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# Ensiling suitability and microbiological quality of Virginia fanpetals biomass

Maja Fijałkowska, Sebastian Wojciech Przemieniecki, Tomasz Kurowski, Krzysztof Lipiński, Zenon Nogalski, and Cezary Purwin

**Abstract:** Virginia fanpetals biomass is relatively suitable for ensiling when stem structure is damaged during harvest. Virginia fanpetals biomass is characterized by extensive lactic acid fermentation with a low proportion of volatile fatty acids (VFA). A microbiological evaluation confirmed the predominance of lactic acid bacteria and the presence of a small group of fungi. Ensiling effectively eliminated toxin-producing *Aspergillus* spp.

**Key words:** *Sida hermaphrodita*, silage, microbiological quality, forage.

**Résumé :** La biomasse de « Virginia fanpetals » convient pour l'ensilage lorsque la structure de la tige est endommagée lors de la récolte. La biomasse de « Virginia fanpetals » est caractérisée par une fermentation extensive de l'acide lactique avec une faible proportion d'acides gras volatils (VFA — « volatile fatty acids »). Une évaluation microbiologique a confirmé la prédominance de bactéries de l'acide lactique et la présence d'un petit groupe de champignons. L'ensilage a effectivement éliminé l'*Aspergillus* spp. producteur de toxines. [Traduit par la Rédaction]

**Mots-clés :** *Sida hermaphrodita*, ensilage, qualité microbiologique, fourrage.

Virginia fanpetals (*Sida hermaphrodita* (L.) Rusby) is a perennial plant of the mallow family (Malvaceae) that is grown mainly for energy biomass (Borkowska and Molas 2012). The plant could also be suitable for forage production due to its high yield potential, resistance to lodging and freezing, low soil nutrient requirements, and high drought tolerance (Franzaring et al. 2014). A disadvantage of Virginia fanpetals is that the plant is difficult to wilt prior to ensiling. The previous research into the practical use of Virginia fanpetals has focused on variations in the chemical composition of herbage in different periods of the growing season and under different fertilization regimes as well as the use of dried plant biomass in ruminant nutrition (Tarkowski 2006). The suitability of Virginia fanpetals for ensiling, fermentation quality, and microbial contamination levels has not been studied to date. The suitability of various plant

species for ensiling is influenced by the content of dry matter and water-soluble carbohydrates (WSC), buffering capacity, physical properties, and the quantitative and qualitative composition of epiphytic microflora (Purwin et al. 2010). In general, analyses of feed and silage contaminations focus on mycotoxins, whereas the presence and survival of toxin-producing fungi that can lead to the secondary contamination of silage under aerobic conditions and animal poisoning are rarely investigated. The main species of toxin-producing fungi include *Aspergillus flavus* (aflatoxin), *Penicillium verrucosum* (ochratoxin), *Fusarium graminearum*, *Fusarium culmorum* (deoxynivalenol and nivalenol), and *Fusarium verticillioides* (fumonisins) (Alonso et al. 2013).

The aim of this study was to analyze the chemical composition and microbiological quality of Virginia fanpetals biomass, and to evaluate its suitability for ensiling.

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The experimental material was first-harvest herbage of Virginia fanpetals (*S. hermaphrodita* (L.) Rusby) grown in a commercial plantation (53°05'27.7"N, 21°11'47.5"E) for 3 yr. At the beginning of the growing season, plants were fertilized with 100 kg N ha<sup>-1</sup>, 50 kg K<sub>2</sub>O ha<sup>-1</sup>, and 80 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup>. Herbage was harvested at the bud formation stage (11 June 2015) with a forage harvester equipped with a crusher, and it was ensiled in 220 L standard open-head high-density polyethylene (HDPE) drums (Brenntag GmbH, Germany) without drainage holes. Herbage was compressed to the density of 832 kg fresh matter m<sup>-3</sup>, covered using plastic wrap and hermetically closed. Samples of herbage ( $n = 3$ ) and 90 d silage ( $n = 3$ ), collected with a probe along the entire length of the barrels, were analyzed to determine their chemical composition (AOAC 2005), including the content of WSC — by the anthrone method (Thomas 1977), fiber fractions according to Van Soest et al. (1991): neutral detergent fiber, acid detergent fiber, and acid detergent lignin. Silage was additionally assayed for ammonia nitrogen, pH, and carboxylic acid, as described by Purwin et al. (2010). The lactic buffering capacity (LBC) of herbage was determined according to the method proposed by McDonald and Henderson (1962).

Silage and herbage samples of 100 g each were ground in an electric mill, and specimens of 250 mg were collected for molecular analysis. The mill was cleaned and disinfected by 10 min exposure to UV 260 nm light after grinding each sample.

