Research Article

Received: 23 September 2016 Revised: 14 December 2016 Accepted article published: 8 March 2017 Published online in Wiley Online Library: 5 April 2017

(wileyonlinelibrary.com) DOI 10.1002/jsfa.8307

A novel feedstuff: ensiling of cowpea (*Vigna unguiculata* L.) stover and apple (*Malus domestica* Borkh.) mixtures. Evaluation of the nutritive value, fermentation quality and aerobic stability

Ederson Andrade,[a,b] Alexandre Gonçalves,[c] Ana Mendes-Ferreira,[d] Valéria Silva,[b] Victor Pinheiro,[b] Miguel Rodrigues[b,c] and Luis Ferreira[b,c*]

Abstract

BACKGROUND: Agro-industrial by-products are of low economic value as foods for human consumption but may have potential value as animal feedstuffs. This study evaluated a novel feedstuff, ensiled discarded apple (85%) and cowpea stover (15%) mixtures with two different ensiling periods (45 and 60 days), regarding the nutritive value, fermentation quality and aerobic stability.

RESULTS: Generally, no differences ($P > 0.05$) were observed between ensiling periods for nutritive value and fermentation characteristics. Silages were stable after ensiling, presenting high lactic acid (77.3 g kg^{-1} dry matter (DM)) and acetic acid (54.7 g kg^{-1} DM) and low ethanol (15.7 g kg^{-1} DM) and NH_3-N (105.6 g kg^{-1} total N) concentrations. No butyric acid was detected in silages, and they were aerobically stable for up to 216 h. Lactic acid bacteria numbers were high at silo opening (7.14 log colony-forming units (CFU) g^{-1}), while Enterobacteriaceae were not detected and yeasts/moulds were low (2.44 log CFU g^{-1}). Yeast/mould and Enterobacteriaceae numbers grew considerably during 12 days of air exposure.

CONCLUSION: A mixture of low calibre discarded apples with cowpea stover can be used as animal feed after the ensiling process owing to its nutritive value and long aerobic stability.

Keywords: discarded apple; legume stover; nutritional valorization; silage

INTRODUCTION

The European Union is now facing the challenge to increase its domestic legume grain production in order to cope with systematic constraints regarding its economic dependence on soybean imports and the volatility of international food commodity prices. In addition, European livestock production systems must face the challenge to meet world animal product demands using fewer resources. The foreseen increase in legume grain production for food and feed, within the frame of sustainable agriculture techniques, will also lead to the production of large amounts of legume stovers that can be used in animal feeding.

The amount of biomass produced by crop stovers is quite high, and straw is one of the main solutions through which these raw materials can be used in animal nutrition,[1] especially in the Mediterranean basin. In fact, although feed legume straws are quantitatively less used than cereal straws, they represent an important feed resource in certain agro-climatic zones.[2,3] Globally, in 2013, the production of cowpea (*Vigna unguiculata* L. Walp) grains was close to 8.0 Mt.[4] Cowpea is one of the most important cultivated legume crops, showing several environmental and economic advantages and improving the diets and incomes of farming families across Africa, Asia and South America.[5] Although cowpea is primarily valued as food for its grain, its stover is an important agro-based by-product that can be used in ruminant production owing to its protein and energy content.[6,7]

Several studies have recently explored the possibility of conserving straw through ensiling given its seasonal availability and the possibility to increase its nutritive value.[8–11] Although these

* *Correspondence to: L Ferreira, University of Trás-os-Montes and Alto Douro, Animal Science Department, Vila Real, Portugal. E-mail: lmf@utad.pt*

a *CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil*

b *Animal and Veterinary Research Centre, University of Trás-os-Montes e Alto Douro (UTAD-CECAV), Department of Animal Science, Vila Real, Portugal*

c *Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes e Alto Douro (UTAD-CITAB), Department of Animal Science, Vila Real, Portugal*

d *BioISI-Biosystems and Integrative Sciences Institute, Lisboa, Portugal*

experiments have been conducted with cereal straws, the nutritive value of legume straws[1] indicates that these feeds may also be evaluated for this purpose. Nevertheless, the high buffer capacity and low water-soluble carbohydrate content of legumes contribute to inadequate fermentation during ensilage, resulting in poor quality silages,[12] thus making it necessary to add a source of soluble sugars prior to ensilage.

Data reveal the high loss of fruit in orchards and the considerable cost involved in its disposal. These residues could be used as a source of fermentable carbohydrates, representing a valuable feed resource in mixed silages. Apple (*Malus domestica* Borkh.) is among the most cultivated and consumed tree fruits in the world, reaching production close to 80.5 Mt.[4] According to Wadhwa and Bakshi,[13] out of the total world production, 30–40% of apples are damaged and are discarded owing to their low calibre, presence of stains and deformations, among others, and therefore not marketed, thus representing a total residue of 24–32 Mt. In northern Portugal, these losses correspond to 15–30% of the total production, representing 13 000–17 000 t. The utilization of apple pomace as livestock feed has been evaluated,[14–17] mainly for ruminant diets owing to the high content of pectins and sugars, which are rapidly fermented in the rumen.[14,16] Rodrigues *et al.*[18] studied the nutritive value of discarded apple and wheat straw mixtures as an alternative ruminant feed and observed that silage mixtures of these two feeds were appropriate for animal feeding. The preparation of silages from apple pomace and straw mixtures has also been suggested in other studies.[19]

The aim of the present study was to use two agricultural by-products, discarded apples and cowpea stover, which through the ensiling process might have an improved nutritive value as animal feed. Hence the chemical and microbiological data, fermentation, aerobic stability and *in vitro* digestibility of an ensiled mixture of these two feeds were assessed in two different ensilage periods.

