

Scotland's Rural College

## **Associative effects of ensiling mixtures of sweet sorghum and alfalfa on nutritive value, fermentation and methane characteristics**

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7 Associative effects of ensiling mixtures of sweet sorghum and alfalfa on nutritive value,  
8 fermentation and methane characteristics

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48 **ABSTRACT**

49           Combining sweet sorghum (**SS**) with alfalfa (**AF**) for ensiling has the potential to  
50 improve the nutritive value and fermentation characteristics of resultant silages. However, the  
51 optimal combination and the associative effects of SS and AF for ensilage have not been  
52 studied. Therefore, the aim of this study was to determine the fermentation characteristic and  
53 nutritive value of silage mixtures with six different SS to AF ratios. The two forages were  
54 ensiled in air free silos for 150 days at room temperature as mixtures containing 0: 100, 20: 80,  
55 40: 60, 60: 40, 80: 20, and 100: 0 of SS : AF on a fresh weight basis. As the proportion of SS  
56 increased in silage, the content of ash, crude protein, saponins, ammonia, acetic acid, propionic  
57 acid and pH decreased, while neutral detergent fiber, acid detergent fiber in organic matter,  
58 acid detergent lignin, water-soluble carbohydrate, starch, total phenolics and condensed  
59 tannins content increased. The silages were evaluated in 24-hour incubations with rumen  
60 liquor. The *in-vitro* rumen degradability of dry matter and organic matter as well as gas  
61 production, pH, ammonia, total volatile fatty acids and methane decreased as the proportion of  
62 SS increased in the silage mixtures. This study suggests that high quality silages can be made  
63 with SS: AF ratios of 20:80 and 40:60. These silage mixtures offer an opportunity to optimize  
64 the nutrient supply for ruminant production.

65 *Keywords:* tannins; saponins; *in-vitro* methane production; volatile fatty acids; gas production;  
66 pH

67  
68 *Abbreviations:* **SS**, sweet sorghum; **AF**, alfalfa; **DM**, dry matter; **OM**, organic matter; **CP**, crude  
69 protein; **aNDFom**, neutral detergent fibre in OM; **ADFom**, acid detergent fibre in OM; **ADL**,  
70 acid detergent lignin; **EE**, ether extract; **IVDMD**, *in-vitro* DM degradability; **IVOMD**, *in-vitro*

71 OM degradability; **tVFA**, total volatile fatty acids; **WSC**, water-soluble carbohydrates; **GP**, gas  
72 production; **CH<sub>4</sub>**, methane; **NH<sub>3</sub>**, ammonia; **SP**, saponins; **TP**, total phenolics; **CT**, condensed  
73 tannins.

74

## 75 **1. Introduction**

76 Sweet sorghum (*Sorghum bicolor*, **SS**) is a promising forage in the arid, semi-arid and  
77 high salinity areas due to its rapid growth, high biomass yield (Qu et al., 2014), drought tolerance  
78 and high water-use efficiency (Wu et al., 2010). Sweet sorghum can be conserved as ruminant  
79 feed through ensilage (Calabrò et al., 2010a). However, the crude protein (**CP**) content in SS fresh  
80 and SS silage (~ 100 g CP/kg DM; Colombini et al., 2012) is insufficient to fulfil the  
81 requirement of growing or lactating ruminants (NRC, 2007). In order to meet the CP requirement  
82 of ruminants, forages with a high CP content, such as legume, can be mixed with low CP forages  
83 before or after ensiling. However, silage only making from legume is often challenging, due to  
84 its low water-soluble carbohydrates (**WSC**) content and high buffering capacity (Fisher and  
85 Burns, 1987) and extensive proteolysis during ensiling (McDonald et al. 1991). Ozturk et al.  
86 (2006) showed that ensiling maize with alfalfa (*Medicago sativa*, **AF**) is a feasible strategy to  
87 increase the CP content and improve the nutritive value of silage. Differently to temperate areas,  
88 maize production is low in the arid and high salinity areas around the world (Qu et al., 2014),  
89 and SS is an attractive alternative in these regions (Wu et al., 2010 ). There have been few  
90 studies to provide detailed investigation of the feasibility of mixing SS and legume forages for  
91 silage making. As a widely grown perennial legume with a deep root system and strong  
92 resistance to drought, AF can be grown well in arid, semi-arid areas. Therefore, AF was selected  
93 as a candidate legume for this study as bases for developing optimal silage mixtures for animal

94 production in arid, semi-arid regions. The aim of this study was to investigate the associative  
95 effects of ensiling mixtures of SS and AF on nutritive value and fermentation characteristics of  
96 resulting silages. It tested the hypothesis that synergies from combining the two forages mean  
97 that the nutritive value and fermentation characteristics of mixed silages are better than would be  
98 predicted from values for silages prepared from the single forages.

99

## 100 **2. Materials and methods**

### 101 *2.1 Forage harvesting and silage making*

102 The cultivars used for SS and AF in this study were *Cowley* with 22.5% Brix value and  
103 *Hetian Big-leaf* respectively. Both SS and AF were sown at the Agricultural Research Station of  
104 Tarim University, XinJiang, China. Whole plant of SS and AF were harvested at milky stage and  
105 at early bloom stage (10% flowering rate), respectively, using a grass hook and leaving a stubble  
106 of 5 cm. Forage sample was chopped into 2.5 cm particle size by a multi-function chopper  
107 (9DF53, Yanbei Animal Husbandry Machinery Group Co. Ltd., Beijing, China). About 500 g  
108 sample of each fresh forage of SS and AF was stored directly at -20°C until analysed for  
109 proximate composition. Plastic silos were used to make chopped forages into six silage types,  
110 with different SS to AF ratios (containing 0, 20, 40, 60, 80 and 100% SS based on fresh weight).  
111 The fresh weight of forages in each silo was 1.5 kg and ten replicates of each silage type were  
112 made. The forage mixtures were manually compressed to remove air before the silos were screw  
113 capped. The silos were stored in the dark at room temperature.

