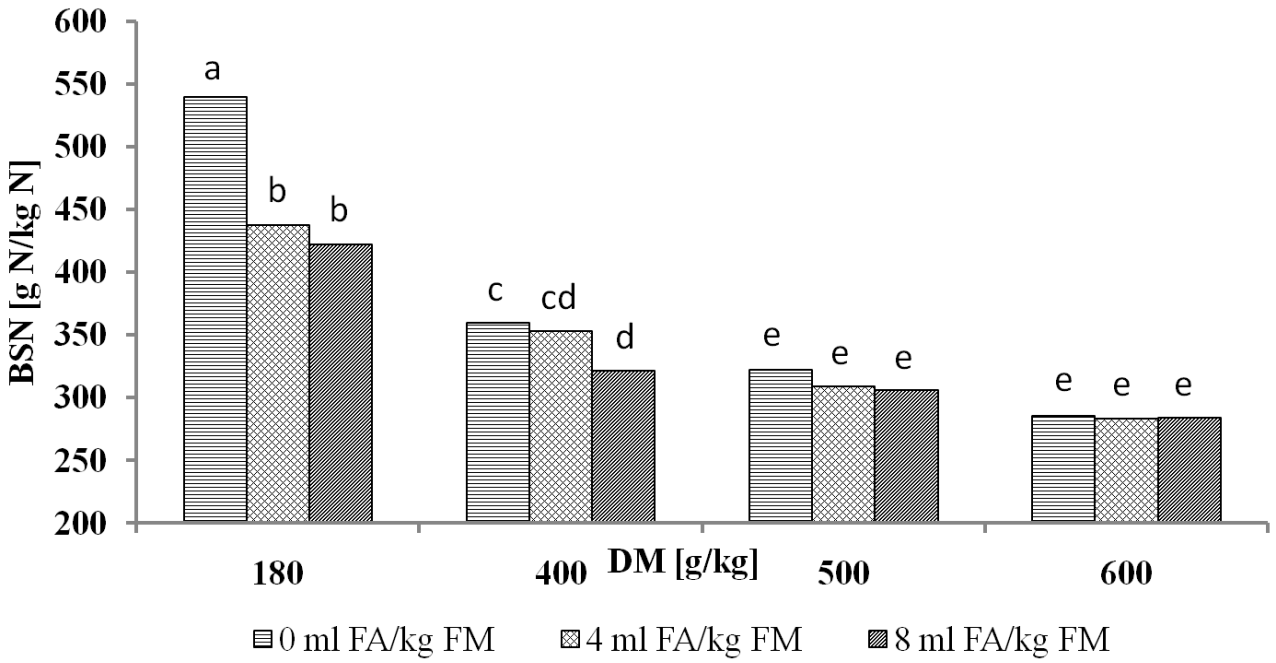
Broderick, G.A., Albrecht, K.A., 1997. Ruminal in vitro degradation of protein in tannin-free and tannin-containing forage legume species. Crop Sci. 37, 1884-1891.