MATERIALS AND METHODS

Treatments and ensiling process

The experiment was conducted using discarded apples from the Douro region of northern Portugal and cowpea (*V. unguiculata* L. Walp, cv. 'fradel') stover collected in Famalicão, northern Portugal. The cowpea stover was obtained after pod collection and was cut and left in the field to dry. The drying process was completed inside a greenhouse to avoid possible damage from rainfall. Apples were ground to pass a 4 mm screen (Pachancho L29025 Cutting Mill, Braga, Portugal) until a homogeneous mash was obtained, while cowpea stover was chopped on a stationary chopper (JN Jensen & Sønner, Agerskov, Denmark) adjusted for a theoretical cut length of 2 cm. The two raw materials were then thoroughly mixed by hand to obtain a mixture containing 85% apple and 15% cowpea stover on a fresh weight basis. This composition was chosen as Rodrigues *et al.*[18] suggested it to be the most appropriate for animal feeding when mixing apple pulp and wheat straw.

Before ensiling, 2 mL L^{-1} propionic acid was added (in fresh matter) to the apple–cowpea stover mixture. This was done as previous studies showed that the ensilability of apple–straw mixtures may be improved by the use of silage additives that limit fermentation while lowering the pH of the mixture.[18] In this context, it has been shown that propionic acid has antimycotic activity while also improving the aerobic stability of silage.[20]

Following the addition of propionic acid, the mixture was conditioned in dark plastic bags, packed in 5 dm^3 plastic buckets (laboratory silos) and packed to an approximate wet density of 600 kg m^{-3} in 15 L laboratory silos. The silos were then sealed with tight lids and maintained at room temperature (26 ± 1.8 °C) for two different ensiling periods (45 and 60 days). Three replicates for each date of sampling were prepared, making a total of six laboratory silos.

After each ensiling period, 5 cm of silage from the surface of each experimental silo was discarded. Cheesecloth and aluminium foil were placed on top to prevent silage dehydration and dust contamination while allowing the entrance of air. Silage aerobic stability was measured and defined by the number of hours that the silage remained stable before its temperature reached 2 °C above ambient temperature.[21] The pH and NH_3-N profiles were monitored at 0, 2, 4, 6, 8, 10 and 12 days of aerobiosis, and yeast/mould, Enterobacteriaceae and lactic acid bacteria (LAB) counts were performed at silo opening and after 12 days of aerobiosis.

Fresh samples of raw materials, pre-ensiled mixtures and ensiled mixtures with 45 and 60 ensiling days, at silo opening and after 12 days, were collected for further microbiological and chemical analysis.

Microbiological analysis of silages

Microbiological analysis was carried out using the standard methodologies described for food and animal feeding stuffs, for the preparation, suspension and dilution of test samples,[22] enumeration of mesophilic LAB,[23] enumeration of Enterobacteriaceae[24] and enumeration of yeasts/moulds.[25] Briefly, 10 g of each sample was aseptically homogenized with 90 mL of buffered peptone water (BPW) using a stomacher (STAR Blender LB 400, VWR, Radnor, PA, USA). Tenfold serial dilutions were made for each sample and used for quantitative microbiological analyses. For LAB, 1 mL of each dilution was inoculated on a double-layered plate of de Man Rogosa Sharpe (MRS) agar (Liofilchem, Roseto degli Abruzzi, Italy) and incubated at 30 °C for 72 h. For Enterobacteriaceae, 1 mL of each dilution was inoculated on a double-layered plate of Violet Red Bile Glucose (VRBG) agar (Liofilchem) and incubated at 37 °C for 24 h. For yeasts/moulds, 0.1 mL of each dilution was inoculated on a double-layered plate of Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Liofilchem) and incubated at 25 °C for 72 h. All microbiological counts were expressed as log colony-forming units (CFU) g^{-1} sample. All analyses were performed in triplicate.

Chemical analysis

Collected samples of raw materials, pre-ensiled mixtures and silages at opening of the laboratory silos and at 12 days after silo opening were dried in a forced air oven at 60 °C and ground to pass a 1 mm screen (Retsch SM1 Cutting Mill, Haan, Germany). Samples were then stored in airtight flasks at room temperature for subsequent chemical analysis.

Dry samples were analysed for ash (942.05) and for total N (954.01) as Kjeldahl N following the methods of the Association of Official Analytical Chemists (AOAC).[26] Neutral detergent fibre (NDFom), acid detergent fibre (ADFom) and lignin (sa) fractions were calculated by the detergent procedures of Robertson and Van Soest[27] and Van Soest *et al.*[28] Sodium sulfite and heat-stable amylase were not used in the sequential analysis, and results were expressed exclusive of residual ash. The acid detergent-insoluble nitrogen (ADIN) of samples was also determined.[29]

The total water-soluble carbohydrate (WSC) and starch contents of samples were determined by the anthrone method.[30] Briefly, soluble sugars were extracted with 800 mL L^{-1} ethanol

from 100 mg of sample in a water bath, and starch was extracted with 300 mL L^{-1} perchloric acid. Next, 3 mL of anthrone solution was added to 200 µL of sample extract and heated in a water bath at 100 °C. Standard curves were prepared with stock glucose solutions. Finally, the absorbance of solutions at 625 nm was read in a spectrophotometer (Shimadzu UVmini 1240, Kyoto, Japan).

The buffering capacity (BC) of freshly macerated samples of the pre-ensiled mixture was measured as the amount of NaOH required to change the pH from 4 to 6, in accordance with the methodology suggested by Playne and McDonald,[31] and expressed as mmol NaOH kg^{-1} dry matter (DM).

Silage pH, NH_3-N, ethanol and organic acids (lactic acid and volatile fatty acids) were determined in water extracts obtained from the silages. Briefly, water extracts were prepared by adding 225 mL of distilled water to 25 g of silage. The pH value was measured using a Metrohm pH Meter 632 (Herisau, Switzerland). The NH_3-N concentration was determined following AOAC method 920.03.[26]

The volatile fatty acid (acetic, propionic and butyric) and ethanol concentrations were analysed according to Czerkawski[32] using a gas–liquid chromatograph (Shimadzu GC-14B, Kyoto, Japan) equipped with a flame ionization detector (FID) and a capillary column (SUPELCO Nukol, 0.25 mm i.d. × 30 m, 0.25 µm), with pivalic acid as the internal standard. Lactic acid was determined using an enzymatic assay procedure (K-DLATE 07/14, Megazyme, Bray, Ireland).