114

### 115 *2.2 Quality analysis of silage*

#### 116 *2.2.1 Chemical analysis*

117 To mimic the silage based livestock production system in arid and semi-arid regions in the  
118 world, where silages are normally made in summer and fed out in autumn and winter when feed  
119 supply is low; the silos were opened 150 days post ensiling and a 500 g fresh weight sample was  
120 collected per silo for analysis. A 15 g fresh weight sample was blended with 135 mL distilled  
121 water for 1 min followed by filtration through two layers of cheesecloth. The supernatant was  
122 then tested for pH using pH meter (pH209, Hanna Instruments., Edge, USA). Two 15 mL  
123 subsamples of the extract were centrifuged at 2500 rpm for 10 min at 4 °C (MSE Mistral 3000,  
124 Sanyo Gallenkamp, Leicestershire, UK), and then acid extraction (Chaudhry and Khan, 2012)  
125 was performed on supernatant before ammonia ( $\text{NH}_3$ ) and organic acids analysis. The  
126 concentration of  $\text{NH}_3$  was analyzed by Pentra 400 (Horriba Ltd, Kyoto, Japan) according to the  
127 method described by Rhine et al. (1998). Lactic, acetic and propionic acids were determined  
128 using GC (Shimadzu Ltd, Kyoto, Japan) according to Fussell and McCalley (1987).

129 Subsamples of 500 g per silage type and fresh forage of SS and AF prior to ensiling were  
130 dried at 65 °C in an oven and then ground through a 1 mm sieve using a mill (Christy and Norris  
131 Co. Ltd., Suffolk, UK), and analysed in triplicate for dry matter (**DM**), ash, ether extract (**EE**)  
132 according to AOAC (2005) procedures. Ash-free neutral detergent fiber in organic matter with  
133 addition of  $\alpha$ -amylase (**aNDFom**), ash-free acid detergent fiber in organic matter (**ADFom**) and  
134 acid detergent lignin (**ADL**) were determined according to the methods of Van Soest (1991).  
135 Crude protein (**CP**) was calculated by multiplying 6.25 with the content of nitrogen (**N**), which  
136 was determined using an Elementar Vario Macro Cube (Elementar, Hanau, Germany). Water-  
137 soluble carbohydrates were determined by Spectrophotometer (Libra S11, Biochrome,  
138 Cambridge, UK) following the method of Koehler (1952). The starch was tested by the method  
139 of Kent-Jones and Amos (1967) as described by Chaudhry and Khan (2012). Total phenolics

140 (TP) of silage samples were measured using the Folin–Ciocalteu method (Singleton and Rossi,  
141 1965). Total condensed tannins (CT) and saponins (SP) of silage samples were measured  
142 according to the method described by Osman (2004) and Khan and Chaudhry (2010),  
143 respectively.

#### 144 *2.2.2 Mineral Analysis*

145 The concentrations of Ca, P, K, Mg, Fe, Zn, Cu, Na, Mn, Mo and Co from each silage type  
146 were determined, in triplicate, using a VISTA-MPX CCD simultaneous ICP-OES (Varian Inc.,  
147 Melbourne, Australia). The samples and the standard solutions for mineral analysis were  
148 prepared according to the methods of Chaudhry and Jabeen (2011) and Ramdani et al. (2013).

149

#### 150 *2.3 Measurement of in-vitro fermentation parameters*

##### 151 *2.3.1 Preparation of rumen fluids and buffered inoculums*

152 Six Texel × Mule castrated lambs ( $45 \pm 1.2$  kg live weight) were fed on nutritionally  
153 balanced perennial ryegrass-concentrate diet prior to slaughter at an abattoir (Linden Food, UK).  
154 The lambs were slaughtered under The Welfare of Animals at the Time of Killing (WATOK)  
155 Regulations of the UK (DEFRA, 2013). Rumen samples were collected immediately post  
156 slaughtering. The rumen fluid was harvested by filtering through double layers of cheesecloth  
157 into pre-warmed (39 °C) thermo bottles and immediately transported to the laboratory. The  
158 rumen fluid was poured into a pre-warmed brown bottle containing artificial saliva (McDougall,  
159 1948) to prepare buffered inoculum. This buffered inoculum was kept anaerobic by flushing it  
160 with anaerobic grade CO<sub>2</sub> before aliquots were added using a dispenser pump, and bottles closed  
161 (Chaudhry and Mohamed, 2011).

##### 162 *2.3.2 In-vitro incubations*

163 A total of 200 mg of each type of dried silage in four replicates were separately weighed into  
164 50 mL graduated glass syringes (KR Analytical Ltd., Sanitex, UK) fitted with plungers. A  
165 mixture of ruminal fluid and buffer (20 mL) was dispensed into each syringe before its  
166 incubation in a shaking water bath (Grant Instruments, Cambridge, UK) at 39 °C for 24 h. At the  
167 same time, incubations without any silage sample of three empty syringes served as the blanks to  
168 correct the final values of respective degradability, gas production (**GP**) and other fermentation  
169 parameters. The volume of GP in each syringe was recorded at 2, 4, 6, 8, 10, 20, 22 and 24 h of  
170 incubation.

171

### 172 *2.3.3 Determination of pH, ammonia, in-vitro dry matter and organic matter degradability*

173 Fermentation in the syringes was terminated at 24 h by transferring the syringes from the  
174 water bath to an ice-filled container. About 15 mL of headspace gas in each syringe was  
175 transferred into a vacuum tube through a three-way valve (Fisher Scientific, Loughborough, UK)  
176 for methane (**CH<sub>4</sub>**) analysis. Each incubated sample was tested for pH and then centrifuged at  
177 2500 rpm for 10 min at 4 °C (MSE Mistral 3000, Sanyo Gallenkamp., Leicestershire, UK). A  
178 total of 2 mL of the supernatant from each centrifuge tube was used for later volatile fatty acid  
179 (**VFA**) analysis. An additional 2 mL of the supernatant from each sample was used for NH<sub>3</sub>  
180 analysis. The remaining supernatant, along with all residues in each centrifugal tube were dried  
181 at 65°C and weighed for *in-vitro* DM degradability (**IVDMD**). The dried residues were decanted  
182 into crucibles and ashed at 550°C for measuring *in-vitro* organic matter degradability (**IVOMD**).

