Protein characteristics in grass-clover silages according to wilting rate and fermentation pattern

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Abstract

Effects of wilting rate and fermentation stimulators and inhibitors on protein characteristics of forages typical for organic production were assessed using traditional analytical methods and a gas production *in vitro* assay. The hypotheses were that the proportion of the crude protein (CP) fraction being soluble would be lowest, and the protein feed value highest, under rapid wilting and restricted fermentation. The solubility of the CP fraction varied according to treatments and between a first and a second cut with moderate and high content of clover, respectively. It was, however, of minor importance for the protein value, both calculated as

amino acids absorbed in the small intestine (AAT_{20}) and estimated as effective utilisable crude protein (uCP_{04}) by the *in vitro* assay. In ensiled herbage, AAT_{20} was highest in rapidly wilted and restrictedly fermented silages made from a first cut dominated by highly digestible grasses. Silages from the second cut dominated by red clover were far lower in AAT_{20}. The *in vitro* assay did not separate silages according to herbage composition or wilting rate, but ranked restrictedly fermented above extensively fermented with regard to protein supply. The assay might still have caught the characteristics that determine the true protein value *in vivo*.

Keywords: *in vitro* gas production, red clover, utilizable protein

Introduction

At northern latitudes, dairy production based on forage from leys of grass and clover is regarded as a sustainable system for production of high quality food from local resources (Janzen, 2011; Norwegian Government, 2011). Still, the utilization of nitrogen in the system is pin pointed as a crucial challenge (Bleken *et al.*, 2005). One of the main reasons for the relatively low nitrogen use efficiency (NUE) is the low marginal response in the animal to increased crude protein (CP) concentration in the diets (Huhtanen and Hristov, 2009). An option to improve the NUE is therefore to enhance the utilisation of forage protein, which is especially relevant in organic dairy production where the proportion of forages in the diets is relatively high (Commission, 2007; Tine Rådgivning, 2014).

Improving the utilization of forage protein in ruminants is challenging. One option is by manipulating the forage quality by i) modifying the botanical composition of the sward, e.g. introducing legumes containing bioactive components such as tannins and polyphenoloxidase (PPO). These compounds may reduce the exposure of the protein to rumen degradation

48 (Coblentz and Grabber, 2013; Eickler *et al.*, 2011; Lee *et al.*, 2008) and increase the flow of
49 feed protein from the rumen (Vanhatalo *et al.*, 2009), ii) increase the wilting rate during
50 preservation and thereby inhibit the natural proteolysis that is induced in plants cells after
51 harvest (Edmunds *et al.*, 2014; Verbič *et al.*, 1999), and iii) adding additives in order to
52 rapidly lower pH and regulate or prevent proteolysis and fermentation of easily degradable
53 carbohydrates (Jatkauskas and Vrotniakiene, 2009; McDonald *et al.*, 1991; Van Soest, 1994).
54 The extent of protein degradation during preservation may theoretically affect both the NUE
55 and the animal performance. For instance, high levels of NH_4-N in the feed may reduce intake
56 and the ability of high yielding ruminants to utilize the N (McDonald *et al.* 1991). However, a
57 meta-analysis of dairy cow production experiments performed by Huhtanen *et al.* (2008a),
58 revealed no evidence that animal yield and NUE were affected by the proportion of soluble
59 non-ammonia N in silages beyond its influence on dry matter intake (DMI).
60
61 The utilisation of forage protein in the animal is most precisely determined in feeding
62 experiments where total inputs and outputs are carefully accounted for. Such studies are,
63 however, laborious and expensive, and several analytical methods have been developed
64 during the last decades to imitate metabolic processes of protein utilisation *in vivo*. One major
65 challenge in developing laboratory methods has been to mimic and account for the complex
66 interactions between feed protein degradation, energy supply and microbial protein synthesis
67 in the rumen (e.g. Broderick, 1987; Lorenz *et al.*, 2011). This complexity was demonstrated in
68 a meta-analysis of Huhtanen and Hristov (2009) who found that metabolizable energy (ME)
69 intake was the main factor influencing milk protein yield and not the protein quality
70 determined by the *in situ* technique (Ørskov and McDonald, 1979).
71

In the Nordic countries of Europe, the protein value of feed for ruminants is described as the concentrations of amino acids absorbed in the small intestine (AAT) and the protein balance in the rumen (PBV). The unit AAT is equivalent to the more internationally used parameter metabolizable protein (MP). In the feed evaluation system NorFor™ (Volden, 2011), standard feed AAT_{20} value is calculated from the concentrations of CP, soluble CP (sCP), and ME at an estimated feed intake of 20 kg dry matter (DM) per day. During the last decades, several alternative analytical methods using *in vitro* gas production and the release of ammonia for feed protein evaluation have been developed and refined (Edmunds *et al.*, 2012; Karlsson *et al.*, 2009; Raab *et al.*, 1983; Zhao and Lebzien, 2000). Edmunds *et al*. (2012) estimated the expected supply of effective utilisable crude protein at the duodenum (uCP) at different rumen passage rates from remaining non-ammonia N after *in vitro* incubations of feed samples in buffered rumen fluid. The method aims to produce a feed value representing an estimate of the sum of microbial *de novo* protein synthesis in the rumen and rumen undegraded feed protein that reaches the small intestine.

Although the effects of wilting and silage additives on the quality of silages have been carefully studied, no investigations have been carried out where the combined effect has been evaluated by the new *in vitro* technique of uCP (Edmunds *et al*. 2012). The objective of the present study was to examine the effects of wilting rate and the addition of fermentation stimulators and inhibitors on protein characteristics of forages typical for organic production, using traditional analytical methods and the new *in vitro* technique described by Edmunds *et al*. (2012).

Our expectations were: 1) The proportion of the CP fraction being solubilized during preservation would be lowest under rapid wilting and restricted fermentation. 2) The effect of

preservation method would be lower in a second cut dominated by red clover than in a first cut dominated by grasses, and 3) the protein feed value would be highest in forages with lowest proportion of solubilized CP. 4) The effective uCP content as analysed by a gas *in vitro* technique would reflect organic matter digestibility and CP characteristics as determined by traditional methods, and be an efficient measure for protein quality in silages.