In vitro digestibility

The *in vitro* organic matter digestibility (IVOMD) of raw materials and pre-ensiled and ensiled samples was determined according to the methodology proposed by Tilley and Terry[33] and modified by Marten and Barnes.[34] Rumen fluid was collected from two non-lactating rumen-cannulated (Bar Diamond Inc., Parma, ID, USA) cows fed a diet composed of maize silage (0.70), concentrate feed (0.25) and meadow hay (0.05) shredded to 20 cm particles through a bale gripper (JN Jensen & Sommer). Diet was offered twice a day in equal amounts in the morning (08:00) and afternoon (16:00). From each cow, rumen fluid was collected 2 h after the morning meal and pooled into a pre-warmed insulated bottle filled with CO_2. Before use in the laboratory, the rumen fluid was strained and filtered through cheesecloth. All manipulations were under continuous flushing with CO_2.

Statistical analysis

Data were analysed using the GLM procedures of SAS Version 9.2.[35] The effects of aerobiosis and ensiling period and their interaction on the chemical composition and *in vitro* digestibility of ensiled mixtures were analysed by two-way analysis of variance (ANOVA) Ensiling period effects on silage stability and characteristics were analysed by one-way ANOVA.

RESULTS

Chemical composition and *in vitro* digestibility

The chemical composition of the materials at ensiling is presented in Table 1. Mashed apple presented higher total carbohydrate content (802.8 *vs* 6.7 g kg^{-1} DM), while cowpea stover showed higher ash, cell wall, protein and starch contents. These differences in chemical composition resulted in different IVOMD results, with the mashed apple showing a higher value (824.4 g kg^{-1}) than the cowpea stover (575.9 g kg^{-1}). As expected, the chemical composition of the mixture before ensiling mainly reflected the composition of the original materials. A buffer capacity of 159 ± 19.1 mmol NaOH kg^{-1} DM was measured for the pre-ensiled mixture.

Table 1. Chemical composition of raw materials (g kg^{-1} DM)

Item	Apple	Cowpea stover	Pre-ensiled mixture
Dry matter (g kg^{-1})	119.6	753.5	242.6
Ash	19.5	88.2	60.8
aNDF	138.6	619.2	393.6
ADF	113.1	544.0	262.4
ADL	41.3	117.2	66.3
Crude protein	28.3	143.8	102.4
WSC	802.8	6.7	432.2
NH_3-N (g kg^{-1} total N)	–	–	14.8
pH	–	–	5.1
BC (mmol NaOH kg^{-1} DM)	–	–	159.0
Starch	56.9	113.0	85.5
IVOMD	824.4	575.9	616.1

aNDF, neutral detergent fibre expressed exclusive of residual ash; ADF, acid detergent fibre; ADL, acid detergent lignin; WSC, water-soluble carbohydrates; BC, buffering capacity: IVOMD, *in vitro* organic matter digestibility.

In general, with the exception of the DM and ADIN fractions, results indicated the absence of effect of the ensiling period (i.e. 45 and 60 days) on the chemical composition and IVOMD of the silage (Table 2). Comparing the chemical composition of the obtained silage with that of the mixture before the ensiling process, a decrease of more than 90% in WSC content could be noted. On the other hand, increases in other chemical components, namely the cell wall fractions (i.e. NDFom, ADFom and lignin) and protein, were observed. However, this may only represent a change in proportion due to the fermentation of soluble constituents.

After opening the silos, during the aerobic period, an increase ($P < 0.05$) in NDFom and lignin fractions was observed, from an average of 525 to 556 g kg^{-1} DM and 100 to 113 g kg^{-1} DM respectively. Again, this may represent a change in proportion due to the consumption of other components such as WSC. In fact, the WSC content decreased from day 0 to day 12 (mean of 33 to 18 g WSC kg^{-1} DM). As a consequence of these modifications in the silage chemical composition, a decrease ($P < 0.05$) in IVOMD was determined during the aerobic period (from 593 to 573 g kg^{-1}).

Fermentation profile

The fermentation characteristics of the ensiled mixture are presented in Table 3. No differences ($P > 0.05$) were observed between silages of 45 and 60 ensiling days, except for ethanol, which presented a higher concentration in silage with 60 days of ensiling (17.9 g kg^{-1} DM). It should be emphasized that butyric acid was not detected in both silages. An upward trend was observed ($P = 0.0964$) for the NH_3-N content of silages at 60 days of ensilage.

Aerobic stability

The variation in pH and temperature difference throughout the 12 day aerobic period is reported in Fig. 1. No differences ($P > 0.05$) were observed between silages of 45 and 60 ensiling days. The silage pH increased from 3.8 at silo opening to 4.6 at the end of the aerobic period. Silage was stable on average for up to 216 h

Table 2. Effects of aerobiosis time and ensiling period on chemical composition and *in vitro* digestibility of ensiled mixtures (g kg^{-1} DM)[a]

Aerobiosis	Ensiling period	DM	Ash	aNDF	ADF	ADL	CP	ADIN	WSC	Starch	IVOMD
0 days	45 days	209.4	73.5	523.1	388.1	106.0	123.6	14.6	30.8	75.6	596.2
	60 days	187.8	74.6	526.5	358.4	94.7	117.7	16.2	34.9	68.7	590.0
12 days	45 days	201.2	85.7	543.1	389.2	112.5	121.3	13.3	14.4	77.5	577.9
	60 days	195.1	77.3	574.5	422.3	112.5	116.4	17.6	21.7	81.7	565.5
SEM		3.98	4.13	9.60	17.52	4.33	4.22	0.96	6.13	4.00	9.06
Effect	Aerobiosis (A)	0.8971	0.0757	0.0047	0.0696	0.014	0.6288	0.9621	0.0271	0.0681	0.0296
	Ensiling period (EP)	0.0051	0.3426	0.0749	0.9144	0.1752	0.1831	0.009	0.3186	0.7108	0.2751
	A × EP	0.0596	0.2209	0.1363	0.0768	0.1754	0.8945	0.1614	0.7694	0.1528	0.7065

DM, dry matter; aNDF, neutral detergent fibre expressed exclusive of residual ash; ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; ADIN, acid detergent-insoluble nitrogen; WSC, water-soluble carbohydrates; IVOMD, *in vitro* organic matter digestibility; SEM, standard error of mean.
[a] Mixture containing 85% apple and 15% cowpea stover on a fresh weight basis.