### 183 *2.3.4 Ammonia, volatile fatty acid and methane analysis*

184 NH<sub>3</sub> was analysed by Pentra 400 (Horriba Ltd., Kyoto, Japan) with a calibrated standard of  
185 NH<sub>3</sub>-N according to Rhine *et al.* (1998). Volatile fatty acids concentration along with relevant



186 standards (Sigma Aldrich, Gillingham UK) was analyzed by a GC (Shimadzu., Kyoto, Japan) as  
187 described by Eun and Beauchemin (2007). Total VFA concentration (mM) was determined by  
188 summing the areas of individual VFA in each sample and each VFA were expressed as % of  
189 total VFA. The CH<sub>4</sub> analysis was performed on a Fisons 8060 GC using a split injection linked  
190 to a Fisons MD800 MS as described by Bhatta et al. (2009).

191

#### 192 *2.4 Calculations and statistical analysis*

193         The GP data for each silage mixture were fitted to the exponential model  $Y = a + b(1 - e^{-ct})$   
194 as described by Ørskov and McDonald (1979) using the Curve Fit software for the estimated  
195 parameters. Where  $a$  = instant GP from rapidly soluble fraction,  $b$  = slow GP from insoluble  
196 fraction,  $c$  = the rate of GP from slowly insoluble fraction (b),  $t$  = incubation time and  $Y$  = GP at  
197 time  $t$ . The SPSS statistical package (SPSS Inc., Chicago, USA) was used for statistical analysis  
198 of all data. One-way ANOVA was used to examine the linear and quadratic effects of silage  
199 types on chemical composition, mineral profile, GP, GP parameters (a, b and c), IVDMD,  
200 IVOMD, CH<sub>4</sub>, pH, NH<sub>3</sub> and VFA adopting a significance level of  $P < 0.05$ . The statistical model  
201 included silage type as treatment effect. The Tukey's post-hoc test was used for multiple  
202 comparisons of means across the monocultures and the mixtures with different ratios of SS and  
203 AF. Treatment differences were considered to be significant when  $P < 0.05$ .

204

### 205 **3. Results**

#### 206 *3.1 Chemical composition of AF and SS prior to ensiling*

207 The chemical composition of AF and SS forages is presented in Table 1. AF was  
208 significantly ( $P<0.001$ ) higher in DM, Ash, CP and EE than SS, whereas SS was significantly  
209 ( $P<0.05$ ) higher in WSC, Starch, aNDFom, ADFom and ADL than AF.

### 210 *3.2 Chemical composition of SS-AF silage mixtures*

211 The chemical composition of the silages is given in Table 2. The concentrations of DM, Ash,  
212 CP, EE, SP in the SS-AF silage mixture significantly ( $P<0.05$ ) decreased, whereas aNDFom,  
213 ADFom, ADL, WSC, starch, TP and CT significantly ( $P<0.05$ ) increased as the proportion of SS  
214 increased in the silage. The CP and WSC content in 0% SS silage was 3.6 times higher and 4.4  
215 times lower than in 100% SS silage, respectively (Table 2). The ash content in 0% SS silage (116  
216 g/kgDM) was about 50% higher than that of 100% SS silage (i.e., 100 % SS silage; 73 g/kg).

217

### 218 *3.3 Fermentation characteristics of SS-AF silage mixtures*

219 The fermentation characteristics of the silage mixtures are shown in Table 2. The pH, NH<sub>3</sub>,  
220 acetic acid and propionic acid content significantly ( $P<0.05$ ) decreased, while lactic acid content  
221 significantly ( $P<0.001$ ) increased as the proportion of SS in the silage mixtures increased from  
222 0% to 100%.

223

### 224 *3.4 Mineral profile of SS-AF silage mixtures*

225 Mineral profile of the silage mixtures are presented in Table 4. The content of K, Ca, P,  
226 Mg, Na, Fe and Zn significantly ( $P<0.001$ ) decreased as more SS was included in the silage  
227 mixtures. No significant differences in the content of Mn, Cu, Mo and Co were observed in the  
228 silage mixtures.

229

### 230 3.5 *In-vitro* fermentation profiles of SS-AF silage mixtures

231 The pH, NH<sub>3</sub>, IVDMD, IVOMD, tVFA and individual VFA except butyrate decreased as the  
232 proportion of SS in silage mixtures was significantly (P<0.05) increased. IVDMD and IVOMD  
233 in the silage mixtures with SS at 0%, 20% and 40% inclusion were significantly (P<0.05) higher  
234 than those with SS at 80% and 100% level (Table 5).

235

### 236 3.5 *In-vitro* gas production, kinetic parameters and methane of SS-AF silage mixtures

237 Methane, GP and values for GP kinetics model of *in-vitro* fermentation are given in Table 6.  
238 *In-vitro* cumulative GP between 2 and 24 h of incubation differed among the silage types. The  
239 AF silage and the silage mixtures containing 20% and 40% of SS produced more gas than the  
240 other silage mixtures. The silage made with 100% SS had the significantly (P<0.05) lowest GP  
241 and CH<sub>4</sub> among all silages used in this study.

242 There were significant (P<0.05) differences between silages in terms of the estimated  
243 parameters from the GP kinetics models. The intercept value (a) for different treatments  
244 representing GP from soluble fractions ranged from -12.75 to 7.09, and the silages with 80% and  
245 100% SS has significantly (P<0.001) higher instant GP from rapidly soluble fraction than other  
246 silages. The GP from the insoluble fraction (b) had a significant (P<0.05) linear increase,  
247 whereas, the rates of GP for the insoluble fraction (c) had a significant (P<0.001) linear decrease  
248 as the proportion of SS increased in the mixture silages.

249

## 250 4. Discussion

### 251 4.1 Chemical compositions of raw materials and SS-AF silage mixtures

252           The content of DM and CP in the silage mixtures is a reflection of the proportions of  
253 the original forages included in each mixture. Alfalfa is a legume and it generally contains higher  
254 level of CP than sorghum (Table 1), because of nitrogen fixation from atmosphere (Ozturk et al.,  
255 2006; Amer et al., 2012). Likewise, many authors showed that the CP content increased in  
256 maize-legume silage mixture when the proportion of legume increased (Titterton and Maasdorp,  
257 1997; Contreras-Govea et al., 2009).

