UNIVERSITY OF TRÁS-OS-MONTES AND ALTO DOURO

Nutritional valorization of cowpea (*Vigna unguiculata*) co-product for animal feeding

Thesis of Ph.D. in Animal Science

EDERSON AMÉRICO DE ANDRADE

Promoters:

Prof. Dr. Miguel António Machado Rodrigues (CITAB, UTAD)

Prof. Dr. Luís Miguel Mendes Ferreira (CITAB, UTAD)

Prof. Dr. Victor Manuel de Carvalho Pinheiro (CECAV, UTAD)

VILA REAL, 2019

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Composition of jury:

Prof. Dr. Jorge Manuel de Azevedo (University of Trás-os-Montes and Alto Douro)

Prof. Dr. Rui Caldeira (University of Lisbon)

Prof. Dr. Javier García Alonso (Universidad Politécnica de Madrid)

Prof. Dr. Jorge Belarmino Ferreira de Oliveira (Instituto Superior Politécnico de Viseu)

Dr. Davi Savietto (Institut National de la Recherche Agronomique de Tolouse)

Dr. Cristina Vitória de Miranda Guedes (University of Trás-os-Montes and Alto Douro)

Prof. Dr. Maria José Marques Gomes (University of Trás-os-Montes and Alto Douro)

Prof. Dr. Miguel António Rodrigues (University of Trás-os-Montes and Alto Douro)

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Promoters

Miguel António Machado Rodrigues, Animal Science, PhD in Animal Science
Associate Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB)
P.O. Box, 5001-801 Vila Real, Portugal

Luís Miguel Mendes Ferreira, Animal Science, PhD in Animal Science
Assistant Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB)
P.O. Box, 5001-801 Vila Real, Portugal

Victor Manuel de Carvalho Pinheiro, Animal Science, PhD in Animal Science
Assistant Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Animal and Veterinary Science Center (CECAV)
P.O. Box, 5001-801 Vila Real, Portugal

Composition of the jury

Jury Presidency

Jorge Manuel de Azevedo, Animal Production, PhD in Zootechnical Engineering
Full Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Animal and Veterinary Science Center (CECAV)
P.O. Box, 5001-801 Vila Real, Portugal

External members

Rui Caldeira, DVM, MSc Animal Prod., PhD Animal Prod.
Full Professor, Faculty of Veterinary Medicine
University of Lisbon
P.O. Box, 1300-477, Lisbon, Portugal

Javier García Alonso, Ing. Agrónomo, PhD en Produción Animal
Full Professor, ETS Inginieria Agronómica, Alimentaria y de Biosistemas
Universidad Politécnica de Madrid
P.O. Box, 28040, Madrid, Spain

Jorge Belarmino Ferreira de Oliveira, Animal Science, PhD in Animal Science
Adjunct Professor, Escola Superior Agrária de Viseu
Instituto Superior Politécnico de Viseu
P.O. Box, 3500-606, Viseu, Portugal

Davi Savietto, Animal Science, PhD en Ciencia y Tecnología de la Producción Animal
Research, Institut National de la Recherche Agronomique de Tolouse (INRA),
GenPhySE.
P.O. Box, 31326, Castanet Tolosan, France

Internal Members

Cristina Vitória de Miranda Guedes, Animal Science, PhD in Animal Science
Research, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Animal and Veterinary Science Center (CECAV)
P.O. Box, 5001-801 Vila Real, Portugal

Maria José Marques Gomes, Animal Science, PhD in Animal Science
Assistant Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
P.O. Box, 5001-801 Vila Real, Portugal

Miguel António Machado Rodrigues, Animal Science, PhD in Animal Science
Associate Professor, Department of Animal Science
University of Trás-os-Montes and Alto Douro (UTAD)
Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB)
P.O. Box, 5001-801 Vila Real, Portugal

Thesis presented to University of Trás-os-Montes and Alto Douro to obtain the PhD Degree in Animal Science.

I Ederson Américo de Andrade, confirm that the work present in this thesis is my own. Where the information has been derived from other sources, I confirm that they were properly referenced in this thesis.

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Abstract

Nutritional valorization of cowpea (*Vigna unguiculata*) co-product for animal feeding

The concept of circular economy is considered a sustainable principle for the reuse of underutilized agro-industrial co-products by shielding global markets against scarcity of resources and volatility of prices. Agro-industrial co-products are of low economic value as foods for human consumption but may have potential value as animal feedstuff, and in the Mediterranean production systems, agricultural co-products have been used as an important feedstuff during periods of shortage of forage. Another possible utilization of these compounds relies on its inclusion on compound feeds, especially in rabbit production. The Eurolegume project, in which this thesis is developed, evaluated cowpea (*Vigna unguiculata*), pea (*Pisum sativum*) and fava (*Vicia faba*) local genetic resources for the development of new varieties for food and feed. Management and valorization of residual biomass for animal feed were also evaluated in the project and created the opportunity to enrol on a PhD program and develop this thesis. Cowpea stover has been shown to be a feedstuff with high cell wall content (666 g kg^{-1} DM), moderate protein value (121 g kg^{-1} DM) and high lignin content (109 g kg^{-1} DM) which in turn influence *in vitro* digestibility values for rabbits (396 g kg^{-1} OM) and ruminants (603 g kg^{-1} OM) and *in sacco* degradability for ruminants (Chapter 1). The leaves of the cowpea stover showed to be the fraction with high nutritional value and its conservation is considered important. Based on these results the thesis advances in two lines of research to evaluate the potential of cowpea stover as animal feed: 1) potential use of cowpea stover treated/untreated with white-rot fungi for animal feeding and 2) conservation of the mixture's treated/untreated cowpea stover and discarded apple by the ensilage process.

In Chapter 2 we evaluated the effect of the incorporation of 0, 20 and 40 g kg^{-1} of untreated cowpea stover in compound feed on the coefficients of total tract apparent digestibility of organic matter, crude protein, neutral detergent fibre and gross energy of growing rabbits. Cowpea stover presented a good potential to be used in rabbit feeding as its inclusion in the experimental diets up to 40 g kg^{-1} did not affect growth performances. However, its inclusion showed to negatively influence protein digestibility values, indicating that its inclusion should be further evaluated so that these possible detrimental effects could be overcome. In this way, chemical or biological pre-treatment of cowpea stover should also be considered as they might improve the digestibility of this feedstuff. As white-rot fungi degrade lignin efficiently, due their extracellular enzymatic system and hyphal penetration power, its potential to improve the nutritive value of cowpea stover was studied. In Chapter 3 five strains of white-rot fungi (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* and *Phlebia rufa*) were tested on the pretreatment of cowpea stover for 22 and 45 days of

incubation. In this study, *P. citrinopileatus* with 22 days of incubation presented the best results in the delignification (-46%) and increase in *in vitro* digestibility for rabbits (+30%) when compared to the control diet. This trial provided enough evidence to produced larger amounts of treated cowpea stover in order to increase the inclusion levels in rabbit´s diets. For this purpose, we formulated five diets containing 50 and 100 g kg^{-1} of treated cowpea stover and 50 and 100 g kg^{-1} untreated cowpea stover, and basal diet (Chapter 4). Animals fed diets with the treated cowpea stover showed a higher final live weight (+5,0%) and a reduction in blood cholesterol levels (-17%) than the ones fed diets with untreated cowpea stover.

As cowpea stover is harvested at the same time that high amounts of discarded apple are normally available, the possibility to use mixtures of both co-products and its potential to be ensiled were evaluated. These silages could be an alternative feedstuff during feed shortage periods. Thus, a novel feedstuff, ensiled discarded apple (85%) and cowpea stover (15%) mixtures with two different ensiling periods (45 and 60 days) on nutritive value, fermentation quality and aerobic stability was evaluated (Chapter 5). The mixtures evaluated could be conserved by the ensiling process. Both silages, with 45 and 60 days of ensiling period, were stable presenting low pH values (≤ 4.0), no butyric acid and high aerobic stability (216h). However, the low residual water-soluble carbohydrate (WSC) concentrations of the resulting silages indicated that lactic acid additives should be used to control microbial fermentation to improve its nutritive value. Furthermore, as fungi treated cowpea stover was already evaluated in previous trials, in Chapter 6, we evaluated the preservation by the ensilage process of treated cowpea stover (15%) and discarded apple (85%) and the effect of the use of a commercial inoculant (Sil-All LV®). The treatment of cowpea stover, before ensilage, with *P. citrinopileatus* modified the chemical composition and *in vitro* digestibility, resulting in an increase of its nutritive value when compared with untreated cowpea stover. Silages, inoculated and non-inoculated with Sil-All, were stable after 45 days of ensiling, presenting low pH values (≤ 4.2) and no butyric acid. Inoculated silage showed higher lactic acid and ethanol concentrations compared to non-inoculated silage. On the other hand, lower acetic acid was observed in the inoculated silage.

Keywords: basidiomycetes, ensilage, feedstuff, legume co-product, lignin

Resumo

Valorização nutricional de co-produto de feijão-caupi (*Vigna unguiculata*) para alimentação animal

O conceito de economia circular é considerado um princípio sustentável para a utilização de co-produtos agroindustriais, protegendo os mercados globais contra a escassez de recursos e a volatilidade dos preços. Co-produtos agroindustriais, em geral, são pouco utilizados na alimentação humana, mas podem apresentar potencial para uso na alimentação animal. Nos sistemas de produção do Mediterrâneo, co-produtos agrícolas são usados como uma importante fonte alimentar aos animais durante períodos de escassez de forragem. Ainda, sua incorporação nos alimentos compostos é usual, especialmente em dietas de coelhos. O projeto Eurolegume, no qual esta tese é desenvolvida, avaliou os recursos genéticos locais do feijão-frade (*Vigna unguiculata*), ervilha (*Pisum sativum*) e da fava (*Vicia faba*) para o desenvolvimento de novas variedades de grãos para alimentação humana. A gestão e valorização da biomassa residual para alimentação animal foi avaliada no projeto e criou a oportunidade de se inscrever no programa de doutorado e desenvolver esta tese. O restolho de feijão-frade tem se mostrado um alimento com alto conteúdo de parede celular (666 g kg^{-1} de MS), moderado teor de proteína (121 g kg^{-1} de MS) e alto teor de lignina (109 g kg^{-1} de MS) que por sua vez influencia valores de digestibilidade *in vitro* para coelhos (396 g kg^{-1} MO) e ruminantes (603 g kg^{-1} MO) e a degradabilidade *in sacco* para ruminantes (Capítulo 1). As folhas do restolho de feijão-frade são uma fração com alto valor nutricional e sua conservação é considerada importante. Com base nesses resultados, a tese avançou em duas linhas de pesquisa para avaliar o potencial do restolho de feijão-frade como alimento para animais, avaliando: 1) potencial uso de restolho de feijão-frade tratado ou não tratado com fungos da podridão-branca e 2) conservação de mistura de restolho de feijão-frade tratado ou não em associação com maçã descarte pelo processo de ensilagem.

No Capítulo 2 avaliamos o efeito da incorporação de 0, 20 e 40 g kg^{-1} restolho de feijão-frade em alimento composto e o efeito no coeficiente de digestibilidade aparente da matéria orgânica, proteína bruta, fibra em detergente neutro e energia bruta de coelhos em crescimento. O restolho de feijão-frade apresentou um bom potencial para ser utilizada na alimentação de coelhos, uma vez que sua inclusão nas dietas experimentais, até 40 g kg^{-1}, não afetou a digestibilidade dos nutrientes. Sua inclusão, entretanto, mostrou influenciar negativamente os valores de digestibilidade da proteína, indicando que deve ser mais bem avaliada para que esses possíveis efeitos prejudiciais possam ser superados. Desta forma, o pré-tratamento químico ou biológico do restolho de feijão-frade também foi considerado, pois pode melhorar a digestibilidade deste alimento. Como os fungos da podridão-branca degradam eficientemente a lignina com seu sistema enzimático extracelular e com seu poder de penetração das hifas, estudou-se seu

potencial para melhorar o valor nutritivo do restolho de feijão-frade. No Capítulo 3, cinco estirpes de fungos da podridão-branca (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* e *Phlebia rufa*) foram testadas no pré-tratamento do restolho de feijão-frade por 22 e 45 dias de incubação. Neste estudo, *P. citrinopileatus* com 22 dias de incubação apresentou os melhores resultados na deslenhificação (-46%) e aumento na digestibilidade *in vitro* de coelhos (+ 30%) quando comparado com o controlo. Este ensaio forneceu evidências suficientes para produzir maiores quantidades do restolho de feijão-frade tratado, a fim de aumentar os níveis de inclusão nas dietas de coelhos. Para tanto, foram formuladas cinco dietas contendo 50 e 100 g kg^{-1} de restolho de feijão-frade tratado e 50 e 100 g kg^{-1} de restolho de feijão-frade não tratado, além da dieta basal (Capítulo 4). Animais alimentados com dietas com restolho de feijão-frade tratado apresentaram um maior peso vivo final (+5%) e uma redução nos níveis de colesterol no sangue (-17%) do que aqueles alimentados com dietas com restolho de feijão-frade não tratado.

Como o restolho de feijão-frade é colhida ao mesmo tempo que quantidades elevadas de maçã descartadas estes estão normalmente disponíveis. Sendo assim a possibilidade de usar misturas de ambos os co-produtos e seu potencial para conservação na forma de ensilagem foram avaliadas. Estas silagens podem ser um alimento alternativo durante os períodos de escassez de alimentação. Assim, avaliou-se um novo alimento, ensilagem de maçã descarte (85%) e restolho de feijão-frade (15%) em dois diferentes períodos de ensilagem (45 e 60 dias) sobre valor nutritivo, qualidade de fermentação e estabilidade aeróbia (Capítulo 5). As misturas avaliadas puderam ser conservadas pelo processo de ensilagem. Ambas as silagens, com 45 e 60 dias de ensilagem, apresentaram-se estáveis com baixos valores de pH (≤ 4,0), ausência de ácido butírico e alta estabilidade aeróbia (216h). No entanto, as baixas concentrações residuais de hidratos de carbono solúveis em água das silagens resultantes indicaram que os aditivos de ácido láctico devem ser utilizados para controlar a fermentação microbiana para melhorar o seu valor nutritivo. Além disso, testamos o restolho de feijão-frade tratado com fungos, já avaliado em ensaios anteriores, o potencial de conservação pelo processo de ensilagem juntamente com a maçã descarte (85%) e o efeito do uso de inoculante (Sil-All LV®, Capítulo 6). O tratamento do restolho de feijão-frade, antes da ensilagem, com *P. citrinopileatus* modificou a composição química e a digestibilidade *in vitro*, resultando em um aumento do seu valor nutritivo quando comparado com o restolho de feijão-frade não tratado. Silagens inoculadas e não inoculadas com Sil-All permaneceram estáveis após 45 dias de ensilagem, apresentando baixos valores de pH (≤ 4,2) e ausência de ácido butírico. A silagem inoculada apresentou maiores concentrações de ácido láctico e etanol em relação à silagem não inoculada. Por outro lado, ácido acético inferior foi observado na silagem inoculada.

Palavras-chave: basidiomicetos, co-produto de leguminosas, ensilagem, lenhina

Summary

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List of acronyms and abbreviations

a	soluble/rapidly degradable fraction (%)
a*	redness (CIELAB colour dimension)
a+b	total potential degradable fraction (%)
ADF	acid detergent fibre
ADIN	acid detergent-insoluble nitrogen
ADL	acid detergent lignin
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
b	slowly degradable fraction (%)
b*	yellowness (CIELAB colour dimension)
BC	buffering capacity
BPW	buffered peptone water
c	fractional degradation rate of fraction (h^{-1})
C	Control
c*	chroma – colour saturation
CCW	chilled carcass weight
cm	centimetre
CO_2	carbon dioxide
CP	crude protein
CS	cowpea stover
CS0	diet containing 0 g kg^{-1} of cowpea stover
CS2	diet containing 20 g kg^{-1} of cowpea stover
CS4	diet containing 40 g kg^{-1} of cowpea stover
CTTAD	coefficients of total tract apparent digestibility
cv.	Cultivar
DE	digestible energy
DM	dry matter
DP	digestible protein
DRBC	dichloran Rose Bengal Chloramphenicol agar
e.g. / i.e.	for example
EU	Europe Union
FAO	Food and Agriculture Organization
FAOstat	Food and Agriculture Organization Corporate Statistical Database
g	Gram
g MJ	kilogram per megajoule
GLM	generalised linear model
h	Hour
h^{-1}	Hour
HC	hemicellulose
IVD	*in vitro* digestibility
IVOMD	*in vitro* organic matter digestibility

kg^{-1}	Kilogram
kg DM ha^{-1}	kilogram of dry matter per hectare
kJ kg^{-1} DM	kilojoule per kilogram of dry matter
L	Litre
L*	lightness (CIELAB colour dimension)
LAB	lactic acid bacteria
log CFU g^{-1}	logarithmic colony-forming unit per gram
m	Meter
min	Minute
MJ kg^{-1}	mega joule per kilogram
mL L^{-1}	millilitre per litter
mm	millimetre
MRS	de man, rogosa and sharpe
Mt	Megaton
N	Nitrogen
NaOH	sodium hydroxide
NDFom	neutral detergent fiber, ash free
NH_3	Ammonia
NH_3-N	ammoniacal nitrogen
ºC	degree Celsius
OM	organic matter
P	probability
pH	potential of hydrogen
pKa	acid dissociation constant at logarithmic scale
SAS	statistical analysis system (software package)
SEM	standard error of mean
SW	slaughter weigh
t ha^{-1}	tonnes per hectare
t^{-1}	tonnes
Tg	Teragrams
TS10	experimental diet containing 100 g kg^{-1} of treated cowpea stover
TS5	experimental diet containing 50 g kg^{-1} of treated cowpea stover
US10	experimental diet containing 100 g kg^{-1} of untreated cowpea stover
US5	experimental diet containing 50 g kg^{-1} of untreated cowpea stover
VFA´s	volatile fatty acids
VRBG	violet red bile glucose
WSC	water-soluble carbohydrate
WSC/BC	ratio between water-soluble carbohydrate and buffering capacity
µg	microgram
µL	Microlitre
µm	micrometre

List of publications

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Andrade, E., Gonçalves, A., Mendes- Ferreira, A., Silva, V., Pinheiro, V., Rodrigues, M., Ferreira, L. (2017). 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. *Journal of the Science of Food and Agriculture*, 97(13), 4306-4313. doi: 10.1002/jsfa.8307

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Andrade, E., Pinheiro, V., Marques, G., Alves, A. B., Cone J. W., Serra, C., Mendes, C., Saavedra, M., Ferreira, L., Rodrigues, M. Incorporation of cowpea stover untreated and pre-treated with *Pleurotus citrinopileatus* on performance, digestibility, health and meat quality of rabbit growing. Submitted to *Animal Feed Science and Technology.*

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General introduction, aims and thesis outline

The livestock production system is in a challenging scenario for the coming decades, due to population growth, urbanization increase and rising in income (FAO, 2011; Gerber *et al*., 2013). FAO (2011) estimates that by 2050 we will need to increase meat production by 73% (from 268.7 to 463.8 million t^{-1}) and milk production by 58% (from 657.3 to 1038.4 million t^{-1}). Added to these difficulties is the cost of animal feed, mainly by the seasonality of production and the volatility of the prices of the main raw materials. In fact, some raw materials, i.e. corn, barley and wheat, used in animal feeding competes with other sectors, human food and biofuels. In animal production systems the feeding represents the highest part of the production costs. In rabbit's production the greatest threats to the producer profitability is the cost of feed, which can reach up to 70% of total costs (Gidenne *et al*., 2017). Therefore, the inclusion of alternative feedstuffs in herbivores feeding to reduce costs and ensure their growth performance has become more important.

Within these feedstuffs, the post-harvest residue of crops is quite abundant, but their nutritive value is low due to high fiber and lignin contents (Bruno-Soares *et al*., 2000; López *et al*., 2005; Tuyen *et al*., 2012). In fact, with the increase of physiological age of the plants the proportion of cellulose, hemicellulose and lignin increases (fiber content), with a consequent reduction of the potentially digestible constituents (soluble carbohydrates, crude protein, vitamins and minerals; Blaser *et al*., 1986; Morrison *et al*., 1998; Ball *et al*., 2001; Filya, 2004) leading to a decline in the nutritive value (Figure 1). Nevertheless, these feedstuffs may be normally included in herbivores diets due to the concentrations in potentially digestible structural carbohydrates (cellulose and

hemicellulose), that may contribute to animal health issues and thus influence animal performance (Montagne *et al*., 2003; Gidenne, 2003; Zebeli *et al*., 2013).

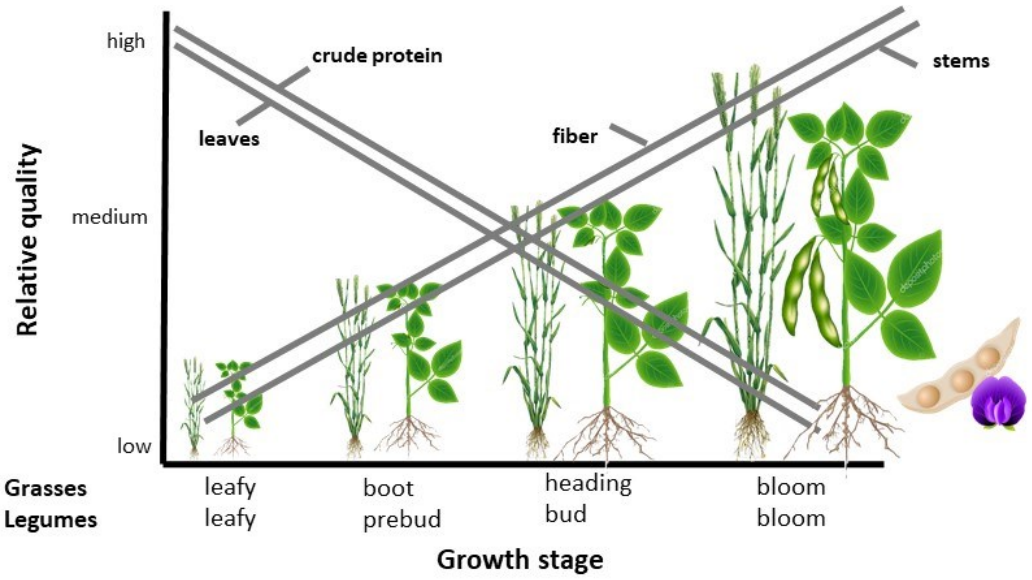

Figure 1. Effect of plant maturity on chemical composition and relative quality.
Source: Adapted of Blaser *et al*., 1986; Ball *et al*., 2001

According to Sadh *et al*. (2018) the use of agro-industrial co-products as feedstuffs can help to reduce the production costs and the pollution load to the environment. These authors pointed that agro-industrial co-products can be divided in two different types: agriculture residues (field residues or process residues) and industrial residues (Figure 2). Field residues may be important feedstuffs as complementary feeds for feeding animals during periods of forage shortage (Anele *et al*., 2011). In Europe, cereal straws are recognized as one of the main feedstuffs for the biobased economy, given the volume annually produced, availability and nutritional importance (Bakker *et al*., 2013). Thus, European Commission proposes the use of agricultural residues as an alternative to preserve biodiversity of ecosystems and as an option to reduce land use and fossil fuels (European Commission, 2011; Gobin *et al*., 2011; Ribeiro *et al*., 2015; Sadh *et al*., 2018).

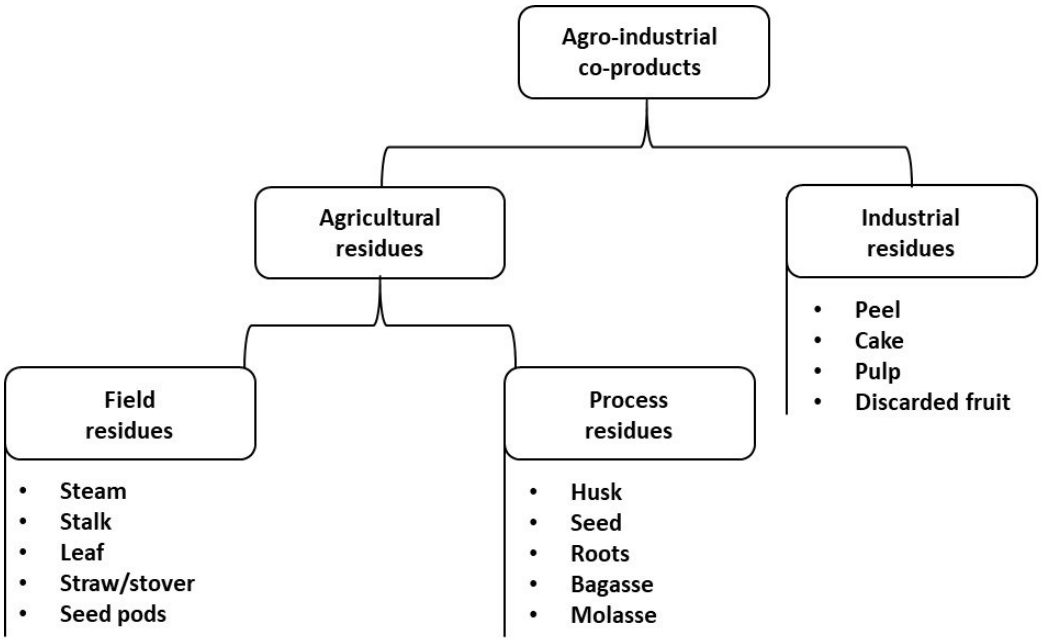

Figure 2. Agro-industrial co-products and origins
Source: Adapted Sadh *et al*. (2018)

The modernization of agriculture and efforts of governments to ban the burning of agricultural residues will lead to more straw and stover being marketed as alternative feedstuffs for the biobased economy (Bakker *et al*., 2013). In many countries, part of the remaining residues after harvest are collected using self-propelled baling machines (Bakker *et al*., 2013) or ensiling machines. The sustainable removal of part of this post-harvest residues (33-50%) has environmental and economic benefits and no harmful effects on land ecology (Ecofys, 2012).