Material and methods

Experimental design

The study comprised two different forage types that originated from two cuts (spring growth and regrowth after first cut) of an organically managed grass-clover ley. After harvest, the crop was wilted at two rates, and fermented without or with additives (formic acid, FA, and lactic acid bacteria, LAB), and later analysed for chemical composition, fermentation products and *in vitro* and *in situ* characteristics. The design was factorial with 2 cuts × 2 wilting rates × 3 additive treatments. There were three field replicates of all wilting rates and additive combinations within harvests.

Establishment of the grass-clover crop

The crop was sown at a seeding rate of 26 kg ha^{-1} in three replicated blocks in June 2011 at the Norwegian Institute of Bioeconomy Research in Stjørdal (63°30'N, 10°54'E). The seed mixture contained (w/w) 38% timothy (*Phleum pratense,* cv.'Grindstad'), 19% meadow fescue (*Festuca pratensis,* cv. 'Stella'), 12% ryegrass (*Lolium perenne,* cvs 'Figgjo', 'Prana', 'Calibra', 'Aston Energy', 'Birger', 'Dunluce', 'Fia' and *Lolium boucheanum*, cv. 'Storm') and 31% red clover (*Trifolium pratense*, cv. 'Varte'). Before sowing, 50 kg total N ha^{-1} was applied from swine manure. The crop was not fertilized further, neither in 2011 nor in 2012.

120 **Harvests and evaluation of botanical composition**

121 At all harvests the crop was cut at a stubble height of 7 cm by a plot harvester without
122 conditioner. In the year of establishment, two harvests were taken on 3 August and 6
123 September. In the experimental year (2012), the first cut was taken on 11 June and the second
124 on 27 July, 614 d° (base temperature 0 °C) later. Botanical composition was evaluated
125 visually, and the proportion of red clover was further determined in dried yield samples by
126 Near-infrared spectroscopy (NIRS) (Fystro and Lunnan, 2006).

127 In the first cut, timothy and perennial ryegrass were the dominating grass species, and
128 constituted 25 and 30% of the DM yield, respectively. Their phenological stage of
129 development was 2.42 and 2.44, according to Mean Stage by Count (MSC) (Moore *et al.*,
130 1991), which corresponds to late stem elongation. Red clover constituted 30% of the DM
131 yield, and the shoots had developed stems with two or more internodes, but no flower buds
132 were visible or palpable. In the second cut, red clover constituted 76% of the DM yield, and
133 perennial ryegrass, with 40% of the tillers elongated or reproductive, was the dominating
134 grass species. The red clover was generative at this harvest, and half of of the shoots had
135 developed buds and/or flowers. The DM yield in the first and second cut was 5000 and 4100
136 kg ha^{-1}, respectively.

137 **Wilting and chopping**

138 The fresh crop was moved indoors for immediate sampling and wilting. For all operations,
139 three replicates (which originated from three different blocks in the field), each of 50 kg fresh
140 matter (FM) were handled successively. Samples (1 kg FM each) of the fresh crop were dried
141 at 60 °C for DM determination and later analyses.

142 The wilting treatments were arranged in a drying hall with forced air inlet on the floor. Swaths
143 of the crop were left on steel frames strapped with a nylon mesh about 1.5 m above the floor.

144 The series of air inlets beneath were switched on and off individually to manage rapid and
145 slow wilting to the targeted DM concentration (240 g kg^{-1}), which was reached after 7.5 and
146 24.5 hours for both cuts. The temperature in the hall and of the forced air was about the same
147 as the ambient outdoor temperature on respective occasions. For the period 11-12 June, the
148 mean air temperature was 10.3°C and for the period 27-28 July it was 15.7°C. The wilted crop
149 was chopped at a length of 1 to 2 cm by a "Hans-Ulrich Hege Saatzuchtmaschinen"
150 (Hohebuck, Waldenburg, Germany). Two kg of each replicate of chopped material was
151 immediately dried at 60°C. The rest was preserved as silage.

152 **Preservation**

153 The wilted and chopped crops were preserved as silage in evacuated and sealed polyethylene
154 bags with 4 ml kg FM^{-1} of different types of additives: 1) tap water (Control treatment (C)), 2)
155 FA (850 g kg^{-1}), and 3) LAB, using Kofasil® Lac (1.25 g L^{-1}) (Addcon Europe, Bonn,
156 Germany). Kofasil® Lac contained two homofermentative strains of *Lactobacillus plantarum*,
157 minimum 2×10^{10} colony forming units (cfu) g^{-1}, and 10^5 cfu were thus applied per g FM of
158 the wilted crop.

159 Each polyethylene bag (6 L) contained the equivalent of about 300 g DM (50 g L^{-1}) of the
160 plant material and was evacuated for 18 seconds and sealed by use of a Magic Vac™ Maxima
161 (Flaem Nuova S.p.A, Brescia, Italy). Two bags were ensiled for each treatment. All silage
162 bags were stored in a dark room with an ambient temperature of 18°C for three months.
163 Thereafter silages from the two bags from each treatment replicate were thoroughly mixed.
164 One subsample of the mixed content from the two bags was immediately frozen at -20°C for
165 later analyses of fermentation products and DM content as outlined below. Another
166 subsample was dried at 60 °C.

167 **Sample preparation and chemical analyses**

168 After drying, the subsamples of fresh and wilted herbage, and silages, were split in two and
169 ground through a 2.0-mm screen for *in situ* incubations and a 1.0-mm screen for *in vitro* and
170 chemical and NIRS analyses using a Tecator Cyclotec 1093 mill (Foss Tecator AB, Höganäs,
171 Sweden). The samples of herbage and silages were thereafter analysed for DM (105ºC for 16
172 h), ash (525ºC for 6 h; AOAC (1984); method 923.03), CP (AOAC 1984; method 7.015) and
173 the concentration of buffer soluble CP (sCP) as described by Hedqvist and Udén (2006).
174 Neutral detergent fibre (NDF) concentrations (Mertens, 2002) were determined using heat
175 stable α-amylase and sodium sulphite using the ANKOM^{200} Fiber Analyser (Ankom
176 Technology Corp., Macedon, NY). The NDF values are expressed exclusive of residual ash.
177 Water-soluble carbohydrates (WSC) and starch were analysed according to the procedure of
178 Larsson and Bengtsson (1983). After thawing, the freshly frozen silage samples were
179 analysed for pH, and content of lactic acid, propionic acid, formic acid , acetic acid, butyric
180 acid and ethanol (Ericson and Andrè, 2010), and NH_4-N (the volatile N fraction in silage juice
181 distilled on a Kjeltech Autosystem 1030, Tecator AB, Höganäs, Sweden). The DM
182 concentrations of the silages were corrected for volatile losses as described by Åkerlind *et al.*
183 (2011).