258           The high levels of residual WSC in the silage mixtures with more SS may be caused by  
259 the high brix and WSC in the initial SS material (Table 1), which had positive correlation with  
260 the residual WSC (Yang et al., 2006). The residual WSC was similar in 0% and 20% SS silages;  
261 this may be because the 20% SS silage provides adequate, but not excessive WSC for  
262 fermentation during ensilage. On the other hand, the increased residual WSC observed from 40%  
263 SS silage to 100% SS silage, despite the decreasing DM content, indicates that these forage  
264 mixtures supplied at least enough WSC for an effective fermentation. The content of starch in  
265 silage mixtures from this study (9 to 80 g/kg DM) is within the wide range observed from other  
266 reports. Though the forage were harvested at similar stages (milk stage for SS and early bloom  
267 stage for AF) as in the current study, Amer et al. (2012) showed lower (51 g/kg DM and 5 g/kg  
268 DM) starch content in SS silage and AF silage than in this study. This may be related to the  
269 starch content of the specific crop prior to ensilage (Table 1), which can be influenced by type of  
270 forages, culture system employed, method for ensilage, and ensilage material. For example,  
271 Colombini et al. (2012) reported a starch content of 34 g/kg DM in forage sorghum silage and  
272 208 g/kg DM in grain sorghum silage. This is in agreement with results showed by Sang et al.  
273 (2008), who suggested that starch is a main chemical component in sorghum grain (~700 g/kg  
274 DM).

275 The fiber content of these silages was in agreement with those reported by other researchers  
276 (Anil et al., 2000; Qu et al., 2013). The higher fiber fractions (i.e., aNDFom, ADFom and ADL)  
277 in the SS and 100% SS silage compared with the AF and 0% SS silage may be because SS is a  
278 C<sub>4</sub> plant and the photosynthetic cells are arranged in Kranz structures and often contain girder  
279 structures, which collectively increases fiber content. Similar anatomical features are lacking in  
280 AF (Wilson, 1994). The higher fiber fractions (Table 1) may be necessary for SS to grow tall and  
281 to produce more biomass. The lower content of fiber in AF silage was also exaggerated by  
282 harvesting at the early-bloom stage. The quadratic effects of SS inclusion on aNDFom and  
283 ADFom indicate that up to 60% of SS can be included in the silage mixtures without increasing  
284 major fiber fractions in the silage mixtures.

285 The multiple phenolic hydroxyl groups in TP and CT lead to the formation of complexes  
286 with proteins, metal ions and other macromolecules like polysaccharides. These effects lead to  
287 the protection of forage proteins from degradation by inhibiting plant and microbial enzymes,  
288 resulting in better quality silages with lower NH<sub>3</sub> levels (Makkar, 2003). SP is a steroid or  
289 triterpene glycoside compound found in different plants. It is the main anti-nutritional  
290 components in AF plant, and their unfavourable effects on ruminant performance (such as bloat  
291 caused by production of slime from AF saponins) can restrict the optimum use of AF as an  
292 animal feed (Sen et al., 1998).

293

#### 294 *4.2 Fermentation characteristics of SS-AF silage mixtures*

295 The fermentation characteristics indicate that adding SS in this study improved overall silage  
296 quality, with a lower pH, higher lactic acid and lower NH<sub>3</sub> concentration (Muck, 1988; Heron et  
297 al., 1989). These effects can be explained by the higher concentration of WSC and starch in the

298 mixtures with a higher proportion of SS. Mono- or disaccharides that are broken down from  
299 starch can also be used as readily fermentable carbohydrate, which help to reduce pH and  
300 increase lactic acid production during the ensiling process (McDonald et al. 2002). On the other  
301 hand, the lower WSC content in silage is related to higher buffering capacity (Fisher and Burns,  
302 1987) and extensive proteolysis during ensiling (Heron et al., 1989) may be attributed to the  
303 higher pH and NH<sub>3</sub> concentration with higher proportions of AF in the silage mixtures. Some  
304 research work showed a higher NH<sub>3</sub> concentration in maize-legume or sorghum-soybean  
305 mixtures than the maize- or sorghum- only silages (Titterton and Maasdorp, 1997; Contreras-  
306 Govea et al., 2009; Lima et al., 2010) and lower pH in Bermuda grass silages prepared from  
307 crops with higher WSC concentrations (Adesogan et al., 2004).

308 The higher content of acetic and propionic acids in AF silage than SS silage indicate that the  
309 legume forage was not well fermented. This was probably due to the comparatively low WSC  
310 and starch concentration in AF. Despite the lower pH was observed in silages containing 80 and  
311 100% SS, little change was found in lactic, acetic and propionic acids. This indicates no benefit  
312 in organic acid production was obtained with more than 60% of SS in the silage mixtures. A  
313 similar change of organic acids in silage mixtures containing maize and legume have been  
314 observed (Sun et al., 2009; Zhu et al., 2011).

315

#### 316 *4.3 Ash and minerals of SS-AF silage mixtures*

317 The higher contents of ash and the minerals such as K, Ca, P, Mg, Na, Fe and Zn in the AF  
318 silage than the SS silage were likely due to the differences that existed between SS and AF in  
319 their ability to absorb and accumulate different minerals during growing. Variation in ash and  
320 mineral concentration among crops are dependent on plant type and environmental factors (Wu

321 et al., 2007), as well as physiological and morphological differences among plants (Hoenig et al.,  
322 1998). Interestingly, Kume (2001) found that CP in AF had a positive correlation with Ca, P, Mg  
323 and K.