Table 3. Effects of ensiling period on silage pH and ethanol, organic acid and NH_3-N concentrations

Ensiling period	pH	Ethanol (g kg^{-1} DM)	Acetic acid (g kg^{-1} DM)	Propionic acid (g kg^{-1} DM)	Butyric acid (g kg^{-1} DM)	Lactic acid (g kg^{-1} DM)	NH_3-N (g kg^{-1} total N)	Aerobic stability (h)
45 days	3.82	13.5	54.3	7.0	ND	71.6	93.6	224
60 days	3.82	17.9	55.1	7.6	ND	82.9	117.6	208
SEM	0.042	0.47	3.90	0.51	–	3.04	7.86	28.8
Effect	1.00	0.0216	0.8886	0.4749	–	0.1204	0.0964	0.7149

ND, not detected; SEM, standard error of mean.

of aerobic exposure when its temperature exceeded 2 °C above ambient temperature.

The counts for LAB, Enterobacteriaceae and yeast/mould populations at silo opening and after the 12 day aerobic period are presented in Fig. 2. The LAB number was high and relatively steady throughout this period, varying from 7.14 to 8.17 log CFU g^{-1}. On the other hand, the Enterobacteriaceae and yeast/mould populations showed a significant increase. At day 0, Enterobacteriaceae were not detected, while yeasts/moulds were determined at 2.44 log CFU g^{-1}. Yeast/mould and Enterobacteriaceae numbers grew considerably during the 12 days of exposure to air, up to 7.13 and 5.67 log CFU g^{-1} respectively.

DISCUSSION

The chemical composition of the discarded apples used in this study is similar to that reported for commercialized regional Portuguese apple cultivars,[36] showing high total sugar content and low fibre and protein concentrations. As expected, fibre and protein contents of cowpea stover were high and within the range of values reported by Savadogo *et al.*[37] and Gonçalves *et al.*[7] Furthermore, chemical composition values of cowpea stover are within the general range of values described for legume straws.[1,2] The chemical composition of the pre-ensiled mixture reflected the composition of the original materials and their proportion in the mixture.

Forage ensilability is known to be mainly influenced by its DM and WSC contents and BC.[38] Based on the ensilability index (EI) developed by Martinez-Fernandez *et al.*,[38] the pre-ensiled mixture used in our study can be classified as having high ensilability (> +28) with an EI of +81.7. Although its DM content falls within the reference range for medium ensilability category (between 200 and 250 g kg^{-1} DM), the WSC content and BC are well within the high ensilability category (higher than 150 g kg^{-1} DM and lower than 250 mmol NaOH kg^{-1} DM respectively). In order to have a general basis of comparison of our data, it should be noted that, according to the same authors, extreme and opposite EI values have been identified for soybean characterized by low EI (−92.16) and maize with high EI (+78.33). The ratio WSC/BC of forage can also be used to characterize its suitability for ensiling. In our study, this ratio was 2.7, which is slightly lower than the minimum value of 3.0 suggested by Dinic *et al.*[39] for obtaining a good ensiling process and a high quality silage.

As stated before, results showed that the ensiling period (i.e. 45 and 60 days) did not influence the chemical composition of the mixture and its IVOMD, indicating that silage was stable at 45 ensiling days. Previous studies have shown that silage from apple pulp and wheat straw may achieve stability after 30 ensiling days.[17,18] Comparison of the pre-ensiled mixture with the silage at silo opening indicated clear changes in the chemical composition as a result of the ensiling process. The DM content of silages after 45 and 60 days of ensiling (209.4 and 187.8 g DM kg^{-1} respectively) was lower than that presented by the pre-ensiled mixture (242.6 g DM kg^{-1}). A similar trend was observed by Rodrigues *et al.*[18] when comparing pre-ensiled mixtures of wheat straw and apple pulp with the resultant silages after 15, 30 and 45 days of the ensiling process. This decrease in DM content can be the result of respiration by aerobic (i.e. dissimilation of carbohydrates to CO_2 and H_2O) and anaerobic or facultative anaerobic (e.g. production of CO_2 by heterolactic fermentation of carbohydrates and/or ethanol production from yeasts) microflora.[40,41] In fact, during the ensiling process, a reduction of 92.5% in WSC was observed as a result

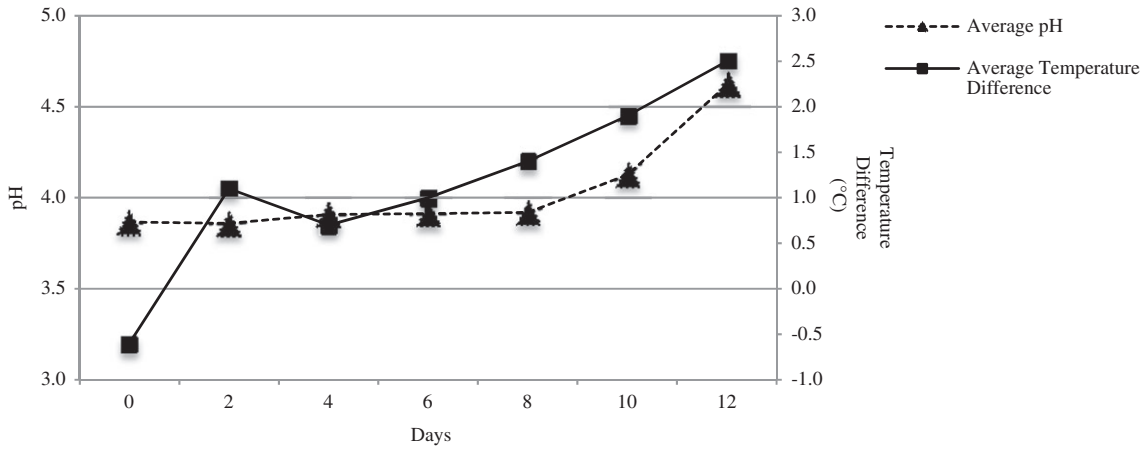

Figure 1. Effect of air exposure on silage pH and temperature difference between silage and ambient.