184 ***In situ* and *in vitro* measurements**

185 All animals used for *in vitro* and *in situ* procedures were treated and kept with permission
186 from the Swedish Ethical Committee on Animal Research represented by the Court of Appeal
187 for Northern Norrland in Umeå, Sweden. Concentrations of indigestible NDF (iNDF) were
188 determined by a 288-h *in situ* incubation (Huhtanen *et al.*, 1994) using three ruminally
189 cannulated lactating Swedish Red cows yielding 27 kg energy corrected milk (ECM) day^{-1}.
190 They were fed grass silage and a commercial concentrate (0.60 : 0.40 on DM basis) in
191 amounts regulated to meet production requirements. Samples of 2 g were weighed into pre-
192 weighed polyester bags with a pore size of 12 µm and a pore area equal to 6 per cent of the

193 total surface (Saatifil PES 12/6; Saatitech S. P.A., Veniano, Como, Italy). The internal
194 dimensions of the nylon bags and sample size were adjusted to give a sample size to surface
195 area ratio of 10 mg cm^{-2}. After removal from the rumen, the bags were rinsed in a domestic
196 washing machine (the rinsing cycle of the wool wash program including three times washing
197 for 2.5 min per washing (including the time for filling of water) using approximately 8°C
198 water (Electrolux Wascator W75MP; AB Electrolux, Stockholm, Sweden), boiled 1 h in
199 neutral detergent solution (NDS) including sodium sulphite (100 ml g^{-1}) of sample (Mertens *et*
200 *al*., 2002), thoroughly rinsed, dried at 60°C for 24 h and weighed. Concentrations of iNDF
201 were expressed exclusive of residual ash.

202 The *in vitro* production of gas (GP) was recorded as described by Hetta *et al.* (2003)
203 simultaneously with determination of the concentration of uCP at 8 h and 48 h as described by
204 Edmunds *et al*. (2012). The *in vitro* procedures were performed with a fully automated system
205 (Cone *et al.*, 1996) recording GP (V) every 12 minutes. The recorded GP was corrected to
206 normal air pressure (1013.5 h Pa). About 400 mg of OM from each sample was incubated in
207 60 ml of buffered rumen fluid during 48 h in 250-ml serum bottles (Schott, Mainz, Germany).
208 The bottles (n=36) were incubated at 39°C and gently agitated continuously in water baths.
209 The inoculum for each run was collected two hours after the morning feeding, from two
210 rumen cannulated lactating dairy cows (Swedish Red) fed as described above. The rumen
211 fluid from the two cows was mixed and filtered through four layers of cheese cloth into a
212 buffered mineral solution, with the ratio of rumen fluid to buffer of 1:2 (V/V). The buffered
213 mineral solution described by Menke and Steingass (1988) was slightly modified with a small
214 alteration in the concentration of NH_4HCO_3 as suggested by Edmunds *et al*. (2012). The
215 concentration of NH_3 in the buffered rumen fluid in the incubation bottles was determined by
216 sampling 0.4 ml of fluid with plastic syringes as described by Karlsson *et al*. (2009) at 8 and
217 48 h after the start of the incubation. The fluid samples were transferred into Eppendorf tubes

kept on ice and thereafter 0.016 ml of 96% H_2SO_4 was added for preservation and stored at -20°C until analysis. Just prior to analysis the sample tubes were thawed at room temperature, centrifuged (12500 × g, 10 min) and 0.1 ml of supernatant was transferred to test tubes and diluted 1:20 with distilled water. The concentration of ammonia-N was analysed using continuous flow analyser (AutoAnalyzer 3 HR, SEAL Analytical Ltd). In each *in vitro* series (run) a blank (buffered rumen fluid without a sample) and standard hay were included in duplicates. The samples were randomly distributed within and between runs and replicated in duplicates within two of total seven runs, resulting four *in vitro* observations per sample. The microbial activity of the rumen fluid was monitored by the results of the blanks and standard hay with known GP profile and uCP values. No anomalies were noted for the standard GP profiles or uCP values for none of the runs. Mean uCP_{04} for standard hay (7 runs and 2 standards per run, altogether 13 samples - one was lost) was 142 g kg^{-1} DM, with a standard deviation of 36 and min and max of 90 and 206, respectively.

Curve fitting of gas production data and modelling of digestion

For the *in vitro* GP measurements, a three pool Gompertz model (Schofield *et al.*, 1994) constructed using the NLIN procedure in SAS (release 9.3, 2002-2010, SAS Institute inc., Cary, NC, USA) was used to fit the averaged (n=3) GP profiles of each feed. The sum of the three GP pools represented the total asymptotic gas production (AGP). The digestibility of potentially digestible organic matter (OM), GP rates of OM, which corresponded to digestion rates (hereafter referred as effective KD), and effective ruminal digestibility of OM (D) were calculated using the modeling approach with two rumen pools as described by Huhtanen *et al.* (2008b).

Calculations of feeding values

241 The potential digestibility of the fibre (pNDFD) was calculated as (NDF–iNDF)/NDF. The
242 first order rate of NDF degradation was calculated as described by Huhtanen *et al*. (2008b).
243 Organic matter digestibility (OMD) was calculated from *in situ* incubation values and NDF
244 concentrations as described by Huhtanen *et al.* (2013):
245
246 $$OMD = 882 - 1.21 \times iNDF - 0.106 \times NDF,$$
247
248 Feed value as content of net energy for lactation (NEL) and metabolisable protein, expressed
249 as amino acids absorbed in the intestine (AAT), was based on Van Es (1978) and Madsen *et*
250 *al.* (1995), respectively. Protein balance in rumen (PBV) was also interpreted according to
251 Madsen *et al*. (l.c). The given values for NEL, AAT and PBV apply for dairy cows with a
252 daily DMI of 20 kg (NEL_{20}, AAT_{20} and PBV_{20}), according to NorFor™, the Nordic feed
253 evaluation system (Volden, 2011).