324

#### 325 *4.4 In-vitro rumen degradability and fermentation of SS-AF silage mixtures*

326 The higher IVDMD and IVOMD of silage mixtures with lower SS content may be due to  
327 their lower fiber fractions, which are known to reduce the degradability of feed (Mustafa et al.,  
328 2000; Sebata et al., 2011; Qu et al., 2013; Calabrò et al., 2010b). Moreover, the presence of  
329 higher content of phenolic compounds and tannins in sorghum silage has been found to be  
330 related to the protection of dietary protein, structural carbohydrates and starch against  
331 degradation by ruminal microorganisms (Tabacco et al., 2006; Oliveira et al., 2007). In this  
332 study, no significant difference was observed in IVDMD and IVOMD for the silage mixtures  
333 containing 0, 20 and 40% of SS. However, they all had higher degradability than 80% SS silage.  
334 This suggests that if a high degradability needs to be achieved, less than 60% SS should be added  
335 into the SS-AF silage mixtures.

336 The higher pH and NH<sub>3</sub> concentrations following the *in-vitro* incubation of low SS  
337 containing silage mixtures were expected. The higher pH and NH<sub>3</sub> from 100% AF silage reflects  
338 that a greater proteolysis occurred during its *in-vitro* incubation than in 100% SS silage. This is  
339 in agreement with Dhiman (1997), who reported that the ruminal NH<sub>3</sub> concentration was higher  
340 in cows fed AF silage than cows fed maize silage. Decreased rumen pH and NH<sub>3</sub> concentration  
341 have been shown in sucrose-supplemented cows (Broderick et al., 2008) and in fructose-  
342 supplemented heifers (Golder et al., 2012).

343 The observed increase in VFA of ruminal liquid with more AF in silage mixtures may be  
344 related to the ruminal microbe species. For example, *Fibrobacter succinogenes* mainly produces  
345 succinate, the major precursor of propionate in the rumen, while *Ruminococcus albus* mostly  
346 produces acetate (Vinh et al., 2011). The increased concentration of acetate and propionate in  
347 silage mixtures containing high level of AF may be due to the higher CP content which leads to a  
348 more favorable fermentation environment (pH, NH<sub>3</sub>) for growth of cellulolytic bacteria. Other  
349 researchers have showed that cellulolytic bacterial population could significantly increased by  
350 higher ruminal NH<sub>3</sub> (Khampa et al., 2006; Vinh et al., 2011) and the cellulolytic activity of  
351 rumen contents could be markedly inhibited by a fall of pH (Terry et al., 1969; Stewart, 1977)  
352 because of their influences on the rumen ecology. Higher ruminal NH<sub>3</sub> level may serve as N  
353 source to improve rumen ecology (Wanapat and Pimpa, 1999). The strong positive relationship  
354 between the number of ruminal cellulolytic bacterial species and the concentration of propionate  
355 and acetate had been observed (Vinh et al., 2011). Therefore, the increase in propionate and  
356 acetate concentration that occurred in the *in-vitro* fermentation with higher AF silage might have  
357 been a consequence of the increase in number of cellulolytic bacterial, such as *Fibrobacter*  
358 *succinogenes*, *Ruminococcus albus*. In addition, the higher concentration of minerals in mixed  
359 silages with more AF might have contribution to the cellulolytic bacterial growth (Kang et al.,  
360 2014). Similar to the findings from the current study, Lettat et al. (2013) also reported that  
361 greater ruminal pH and concentration of acetate in the rumen fluid of cows fed diet with high  
362 level of AF silage. The present finding of lower acetate production from more SS inclusion in the  
363 silages is similar to the findings from Kaplan (2011). This was likely due to the fibre type in SS  
364 that was less fermentable than that in AF.



365 Branched-chain VFA can be derived from the fermentation of branched-chain amino acids  
366 (Saro et al., 2014), so the higher iso-butyrate and iso-valerate concentration for AF silage in this  
367 study could be due to higher CP content and its great degradation. Hassanat et al. (2014) found  
368 the ruminal concentration of branched-chain VFA increased as cows were fed higher proportions  
369 of AF silage in the diet. Also, in agreement with our results, other researchers (Haddad, 2000;  
370 Saro et al., 2014) have reported that the rumen total VFA were increased as proportions of AF  
371 were increased in diets.

372

#### 373 *4.5 Methane and gas production of SS-AF silage mixtures*

374 CH<sub>4</sub> is an end-product of rumen carbohydrates fermentation and it has been recognized as a  
375 potent greenhouse gas (Moss et al., 1994). The higher CH<sub>4</sub> production from silages containing  
376 less SS may have resulted from more digestible portions and lower fiber content. Blaxter and  
377 Clapperton (1965) reported that CH<sub>4</sub> emission was positively correlated with the amount of  
378 digestible OM. Chaudhry and Khan (2012) proved less CH<sub>4</sub> production for the high fibrous  
379 substrates during *in-vitro* rumen fermentation. In addition, other researchers (Tavendale et al.,  
380 2005; Bhatta et al., 2009) confirmed that tannins could suppress methanogenesis by reducing the  
381 protozoa population, which had inhibitory effects on methanogens. Methane production is higher  
382 when protozoa are present in greater numbers in the rumen than when they are present in low  
383 numbers (Bhatta et al., 2009). Thus, the lower CH<sub>4</sub> production in silage mixtures with lower SS  
384 had likely contributed to the stronger anti-methanogenic activity from the presence of more CT  
385 content in SS.

386 Over 24 hours of incubation, a higher GP was observed from the AF silage than the SS  
387 silage, this mostly likely reflected that AF had lower aNDFom, ADFom and ADL

388 concentrations, as the negative correlation between fiber and GP was observed by Zerbini et al.  
389 (2002) and Sebata et al. (2011). Higher structural carbohydrates content can inhibit GP by  
390 limiting microbial fermentation or enzymatic hydrolysis of forage polysaccharides (Jung and  
391 Allen, 1995; Sebata et al., 2011). Sebata et al. (2011) also observed that GP was positively  
392 correlated with IVDMD and negatively correlated with CT. The trend of gas production in  
393 current study was opposite to the report from Kaplan (2011). It is likely that the AF used in this  
394 study was higher in CP content that resulted in more NH<sub>3</sub> production, which contributed to the  
395 total gas production. On the other hand, AF was low in fibre which might have caused a higher  
396 production in CH<sub>4</sub> production compared with SS. It is important to note that the GP were not  
397 different among 0%, 20%, and 40% SS silage mixtures at all times measured in this study. The  
398 shift from higher to lower GP was observed between 40% and 60% SS silage mixtures at the end  
399 of 24 hours incubation.