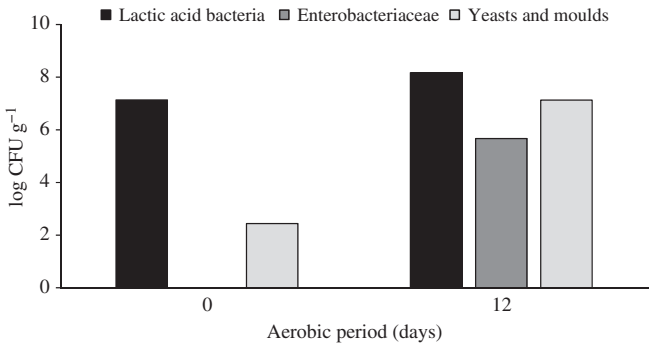

Figure 2. Lactic acid bacteria, Enterobacteriaceae and yeast/mould populations in apple and cowpea straw silages at silo opening and after 12 days of aerobic exposure.

of the activity of the microbial population. Slightly lower reduction of WSC (average of 82%) was found by Ke *et al.*[15] when ensiling alfalfa with apple or grape pomace, while Rodrigues *et al.*[18] observed an average reduction of 50% in WSC. High residual WSC concentrations in silages are required as they indicate more efficient fermentation.[42] On the other hand, an increase in the fibre fraction and protein contents of the silage was detected, possibly as a result of the decrease in its WSC content.[18] Nevertheless, Beigh *et al.*[17] suggested that an increase in silage protein content can also originate from the increase in silage microbial population. Although the ensiling altered the chemical composition of the silage, its IVOMD did not differ from that of the pre-ensiled mixture. The silage IVOMD values found in the present study were higher than those obtained by Rodrigues *et al.*[18] for apple pulp and wheat straw silages. The higher cell wall (713 *vs* 525 g kg^{-1} DM) and lower crude protein (28 *vs* 121 g kg^{-1} DM) contents of their silages, with 45 ensiling days, compared with those used in the present study may explain these differences.

Results on the fermentation parameters observed in the present study suggest that silages at 45 and 60 days of ensiling were well preserved, showing low pH values and high lactic acid concentrations. Similar pH values were observed by Rodrigues *et al.*[18] in silages of wheat straw with inclusion of 15% apple pulp, resulting from intense fermentation of WSC of the silage by the epithytic microbial population, especially LAB. These strict anaerobic bacteria should dominate this fermentation phase and mainly convert WSC into lactic acid (lower pK_a), decreasing pH values more efficiently.[40,43] Data on microbial populations at silo opening are consistent with these results, as a high number of LAB was determined (7.14 log CFU g^{-1}; Fig. 2). Similar LAB numbers (7.86 log CFU g^{-1}) were observed by Ke *et al.*[15] when ensiling dried apple pomace with wilted alfalfa (100 g dried apple pomace kg^{-1} wilted alfalfa). Although lactic acid concentrations of silages are dependent on their moisture content, values obtained in the present study are much higher than those observed by Alibes *et al.*[44] when ensiling apple pomace and barley straw, but are within the range of values reported by Ke *et al.*[15] Higher lactic acid concentrations were found by Fraser *et al.*[45] when ensiling two different varieties of white lupin (*Lupinus albus*) as a whole crop with or without inoculation (*Lactobacillus plantarum*). The ratio between lactic and acetic acids is also used to assess the ensiling process and silage quality. Chahine *et al.*[46] suggested that this ratio should vary between 1.5 and 4.0 for corn silages. This ratio was slightly lower in our silages, varying between 1.32 (45 days) and 1.50 (60 days) as a result of the high acetic acid concentrations, and may indicate that fermentation was less efficient.[47] Acetate found in silages may result from the activity of epiphytic Enterobacteriaceae and of both obligate and facultative heterofermentative LAB.[41,48] Arriola *et al.*,[42] Kung and Ranjit[49] and Kleinschmit and Kung[50] suggested that high acetate concentrations are normally found in silages inoculated with *Lactobacillus buchneri*. According to Oude Elferink *et al.*,[48] this obligate heterofermentative species converts some lactic acid into equimolar amounts of acetic acid and 1,2-propanediol, compounds generally associated with higher aerobic stability of silages owing to their inhibitory effects on yeasts. Although identification/distinction of these LAB species was not performed, results suggest a high presence of epiphytic populations of *L. buchneri* or other obligate heterofermentative LAB on the raw materials used in the present study. As stated before, acetate is the main fermentation product of enterobacteria, and their growth in silage is undesirable as they compete with LAB for nutrients, including sugars. According to Muck,[51] Enterobacteriaceae are inhibited once the pH drops below 4.5–5.0, and their populations become undetectable. Our results are consistent with this suggestion, as the number of Enterobacteriaceae was below the detectable levels at silo opening for silages with both 45 and 60 days of ensiling period.