254 The concentration of uCP at 8 and 48 h was calculated as by Edmunds *et al*. (2012) utilising
255 the formula:

256 $$uCP\ (g\ kg\ DM-1) = \frac{NH_3N_{blank} + N_{sample} - NH_3N_{sample}}{\text{weight (mg DM)}} \times 6.25 \times 1000$$

257 Where NH_3N_{blank} is the average amount (mg) of NH_3N in the two blanks, N_{sample} is the amount
258 (mg) of N in the sample at the start of the incubation and NH_3N_{sample} is the amount (mg) of
259 NH_3N in the incubation bottles with samples. The GP and the uCP estimates are presented as
260 least square means (LSmeans) of the four observations calculated with the general linear
261 model with effects of run as a random factor. The uCP values from 8 and 48 h were plotted
262 against ln time (h) and the intercept (y) and the slope (a) were used to calculate the effective
263 uCP using the formula where Kp is the assumed rate of passage (/h).

264 $$effective\ uCP\ = \mathrm{y} + \mathrm{a}\ + ln\ (1/Kp)$$

265 **Statistical analyses**

266 Analyses of variance of data for the whole range of constituents in the forages were derived
267 from linear mixed-effects models using the procedure MIXED in SAS (release 9.3, 2002-
268 2010, SAS institute inc., Cary, NC, USA).

269 The constituents in herbage were modelled with cut (first and second) and wilting (no wilt,
270 rapid wilt or slow wilt) as fixed factors and field replicate (1-3) as random factor. The
271 interaction between cut and wilting rate was included in the model. Cut was analysed as
272 repeated to check for covariance that may occur for first and second cut harvested on the same
273 plots. The optimal covariance structure among Variance Components and Unstructured was
274 assessed for each forage constituent with attention to Akaike information criterion and
275 Schwarz Bayesian criterion (Littell *et al*., 1998). Tukey's test was used for pairwise
276 comparisons of means within cuts ($P \leq 0.05$). Residual normality was assessed using the tests
277 performed in the procedure UNIVARIATE in SAS (release 9.3, 2002-2010, SAS institute
278 inc., Cary, NC, USA), with no data showing deviation from normal distribution.

279 For silages, the constituents and quality parameters were modelled with cut (first or second),
280 wilting rate (rapid or slow) and additive (C, FA or LAB) as fixed factors and replicate (1-3) as
281 random factor. All two- and three-factor interactions were included in the model. Cut was
282 analysed as repeated with covariance structure as described above, and Tukey's test was used
283 for pairwise comparisons of means within cut, wilting rate and additive, respectively ($P \leq$
284 0.05). Residual normality was assessed as described above. For data showing deviation from
285 normal distribution (AGP, KD and D) square root transformations were applied. Correlations
286 between pairs of feed parameters were analysed by the procedure CORR in SAS (release 9.3,

2002-2010, SAS institute inc., Cary, NC, USA) and expressed as Pearson's product moment correlation coefficients.

Results

Chemical and nutritive characteristics of fresh and wilted crops

Concerning the herbage (pre-ensiling), there were differences in the chemical composition between first and second cut and according to wilting rate (Table 1). At harvest, the first cut was, on DM basis, higher than the second cut in concentrations of NDF, WSC and OMD, and lower in CP, starch and iNDF. The AGP, the proportion of the CP fraction being soluble and the calculated concentration of NEL_{20} and AAT_{20} were also higher in first compared to second cut. The concentration of PBV_{20} was negative in first cut, but positive, and significantly higher in the second cut. During wilting, the concentration of non-structural carbohydrates decreased. Parallel to this, the concentration of iNDF increased, whereas the concentration of AAT_{20} and NEL_{20} decreased. The buffer soluble CP fraction of total CP was higher after slow compared to rapid wilting, whereas the concentration of utilisable protein calculated as uCP_{04} did not differ between cuts, nor wilting rates.

Fermentation pattern and nutritive characteristics of silages

All silages were well fermented as evaluated from the pH and the concentration of NH_3-N (Table 2) (Eurofins, 2015). Eleven out of 36 samples contained traces of butyric acid (detection limit 0.3 g kg DM^{-1}), but none of them more than 1 g kg DM^{-1} (data not shown). All eleven were from the first cut and from treatments with no additive or addition of LAB. No single sample contained more than 72 g NH_3-N kg N^{-1} and none had pH higher than 4.50.

309 Silages from second cut were more extensively fermented than silages from first cut (Table
310 2). Slow wilting increased acetic acid concentration compared with rapid wilting; otherwise
311 no effect of wilting rate on fermentation quality was found. Addition of LAB caused the most
312 extensive fermentation and addition of FA the most restricted fermentation. For most of the
313 constituents, there was a significant cut × additive-interaction (split data not presented in
314 Table 2), involving a greater difference in fermentation pattern between additives in the
315 second than in the first cut. No two- or three factor interactions for the effects of cut, wilting
316 rate and additive modified the conclusions for significant main effects as presented in Table 2.

317 The different fermentation patterns caused by the additives also affected the composition and
318 nutritive value of the silages (Table 3). Extensively fermented silages, added LAB or no
319 additive, had lower concentrations of non-structural carbohydrates, AAT_{20} and NEL_{20} and a
320 higher concentration of PBV_{20} than the restrictedly fermented silages added FA. The *in vitro*
321 gas production expressed as AGP was higher from silages added FA than from silages added
322 LAB. The rate of degradation of OM as estimated from the GP recordings did not differ
323 between cuts, wilting rates or additives. The OMD and concentration of NEL_{20} were higher in
324 silages from first than from second cut, and in rapidly compared to slowly wilted silages,
325 whereas the concentration of iNDF was lower in the first than in the second cut, and lower in
326 rapidly than in slowly wilted silage (Table 3).

327 There were hardly any significant interactions for effects of cut, wilting rate and additive on
328 constituents and quality parameters (Table 3). For the concentration of WSC, however, the
329 differences according to wilting rate and additive were higher in the first compared to the
330 second cut.