In Europe, the legume crop currently accounts for less than 4% of arable area (Voisin *et al*., 2014). The legume production offers a range of benefits such as high protein content for human and animal feed, atmospheric nitrogen fixation, reductions of pests in the cropping system, improvement of the soil quality and reduction of greenhouse gases emissions (Zander *et al*., 2016). In the last decade, the European Union has emphasized the need to increase the production of local legumes in order to

reduce soybean imports from America and to reduce the impact of intensive cereal production (Cernay *et al*., 2016; Carvalho *et al*., 2017; Rosa *et al*., 2017).

To promote the development and production of new cultivars of legumes adapted to European conditions several projects such as, mention the financing of the Eurolegume and Legato projects have been recently supported by European funding's (Carvalho *et al*., 2017; Rosa, 2017). The Eurolegume project, in which this thesis is included, evaluated of cowpea (*Vigna unguiculata*), pea (*Pisum sativum*) and fava (*Vicia faba*) local genetic resources for the development of new varieties for food and feed. Management and valorization of residual biomass for animal feed were also evaluated in the project.

Although used on a smaller scale when compared to cereal residues, residues of legume crops have high production potential and generally present higher nutritive value due to their higher protein and lower fibre content (Suttie, 2000; Bruno-Soares *et al*., 2000¸ López *et al*., 2005). In fact, López *et al*., 2005 evaluating different residues of legume and cereals concluded that legume residues are degraded in the rumen at a faster rate and presented higher dry matter digestibility when compared with cereals straws. As the plant cell develops, a reduction in leaf/stem ratio is observed, and at the same time phenolic acids and lignin are deposited in the maturing cell wall (Van Soest, 1994; Jung and Allen, 1995). These changes directly affect the nutritional value and may limit its utilization in animal feed (Van Soest, 1994; Jung and Allen, 1995; Tuyen *et al*., 2012). Among the different antinutritional factors in the agricultural residues, lignin is considered the most important. In animal feeding, the lignin content can limit the digestion of polysaccharides by three possible mechanisms: 1) toxic effect of lignin components (e.g. p-coumaric acid) on microbiota; 2) physical impediment in lignin-polysaccharide link, limiting the action of fibrolitic enzymes; and 3) limitation of

hydrophilic enzymes due the hydrophobicity of lignin polymers (Jung and Deetz, 1993). The reduction in the indigestible cell-wall fraction (lignin) is beneficial because this will increase digestibility (Jung and Allen, 1995).

The pretreatment of post-harvest residues may reduce lignin in the cell wall resulting in a substrate easily hydrolysed by enzyme-producing microorganisms wich release sugars for fermentation (Bajpai, 2016). Among these technologies are the physical treatments through grinding, steam and granulation (Liu *et al*., 1999; Wan Zahari *et al*., 2003); the chemical treatments with the use of alkaline substances, such as NaOH and NH_3 (Sundstøl and Owen, 1984; Dias-da-Silva et al., 1990; Guedes et al., 2006; Sarnklong *et al*., 2010), leading to an improvement of intake and digestibility of these residues. However, these treatments are considered expensive, risky for users, and have a high environmental cost (Van Soest, 2006). In this way, biological treatments using white-rot fungi have shown promising results. These are selective to lignin degradation, disrupting the plant cell wall, converting polysaccharides into sugars of high assimilation, and the fungal mass that develops in the material can serve as a source of protein (Villas-Bôas *et al*., 2002; Bajpai, 2016). Recent studies have pointed out an improvement in the nutritional value of low-quality feeds (Sarnklong *et al*., 2010; Shrivastava *et al*., 2011; Tian *et al*., 2012; Shrivastava *et al*., 2012; Tuyen *et al*., 2012; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015; Sharma and Arora, 2015; Mao *et al*., 2018). However, this technology requires optimization to become competitive with conventional treatments such as alkaline and urea treatments (Van Kuijik *et al*., 2015). In this respect, future research should focus on the selection of candidate strains for certain biomass materials, optimization of cultivation methods (Tian *et al*., 2012) and evaluating the incorporation of pre-treated residues in *in vivo* trials (Van Kuijk *et al*.,

2015), including performance studies, digestibility of nutrients, health evaluation and meat quality measurements.

Mature plants have a lower leaf/stem ratio with a reduction in leafiness. negatively affecting the nutritive values to (Van Soest, 1994; Ball *et al*., 2001). At the time of crop harvesting the plant presents a high amount of dry matter and woody stems. In legumes, the stem has a slower rate of moisture loss, due to its composition and thickness, in comparison to the leaves that tend to detach from the stem very easily, thus decreasing the nutritive value of dried feedstuffs (Harris and Tullberg, 1980; MacDonalds and Clark, 1987; Ball *et al*., 2001). Another difficulty in hay production is in the "extreme hardness" of this material that may injure an animal's mouth, lowering intake (Ball *et al*., 2001). Silage of residues may be an alternative for the conservation of biomass and use during the season of low-growth and low-quality of pasture (Reyes-Gutiérrez et al., 2015). Recent works associate the fibrous material with an energetic source to favor the process and to produce a material of interesting alimentary value (Rodrigues *et al*., 2008; Ke *et al*., 2015; Wang *et al*., 2017; Mao *et al*., 2018). Sugarcane molasses and residues from the fruit industry have been shown to be promising (Rodrigues *et al*., 2008, Ke *et al*., 2015; Wang *et al*., 2017, Mao *et al*., 2018). Apple (*Malus domestica*) and its derived compound one of the major fruit industries all over the world (Simitzis and Deligeorgis, 2018). Rejected or discarded apples may account for 30% of total production (Kennedy *et al*., 1999; Wosiacki and Nogueira, 2001).

Aims and thesis outline

The potential use of post-harvest residues of cowpea (*Vigna unguiculata*) without (intact stover) or with biological pre-treatment (white-rot fungi and ensilage process) for animal feeding was investigated in this thesis. The thesis is structured in six chapters based on published scientific articles (3) and / or submitted (3), Figure 3.

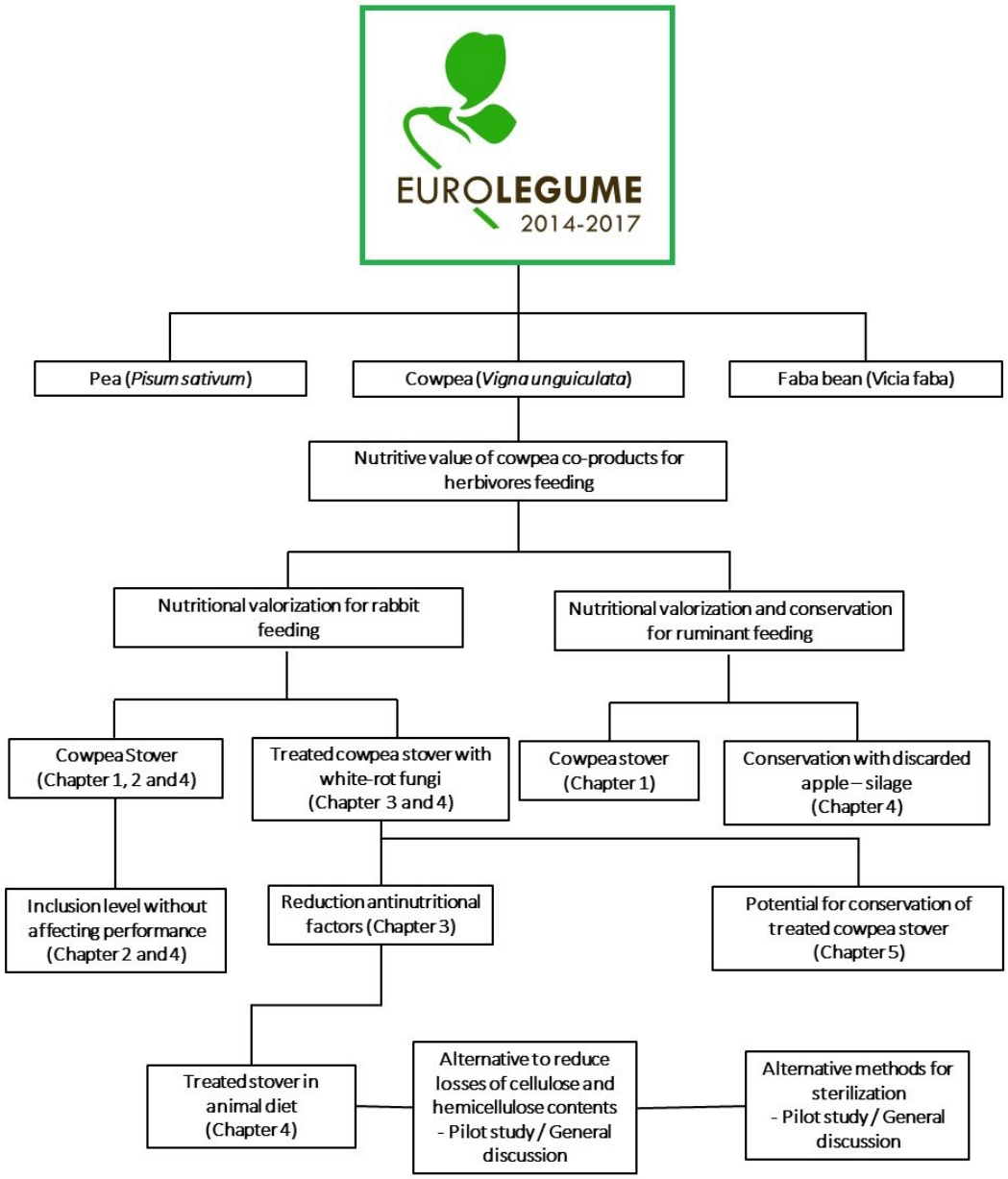

Figure 3. Fluxogram of experimental trials

In Chapter 1, general considerations about cowpea stover and its main constituents (leave and stem) are investigated. This chapter includes the chemical composition, *in vitro* digestibility for rabbits and ruminants and *in sacco* degradability for ruminants. In Chapter 2, three diets for growing rabbits were produced with substitution of raw materials normally used (alfalfa hay, beet pulp and wheat straw) with inclusion of 20 or 40 g kg^{-1} of cowpea stover and wheat bran on total tract apparent digestibility of of organic matter, crude protein, neutral detergent fibre and gross energy nutrients. The cowpea grain is harvested at an advanced stage of development (physiological maturation stage, R5 stage, 80-90 days), and its stover includes a highly lignified cell wall that may limit its fermentation by the digestive tract of the animals. Studies with white-rot fungi show its ability to increase the nutritional value of feedstuffs through changes in lignin structure and increased access to structural polysaccharides that are potentially digestible. Thus, pre-treatment with white-rot fungi was studied in Chapters 3 and 4. The effects of different white-rot strains (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* and *Phlebia rufa*) and incubation periods (22 and 45 days) were evaluated on the chemical composition and *in vitro* digestibility for rabbits, in Chapter 3.In Chapter 4, given the best results of the previous chapter (*Pleurotus citrinopileatus* at 22 days of incubation), five experimental diets, namely: control diet; diets with pre-treated cowpea stover (5 and 10%) and untreated cowpea stover (5 and 10%) were tested. The variables analysed in this chapter were growth performance, digestibility, blood parameters and carcass quality of growing rabbits.

The last two Chapters (5 and 6) we intended to evaluate the potential of conservation of cowpea stover by the ensiling process, using the inclusion of discarded apple as a source of fermentable sugars, in order to facilitate the fermentation process.

Chapter 5 presents the nutritive value, fermentative parameters and aerobic stability of cowpea stover and discarded apple silage. Based on these results, Chapter 6 shows the potential of using cowpea stover treated with white-rot fungi with apple discard by the ensilage process. In this chapter a commercial inoculant was tested as an alternative to favour the fermentation process and preserve sugars, increasing the nutritive value of mixtures.

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Experimental trials

EXP 1401 – Effect of cowpea stover (*Vigna unguiculata* (L.) Walp.) dietary inclusion level on performance and digestibility of growing rabbits

EXP 1501 – Potential use of cowpea (*Vigna unguiculata* (L.) Walp.) stover treated with white-rot fungi as rabbit feed

EXP 1502 – A novel feedstuff: ensiling of cowpea (*Vigna unguiculata* (L.) Walp.) stover and apple (*Malus domestica* Borkh.) mixtures. Evaluation of the nutritive value, fermentation quality and aerobic stability

EXP 1601 – Effects of the dietary incorporation of untreated and white-rot fungi (*Pleurotus citrinopileatus*) pre-treated cowpea stover on performance, digestibility, health and meat quality of rabbits

EXP 1602 – Preservation of cowpea (*Vigna unguiculata*) stover treated with *Pleurotus citrinopileatus* and discarded apple (*Malus domestica*) by the ensilage process

EXP 1701 – Potential use of cowpea (*Vigna unguiculata* (L.) Walp.) co-products in animal feeding

EXP 1801 – Alternative methods to sterilization of the pre-treatment of cowpea (*Vigna unguiculata* (L.) Walp.) stover with white rot fungi

EXP 1802 – Effect of itaconic acid on pretreatment of cowpea stover

Chapter 1 – Chemical composition, *in vitro* digestibility and *in sacco* degradability of cowpea stover and its different fractions

Andrade E,[1,2] Silva V,[2] Pinheiro V,[2] Gomes M.J,[2] Guedes C,[2] Ferreira L[3] and Rodrigues M[3*]

[1]CAPES Foundation, Ministry of Education of Brazil, Brasília - DF 70040-020, Brazil

[2]Animal and Veterinary Research Centre, University of Trás-os Montes and Alto Douro, (CECAV-UTAD), Portugal

[3]Centre for the Research and Technology of Agro Environmental and Biological Sciences, University of Trás-os Montes and Alto Douro, (CITAB-UTAD), Portugal

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Abstract

In Mediterranean production systems, agricultural co-products, e.g. straw, stover and hulls, may represent an important feedstuff as complementary source for feeding animals during periods of forage shortage or addition of fiber to compound feeds. Therefore, the aim of this study was to evaluate the nutritive value of cowpea (*Vigna unguiculata*) co-products for ruminant and rabbit feeding. Chemical composition, *in vitro* digestibility and *in sacco* NDFom degradability of stover and its different fractions, stems and leaves, were analysed. As expected, leaves showed higher ($P<0.001$) crude protein (CP) and lower neutral detergent fiber (ash free; NDFom) contents comparing to stover and stems (214.7 vs. 121.5 and 120.4 g kg^{-1} DM; 421.6 vs. 666.4 and 685.4 g kg^{-1} DM, respectively). Additionally, leaves had higher ($P<0.001$) *in vitro* digestibility of organic matter for ruminants and rabbits and total potential degradable NDFom fraction (796.7 vs. 603.8 and 586.1 g kg^{-1} DM; 595.6 vs. 396.5 and 357.2 g kg^{-1} DM, 76.6 vs. 50.3 and 51.3%). The concentration of water-soluble carbohydrates and starch was low in all the co-products (10.2 and 8.6 g kg^{-1} DM, respectively). Data indicate that cowpea co-products have potential to be use in animal feeding and, future studies should be performed to evaluate the effect of its inclusion in animal diets. Furthermore, the high NDFom and lignin contents indicated that pre-treatments should be considered as a mean to increase its nutritive value. Due to the high nutritional of the leaves, future studies should evaluate their potential of conservation.

Keywords: nutritive value, rabbit, ruminants, *Vigna unguiculata*

Introduction

Cowpea (*Vigna unguiculata* L. Walp), fabaceae original from Africa, has a world grain production estimated at 8 million tonnes in 2013 (FAOStat, 2015). The main production areas are distributed by several countries in Africa, America and Asia (Singh, 2014; Figure 4). In Europe, this crop is cultivated in the Mediterranean region, main Portugal, Spain, Italy and Greece, be responsible for only 0.4% of worldwide cowpea production (FAOStat, 2015; Carvalho *et al*., 2017). In three decades, the world production of its grain has increased six times, from 1.3 in 1981 to 8.0 million tons in 2013 (FAOStat, 2015). This increase is related to a number of factors, namely its agronomic characteristics, environmental adaptability, and the dual-purpose utilization of this crop (grain and forage). The plant adapts to a wide variety of soils, with shade tolerance. It develops between latitudes 40ºN and 30ºS, which favor its cultivation in more than two-thirds of the world (Vasconcelos *et al*., 2010; FAOStat, 2015).

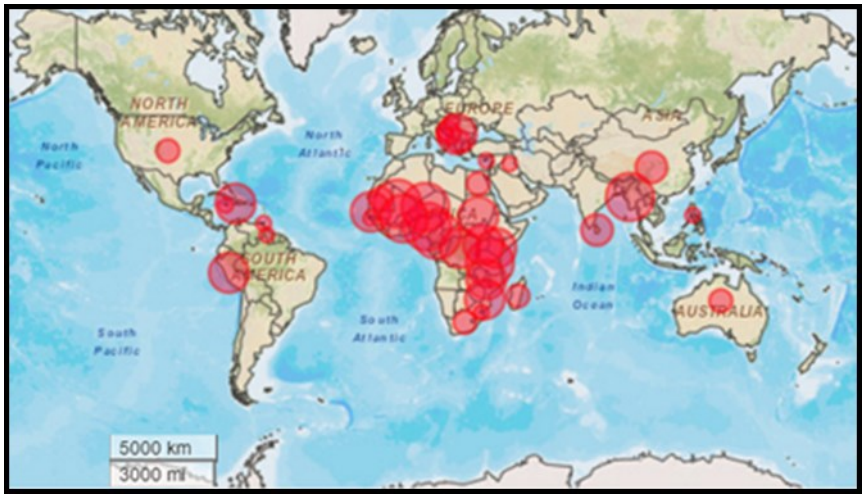

Figure 4. World cowpea production (*Vigna unguiculata*)
Source: FAOStat, 2015.

Furthermore, the global increasing demand for protein sources and legume grains could contribute to rebalancing the protein profile of human diets. Due to its high

nutritive value (high protein content and low-fat content) cowpea and its main products (mature and green beans, leaves and green pods) can be considered as one of the options for using in human feeding (Gonçalves *et al*., 2016). In Europe, an increase in cowpea production and consumption is highly desirable (Carvalho *et al*., 2017). Moreover, the residual mass of this crop can be also valorized in animal feeding (Gebremeskel *et al*., 2011). In Africa and the Mediterranean region, cowpea residues, around 2.0 to 3.0 t ha^{-1} (Figure 5), have been studied as a complementary source for feeding animals during periods of forage shortage or addition of fiber to compound feeds (Savadogo *et al*., 2000; Baloyi *et al*., 2008; Anele *et al*., 2011, Andrade *et al*., 2017).

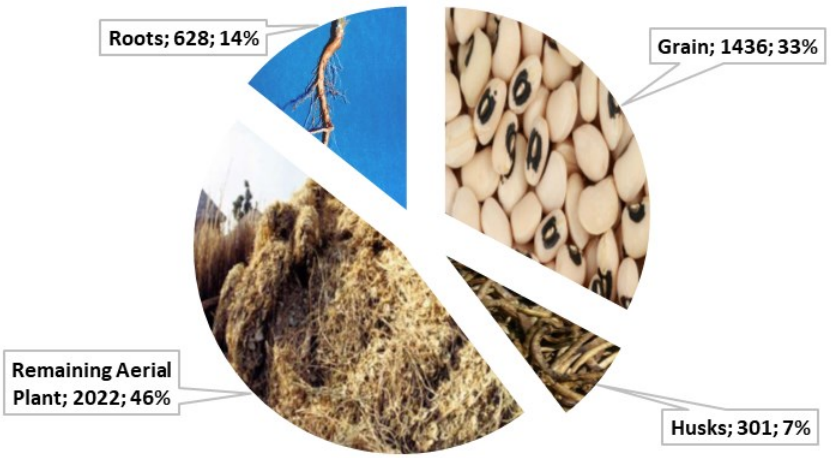

Figure 5. Cowpea mature production, cultivar Fradel, cultivated in the north of Portugal. Mean in the years 2015 and 2016. (Part; kg DM ha^{-1}; percental). Source: Eurolegume Project (unpublished data)

The objective of this study was evaluated the nutritive value of cowpea stover and its different fractions (leave and stems), identifying the potential for use in animal feed. Thus, the parameters evaluated were chemical composition, *in vitro* digestibility of rabbits, *in vitro* digestibility of ruminants and *in sacco* degradability (DM and NDFom).

Materials and methods

Cowpea stovers and its different fractions (stems and leaves) collected (stage R5, 80-90 days of cultivation).in Famalicão (41°24′N and 8°31′) / northeast of Portugal (summer 2015) was evaluated.

Chemical composition

Samples of the cowpea stover and its different fractions (leaves and stems) were dried in an air-forced oven at 50ºC and grounded to 1 mm sieve prior to chemical analysis and 4 mm for degradability *in sacco* assay. Ash content was analysed by the method no. 942.05 (AOAC, 1990). Dry samples were analysed for total N as Kjeldahl N following the method no. 954.01 (AOAC, 1990). The crude protein (CP) content was calculated as N × 6.25. Neutral detergent fibre (NDFom), acid detergent fibre (ADF) and lignin (ADL) fractions were obtained by the detergent methodologies without the use of sodium sulphite (Robertson and Van Soest, 1981; Van Soest *et al*., 1991). The total water-soluble carbohydrates (WSC) content of the samples was determined by the anthrone method (Irigoyen *et al*., 1992). Total starch was quantified using an enzymatic assay procedure (K-TSTA-100A, Megazyme, Ireland).

In vitro digestibility

Dry samples were analysed for *in vitro* digestibility of organic matter (IVOMD) for ruminants and rabbits. IVOMD for ruminants was determined according to the methodology proposed by Tilley and Terry (1963) and modified by Marten and Barnes (1980). Rumen fluid was collected from three non-lactating rumen-cannulated (Bar Diamond Inc., Parma, ID, USA) cows fed a diet composed of maize silage, meadow hay and concentrate feed (0.70:0.05:0.25; DM basis proportion). 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.

For IVOMD determination for rabbits followed a three-step methodology using several enzymes that simulate the digestibility process in the stomach, small intestine and caecum (Ramos *et al*., 1992).

In sacco degradability

Three non-lactating rumen-cannulated (Bar Diamond Inc., Parma, ID, USA) cows were used to measure the degradability of dry matter (DM) and NDFom of the cowpea stover and its different fraction (leaves and stems). The degradability of samples was measured using the nylon bag technique (Ørskov *et al*., 1980). Samples of approximately 7.0 g, in duplicate for cow, were put into nylon bags, porosity 53±10 μm (ref #BG120, Bar Diamond® EUA,) measuring 10 cm × 20 cm, incubated at 9:00 h and withdrawn after 2, 4, 8, 16, 24, 48, 72, 96 and 120 h. After the incubation period, the bags were washed with tap water in a washing machine, dried at 60ºC for 48 h and the residues were weighed and analysed for NDFom. The bags containing un-incubated samples for each co-product of cowpea were also washed, dried and residues were analyzed for NDF to represent 0 h values. Data were fitted to the model proposed by Ørskov e MacDonald 1979: $p = a + b\ [1 - \exp(-ct)]$, where p is the degradation after t hours, *a* the soluble/rapidly degradable fraction (%); b the insoluble but potentially degradable fraction, and *c* the fractional degradation rate of fraction *b*.

Statistical analysis

Data were analysed with GLM procedure of SAS, (2009), version 9.2, as a completely randomized design experiment using one-way analysis of variance (ANOVA), considering the type of cowpea stover and its different fractions (leaves and stems) as the main effect. When the F test was significant ($P<0.05$), multiple comparisons among means were analysed by the Tukey test.

Results and discussion

Several legume co-products, such as green and mature fodder, hay and silage can be used in animal feeding (Anele *et al*., 2011; Bruno-Soares *et al*., 2000; López *et al*., 2005). Depending on harvest technology of cowpea grains, different residues can be obtained such as leaves, stems and pods. These residues may have potential nutritive value as they can be used as a source of digestible fiber (Andrade *et al*., 2017) or as an alternative to low quality forages for lactating dairy cattle during the dry season (Anele *et al*., 2011).