NH_3-N concentrations in the ensiled mixture (mean of 105.6 g NH_3-N kg^{-1} total N) indicate to some extent the activity of Enterobacteriaceae, as they can degrade proteins, increasing NH_3-N levels.[41,51] Proteolytic clostridia may also be responsible for the

appearance of NH_3-N on silages as a result of the deamination or coupled oxidation reduction (Stickland reaction) of amino acids.[41,51,52] Nevertheless, the absence of butyric acid indicates that clostridia did not develop in large numbers.[53] NH_3-N concentrations found in the present study are within the range of values reported by Pirmohammadi *et al.*[19] when ensiling apple pomace and wheat straw. A broader band of values (67–179 g NH_3-N kg^{-1} total N) was obtained by Fraser *et al.*[45] for *L. albus* silages. In general, NH_3-N concentrations found in grass and corn are slightly lower, ranging between 80 and 100 g NH_3-N kg^{-1} total N.[42,54] The presence of *L. buchneri* on the raw materials may also be responsible for an increase in NH_3-N concentrations. Increased NH_3-N concentrations were observed by Driehuis *et al.*[54,55] in grass and corn silages as a result of their inoculation with *L. buchneri*.

Ethanol concentrations observed in silages with 45 and 60 days of ensiling period were low, indicating that the application of propionic acid in the pre-ensiled mixture fulfilled its role of preventing yeast development. Propionic acid is recognized as a very powerful fungicidal agent.[41] Ethanol concentrations were five to seven times lower than those previously observed by Rodrigues *et al.*[18] and Alibes *et al.*[44] in silages with comparable levels of apple pomace incorporation. This inhibition effect can also be observed in the low number of yeasts/moulds (2.44 log CFU g^{-1}) detected at silo opening, below the threshold typically associated with silage spoilage.[42] The high acetic acid concentrations found in our silages may also have affected yeast survival during the ensiling period.[48] In fact, at silo opening, yeast numbers were quite low. Kleinschmit and Kung[50] reported a significant reduction in yeast numbers at 56 days of the ensiling period of corn silage with greater acetic acid concentrations as a result of inoculation with *L. buchneri* and *Pediococcus pentosaceus*.

One of the main problems affecting silage quality is its aerobic deterioration after silo opening, caused by the activities of aerobic microbial populations such as bacteria, moulds and yeasts.[51] These activities result in modifications of the chemical composition of the silage as a result of the consumption of residual sugars, organic acids and ethanol, and increase the risk of proliferation of other undesirable microorganisms.[56,57] In the present study, aerobic stability of the silages was high, reaching almost 10 days. Data presented by Ke *et al.*[15] when ensiling alfalfa with apple pomace (246 h) or grape pomace (254 h) are within the same range of values. This relatively high aerobic stability may be due to the high acetic acid concentrations found in the silages. According to Woolford,[41] acetic acid has strong antifungal properties, and its high concentrations were probably the main reason for improvements in the aerobic stability of corn silages and wheat silage inoculated with *L. buchneri*.[55,58,59]

As stated before, the number of yeasts/moulds was quite low (2.44 log CFU g^{-1}) at silo opening and increased to 7.13 log CFU g^{-1} at 12 days after silo opening This level is above the threshold (5 log CFU g^{-1}) proposed by Woolford[41] for silages more prone to aerobic deterioration. Although it is not possible to discriminate between yeast and mould populations in this study, it is expected that the aerobic deterioration was initiated by yeasts, as they are acid-tolerant and some are lactate oxidizers,[60] and after this initial phase, moulds start to grow.[40] Besides using lactic acid, epiphytic yeasts are able to oxidize residual WSC into CO_2 and H_2O and other compounds that impair silage quality.[43] Consequently, silage pH increases and allows the growth of less acid-tolerant and harmful microorganisms that are involved in deterioration of silage. Our results are consistent with these microbial action mechanisms, as silage pH increased from 3.8 (day 0) to 4.6 (day 12). During the same period, WSC concentrations decreased from 32.9 to 18.1 g kg^{-1} DM, resulting from the activity of both yeasts and moulds, and later from Enterobacteriaceae activity. Indeed, as Enterobacteriaceae are less tolerant to acidic conditions, it is expected that the utilization of WSC by these bacteria occurred in the final days of the aerobic period when silage pH was near 4.5. According to Muck,[51] Enterobacteriaceae are inhibited below pH 4.5, although Östling and Lindgren[61] found that most enterobacteria species are able to grow at pH values above 4.0.

CONCLUSIONS

Results obtained in the present study showed that mixtures of discarded apples with cowpea stover could be conserved by the ensiling process. Further studies using animal trials should be conducted to evaluate the mixture acceptability as well as its incorporation levels in diets. However, the low residual WSC concentrations of the resulting silages indicate that lactic acid additives should be used to control microbial fermentation in order to improve the nutritive value. These silages were also characterized by long aerobic stability. Further studies should be conducted in order to evaluate different levels of cowpea stover incorporation in order to obtain mixtures with higher crude protein content without compromising the efficiency of the ensiling process.

ACKNOWLEDGEMENTS

This work received support by the European Project EUROLEGUME (Seventh Research Framework Programme of the European Union – FP7 research project no. 613781). EA is recipient of a predoctoral fellowship from the CAPES Foundation (grant number BEX-13521/13-6).

REFERENCES

1 López S, Davies DR, Giráldez FJ, Dhanoa MS, Dijkstra J and France J, Assessment of nutritive value of cereal and legume straws based on chemical composition and *in vitro* digestibility. *J Sci Food Agric* **85**:1550–1557 (2005).
2 Bruno-Soares AM, Abreu JMF, Guedes CVM and Dias-da-Silva AA, Chemical composition, *DM and NDF degradation kinetics in rumen of seven legume straws. Anim Feed Sci Technol* **83**:75–80 (2000).
3 Capper BS, The role of food legume straw and stubble in feeding livestock, in *The Role of Legumes in the Farming Systems of the Mediterranean Areas*, ed. by Osman AE, Ibrahim MM and Jones MA. Kluwer Academic, Dordrecht, pp. 151–162 (1990).
4 FAO, *Food and Agriculture Organization Corporate Statistical Database (FAOSTAT)*. Food and Agriculture Organization, Rome (2015).
5 Singh BB, *Cowpea. The Food Legume of the 21st Century*. Crop Science Society of America, Madison, WI (2014).
6 Anele UY, Hummel J, Arigbede OM, Böttger C and Südekum K-H, Chemical composition and nutritive value of some cowpea (*Vigna unguiculata* L. Walp) haulm varieties. *J Anim Sci* **88**(Suppl 2):621 (2010).
7 Gonçalves A, Goufo P, Barros A, Domínguez-Perles R, Trindade H, Rosa EAS *et al.*, Cowpea (*Vigna unguiculata* L. Walp), a renewed multipurpose crop for a more sustainable agri-food system: nutritional advantages and constraints. *J Sci Food Agric* **96**:2941–2951 (2016).
8 Gado HM, Salem AZM, Camacho LM, Elghandour MMY and Salazar MC, Influence of exogenous enzymes on *in vitro* ruminal degradation of ensiled rice straw with DDGS. *Anim Nutr Feed Technol* **13**:569–574 (2013).
9 Liu J, Liu X, Ren J, Zhao H, Yuan X, Wang X *et al.*, The effects of fermentation and adsorption using lactic acid bacteria culture broth on the feed quality of rice straw. *J Integr Agric* **14**:503–513 (2015).