331 **Protein characteristics in silages**

332 The proportion of the CP being soluble (sCP) increased from wilted crops to silages (Tables 1
333 and 3), and it was highest (602 and 600 g kg CP^{-1}) in extensively fermented (C and LAB)
334 silages from the first cut. The NH_3-N concentration in these silages was 63 and 61g kg N^{-1},
335 respectively, which amounted to ca. 200 g kg^{-1} N of the crude protein that was solubilized
336 during extensive fermentation (600 g kg CP^{-1} in silages minus 330 g kg CP^{-1} in wilted crops).
337 The proportion of sCP was higher in slowly wilted than in rapidly wilted silages (Table 3), but
338 the proportion of NH_3-N of total N did not differ according to wilting rate (Table 2). From a
339 separate test for the increment in sCP from wilted crops to silages, it appeared that it was
340 significantly higher (P<0.01) in the first cut than in the second cut for all types of silages.

341 The concentration of uCP_{04} did not differ between first and second cut or according to wilting
342 rate, but was higher in FA silages than in the control and LAB silages (Table 3). The
343 concentration of AAT_{20} was higher in first than in second cut silages, in rapidly versus slowly
344 wilted, and in restrictedly versus extensively fermented silages (Table 3).

345 Correlation analyses revealed no significant relationship between uCP_{04} in silages and any of
346 the other measured or calculated characteristics of the protein fraction (CP, sCP, NH_3-N,
347 AAT_{20}, PBV_{20}) (Table 4). There was, however, a negative relationship between the
348 concentration of total acids and uCP_{04} and a positive relationship between silage pH and
349 uCP_{04}. The other feed characteristics correlated with total acids, such as concentration of
350 WSC, iNDF and OMD, were not statistically related to uCP_{04}, suggesting a direct relationship
351 between pH (concentration of acids) and uCP_{04}.

352

353 ## Discussion

354 The herbage harvested in the two successive cuts of the grass-clover ley in the present study,
355 were typical for organic or low N-input forage production in temperate regions regarding the
356 disproportionate content of clover, CP and ME (Eriksen *et al.*, 2012; Kunelius *et al.*, 2006;
357 Riesinger and Herzon, 2008; Steinshamn and Thuen, 2008). The results from the experiments
358 will therefore be relevant for silage production and feeding on that type of commercial farms.

359

360 The initial hypotheses that the proportion of the CP fraction being solubilized during
361 preservation would be affected by wilting rate and fermentation pattern, and less so in a
362 second cut dominated by clover than in a first cut dominated by grasses, were supported by
363 the results. Proteolysis during the first step of preservation (wilting) appeared, however, to be
364 numerically small, and the proportion of sCP increased significantly only for herbage from
365 the first cut at the slowest rate of wilting. Still, previous wilting rate mattered for the
366 proportion that later was solubilized during fermentation, irrespective of cut and additive,
367 indicating that changes had occurred in the protein fraction that were not necessarily reflected
368 in the analyzed pre-ensiling sCP concentration. Our results were in agreement with the
369 findings of Edmunds *et al*. (2014) who found a lower non-protein nitrogen concentration in
370 rapidly than in slowly wilted silages. They explained the difference by differing duration of
371 exposure to proteolytic plant enzymes. Differing exposure to plant proteases were very likely
372 the cause for the observed differences in protein degradation according to fermentation
373 pattern, also. As reviewed by McDonald *et al*. (1991), numerous studies have confirmed that
374 an initial rapid drop in pH after addition of acids retards proteolysis during conservation. They
375 also referred to studies having shown that grass proteases were active below pH 4, so the FA
376 silages in the present study with an average final pH of 4.3 would have been an environment
377 allowing proteolytic activity. Still, they contained less sCP than the extensively fermented
378 control and LAB silages. The reason why the protein fraction seemed more stable during

379 wilting and fermentation in the second than in the first cut, might be the high red clover
380 content in the second. The enzyme PPO in red clover tissues, might have catalyzed the
381 synthesis of protein bound phenolics and thus limited protein degradation (Lee *et al*., 2008).

382

383 The third hypothesis that the protein feed value of the silages would vary according to the
384 solubility of CP was not unambiguously confirmed by the results. Statistically, sCP was
385 neither correlated to AAT_{20} nor to uCP_{04}, and although silages from the second cut contained
386 more CP and less that had been solubilized during preservation, the AAT_{20} was lower than in
387 the corresponding silages made from the first cut. The main explanation for this was the lower
388 digestibility of OM and the lower content of WSC in silages dominated by mature red clover.
389 According to the NorFor feed evaluation system (Volden, 2011), the potential for microbial
390 protein synthesis in the rumen would have been limited by the low digestibility of the second
391 cut silages.

392

393 Differences in characteristics of the carbohydrate fractions were also the reason for the higher
394 concentration of AAT_{20} in restrictedly (FA) compared to extensively fermented silages
395 (control and LAB), and in rapidly versus slowly wilted silages. More WSC and starch
396 remained after rapid wilting and restricted fermentation. The digestibility of OM did,
397 however, not differ according to type of additive, and the lower sCP in FA silages contributed
398 to their higher AAT_{20} concentration relative to the other two types. Because AAT_{20} is a
399 calculated measure of protein feed value based on the presumption that the proportion of sCP
400 is important (Volden, 2011), its scores here and elsewhere, constitutes no basis for a
401 discussion of the issue of whether sCP is important for the forage protein value or not *in vivo*.
402 However, the present study illustrates what compounds and characteristics that are important
403 for the ranking of different typical types of silages according to the AAT_{20} definition.

Protein quality evaluated as effective uCP_{04} determined by the gas *in vitro* analyses was expected to reflect and express characteristics that determine forage protein values *in vivo*, such as OM digestibility. Here, uCP_{04} turned out to be rather insensitive to differences in carbohydrate and CP characteristics obtained under the different cuts and treatments. The FA silages contributed more utilizable uCP_{04} per kg DM than LAB silages, but very few of the attributes differing between these types of forages were statistically correlated to uCP_{04}. The fact that sCP proportion and concentration did not influence uCP_{04} is in agreement with the meta-analysis of experiments analysing milk protein yield performed by Huhtanen and Hristov (2009). It is, however, more difficult to explain why OM digestibility and carbohydrate concentration seemed to have no relationship to microbial protein synthesis during the *in vitro* incubation, when gas production *in vitro* (AGP) was closely related to these parameters.