The nutritive value of cowpea stover and its different fractions (stems and leaves), grown in Portugal is presented in Table 1. The leaf/haulm ration of stover was 1:9 (DM basis). The chemical composition of leaves showed higher CP (214.7 vs. 121.5 and 120.4 g kg^{-1} DM; $P<0.001$) and lower NDFom (421.6 vs. 666.4 and 685.4 g kg^{-1} DM; $P<0.001$) when compared to stover and stems, respectively (Table 1). Consequently, the leaves had higher ($P<0.001$) *in vitro* digestibility for ruminants (796.7 vs. 603.8 and 586.1 g kg^{-1} DM) and rabbits (595.6 vs. 396.5 and 357.2 g kg^{-1} DM). The concentration of WSC and starch were low in all substrates (10.2, 8.6 g kg^{-1} DM for WSC and starch, respectively). This feature may have been influenced by the maturity stage of cowpea (physiological maturation stage R5, 80-90 days). In fact, the grain harvest is normally performed in advanced maturity stages in which high concentrations of structural carbohydrates and lignin are observed.

In sacco degradability of DM and NDFom is presented in Table 2. As expected, the leaves were characterized by a higher total potential degradable (*a*+*b*) of DM and NDFom fraction (84.6 vs. 65.8 and 63.7%; 76.5 vs. 50.3 and 51.3%; P<0.001), slowly degradable (*b*) of DM and NDFom fraction (66.1 vs. 46.2 and 44.8%; 75.6 vs. 48.2 and 49.4%; P<0.01) and degradation rate (*c*) of DM and NDFom (0.058 vs. 0.043 and 0.039; 0.048 vs. 0.032 and 0.033 h^{-1}; P<0.01) when compared to stover and stems, respectively. The lower lignin content of the leaves (69.4 g kg^{-1} DM) compared to the stems and stover (109.7 and 131.9 g kg^{-1} DM, respectively) clearly influenced the distinct degradation kinetics. Moore and Jung (2001) have referred that the lignification process tends to be higher in structural tissues such as xylem and sclerenchyma. In this way, stems, containing higher concentrations of these tissues will be less digestible.

Table 1. Chemical composition (g kg^{-1} DM) and *in vitro* digestibility of organic matter (IVDOM) of cowpea (*Vigna unguiculata*, cv. fradel) stover and its different fractions (stems and leaves), post-harvest of grains, collected in Douro region, Portugal.

	Stover	Stems	Leaves	SEM	P value
Chemical composition					
Crude protein	121.5^{a}	120.4^{a}	214.7^{b}	2.60	<0.0001
NDFom	666.4^{b}	685.4^{b}	421.6^{a}	7.79	<0.0001
ADF	502.1^{b}	544.7^{c}	275.9^{a}	8.76	<0.0001
Lignin	109.7^{ab}	131.1^{b}	69.4^{a}	11.00	0.0055
Hemicellulose	164.3	140.7	145.7	11.70	0.3593
Cellulose	392.4^{b}	413.6^{b}	206.5^{a}	11.10	<0.0001
Ash	85.2^{a}	81.6^{a}	140.0^{b}	2.44	<0.0001
WSC	10.2	11.7	8.6	0.81	0.0783
Starch	8.6	10.9	6.3	1.07	0.0626
IVDOM (g kg^{-1} OM)					
Ruminants	603.8^{a}	586.1^{a}	796.7^{b}	9.15	<0.0001
Rabbits	396.5^{a}	357.2^{a}	595.6^{b}	14.4	<0.0001

SEM, standard error of means; mean values in the same line with different letters are significantly different (*P*<0.05). WSC, water-soluble carbohydrates

Although available data on the degradability characteristics of legume stover fractions are scarce, results found in the present study for stems and stover are consistent with those reported by Bruno-Soares *et al*. (2000) for seven legume straws

(*Cicer arietinum*, *Vicia benghalensis*, *Vicia sativa*, *Vicia villosa*, *Vicia faba*, *Lens culinaris* and *Pisum sativum*), with *a* varying from 0.96 to 8.1%, *b* ranging from 32.7 to 45.1%, and *c* varying from 0.038 to 0.047 h^{-1}. Higher values of NDF degradability fractions were reported by Gebremeskel *et al*. (2011) for different varieties of *Vicia faba* straws, with *a* and *c* varying between 7.9 to 27.8% and 0.057 to 0.190 h^{-1}, respectively. It should be noted that a fraction should be considered as only representing physical particle losses from the nylon bags. In fact, NDF should not present any soluble fraction. In this way, differences observed in the previously mentioned works must be attributed to different particles sizes and nylon bag porosity.

Table 2. Degradation parameters (*a*, *b*, *a*+*b* and *c*) of dry matter and neutral detergent fiber, ash free, (NDFom) of cowpea (*Vigna unguiculata*, cv. fradel) stover and its different fractions (stem and leaves), post-harvest of grains, collected in Douro region, Portugal.

Component	*a*	*b*	*a*+*b*	*c*
Dry matter				
Stover	19.6	46.2[a]	65.8[a]	0.043[a]
Stems	18.9	44.8[a]	63.7[a]	0.039[a]
Leaves	18.5	66.1[b]	84.6[b]	0.058[b]
SEM	0.38	1.00	0.65	0.0020
P value	0.2374	0.0011	0.0003	0.0067
NDFom				
Stover	2.1	48.2[a]	50.3[a]	0.032[a]
Stems	1.9	49.4[a]	51.3[a]	0.034[a]
Leaves	0.9	75.6[b]	76.5[b]	0.048[b]
SEM	0.03	1.25	1.26	0.0013
P value	0.2201	0.0018	0.0010	0.0068

a, soluble/rapidly degradable fraction (%); *b*, slowly degradable fraction (%); *a*+*b*, total potential degradable fraction (%); *c*, fractional degradation rate of fraction (h^{-1}). SEM, standard error of means; mean values in the same column with different letters are significantly different ($P < 0.05$)

Final considerations

Data indicate that cowpea co-products have potential to be use in animal feeding and, future studies should be performed to evaluate the effect of its inclusion in animal diets.

The high NDFom and lignin contents indicated that pre-treatments should be considered as a mean to increase its nutritive value. The reduction in the indigestible cell-wall fraction, as lignin, is beneficial because this will decrease fill and increase digestibility (Jung and Allen, 1995). The pre-treatment of fibrous feedstuffs with white-rot fungi has showed efficiency of depolymerizing of lignin with increase in nutritive value for animal feeding (Tuyen *et al*., 2012; Tuyen *et al*., 2013).

Another important factor in this study is the high nutritive value, main high crude protein, low lignin content and high *in vitro* digestibility, of the leaves compared to the stems. In the natural process of dehydration of the legumes, the stem has greater difficulty in the loss of moisture, because it is thicker, thus the leaves, more nutritious part, becomes highly brittle, thus causing its detachment from the stem and hindering the hay process. Thus, conservation methods to preserve this important fraction and increase their participation as ingested forage should be studied.

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Chapter 2 - Effect of cowpea (*Vigna unguiculata* (L.) Walp.) stover dietary inclusion level on total tract apparent digestibility of nutrients of growing rabbits

Andrade E,[1,2] Rodrigues M,[3] Ferreira L,[3] Mendes C,[4] Ribeiro L[5] and Pinheiro V.[2]

[1]CAPES Foundation, Ministry of Education of Brazil, 70.040-020, Brasília- DF, Brazil.

[2]Animal and Veterinary Research Centre (CECAV), Dept. of Animal Science, University of Trás-os-Montes and Alto Douro, 5000-801, Vila Real, Portugal.

[3]Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Dept. of Animal Science, University of Trás-os-Montes and Alto Douro, 5000-801, Vila Real, Portugal.

[4]Faculty of Agronomy and Veterinary Medicine, University of Brasília FAV/UnB), 70.910-900, Brasília - DF, Brazil.

[5]Alimentação Animal Nanta, SA, 4630-221, Marco de Canaveses, Portugal.

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Abstract

Although agro-industrial co-products have low economic value as foods for human consumption they may have potential value as animal feedstuffs. This experiment was carried out to evaluate the effect of cowpea stover inclusion in rabbits' diet on growth performance and nutrient digestibility. A total of 180 animals were randomly assigned to three treatments (CS0, CS2 or CS4, with no inclusion, 20 or 40 g kg^{-1} of cowpea stover, respectively). Animal performance was evaluated between the 53rd and 67th d of age in 48 animals per treatment. The coefficients of total tract apparent digestibility (CTTAD) of organic matter, crude protein, neutral detergent fibre and gross energy were measured between 63 to 67 d of age in 12 animals per treatment. Results showed that in general CTTAD values were not affected by the inclusion of cowpea stover. Nevertheless, a trend for a decrease in crude protein digestibility (P=0.0848) was observed when including cowpea stover. This had a negative influence on digestible protein (P=0.0240) and on the ratio between digestible protein and digestible energy (P=0.0231) for diet CS4. Rabbits showed normal figures for growth rate (on av. 46.8 g d), feed intake (on av. 168.3 g d) and conversion ratio (on av. 3.61). Future studies should evaluate the possibility of incorporating higher levels of cowpea stover while analysing the economic impact of this inclusion.

Keywords: digestibility, legume co-products, nutrition, performance, rabbit

Introduction

Different feed ingredients with high fibre content are frequently used in rabbits' feeding, e.g. alfalfa hay, grape seed, olive leave, paprika meal, soybean hull, sunflower hull, and wheat straw (García *et al*., 2000; Nicodemus *et al*., 2007; Ribeiro *et al*., 2012). Alfalfa (Medicago sativa) hay is the preferred fibre source and is usually included up to 20-40% in feeds. However, the rising prices of ingredients frequently used in animal feeding has led to the evaluation of alternative and less costly ingredients since feed can represent up to 70% of the total costs of rabbit production (Gidenne *et al*., 2017). Although it could be argued that feed cost minimization could be attained by the use of untraditional cheaper feed ingredients, the inclusion of these feed resources is limited as most of the times data on their nutritive value are scarce and highly variable (Lima *et al*., 2017; de Blas *et al*., 2018; Uhlirová *et al*., 2018). Furthermore, possible effects on animal growth performance must also be evaluated.

Cowpea (*Vigna unguiculata*) is grown primarily for human nutrition and feeding. Its production results in large amounts of mature plants, with high fibre contents, that are generally discarded as organic waste or can be partially used in animal feeding (Gonçalves *et al*., 2016). A source of dietary fibre is essential to prevent digestive disorders in growing rabbits and several classes of fibre, including low-digested and digestible fibre, have been recommended for use in rabbit feeds (Gidenne, 2003).

Although it is accepted that cowpea stover may have the potential to be used as animal feed (Anele *et al*., 2011; Anele *et al*., 2012; Samireddypalle *et al*., 2017) to the authors knowledge no studies have been conducted using it as a feed ingredient in rabbit's diets.

This study aimed to assess the effect of the inclusion of different levels of cowpea stover on performance and nutrient digestibility of growing rabbits.

Materials and methods

Animals and diets

The trial was carried out in the animal facility of the ESTIRPE - Estirpe D'Honra, Unipessoal Lda at Bragança, Portugal, between November and December of 2014. New Zealand × Californian rabbits were kept in a closed air-conditioned building maintained between 18 and 23ºC and received 12 h of light daily (07:00 to 19:00 h). Rabbits were handled according to the Portuguese legislation (Ports no. 1005/92, 214/08, and 635/09) on animal welfare. Rabbits were weaned at 35 d of age and between this date and the beginning of the trial (53 d of age) were fed a commercial diet.

Cowpea stover (comprising the aerial part after grain harvest) was collected after harvesting of cowpea grains in Famalicão, Portugal. The cowpea stover was dried and chopped into lengths of 1-2 cm and stored in a cool environment until analysis and utilization as raw material in the manufacture of the experimental diets (Table 3). Three dietary treatments (isoenergetic and isonitrogenous) were formulated according to De Blas and Mateos (2010) to meet the requirements of rabbits at fattening stage. The three diets were prepared by including, 20 or 40 g kg^{-1} (CS2 and CS4, respectively) of cowpea stover into a basal diet (CS0), as presented in Table 3. In CS2 and CS4 diets alfalfa hay, beet pulp and wheat straw were partially replaced by cowpea stover and wheat bran. During the experiment the rabbits had access to feed and water ad libitum and neither the diets or the water contained any drug supplementation.

Experimental design, growth performances and in vivo digestibility

A total of 180 rabbits of both sexes with 53 d of age and similar live weight (1816 g, on average) were reared until slaughter (67 d of age) and the growth performance and nutrient digestibility were measured. One of the reasons why the experimental period

started when animals reached 53 d of age is related to the higher efficiency in fibre digestibility in older animals (Ribeiro *et al*., 2012). The second reason is related to the risk of mortality caused by mucoid enteropathy that is generally high up to 50 d of age (Bennegadi *et al*., 2003; Martinez-Paredes *et al*., 2009).

Table 3. Ingredients and chemical composition of cowpea (*Vigna unguiculata*) stover (CS) and experimental diets containing 0 (CS0), 20 (CS2) or 40 g kg^{-1} (CS4) of cowpea stover.

		Experimental diets		
	CS	CS0	CS2	CS4
Ingredients (g kg^{-1}, as fed)				
Cowpea stover	-	0	20 (+20)	40 (+40)
Alfalfa hay	-	223	218 (-5)	213 (-10)
Sunflower meal	-	200	200	200
Wheat	-	150	150	150
Beet pulp	-	103	93 (-10)	83 (-20)
Wheat bran	-	85	90 (+5)	95 (+10)
Wheat straw	-	66	56 (-10)	46 (-20)
Corn gluten feed	-	35	35	35
Palm kernel	-	35	35	35
Defatted grape seed	-	30	30	30
Soybean oil	-	18	18	18
Colza meal	-	10	10	10
Sugarcane molasses	-	10	10	10
Minerals, vitamins and additives[1]	-	35	35	35
Chemical composition (g kg^{-1}, as fed)				
Dry matter	917	923	916	921
Organic matter	836	821	817	828
Crude protein	127	153	151	150
Crude fibre	303	213	213	216
Starch	11	83	74	73
Neutral detergent fibre	541	383	387	393
Acid detergent fibre	432	226	244	246
Lignin	83	62	70	71
ADIN	2.5	2.4	2.1	2.3
Ether extract	-	29	27	28
Gross energy (MJ kg^{-1} DM)	17.5	18.1	18.3	18.2

[1]Calcium carbonate, Luctarom 1408-Z, Sepiolite, NL-510-R, salt, Biolys 70, Sodium bicarbonate and Bio-Mos.

For each experimental diet growth performance was measured in 48 rabbits distributed into 16 collective cages (3 rabbits per cage). During the experimental period,

individual live weight and feed intake per cage were recorded and the weight gain, daily feed intake and feed conversion rate were calculated. It should be noted that the mortality rate was not calculated as only four animals died during the trial.

After the first 10 d of the beginning of the experiment, samples of faeces and experimental diets were collected during 4 d (63 to 67 d of age) from other 36 rabbits (12 per treatment) housed in individual cages from the beginning of experiment (53 d of age) to determine *in vivo* digestibility according to the European reference method (Perez *et al*., 1995).

Chemical analysis

Samples of the experimental diets and faeces were dried in an air-forced oven at 50ºC and grounded to 1 mm sieve prior to chemical analysis. AOAC (1990) procedures were used to determine dry matter, (DM; 934.01), organic matter (OM; 942.05), crude protein (CP; 954.01), crude fibre (962.09) and ether extract (920.39). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin fractions were obtained by the detergent methodologies without the use of sodium sulphite and expressed exclusive ash (Robertson and Van Soest, 1981; Van Soest *et al*., 1991). The acid detergent-insoluble nitrogen (ADIN) was determined according to Goering and Van Soest (1990). Total starch was quantified using an enzymatic assay procedure (K-TSTA-100A, Megazyme, Ireland). Gross energy was determined in an adiabatic oxygen bomb calorimeter (Parr 6300, Moline, IL, USA).

Statistical analysis

Data analysis was performed using SAS 9.2 program (SAS, 2009). The effect of the inclusion of different levels of cowpea stover on growth performance and *in vivo* digestibility was analysed using one-way ANOVA. For multiple mean comparisons the

Tukey test was used. Significant differences were set up at $P<0.05$ and trends were considered when 10% ($P<0.10$).

Results and discussion

Cowpea stover showed high fibre (541 g kg^{-1}; NDF) and a reasonable concentration of protein (127 g kg^{-1}; CP). Results on chemical composition of legume straws are rather scarce when compared to cereal straws. Nevertheless, data presented by Bruno-Soares *et al*. (2000) evaluating the chemical composition of seven different legume straws reported NFD values varying from 580 to 765 g kg^{-1} DM., Additionally, López *et al*. (2005) have showed that for 11 legume straws NDF fraction varied from 454 to 669 g kg^{-1} DM. Although NDF values of cowpea straw are within the reported variation, data regarding CP concentration (127 g kg^{-1} DM) is higher that the values reported by those authors, ranging from 43 to 114 g kg DM. Foster *et al*. (2009) evaluating cowpea hay have also reported a relative lower value of CP averaging 117 g kg^{-1} DM. Nevertheless, Anele *et al*. (2011, 2012) reported CP values around 190 g kg^{-1} DM. It is well known that the maturity stage of plants will influence the nutritive value of forages and fibrous feedstuffs. However, in the case of cereal straws, typically harvested when the grain evenly dries to the desired moisture concentration, NDF and CP contents are not that variable. For legume stovers, as pulses are collected when 80-90% of the pods reach the adequate maturity stage and the quantity of immature pods and grains that remain in the residue might be quite variable, variations in NDF and CP contents could be significant. Furthermore, as flowering time (Catt and Paull, 2017) and number of flowers (Gray and Brady, 2016) are influenced by genotype and environmental conditions thus conditioning the number of pods and its maturation, higher variation in the chemical composition of legume stovers is expected. This variation has been reported by Anele *et al*. (2011, 2012) who verified that cowpea haulms obtained from six varieties in the

rainy season presented lower CP values (P<0.001) than the haulms of the same varieties cultivated in the dry season (164 and 215 g kg^{-1} DM, respectively).

Altering these ingredients proportion in the diet in order to obtain 20 (CS2) and 40 g kg^{-1} (CS4) of cowpea stover inclusion did not change the coefficients of total tract apparent digestibility (CTTAD) of OM, CP, NDF and gross energy (Table 4). However, a trend (P=0.0848) towards lower CP digestibility with the increase of cowpea stover inclusion was detected with a decrease from 68.9% to 64.4% as the level of cowpea inclusion increased from 0 to 40 g kg^{-1} (CS4). This variation could be the result of the change in the dietary protein quality linked to the variation in the ingredients (Villamide *et al*., 2010). Furthermore, the indigestible protein concentrations could also have influenced CP digestibility. Although several authors have reported negative correlations between ADIN and CP digestibility (Martinez and Fernández, 1980; Villamide and Fraga, 1998) our data show no variation within the ADIN concentrations of the diets, thus excluding this hypothesis. On the other hand, changes in the fibre constituents (compared to CS0, ADF and lignin increased in CS2 and CS4) could also have contributed to reduce CP digestibility by affecting the passage rate along the gastrointestinal tract (Gidenne, 2003). This negative influence was clear for the data on digestible protein (DP) and for the data on the ratio between DP and digestible energy (DE), so that higher inclusion levels of cowpea stover had a negative impact on DP (P=0.0240) and on DP/DE (P=0.0231) values.

The digestible protein (DP) contents were below the recommended values for growth (101 vs. 105-110 g kg^{-1}) and the digestible energy (DE) contents were above the the recommended values (10.9 vs. 10.0-10.5 MJ kg^{-1}) proposed by Xiccato and Trocino (2010). Consequently, the DP to DE ratio of the experimental diets was lower than the recommended values (9.3 vs. 10.5-11.0 g MJ) proposed by the same authors.

Nevertheless, cowpea stover inclusion in the diets did not affect the growth performance (live weight at the 67 d, daily weight gain, daily feed intake and feed conversion rate), which remained within normal values (Table 5).

Table 4. Daily feed intake, coefficients of total tract apparent digestibility (CTTAD) and dietary nutritive value digestibility of the experimental diets with different levels of cowpea (*Vigna unguiculata*) stover (CS) in growing rabbits between 59 to 63 days of age. n = 12 rabbits (individual cage) per experimental diet.

	Experimental diets[1]			SEM	*P*-value
	CS0	CS2	CS4		
Average daily feed intake (g d)	173.5	180.6	187.3	6.36	0.2417
CTTAD (%)					
Organic matter	56.3	56.8	55.3	1.98	0.8557
Crude protein	68.9	67.7	64.4	1.68	0.0848
NDF	30.8	29.1	27.3	2.58	0.5750
Gross energy	56.3	55.2	54.5	2.14	0.7623
Dietary nutritive value					
Digestible protein (DP) (g kg^{-1})	105.4^{b}	101.3^{ab}	96.6^{a}	2.73	0.0240
Digestible energy (DE) (MJ kg^{-1})	11.0	11.0	10.8	0.42	0.8343
Ratio DP/DE (g MJ^{-1})	9.6^{b}	9.2^{ab}	9.0^{a}	0.20	0.0231

[1]CS0, CS2 and CS4 diets containing 0, 20 or 40 g kg^{-1} (as fed) of cowpea stover; SEM: standard error of the mean. Values with different letters within a line differ significantly by the Tukey test ($P < 0.05$)

Table 5. Growth performance of rabbits fed with the experimental diets with different levels of cowpea (Vigna unguiculata) stover (CS), n = 48 rabbits (16 cage) per experimental diet.

	Experimental diets[1]			SEM	*P*-value
	CS0	CS2	CS4		
Live weight (g)					
53 d	1820	1828	1802	35.9	0.8593
60 d	2172	2158	2155	36.6	0.9318
67 d	2495	2447	2474	36.6	0.5926
Daily weight gain (g/d)					
53-60 d	50.2	47.1	50.4	2.23	0.4873
60-67 d	46.2	41.3	45.6	2.36	0.2728
53-67 d	48.2	44.2	48.0	1.78	0.1964
Daily feed intake (g/d)					
53-60 d	152.1	149.6	152.3	7.42	0.3696
60-67 d	182.0	184.6	189.3	6.61	0.3819
53-67 d	167.0	167.1	170.8	5.59	0.3698
Feed conversion rate					
53-60 d	3.03	3.18	3.02	0.203	0.9802
60-67 d	3.94	4.46	4.15	0.253	0.2851
53-67 d	3.47	3.78	3.57	0.140	0.3268

[1]CS0, CS2, and CS4, diets containing 0, 20, or 40 g/kg (as fed) of cowpea stover, respectively; SEM, standard error of mean

Conclusion

Cowpea stover may present a good potential to be used in rabbit feeding. However, protein digestibility values indicate that its inclusion should be further evaluated so that possible detrimental effects of the fibre fraction can be overcome. In this way, chemical or biological pre-treatments of cowpea stover should also be considered as they might improve the digestibility of this feedstuff.

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Chapter 3 - Potential use of cowpea (*Vigna unguiculata* (L.) Walp.) stover treated with white-rot fungi as rabbit feed

Andrade E,[1,2] Pinheiro V,[2] Gonçalves A,[3] Cone J.W,[4] Marques G,[3] Silva V,[2] Ferreira L[2,3] and Rodrigues M[2,3]

[1] CAPES Foundation, Ministry of Education of Brazil, Brasília, DF 70040-020, Brazil

[2] The Animal and Veterinary Research Centre, University of Trás-os-Montes and Alto Douro, (UTAD-CECAV), Department of Animal Science, Quinta de Prados, 5001-801, Vila Real, Portugal

[3] Centre for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro, (UTAD-CITAB), Quinta de Prados, 5000-801, Vila Real, Portugal

[4] Animal Nutrition Group, Department of Animal Sciences, Wageningen University, Wageningen, the Netherlands

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Abstract

Lignin inhibitory effects with in the cell wall structure constitute a serious drawback in maximizing the utilization of fibrous feedstuffs in animal feeding. Therefore, treatments that promote efficient delignification of these materials must be applied. This study evaluated the potential of white-rot fungi to upgrade the nutritive value of cowpea stover for rabbit feeding. There was an increase in the crude protein content of all substrates as a result of fungi treatments, reaching a net gain of 13% for *Pleurotus citrinopileatus* incubation. Overall, net losses of dry and organic matter occurred during fungi treatments. Although the fiber content remained identical, higher consumption of cell wall contents was measured for *P. citrinopileatus* incubation (between 40 and 45%). The incubation period did not influence lignin degradation for any of the fungi treatments. Differences within the fungal degradation mechanisms indicate that *P. citrinopileatus* treatment was most effective, enhancing invitro organic matter digestibility by around 30% compared with the control. Treatment of cowpea stover with *P. citrinopileatus* led to an efficient delignification process which resulted in higher *in vitro* organic matter digestibility, showing its potential in the nutritional valorization of this feedstuff.