400 The higher ( $P < 0.001$ ) instant GP from rapidly soluble fraction (a) in 80% and 100% SS  
401 might reflect the more soluble fraction in SS, such as WSC. However, the negative “a” values,  
402 which are difficult to interpret in biological terms, might due to no gas production recordings  
403 between 10 to 20 hours of incubations or possible delays in the onset of fermentation due to slow  
404 microbial colonization (Kang and Wanapat, 2013). The greater GP rate constants (c) from the  
405 insoluble but slowly degradable fraction could be a subsequence of the greater availability to the  
406 microorganisms of fermentable nutrients in the silages with more AF. The greater insoluble  
407 fractions (b) in the silages with 80% and 100% SS may be related to their higher contents of  
408 more slowly fermented fibres, such as aNDFom and ADFom, which could produce more GP  
409 with longer incubation times.

410

## 411 **5. Conclusions**

412        Ensiling AF alone is not practical due to its high buffer capacity, pH and low WSC  
413 concentration, which make it unsuitable for producing high-quality silage. On the other hand,  
414 ensiling SS alone results in low IVDMD and IVOMD, and it indicates that the overall quality of  
415 SS-AF silage mixtures were better than would be predicted on the basis of proportional  
416 combinations of the silages prepared from SS or AF alone. Our results have demonstrated the  
417 interesting effect of mixing SS and AF for silage making on nutritive value and fermentation  
418 characteristics; it indicates that the overall quality of SS-AF silage mixtures was better than the  
419 silages prepared from SS or AF alone. The silage mixtures with SS to AF ratios of 20:80 and  
420 40:60 have the potential to be used for ruminant production. However, additional research is  
421 needed to study the effect of feeding such silage mixtures to ruminants on their voluntary feed  
422 intake and production performance.

423

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428

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**Table 1**

Chemical composition (g/kg DM) of SS (sweet sorghum) and AF (alfalfa) prior to ensiling.

Crop	DM	Ash	CP	WSC	EE	Starch	aNDFom	ADFom	ADL
AF	385.93	95.83	227.05	81.63	28.14	14.95	215.82	207.24	38.58
SS	282.06	67.10	72.47	186.69	17.76	93.35	481.37	287.59	57.45
SME	23.269	6.433	34.671	23.711	5.84	17.639	59.67	18.474	4.762
<i>P-value</i>	<0.001	<0.001	<0.001	<0.001	0.001	0.001	0.001	<0.001	0.019

DM, dry matter; CP, crude protein; WSC, water-soluble carbohydrates; EE, ether extract; OM, organic matter; aNDFom, neutral detergent fibre in OM; ADFom, acid detergent fibre in OM; ADL, acid detergent lignin.



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**Table 2**

Chemical composition (g/kg DM) of SS-AF (sweet sorghum-alfalfa) silage mixtures\*.

Items	0%SS	20%SS	40%SS	60%SS	80%SS	100%SS	SEM	linear	quadratic
DM	393.06 <sup>a</sup>	386.83 <sup>a</sup>	354.70 <sup>b</sup>	333.9 <sup>c</sup>	305.90 <sup>d</sup>	286.67 <sup>e</sup>	9.527	<0.001	0.036
Ash	115.66 <sup>a</sup>	108.29 <sup>b</sup>	96.67 <sup>c</sup>	85.51 <sup>d</sup>	78.79 <sup>e</sup>	72.75 <sup>f</sup>	3.748	<0.001	<0.001
CP	222.77 <sup>a</sup>	191.35 <sup>b</sup>	149.79 <sup>c</sup>	125.20 <sup>d</sup>	84.88 <sup>e</sup>	62.32 <sup>f</sup>	13.628	<0.001	0.048
WSC	18.34 <sup>e</sup>	17.66 <sup>e</sup>	42.35 <sup>d</sup>	46.62 <sup>c</sup>	55.63 <sup>b</sup>	80.92 <sup>a</sup>	5.309	<0.001	<0.001
Starch	9.19 <sup>f</sup>	17.56 <sup>e</sup>	27.49 <sup>d</sup>	34.06 <sup>c</sup>	48.83 <sup>b</sup>	79.69 <sup>a</sup>	5.617	<0.001	<0.001
EE	33.25 <sup>a</sup>	29.74 <sup>b</sup>	27.87 <sup>b</sup>	24.73 <sup>c</sup>	21.26 <sup>d</sup>	20.34 <sup>d</sup>	1.138	<0.001	0.341
aNDFom	228.33 <sup>f</sup>	275.44 <sup>e</sup>	320.92 <sup>d</sup>	337.80 <sup>c</sup>	445.57 <sup>b</sup>	504.08 <sup>a</sup>	23.101	<0.001	<0.001
ADFom	211.58 <sup>c</sup>	221.46 <sup>c</sup>	226.57 <sup>c</sup>	221.25 <sup>c</sup>	273.10 <sup>b</sup>	305.44 <sup>a</sup>	8.475	<0.001	<0.001
ADL	39.94 <sup>d</sup>	42.56 <sup>cd</sup>	47.25 <sup>bc</sup>	46.03 <sup>bc</sup>	49.14 <sup>b</sup>	55.72 <sup>a</sup>	1.264	<0.001	0.175
SP	91.29 <sup>a</sup>	88.43 <sup>a</sup>	89.85 <sup>a</sup>	90.12 <sup>a</sup>	65.73 <sup>ab</sup>	54.84 <sup>b</sup>	3.994	0.001	0.019
TP	10.62 <sup>c</sup>	14.64 <sup>bc</sup>	20.39 <sup>a</sup>	18.11 <sup>ab</sup>	21.10 <sup>a</sup>	20.47 <sup>a</sup>	0.971	<0.001	0.002
CT	11.34 <sup>b</sup>	12.01 <sup>ab</sup>	12.38 <sup>ab</sup>	12.72 <sup>ab</sup>	14.75 <sup>ab</sup>	16.06 <sup>a</sup>	0.519	0.034	0.286

602 \* Values within rows with different superscripts (<sup>a</sup>, <sup>b</sup>, <sup>c</sup>, <sup>d</sup>, <sup>e</sup> and <sup>f</sup>) are significantly different (P<0.05).  
603 DM, dry matter; CP, crude protein; WSC, water-soluble carbohydrates; EE, ether extract; OM,  
604 organic matter; aNDFom, neutral detergent fibre in OM; ADFom, acid detergent fibre in OM;  
605 ADL, acid detergent lignin; SP, saponins; TP, total phenolics; CT, condensed tannins.