Broderick, G.A., Kang, J.H., 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63, 64-75.
Buxton, D.R., Muck, R.E., Harrison, J.H., 2003. Silage Science and Technology. American Society of Agronomy, Inc., Crop Science Society of America, Inc., Soil Science Society of America, Inc., Madison, Wisconsin, USA.
Charmley, E., 2001. Towards improved silage quality - A review. Can. J. Anim. Sci. 81, 157-168.
Davies, D.R., Merry, R.J., Williams, A.P., Bakewell, E.L., Leemans, D.K., Tweed, J.K.S., 1998. Proteolysis During Ensilage of Forages Varying in Soluble Sugar Content. J. Dairy Sci. 81, 444-453.
Dhanoa, M.S., 1988. On the Analysis of Dacron Bag Data for Low Degradability Feeds. Grass Forage Sci. 43, 441-444.
Gross, F., Grub, G.A., 1968. Losses of dry matter in ensiling feedstuffs with a high protein content [Über die Trockensubstanzverluste beim Silieren eiweissreicher Futtermittel]. Das betriebseigene Futter 14, 194-205.
Henderson, A.R., McDonald, P., 1971. Effect of formic acid on the fermentation of grass of low dry matter content. J. Sci. Food Agric. 22, 157-163.
Henderson, A.R., McDonald, P., Woolford, M.K., 1972. Chemical changes and losses during the ensilage of wilted grass treated with formic acid. J. Sci. Food Agric. 23, 1079-1087.
Henderson, N., 1993. Silage additives. Anim. Feed Sci. Technol. 45, 35-56.
Hristov, A.N., Sandev, S.G., 1998. Proteolysis and rumen degradability of protein in alfalfa preserved as silage, wilted silage or hay. Anim. Feed Sci. Technol. 72, 175-181.
Jones, D.I.H., Jones, R., 1995. The Effect of Crop Characteristics and Ensiling Methodology on Grass Silage Effluent Production. J. Agr. Eng. Res. 60, 73-81.
Jones, G.A., McAllister, T.A., Muir, A.D., Cheng, K.J., 1994. Effects of Sainfoin (Onobrychis viciifolia Scop.) Condensed Tannins on Growth and Proteolysis by Four Strains of Ruminal Bacteria. Appl. Environ. Microbiol. 60, 1374-1378.
Jones, W.T., Mangan, J.L., 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. J. Sci. Food Agric. 28, 126-136.
Karnezos, T.P., Matches, A.G., Brown, C.P., 1994. Spring Lamb Production on Alfalfa, Sainfoin, and Wheatgrass Pastures. Agron. J. 86, 497-502.
Koivisto, J.M., Lane, J.P.F., 2001. Sainfoin - Worth another look., Food and Agriculture Organisation of the United Nations. www.fao.org/ag/AGP/AGPC/doc/Gbase/AddInfo/sainfoin.pdf.
Lancaster, R.J., Brunswick, L.F.C., 1977. Optimum rate of application of formic acid during ensiling of unwilted lucerne. N. Z. J. Agric. Res. 20, 151-156.
Licitra, G., Hernandez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347-358.
Lorenz, M.M., Eriksson, T., Uden, P., 2010. Effect of wilting, silage additive, PEG treatment and tannin content on the distribution of N between different fractions after ensiling of three different sainfoin (Onobrychis viciifolia) varieties. Grass Forage Sci. 65, 175-184.
Lorenzo, B.F., O'Kiely, P., 2008. Alternatives to formic acid as a grass silage additive under two contrasting ensilability conditions. Irish J. Agr. Food Res. 47, 135-149.
Mann, E.M., McDonald, P., 1976. Effect of Formalin and lower volatile Fatty-acids on Silage Fermentation. J. Sci. Food Agric. 27, 612-616.
McDonald, P., Henderson, A.R., 1974. The use of fatty acids as grass silage additives. J. Sci. Food Agric. 25, 791-795.
McDonald, P., Hernderson, N., Heron, S., 1991. The Biochemistry of Silage. Chalcombe Publications, Bucks, UK.

Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86, 2010-2037.
Oh, H.-I., Hoff, J.E., 1987. pH Dependence of Complex Formation Between Condensed Tannins and Proteins. J. Food. Sci. 52, 1267-1269.
Pursiainen, P., Tuori, M., 2008. Effect of ensiling field bean, field pea and common vetch in different proportions with whole-crop wheat using formic acid or an inoculant on fermentation characteristics. Grass Forage Sci. 63, 60-78.
Randby, Å.T., 2000. The effect of some acid-based additives applied to wet grass crops under various ensiling conditions. Grass Forage Sci. 55, 289-299.
Rochfort, S., Parker, A.J., Dunshea, F.R., 2008. Plant bioactives for ruminant health and productivity. Phytochemistry 69, 299-322.
Ross, G.J.S., 1987. MLP: Maximum Likelihood Program (a manual). Rothamsted Experimental Station, Harpenden, UK, p. 228 pp.
Salawu, M.B., Acamovic, T., Stewart, C.S., Hvelplund, T., Weisbjerg, M.R., 1999. The use of tannins as silage additives: effects on silage composition and mobile bag disappearance of dry matter and protein. Anim. Feed Sci. Technol. 82, 243-259.
Salawu, M.B., Warren, E.H., Adesogan, A.T., 2001. Fermentation characteristics, aerobic stability and ruminal degradation of ensiled pea/wheat bi-crop forages treated with two microbial inoculants, formic acid or quebracho tannins. J. Sci. Food Agric. 81, 1263-1268.
SAS, 2003. SAS, Statistical Analysis System, Cary, NC, USA.
Thomson, D.J., Beever, D.E., Harrison, D.G., Hill, I.W., Osbourn, D.F., 1970. The digestion of dried lucerne (*Medicago sativa* L.) and dried sainfoin (*Onobrychis viciifolia* Scop.) by sheep. Proc. Nutr. Soc. 30, 1791.
Valentine, S., Brown, D., 1973. Formaldehyde as a silage additive. II. The chemical composition and nutritive value of lucerne hay, lucerne silage, and formaldehyde and formic acid-treated lucerne silages. Aust. J. Agric. Res. 24, 939-946.
Wang, Y., Barbieri, L.R., Berg, B.P., McAllister, T.A., 2007. Effects of mixing sainfoin with alfalfa on ensiling, ruminal fermentation and total tract digestion of silage. Anim. Feed Sci. Technol. 135, 296-314.
Woolford, M.K., Wilkinson, J.M., Cook, J.E., 1983. Investigations on the effect of sodium bentonite and other moisture absorbents on the production of effluent from grass silage. Anim. Feed Sci. Technol. 8, 107-118.