Keywords: cowpea stover; white-rot fungi; nutritional valorization; rabbit feeding

Introduction

Global demand for food sources has been constantly increasing and must be met using fewer available natural resources. This scenario has led to the implementation of policies that enhance the production of legumes for human food within European agriculture. Cowpea is one of the most productive heat-adapted cultivated legumes showing high nutritive value (Gonçalves *et al*., 2016) and has been given increased importance recently owing to its adaptability to different environmental conditions. Therefore, global intensification of its production is expected.

Besides the agronomic, environmental and economic advantages of including legumes in cropping systems, the production of grains will also generate large amounts of stovers that may have a negative environmental impact if not properly discarded. One of the possible alternatives for the utilization of these disposable resources is their valorization as animal feed. In fact, in extensive Mediterranean production systems, fibrous feeds such as straws and stovers are considered a valuable feed resource in certain periods of the year (Bruno-Soares *et al*., 2000). Nevertheless, as with many lignocellulosic biomass materials, lignin inhibitory effects within the cell wall structure constitute a serious drawback in maximizing their utilization (López *et al*., 2005). Therefore, treatments that promote efficient delignification of these materials must be applied. The colonization of fibrous feedstuffs with fungi, more specifically the white-rot wood basidiomycetes has been studied for some time (Zadrazil, 1985; Agosin *et al*., 1986), and recent results have pointed out its efficiency in depolymerizing lignin (Rodrigues *et al*., 2008; Dinis *et al*., 2009; Camarero *et al*., 2014; Van Kuijk *et al*., 2015a). However, the complexity and specificity of the ligninolytic enzyme systems involved in lignin degradation mechanisms promote variable responses in terms of fungi colonization efficiency. Furthermore, in a recent review, Van Kuijk *et al*. (2015a) have

pointed out that the combined effect of fungi on delignification and digestibility is mostly dependent on the fungal strain, the substrate and the extent of fungal biodegradation. Within the substrate level, one must highlight that most studies reporting on the effect of fungi treatments have been conducted using wheat straw. As there are substantial differences between grasses and legumes in lignin structure (Vanholme *et al*., 2010) and in linkages between lignin and other cell wall components (Cornu *et al*., 1994), mechanisms of fungal degradation should also be different.

Therefore, this study aimed to compare the effectiveness of treatments with five white-rot fungal species (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* and *Phlebia rufa*), using two incubation periods, on the chemical composition and *in vitro* digestibility of cowpea stover for rabbit nutrition and feeding.

Materials and methods

Fungal species and spawn preparation

The five fungal species used, *G. lucidum* (UF20707), *L. edodes* (UF21403), *P. citrinopileatus* (UF21401), *P. eryngii* (UF21402) and *P. rufa* (156), were preserved in the culture collections of the Laboratory of Mycology and Soil Microbiology and the Laboratory of Biochemistry of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal. Cultivation and spawn preparation were carried out as previously described (Pinto *et al*., 2012). To prepare the fungal inoculants, the fungi were transferred to potato dextrose agar plates and incubated at 28ºC until the mycelia colonized most of the plate surface. Subsequently, the colonization of the spawn was carried on wheat grain. Briefly, the wheat grain was hydrated in water and drained. Next, the grain was placed in glass flasks and sterilized in an autoclave at 121ºC for 30

min. After cooling, each flask was inoculated with a 3 cm diameter agar disk containing mycelium and incubated at 25ºC in full darkness for 3 weeks.

Preparation and fungi cultivation on substrate

Cowpea (*Vigna unguiculata*) stover was collected after harvesting of cowpea grains in the south of Spain. The cowpea stover was chopped into lengths of 1–2 cm, then water was added to approximately three times the weight of the stover and left overnight for water absorption by the inner structures. Approximately 50 g portions of cowpea stover were weighed into 250 mL Erlenmeyer flasks and autoclaved at 121ºC for 30min. In a laminar flow chamber, 2 g of previously prepared wheat spawn was inoculated into each Erlenmeyer flask. The inoculated stover was incubated in triplicate along with the control (autoclaved stover with 2 g of non-inoculated wheat grain) at 28ºC and 75% relative humidity for 22 and 45 days in an incubation chamber (Conviron CMP 3244, Controlled Environments Ltd, Winnipeg, Canada).

Chemical analysis and in vitro digestibility

To determine the dry matter (DM) content, samples were dried to constant weight in an air-forced oven at 50ºC and ground over a 1mm screen (Tecator Cyclotec 1093 Sample Mill, FOSS, Hillerød, Denmark). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) fractions were determined by detergent methodologies without the use of sodium sulfite (Robertson and Van Soest, 1981; Van Soest *et al*., 1991). The concentration of hemicellulose was calculated as the difference between NDF and ADF, and that of cellulose as the difference between ADF and ADL.

Dried samples were analyzed for ash (no. 942.05) and total N as Kjeldahl N (no. 954.01) according to AOAC, 1990 methods. The crude protein (CP) content was

calculated as N×6.25. Following a three-step methodology, the *in vitro* digestibility (IVD) of organic matter (OM) in rabbits was determined using several enzymes that simulate the digestibility process in the stomach, small intestine and caecum (Ramos *et al*., 1992).

Losses in the chemical composition following fungal incubation were calculated as the difference between the control and the inoculated substrate.

Statistical analysis

Data were analyzed with the GLM procedure of SAS (2009) as a completely randomized design experiment using one-way analysis of variance (ANOVA), considering fungal treatments (performed in triplicate) as the main effect. When the F test was significant ($P<0.05$), multiple comparisons among means were examined by the Tukey test. All chemical and *in vitro* digestibility analyses were performed in triplicate.

Results and discussion

The autoclaving process resulted in a substrate (control) with higher ($P<0.05$) ADL content and decreased ($P<0.05$) IVOMD (Table 6). The application of pressurized steam to fibrous feeds promotes changes in the cell wall architecture, depolymerizing lignin and partially hydrolysing cellulose and hemicellulose molecules (Kumar *et al*., 2009). However, this process is dependent on the types of lignocellulosic materials and on the pressure and temperature conditions of the autoclave (Lawther *et al*., 1996; Liu *et al*., 1999). Therefore, although a decrease in cell wall components is normally reported for substrates treated with pressurized steam (Sarnklong *et al*., 2010), the data we have obtained may result from DM losses associated with steam treatment of substrates (Baugh *et al*., 1988). In fact, sugars and other soluble components may volatilize, leading to what may only represent a change in proportion of other compounds such as

lignin. Nevertheless, we should also point out that polymerization reactions between hydrolysed components and lignin may also occur during the application of pressurized steam (Han *et al*., 2009), contributing to an increase in its concentration. Furthermore, the decrease in IVOMD indicates that changes have occurred in the substrate during the sterilization process using autoclaving, suggesting possible modifications in the polymer structure limiting its accessibility to enzymes. Taking into account that lignin composition and existing linkages between it and carbohydrates may modify the efficiency of fungal colonization (Van Kuijk *et al*., 2015a), possible alternative methodologies such as chemical sterilization, already reported by Pandey *et al*. (2012) need to be evaluated.

With the exception of *P. eryngii* (22 days of incubation), an increase ($P<0.05$) in the CP content (Table 6) was measured for all substrates at the end of both incubation periods. Although initial studies (Rangaswami *et al*., 1975; Ginterová and Gallin, 1979) have pointed out that fungi could fix atmospheric nitrogen, earlier data reported by Millbank (1969) indicated exactly the opposite. However, this issue was quite controversial, because the evidence supporting this capability was not completely irrefutable. Furthermore, data published by Kurtzman (1979) raise the possibility that increases in the nitrogen content of incubated substrates might be due to the presence of nitrogen - fixing bacteria, a suggestion also supported by Pandey *et al*., (2012). More recently, Walker and White (2005) considered fungi to be non-diazotrophic (cannot fix nitrogen) and need to be supplied with nitrogen-containing compounds. Thus, although increased protein content during fungal incubation has been reported elsewhere (Shrivastava *et al*., 2011; Tuyen *et al*., 2013), one must be judicious in its interpretation. Another suggestion might be that, while the total Kjeldahl N method does not quantify nitrates and nitrites (Van Camp and Dierckx, 2004), some white-rot fungi species

possess the ability to use these inorganic nitrogen sources, degrading them to ammonium ions that can be assimilated into glutamate and glutamine (Walker and White, 2005; Bumpus, 1993); thus the net increase in nitrogen content (Table 6) might be related to the transformation of nitrates and nitrites during fungal incubation, which will then be quantified by the total Kjeldahl N method. Ultimately, the net increase in CP content is considered an advantage of the fungi- treated fibrous substrates (Tuyen *et al*., 2012). Our data show that for *P. citrinopileatus* the net gain in CP, in comparison with the intact stover, was around 13% at 22 days of incubation (Table 6).

Only *P. citrinopileatus* (22 and 45 days) and *P. rufa* (22 and 45 days) treatments decreased ($P<0.05$) the NDF and ADF proportion of cowpea stover, while the other treatments did not promote any changes in these cell wall components (Table 7). In relation to the ADL fraction, only the treatment with *P. citrinopileatus* (22 and 45 days) was able to decrease its content ($P<0.05$), and an increase in the proportion of ADL was even obtained for *G. lucidum* treatment at 45 days of incubation ($P<0.05$). As reported before (Tuyen *et al*., 2012), the increase in the contents of this fraction due to fungi treatments can be due to its smaller losses in relation to OM losses (Table 7). Differences obtained in the relative proportions of the different analyzed fractions between 22 and 45 days of incubation might also be explained by an increase in OM losses during the incubation period (Table 7).

Table 6. Chemical composition (g kg^{-1} DM) and *in vitro* digestibility (g kg^{-1} OM) of cowpea stover autoclaved, intact and incubated with different fungi for 22 and 45 days.

Fungi/ Sample	Incubation	CP	NDF	ADF	ADL	HC	Cellulose	Ash	IVDOM
Ganoderma lucidum	22	168.3bcd	618.6cd	454.6cd	125.9de	164.1a	328.6bc	100.6f	283.4b
Lentinula edodes	22	163.0b	670.0ef	493.4e	112.4bc	176.5a	381.1e	86.0ab	372.3cde
Pleurotus citrinopileatus	22	207.5f	472.5a	347.5a	81.5a	125.0a	266.0a	122.1h	465.5h
Pleurotus eryngii	22	149.7a	618.3cd	464.8de	116.6bcd	153.5a	348.2cde	89.4bc	369.2cd
Phlebia rufa	22	173.6cde	593.0c	426.1bc	121.0cde	166.9a	305.1ab	97.8ef	381.6def
Ganoderma lucidum	45	179.2e	632.7de	482.0de	145.6f	150.7a	336.4bcd	108.8g	233.0a
Lentinula edodes	45	175.2de	673.6f	487.0de	109.1bc	186.5a	377.9de	84.9a	381.5def
Pleurotus citrinopileatus	45	224.7g	499.8ab	358.5a	94.2a	141.3a	264.3a	131.2i	404.5g
Pleurotus eryngii	45	165.4bc	612.0cd	466.2de	112.0bc	145.8a	354.2cde	95.7e	381.9def
Phlebia rufa	45	203.7f	534.6b	401.9b	131.1e	132.7a	270.8[a]	123.6h	387.9efg
Autoclaved straw (control)	-	147.5a	643.6def	470.4de	121.5cde	173.2a	348.9cde	94.2de	356.8c
Intact straw	-	143.7a	618.4cd	474.1de	107.5b	144.3a	366.5cde	91.1cd	398.8fg
SEM		1.68	6.87	6.77	2.35	12.32	7.54	0.75	3.50

Values with different letters within a column are significantly ($P < 0.05$) different. DM, dry matter; OM, organic matter; CP, crude protein; NDF, ash-free neutral detergent fiber; ADF, ash-free acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; IVDOM, digestibility of organic matter; SEM: standard error of the mean.

Table 7. Loss of nutrients (%) in cowpea stover incubated with different fungi for 22 and 45 days compared with control.

	Incubation	DM	OM	CP[a]	NDF	ADF	ADL	HC	Cellulose
Ganoderma lucidum	22	3.7b	4.3b	-9.8bc	7.5a	7.3ab	0.3a	8.0ab	9.7a
Lentinula edodes	22	8.4c	7.8c	-1.1de	4.7a	4.3ab	15.4bc	5.8a	0.4a
Pleurotus citrinopileatus	22	19.6f	22.1f	-13.0a	41.0d	40.9f	46.1d	41.5b	39.0c
Pleurotus eryngii	22	0.0a	0.0a	-2.5de	3.0a	0.5a	3.1ab	11.3ab	0.1a
Phlebia rufa	22	8.7c	9.1c	-7.3c	15.9b	17.7d	9.2abc	38.4ab	20.6b
Ganoderma lucidum	45	17.4e	18.7e	-0.3e	18.8b	15.6cd	1.1ab	27.5ab	20.7b
Lentinula edodes	45	12.6d	13.9d	-3.7d	8.6a	9.9bd	21.7c	5.0a	5.8a
Pleurotus citrinopileatus	45	27.0g	30.0g	-11.1ab	43.3d	44.6f	43.5d	39.9ab	45.0c
Pleurotus eryngii	45	2.6b	2.8b	-9.1bc	7.4a	3.9ab	10.4abc	17.3ab	1.6a
Phlebia rufa	45	20.3f	22.9f	-10.0bc	33.8c	32.2e	14.1abc	38.4ab	38.5c
SEM		0.24	0.36	0.50	1.01	1.21	2.61	6.27	1.83

Values with different letters within a column are significantly ($P < 0.05$) different. DM, dry matter; OM, organic matter; CP, crude protein; NDF, ash-free neutral detergent fiber; ADF, ash-free acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; SEM: standard error of the mean.

[a]The negative sign indicates an *increase* in proportion

All fungi, except *P. eryngii* at 22 days of incubation, caused a net loss of DM and OM (Table 7), with the highest consumption occurring at 45 days of incubation for all fungi treatments ($P<0.05$). These results were expected, as later development of fungi during the colonization process will implicate the degradation of structural cell wall components compared with the utilization of available soluble compounds during the initial stage. Higher losses ($P<0.05$) of DM and OM where observed in incubations with *P. citrinopileatus* (22 and 45 days). For cell wall contents NDF, ADF and ADL, higher depletion (between 41 and 46%) was also detected for the treatment with *P. citrinopileatus* ($P<0.05$). Nevertheless, the degradation pattern between 22 and 45 days of incubation was not similar for all fungi treatments, with no differences identified for *L. edodes*, *P. citrinopileatus* and *P. eryngii* treatments but higher losses ($P<0.05$) detected for *G. lucidum* and *P. rufa* treatments in NDF and ADF fractions. The incubation period did not influence ADL utilization for any of the fungi treatments ($P>0.05$). The treatments with *L. edodes* and *P. eryngii* resulted in lower ($P<0.05$) hemicellulose and cellulose consumption. In contrast, these losses were higher for *P. citrinopileatus* and *P. rufa* treatments ($P<0.05$). Interestingly, in all fungi, increasing the incubation period did not affect hemicellulose concentrations, and only *G. lucidum* and *P. rufa* treatments increased ($P<0.05$) cellulose depletion through the incubation period. These changes within the cell wall contents promoted higher ($P<0.05$) values of *in vitro* digestibility (IVDOM) for all fungi treatments at 45 days of incubation compared with the control, with the exception of *G. lucidum*. In fact, the treatment with this fungus negatively influenced ($P<0.05$) the IVOMD. At 22 days of incubation, only *P. citrinopileatus* and *P. rufa* showed higher IVOMD values. It should also be noted that only the treatment with *P. citrinopileatus* at 22 days of incubation was able to improve

the IVOMD of intact stover by 17%. Furthermore, a decrease in IVOMD was measured for *G. lucidum* and *P. citrinopileatus* along the incubation period.

Various fungal species have been used to valorized different lignocellulosic substrates for animal nutrition. In a recent review, van Kuijk *et al*. (2015a) highlighted the main biomass sources that can be used as animal feed ingredients after fungal pretreatment, mentioning that these substrates are mainly selected for their potential nutritive value but also because of their geographical availability. These substrates are mainly constituted by grass straws and stovers and residues from other grasses such as bamboo and sugarcane. Data on fungi treatment of legume residues are scarce, and to our knowledge there are no references on legume straws. Furthermore, owing to their nutritional characteristics, these substrates have been evaluated as ruminant feeds, and data on rabbit feeding studies are also in frequent.

In general, data from these studies have indicated that fungi treated material is adequate for use in animal feeding, enhancing changes in the cell wall chemical composition and digestibility (Arora *et al*., 2011; Tuyen *et al*., 2012; Tuyen *et al*., 2013; Sharma and Arora, 2015; Shirivastava *et al*., 2012; Van Kuijk *et al*., 2016). Although the effectiveness of fungi treatments depends on several factors such as the fungal strain, the chemical and structural features of the substrate and the culture conditions, it has been proposed that fungi should be selected according to their specificity in degrading lignin without substantially depleting cellulose and hemicellulose concentrations, as these structural carbohydrates may be extensively used by herbivore animals, increasing the digestibility of the substrate (Van Kuijk *et al*., 2015a). The data presented in this study clearly show that there are differences within the fungal degradation mechanisms, indicating that *P. citrinopileatus* treatment was the most effective in enhancing IVOMD. Although it may be argued that this is due to the

decrease in ADL contents, the high losses of hemicellulose and cellulose during this treatment do not agree with previous data reported by Tuyen *et al*. (2012). The results obtained for *P. rufa* treatment also point to higher *in vitro* digestibility values in spite of high losses of hemicellulose and cellulose fractions. Tuyen *et al*. (2012) pointed out that for wheat straw, only fungi characterized by high lignin degradation potential, but low depletion of cellulose would be able to improve its nutritive value. Other authors working with the same fungi used in this study have also reported different results (Kamra *et al*., 1993; Shrivastava *et al*., 2011; Pandey *et al*., 2012; Tuyen *et al*., 2013). Again, differences in the degradation patterns of cell wall components might be attributed to morphological differences within the cell wall structure of the substrates used in the different experiments or to any of the other above-mentioned factors. However, it should be noted that the lignin composition of legumes comprises only guacyl and syringyl units, while that of grasses also possesses high amounts of p-hydroxyphenyl units (Vanholme *et al*., 2010). Furthermore, legumes have smaller amounts of ferulate and p-coumarate esters (Jung and Deetz, 1993), phenolic acids that allow the formation of linkages between lignin and carbohydrates, thus influencing the fungal degradation patterns. In addition, NDF is also lower in legumes, while lignin is present in higher concentrations (Buxton and Russell, 1988). Moreover, no ferulate-mediated crosslinks of lignin to cell wall polysaccharides have been observed in legumes (Rodrigues *et al*., 2007). Therefore, the variations in cell wall degradation observed in studies using fungal incubation in legume substrates might not be similar to the results obtained when using grasses.

Some studies have pointed out the advantages of prolonged incubation times that will positively influence the nutritive value of substrates (Tuyen *et al*., 2012; Tuyen *et al*., 2013; Khan *et al*., 2015), showing an increase in the *in vitro* digestibility and/or gas

production, in spite of higher DM losses that might compromise the efficacy of utilization of these fungi (Rodrigues *et al*., 2008). In contrast, Shrivastava *et al*. (2011) identified higher *in vitro* gas production and IVOMD for shorter incubations times (10 and 20 days) in wheat straw treated with *Pleurotus ostreatus* and *Trametes versicolor* respectively. Also, Lynch *et al*. (2014), evaluating the effect of the same fungal species on the DM and NDF digestibility of maize stovers, only reported a positive effect for P. ostreatus. Therefore, it seems that the influence of the incubation period might depend on the nature of the substrate, the incubation procedures and the specific fungi growth patterns. Our results also confirm these data, showing that fungi present different colonization strategies during the incubation period. Thus, the most effective treatment was attained at 22 days of incubation for *P. citrinopileatus*, allowing for an increased digestibility of around 30.4% compared with the control. In a recent study, Van Kuijk *et al*. (2015b) also describe this problem, showing that results could even vary depending on the utilization of different batches, cultivars and growth conditions of substrates.

Conclusions

Data presented in this study show clear differences between the colonization patterns of fungi on cowpea stover. The chemical composition of the substrate and the incubation conditions used allowed the *P. citrinopileatus* strain an optimal growth and a more efficient delignification process, which resulted in higher *in vitro* digestibility. This work highlights the need for future work in order to optimize the incubation process, seeking to increase the lignin degradation efficiency while limiting the degradation of cellulose and hemicellulose.

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Chapter 4 - Incorporation of cowpea stover untreated and pre-treated with *Pleurotus citrinopileatus* on performance, digestibility, health and meat quality of rabbit growing

Ederson Andrade[1,2], Victor Pinheiro[2,3], Valéria Costa-Silva[2], Guilhermina Marques[4], Anabela Alves[3], Cláudia Serra[5], John W. Cone[6], Maria José Saavedra[3], Ana Barros[4], Luís Ferreira[2,3] and Miguel Rodrigues[2,3*]

[1]CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil.
[2]University of Trás-os-Montes e Alto Douro (UTAD), Animal Science Department, Vila Real, Portugal
[3]Animal and Veterinary Research Centre (CECAV), Vila Real, Portugal.
[4]Centre for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), Vila Real, Portugal.
[5]Interdisciplinary Centre of Marine and Environmental Research - University of Porto, Matosinhos, Portugal
[f6]Animal Nutrition Group, Wageningen University & Research, Wageningen, The Netherlands

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Abstract

Legume stovers may be an important feedstuff resource for animals, especially in the Mediterranean production systems. However, these stovers contain antinutritional factors, such as lignin, that may affect the growth performance of the animals. Studies with white-rot fungi showed their ability to increase the nutritional value of several agricultural wastes through changes in lignin structure facilitating the access to structural polysaccharides that are potentially digestible. This contributes to a better utilization of the diet's nutrients and, consequently, to a better animal performance and health. The present work aimed at evaluating the effect of cowpea stover (*Vigna unguiculata*), untreated (US) or treated (TS) with *Pleurotus citrinopileatus*, on performance, digestibility, health and meat quality of growing rabbits. The TS treatment resulted from the inoculation of cowpea stover with *Pleurotus citrinopileatus* under solid-state fermentation per 22 days. Five experimental diets incorporating 0 (C), 50 (US50) and 100g/kg (US100) of US and 50 (TS50) and 100g/kg (TS100) of TS were used. Trials were conducted using 80 rabbits (40 males and 40 females) with 35 days old. The animals were blocked on sex and randomly assigned to one of the 5 treatments and were slaughtered at 63 days of age. The incorporation of cowpea stover did not affect animal growth performances or digestibility. The orthogonal contrasts showed that the animals fed with US had a final live weight 5.0% lower ($P = 0.04$) than the ones fed TS (2214 vs. 2323g). The TS diet allowed a 17.4% reduction ($P = 0.03$) in blood cholesterol levels. No influence ($P > 0.05$) of diets was detected for carcass traits, meat quality parameters and caecal microbiota. The incorporation up to 10% of treated cowpea stover with *Pleurotus citrinopileatus* in the rabbits' compound feed showed potential to overcome the detrimental effect that the incorporation of the US has on the final live weight of growing rabbits. Further research on the specific effect of TS diets on cholesterol blood levels and shifts on the microbial population of the gastrointestinal tract should be assesed.

Keywords: biological treatment, agricultural waste, *Vigna unguiculata*, white-rot fungi

Introduction

The disparity in the prices of raw materials used in the compound feed industry is a serious disadvantage in rabbit production systems as feeding represents about 70% of the total costs (Gidenne et al., 2017). Thus, search for alternative raw materials that allow a reduction of these costs and, simultaneously, ensures the maintenance of growth performance of the animals is required. Due to their nutritional characteristics and abundance, agricultural wastes from the legume production, particularly stovers, hulls and pods, are materials that can be incorporated into compound feeds for herbivores. Previously developed studies indicated that cowpea (*Vigna unguiculata*) stover presented good potential for use in rabbit feeding (Andrade et al., 2017). However, this material contains high lignin content and, for that reason biological and/or chemical pretreatments of cowpea stover should be considered, as they might improve the digestibility of this feedstuff (Andrade et al., 2017).

Several pretreatments (chemical, physical and biological) have been used to improve the nutritional value of agricultural wastes. Application of white-rot fungi through solid-state fermentation may be a viable pretreatment to increase the nutritional value of the stover as it promotes changes in its lignin structure and facilitates access to structural polysaccharides (Ribeiro et al., 2012; Andrade et al., 2017). Many studies have evaluated the effect of pretreatment of different agricultural waste (wheat straw, rice straw, cowpea stover, maize stover, sugarcane bagasse) with white-rot fungi on its chemical composition and *in vitro* digestibility (Tuyen et al., 2012; Tuyen et al., 2013; Van Kuijk et al., 2015; Andrade et al., 2017). However, few *in vivo* rabbit trials have been performed to assess the effect of its incorporation in compound feeds. In this context, the objective of this study was to evaluate the incorporation of untreated and

treated cowpea stover with *Pleurotus citrinopileatus* in compound feed and its effects on growth performance, digestibility, health and meat quality of rabbits.