Edmunds *et al*. (2014) evaluated the effect of wilting on grass silage using the same *in vitro* technique in combination with an *in situ* method and the Cornell system for protein evaluation of forages (Sniffen *et al.*, 1992). They concluded that the degree and rate of wilting influenced the concentration of uCP_{04} and rumen undegraded protein in grass silage, and that the method may be a useful tool in predicting animal response to the forage because it considers the interaction of energy and protein metabolism. They evaluated, however, silages with a higher DM content (350 – 650 g kg^{-1}) than those in the present study and wider ranges in wilting duration.

It is possible that methodological discrepancies are the reason for different results regarding the appearing sensitivity and accuracy of the gas *in vitro* method. Still, the analytical variation

429 and errors were about the same in the two studies, and the concentration of $uCP_{0.04}$ was within
430 the same range for quite similar forages (120-140 g kg^{-1} DM in the study of Edmunds *et al*.
431 (2014), and 120-150 g kg^{-1} DM in the present study).
432
433 Alternative explanations for what seemed to be a low sensitivity of the gas *in vitro* method in
434 the present study may be found in what was limiting factors for microbial protein synthesis in
435 the silages compared, e.g. those from the first versus those from the second cut. In the first
436 one, the CP in the silage might have been limiting and very efficiently utilized for *in vitro*
437 protein synthesis because of surplus supply of metabolizable energy. In contrast, a low energy
438 supply probably limited the protein synthesis from second cut silages that contributed surplus
439 CP. In the end, these relationships resulted in equal supply uCP_{04} from the two cuts. In the
440 comparison of *in vitro* protein synthesis from silages with more equal CP concentration and
441 still differing energy supply to microbes (FA silages versus extensively fermented LAB
442 silages) the *in vitro* method revealed differences in uCP_{04} and ranked FA silages highest in
443 terms of protein supply.
444
445 The study of Jaakkola *et al.* (2006) may contribute further explanations for the higher $uCP_{0.04}$
446 in FA silages. They suggested that restrictedly fermented silages were efficient substrates for
447 *de novo* protein synthesis not only because of higher concentrations of WSC and other energy
448 substrates but also because of higher levels of free amino acids and peptides in the non-
449 protein N fraction than in more extensively fermented silages. The authors further added that
450 the organic acid profile of extensively fermented silages makes them less efficient as energy
451 substrates for microbial protein synthesis because lactate does not supply energy for microbial
452 growth. In the LAB silages in the present study, lactate constituted nearly 15% of the
453 digestible OM, whereas it constituted 4% in FA silages.

The last issue we wanted to address and discuss in the present study was whether the *in vitro* uCP technique could be an analytically efficient and precise measure for protein quality in forages. As mentioned above, the estimated concentrations of uCP_{04} in the present study covered about the same range as the silage analyses performed by Edmunds *et al*. (2012; 2014) did. This fact indicates reproducibility of the method in between laboratories, but as Edmunds *et al*. (2012) concluded in their study, it is difficult to estimate how precisely the uCP_{04} figures represent *in vivo* conditions because no reference values exist. However, preliminary results from recent studies at the Swedish University of Agricultural Sciences in Umeå, indicate a positive and consistent correlation between the concentration of uCP in feeds and their supply of protein to the small intestine as analysed by *in vivo* flow studies (Huhtanen *et al*., 2016).

It is clear that the accuracy and reproducibility of the method are challenged by the natural variation in ammonia concentration and microbial activity in the rumen fluid between animals and sampling occasions (Lorenz *et al.,* 2011). This variation may be reduced by using pre-incubation of the rumen fluid with carbohydrates as described by Lorenz *et al*. (2011) to normalize the ammonia concentration in the inoculum prior to incubations. Our experience from this study was that we needed a relatively large number *in vitro* replicates to obtain reliable results. This was reflected in the relatively large standard errors of means for uCP_{04}, and it suggests that the analytical protocol for the assay should be expanded with a normalisation of the rumen fluid and runs of a standard protein feed previously analysed for protein flow to the small intestine *in vivo*.

Conclusions

The silages investigated in this study covered a range in chemical composition and nutritive characteristics that were representative of forages produced at low rates of external N supply on commercial farms in Northern Europe. The solubility of the CP fraction varied pre and post ensiling, but this parameter did not determine the protein value of the forages, neither according to calculated AAT_{20}, nor to the gas *in vitro* assay estimating uCP_{04} at the small intestine. The concentration of AAT_{20} was highest in rapidly wilted and restrictedly fermented silages made from an early first cut dominated by grasses with highly digestible OM. Corresponding silages from a mature second cut dominated by red clover and with a higher content of less soluble CP, were far lower in AAT_{20}. The *in vitro* protein assay did not separate silages according to initial herbage composition and wilting rate, but ranked restrictedly above extensively fermented crops with regard to protein supply to the animal. The assay might still have caught the characteristics that determine the true protein value *in vivo.* In this matter, animal experiments rather than AAT_{20} calculations will contribute the ground truth for further method evaluation and development.

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652 **Table 1** Composition and nutritive characteristics of herbage from a first and second cut of a grass-clover crop at harvest (fresh) and after 7.5 h
653 (rapid) and 24.5 h (slow) wilting, and levels of significance for the effects of different treatments.

Herbage	n	DM	Ash	CP	sCP	WSC	Starch	NDF	iNDF	OMD	AAT_{20}	PBV_{20}	NEL_{20}	uCP_{04}	AGP	D	KD
First cut																	
Fresh	3	163[b]	70	101	314[b]	264[a]	16	391	59	769	89	-34	6.2	133	440	0.924	0.106
Rapidly wilted	3	233[a]	75	113	314[b]	228[b]	27	392	61	767	89	-24	6.2	128	434	0.929	0.112
Slowly wilted	3	235[a]	76	114	338[a]	221[b]	18	396	67	759	88	-20	6.1	150	433	0.926	0.109
SEM		3.3	1.8	4.2	5.9	3.5	3.6	10.9	3.5	5.2	0.64	3.1	0.05	12.0	12.7	0.003	0.004
Second cut																	
Fresh	3	139[b]	99	133	301[a]	112	45[a]	361	113	707	81	9	5.4	130	419	0.927	0.110
Rapidly wilted	3	231[a]	99	133	251[b]	78	36[a]	375	119	698	80	10	5.2	152	389	0.929	0.113
Slowly wilted	3	232[a]	102	138	294[a]	87	22[b]	383	121	695	79	16	5.2	124	364	0.934	0.118
SEM		6.3	1.0	2.8	8.2	9.1	2.8	5.5	2.1	2.5	0.58	2.6	0.05	8.6	10.2	0.003	0.004
Effects																	
Cut		0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	<0.01	<0.01	<0.01	<0.01	<0.01	NS	<0.01	0.18	NS
Wilting rate		<0.01	0.08	0.18	<0.01	<0.01	0.02	NS	0.05	0.06	<0.01	0.07	0.03	NS	0.17	NS	NS
Cut×Wilt		0.03	0.12	0.20	0.07	NS	0.02	NS	NS	NS	NS	0.13	NS	0.16	0.09	NS	NS