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628 **Table 3**  
 629 Fermentation characteristics (g/kg DM) of SS-AF (sweet sorghum-alfalfa) silage mixtures\*.  
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Items	0%SS	20%SS	40%SS	60%SS	80%SS	100%SS	SEM	linear	quadratic
pH	5.03 <sup>a</sup>	4.92 <sup>b</sup>	4.75 <sup>c</sup>	4.62 <sup>d</sup>	4.51 <sup>e</sup>	4.16 <sup>f</sup>	0.069	<0.001	<0.001
NH <sub>3</sub>	108.49 <sup>a</sup>	78.17 <sup>b</sup>	62.73 <sup>c</sup>	50.49 <sup>d</sup>	24.89 <sup>e</sup>	7.66 <sup>f</sup>	0.952	<0.001	0.109
Lactic acid	58.83 <sup>c</sup>	66.65 <sup>c</sup>	59.95 <sup>c</sup>	92.81 <sup>b</sup>	132.06 <sup>a</sup>	137.27 <sup>a</sup>	7.949	<0.001	<0.001
Acetic acid	65.57 <sup>a</sup>	68.59 <sup>a</sup>	67.10 <sup>a</sup>	66.86 <sup>a</sup>	63.26 <sup>ab</sup>	57.06 <sup>b</sup>	1.027	<0.001	0.001
Propionic acid	0.65 <sup>a</sup>	0.63 <sup>ab</sup>	0.56 <sup>bc</sup>	0.49 <sup>c</sup>	0.57 <sup>bc</sup>	0.52 <sup>c</sup>	0.015	<0.001	0.013

631 \* Values within rows with different superscripts (<sup>a, b, c, d, e</sup> and <sup>f</sup>) are significantly different (P<0.05).  
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666 **Table 4**  
 667 Mineral profile (mg/kg DM) of SS-AF (sweet sorghum-alfalfa) silage mixtures\*.  
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Items	0%SS	20%SS	40%SS	60%SS	80%SS	100%SS	SEM	linear	quadratic
K	25908.21 <sup>a</sup>	22412.23 <sup>b</sup>	16066.18 <sup>c</sup>	16688.41 <sup>c</sup>	14155.02 <sup>cd</sup>	12106.15 <sup>d</sup>	1173.507	<0.001	0.001
Ca	14340.46 <sup>a</sup>	12807.43 <sup>b</sup>	11824.76 <sup>c</sup>	6590.70 <sup>d</sup>	5324.94 <sup>e</sup>	3572.38 <sup>f</sup>	989.563	<0.001	0.051
P	2967.30 <sup>a</sup>	2598.49 <sup>b</sup>	1910.01 <sup>c</sup>	1831.19 <sup>cd</sup>	1672.83 <sup>de</sup>	1491.05 <sup>e</sup>	128.316	<0.001	<0.001
Mg	3940.92 <sup>a</sup>	3945.68 <sup>a</sup>	4064.72 <sup>a</sup>	3194.58 <sup>b</sup>	3100.73 <sup>bc</sup>	2969.69 <sup>c</sup>	111.118	<0.001	0.002
Na	1679.69 <sup>b</sup>	2015.40 <sup>a</sup>	1782.78 <sup>ab</sup>	903.41 <sup>c</sup>	736.41 <sup>cd</sup>	527.59 <sup>d</sup>	139.571	<0.001	<0.001
Fe	717.12 <sup>a</sup>	696.82 <sup>b</sup>	622.01 <sup>c</sup>	505.18 <sup>d</sup>	444.17 <sup>e</sup>	423.78 <sup>f</sup>	28.330	<0.001	0.004
Zn	36.61 <sup>a</sup>	35.74 <sup>a</sup>	25.33 <sup>b</sup>	14.20 <sup>c</sup>	13.97 <sup>cd</sup>	10.54 <sup>d</sup>	2.569	<0.001	0.001
Mn	31.84	30.46	30.59	29.94	31.94	30.48	0.291	0.602	0.265
Cu	10.13	9.75	9.33	8.71	8.38	8.49	0.233	0.141	0.552
Mo	1.48	0.91	0.98	0.91	0.59	0.53	0.101	0.060	0.597
Co	0.25	0.25	0.23	0.22	0.19	0.19	0.008	0.081	0.874

669 \* Values within rows with different superscripts (<sup>a, b, c, d, e</sup> and <sup>f</sup>) are significantly different (P<0.05).  
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**Table 5**

*In-vitro* degradability (g/kg DM), ammonia (g/kg DM), pH, total volatile fatty acids (mM) and volatile fatty acids (mol/100mol) after 24 h incubation of SS-AF (sweet sorghum-alfalfa) silage mixtures\*.