The DNA was isolated with the use of the GeneMATRIX Food-Extract DNA Purification Kit (EurX, Poland), and was quantified with the Qubit<sup>®</sup> 2.0 fluorometer (Invitrogen Life Technologies, USA), and its quality was determined with the use of the NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

Total counts of *Lactobacillus* bacteria were determined by real-time PCR with the use of F\_allact\_IS and R\_allact\_IS primers and the P\_allact\_IS probe developed by Haarman and Knol (2006). Fungal counts were determined in the SYBR Green assay with NS1 and 58A2R primers (Martin and Rygiewicz 2005).

Toxin-producing fungi of the genera *Penicillium* and *Aspergillus* were detected by real-time duplex PCR with the use of ITSPF and ITSPR primers and PENP and ASPIP probes (Sunanthie et al. 2009). Duplex PCR was used instead of multiplex PCR, but no changes were made in the reagent concentrations given by the authors.

A quantitative analysis of *Lactobacillus* spp. was performed under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The following protocol was applied for the quantification of fungi: initial denaturation at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 52 °C for 30 s, and 72 °C for 30 s. The fluorescence signal was read at 78 °C for 15 s. Melting curve analyses involved a denaturing step at 95 °C for 15 s, annealing at 60 °C for 1 min, and melting in 0.3 °C steps up to 95 °C for 15 s.

Duplex PCR involved initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 20 s and 70 °C for 60 s. Reference strains of *Lactobacillus brevis*, *Penicillium chrysogenum*, and *Aspergillus niger* were used. Reaction efficiency ranged from 90% to 107%.

Three herbage samples were randomly collected from a pooled sample of harvested forage, and ensiling was performed in three replications. Each silage sample was quantified in duplicate. The results were analyzed statistically by Tukey's HSD test ( $\alpha = 0.05$ ) in the Statistica version 12 program (ANOVA).

The content of total protein, crude ash, WSC, and structural carbohydrates in Virginia fanpetals herbage (Table 1) corresponded to the values reported in alfalfa green forage at the bud formation stage (Purwin et al. 2014). The content of dry matter and crude ash was higher than that noted in previous studies investigating the suitability of Virginia fanpetals biomass for biogas production (Tarkowski 2006; Kwiatkowski et al. 2012). The WSC/LBC ratio was determined at 1.05, and it was higher than that reported in alfalfa (0.8) by Weissbach and Auerbach (2013) and lower than barley silage (2.0) and maize silage (2.7). Thus, it could be classified as relatively difficult to ensile. Virginia fanpetals silage was characterized by a low pH, a high proportion of lactic acid in total acids (>0.7), and a low proportion of acetic acid. Huhtanen et al. (2002) described the fermentation process as extensive lactic acid fermentation with relatively low concentrations of volatile fatty acids (VFAs). However, the share of ammonia N in total N exceeded the safe level by 3% (11% vs. 8%) as defined by Huhtanen et al. (2002). In a previous study, Virginia fanpetals herbage was characterized by a significantly less favorable fermentation profile (Kwiatkowski et al. 2012). The improvement in fermentation quality, observed in the present experiment, could be attributed to the damage of stem structure, which improved the substrate availability and increased the density of ensiled biomass.

An analysis of genetic material in the examined samples revealed 201.1 ± 69.4 µg DNA g<sup>-1</sup> in herbage and 19.6 ± 1.7 µg DNA g<sup>-1</sup> in silage, on average. The amount of DNA isolated from silage was 10-fold lower, which suggests that most DNA in herbage originated from plant cells in which genetic material had not been degraded. Ensiling contributed to the degradation of genetic material, which significantly decreased DNA yield to a level corresponding to that of microbial DNA.

Silage samples were characterized by a significant increase in the number of *Lactobacillus* spp. gene copies, and the increase in *Lactobacillus* spp. counts in silage was nearly 20-fold higher than in fresh forage samples. An analysis of fungal counts demonstrated that this group of microorganisms was significantly reduced during ensiling. Toxin-producing fungi of the genera *Aspergillus* and *Penicillium* were identified in each of the three herbage samples, whereas the presence of *Penicillium* spp. was noted in only one silage sample.

**Table 1.** Chemical composition (mean  $\pm$  SD) and microbiological parameters of Virginia fanpetals herbage and silage.