Materials and methods

The Ethical Committee of the University of Trás-os-Montes and Alto Douro (ORBEA: Órgão Responsável pelo Bem-Estar dos Animais) approved the experimental protocol (Ref. 667-e-DZ-2018, 13/02/2018). The trial was handled according to the Portuguese legislation (Ports. no. 1005/92, 214/08, 635/09) on animal welfare.

Untreated and treated cowpea stover

Cowpea stover (~10%, w/w moisture) was collected from a cowpea field in the north of Portugal. Cowpea stover was divided in two equal parts, one part denominate untreated stover (US) was stored until the feed production, and the other part was treated (treated stover, TS) with one fungal strain (*Pleurotus citrinopileatus*, UF21401 - Laboratory of Mycology and Soil Microbiology of the University of Trás-os-Montes and Alto Douro). The *P. citrinopileatus* was selected as previous studies (Andrade *et al*., 2017) showed that this fungal specie presents an efficient delignification process and a greater potential for the improvement of the nutritional value of cowpea stover for rabbit feeding. Aproximally 0.8kg of the humidified cowpea stover was placed each box TP3000 + TPD3000 XXL (Microbox Combiness, Nevele, Belgium), autoclaved (121ºC for 30 min), cooled and inoculated (32g of spaw) in solid state fermentation with the fungal per 22 days, as previously described by Andrade *et al*. (2017).

Diets

Five experimental diets were prepared (Table 8) containing 0 g kg^{-1} (control, C) and 50 g kg^{-1} and/or 100 g kg^{-1} of cowpea stover untreated or treated with *Pleurotus citrinopileatus*, (US5, US10, TS5 and TS10, respectively). The diets were formulated according to the recommendations of De Blas and Mateos (2010) for growing rabbits. The level of crude protein and crude energy were similar in all diets (Table 8). During this trial antibiotics were administrated in feeds and drinking water.

Table 8. Ingredient and chemical composition of experimental diets

	Experimental diets[1]				
	C	**US5**	**US10**	**TS5**	**TS10**
Ingredients (g kg^{-1}, as fed)					
Cowpea stover untreated	0	50	100	0	0
Cowpea stover treated	0	0	0	50	100
Wheat bran	265	300	300	300	300
Alfalfa hay	220	170	120	170	120
Sunflower	210	210	219	210	219
Beet pulp	159	131	107	126	100
Barley	75	75	72	76	76
Sugarcane molasses	16	15	16	14	17
Soybean oil	3	2	3	2	3
Soybean meal 47%	0	0	9	0	9
Minerals, vitamins and additives[2]	54	54	54	54	54
Chemical composition (g kg^{-1}, as fed)					
Dry matter	895	902	902	902	904
Organic matter	883	888	883	886	883
Crude protein	162	162	162	162	162
Gross energy (kJ kg^{-1}, dry matter)	18,0	18,0	18,1	17,9	18,0
Neutral detergent fibre	391	406	412	418	420

[1]Diets C, US5 and US10 containing 0 g kg^{-1}, 50 g kg^{-1} and 100 g kg^{-1} of the untreated cowpea stover, respectively. Diets TS5 and TS10 containing 50 g kg^{-1} and 100 g kg^{-1} of the cowpea stover treated with *Pleurotus citrinopileatus*, respectively.; [2]Calcium carbonate, Luctarom 1408-Z, Sepiolita, NL-510-R, salt, Biolys 70, Sodium bicarbonate and Bio-Mos.

Growth trial

The trial was carried out in the animal facility of University of Trás os Montes and Alto Douro, Vila Real, Portugal, between March and April of 2018. New Zealand × Californian rabbits were kept in a closed air-conditioned building maintained between 18 and 23ºC and received 12 h of light daily (07:00 to 19:00 h).

A total of 80 rabbits (40 males and 40 females) of both sexes with 35 days of age and similar live weight (1038 ± 147,7g) were reared until slaughter (63 days of age). For each experimental diet, 16 rabbits (eight males and eight females) were distributed in individual cages. Live weight and feed consumed were registered during growing period daily and the weight gain, the daily feed intake and the feed conversion rate (ratio between the daily weight gain and the daily feed intake) were calculated.

The coefficients of total tract apparent digestibility (CTTAD) of DM, OM, NDFom and CP of each experimental diet were also measured on 50 rabbits among those being tested (10 animals of both sexes per diet) according to the European standardized method (Perez *et al*., 1995). Digestibility measurement started at 55 d of age with a 4-d collection period of feed samples and refusals. Animals were fed ad libitum and total faecal excretion was quantified daily from each cage. Samples of feeds, refusal and faeces were then stores at – 20ºC for subsequent chemical analysis. All samples were then dried at 60°C to a constant weight (approximately 3 days), and from these samples, a representative pool sample was grounded over a 1 mm screen (Tecator Cyclotec 1093 Sample Mill, Foss SA, Sweden) and prepared for chemical analysis.

Chemical analysis

All samples were analysed for dry matter (DM), ash, organic matter and cell wall components (NDFom and lignin). Neutral detergent fibre, ash free (NDFom) and lignin fractions were determined by the detergent methodologies without the use of sodium sulphite (Robertson and Van Soest, 1981; Van Soest *et al*., 1991). Dried samples were analysed for ash (no. 942.05) and total N as Kjeldahl N (no. 954.01) following the

methods proposed by AOAC (1990). The crude protein (CP) content was calculated as N × 6.25.

Blood analysis

Before the end of the feeding trial, 10 animals per treatment was assigned for blood examination. To determine haematological parameters, blood samples were collected into tubes containing ethylenediaminetetraacetic acid tripotassium (K3EDTA; Sigma Company, St Louis, MO, USA). All of blood haematology and serum biochemistry were analysed using diagnostic kits (Daytona, Randox Laboratories Ltd. Crumlin, United Kingdon).

Carcass traits, meat quality and gastrointestinal tract analysis

At the end of the growth trial, rabbits used for the digestibility measurement (10 animals per dietary treatment) were slaughtered for gastrointestinal tract assessment, carcass traits and meat quality evaluation. Animals were weighed at slaughter (SW) and then bled and their skin, genitals, urinary bladder, gastrointestinal tract, and the distal part of legs were removed to determine hot carcass weight. Gastrointestinal tract organs were measured and weighed individually and expressed as percentage of the SW. Carcasses were then chilled at +4 °C for 24 h and their weight (CCW) was recorded. The weight of the hindleg, each thigh, loin, rib, paw, head, liver, kidneys, heart, lungs, and fat were recorded for each carcass and calculated their ratio to the CCW. The colour, pH and cooking loss analyses in CCW was determined in the L. dorsi muscles (between the first and seventh lumbar vertebra). Also, the pH of the thighs was calculated. The pH value was determined using a Metrohm pH Meter 632 (Herisau, Switzerland). A colorimeter Minolta CR-10 was used to assess meat colour according to the L*, a*, and b* system

(CIE, 1986). The cooking loss was determined weighing the sample and vacuum packaged in a plastic bag. The samples were cooked in a water bath at 80°C until when reaching 75°C (monitored with a food thermocouple) at core sample (Honikel, 1998).

Gut fragments (jejunum and ileum) from the slaughtered animals were fixed by immersion in 10% neutral formalin. Tissues of the duodenum and jejune were processed in an automatic tissue processor (Shandon® - Hipercenter XP) and embedded in paraffin wax (Histoplast - Shandon®). Slides were evaluated on a Nikon DXM1200 digital still camera utilizing optical lens no 4 to measure the height, tip width, junction width and crypt depth. Fifteen villi per animal were assessed and the reported mean values were based in these measurements. The program Digimizer® was used to determine the characteristics of crypts.

Microbial diversity analysis

Hard faeces of caecal content were collected from 10 rabbits from each experimental diet. DNA extraction, PCR-DGGE (polymerase chain reaction-desaturating gradient gel electrophoresis), were performed of each sample as previously described by Pitcher *et al*., (1989) and Serra *et al*., (2018).

Statistical analysis

Data were analyzed with the GLM procedure of SAS, (2009) as a completely randomized design experiment using one-way ANOVA. Non-orthogonal contrasts (C vs. US5 vs. US10 and C vs. TS5 vs. TS10) were performed to evaluate the effect of the incorporation of untreated and treated cowpea stover, respectively, on growth performance and digestibility. Additionally, orthogonal contrasts (US vs. TS) were

carried out to compare the effects of the incorporation of untreated vs. treated cowpea stover on the same parameters. Differences were considered significant at $P < 0.05$.

Results

Diets were similar in terms of main crude protein (on average 146 g/kg, as fed) and gross energy contents (18.0 MJ/kg DM), despite some differences in NDF content (Table 8). The incorporation of both untreated (US) and treated cowpea stover (TS) in the diets was followed by an increase of wheat bran and a decrease in alfalfa and beet pulp proportions.

During the trial only two animals died, and, for that reason, the mortality rate was not calculated. Data presented in Table 9, indicate that live weight at 63 days was negatively affected by the incorporation of untreated cowpea stover ($P = 0.04$). Animals fed with US100 diet had 10% lower final live weight compared with the control (C). By contrast, the final live weight of animals fed the control diet and those with treated cowpea stover did not differ. Furthermore, animals fed the diet containing treated cowpea stover (TS) presented higher final live weight compared to the animals fed with untreated cowpea stover (TS vs. US; 2323 vs 2214 g; $P=0.04$). These results suggest that the treatment of cowpea stover with P. citrinopileatus (TS) diminished the negative effects of the incorporation of US on rabbit performance. The CTTAD of DM, OM, NDF and CP digestibility did not differ between the experimental diets. Data on the digestive tract histology indicates that the incorporation of both treated and untreated cowpea stover did not influenced the morphology characteristics of villi and crypts of the jejunum and ileum tissues (Table 9).

Table 9. Effect of the control diet (C) and diets containing untreated cowpea stover (US5 and US10) and treated cowpea stover (TS5 and TS10) with *Pleurotus citrinopileatus* on growth performance, coefficients of total tract apparent digestibility (CTTAD) and digestive tract histology in rabbits.

	Experimental diets[1]						*P*-value			
	C	**US5**	**US10**	**TS5**	**TS10**	**SEM**	**General**	**C vs. US5 vs. US10**	**C vs.TS5 vs. TS10**	**US vs. TS**
Growth performance (n = 16 group)										
Initial BW (g)	1061	1022	995	1045	1058	37.5	0.7080	0.4353	0.9588	0.2496
Live weight at 63 days (g)	2365	2233	2196	2290	2356	52.5	0.1099	0.0408	0.6426	0.0391
Daily weight gain (g day)	46	43	43	44	46	1.4	0.2563	0.1406	0.6108	0.0983
Daily feed intake (g day)	155	148	153	151	163	4.9	0.2426	0.5870	0.2291	0.1881
Feed conversion rate	3.35	3.44	3.57	3.42	3.52	0.074	0.2238	0.1209	0.2466	0.0627
CTTAD (%) (n = 10 group)										
Dry matter (g kg^{-1})	567	566	566	569	556	11.2	0.9421	0.9934	0.6330	0.7918
Organic matter (g kg^{-1})	578	571	568	573	562	11.5	0.8973	0.8309	0.5450	0.8476
Neutral detergent fiber (g kg^{-1})	362	375	378	385	359	18.2	0.8281	0.7724	0.5616	0.7971
Crude protein (g kg^{-1})	692	689	689	687	670	8.3	0.3585	0.9675	0.1649	0.2050
Digestive tract histology (n = 10 group)										
Jejune										
Height (µm)	344	339	318	338	322	19.0	0.8211	0.6610	0.6327	0.9307
Tip width (µm)	66	67	67	66	61	4.9	0.8999	0.9791	0.7170	0.4909
Juction width (µm)	102	99	97	95	97	5.0	0.8337	0.8274	0.5875	0.5145
Crypt depth (µm)	225	194	220	241	218	13.9	0.2272	0.1495	0.5570	0.1149
Ileum										
Height (µm)	369	336	305	338	335	24.2	0.4646	0.1968	0.5830	0.5177
Tip width (µm)	72	75	79	76	76	3.6	0.7730	0.4472	0.5808	0.7993
Juction width (µm)	95	98	102	99	98	3.9	0.7545	0.4881	0.6307	0.6834
Crypt depth(µm)	253	234	216	237	242	16.9	0.6159	0.2973	0.9334	0.3908

[1]Diets C, US5 and US10 containing 0 g kg^{-1}, 50 g kg^{-1} and 100 g kg^{-1} of the untreated cowpea stover (US), respectively. Diets TS5 and TS10 containing 50 g kg^{-1} and 100 g kg^{-1} of the cowpea stover treated (TS) with *Pleurotus citrinopileatus*, respectively

Similarly, to that reported for the gastrointestinal histology, no differences were observed for the haematology parameters between diets (Table 10). Except for the cholesterol data, data on the serum biochemistry was similar in animals fed treated or untreated cowpea stover. In fact, animals fed diets containing treated cowpea stover tended to present lower (57.6 vs. 47.6 mg/dl; P=0.03) cholesterol levels than those fed with untreated cowpea stover diets.

Data on carcass traits and meat quality indicates that the incorporation of both treated and untreated cowpea stover did not influenced any of the parameters evaluated (Table 11). Our results pointed out to medium carcass weights of 1314 g, carcass yield of 59.6%, hindleg of chilled carcass of 29.4% and chilling losses around 2.2%.

Microbial diversity analysis

The effect of incorporation of untreated and treated cowpea stover on gut microbiota of rabbits' is presented in Table 12 and Figure 6. The microbial community profiling of fecal samples recovered from the intestines of rabbits' fed the experimental diets was studied by polymorphism analyses of the variable V3 region of the 16S rRNA gene using DGGE. The Bray–Curtis dendrogram (Figure 6) did not reveal similar banding patterns between all the 10 replicates of each feeding condition, resulting in the absence of clear distinct clusters (Figure 1). Despite these observations, a decrease (P =0.0148) of the SIMPER similarity indices was observed (Table 12) when rabbits were fed with diets containing treated cowpea stover compared with animals fed the untreated cowpea stover diets (US vs. TS).

Table 10. Effect of the control diet (C) and diets containing untreated cowpea stover (US5 and US10) and treated cowpea stover (TS5 and TS10) with *Pleurotus citrinopileatus* on blood haematology and serum biochemistry in rabbits (n = 10 group).

	Experimental diets[1]					SEM	*P value*			
	C	US5	US10	TS5	TS10		General	C vs. US5 vs. US10	C vs. TS5 vs. TS10	US vs. TS
Haematology										
Haemoglobin (g dL^{-1})	12.2	11.9	12.3	11.5	12.0	0.46	0.7124	0.7163	0.5330	0.4637
Haematocrit (%)	39.5	38.8	40.3	37.2	38.7	1.40	0.5174	0.6663	0.4362	0.2417
Lymphocytes (%)	57.1	46.7	55.4	65.4	58.2	6.36	0.3161	0.2548	0.6484	0.1037
Monocytes (%)	8.25	9.66	9.29	7.10	8.20	1.021	0.3583	0.5670	0.5723	0.0635
Eosinophils (%)	1.27	1.94	1.06	1.00	0.60	0.509	0.3896	0.3888	0.6756	0.0919
Basophils (%)	4.38	7.48	4.78	3.80	5.32	0.838	0.0251	0.0263	0.2279	0.0803
RDW[2] (%)	17.4	16.7	17.5	16.0	15.65	1.023	0.5509	0.8510	0.3413	0.1558
Reticulocyte cout (%)	4.57	4.40	3.70	4.07	3.25	0.792	0.6944	0.6675	0.2987	0.5947
Serum biochemistry										
Triglycerides (mg dL^{-1})	94.5	98.0	101.9	100.2	97.8	9.89	0.9331	0.8061	0.8429	0.7385
Urea	27.6	30.9	29.1	30.3	25.7	2.54	0.4822	0.3692	0.4926	0.3960
Creatine (mg dL^{-1})	0.67	0.68	0.74	0.70	0.67	0.04	0.5659	0.3325	0.7923	0.5900
Cholesterol (mg dL^{-1})	56.8	61.7	53.5	45.9	49.3	5.04	0.1522	0.4889	0.1072	0.0344
AST[3] (mg dL^{-1})	33.6	32.8	34.1	32.5	32.6	4.74	0.9977	0.9753	0.9671	0.8289
ALT[4] (mg dL^{-1})	42.9	39.2	58.3	41.5	42.2	6.46	0.0865	0.0275	0.9821	0.2413
Albumin (mg dL^{-1})	3.61	3.31	3.69	3.66	3.60	0.178	0.4359	0.2094	0.9361	0.4293
Total protein (mg dL^{-1})	5.61	5.88	5.74	5.53	6.05	0.259	0.5051	0.5496	0.2464	0.9353
Globulin (mg dL^{-1})	2.00	2.13	2.05	1.88	2.45	0.170	0.1200	0.5800	0.0726	0.6261

[1]Diets C, US5 and US10 containing 0 g kg^{-1}, 50 g kg^{-1} and 100 g kg^{-1} of the untreated cowpea stover (US), respectively. Diets TS5 and TS10 containing 50 g kg^{-1} and 100 g kg^{-1} of the cowpea stover treated (TS) with *Pleurotus citrinopileatus*, respectively; [2]RDW, red blood cell; [3]AST, asparate aminotransferase; [4]ALT, alamine aminotransferase.

Table 11. Effect of the control diet (C) and diets containing untreated cowpea stover (US5 and US10) and treated cowpea stover (TS5 and TS10) with *Pleurotus citrinopileatus* on carcass traits and meat quality in rabbits (n = 10 group).

	Experimental diets[1]						*P value*			
	C	**US5**	**US10**	**TS5**	**TS10**	**SEM**	**General**	**C vs. US5 vs. US10**	**C vs. TS5 vs. TS10**	**US vs. TS**
Carcass traits										
Slaugter weight	2323	2190	2173	2222	2286	80.9	0.6219	0.2624	0.7096	0.3545
Hot carcass weight	1418	1341	1292	1325	1342	50.0	0.4989	0.1882	0.4519	0.7276
Chilled carcass weight (CCW)	1377	1291	1288	1309	1307	51.4	0.7382	0.3944	0.5752	0.7178
Hindleg (g 100g CCW)	29.5	30.2	29.9	28.6	28.8	0.75	0.5185	0.7569	0.6808	0.1104
Thigh (g 100g CCW)	11.7	11.8	11.9	11.4	11.3	0.45	0.8609	0.9474	0.8284	0.2767
Loin (g 100g CCW)	17.5	17.7	17.2	16.0	16.4	0.66	0.3181	0.8510	0.1896	0.0814
Rib (g 100g CCW)	31.9	32.0	31.3	31.4	32.1	0.89	0.9586	0.8702	0.7788	0.9601
Paw (g 100g CCW)	1.83	1.95	2.01	1.91	1.95	0.095	0.7412	0.4055	0.6241	0.6440
Head (g 100g CCW)	8.39	8.49	8.54	8.66	8.54	0.219	0.9330	0.8800	0.7085	0.6822
Liver (g 100g CCW)	6.70	6.69	6.72	6.85	6.85	0.300	0.9908	0.9979	0.9014	0.6357
Kidneys (g 100g CCW)	1.33	1.33	1.33	1.33	1.38	0.041	0.8990	0.9965	0.4821	0.6774
Heart (g 100g CCW)	0.55	0.55	0.57	0.56	0.55	0.024	0.9861	0.8880	0.9710	0.7848
Lungs (g 100g CCW)	2.00	2.16	2.07	2.00	2.07	0.146	0.9353	0.7196	0.9341	0.5823
Fat (g 100g CCW)	1.72	1.94	1.60	1.83	1.82	0.200	0.8138	0.4464	0.9185	0.7898
Meat quality										
Lightness (L*)	48.3	49.6	50.3	49.7	49.9	2.18	0.9685	0.8286	0.8699	0.9893
Redness (a*)	3.4	3.8	4.5	3.4	3.9	0.83	0.8780	0.8916	0.5142	0.4077
Yellowness (b*)	10.3	10.9	11.6	11.3	11.3	11.57	0.9002	0.7721	0.7279	0.7994
pH_{48} thighs	5.8	5.8	5.8	5.7	5.7	0.03	0.9713	0.7849	0.9916	0.8756
pH_{48} *L. dorsi*	5.7	5.6	5.7	5.7	5.6	0.03	0.0736	0.2820	0.1683	0.5380
Cooking losses (%)	17.4	17.8	17.9	17.5	17.8	1.09	0.8916	0.6647	0.9740	0.6907

[1]Diets C, US5 and US10 containing 0 g kg^{-1}, 50 g kg^{-1} and 100 g kg^{-1} of the untreated cowpea stover (US), respectively. Diets TS5 and TS10 containing 50 g kg^{-1} and 100 g kg^{-1} of the cowpea stover treated (TS) with *Pleurotus citrinopileatus*, respectively.

Table 12. Effect of the control diet (C) and diets containing untreated cowpea stover (US5 and US10) and treated cowpea stover (TS5 and TS10) with *Pleurotus citrinopileatus* on ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in faeces (n = 10 group).

	Experimental diets[1]					**SEM**	***P value***			
	C	**US5**	**US10**	**TS5**	**TS10**		**General**	**C vs. US5 vs. US10**	**C vs. TS5vs. TS10**	**US vs. TS**
Gut microbiota										
OTUs[1]	12	13	12	14	12	0.9	0.2824	0.2635	0.2049	0.8620
Richness[2]	0.7	0.8	0.7	0.8	0.7	0.05	0.2789	0.3170	0.1820	0.9662
Diversity[3]	2.1	2.3	2.1	2.1	2.1	0.08	0.4144	0.2076	0.8310	0.4624
SIMPER Similarity (%)[4]	38^{ab}	44^{b}	43^{ab}	26^{a}	35^{ab}	3.8	0.0148	0.4784	0.1284	0.0012

[1]Diets C, US5 and US10 containing 0 g kg^{-1}, 50 g kg^{-1} and 100 g kg^{-1} of the untreated cowpea stover, respectively. Diets TS5 and TS10 containing 50 g kg^{-1} and 100 g kg^{-1} of the treated cowpea stover with *Pleurotus citrinopileatus*, respectively. The values are means (n = 10 per diet); Significant differences within the diets are indicated by different superscript letters ($p < 0.05$). [1]OTUs: Average number of operational taxonomic units. [2]Margalef species richness: d=(S-1)/log(N), [3]Shannons diversity index: H'=-∑(pi(lnpi)), [4]SIMPER, similarity percentage within group replicates, SEM: standard error of mean.

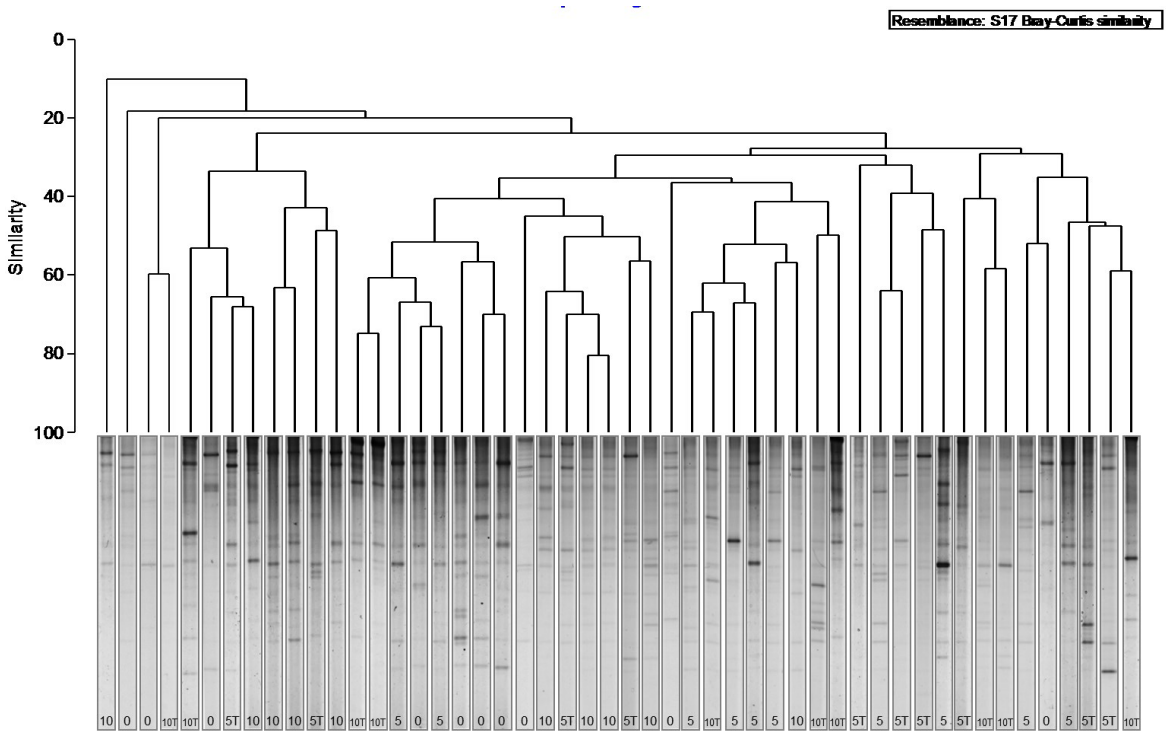

Figure 6. Dendrogram of effect of the control diet (C) and diets containing untreated cowpea stover (US5 and US10) and treated cowpea stover (TS5 and TS10) with *Pleurotus citrinopileatus* on ecological parameters obtained from PCR-DGGE fingerprints of the microbiota found in faeces (n = 10 group).