654
655 n, number of samples; DM, dry matter (g kg^{-1}); CP, crude protein (g kg DM^{-1}); sCP, soluble CP (g kg CP^{-1}); WSC, water soluble carbohydrates (g kg DM^{-1});
656 NDF, neutral detergent fibre (g kg DM^{-1}); iNDF, indigestible NDF (g kg DM^{-1}); OMD, estimated in vivo digestibility of organic matter (g kg^{-1}); AAT_{20},
657 amino acids absorbed in the small intestine (g kg DM^{-1}); PBV_{20}, protein balance in the rumen (g kg DM^{-1}); NEL_{20}, net energy for lactation (MJ kg DM^{-1});
658 uCP_{04}, utilisable crude protein (g kg DM^{-1}); AGP, asymptotic gas production (ml gas mg OM^{-1}); D, predicted ruminal digestibility of potentially digestible
659 organic matter (OM) calculated using a two compartment, mechanistic and dynamic rumen model; KD, effective OM digestion rates (1/h) calculated using a
660 two compartment mechanistic dynamic rumen model; SEM, standard error of the mean; NS, not significant ($P > 0.20$). Means within cuts with different
661 superscripts were significantly different ($P \leq 0.05$).

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665 **Table 2** Concentrations of dry matter (DM) and fermentation products and pH, in grass-clover silages according to cut, wilting rate (duration 7.5
666 or 24.5 h) and additive, and levels of significance for the effects of different treatments.

Silage	n	pH	DM	NH_3-N	Lactic acid	Formic acid	Acetic acid	Propionic acid	Total acids	Ethanol
Cut										
First cut	18	4.22[a]	244[a]	53[a]	59[b]	3.9[b]	21.3[b]	2.3[a]	87[b]	5.4
Second cut	18	4.20[b]	234[b]	42[b]	96[a]	6.7[a]	28.0[a]	1.2[b]	132[a]	4.5
SEM		0.009	2.8	1.5	1.9	0.28	0.32	0.21	2.0	0.54
Wilting rate										
Rapidly wilted	18	4.20	236	46	78	5.1	23.5[b]	1.6	109	4.6
Slowly wilted	18	4.22	242	49	77	5.5	25.8[a]	1.9	110	5.3
SEM		0.013	3.5	1.8	1.7	0.46	0.57	0.25	2.2	0.44
Additive										
Control	12	4.19[b]	240	56[a]	97[b]	0.3[b]	32.2[a]	1.5	132[b]	5.9[a]
FA	12	4.31[a]	239	34[b]	32[c]	14.7[a]	9.3[b]	1.6	57[c]	3.2[b]
LAB	12	4.14[c]	238	54[a]	104[a]	0.9[b]	32.4[a]	2.2	140[a]	5.8[a]
SEM		0.015	1.8	2.2	2.1	0.56	0.70	0.30	2.6	0.54
Effects										
Cut		0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.13
Wilting rate		0.11	NS	NS	NS	NS	<0.01	NS	NS	0.16
Additive		<0.01	NS	<0.01	<0.01	<0.01	<0.01	0.10	<0.01	<0.01
Cut×Wilt		NS	NS	NS	NS	NS	0.08	NS	NS	NS
Cut×Additive		<0.01	0.10	0.01	<0.01	<0.01	0.07	<0.01	NS	NS
Wilt×Additive		0.01	NS	0.15	0.08	NS	NS	NS	0.17	NS
Cut×Wilt×Additive		0.02	NS	0.19	NS	0.03	0.03	NS	NS	NS

667 n, number of samples; DM, dry matter (g kg^{-1}); NH_3-N (g kg N^{-1}); Fermentation products (g kg DM^{-1}); Control, addition of water; FA, addition of formic acid;
668 LAB, addition of a suspension of lactic acid bacteria. SEM, standard error of the mean; NS, not significant ($P > 0.20$). Means within treatment categories with
669 different superscripts were significantly different ($P \leq 0.05$).

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671

672 **Table 3** Composition, nutritive characteristics and *in vitro* gas production from grass-clover silages according to harvest, wilting rate (duration
673 7.5 or 24.5 h) and additive, and levels of significance for the effects of different treatments.