10 Qiu X, Guo G, Yuan X and Shao T, Effects of adding acetic acid and molasses on fermentation quality and aerobic stability of total mixed ration silage prepared with hulless barley straw in Tibet. *Grassl Sci* **60**:206–213 (2014).
11 Wang Y-S, Shi W, Huang L-T, Ding C-L and Dai C-C, The effect of lactic acid bacterial starter culture and chemical additives on wilted rice straw silage. *Anim Sci J* **87**:525–535 (2016).
12 Nkosi BD, Meeske R, Langa T, Motiang MD, Modiba S, Mkhize NR *et al.*, Effects of ensiling forage soybean (*Glycine max* (L.) Merr.) with or without bacterial inoculants on the fermentation characteristics, aerobic stability and nutrient digestion of the silage by Damara rams. *Small Ruminant Res* **134**:90–96 (2016).
13 Wadhwa M and Bakshi MPS, *Utilization of Fruit and Vegetable Wastes as Livestock Feed and as Substrates for Generation of Other Value-added Products*. Food and Agriculture Organization of the United Nations, Bangkok (2013).
14 Fang J, Cao Y, Matsuzaki M and Suzuki H, Effects of apple pomace proportion levels on the fermentation quality of total mixed ration silage and its digestibility, preference and ruminal fermentation in beef cows. *Anim Sci J* **87**:217–223 (2015).
15 Ke WC, Yang FY, Undersander DJ and Guo XS, Fermentation characteristics, aerobic stability, proteolysis and lipid composition of alfalfa silage ensiled with apple or grape pomace. *Anim Feed Sci Technol* **202**:12–19 (2015).
16 Shalini R and Gupta DK, Utilization of pomace from apple processing industries: a review. *J Food Sci Technol* **47**:365–371 (2010).
17 Beigh YA, Ganai AM and Ahmad HA, Utilisation of apple pomace as livestock feed: a review. *Indian J Small Ruminants* **21**:165–179 (2015).
18 Rodrigues MAM, Guedes CM, Rodrigues AL, Cone JW, Van Gelder AH, Ferreira LMM *et al.*, Evaluation of the nutritive value of apple pulp mixed with different amounts of wheat straw. *Livest Res Rural Dev* **20**:6 (2008).
19 Pirmohammadi R, Rouzbehan Y, Rezayazdi K and Zahedifar M, Chemical composition, digestibility and *in situ* degradability of dried and ensiled apple pomace and maize silage. *Small Ruminant Res* **66**:150–155 (2006).
20 Kung L, Robinson JR, Ranjit NK, Chen JH, Golt CM and Pesek JD, Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. *J Dairy Sci* **83**:1479–1486 (2000).
21 Conaghan P, O'Kiely P and O'Mara FP, Conservation characteristics of wilted perennial ryegrass silage made using biological or chemical additives. *J Dairy Sci* **93**:628–643 (2010).
22 International Organization for Standardization, *Microbiology of Food and Animal Feeding Stuffs – Preparation of Test Samples, Initial Suspension and Decimal Dilutions for Microbiological Examination – Part 1: General Rules for the Preparation of the Initial Suspension and Decimal Dilutions*. ISO 6887-1:1999 (1999).
23 International Organization for Standardization, *Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Mesophilic Lactic Acid Bacteria – Colony-count Technique at 30 Degrees C*. ISO 15214:1998 (1998).
24 International Organization for Standardization, *Microbiology of Food and Animal Feeding Stuffs – Horizontal Methods for the Detection and Enumeration of Enterobacteriaceae – Part 2: Colony-count Method*. ISO 21528-2:2004 (2004).
25 International Organization for Standardization, *Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Yeasts and Moulds – Part 1: Colony Count Technique in Products with Water Activity Greater Than 0,95*. ISO 21527-1:2008 (2008).
26 AOAC, Official Methods of Analysis *(15th edn)*. Association of Official Analytical Chemists, Arlington, VA (1990).
27 Robertson JB and Van Soest PJ, The detergent system of analysis and its application in human foods, in *The Analysis of Dietary Fiber in Food*, ed. by James WPT and Theander O. Marcel Dekker, New York, NY, pp. 123–158 (1981).
28 Van Soest PJ, Robertson JB and Lewis BA, Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci* **74**:3583–3597 (1991).
29 Goering HK and Van Soest PJ, *Forage Fiber Analyses (Agricultural Handbook No 379)*. ARS-USDA, Washington, DC (1970).
30 Irigoyen JJ, Emerich DW and Sánchez-Díaz M, Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. *Physiol Plant* **84**:55–60 (1992).
31 Playne MJ and McDonald P, The buffering constituents of herbage and of silage. *J Sci Food Agric* **17**:264–268 (1966).
32 Czerkawski JW, The use of pivalic acid as a reference substance in measurements of production of volatile fatty acids by rumen micro-organisms *in vitro*. *Br J Nutr* **36**:311–316 (1976).
33 Tilley JMA and Terry RA, A two-stage technique for the *in vitro* digestion of forage crops. *Grass Forage Sci* **18**:104–111 (1963).
34 Marten GC and Barnes RF, Prediction of energy digestibility of forages with *in vitro* rumen fermentation and fungal enzyme systems, in *International Workshop on Standardization of Analytical Methodology for Feeds*, ed by Pigden WJ, Balch CC and Graham M. International Development Research Center, Ottawa, pp. 61–71 (1980).
35 SAS, SAS User's Guide 9.2 *(2nd edn)*. SAS Institute, Cary, NC (2009).
36 Guiné RPF, Sousa R, Alves A, Figueiredo C, Fonseca S, Soares S *et al.*, Composition of regional Portuguese apple cultivars in different harvest years. *Int J Fruit Sci* **9**:360–371 (2009).
37 Savadogo M, Zemmelink G and Nianogo AJ, Effect of selective consumption on voluntary intake and digestibility of sorghum (*Sorghum bicolor* L. Moench) stover, cowpea (*Vigna unguiculata* L. Walp.) and groundnut (*Arachis hypogaea* L.) haulms by sheep. *Anim Feed Sci Technol* **84**:265–277 (2000).
38 Martinez-Fernandez A, Soldado A, de la Roza-Delgado B, Vicente F, Gonzalez-Arrojo MA and Argamenteria A, Modelling a quantitative ensilability index adapted to forages from wet temperate areas. *Span J Agric Res* **11**:455–462 (2013).
39 Dinic B, Radović J and Jevtić G, Procedures for improvement of the quality of fermentation process and increase nutritive value of silages, XII International Symposium on Forage Crops of Republic of Serbia. *Biotechnol Anim Husb* **26**:261–274 (2010).
40 McDonald P, Henderson AR and Heron SJE, *The Biochemistry of Silage*. Chalcombe Publications, London (1991).
41 Woolford MK, *The Silage Fermentation*. Marcel Dekker, New York, NY (1984).
42 Arriola KG, Kim SC and Adesogan AT, Effect of applying inoculants with heterolactic or homolactic and heterolactic bacteria on the fermentation and quality of corn silage. *J Dairy Sci* **94**:1511–1516 (2011).
43 Dunière L, Sindou J, Chaucheyras-Durand F, Chevallier I and Thévenot-Sergentet D, Silage processing and strategies to prevent persistence of undesirable microorganisms. *Anim Feed Sci Technol* **182**:1–15 (2013).
44 Alibes X, Muñoz F and Rodriguez J, Feeding value of apple pomace silage for sheep. *Anim Feed Sci Technol* **11**:189–197 (1984).
45 Fraser MD, Fychan R and Jones R, The effect of harvest date and inoculation on the yield and fermentation characteristics of two varieties of white lupin (*Lupinus albus*) when ensiled as a whole-crop. *Anim Feed Sci Technol* **119**:307–322 (2005).
46 Chahine M, Fife TE and Shewmaker GE, Target values for corn silage. *Idaho Alfalfa and Forage Conf. Proc.*, pp. 1–5 (2009).
47 Danner H, Holzer M, Mayrhuber E and Braun R, Acetic acid increases stability of silage under aerobic conditions. *Appl Environ Microbiol* **69**:562–567 (2003).
48 Oude Elferink SJWH, Driehuis F and Gottschal JC, Silage fermentation processes and their manipulation. *Proc. FAO Electronic Conf. on Tropical Silage*, pp. 17–30 (2000).
49 Kung L and Ranjit NK, The effect of *Lactobacillus buchneri* and other additives on the fermentation and aerobic stability of barley silage. *J Dairy Sci* **84**:1149–1155 (2001).
50 Kleinschmit DH and Kung L, The effects of *Lactobacillus buchneri* 40788 and *Pediococcus pentosaceus* R1094 on the fermentation of corn silage. *J Dairy Sci* **89**:3999–4004 (2006).
51 Muck RE, Silage microbiology and its control through additives. *Rev Bras Zootec* **39**(supl. especial):183–191 (2010).
52 McDonald P, Silage fermentation. *Trends Biochem Sci* **7**:164–166 (1982).
53 Driehuis F and van Wikselaar PG, The occurrence and prevention of ethanol fermentation in high-dry-matter grass silage. *J Sci Food Agric* **80**:711–718 (2000).
54 Driehuis F, Oude Elferink SJWH and van Wikselaar PG, Fermentation characteristics and aerobic stability of grass silage inoculated with