Discussion

The average crude protein content (146 g/kg, as fed) was similar for the five diets (Table 8) and within the variation of 142-160 g/kg (as fed) reported by de Blas and Mateos (2010) for growing rabbits. Although the NDF content of the control diet (350 g/kg, as fed) is within the values proposed by de Blas and Mateos (2010), diets including untreated or treated cowpea stover presented slightly higher concentrations (374 g/kg, as fed). This variation is the reflex of alfalfa hay and beet pulp partially replacement by cowpea stover and wheat bran. Many studies have evaluated the potential incorporation of agricultural wastes on rabbit feeding, as bilberry pomace (Dabbou *et al*., 2018), cowpea husk (Oduguwa *et al*., 2008), cowpea stover (Andrade *et al*., 2017), olive leaves (Ribeiro *et al*., 2012; Mattioli *et al*., 2018), sugarcane bagasse

(Ferreira *et al*., 2017), soybean hull (Garcia *et al*., 2000; Nicodemus *et al*., 2007), sunflower hull (Garcia *et al*., 2000; Liu et al., 2018), pea straw (Omer and Badr., 2013) and wheat straw. Most of these trials were unable to cope with the simultaneous balancing of protein, energy and fibrous content of the diets.

Diets including white-rot fungi pretreated feedstuffs have been tested in cattle (Laconi and Jayanegara, 2015; Shrivastava *et al*., 2014), lambs (Calzada *et al*., 1987), goats (Chanjula *et al*., 2017; Hamchara et al., 2018) and rabbits (Ribeiro *et al*.,2012). Additionally, *in vitro* trials evaluating co-products pretreated with white-rot fungi have also pointed out the potential of this treatment for ruminants (Tuyen *et al*., 2012; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015; Laconi and Jayanegata, 2015) and rabbits (Andrade et al., 2017). Studies have been conducted in herbivorous animals using feeds treated with different *Pleurotus* strains, with positive results on the performance parameters of lambs and cattle, more specifically on weight gain (Calzada *et al*., 1987; Shrivastava *et al*., 2014). Most of the published data indicate inconclusive results on animal performance studies conducted with lambs and goats fed diets contain different levels of treated substrates with several strains of white-rot fungi (Chanjula *et al*., 2017). In rabbits, Ribeiro *et al*. (2012) compared the performance of growing rabbits fed on untreated or treated olives leaves with *Ganoderma resinaceum* and reported no differences between the diets. These data clearly show that results of white-rot treated feeds on animal performance are dependent on the initial substrate, strain of fungi and level of incorporation.

The morphology of villi and crypts is associated to the gastrointestinal function and with growth performance of animals (Tufarelli *et al*., 2010) influencing the intestinal health status and the absorptive capacity. In this study the analysis of the digestive tract histology, showed that there were no differences between jejune and

ileum measurements (Table 9), thus indicating that the intestinal health performance was similar across the diets.

In general, our data indicated that measured blood parameters were within the normal range for rabbits found in reference studies with New Zealand white rabbits (Hewitt *et al*., 1989; Ozkan *et al*., 2012; Ogbuewu *et al*., 2017). Haematological parameters are currently used as indicators of health and variations of these indicators may reflect bacterial, viral, parasitic, or fungal infections, as well as intoxication problems (Iser *et al*., 2016). The potential for certain strains of white-rot fungi, namely *Pleurotus* spp., to decrease blood cholesterol levels has been pointed out (Wasser and Weis, 1999) and this effect has been attributed to a possible increase in the excretion of total lipids and cholesterol through faeces and/or lower cholesterol synthesis (Patel et al., 2012). These action mechanisms are associated to the production of statins that might inhibit the activity of HMG-CoA reductase an enzyme from the metabolic pathway of cholesterol synthesis (Amirullah *et al*., 2018). Alam *et al*. (2011) have reported data enhancing a 30% reduction of total plasma cholesterol of rats fed a diet containing 5% of *Pleurotus ostreatus* (fruiting body) and more recently several studies have enhanced this mechanism of action for white-rot fungi also using rats as an animal model (Morales *et al*, 2018; Jin *et al*., 2018). In broilers, similar hypocholesterolaemic effects have been attributed to eritadenine a compound produced by *Lentinula edodes* (Djulardi *et al*., 2018). In the present study, medium values of ether extract excretion (data not shown) between treatments were similar (C – 4.0 g/kg DM; US – 3.8 g/kg DM; TS – 3.8 g/kg DM; $P > 0.05$), indicating that the previously mentioned mechanism of lower cholesterol synthesis might be responsible by the lower blood cholesterol concentrations of rabbits fed the fungi treated diets.

As shown in Table 11 the carcass traits and meat quality were not different beetween diets (P > 0.05). Similarly, Ribeiro *et al*. (2012) did not find any effect between treatments when they evaluated 50 g kg−1 of olive leaves treated with fungi. In the same way, Hernandez-Martinez *et al*. (2017) analyzing the effect of enzymatic extract of Trametes maxima CU1 on productive parameters and carcass yield of rabbits dis not report any differences for these parameters. Nevertheless, results obtained for slaughter (SW), hot carcass (HCW) and chilled carcass weights (CCW) in this study, are lower than those reported by other authors (Trocino *et al*., 2011; Trocino *et al*., 2015; Kovitvadhi *et al*., 2016; Szendrõ *et al*., 2016; Cullere and Dalle Zotte, 2018; Volek *et al*., 2018). It is well established that the adult weight and the maturity of rabbits at slaughter age are factors that influence carcass weight (Dalle Zotte *et al*., 2002; Metzger *et al*., 2003; Trocino *et al*., 2015). Thus, our results could be explained by the age at slaugher, that in our study was 63 days while the age at slaughter on the above mentioned studies averaged 75-90 days. In this way, animals slaughtered at lower maturity stages will present lower values of carcass weight (Trocino *et al*., 2015).

The utilization of molecular techniques applied to microbiology studies have allowed a more detailed understanding of microbiota main physiological roles, including the hydrolysis and fermentation mechanisms and its contribution to the immune system regulation by controlling infectious agents (Combes *et al*. 2013). In this way, possible shifts in the gastrointestinal tract microbiota could be related to differences in the digestive efficiency and digestive health of animals (Walsh *et al*. 2014). Within the factors that could influence commensal microbiota development, dietary fibre content and composition as well as utilization of prebiotics are often referred. The anaerobic symbiotic flora from the caecum enables the rabbit to take advantage of fibrous feeds and because of caecal location in the digestive tract, mainly

fibrous particles and endogenous material are found in caecum contents, with a scarce contribution of starch and other digestible dietary components. Our results indicate that although there are slight changes in the NDF content of the diets no differences were observed between treated and untreated diets in relation to NDF digestibility. Thus, it seems that by itself the fibre content could not explain the differences on the SIMPER similarity indices when rabbits were fed with diets containing treated cowpea stover compared with animals fed the untreated cowpea stover diets. A possible alternative could be related to the effects of fungal treatment. Solid state fermentation using white-rot fungi will promote cell wall degrading mechanisms, hydrolysing lignin as well as structural carbohydrates such as cellulose and hemicelluloses. This mode of action will consequently release different types of oligosaccharides. In addition, during fungal growth, exopolysaccharide production could also contribute to an increase in the concentration of compounds that are not hydrolyzed in the small intestine and arrive unchanged in the caecum, acting as prebiotics and affecting the balance of microbial communities. Recent studies have demonstrated these beneficial effects on rabbits (Sun *et al*., 2016).

Conclusions

The results obtained in this study conclude that the incorporation of cowpea stover did not affect the general parameters of animal performance and digestibility of the diets, except for the final weight of the animals. In addition, results also point out that the treated cowpea stover with *Pleurotus citrinopileatus* inhibited the negative effects of incorporation of untreated cowpea stover into the diet of growing rabbits, increasing the final live weight. The treated cowpea stover diet allowed a reduction in blood cholesterol levels when compared with untreated cowpea stover diet. Furthermore, data

from growth performances, gastrointestinal tract histology, blood parameters and carcass traits indicate that the threshold level of 10% incorporation of treated cowpea stover can be set at higher levels.

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Chapter 5 - 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

Andrade E,[1,2] Gonçalves A,[3] Mendes-Ferreira A,[4] Silva V,[2] Pinheiro V,[2] Rodrigues M[2,3] and Ferreira L[2,3]

[1]CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil.

[2]Animal and Veterinary Research Centre, University of Trás-os-Montes and Alto Douro (UTAD-CECAV), Department of Animal Science, Vila Real, Portugal.

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

[4]BioISI-Biosystems and Integrative Sciences Institute, Lisboa, Portugal.

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Abstract

Agro-industrial co-products have low economical value as foods for human consumption however might represent a potential value as animal feedstuffs. This study evaluated a novel feedstuff, ensiled discarded apple (85%) and cowpea stover (15%) mixtures with two distinct ensiling periods (45 and 60 days) regarding the nutritive value, fermentation quality and aerobic stability. Generally, no differences ($P>0.05$) were observed between ensiling periods for its nutritive value and fermentation characteristics. Silages were stable after ensiling, presenting high lactic (77.3 g kg^{-1} DM) and acetic (54.7 g kg^{-1} DM) acids 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 (LAB) numbers were high at silo opening (7.14 log CFU g^{-1}), whilst Enterobacteriaceae were not detected and yeasts and moulds were low (2.44 log CFU g^{-1}). Yeasts and moulds and Enterobacteriaceae numbers grew considerably during the 12 d of air exposure. The mixtures of low-calibre discarded apples with cowpea stover can be used as an animal feed after the ensiling process due 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 (López *et al*., 2005), 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 (Capper, 1990; Bruno-Soares *et al*., 2000). Globally, in 2013, the production of cowpea (*Vigna unguiculata* L. Walp) grains was close to 8.0 Mt (FAO., 2015). 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 (Singh, 2014). Although cowpea is primarily valued as food for its grain, its stover is an important agro-based co-product that can be used in ruminant production owing to its protein and energy content (Anele *et al*., 2010; Gonçalves *et al*., 2016.

Several studies have recently explored the possibility of conserving straw through ensiling given its seasonal availability and the possibility to increase its nutritive value (Gado *et al*., 2013; Qiu *et al*., 2014; Liu *et al*., 2015; Wang *et al*., 2016). Although these experiments have been conducted with cereal straws, the nutritive value of legume

straws (Lopéz *et al*., 2005) 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 (Nkosi *et al*., 2016), 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. (FAO, 2015). According to Wadhwa and Baksh, (2013), 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 (Shalini and Gupta, 2010; Fang *et al*., 2015; Ke *et al*., 2015; Beihgh *et al*., 2015), mainly for ruminant diets owing to the high content of pectins and sugars, which are rapidly fermented in the rumen (Ke *et al*., 2010; Fang *et al*., 2015). Rodrigues *et al*. (2008) 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 (Pirmohammadi *et al*., 2006).

The aim of the present study was to use two agricultural co-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*. (2008) 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 (Rodrigues *et al*., 2008). In this context, it has been shown that propionic acid has antimycotic activity while also improving the aerobic stability of silage (Kung *et al*., 2000).

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 ± 1.8ºC) 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 2ºC above ambient temperature (Conaghan *et al*., 2010). 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 (ISO 6887-1:1999), enumeration of mesophilic LAB (ISO 15214:1998), enumeration of Enterobacteriaceae (ISO 21528-2:2004) and enumeration of yeasts/moulds (ISO 21527-1:2008). 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 30ºC 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 37ºC 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 25ºC 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 60ºC 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, 1990). Neutral detergent fibre (NDFom), acid detergent fibre (ADFom) and lignin (sa) fractions were calculated by the detergent procedures of Robertson and Van Soest, (1981) and Van Soest *et al*., (1991) 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 (Goering and Van Soest, (1970). The total water-soluble carbohydrate (WSC) and starch contents of samples were determined by the anthrone method (Irigoyen *et al*., 1992). 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 100ºC. 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, (1966) and expressed as mmol NaOH kg^{-1} dry matter (DM).

Silage pH, NH3-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 (AOAC, 1990).

The volatile fatty acid (acetic, propionic and butyric) and ethanol concentrations were analysed according to Czerkawski (1976) 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, (1963) and modified by Marten and Barnes, (1980). 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 (SAS, 2009). 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 13. 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.

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 14). 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.

Table 13. 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 excusive of residual ash; ADF, acid detergent fibre; ADL, acid detergent lignin; WSC, water-soluble carbohydrates lignin; BC, buffering capacity; IVOMD, *in vitro* organic matter digestibility.

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}).

Table 14. 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	74.5	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	0.681	0.0296
	Ensiling period (EP)	0.0051	0.3426	0.0749	0.9144	0.1752	0.1831	0.0090	0.3186	0.7108	0.2751
	A x P	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 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 15. Effects of ensiling period on silage pH and ethanol, organic acid and NH_3-N concentration.

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)	NH3-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.

Fermentation profile

The fermentation characteristics of the ensiled mixture are presented in Table 15. 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 Figure 7. 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 of aerobic exposure when its temperature exceeded 2ºC above ambient temperature.

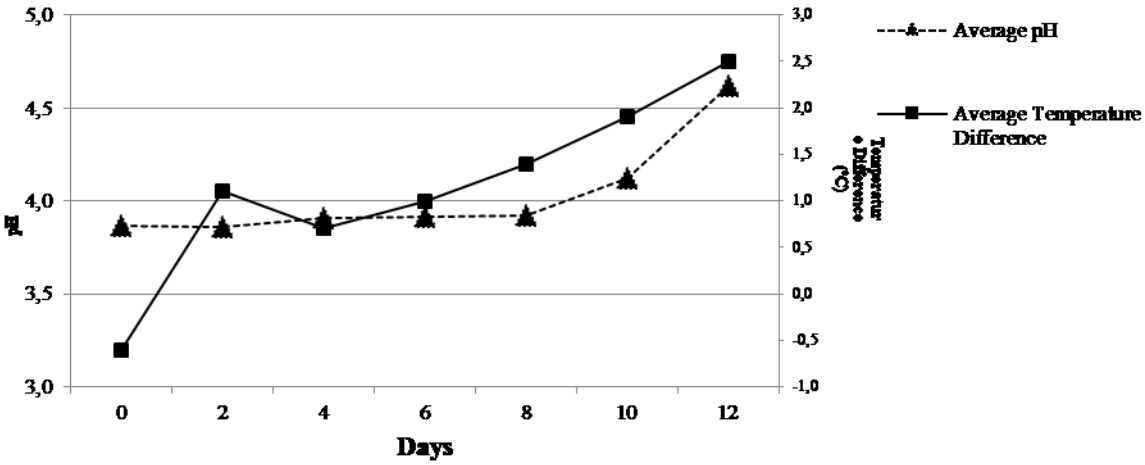

Figure 7. The effect of air exposure on silages' pH and temperature difference between silage and ambient.

The counts for LAB, Enterobacteriaceae and yeast/mould populations at silo opening and after the 12 days aerobic period are presented in Figure 8. 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.

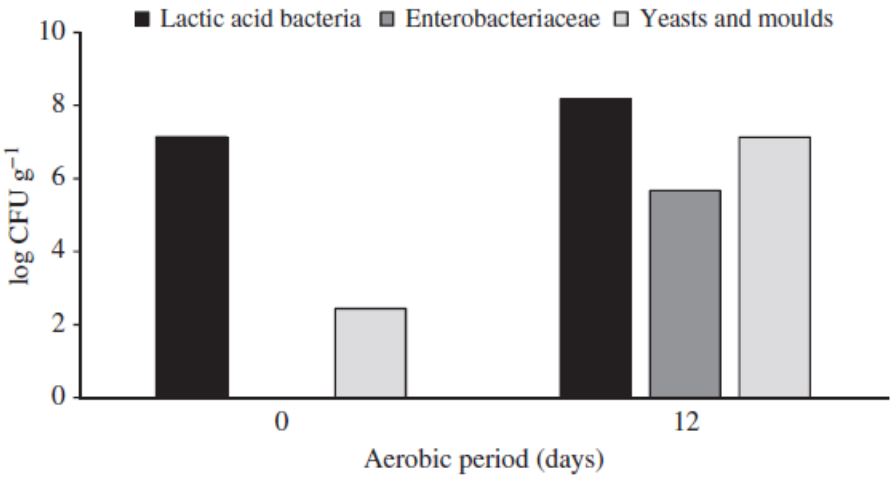

Figure 8. Lactic acid bacteria, Enterobacteriaceae, and yeast and moulds populations in apple and cowpea straw silages at silo opening and after 12 days of aerobic exposure.

Discussion

The chemical composition of the discarded apples used in this study is similar to that reported for commercialized regional Portuguese apple cultivars (Guiné *et al.*, 2009), 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*., (2000) and Gonçalves *et al*., (2016). Furthermore, chemical composition values of cowpea stover are within the general range of values described for legume straws (Bruno-Soares *et al*., 2000; López *et al*., 2005). 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 (Martinez-Fernandez *et al*., 2013). Based on the ensilability index (EI) developed by Martinez-Fernandez *et al*. (2013) 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*. (2010) 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 (Rodrigues *et al*., 2008; Beigh *et al*., 2015). 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*. (2008) 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 (Woolford, 1984; McDdonald *et al*., 1991). In fact, during the ensiling process, a reduction of 92.5% in WSC was observed as a result of the activity of the microbial population. Slightly lower reduction of WSC (average of 82%) was found by Ke *et al*. (2015) when ensiling alfalfa with apple or grape pomace, while Rodrigues et al. (2008) observed an average reduction of 50% in WSC. High residual WSC concentrations in silages are required as they indicate more efficient fermentation (Arriola *et al*. 2011). 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 (Rodrigues *et al*., 2008). Nevertheless, Beigh *et al*. (2015) 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*. (2008) 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*. (2008) in silages of wheat straw with inclusion of 15% apple pulp, resulting from intense fermentation of WSC of the silage by the epiphytic microbial population, especially LAB. These strict anaerobic bacteria should dominate this fermentation phase and mainly convert WSC into lactic acid (lower pKa), decreasing pH values more efficiently (McDonald *et al*., 1991; Dunière *et al*., 2013). 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*. (2015) 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*. (1984) when ensiling apple pomace and barley straw, but are within the range of values reported by Ke *et al*. (2015) Higher lactic acid concentrations were found by Fraser *et al*. (2005) 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*. (2009) 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 (Danner *et al*., 2003). Acetate found in silages may result from the activity of epiphytic Enterobacteriaceae and of both obligate and facultative heterofermentative LAB (Wolford, 1984; Ounder Elferink *et al*., 2000). Arriola *et al*. (2011) Kung and Ranjit,

(2001) and Kleinschmit and Kung, (2006) suggested that high acetate concentrations are normally found in silages inoculated with *Lactobacillus buchneri*. According to Oude Elferink *et al*. (2000) 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, (2010) 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 (Wolford, 1984; Muck, 2010). Proteolytic clostridia may also be responsible for the appearance of NH3-N on silages as a result of the deamination or coupled oxidation reduction (Stickland reaction) of amino acids (McDonald, 1982; Wolford, 1984; Muck, 2010). Nevertheless, the absence of butyric acid indicates that clostridia did not develop in large numbers (Driehuis and Wikselaar, 2000. NH_3-N concentrations found in the present study are within the range of values reported by Pirmohammadi *et al*. (2006) 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*. (2005) 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 (Driehus *et al*., 2001; Arriola *et al*., 2011). 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*. (2000; 2001) 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 (Woolford, 1984).

Ethanol concentrations were five to seven times lower than those previously observed by Rodrigues *et al*. (2008) and Alibes *et al*. (1984) 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 (Arriola *et al*., 2011). The high acetic acid concentrations found in our silages may also have affected yeast survival during the ensiling period (Oude Elferink *et al*., 2000). In fact, at silo opening, yeast numbers were quite low. Kleinschmit and Kung, (2006) 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 (Muck, 2010). 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 (Gelach *et al*., 2013; Woolford, 1990). In the present study, aerobic

stability of the silages was high, reaching almost 10 days. Data presented by Ke *et al*. (2015) 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, (1984) 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* (Driehuis *et al*., 1999; Werinberg *et al*., 1999; Ranjit and Kung, 2000).

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, (1984) 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 (Pahlow *et al*., 2003), and after this initial phase, moulds start to grow (McDonald *et al*., 1991) 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 (Dunière *et al*., 2013). 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,

(2010) Enterobacteriaceae are inhibited below pH 4.5, although Östling and Lindgren, (1993) 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.

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Chapter 6 - Ensilability, fermentation quality, aerobic stability and nutritive value of cowpea (*Vigna unguiculata*) stover treated with *Pleurotus citrinopileatus* in association with discard apple (*Malus domestica*) and commercial inoculant

Andrade E,[1,2] Mendes-Ferreira A,[3] Botelho S,[2] Marques G,[4] Mendes C,[5] Rosa E,[4] Rodrigues M[2,4] and Ferreira L[2,4]

[1] CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil

[2] University of Trás-os-Montes and Alto Douro (UTAD), Animal Science Department, Vila Real, Portugal

[3] BioISI-Biosystems and Integrative Sciences Institute, Lisboa, Portugal

Sciences (CITAB)

[4] Centre for the Research and Technology of Agro-Environmental and Biological

[5] Faculty of Agronomy and Veterinary Medicine, University of Brasília (FAV/UnB), Brasília -DF, Brazil.

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Abstract

Previous studies showed that cowpea stover could be valorized as animal feedstuff either by the application of biological treatments or by ensiling it together with discarded apple from the fruit industry. This study assessed the ensilability of cowpea stover (15%, as fresh) treated with *Pleurotus citrinopileatus* in association with discarded apple using or not a commercial inoculant. At silo opening silages were stable and presented low pH values (≤4.2), high lactic acid concentration, no butyric acid and aerobic stability averaged 134 h. The inoculated silage presented higher ($P<0.01$) neutral detergent fibre, water-soluble carbohydrate (WSC), lactic acid and ethanol, but lower ($P<0.01$) acetic acid concentrations. The use of the commercial inoculant increased the amount of lactic acid bacterial in the pre-mixture and at the time of opening the silo. No differences were observed in enterobacteriaceae, yeast and moulds count in both treatments until 288 h of air exposure. Cowpea stover valorized with biological treatment (*P. citrinopileatus*) could be conserved by the ensiling process when mixed with low grade discarded apples. The utilization of commercial inoculant allowed the conservation of greater amounts of WSC.

Keywords: silage; legume stover; nutritional valorization; white-rot fungi

Introduction

Leguminous cultivation in Europe is seen as a priority to ensure food security and to avoid dependence on grain imports (Carvalho *et al*., 2017; Watson *et al*., 2017). Legume species adapted to local edaphoclimatic conditions and resistant to the effects of the environmental changes are fundamental (Iglesias and Garote, 2015; Carvalho *et al*., 2017). Cowpea (*Vigna unguiculata*) is adapted to regions with high temperatures and drought, tolerates low fertility soils and wide range of soil pH (Carvalho *et al*., 2017) showing high productivity in the Mediterranean region. The remaining post-harvest aerial part of cowpea crop, featured by high cell wall contents and moderate protein values, can represent an important animal feedstuff (Anele *et al*., 2011; Andrade *et al*., 2017a). The remaining post-harvest of cowpea crop is characterized with high cell wall content and good protein value. However, the high lignin content is a serious drawback in maximizing its utilization in animal feeding (Andrade *et al*., 2017a).

Currently, techniques that promote the valorization of this co-product using white-rot fungi degrade lignin efficiently due their extracellular enzymatic system and hyphal penetration power are being studied (Sharma and Arora, 2015). Modifications in structure, chemistry and enzymatic hydrolysis of lignocellulosic biomass after white-rot fungi treatment have been observed (Tuyen *et al*., 2012; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015; Andrade *et al*., 2017b). Results obtained by Andrade *et al*. 2017b showed that treatment of cowpea stover with fungi *Pleurotus citrinopileatus* decreased its lignin content and, consequently, enhanced its *in vitro* digestibility.

So far preservation of fungal-treated co-products for longer periods have not been assessed in depth (Mao *et al*., 2018). Andrade *et al*. (2017a) evaluated the potential of conservation this crop residue as silage in association with discarded apples (*Malus domestica*). Results showed that stable silages and high aerobical stability can be

obtained. However, a significant reduction of the water-soluble carbohydrate content was observed, suggesting that the use of additive to increase the acidification will contribute to the control of fermentation and enhance the quality of the silage produced.