383 Figure 1. Buffer soluble N (BSN) in acidified and wilted silages (BSN values in Table 1). Values with

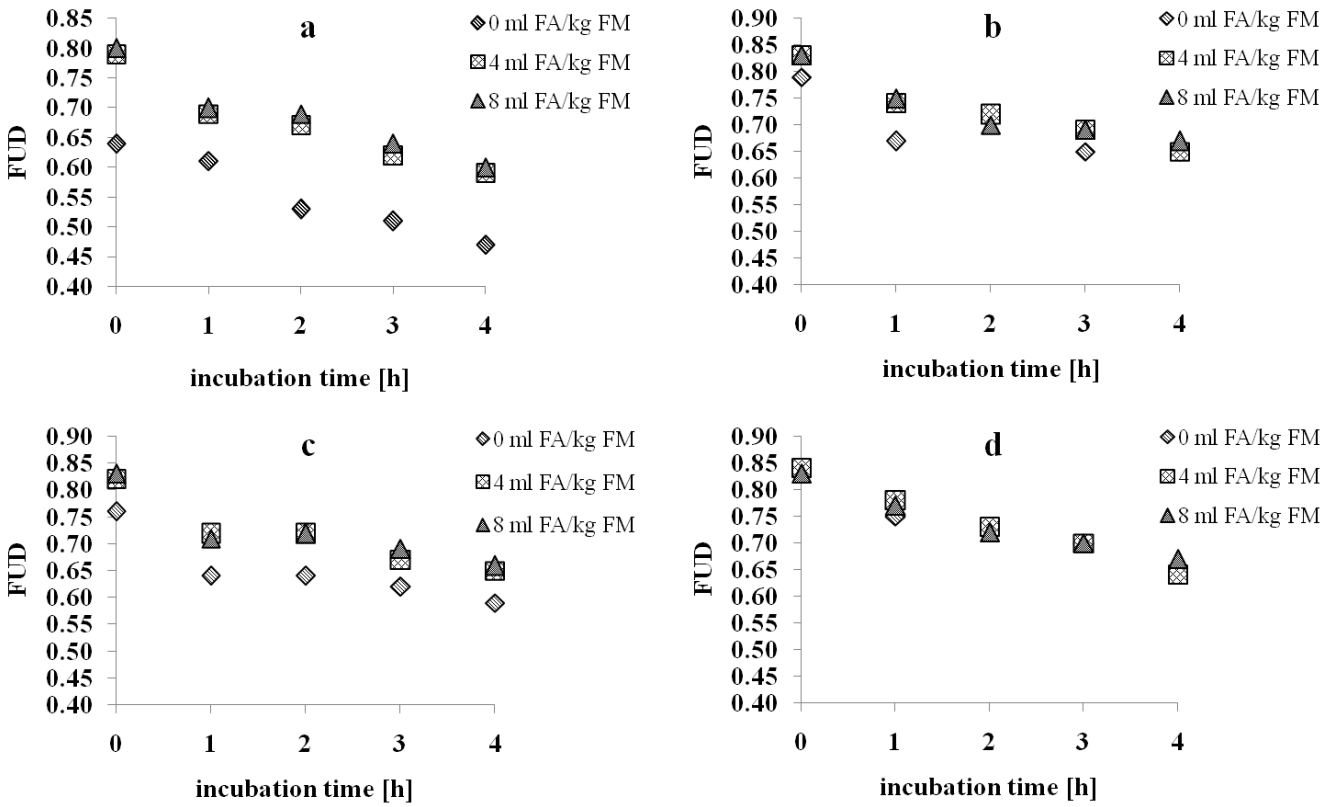
384 different superscripts differ (P<0.05).



385

386 Figure 2. Fraction of undegraded protein (FUD) after *in vitro* degradation at: a) 180 g/kg DM; b) 400 g/kg

387 DM; c) 500 g/kg DM; d) 600 g/kg DM (FUD values in Table 2).



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