Silage	n	Ash	CP	sCP	WSC	Starch	NDF	iNDF	OMD	AAT_{20}	PBV_{20}	NEL_{20}	uCP_{04}	AGP	D	KD
Cut																
First cut	18	84^{b}	122^{b}	570^{a}	88^{a}	15.8	387	66^{b}	761^{a}	81^{a}	3.5^{b}	6.0^{a}	137	438^{a}	0.923	0.106
Second cut	18	110^{a}	152^{a}	441^{b}	25^{b}	20.3	379	114^{a}	705^{b}	68^{b}	51.7^{a}	5.3^{b}	137	375^{b}	0.926	0.109
SEM		0.6	1.7	4.9	1.9	2.0	6.0	1.7	2.4	0.6	1.5	0.04	6.9	6.7	0.001	0.001
Wilting rate																
Rapidly wilted	18	96	135	496^{b}	63^{a}	23.5^{a}	379	87^{b}	736^{a}	76^{a}	23.6	5.7^{a}	134	414	0.926	0.108
Slowly wilted	18	98	140	515^{a}	50^{b}	12.7^{b}	387	92^{a}	730^{b}	74^{b}	31.6	5.6^{b}	140	399	0.924	0.107
SEM		0.6	1.6	5.3	2.5	1.2	7.1	1.7	2.2	0.5	2.0	0.03	7.3	8.1	0.002	0.002
Additive																
Control	12	98^{a}	141^{a}	534^{a}	20^{b}	16.7^{b}	381	89	734	70^{b}	38.2^{a}	5.6^{b}	138^{ab}	404^{ab}	0.926	0.109
FA	12	96^{b}	133^{b}	455^{b}	129^{a}	21.3^{a}	383	89	733	84^{a}	8.3^{b}	5.7^{a}	151^{a}	421^{a}	0.922	0.105
LAB	12	97^{a}	138^{a}	528^{a}	20^{b}	16.3^{b}	385	90	732	70^{b}	36.4^{a}	5.6^{b}	123^{b}	393^{b}	0.925	0.108
SEM		0.5	1.0	4.4	3.0	1.4	8.7	2.1	2.6	0.7	0.8	0.04	8.9	9.9	0.002	0.002
Effects																
Cut		<0.01	<0.01	<0.01	<0.01	0.05	NS	<0.01	<0.01	<0.01	<0.01	<0.01	NS	<0.01	0.07	0.13
Wilting rate		0.12	0.10	<0.01	<0.01	<0.01	NS	0.02	0.01	<0.01	0.06	<0.01	NS	0.08	NS	NS
Additive		0.01	<0.01	<0.01	<0.01	<0.01	NS	NS	NS	<0.01	<0.01	<0.01	0.03	0.05	0.13	0.14
Cut×Wilt		NS	NS	NS	<0.01	NS	NS	0.07	0.14	NS	NS	NS	NS	NS	0.09	0.08
Cut×Additive		NS	NS	0.05	<0.01	NS	NS	0.19	NS	NS	NS	NS	0.15	NS	0.06	0.06
Wilt×Additive		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Cut×Wilt×Additive		NS	NS	NS	0.16	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

674 n, number of samples; CP, crude protein (g kg DM^{-1}); sCP, soluble CP (g kg CP^{-1}); WSC, water soluble carbohydrates (g kg DM^{-1}); NDF, neutral detergent
675 fibre (g kg DM^{-1}); iNDF, indigestible NDF (g kg DM^{-1}); OMD, estimated in vivo digestibility of organic matter (g kg^{-1}); AAT_{20}, amino acids absorbed in the
676 small intestine (g kg DM^{-1}); PBV_{20}, protein balance in the rumen (g kg DM^{-1}); NEL_{20}, net energy for lactation (MJ kg DM^{-1}); uCP_{04}, utilisable crude protein (g
677 kg DM^{-1}); AGP, asymptotic gas production (ml gas mg OM^{-1}); D, predicted ruminal digestibility of potentially digestible organic matter (OM) calculated
678 using a two compartment, mechanistic and dynamic rumen model; KD, effective OM digestion rates (1/h) calculated using a two compartment mechanistic
679 dynamic rumen model; SEM, standard error of the mean; NS, not significant ($P > 0.20$). Means within treatment categories with different superscripts were
680 significantly different ($P \leq 0.05$).

Table 4 Matrix of Pearson's product moment coefficients for pairwise correlations between nutritive and fermentation parameters analysed in 36 silages (two cuts × two wilting rates × three additives × three replicates).

	CP	sCP	WSC	NDF	iNDF	OMD	pH	DM	TA	NH_3-N	AAT_{20}	PBV_{20}	NEL_{20}	KD	AGP	D
sCP	**-0.71**															
WSC	**-0.60**	-0.01														
NDF	**-0.41**	0.31	0.07													
iNDF	**0.90**	**-0.81**	**-0.47**	-0.16												
OMD	**-0.88**	**0.80**	**0.47**	0.10	**-1.00**											
pH	-0.26	-0.24	**0.77**	0.11	-0.10	0.09										
DM	**-0.56**	**0.44**	0.31	**0.33**	**-0.47**	**0.46**	**0.37**									
TA	**0.61**	-0.04	**-0.92**	-0.11	**0.51**	**-0.50**	**-0.71**	-0.25								
NH_3-N	-0.25	**0.79**	**-0.51**	0.16	**-0.39**	**0.38**	**-0.49**	0.22	**0.46**							
AAT_{20}	**-0.75**	0.25	**0.91**	0.07	**-0.72**	**0.72**	**0.56**	0.30	**-0.95**	-0.29						
PBV_{20}	**0.92**	**-0.49**	**-0.81**	-0.27	**0.85**	**-0.84**	**-0.45**	**-0.44**	**0.85**	0.05	**-0.94**					
NEL_{20}	**-0.87**	**0.66**	**0.61**	0.08	**-0.96**	**0.96**	0.15	**0.38**	**-0.60**	0.18	**0.82**	**-0.89**				
KD	0.31	-0.07	**-0.32**	**-0.46**	0.19	-0.17	**-0.38**	-0.26	**0.35**	0.11	-0.31	**0.34**	-0.20			
AGP	**-0.78**	**0.53**	**0.57**	0.04	**-0.80**	**0.81**	0.21	**0.33**	**-0.63**	0.10	0.75	**-0.81**	**0.81**	**-0.39**		
D	**0.35**	-0.10	**-0.35**	**-0.50**	0.22	-0.19	**-0.38**	-0.28	**0.37**	0.10	-0.33	**0.37**	-0.22	**0.99**	**-0.41**	
uCP04	-0.01	-0.15	0.25	0.08	-0.03	0.03	**0.38**	0.11	**-0.38**	0.19	0.27	-0.16	0.01	-0.32	0.29	-0.29

CP, crude protein (g kg DM^{-1}); sCP, soluble CP (g kg CP^{-1}); WSC, water soluble carbohydrates (g kg DM^{-1}); NDF, neutral detergent fibre (g kg DM^{-1}); iNDF, indigestible NDF (g kg DM^{-1}); OMD, estimated in vivo digestibility of organic matter (g kg^{-1}); DM, dry matter (g kg^{-1}); TA, total acids (g kg DM^{-1}); AAT_{20}, amino acids absorbed in the small intestine (g kg DM^{-1}); PBV_{20}, protein balance in the rumen (g kg DM^{-1}); NEL_{20}, net energy for lactation (MJ kg DM^{-1}); KD, effective OM digestion rates (1/h); AGP, asymptotic gas production (ml gas mg OM^{-1}); D, predicted ruminal digestibility of potentially digestible matter (OM) calculated using a two compartment, mechanistic and dynamic rumen model; uCP_{04}, utilisable crude protein (g kg DM^{-1}). Coefficients with bold typing were significant ($P \leq 0.05$).