The objective of this study was to evaluate the ensilability, fermentation quality, aerobic stability and nutritive value of cowpea stover treated with *P. citrinopileatus* in association with discarded apple, using or not a commercial inoculant.

Materials and methods

Raw materials

The cowpea (*Vigna unguiculata* L. Walp, cv. 'fradel') stover was produced in the north of Portugal and was collected after grain harvest (summer of 2015). After collection, cowpea stover was chopped on a stationary chopper (Pachancho Cutting mill, model L29025, Braga, Portugal) adjusted for a cut length of 2 cm. Low grade discarded apples (*Malus domestica* Borkh cv. 'golden delicious') were collected from orchards located in the north of Portugal and were ground to pass a 2 cm screen (Pachancho Cutting mill, model L29025, Braga, Portugal) until a homogeneous mash was obtained.

Fungi biological pre-treatment

Before ensiling, cowpea stover was pre-treated with *Pleurotus citrinopileatus* as previous studies (Andrade *et al*., 2017a) observed that this fungus presented an efficient delignification process and a greater potential for the improvement of its nutritive value. Incubations were processed in several plastic containers (TP3000 + TPD3000 XXL, Microbox Combiness, Nevele, Belgium) for 22 days, as described previously (Andrade *et al*., 2017b). Briefly, before incubation the cowpea stover was submerged in water (1:3 of cowpea stover and water, respectively) for 12 hours and then drained over 2-hour period. Approximately 0.8 kg^{-1} of the humidified cowpea stover was placed in

each container and then autoclaved at 121ºC for 30 min. After cooling, 32 g of spawn were added to each container and conditioned in an incubation chamber at 28ºC and 75% relative humidity for 22 days.

Ensiling process

Pre-treated cowpea stover was thoroughly mixed by hand with the homogenous apple mash aiming to obtain a mixture containing 15% of treated cowpea stover on a fresh weight basis. This level of incorporation of cowpea stover was chosen based on a preliminary study that indicated a greater nutritive value and aerobic stability of the resulting silages of mixtures of these raw materials (Andrade *et al*., 2017a).

Before ensiling 0.2% of propionic acid was added (in fresh matter) to the apple-treated cowpea stover mixture to limit undesirable fermentation while lowering the pH of the mixture according to Kung *et al*. (2018). After the application of propionic acid, the mixture was divided in two equal amounts: 1) one part was treated (application rate of 5 g t of fresh mixture) with an commercial inoculant (SIL-ALL® - LV) containing *Lactobacillus plantarum* (4×10^{10} CFU g^{-1}), *Pediococcus acidilactici* (2×10^{10} CFU g^{-1}), *Pediococcus pentosaceus* (2×10^{10} CFU g^{-1}), *Propionibacterium acidipropionici* (2×10^{10} CFU g), α-amylase from *Bacillus amyloliquefaciens* (3600 BAU g^{-1}), cellulase from *Trichoderma reesei* (60 CMC g^{-1}), β-glucanase from *Aspergillus niger* (1000 IU g^{-1}) and xylanase from *Trichoderma longibrachiatum* (1500 IU g^{-1}), and 2) the other one was left non-inoculated. The additive was applied uniformly to the mixtures using a hand sprayer with constant mixing.

Approximately 3.0 kg^{-1} of each mixture was packed into dark plastic bags, placed in plastic buckets of 10 L^{-1} to an approximated wet density of 525 kg fresh matter/m^3, and sealed immediately with tight lids. The ensiled mixtures were weighed and stored in

a conditioned room (26 ± 1.3ºC) for an ensiling period of 45 days. Five replicates of each mixture were prepared resulting in a total of 10 laboratory silos.

After the ensiling period, 3 cm of silage from the surface of each experimental silo was discarded. Each silo was covered by cheesecloth and aluminium foils to prevent silage dehydration and dust contamination while allowing the entrance of air and, consequently, assess the aerobic stability of the silages. Aerobic stability of the silages was defined by the number of hours that the silages remained stable before its temperature reached 2°C above the ambient temperature (Conaghan *et al*., 2010). Temperature, ambient and silage, was recorded every 6 hours, and pH was monitored at 0, 2, 4, 6, 8, 10 and 12 days after silo opening. Fresh samples from initial materials (i.e., apple and treated cowpea stover), pre-ensiled mixtures and silages at silo opening and after 12 days of aerobic exposure were collected for further analysis.

Chemical analysis

All samples collected for chemical analysis were dried in an air forced oven at 55ºC for 4 days, and then ground to pass 1mm sieve (Retsch, cutting mill, model SM1, Germany) and then stored in airtight flasks at room temperature for subsequent analysis.

Samples were analysed for ash (nº. 942.05) and total N (nº. 954.01) as Kjeldahl N following the methods of the Association of Official Analytical Chemists (AOAC, 1990). Neutral detergent fibre (NDFom), acid detergent fibre (ADFom) and lignin fractions were calculated by the detergent procedures of Robertson and Van Soest, (1981) and Van Soest *et al*. (1991). Sodium sulfite and heat stable amylase were not used in the analysis and results were expressed exclusive of residual ash. The acid detergent insoluble nitrogen (ADIN) of samples was also determined following the procedures of Goering and Van Soest, (1970).

The total water-soluble carbohydrates (WSC) content of the samples was determined by the anthrone method (Irigoyen *et al*., 1992). Standard curves were prepared with stock glucose solutions, and the solutions were read in a spectrophotometer (Shimadzu UVmini 1240, Kyoto, Japan) at 625 nm. Total starch was quantified using an enzymatic assay procedure (K-TSTA-100A, Megazyme, Ireland). The buffering capacity (BC) of the pre-ensiled mixtures was measured in fresh macerated samples and was expressed as mmol required to change the pH from 4 to 6 (Playne and McDonald, 1966).

The pH, NH_3-N, ethanol and organic acids (lactic acid and volatile fatty acids) on the obtained silages were determined in water extracts obtained by mixing thoroughly 225 mL of distilled water to 25 g of fresh silages. The pH value of the silage samples collected at 0, 2, 4, 6, 8, 10 and 12 days of aerobic exposure was measured using pH meter equipment (pH meter 632, Metrohm Ltd., Herisau, Switzerland). The NH_3-N was determined following the method (no. 920.03) proposed by the AOAC (1990) and expressed as proportion of total N.

Concentrations of acetic, propionic and butyric acids, and ethanol were analysed by gas-liquid chromatography (Shimadzu GC141 B, Kyoto, Japan) following the procedures of Czerkawski (1976) using pivalic acid as the internal standard. Chromatographer was equipped with a flame ionization detector (FID) and a capillary column (SUPELCO Nukol, 0.25 mm i.d. x 30 m x 0.25 μm). Lactic acid was determined using an enzymatic kit assay procedure (K-DLATE 07/14, Megazyme, Ireland).

In vitro digestibility

The i*n vitro* organic matter digestibility (IVOMD) of raw materials (apple, treated cowpea stover and cowpea stover autoclaved), pre-ensiled mixtures and silages was determined according to the methodology of Tilley and Terry (1963) and modified by Marten and Barnes (1980). Two non-lactating rumen cannulated (Bar Diamond Inc., Parma, Idaho, USA) cows were used as rumen fluid donors and were fed twice a day with a diet of corn silage (0.70), a concentrate feed (0.25) and a meadow hay (0.05) shredded to 20 cm particles through a bale gripper (JN Jensen & Sommer APS, model DK 6534 Agerskov, Denmark). Rumen fluid was collected 2 h after the morning meal and pooled into one in a pre-warmed insulated bottle filled with CO_2 and was strained and filtered through cheesecloth. All manipulations were under continuous flushing with CO_2.

Microbiological analysis

Microbiological analysis was carried out in pre-ensiled mixtures and silage samples collected at 0, 3, 6, 9 and 12 days of aerobic exposure, following the procedures described by Andrade *et al*. (2017a) For this purpose, standard methodologies described for food and animal feeding stuffs, for the preparation, suspensions and dilutions of test samples (ISO 6887-1, 1999), enumeration of mesophilic Lactic acid bacteria (LAB) (ISO 15214, 1998), enumeration of *Enterobacteriaceae* (ISO 21528-2, 2004), and enumeration of yeasts and moulds (ISO 21527-1, 2008) were used.

Statistical analysis

Data were analysed using the GLM procedures of SAS, version 9.2 (SAS, 2009). Data relating with cowpea stover autoclaved, raw materials and pre-ensiled mixture on chemical composition and IVOMD was analysed by a one-way analysis of variance

(ANOVA). Similar procedure was used for assessing the effect of silage non-inoculated and inoculated on chemical composition, IVOMD, aerobic stability and microbiology population. When the F test was significant ($P < 0.05$), multiple comparisons among means were examined by the Tukey test.

Results and discussion

The pre-treatment with *P. citrinopileatus* modified the chemical composition of the cowpea stover, resulting in an increase of its nutritive value (Table 16). In fact, a reduction in the NDFom and lignin content was observed. These modifications on the cell wall content are consistent with those previously reported by Andrade *et al*. (2017b) when treating also cowpea stover with this fungi strain. An increase in the crude protein content was also observed as a result of the fungal treatment. Similar results were observed elsewhere (Tuyen *et al*., 2012; Andrade *et al*., 2017b; Mao *et al*., 2018) and mainly attributed to 1) a proportional reduction of other components (e.g. lignocellulose content; Tuyen *et al*., 2012; Tuyen *et al*., 2013) and/or 2) the inability of the Kjeldahl method to quantify nitrates and nitrites as total N in the untreated cowpea stover (Van Camp and Dierckw, 2004). These distinct N chemical forms can be assimilated by the fungus to glutamate and glutamine (Han *et al*., 2009) during the incubation, making them measurable as N by the Kjeldahl method in the treated cowpea stover (Andrade *et al*., 2017b).

As a consequence of the modification on the cell wall structure mentioned above, the pre-treatment of cowpea stover with *P. citrinopileatus* increased its IVOMD. A similar increase in the nutritive value has been observed in various raw materials as result of the application of different fungi spawn (Mao *et al*., 2018; Shrivastava *et al*., 2011). Tuyen *et al*. (2012) found an increase in the *in vitro* gas production of wheat

straw treated with *Ceriporiopsis subvermispora* after 49 days of incubation. These results are possibly attributed to the delignification process promoted by the different fungi strains on the raw materials, either decreasing its lignin content (Chen *et al*., 2010) and/or reducing covalent and ester or ether linkages between lignin and structural carbohydrates (Van Kuijk *et al*., 2015). These authors referred that this feature will increase the accessibility of rumen microbial enzymes to these polysaccharides with a subsequent positive effect on the IVOMD.

Table 16. Chemical composition and IVOMD (*in vitro* organic matter digestibility) of autoclaved cowpea stover, raw materials and pre-ensiled mixture.

Items	Untreated cowpea stover[1]	Apple	Treated cowpea stover[2]	Pre-ensiled mixture[3]	SEM	p-value
Dry Matter (DM), g kg^{-1}	214.4^{b}	164.1^{a}	922.0^{d}	238.8^{c}	3.25	<0.0001
Ash, g kg^{-1} DM	72.7^{c}	20.1^{a}	96.8^{d}	67.4^{b}	0.65	<0.0001
NDFom, g kg^{-1} DM	748.6^{d}	123.7^{a}	669.4^{c}	405.8^{b}	7.84	<0.0001
ADF, g kg^{-1} DM	637.3^{d}	87.3^{a}	542.6^{c}	342.2^{b}	4.49	<0.0001
Lignin, g kg^{-1} DM	153.8^{d}	34.7^{a}	127.2^{c}	89.9^{b}	1.06	<0.0001
Crude Protein, g kg^{-1} DM	96.6^{c}	19.9^{a}	121.8^{d}	70.6^{b}	1.95	<0.0001
WSC, g kg^{-1} DM	6.4^{a}	804.2^{c}	7.2^{a}	359.0^{b}	6.34	<0.0001
Starch, g kg^{-1} DM	34.7^{a}	77.6^{c}	36.0^{a}	56.8^{b}	0.64	<0.0001
ADIN, g kg^{-1} DM	2.2^{a}	0.4^{a}	2.3^{b}	2.2^{b}	0.05	<0.0001
pH	8.4^{c}	4.0^{a}	8.3^{c}	6.3^{b}	0.04	<0.0001
Malic acid, g kg^{-1} DM	ND	13.2^{b}	ND	7.5^{a}	0.08	<0.0001
IVOMD, g kg^{-1} OM	381.4^{a}	866.3^{d}	436.7^{b}	692.7^{c}	2.98	<0.0001
N-NH_3, g kg^{-1} total N	-	-	-	23.8	-	-
BC, mmol NAOH	-	-	-	247.0	-	-

[1]Untreated cowpea stover: cowpea stover before fungi treatment; [2]Treated cowpea stover: cowpea stover treated with fungi *Pleurotus citrinopileatus* during 22days; [3]Pre-ensiled mixture containing 85% of discarded apple and 15% of cowpea stover treated with *Pleurotus citrinopileatus* on a fresh weight basis. aNDF, neutral detergent fibre expressed exclusive of residual ash; ADF, acid detergent fibre; ADL, acid detergent lignin; WSC, water soluble carbohydrates; ADIN, acid detergent insoluble nitrogen; N-NH_3, ammoniacal nitrogen; BC, buffering capacity; IVOMD, *in vitro* organic matter digestibility; ND, not detected.

It should be noted that the pre-treatment of cowpea stover with *P. citrinopileatus* did not promote a decrease on its pH (Table 16). Most of the studies have found a reduction in the pH of treated raw materials after its colonization with white-rot fungi (Agosin and Odier, 1985; Mao *et al*., 2018) as a result of the production of organic acids during the solid-state fermentation (Mao *et al*., 2018). These data can be the result of the distinct buffer capacity of the raw materials used in distinct studies, being higher for legumes than grasses (Dewhurst *et al*., 2003). In the present study, the absence of pH reduction may not have had a direct negative impact on the synthesis of ligninolytic enzymes produced by the fungi. However, it may have influenced the initial colonization period as observed after 5 days of incubation. Nevertheless, the increase in the IVOMD suggests that this colonization process was efficient.

The mashed apple presented a distinct chemical composition compared to that of the cowpea stover, namely a lower fibre content, and a higher WSC content and IVOMD. It should also be pointed out the acidic nature of the mashed apple with a low pH (4.0) and a high malic acid concentration (13.2 g kg^{-1} DM). As expected, the chemical composition of the pre-ensiled mixture mainly reflected the composition of the raw materials and suggests its high ensilability. In fact, based on its DM and WSC content and BC, the ensilability of the pre-ensiled mixture can be considered high, as its ensilability index (EI, +43.1) falls within the category defined by Martinez-Fernandez *et al*. (2013) for forages with high ensilability (EI >28). It should be pointed out that the BC (247.0 mmol NaOH kg^{-1} DM) was higher than that (159 mmol NaOH kg^{-1} DM) observed by Andrade *et al*. (2017a) for similar mixtures of mashed apple and untreated cowpea stover. These results suggest that the fungus activity on the cowpea stover during the incubation process resulted in a material with a higher buffer capacity, possibly related to the increase in its protein content.

Table 17. Chemical composition, aerobic stability and *in vitro* organic matter digestibility (IVOMD), aerobic stability of the ensiled mixture.

Parameter	Non-inoculated	Inoculated[1]	SEM	P-value
DM, g kg^{-1}	217.1	197.2	1.20	<0.0001
Loss DM, g kg^{-1}	24.2	32.3	0.08	0.0005
Ash, g kg^{-1} DM	78.3	84.1	1.47	0.0308
NDFom, g kg^{-1} DM	465.8	504.2	7.63	0.0091
ADF, g kg^{-1} DM	404.5	429.1	6.40	0.0346
Lignin, g kg^{-1} DM	110.8	123.4	2.87	0.0210
CP, g kg^{-1} DM	84.2	90.6	3.34	0.2279
ADIN	3.2	3.2	0.25	0.9180
WSC, g kg^{-1} DM	58.5	76.0	2.55	0.0029
Starch, g kg^{-1} DM	22.0	27.0	1.38	0.0405
$N\text{-}NH_3$, g kg^{-1} total N	31.3	29.7	1.85	0.5747
pH	4.2	4.0	0.06	<0.0001
Ethanol, g kg^{-1} DM	17.6	33.4	2.94	0.0051
Acetic acid, g kg^{-1} DM	20.2	9.7	1.28	0.0012
Propionic acid, g kg^{-1} DM	4.2	5.2	0.42	0.1621
Butyric acid, g kg^{-1} DM	ND	ND	-	-
Lactic acid, g kg^{-1} DM	53.4	84.7	0.83	<0.0001
Malic acid, g kg^{-1} DM	ND	ND	-	-
Aerobic stability, h	144	125	13.4	0.3367
IVOMD, g kg^{-1} DM	591.4	584.5	7.07	0.5165

[1]Inoculat containing *Lactobacillus plantarum* (4×10^{10} CFU g^{-1}), *Pediococcus acidilactici* (2×10^{10} CFU g^{-1}), *Pediococcus pentosaceus* (2×10^{10} CFU g^{-1}), *Propionibacterium acidipropionici* (2×10^{10} CFU g^{-1}), α-amylase from *Bacillus amyloliquefaciens* (3600 BAU g^{-1}), cellulase from *Trichoderma reesei* (60 CMC g^{-1}), β-glucanase from *Aspergillus niger* (1000 IU g^{-1}) and xylanase from *Trichoderma longibrachiatum* (1500 IU g^{-1}). aNDF, neutral detergent fibre expressed exclusive of residual ash; ADF, acid detergent fibre; ADL, acid detergent lignin; WSC, water soluble carbohydrates; ADIN, acid detergent insoluble nitrogen; N-NH3, ammoniacal nitrogen; BC, buffering capacity

Results indicated that both non-inoculated and inoculated silages were stable after 45 days of ensiling, presenting low pH values (≤ 4.2) and no butyric acid (Table 17). These results are within those reported for mixed silages containing wheat straw/mashed apple (Rodrigues *et al*., 2008) and cowpea stover/mashed apple (Andrade *et al*., 2017a). It is well known that the critical pH value for stable silages is mainly dependent on its dry-matter content.

The pH of both silages is within the range (4.10 and 4.35) referred by Weissbach and Muck, (1996) for stable silages with DM content between 150 and 250 g kg^{-1}.

Silage pH is highly correlated with the amount of lactic acid (low pK_a) produced during the fermentation period of the ensiling process (Pahlow *et al*., 2003). This result from the activity of lactic acid bacteria (LAB) from epiphytic and added by the inoculants on the water-soluble carbohydrates (WSC). As expected, inoculated silage had higher lactic acid concentrations than non-inoculated silages as facultative heterofermentative LAB (*L. plantarum*, *P. acidilactici* and *P. pentosaceus*) were added. Muck *et al*. (2018) referred that, generally, silages inoculated with these LAB species present higher lactic acid content as they are able to ferment both hexoses and pentoses (owning phosphoketolase) producing almost entirely lactic acid and primarily lactic and acetic acids, respectively.

The chemical composition of the pre-ensiled mixture indicates a high WSC content that could have been used as substrate by the LAB population. Furthermore, sugar concentration in the pre-ensiled mixture is mainly dependent on the chemical composition of the discarded apple which contains mainly fructose (40 to 90 g kg^{-1}, as fresh), glucose (15 to 40 g kg^{-1}, as fresh) and sucrose (15 to 30 kg^{-1}, as fresh; Fertonani *et al*., 2006; Wu *et al*., 2007). In this way, it was expected that the predominant biochemical pathway used by the LAB population would be the fermentation of hexoses, thus resulting in mainly lactic acid. Nevertheless, lactic acid concentration in the non-inoculated mixtures is relatively high indicating LAB epiphytic activity. Data from microbial population counts (Table 18) are consistent with these results, as a higher number of LAB was determined for the inoculated (8.2 log CFU g^{-1}) compared to non-inoculated (5.7 log CFU g^{-1}) silages.

Recently, on a meta-analysis evaluating the effect of LAB inoculation in silage fermentation, Oliveira *et al*. (2017) reinforced the capability of LAB inoculants to improve fermentation of legume silages, attributing this effect to their low epiphytic

flora numbers, low WSC concentration and high buffering capacity. Our mixed silages cannot be compared to legume silages as mashed apple was added, increasing the WSC content to values higher than the ones normally present by legume silages. In fact, these values are even higher than pre-ensiled corn forage (Martinez-Fernandes *et al*., 2013). Nevertheless, considering the low epiphytic flora and high buffer capacity of our silage mixtures LAB inoculation was effective. These results indicate that WSC content was not as relevant as the epiphytic microbial population of the substrates in controlling the fermentation processes.

Table 18. Lactic acid bacteria, enterobacteriaceae and yeast/moulds population (log CFU g^{-1}) of pre-ensiled and ensilage non-inoculated or inoculated at silo opening until 288h of aerobic exposure.

	Non-inoculated	Inoculated[1]	SEM	P-value
Lactic acid bacteria				
Pre-ensiled	ND	4.1	0.00	<0.0001
0h	5.7	8.2	0.14	<0.0001
72h	7.8	7.3	0.18	0.1039
144h	9.8	9.6	0.26	0.4673
216h	9.5	9.6	0.22	0.8364
288h	10.1	9.0	0.19	0.0047
Enterobacteriaceae				
Pre-ensiled	3.3	3.3	0.01	0.9989
0h	ND	ND	-	-
72h	ND	ND	-	-
144h	ND	ND	-	-
216h	3.4	3.1	0.37	0.6300
288h	3.1	3.7	0.27	0.2039
Yeast and moulds				
Pre-ensiled	4.1	4.1	0.01	0.9993
0h	4.7	5.0	0.26	0.3868
72h	6.8	6.4	0.18	0.2591
144h	8.2	7.9	0.30	0.5387
216h	6.8	7.3	0.16	0.0689
288h	6.8	6.6	0.28	0.7659

[1]Inoculat containing *Lactobacillus plantarum* (4×10^{10} CFU g^{-1}), *Pediococcus acidilactici* (2×10^{10} CFU g^{-1}), *Pediococcus pentosaceus* (2×10^{10} CFU g^{-1}), *Propionibacterium acidipropionici* (2×10^{10} CFU g^{-1}), α-amylase from *Bacillus amyloliquefaciens* (3600 BAU g^{-1}), cellulase from *Trichoderma reesei* (60 CMC g^{-1}), β-glucanase from *Aspergillus niger* (1000 IU g^{-1}) and xylanase from *Trichoderma longibrachiatum* (1500 IU g^{-1}).

Another parameter normally used to evaluate silage quality is the lactic/acetic ratio. While Chahine *et al*. (2009) suggested that this ratio should vary between 1.5 and 4 for corn silages, Ward (2000) evaluating 3.600 samples of corn, legume and grass silages, reported ratio values of 1.6, 2.0 and 1.9, respectively. Ratios obtained in this study point out to relatively higher values for non-inoculated mixed silages (2.6) and too much higher values for inoculated mixed silage (8.7). Compared to previous studies[5] it is clear that no differences were obtained for the lactic acid (77.3 vs. 69.1 g kg^{-1} DM) but acetic acid concentrations are much lower (54.7 vs. 15.0 g kg^{-1} DM).

According to Pahlow *et al*. (2003) acetic acid may result from the fermentative activity of epiphytic Enterobacteriaceae and of both obligate and facultative heterofermentative LAB. Data (Table 18) indicates that pre-ensiled mixtures had similar Enterobacteriaceae counts but only the inoculated mixtures presented quantifiable LAB. In this way, it seems that the initial bacterial population influenced the fermentation process, leading to a lower acetic acid production during the fermentation phase as a result of a rapid pH decrease influenced by the intense lactic acid production in the inoculated mixed silages. By the contrary, in the non-inoculated silages the absence of LAB in the pre-ensiled mixture may have promoted a longer fermentation phase allowing higher activities of Enterobacteriaceae. This activity during the initial fermentation phase of the ensiling process could have led to a competition with LAB for the nutrients including WSC. Values presented in Table 17 are consistent with this hypothesis as less WSC were found in the non-inoculated mixed silages.

Although data on acetic acid concentrations pointed out to a more prolonged acetic fermentation phase, this was not reflected on the $N\text{-}NH_3$ concentrations as no differences between inoculated and non-inoculated silages were observed (averaging 30 g $N\text{-}NH_3$ kg^{-1} of total N), indicating a low microbial proteolytic process in both silages.

The observed values are well below those reported by Kung *et al*. (2018) for legume silages with less than 35% DM (100-150 g $N\text{-}NH_3$ kg^{-1} of total N) and even for corn silages with 30-40% DM (50-70 g $N\text{-}NH_3$ kg^{-1} of total N). As DM content of our silages was relatively low (238.8 g kg^{-1}) higher $N\text{-}NH_3$ concentrations were expected. Similar $N\text{-}NH_3$ concentrations (25 to 35 g $N\text{-}NH_3$ kg^{-1} of total N) are reported for silages (i.e. sugarcane) showing a rapid decrease of pH (Reyes-Gutiérrez *et al*., 2015). It should be pointed out that $N\text{-}NH_3$ concentrations found in both inoculated and non-inoculated silages are much lower (30 vs. 106 g $N\text{-}NH_3$ kg^{-1} of total N) than those observed in a previous experiment[5] performed with similar mixtures of mashed apple and untreated cowpea stover. These results might be explained by the production of secondary compounds, during the cowpea stover fungi colonization, that are known to have an antimicrobial property that could have limited the development of the Enterobacteriacea. In fact, several studies (Lang *et al*., 1997; Folman *et al*., 2008; de Boer *et al*., 2010) have reported that white-rot fungi present bacteriostatic and bactericidal effects that comprise the production of toxic secondary metabolites.