Items	0%SS	20%SS	40%SS	60%SS	80%SS	100%SS	SEM	linear	quadratic
pH	6.81 <sup>a</sup>	6.81 <sup>a</sup>	6.80 <sup>a</sup>	6.76 <sup>ab</sup>	6.74 <sup>b</sup>	6.73 <sup>b</sup>	0.009	0.009	0.596
NH <sub>3</sub>	98.46 <sup>a</sup>	89.31 <sup>a</sup>	59.65 <sup>b</sup>	43.79 <sup>c</sup>	38.90 <sup>c</sup>	18.39 <sup>d</sup>	5.972	<0.001	0.152
IVDMD	666.56 <sup>a</sup>	665.44 <sup>a</sup>	603.75 <sup>ab</sup>	520.69 <sup>bc</sup>	494.25 <sup>c</sup>	457.89 <sup>c</sup>	18.559	<0.001	0.850
IVOMD	719.91 <sup>a</sup>	749.03 <sup>a</sup>	676.10 <sup>ab</sup>	580.23 <sup>bc</sup>	552.23 <sup>c</sup>	498.01 <sup>c</sup>	21.346	<0.001	0.341
tVFA	49.79 <sup>a</sup>	46.89 <sup>ab</sup>	45.79 <sup>ab</sup>	45.24 <sup>ab</sup>	44.82 <sup>ab</sup>	42.43 <sup>b</sup>	0.793	0.003	0.840
Acetate	66.48 <sup>a</sup>	66.75 <sup>a</sup>	66.17 <sup>ab</sup>	66.09 <sup>ab</sup>	66.04 <sup>ab</sup>	64.58 <sup>b</sup>	0.853	0.014	0.925
Propionate	17.68 <sup>a</sup>	17.69 <sup>a</sup>	17.18 <sup>ab</sup>	16.87 <sup>ab</sup>	16.85 <sup>ab</sup>	16.50 <sup>b</sup>	0.259	0.039	0.420
Butyrate	9.38	9.94	10.57	11.18	11.89	12.01	0.417	0.410	0.885
iso-Butyrate	1.83 <sup>a</sup>	1.73 <sup>a</sup>	1.51 <sup>ab</sup>	1.48 <sup>ab</sup>	1.45 <sup>ab</sup>	1.51 <sup>b</sup>	0.053	0.008	0.219
Valerate	3.90 <sup>a</sup>	3.48 <sup>ab</sup>	2.99 <sup>b</sup>	2.76 <sup>b</sup>	2.79 <sup>b</sup>	2.90 <sup>b</sup>	0.119	0.007	0.148
iso-Valerate	3.00 <sup>a</sup>	2.77 <sup>ab</sup>	2.29 <sup>b</sup>	2.21 <sup>b</sup>	2.23 <sup>b</sup>	2.33 <sup>b</sup>	0.092	0.007	0.127

704 \* Values within rows with different superscripts (<sup>a</sup>, <sup>b</sup>, <sup>c</sup> and <sup>d</sup>) are significantly different (P<0.05).  
705 DM, dry matter; IVDMD, *in-vitro* DM degradability; OM, organic matter; IVOMD, *in-vitro* OM  
706 degradability; tVFA, total volatile fatty acids.

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734**Table 6***In-vitro* gas production, estimated parameters of gas production and methane production (mL/g DM) of SS-AF (sweet sorghum-alfalfa) silage mixtures over 24 hours incubation\*.

Items	0%SS	20%SS	40%SS	60%SS	80%SS	100%SS	SEM	linear	quadratic
CH <sub>4</sub>	25.7 <sup>a</sup>	24.3 <sup>a</sup>	23.3 <sup>a</sup>	24.0 <sup>a</sup>	20.5 <sup>b</sup>	21.1 <sup>b</sup>	0.44	<0.001	0.949
2h	23.13 <sup>ab</sup>	26.87 <sup>a</sup>	24.37 <sup>ab</sup>	22.50 <sup>ab</sup>	20.00 <sup>b</sup>	19.37 <sup>b</sup>	0.736	0.015	0.108
4h	48.75 <sup>a</sup>	46.88 <sup>a</sup>	43.75 <sup>ab</sup>	40.63 <sup>ab</sup>	37.50 <sup>b</sup>	35.63 <sup>b</sup>	1.216	0.001	0.993
6h	69.37 <sup>a</sup>	68.75 <sup>a</sup>	62.50 <sup>ab</sup>	59.38 <sup>b</sup>	50.63 <sup>c</sup>	40.00 <sup>d</sup>	2.256	<0.001	<0.001
8h	90.00 <sup>a</sup>	90.00 <sup>a</sup>	83.75 <sup>a</sup>	73.75 <sup>b</sup>	60.00 <sup>c</sup>	48.75 <sup>d</sup>	3.123	<0.001	0.001
10h	107.50 <sup>a</sup>	103.75 <sup>a</sup>	98.13 <sup>a</sup>	88.13 <sup>b</sup>	68.75 <sup>c</sup>	58.75 <sup>d</sup>	3.848	<0.001	0.001
20h	146.87 <sup>a</sup>	145.63 <sup>a</sup>	140.63 <sup>ab</sup>	133.13 <sup>b</sup>	122.50 <sup>c</sup>	109.38 <sup>d</sup>	2.889	<0.001	<0.001
22h	148.75 <sup>a</sup>	149.36 <sup>a</sup>	148.75 <sup>a</sup>	136.88 <sup>b</sup>	131.87 <sup>b</sup>	116.87 <sup>c</sup>	2.549	<0.001	0.001
24h	151.25 <sup>a</sup>	154.38 <sup>a</sup>	153.75 <sup>a</sup>	141.88 <sup>b</sup>	130.00 <sup>c</sup>	121.25 <sup>d</sup>	2.716	<0.001	0.021
Estimated parameters <sup>#</sup>									
a	-12.75 <sup>c</sup>	-5.35 <sup>c</sup>	-3.84 <sup>b</sup>	-1.48 <sup>b</sup>	5.92 <sup>a</sup>	7.09 <sup>a</sup>	1.618	<0.001	0.708
b	179.12 <sup>b</sup>	179.69 <sup>b</sup>	188.23 <sup>ab</sup>	179.29 <sup>b</sup>	231.46 <sup>a</sup>	233.42 <sup>a</sup>	8.928	0.003	0.062
c	0.107 <sup>a</sup>	0.092 <sup>a</sup>	0.077 <sup>ab</sup>	0.070 <sup>ab</sup>	0.034 <sup>b</sup>	0.026 <sup>b</sup>	0.0063	<0.001	0.298

735 \* Values within rows with different superscripts (<sup>a</sup>, <sup>b</sup>, <sup>c</sup> and <sup>d</sup>) are significantly different (P<0.05).736 <sup>#</sup> a= instant gas production from rapidly soluble fraction (mL/g DM), b = slow gas production  
737 from insoluble fraction (mL/g DM), c = the rate of gas production from slowly insoluble fraction  
738 (mL/h).

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