Parameter	Herbage	Silage
DM (g kg <sup>-1</sup> )	197 $\pm$ 2.01	160 $\pm$ 1.85
Crude ash (g kg <sup>-1</sup> DM)	83.5 $\pm$ 1.67	86.0 $\pm$ 1.87
Crude protein (g kg <sup>-1</sup> DM)	199 $\pm$ 6.03	193 $\pm$ 5.79
WSC (g kg <sup>-1</sup> DM)	73.0 $\pm$ 1.01	6.0 $\pm$ 0.12
NDF (g kg <sup>-1</sup> DM)	403 $\pm$ 11.96	423 $\pm$ 12.69
ADF (g kg <sup>-1</sup> DM)	308 $\pm$ 6.16	371 $\pm$ 7.42
ADL (g kg <sup>-1</sup> DM)	38.0 $\pm$ 0.42	61.0 $\pm$ 0.59
LBC (g kg <sup>-1</sup> DM)	69.8 $\pm$ 0.71	ND
pH	ND	4.08 $\pm$ 0.05
Lactic acid (g kg <sup>-1</sup> DM)	ND	125 $\pm$ 2.5
Acetic acid (g kg <sup>-1</sup> DM)	ND	21.4 $\pm$ 0.64
Butyric acid (g kg <sup>-1</sup> DM)	ND	9.39 $\pm$ 0.12
N-NH <sub>3</sub> (g kg <sup>-1</sup> TN)	ND	110 $\pm$ 0.33
Abundance of <i>Lactobacillus</i> spp. <sup>a</sup>	8.7 $\times 10^5$ $\pm$ 3.1 $\times 10^5$ B	1.5 $\times 10^7$ $\pm$ 5.8 $\times 10^6$ A
Abundance of fungi <sup>b</sup>	1.3 $\times 10^3$ $\pm$ 3.3 $\times 10^2$ A	1.0 $\times 10^2$ $\pm$ 9.8B
Toxigenic fungi <sup>c</sup>		
<i>Penicillium</i> spp.	+	-
<i>Aspergillus</i> spp.	+	+

**Note:** WSC, water soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; LBC, lactic buffering capacity; N-NH<sub>3</sub>, ammonia nitrogen; DM, dry matter; TN, total nitrogen; ND, not determined; SD, standard deviation; microbiological parameters: means with different uppercased letters in the same row differed at  $P < 0.01$  — AB; “+” — detected; “-” — not detected.

<sup>a</sup>Population size of *Lactobacillus* spp. calculated based on the number of copies of 16S rRNA genes.

<sup>b</sup>Population size of total fungi calculated based on the number of copies of ITS sequences.

<sup>c</sup>All of the detected toxigenic fungi were present in only trace amounts.

Due to the limitations of the applied method and a very low number of gene copies ( $\sim 10$ ), only qualitative results were presented for toxin-producing fungi (Table 1).

Potentially toxic fungi have been identified in silages by many authors, but most studies analyzed maize and grasses. The most commonly detected genera of fungi were *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Bysochlamys* sp., *Cladosporium* sp., *Mucor* sp., *Rhizopus* sp., *Paecilomyces* sp., *Scopularopsis*, and numerous yeast species (Alonso et al. 2013).

In a study of baled grass silage from 35 Irish farms, O’Brien et al. (2005) show a significant problem of fungal contamination of tested samples. The results of this study indicate that the presence of toxigenic fungi in the analyzed samples was traceable so that Virginia fanpetals could potentially be more resistant to the contamination with undesirable microorganisms.

The counts of *Lactobacillus* spp. bacteria determined in this study were similar to LAB counts reported in grass silage, whereas fungal counts corresponded to the number of yeast cells. Total microbial counts in herbage were relatively low, which could be attributed to drought during the growing season. A comparison of the quantity of microbial DNA and total DNA isolated in each

experimental treatment (data not shown) revealed that in silage samples, fungi, and *Lactobacillus* spp. accounted for nearly 100% of total DNA. The above could point to the elimination of other bacterial groups, such as spore-forming bacteria, from the microbial community, and decontamination during ensiling, which is difficult to achieve in grass silage (McEniry et al. 2008).

Virginia fanpetals biomass harvested before flowering stage has a similar chemical composition and a more desirable WSC/LBC ratio than alfalfa herbage at the bud formation stage. Virginia fanpetals silage without additives is characterized by extensive lactic acid fermentation with low concentrations of VFAs. A microbiological analysis confirmed the predominance of lactic acid bacteria and the presence of a small group of fungi. Potentially pathogenic fungi were identified in herbage and in one of the three silage samples, but only several cells per gram of fresh weight were noted. Further research is needed to analyze potential routes of contamination and possible decontamination methods for Virginia fanpetals herbage, especially, its high protein content and desirable carbohydrate composition point to its suitability for ruminant nutrition. Our previous study of sheep (C. Purwin and M. Fijałkowska,

unpublished data) confirmed high organic matter digestibility (above 66%) and high palatability of Virginia fanpetals comparing with grass and alfalfa silage.

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