Lactobacillus buchneri, with or without homofermentative lactic acid bacteria. *Grass Forage Sci* **56**:330–343 (2001).
55 Driehuis F, Oude Elferink SJWH and Spoelstra SF, Anaerobic lactic acid degradation during ensilage of whole crop maize inoculated with *Lactobacillus buchneri* inhibits yeast growth and improves aerobic stability. *J Appl Microbiol* **87**:583–594 (1999).
56 Gerlach K, Roß F, Weiß K, Büscher W and Südekum K-H, Changes in maize silage fermentation products during aerobic deterioration and effects on dry matter intake by goats. *Agric Food Sci* **22**:168–181 (2013).
57 Woolford MK, The detrimental effects of air on silage. *J Appl Bacteriol* **68**:101–116 (1990).
58 Weinberg ZG, Szakacs G, Ashbell G and Hen Y, The effect of *Lactobacillus buchneri* and *L. plantarum*, applied at ensiling, on the ensiling fermentation and aerobic stability of wheat and sorghum silages. *J Ind Microbiol Biotechnol* **23**:218–222 (1999).
59 Ranjit NK and Kung L, The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. *J Dairy Sci* **83**:526–535 (2000).
60 Pahlow G, Muck RE, Driehuis F, Oude Elferink SJWH and Spoelstra SF, Microbiology of ensiling, in *Silage Science and Technology*, ed by Buxton DR, Muck RE and Harrison JH. American Society of Agronomy, Madison, WI, pp. 31–93 (2003).
61 Östling CE and Lindgren SE, Inhibition of enterobacteria and *Listeria* growth by lactic, acetic and formic acids. *J Appl Bacteriol* **75**:18–24 (1993).