Results showed that inoculated silages had higher (P=0.0051) ethanol concentrations compared to non-inoculated silages (Table 18). These ethanol concentrations are higher than those usually reported by Kung *et al*. (2018) for legumes silages (5-10 g kg^{-1} DM), but are within the range of that suggested for high fermentable silages as corn silages with 30-40% DM (10-30 g kg^{-1} DM). Much higher mean ethanol concentrations (>30 g kg^{-1} DM) are generally found for sugarcane silages (Mendes *et al*., 2008; Daniel *et al*., 2013; da Silva *et al*., 2017; Jacovaci *et al*., 2017; Kung *et al*., 2018) as a result of large numbers of epiphytic yeasts and high WSC concentrations (Mendes *et al*., 2008; Daniel *et al*., 2013; da Silva *et al*., 2017). The high WSC concentrations found in our pre-ensiled mixture (359 g kg^{-1} DM) and the fact that apples

are known to have a rich population of epiphytic yeasts (Wei *et al*., 2017) may explain our high ethanol concentrations.

Several additives, namely propionic acid, calcium oxide, potassium sorbate, sodium benzoate, sodium nitrite, have been suggested to control yeast development (Kleinschmit *et al*., 2005; Knicky and Spörndly, 2011; Javovaci *et al*., 2017; Kung *et al*., 2018). Based on the recommendations of Kung *et al*. (2018) 0.2% of propionic acid was added to our pre-ensiled mixture aiming to limit yeast development. Results showed that this level of addition was not sufficient to control yeast development during the ensiling process. This could be the result of the already mentioned high epiphytic yeasts population and also due to the low dry matter content of our mixtures. In fact, the recommendation proposed by Kung *et al*. (2018) is mainly applied to silages with a dry matter content around 35%.

Data show that ethanol concentration in the inoculated silages was almost two times higher than that found in the non-inoculated silages. The higher acetic acid concentrations observed in the non-inoculated silages may explain these results. In fact, the acetic acid is the main organic acid that controls yeast (Oude *et al*., 2000; Muck 2010; Kung *et al*., 2018) and, in its undissociated form, this acid diffuses into the yeast cell reducing the intracellular pH by releasing H^+ ions, killing the yeast cell (Pahlow *et al*., 2003).

The aerobic stability of silages was not affected by use of the inoculant, both presenting a mean stability value of 134h (Table 17 and Figure 9). Comparing the mean reported values for high fermentable silages (i.e. corn and sugarcane silages; Kleinschmit *et al*., 2006; Jacovaci *et al*., 2017) our data is quite similar (mean of 100-150h). However, for alfalfa silages (Tao *et al*., 2016) and mixtures of alfalfa with apple pomace or alfalfa with grape pomace (Ke *et al*., 2015) our results are much lower

(between 50 and 60%). Previous results verified by Andrade *et al*. (2017a) using silage mixtures of cowpea stover and discarded apple, indicate mean aerobic stability values of 216 h. These results can be explained by different chemical composition of the silages. In fact, lower concentration of acetic acid and higher concentration of WSC were observed compared to the data presented by Andrade *et al*. (2017a) The same reasoning can be established for legume and alfalfa mixture silages. According to Woolford (1990) acetic acid has strong antimicrobial properties and is well correlated to the improvement of the aerobic stability of the silages. Woolford (1990) reported that proliferation of undesirable microorganisms and consequently the decrease in aerobic stability is affected by the chemical composition of the silage, highlighting the use/consumption of residual sugars and organic acids. Silages with high lactic acid and residual carbohydrates contents are more susceptible to aerobic deterioration, as they are the main substrates for the yeasts that initiate the deterioration process (da Silva *et al*., 2017). At silo opening, both silages presented similar values of yeast and moulds (Table 18) and this similarity was maintained over the 288h evaluated.

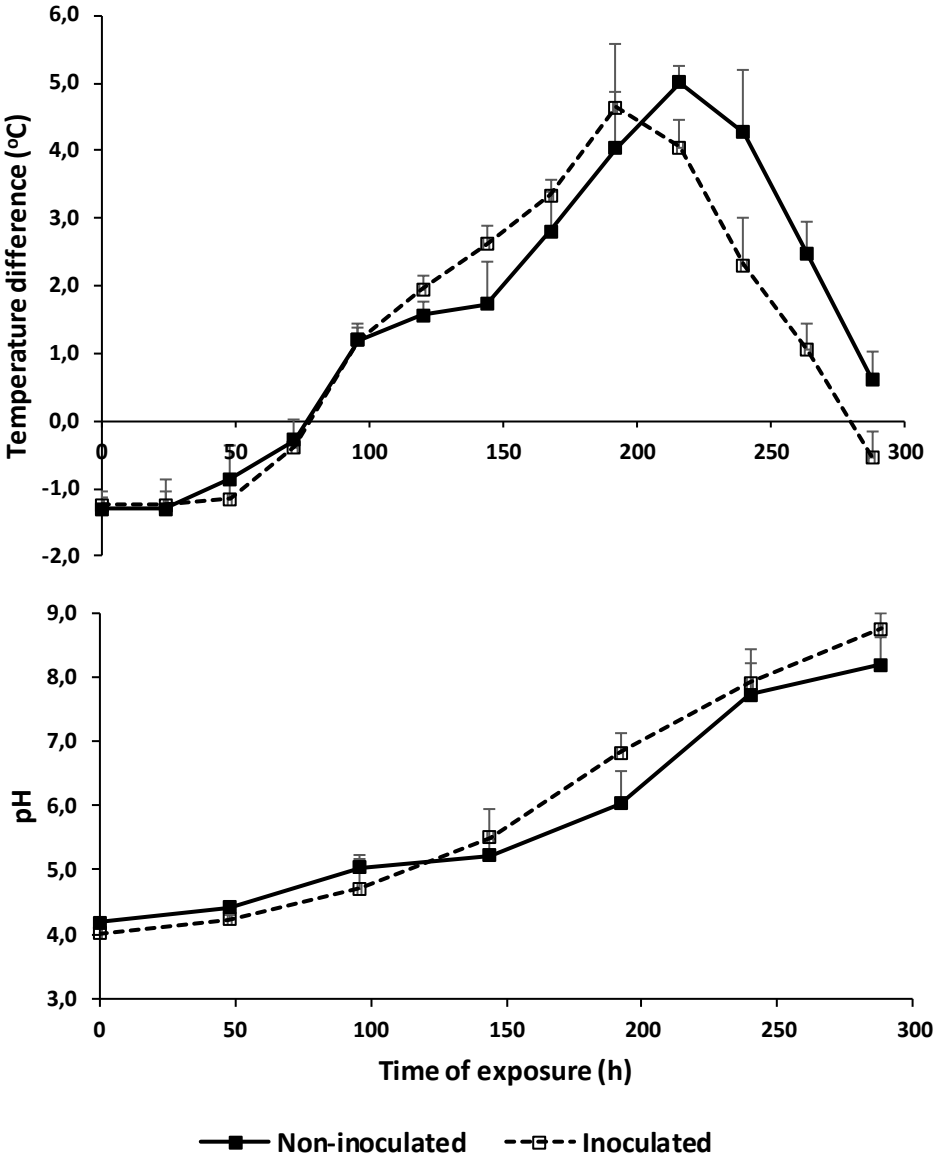

Figure 9. The effect of air exposure on silage non-inoculated and inoculated on pH (a) and temperature difference between silage and ambient (b).

Conclusion

The pre-treatment with *Pleurotus citrinopileatus* modified the chemical composition of the cowpea stover, improving its nutritive value. Cowpea stover treated with *P. citrinopileatus* in association with low grade discarded apple can be given an added value by the ensiling process. The utilization of commercial inoculant SIL-ALL® - LV influences the chemical composition improving the conservation of greater amounts of water-soluble carbohydrate.

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General discussion and perspectives

Agricultural and livestock production systems face a challenging scenario with the increase of global demand for protein sources, namely legume grains and animal protein (FAO, 2011, Rosa, 2017). Leguminous cultivation in Europe is seen as essential to ensure food security and avoid dependence on grain imports as the continent has a deficit of about 70% high-protein materials (Carvalho *et al*., 2017; Watson *et al*., 2017). Thus, species adapted to the local edaphoclimatic conditions and cultivars resistant to the effects of the environmental changes that are expected for the next decades (e.g. increased temperature, reduction of water availability) are fundamental for Mediterranean region (Iglesias and Garrote, 2015; Rosa, 2017).

Cowpea is a legume originating in Africa that adapts to regions with high temperature and drought, tolerates to low fertility soils and wide range of soil pH (Fery, 1990; Hall, 2004; Carvalho *et al*., 2017). Thus, in the Mediterranean region this species develops well and the seasonality of its production can represent an important feedstuff (Anele *et al*., 2011). Agricultural co-products, e.g. straw, stover and hulls, could represent an important feedstuff as complementary source for animal feeding during periods of forage shortage or addition of fiber to compound feeds (Bruno-Soarez *et al*., 2000; López *et al*., 2005; Anele *et al*., 2011; Anele *et al*., 2012). Depending on harvest technology of cowpea grains, different residues, such as leaves, stems and pods, can be obtained. These residues may have potential nutritive value as they can be used as a source of digestible fiber or as an alternative to low quality forages for ruminant feed during the dry season (Anele *et al*., 2011).

The nutritive value of cowpea stover *cv.* fradel and its main fractions (leaves and stems) was studied in Chapter 1. As expected, results showed a trend for leaves to

present higher crude protein and lower cell wall contents comparing to the fractions (stover and stems) that are characterized to have higher concentrations in structural tissues such as xylem and sclerenchyma (Moore and Jung, 2001). Consequently, the stover and stems had lower *in vitro* digestibility for both ruminants and rabbits, and lower *in sacco* degradability parameters of DM and NDFom fractions. It should be highlighted that the low nutritive value of the stover results from the advanced stage of development (90 to 95% of the dried pods (physiological maturation stage, R5 stage, 80-90 days) of cowpea at harvesting. In fact, as the plant advances in its physiological stage cellulose, hemicellulose and lignin content increases, with a consequent reduction of the potentially digestible constituents (soluble carbohydrates, crude protein, vitamins and minerals; Blaser *et al*., 1986; Morrison *et al*., 1998; Ball *et al*., 2001; Filya, 2004) and of the *in vitro* digestibility. Results obtained in Chapter 1 indicated that: 1) cowpea co-products (stover, leaves and stems) have potential to be use in animal feeding and, future studies should be performed to evaluate the effect of its inclusion in animal diets; 2) the high NDFom and lignin contents indicated that pre-treatments should be considered as a mean to increase its nutritive value; 3) the leaves of the cowpea represent the fraction of the higher nutritional value (higher CP and lower NDFom that stover and stems) promoved higher extent of degradation and higher dry matter digestibility and its conservation is important.

Based on the results showed in Chapter 1, the effect of the incorporation of cowpea stover (0, 20 and 40 g kg^{-1}) on total tract apparent digestibility of nutrients of growing rabbits was evaluated in Chapter 2. The effects of substitution of traditional raw material, alfalfa-beet pulp-wheat straw, by a combination of cowpea stover-wheat bran in rabbits in the final stages of fattening (53 to 67 days) was assessed. In rabbit feeding, different feed ingredients with high fibre content are frequently used, e.g.

alfalfa hay, grape seed, olive leave, paprika meal, soybean hull, sunflower hull, and wheat straw (García *et al*., 2000; Nicodemus *et al*., 2007; Ribeiro *et al*., 2012). The results obtained in this Chapter 2 clearly demonstrated that rabbits' performance (i.e., growth rate and feed conversion ratio) was not affected by the incorporation (up to 4%) of cowpea stover in the experimental diet. Although total tract apparent digestibility of organic matter, neutral detergent fibre and gross energy did not differ between diets (control vs. 20 and 40 g kg^{-1} incorporation) a trend for lower crude protein digestibilities was observed as cowpea stover inclusion increased.

In Chapter 1 and 2, the high lignification of the cell wall of cowpea stover was evidenced as well as the need to study methods that promote delignification with the objective of improving the nutritive value of this co-product. Physical, chemical and, more recently, biological pre-treatments are evaluated for altering the cell wall structure, especially lignin, of agroindustrial residues with subsequent use in animal feed and/or in the biofuels sector (Pandey *et al*., 2012; Tuyen *et al*., 2012; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015). Pretreatment with white-rot fungi presented unique mechanism of change in the cell wall structure and consequently lignin and in many cases increase the nutritional value (Tuyen et al., 2012; Tuyen et al., 2013; Van Kuijk et al., 2015). Biological pretreatments present potential for scaling as the material needs to be stored in the harvest period to be supplied at a specific period of forage shortage. In this way, in Chapter 3 the pre-treatment of cowpea stover with five fungal strains (*Ganoderma lucidum*, *Lentinula edodes*, *Pleurotus citrinopileatus*, *Pleurotus eryngii* and *Phlebia rufa*) with two incubation periods (22 and 45 days) was evaluated. Data on chemical composition and *in vitro* digestibility of rabbits of the cowpea stover after incubation with fungi suggested that: 1) the *P. citrinopileatus* strain showed optimal growth and a more efficient delignification process, which resulted in higher in *vitro*

digestibility for rabbits; 2) *P. citrinopileatus* at 22 days promoted a reduction in lignin content of 45% and an increase in *in vitro* digestibility for rabbits of the order of 38% comparing with control. *Pleurotus* spp., *Lentinula edodes* and *Ceriporiopsis subvermispora*, have been reported as promising strains in the valorization of different co-products in animal feeding (Tuyen *et al*., 2012; Tuyen *et al*., 2013).

Based on the results obtained in Chapter 3, in our opinion two aspects need particular attention to enhance the biological pretreatment technique: 1) develop an alternative sterilization process to autoclaving and 2) limit the consumption of structural polysaccharides (cellulose and hemicellulose) during colonization period of fungi. Several authors (Tuyen *et al*., 2012; Tuyen *et al*., 2013) suggested that the application of this sterilization process promotes changes in the cell wall architecture, limiting enzymes accessibility to digestible compounds, with a decrease of the nutritive value of different co-products. Alternative methodologies such as chemical sterilization, already reported by Pandey *et al*., 2012 need to be evaluated. Thus, our research group advanced with a pilot study (data not published) aiming to study alternative (physical and chemical) sterilization methods of the cowpea stover. In this pilot study we tested different concentrations of hydrogen peroxide (1.5, 3, 6 and 9%), hydrated lime (4% for 24 hours) and hot water (100°C for one hour) in the control of microorganisms (yeast and molds and total aerobic). The more promising results were obtained with the utilization of hydrogen peroxide at 6 and 9% (Figure 10). Nevertheless, it is necessary to assess in future studies that the colonization process by white-rot fungi in this sterilized material is not compromised.

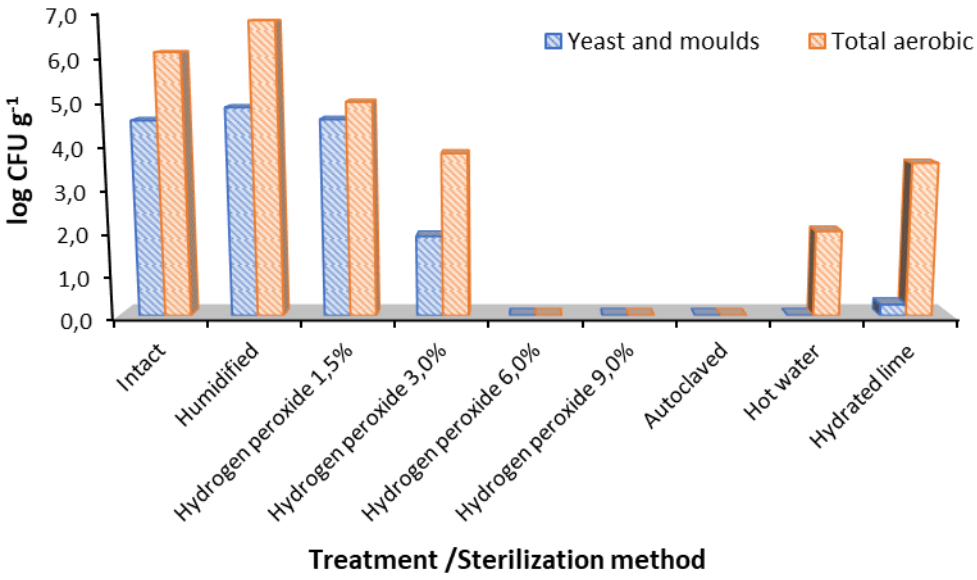

Figure 10. Physical and chemical methods for sterilization of cowpea stover (data not shown)

As mentioned before the need to control the consumption of structural of structural polysaccharides (cellulose and hemicellulose) during the fungi colonization period is required. One possible approach is to use additives such as cooper, manganese, urea, veratryl alcohol and conjugated linoleic acid (Pandey *et al*., 2012; Tuyen *et al*., 2012; Tuyen *et al*., 2013; Van Kuijk *et al*., 2015) that increase the production of peroxidases during this phase. Nishimura *et al*. (2012) reported higher lignin degradation when adding alkylitaconic acids to fungal treatments comparing to linoleic acid, possibly as a result of a greater lifespan of the radical in the lipid peroxidation. Moreover, Rahmawati *et al*. (2005) suggested that alkylitaconic acids may act as a neutralization agent of products produced during the Fenton reaction. This reaction is one of the few pathways used by white-rot fungi to degrade cellulose (Tanaka *et al*., 2009), and its blocking by alkylitaconic acids could make fungus very selective for lignin (Van Kuijk *et al*., 2016). In this context, the effect of adding itaconic acid (0, 2.5 and 5.0mM L^{-1}, after and before the autoclaved process) to cowpea stover inoculated with *Pleurotus citrinopileatus* on the chemical composition, *in vitro* digestibility and

enzymatic kinetics was assessed. Preliminary results (data not shown) on enzymatic kinetics indicate that the use of itaconic acid increased the production of lignolytic enzymes (laccase and manganese peroxidase) and decreased the production of cellulolytic enzymes (carboxymethylcellulose and avicellase).

Results obtained in Chapter 2 suggested that higher incorporation of cowpea stover in rabbit feeding without compromising animal performance and that its biological treatment of of cowpea stover with *P. citrinopileatus* enhances its nutritive value. Consequently, in Chapter 4 the incorporation of treated (TS) and untreated (US) cowpea stover with *P. citrinopileatus* on rabbit feeding (performance, digestibility, health and meat quality) in proportion of 50 and 100 g kg^{-1} of boths cowpea stover, was assessed. Few *in vivo* trials (Ribeiro *et al*., 2012) have been carried our to evaluate the incorporation of co-products pretreated with white-rot fungi in rabbit feeding. Currently, the studies with laboratory rats evaluated the effect of white-rot fungi, in fruiting body, and their effect on health of animal (Alam *et al*., 2011; Patel *et al*., 2012). In Chapter 4 the results indicated that 1) animals fed with untreated stover had a final live weight 5% lower than the ones fed treated stover, 2) the treated stover diet allowed a 17% reduction in blood cholesterol levels, and 3) carcass traits, meat quality parameters and caecal microbiota were not influenced by the incorporation of both treated and untreated cowpea stover. Alam *et al*. (2011) have reported data showing a reduction of 30% of total plasma cholesterol on rats fed a diet containing 5% of *Pleurotus ostreatus* (fruiting body). Similar results were described in a review work by Patel *et al*. (2012) where a reduction in the arterial pressure and blood cholesterol level was observed in rabbits and rats fed on diets containing 4-10% dried fruiting body of *Pleurotus* spp. compared to animals receiving normal diets.

As mentioned before, results obtained in Chapter 1 indicated that the preservation of leaves of cowpea stover is vital, as this is the fraction with higher nutritive value. In fact, leafiness is particularly important as leaf content is positively correlated with forage quality (Ball *et al*., 2001). In the hay making process of legumes, the stem has greater difficulty in losing moisture, as it is thicker, prolonging this process. As a result, leaves become highly brittle, causing its physical detachment from the stem (Harris and Tullberg, 1980; Ball *et al*., 2001) and a decrease in the hay nutritive value. Ensiling may be an alternative for biomass conservation (Reyes-Gutiérrez *et al*., 2015). In the last two Chapters (5 and 6) we evaluated the potential of conserving cowpea stover in association with a rich source of soluble sugars (discarded apple) by the ensiling process. The harvesting season of cowpea and apple generally occurs in the same period, and as such huge amounts of residues from these two crops are available at the same time. Most of the times, elimination of these residues has a high environmental impact and represents an additional financial cost. In this way, the possibility to use mixtures of these two co-products might be attractive in terms of animal feeding.

In Chapter 5 the ensilability and nutritive value of mixtures of cowpea stover (15%) and discarded apple (85%) with two different ensiling periods (45 and 60 days) were evaluated. Results suggested that stable mixtures, with low pH values, no butyric acid and high aerobic stability (216h), can be obtained. However, low residual water-soluble carbohydrate (WSC) concentrations (92.5% reduction of WSC content) of the resulting silages were obtained as a result of the activity of the epiphytic microbial population. These results suggested that lactic acid additives should be used to control microbial fermentation and improve the nutritive value of the obtained silages. Based on these results, the effect of using of commercial inoculant contain *Lactobacillus plantarum*, *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Propionibacterium*

acidipropionici, α-amylase from *Bacillus amyloliquefaciens*, cellulase from *Trichoderma reesei*, β-glucanase from *Aspergillus niger* and xylanase from *Trichoderma longibrachiatum* on the ensilability, fermentation parameters and aerobic stability was studied in Chapter 6. Additionally, the potential of conservation of cowpea stover previously treated with *Pleurotus citrinopileatus* (22 days of incubation period) with discarded apple by the ensilage process was evaluated. Results indicated that silages of both inoculated and non-inoculated cowpea stover mixed with discarded apple were stable after 45 days of ensiling, presenting low pH values and no butyric acid. Moreover, addition of the commercial inoculant affected the chemical composition allowing the conservation of greater amounts of water-soluble carbohydrate, increasing the nutritive value of the obtained silages.

This work showed that cowpea co-products could be used in herbivore feeding. Nevertheless, processes involving the application of biological treatments must be subjected to a broader evaluation. In this way, although the use of agricultural residues might be an alternative, the overall process assessment identifying all the economic and environmental costs involved in the different steps (material collection and processing, pre-treatment, storage and transportation aspects) needs to integrated in future studies.

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Conclusions and future perspectives

The results obtained in this thesis evidence that cowpea stover *cv.* fradel has potential to be used in animal feeding. Although, this co-product presents high lignified cell wall content, results demonstrated that its incorporation upto 40 g kg^{-1} did not affect total tract apparent digestibility of organic matter, crude protein, neutral detergent fibre and gross energy of the diets fed to growing rabbits.

Data also indicate the benefits of a biological pretreatment of cowpea stover, especially the strain *Pleurotus citrinopileatus* at 22 days of incubation period, to improve its nutritive value. In fact, treatment of cowpea stover with this white-rot fungus strain led to an efficient delignification process, resulting in a higher *in vitro* organic matter digestibility. Although the technology has proven to be effective, future studies should be carried out to evaluate alternative methods to autoclaving (i.e. hot water, hydrogen peroxide and hydrated lime) and techniques to control the consumption of cellulose and hemicellulose during biological pretreatment (i.e. additive and secondary compounds).

In general, data obtained in the animal trials shows that the incorporation up to 100 g kg^{-1} of both treated and untreated cowpea stover in rabbit diets did not compromised their performance and nutrient digestibilities. Moreover, rabbits fed with treated cowpea stover had a higher final live weight and a reduction in blood cholesterol levels than the ones fed untreated cowpea stover. Further research is needed to elucidate the biological processes underlined this effect of lowering blood cholesterol and to evaluate the impact of incorporating treated cowpea stover on animal health and carcass quality.

Results obtained in this thesis demonstrates the potential of conserving of mixtures of treated and untreated cowpea stover with discarded apples by the ensiling process. Stable silages with low pH, high lactic acid with no butyric acid and long aerobic stability can be obtained by ensiling these mixtures. Moreover, data obtained evidenced the benefits of adding a commercial inoculant by allowing the conservation of greater amounts of water-soluble carbohydrate and, consequently, obtain silages with higher nutritive value. Further research should be conducted evaluate the incorporation of higher levels of cowpea stover incorporation aiming to obtain silages with higher crude protein content without compromising the efficiency of the ensiling process. Moreover, the palatability of these silage mixtures should be assessed in *in vivo* animal trials.